Role of Alpha$_{2a}$-Adrenergic Heteroreceptors in Stress-Induced Reinstatement of Cocaine Associated Behaviors: Implications for the Pharmacological Treatment of Stress-Driven Relapse of Drug Use

by

Rafael Eugenio Perez

Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
for the degree of
DOCTOR OF PHILOSOPHY
in
Pharmacology
May 31, 2020
Nashville, TN

Approved:

Brad A. Grueter, Ph.D. (Chair)
Fiona E. Harrison, Ph.D.
Erin S. Calipari, Ph.D.
Sachin Patel, M.D./Ph.D.
Danny G. Winder, Ph.D. (Thesis Advisor)

Dedication

Para Mami, Papi, y Tia Margo, por su dedicación, amor, y sacrificio

And

To Oakleigh, for inspiring me every day to be a better scientist and human being, I could not have done this without you.

Acknowledgement

This work would not have been possible without the financial support provided by the National Institutes of Health. This work was supported by grants from the National Institute of General Medical Sciences awarded to the Initiative for Maximizing Student Development (GM062559) and the Vanderbilt Pharmacology Department (GM007628). The work on this thesis was also supported by grants from the National Institute on Drug Abuse to Danny Winder (DA042475 and DA04247504S1). Portions of the work were performed in part through the use of the Vanderbilt Cell Imaging Shared Resource (CA68485, DK20593, DK58404, DK59637 and EY08126), and the Vanderbilt Mouse Neurobehavioral Core (U54HD083211).

Thank you to my thesis advisor Danny Winder, for giving me a chance and the room to grow, and for believing in me even at times when I did not have the strength to believe in myself. Through your patience, diligence, and grace, you have taught me how to be a scientist with integrity, a supportive mentor, and leader. I could not have asked for a better mentor or role model.

Thank you to the members of my thesis committee: Brad Grueter (Chair), Fiona Harrison, Erin Calipari, and Sachin Patel. Thank you for pushing me out of my comfort zone and encouraging me along the way. Your science and career advice have been instrumental in bringing this moment into fruition.

Thank you to the members of the Winder lab, past and present, for your intellectual support and easy laughs. Thank you to Nick Harris and Bob Matthews for showing me the ropes. Thank you to Greg Salimando for being a great friend during this strange ride that is grad school. Thank you to Elana Milano for holding the lab together and showing me how to do surgeries. Thank you to Anel Jaramillo, Sam Centanni, and James Melchior for being so generous with your time and input. Thank you to Aakash Basu and Brett Nabit for working so hard on this project, seeing you grow into independent, creative, and fearless scientists has been a blast. Thank you to Kelly Williford, Jordan Brown, Joe Luchsinger, and Nick Petersen, and Bridget Morris for your friendship and willing ears.

Quiero darle las gracias a mis padres Marino Pérez y Grecia Holguín, por todo sus sacrificios durante todos estos años, y a mi tía, Margarita Gómez por abrirme su puerta y su corazón. Este éxito es tanto de ustedes como mío.

Thank you to Mika, Marika, Rolfy, Argeniz, and Steven for being there from the beginning. Thank you to Alex, Shellie, Alezander, Cristian and Junior for your continued love and support. Thank you to CRISPR R.G.S. Pérez Folkes for all the snuggles, long walks, and for keeping me sane through grad school.

Most importantly, thank you to my wife Oakleigh, whom I owe so much. Your love has made this possible.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Table Page

Chapter 1

Introduction

Cocaine use disorder (CUD)

History of cocaine

Cocaine is an ester alkaloid extracted from the coca plant (*Erythroxylum Spp.),* which is endemic to the vast Andes mountain region, but grows and is cultivated all over South and Central America [1,2]. Indigenous populations of South and Central America have been consuming coca for millennia, due to the plants mild stimulant and medicinal properties and its role in religious practices [2,3]. Coca was introduced to Europe following the 14th century invasion, conquest, and subjugation of the Inca empire by the Spaniards [3]. The first European medical description of the plant was published by the Spanish doctor Nicholas Modarnes in 1565 [3]. While the use of coca leaves and paste remained popular for the next 300 years, cocaine was first separated and extracted by the German chemist Friedrich Gaedcke in 1855 [1]. The process of cocaine purification was later refined and perfected by Albert Neumann, who also described the local anesthetic properties of the substance [1].

During the late 19th century, cocaine was widely prescribed in Europe and America as a remedy for many ailments including fatigue, pain, and even opioid addiction [3]. In 1863, the Italian chemist Angelo Mariani, developed Vin Coca Mariani, a wine containing cocaine that was endorsed by prominent figures of the time, most notably the Pope [3]. By 1886, following the success of Vin Mariani, the American biochemist John Stith Pemberton patented a formula for a medicinal drink containing the extract of coca leaves termed Coca-Cola, which remains massively popular, although by 1916, most cocaine had been eliminated from the drink [3]. The

most influential proponent of the use of cocaine during this period was the famed psychotherapist Sigmund Freud, who experimented with the substance during the 1880s and published a series of paper including *uber coca* in 1884 [1]. Freud received financial compensation and promoted the cocaine formulations of Merck and Parke-Davis, the leading pharmaceutical companies of that period [1].

By the beginning of the 20th century, as cocaine's addictive properties became widely recognized, the use of the substance began to be strictly regulated [3]. Cocaine stopped being prescribed as an over the counter medication in 1916 [3]. The illicit use of cocaine also waned during this period, due to the rise amphetamine use, a cheaper stimulant, during the 1920s and 1930s, and remained low until the 1970s when consumption began to increase again [3].

Mechanisms of action of cocaine

At higher doses or following repeated use, cocaine produces adverse effects, including jitteriness, aggressiveness, irritability, anxiety, and paranoia [4]. Clinically, cocaine is classified as a local anesthetic due to its ability to produce numbing [5]. Cocaine is also classified as a psychostimulant due to its enhancing effects on mood, arousal, alertness and energy levels [6]. Cocaine produces analgesia by blocking voltage-gated sodium channels, which decreases the excitability of nerve terminals [7–9]. By increasing blood levels of the catecholamine neurotransmitters dopamine (DA) and norepinephrine (NE), cocaine induces vasoconstriction, which leads to elevated blood pressure and heart rate [10]. Centrally, by blocking the DA, NE, and serotonin (SER) transporters (DAT, NET, and SERT), cocaine prevents the reuptake and vesicular packaging of these neurotransmitters, resulting in increased levels at the synapse [11–14].

Early work suggested that the acute psychostimulant and reinforcing effects of cocaine in animals, and the euphoric effects of the drug in humans, are dependent on dopamine signaling,

leading to the "DAT-is-it" theory, which stated that cocaine's psychoactive effects are mediated by DAT-blockade [13,15–19]. Microdialysis studies in rodents, and imaging studies in humans, provided further support for this theory. Cocaine rapidly elevates dopamine, presumably due to DAT blockade, particularly in the mesolimbic pathway consisting of the ventral tegmental area (VTA) to nucleus accumbens (NAc) and prefrontal cortex (PFC) projections, a feature shared by many other addictive substances and stimuli [20–23].

However, early studies in knockout and transgenic mouse lines uncovered a complex role for NET and SERT in the reinforcing and aversive effects of cocaine in mice with life-long somatic deletion of DAT [6,24]. While mice lacking DAT do not display cocaine-induced hyperlocomotion, they still exhibit cocaine conditioned place preference (CPP) and self-administration (SA), which are measures of the reinforcing properties of this drug [6,25–27]. Since the deletion of DAT induces profound changes in dopamine homeostasis and plasticity in catecholinergic systems, a potential role of SERT and NET on dopamine reuptake has been investigated. However these transporters were found to not contribute to the reuptake of dopamine in the NAc of DAT knockout mice[27–29].

Mice lacking either SERT or NET also exhibit CPP, suggesting that compensatory mechanisms in single-transporter knockout mice and functional redundancy amongst the transporters may play a role in mice with germline mutations[6]. Using combinatorial knockdown and pharmacological approaches, researchers found that eliminating DAT and SERT simultaneously decreases the reinforcing effects of cocaine and that SERT/NET knockout increases the reinforcing effects of cocaine [30,31]. These findings, alongside evidence showing that NET knockout leads to cocaine conditioned aversion (CPA), suggest that the reinforcing and stimulant effects of cocaine on single transporter knockout mice are likely mediated by the

blockade of DAT and SERT, whereas the aversive effects reported by human subjects at higher doses are likely the result of NET blockade [4,13]. Selective manipulations such as single-point mutations which eliminate cocaine's ability to interact with DAT, revealed that in mice with functional DAT, cocaine's rewarding and psychomotor effects are indeed mediated by this transporter[32].

Cocaine use, particularly chronic use, has substantial health risks, including the development of destructive patterns of compulsive thoughts and behaviors leading to the psychiatric disorder known as cocaine use disorder (CUD), described below.

Diagnosis and prevalence of CUD

The Diagnostic Manual of Mental Disorders (DSM-5) defines CUD using the following 11 criteria [33]: 1) Increased or prolonged use of cocaine, 2) desire to stop using cocaine but not being able to, 3) increased time spent acquiring, using, or recovering from the use of cocaine, 4) craving or strong desire for cocaine, 5) failure to fulfill work or family obligations, 6) continued use despite social, occupational, or relationship problems, 7) continued use despite physical problems, 8) hazardous cocaine use, 9) reduced engagement in social, occupational, or recreational activities due to cocaine use, 10) tolerance (increased use of the drug needed to obtain same effects), and 11) withdrawal. Mild CUD is defined by meeting 2-3 of the diagnostic criteria, moderate CUD by meeting 4-5, and severe CUD by meeting 6 or more of the criteria [33]. As of 2018, approximately 1 million individuals have a diagnosis of CUD in the united states, according to the National Survey on Drug Use and Health (NSDUH) [34]. Furthermore, from 2011 to 2015 there was 4-fold increase in the prevalence of CUD among the general population suggesting a resurgence in CUD as a potential epidemic health concern [35].

Health risks associated with CUD

Cocaine use and CUD produce a number health issues that contribute to increased mortality including overdose [36,37]. Cocaine users experience a number of cardiac and cerebrovascular complications associated with acute or chronic cocaine consumption, including: arrhythmias, cardiomyopathy, and coronary arterial disease [10,38]. These cardiac complications are also worsened by the high degrees of comorbidity between CUD and alcohol use disorder (AUD), which further impacts cardiovascular health [10]. Both cocaine's ability to block sodium channels and increase NE levels in sympathetic tissue lead to vasoconstriction and increased blood pressure and heart rate in the short term. Whereas chronic cocaine exposure leads to toxic accumulation of free radicals and oxidative damage to myocardial tissue [10]. However, adrenergic compounds that show efficacy in non-CUD patients, such as beta-adrenergic receptor (β-AR) antagonists, can have adverse and potentially lethal effects in the presence of cocaine due to unopposed alpha-adrenergic (α-AR) receptor signaling. Therefore, the contraindication of β-AR antagonists further complicates the treatment of cardiac issues in CUD [38].

Cognitive ability is another potential area that might be affected in patients with CUD which has been extensively studied[39]. However, reports have been inconsistent, with clinical laboratory and retrospective studies showing a wide range of decreases, increases, or lack of differences in the cognitive performance of CUD or cocaine-using individuals [39]. In conjunction with these psychometric analyses, studies evaluating differences in brain structure between healthy individuals and cocaine users also show a wide range of results, with reported differences in gray and white or no changes[39]. There are many potential reasons for the discrepancies in these results, but differences in educational level, age, polydrug use, and comorbid AUD, play a role in the wide variability in results from cognitive assessments [39,40].

Current treatments for CUD and their limitations

Psychosocial therapies

Currently, there are no FDA-approved medications for the treatment of CUD, therefore psychologically- and socially-based treatment strategies are the preferred options [36]. Group, and individual, drug counseling therapy show efficacy, particularly in establishing and sustaining early abstinence [36]. Additionally, cognitive behavioral therapy and voucher-based contingency management have been shown to be efficacious in increasing days of abstinent in CUD patients that complete these treatments [41–45]. However, these programs are expensive, time-consuming, and difficult to access, particularly for low socioeconomic status, racially-minoritized, or rural communities, which are disproportionally more affected by cocaine overdoses and lethality [46,47]. Thus, these treatment modalities show high drop-out rates, as patients report high levels of re-occurring relapse of cocaine use [45]. Many pharmacotherapies have been investigated as potential treatments for CUD, however, due to the effects of cocaine on the catecholaminergic system, drugs that target dopamine and norepinephrine receptors have been of particular interest [36].

Dopaminergic ligands

The dopaminergic agents dextroamphetamine and amphetamine, which, due to different routes of administration, have a slower pharmacokinetic profile than cocaine, have been investigated in laboratory settings and clinical trials [48–52]. While patients in these studies showed a reduction in cocaine use, particularly patients with comorbid attention deficit/hyperactivity disorder, dropout rates were high [50,52]. Modafinil, a mild stimulant that binds to the same site in DAT as cocaine but with lower affinity, decreased subjective feelings of euphoria or "high" after cocaine use or cocaine administration in clinical laboratory studies [53]. However, in clinical trials,

the efficacy of modafinil has been mixed, with a small trial reporting an increase in days abstinent, while larger follow-up studies have reported negative results [54,55].

Noradrenergic ligands

Due to their ability to regulate many of the psychological and physiological effects produced by cocaine, as well as anxiety, a common feature of CUD withdrawal, adrenergic ligands have been investigated as potential treatments for CUD [56–59].

α_1-AR antagonists

The α_1-AR blocker doxazosin blocked the subjective effects of cocaine in clinical laboratory studies as well as cocaine-positive urine samples in a small clinical trial [59,60]. However, the efficacy of doxazosin has been shown to be regulated by polymorphisms in the gene coding the α_{1D}-AR, suggesting that this compound should be used in specific CUD subpopulations in order to enhance effectiveness [61].

β-AR antagonists

β-AR antagonists such as propranolol show some efficacy as a treatment for CUD, particularly in reducing withdrawal-induced anxiety and craving [56,57]. Patients with higher withdrawal severity report the most benefits [57]. However, potential cardiovascular complications, due to unopposed α_1-AR signaling, limit the clinical utility of these compounds [10]. Therefore, researchers have also investigated the dual β-AR/α_1-AR antagonist carvedilol which is used for the treatment of congestive heart failure and hypertension and has been shown to be safe in CUD patients [62]. Although carvedilol blocked cocaine-induced elevations in blood pressure and cocaine-self administration in laboratory studies, in a small clinical trial, this compound did not reduce cocaine use in a population of CUD patients with comorbid opioid use disorder (OUD) undergoing methadone maintenance treatment [62]. Given the safety and tolerability of carvedilol,

studies with larger sample sizes and in populations that do not have comorbid OUD are needed to better determine the clinical efficacy of this compound for the treatment of CUD.

α_{2A}-AR agonists

Given the prominent role of stress, hypothalamic-pituitary-adrenal (HPA) axis, sympathetic, and adrenergic activation on relapse in CUD, drugs that decrease NE release by activating α_2-ARs have been investigated as treatment for stress-driven symptoms in CUD [58,63–65]. α_2-AR agonists have been of particular interest due to their well-characterized safety and pharmacodynamic profiles, as well as their wide-spread clinical use [66,67].

In preclinical studies, α_2-AR agonists such as clonidine, lofexidine, and guanfacine decrease NE release, stress-induced sympathetic activation, and stress-induced reinstatement of cocaine self-administration (SA) and conditioned place preference (CPP) which are animal models useful in the study of relapse [68–72]. Conversely, the non-selective α_2-AR antagonist yohimbine, and the selective α_{2A}-AR antagonist BRL-4448, reinstate cocaine SA and CPP, providing more evidence for the role of α_2-ARs in stress-induced cocaine seeking [70,73,74]. α_2-AR agonists also decrease cocaine-induced anxiety, an important finding given the strong correlation between withdrawal severity, anxiety, and relapse in patients with CUD [63,75]. However, the implementation of α_2-AR agonists, particularly the non-selective agonists clonidine and lofexidine has been limited due to their potential adverse effects precluding their chronic use, including hypotension, sedation, somnolence, sexual problems, and decreases in cognitive performance [66].

In light of these results, the subtype-selective α_{2A}-AR agonist guanfacine has been investigated as a pharmacological treatment for stress-related symptoms and complications in CUD [63]. A low dose of guanfacine decreases anxiety-like behaviors and blocks stress-induced

reinstatement of cocaine CPP [76–78]. In clinical laboratory studies, guanfacine decreases stress-induced elevations in blood pressure, cue-evoked and stress-induced cocaine craving, anxiety and arousal in subjects with CUD [58,79]. Guanfacine was more effective in reducing cue- and stress-induced craving and anxiety in female subjects, as compared to male subjects with CUD [79].

In addition to anti-craving effects, guanfacine also enhances cognitive flexibility and inhibitory control following stress in subjects with CUD [80]. These cognitive improvements are likely due to activation and strengthening of prefrontal cortex (PFC) networks, which are observed in rodents and primate models [81–83]. In subjects with CUD, guanfacine increases activation of medial and lateral PFC regions in response to stress, suggesting strengthening of inhibitory networks that may decrease stress-precipitated craving [58].

While α_{2A}–ARs show beneficial effects in clinical trials, such as reducing withdrawal-induced anxiety and irritability, these compounds have failed to increase treatment retention or drug-free urine samples in clinical trials [84,85]. The factors underlying the observed lack of efficacy of these compounds is currently unknown, but reported dose-dependent negative side effects such as sedation and dry mouth, as well as underlying mechanisms mediating adrenergic regulation of relapse of cocaine use are implicated [63,79,86,87]. The following section will discuss the adrenergic system and contributions of different α_{2A}-AR subpopulations to the pharmacological effects of α_{2A}-AR agonists.

Overview on the noradrenergic system

The noradrenergic system is composed of neurons from the locus coeruleus (LC), a brain stem cluster in A6, and the lateral tegmental nuclei, located in the nucleus tractus solitarii (NTS) in A1, A2, and A4. These regions send dense projections across the brain through the ventral and

dorsal noradrenergic bundles [88–91]. Upon stimulation, nerve terminals from these bundles release norepinephrine (NE), which binds to 3 classes of heterotrimeric G-protein coupled receptors (GPCRs): beta- (β-ARs), alpha$_1$- (α_1-ARs), and alpha$_2$-adrenergic receptors (α_2-ARs) [92]. α-ARs and β-ARs were originally characterized as such due to the opposite potency of adrenergic ligands in soft-tissue contraction and relaxation assays. Ligands with higher affinity for α-ARs show stronger potency in contraction assays while β-AR ligands show stronger potency in relaxation assays [92,93]. β-ARs couple to heterotrimeric $G_{\alpha s}$ proteins which induces adenylyl cyclase (AC) activity, cyclic AMP (cAMP) production, protein kinase A activation and second messenger signaling. α_2-ARs in contrast, couple to $G_{\alpha i/o}$ proteins which inhibits cyclic AMP (cAMP) production, leading to a variety of intracellular events which result in decreased or increased cellular activity depending on signaling partners [92,94]. Within the nervous system $G_{\alpha i/o}$-coupled GPCRs inhibit neurotransmitter release primary through three different pathways: 1) through the activation of G protein-coupled inwardly rectifying potassium channels (GIRKs), 2) the inhibition of voltage-gated calcium channels (VGCCs), and 3) via direct inhibitory interactions between $G_{\beta\gamma}$ and the vesicular release machinery [92,95,96]. α_1-ARs couple to $G_{\alpha q/11}$ to activate the hydrolysis of phospholipids by the phospholipase c family of proteins, which leads to the release of intracellular calcium and subsequent cellular processes such as cardiomyocyte contraction, and long-term depression of excitatory transmission in neurons [92,97]. Adrenergic receptors can also activate other non-G_α-mediated pathways such as the beta-arrestin-ERK (β-Arr-ERK) signaling cascade [92].

α_{2A}-ARs

The α_2-AR family is composed of three distinct subtypes of receptors based on pharmacological and physiological profiles α_{2A}-, α_{2B}-, and α_{2C}-ARs [98–100]. While the three

subtypes show a large degree of overlapping and compensatory functions, α_{2a}-ARs appear to be the most prominent subtype in the regulation of physiological processes including increases in neuronal activity, working memory networks, sedation, anesthesia, seizures, bradycardia, hypotension, and blockade of insulin secretion, among others [99]. α_{2A}-ARs can be further divided into autoreceptors, expressed in adrenergic neurons, and heteroreceptors, expressed in non-adrenergic cells [99]. While deciphering the relative contributions of auto- and heteroreceptors to the effects of α_2-AR agonists, such as clonidine and guanfacine, has been difficult due to the non-specific nature of pharmacological manipulations, results from adrenergic denervation studies using toxins in the DSP family, gene knockout, and cell-targeted RNA-knockdown experiments provide insight into the relative function of auto- and heteroreceptor populations [76,101,102]. The following section summarizes the results from these experiments and outlines the relative contributions of auto- and heteroreceptor populations to the effects of α_{2A}-AR agonists, which are widely used in the regulation of blood pressure and attention deficit/hyperactivity disorder (ADHD)[67,87].

<u>α_{2A}-AR autoreceptors</u>

α_{2A}-ARs are among the most well characterized and abundant autoreceptors in the central and peripheral nervous systems [103]. Early pharmacological studies reported agonist-mediated decreases in nerve stimulation of cardiac and soft muscle tissue, and adrenergic release [104,105]. These studies were followed by experiments in acutely prepared brain slices which later defined the presynaptic α_2-ARs expressed in the brain, which are predominantly the α_{2A}-AR and α_{2C}-AR subtypes [106–108].

In more recent studies aimed at further dissecting the specific function of α_{2A}-AR autoreceptors, researchers re-expressed α_{2a}-ARs under the promoter for dopamine-β-hydroxylase

(DβH), an enzyme that synthetizes NE, in mice lacking all α_{2A}-ARs, allowing for the functional expression of autoreceptors [102]. Surprisingly, these studies have uncovered relatively minor contributions of α_{2A}-AR autoreceptors in the effects of α_2-AR agonists on physiology, which are discussed below.

Autoreceptor functions in the cardioprotective effects of α_2-AR agonists

α_2-AR knockout mice selectively re-expressing autoreceptors show agonist-mediated decreases in NE release from nerve terminals into cardiac tissue, and inhibition of voltage gated calcium channels, confirming the previously assigned role of this population as a feedback regulator of adrenergic signaling [102,109]. The ability of α_{2a}-AR agonists to decrease NE release and circulating NE levels had been previously ascribed to autoreceptors [110–113]. Elevated levels of circulating NE are associated with cardiac damage and negative outcomes including fibrosis, hypertrophy and heart failure. Therefore, activation of autoreceptors has been hypothesized to be cardioprotective [114]. The negative cardiac outcomes associated with hyperadrenergic tone in patients are recapitulated by the full α_{2a}-AR knockout mouse line and rescued in knockout mice re-expressing α_{2a}-AR autoreceptors [109].

Autoreceptor functions in depression

Centrally, α_{2a}-AR autoreceptor regulation of NE has been implicated in the pathology of depression [115]. The link between depression and central NE was established by the observation that compounds that increase NE levels, such as tricyclic antidepressants, monoamine oxidase inhibitors, and NE reuptake inhibitors, have antidepressant actions [115]. Radioligand assays of circulating platelets from patients with major depressive disorder (MDD) indicate that the levels of high affinity-state, supersensitive α_{2a}-ARs are increased in this patient population [116]. Postmortem analyses of mRNAs from patients with MDD that committed suicide also show an

increase in α_{2a}-AR mRNAs when compared to samples from healthy controls [117,118]. Chronic antidepressant administration prevents stress-induced, and depression-like, upregulation of α_{2a}-ARs in the LC, suggesting a causal relationship between stress, depression and LC autoreceptor expression [119]. Data from functional studies in animal models support the results of these expression studies. Following chronic unpredictable stress, there is a decrease in NE release from LC terminals into the paraventricular nucleus of the hypothalamus, a brain region that has been implicated in depression, which correlates with decreased sucrose preference, a measure of anhedonia-like responses [119]. The selective α_{2a}-AR antagonist BRL-4448 malate normalized behavioral responses to stress and LC-terminal NE release [119]. Based on these observations, it has been postulated that in MDD, compensatory mechanisms upregulate the expression and function of α_{2a}-AR autoreceptors on LC terminals, leading to a hypo-noradrenergic state that drives depressive symptoms [115]. Interestingly, results from gene deletion studies appear to contradict this model. Mice lacking all α_{2a}-ARs show increased time in the closed arm in the elevated plus maze model of anxiety-like behaviors and increased immobility in the forced swim model of learned helplessness and depression-like behaviors [120]. However, in a recent study, the selective RNA knockout of α_2-AR heteroreceptors in the basolateral amygdala prevented guanfacine-induced antidepressant-like responses in the forced swim assay, suggesting that the depressive-like responses observed in α_{2a}-AR knockout mice may be heteroreceptor-dependent [76].

Autoreceptor functions in the somniferous effects of α_2-AR agonists

α_{2a}-AR autoreceptors also play a major role in the sleep-promoting effects of α_2-AR agonists. NE is a key regulator of arousal, alertness and sleep and compounds that decrease NE release, such as clonidine, produce somnolence and increase sleeping time in human subjects [121–

[123]. The LC controls wakefulness and arousal through noradrenergic release onto to the ventral periaqueductal gray and ventrolateral preoptic area [122,124–128]. α_2-AR agonists, presumably NE release into these areas, which produces drowsiness and sleep [127]. Full α_{2a}-AR knockout mice show spontaneous hyperlocomotion during the night period but animals that selectively express autoreceptors do not display this phenotype [102].

Autoreceptor functions in seizure disorders

α_{2a}-AR autoreceptors also display proconvulsant effects, which can potentially limit the use of agonist targeting these receptors in populations with comorbid epilepsies [129,130]. Toxin denervation studies, showed that NE depletion increases the severity and duration of seizures in animal models of epilepsy [130]. Furthermore, LC lesions also increase seizures, which points towards a protective role of central NE in seizure disorders [131]. Taken together, these findings suggest that α_{2a}-AR autoreceptors may increase the propensity of seizures by decreasing NE release from the LC. However, results from studies using α_2-AR agonists have been inconsistent, with anti-, proconvulsant, or no effects reported. Work in mice lacking DβH suggest that the proconvulsant effects of these compounds is autoreceptor dependent [129,130]. Further work of the role of autoreceptors in seizure disorders is warranted.

α_{2A}-AR heteroreceptors

While few studies have specifically evaluated the function of α_{2A}-AR heteroreceptors in physiological and pharmacological responses, these receptors have nonetheless been implicated in many of the α_{2A}-AR actions, including the regulation of blood pressure, pain, and cognition [99]. These actions are discussed below.

Heteroreceptor functions in the hypotensive effects of α_2-AR agonists

Clever denervation manipulations in which NE nerves are ablated using toxins, thus eliminating NE release and autoreceptor function, showed that α_2-AR agonists decrease blood pressure, and heart rate by activating heteroreceptors [110]. Mice lacking all α_2-ARs show basal elevated blood pressure and heart rate, and do not respond to the hypotensive and bradycardic effects of α_2-AR agonists [102]. Re-expression of α_{2A}-AR autoreceptors does not rescue the deficits in α_2-AR agonist regulation of blood pressure or heart rate, further suggesting that heteroreceptors are required for the hypotensive effects of these compounds [102]. While the heteroreceptor-driven hypotensive mechanism is currently unknown, heteroreceptor inhibition of GABAergic projections onto vagal neurons has been implicated [132]. Disinhibition of vagal neurons that innervate cardiac muscle leads to greater sympathetic control of cardiac output, bradycardia, and hypotension [132].

Heteroreceptor functions in the analgesic effects of α_2-AR agonists and neuropathic pain

α_{2A}-AR heteroreceptors also play a significant role in the analgesic effects of α_2-AR agonists. α_{2A}-AR receptors are highly expressed in the superficial layers of the spinal cord, particularly in glutamatergic projections onto dorsal horn neurons in the outer zone of the layer II/III lamina [133–135]. *Ex vivo,* α_{2A}-AR agonist administration decreases evoked excitatory postsynaptic currents (EPSC) into dorsal horn neurons [135]. *In vivo,* denervation of NE fibers, which results in functional elimination of autoreceptors, actually increases acute agonist-induced analgesia, further implicating heteroreceptors in this process [136]. In accordance with these studies, full α_{2A}-AR and heteroreceptor knockout mice do not show α_{2A}-AR agonist induced analgesia [102]. Taken together, these studies suggest that targeting α_{2A}-AR heteroreceptors in glutamatergic terminals on the spinal cord might be beneficial in acute pain. However, in models of chronic and neuropathic pain, heteroreceptor activation may increase pain responses [136].

Following root constriction, a model of neuropathic pain, rats show increase expression of α_{2A}-AR mRNA in dorsal root ganglion cells [136]. Furthermore, in this model, systemic administration or local spine infusions of α_{2A}-AR antagonists reduce pain responses [137]. *In vitro* experiments suggest that activation of α_{2A}-AR heteroreceptors in dorsal root ganglion cells may lead to the release of neuropeptide factors and reduced desensitization of transient receptor potential vanilloid type 1 (TRPV1) channels, leading to increased pain and sensitivity [138].

Heteroreceptor functions in the pro-cognitive effects of α_{2A}-AR agonists

Early studies defined a role of catecholaminergic regulation the dorsolateral prefrontal cortex (dlPFC) in attention and cognitive control, particularly pyramidal neurons from layers II/III [139]. Elevated levels of DA or NE, which can occur following chronic drug or stress exposure, decrease the activity of layer II/III pyramidal neurons [139]. Due to their actions as inhibitory autoreceptors, α_2-AR agonists were investigated as potential regulators of dlPFC function and cognitive pathology [139]. In studies in aged monkeys, clonidine and guanfacine were found to strengthen dlPFC network dynamics and improve performance in tests of cognition, attention, and impulsivity [140]. These results have been recapitulated in rats and monkeys with a history of cocaine exposure [141]. Guanfacine ameliorated cognitive and attentional deficits in animals chronically treated with cocaine [141]. Human laboratory studies found that guanfacine increases dlPFC activation and attentional bias to emotional cues in healthy subjects, suggesting greater top-down cognitive control in response to emotionally salient cues [142]. Guanfacine also increases dlPFC activity and cognitive flexibility during a stressful imaginary exercise in subjects with CUD [58,143,144]. Based on these studies, clinical trials evaluated the potential use of clonidine and guanfacine as ADHD medications and found efficacy for both agents although

guanfacine is preferred due to the more favorable therapeutic profile of its extended release formulation [67].

Studies on the effects of guanfacine on cortical activity have revealed novel mechanisms of action of α_{2A}-AR heteroreceptors. Toxin denervation experiments in rats showed that the α_2-AR agonists-mediated improvements in PFC function are heteroreceptor-dependent [145]. α_{2A}-AR heteroreceptors are expressed in dendritic spines in cortical layers II/III and show a high degree of colocalization with hyperpolarization cyclic nucleotide-gated (HCN) channels [140]. HCN channels are gated by cAMP, and during periods when cAMP is elevated, these channels open and decrease neuronal excitability, resulting in a "weakening" of PFC networks [139,140]. Activation of α_{2A}-AR heteroreceptors and $G_{\alpha i}$-signaling leads to a decrease in cAMP, closing of HCN channels, and restoration of neuronal excitability [140]. Pharmacological blockade or genetic deletion of cortical HCN channels mimics the pro-cognitive effects of guanfacine [140]. Conversely, intracortical infusions of cAMP analogs abolishes the pro-cognitive effects of guanfacine [82,140] In addition to their acute effects on HCN channel gating, chronic guanfacine administration prevents stress- and hypoxia-mediated dendritic spine loss in layers II/III, suggesting that this compound may be effective for the treatment of chronic conditions that impact the cortex [81,146].

Stress as a risk factor for reinstatement in CUD

Stress is a broad concept that encompasses a wide range of stimuli that challenges the coping abilities of organisms [147,148]. Stress can be positive (eustress) or negative (distress) in nature, and in humans it can take many forms raging from psychological, sexual, economic, social, or environmental [148]. The link between drug use disorders, including CUD, and stress has

been investigated for many decades, some of the primary stressors that have been implicated in CUD are discussed below.

Posttraumatic stress disorder (PTSD) and CUD

PTSD is a chronic stress disorder characterized by anxiety, sleep disturbances, perseverative and intrusive thoughts, and flashbacks [149]. Following a traumatic incident, a fraction of individuals, as high as 8%, will experience PTSD [150]. Among patients with CUD, the life-time prevalence of PTSD is significantly higher than the general population, with reports raging from 8-43% [151,152]. The wide range in the reported CUD and PTSD co-occurrence is due in part to methodological differences among studies as well as gender differences [151–153]. For example, female patients with CUD are twice as likely to report experiencing traumatic events and PTSD when compared to male patients [154]. CUD patients with comorbid PTSD report greater cocaine use following unpleasant or negative situations when compared to patients with CUD alone [153]. PTSD and CUD comorbidity has also been associated with worse CUD symptom severity, decreases in treatment adherence and days-abstinent, and increased relapse rates [152].

Childhood trauma and CUD

Data from preclinical models and correlational clinical studies show that early childhood trauma alters HPA axis function, stress responsiveness, coping, and susceptibility to drug use relapse [155,156]. CUD patients report higher incidence of childhood trauma and neglect compared to the general population, conversely individuals with childhood trauma are more likely to experience CUD [157]. CUD patients with childhood trauma report more negative responses to daily life stressors and more severe withdrawal symptoms during detoxification treatment, which may lead to increased relapse [156]. Indeed, the severity and duration of childhood trauma has been

strongly correlated with poorer treatment outcomes and abstinence in female CUD patients [158,159].

Stress-induced craving and relapse

Stressful events are often cited as precipitating factors in cocaine use relapse, with female patients reporting a higher propensity to relapse due to stressful or negative affective stimuli compared to male patients, which show a higher rate of craving and relapse due to drug-associated cues [79,158,160]. In clinical laboratory settings, female CUD patients also show greater stress-induced elevations in circulating cortisol and corticotropin releasing factor (CRF), hormones that play a key role in the HPA axis response to stressors [158,161]. The pharmacological stressor yohimbine, which induces anxiety and stress-like responses, also increases craving in cocaine users [158]. Infusions of CRF also lead to craving, particularly in female patients with CUD [161]. Elevated cortisol levels in stressful clinical laboratory exercises are associated with increased cocaine use relapse rates [162].

Stress-driven craving and relapse has been studied using preclinical models, allowing for more invasive exploration of the brain regions, cellular populations, and molecular signaling mechanism underlying relapse, these studies are summarized below.

Stress-induced reinstatement procedures: preclinical research models of relapse

For decades, stress-induced reinstatement procedures has been the primary framework used for the preclinical modeling of stress-driven relapse [73]. In these studies, rodents are typically trained to either self-administer drug through operant responding by nose poking or level pressing (self-administration (SA)), or learn to associate a distinct place within a chamber with the drug (conditioned place preference (CPP)) [70,163]. Drug or conditioned place seeking can be suppressed through extinction training, a process analogous to abstinence in patients [73]. Once

extinction is achieved, a variety of stimuli including contextual or drug associated cues, the drug itself, or stressors, can induce the reinstatement of responding in SA or CPP [70,73,164]. Researchers have found that stress-induced reinstatement procedures have a high degree of validity, with studies showing translatability among stressor, brain region-specific, and pharmacological findings [73]. For example, foot shock, predator odor, and single prolonged stress, which are models analogous to PTSD, induce reinstatement of cocaine SA [165]. Pharmacological stressors that produce discomfort, anxiety, and craving in patients with CUD, such as yohimbine and CRF, reinstate cocaine SA and CPP in animals [70,166–168]. Also, studies have reported sex-differences in stress-induced reinstatement behavior, findings that converge with reports of differences in the magnitude of stress-induced craving and relapse among male and female patients with CUD [168].

Stress-induced reinstatement procedures have also identified many putative targets for the potential pharmacological treatment of stress-driven relapse [73]. In corroboration with clinical observations, researchers have found that CRF, β-AR, and α_1−AR inhibitors block stress-induced reinstatement of SA and CPP in rodents [69,158]. Additionally, compound classes that target receptors or processes predicted to play a role in stress-induced relapse but have not been as extensively tested in human populations have also been empirically evaluated in these procedures, including: serotonin receptor agonists, GABA receptor agonists, subtype-selective metabotropic glutamate receptor agonists, and neuropeptide ligands, among others [73].

Brain regions and neurotransmitter systems that regulate stress-induced reinstatement

Ventral tegmental area (VTA)

Although the VTA has been traditionally associated with the reinforcing and hedonistic properties of addictive substances, results from stress-induced reinstatement studies show a role for this brain region in stress-induced neuronal activity and reinstatement [17,22,69]. The VTA

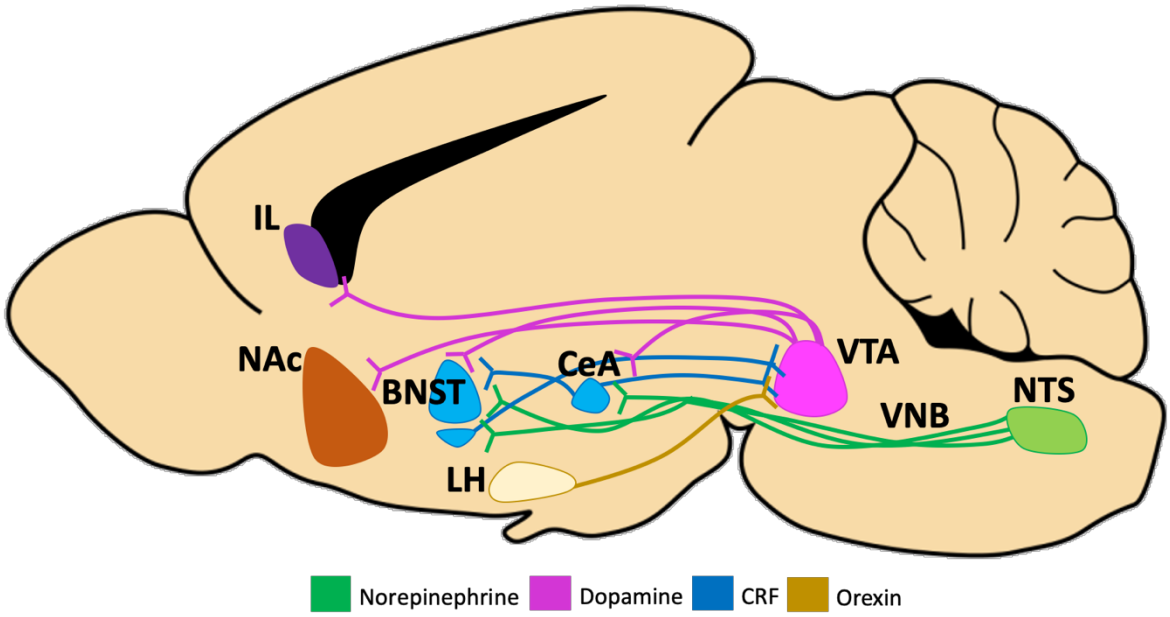

Figure 1. Brain regions and neurotransmitter systems that contribute to stress-induced reinstatement of cocaine seeking behavior. IL = infralimbic cortex, NAc = nucleus accumbens, BNST = bed nucleus of the stria terminalis, CeA = central amygdala, LH = lateral hypothalamus, VTA = ventral tegmental area, VNB = ventral noradrenergic bundle, NTS = nucleus tractus solitarii.

receives ascending inputs from areas involved in stress processing including the extended amygdala (discussed below), and lateral hypothalamus [69,169,170]. The VTA also projects to many areas that are important for drug seeking and stress-induced reinstatement including the NAc, infralimbic and prefrontal cortex, BNST and CeA (**Fig. 1**)[171,172]. VTA dopamine neurons are activated by acute and chronic stressors [173,174]. Stress increases excitatory, and decreases inhibitory, input onto VTA dopaminergic neurons [173]. Elevated excitatory transmission in the VTA is associated with stress-induced reinstatement and blockade of ionotropic glutamate receptors within this region blocks stress-induced reinstatement [175,176]. Inactivation of the VTA through the infusion of a GABA receptor cocktail consisting of muscimol and baclofen, prevents stress-induced reinstatement of SA [177]. Suppressing the output of VTA dopaminergic neurons,

using chemogenetic or pharmacological approaches, also abolishes stress-induced reinstatement [29,171].

Stress increases CRF within the VTA, and in cocaine-experienced rodents, CRF increases VTA glutamatergic input into dopamine neurons (**Fig. 2**)[176]. Intra-VTA application of CRF is sufficient to reinstate cocaine SA in manner similar to stress [178]. Attempts at identifying the precise site of the reinstating actions of CRF in the VTA have yielded conflicting results [176,178].

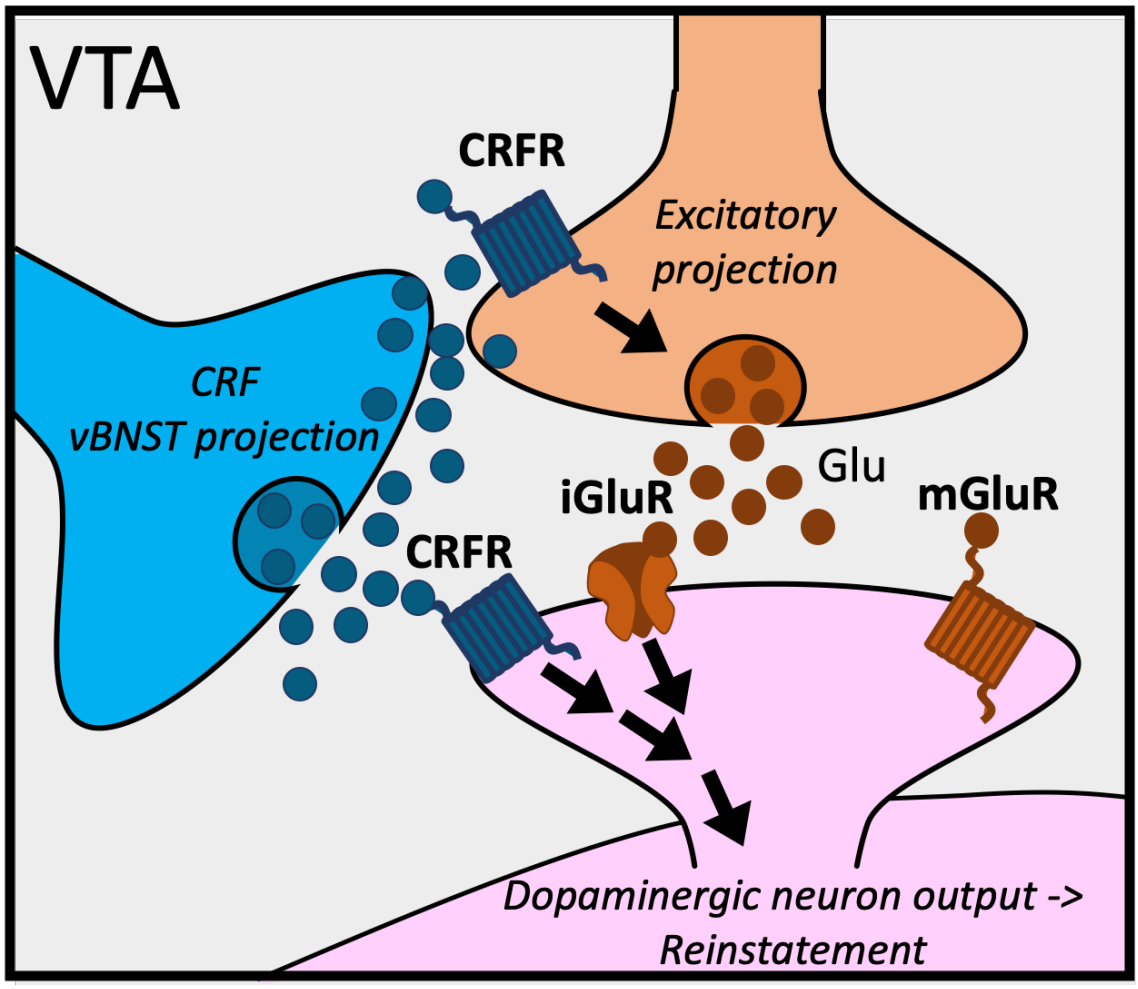

Figure 2. Excitatory signaling pathways in the VTA implicated in stress-induced reinstatement. Stress increases the release of CRF into the VTA from extended amygdala projections. CRF binds to CRF receptors to synergistically increase glutamate release from excitatory terminals and the activity of dopaminergic projecting neurons, leading to reinstatement. VTA = ventral tegmental area, vBNST = ventral bed nucleus of the stria terminalis, CRFR = CRF receptor 1 or 2, Glu = glutamate, iGluR = ionotropic glutamate receptor, mGluR = metabotropic glutamate receptor.

Some studies have reported that bilateral infusions of CRF receptor 1 (CRF-R1) antagonists can block reinstatement, with no involvement of CRF receptor 2 (CRF-R2) [176,178]. While other studies have reported that CRR-R2 receptor is the primary receptor in the VTA mediating the reinstating effects of stress and CRF [179,180]. These discrepancies might be due to differences in methodology and in the pharmacological profiles of the CRF receptor ligands used in these studies, since reports suggest that ligands that activate CRF biding-protein (CRF-BP) in the VTA may be more efficacious at regulating reinstatement [180]. However, non-pharmacological viral knockdown of CRF-R1 abolishes stress-induced cocaine SA suggesting that this receptor might play a more prominent role in this proccess [181].

CRF-R2 induced reinstatement requires $GABA_B$ signaling in the VTA, and slice electrophysiology studies have uncovered that CRF-R2 agonists increase GABA release [179]. In stress-naïve mice, CRF-R2 agonists increase inhibitory postsynaptic currents (IPSCs) in the VTA, however following stress, these compounds increase EPSCs, suggesting a mechanisms by which stress alters CRF system dynamics to increase VTA dopaminergic output and drive reinstatement [179]. Kappa opioid receptors (KORs) also mediate stress-induced reinstatement through actions that converge on VTA GABAergic signaling (**Fig. 3**) [182]. Stress blocks long term potentiation (LTP) of GABAergic synapses onto VTA dopaminergic cells, which disinhibits them and results in increased output through activation of kappa opioid receptors (KORs) in GABA interneurons [182]. Consequently, KOR antagonists block stress-induced deficits in VTA GABA LTP and stress-induced reinstatement of CPP [173,182]. Orexin signaling from lateral hypothalamic terminals into the VTA also regulates GABA dynamics in this region and stress induced reinstatement [169,183]. Orexin receptor 2 or 1 (OX2R and OX1R) agonist infusion into the VTA induces reinstatement, while OX1R antagonist infusion blocks stress-induced reinstatement

[169,183]. Activation of OX2R in VTA dopamine cells induces $G_{\alpha q}$ -dependent retrograde cannabinoid receptor (CB1) activation in GABA neurons, which decreases their output to disinhibit dopamine cells [183]. Taken together, these findings highly the critical role VTA

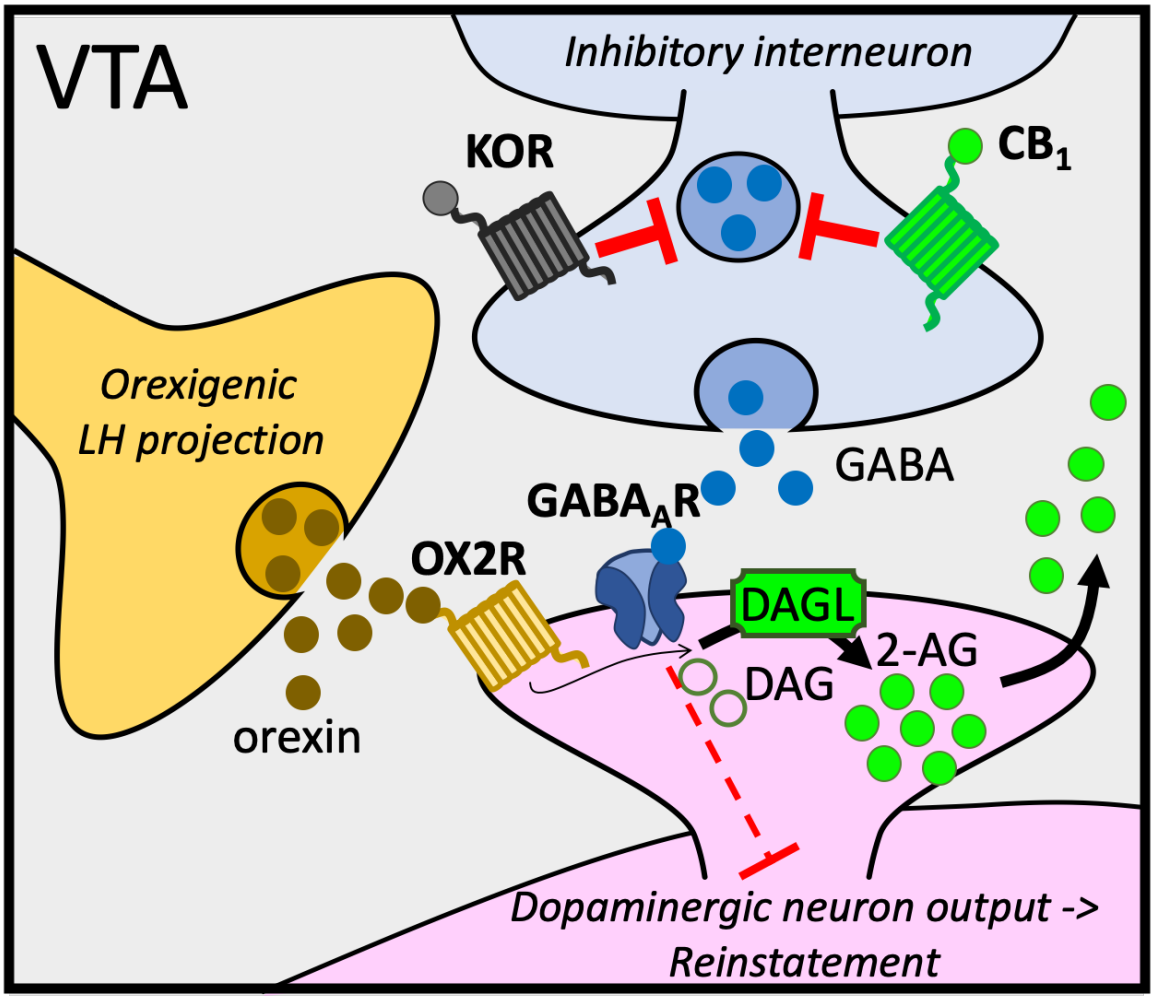

Figure 3. Inhibitory signaling pathways in the VTA implicated in stress-induced reinstatement. Stress increases the release of orexin into the VTA from LH projections, which bind to OX2R in dopamine neurons and induces the production of 2-AG, an endocannabinoid. 2-AG retrogradely binds to CB1 receptors in inhibitory interneurons to decrease GABA release onto dopamine neurons. KORs binds dynorphin or other opioid ligands to decrease GABA release onto VTA dopamine neurons to increase relapse.VTA = ventral tegmental area, LH = lateral hypothalamus, OX2R = orexin receptor 2, $GABA_A$ R = GABA A receptor, DAG = diacylglycerol, DAGL = diacylglycerol lipase, 2-AG = 2-Arachidonoylglycerol, CB_1 = cannabinoid receptor 1, KOR = kappa opioid receptor.

dopaminergic and GABAergic signaling in stress-induced reinstatement of cocaine-associated behaviors (**Fig. 3**).

Extended amygdala

The extended amygdala is composed of the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the nucleus accumbens shell [184]. The CeA and BNST are highly interconnected and show a functional role in stress, anxiety, craving, and fear responses [185–187]. The role of the CeA and BNST in stress-induced reinstatement of drug-seeking behaviors, including cocaine, has been extensively characterized [70,73,165,166,188,189]. Intermittent foot-shock, restraint, and forced swim, stressful manipulations that reinstate cocaine CPP and SA also increase levels of the immediate early gene and proxy marker of cellular activity cFOS in the CeA and BNST [190–192]. Forced swim also increases levels of CREB phosphorylation, another marker of activity, in the CeA [193]. Reversible pharmacological inactivation of the CeA or BNST via intracranial infusions of tetrodotoxin (TTX), a sodium channel blocker, or a $GABA_A$ and $GABA_B$ receptor agonist cocktail (baclofen + muscimol) block stress-induced reinstatement of SA [73,177].

In vivo studies provide a link between NE in the extended amygdala and stress-induced reinstatement. Lesions to the ventral noradrenergic bundle, the main noradrenergic pathway into the BNST, prevents stress-induced reinstatement [73]. Infusions of clonidine into the BNST, block stress-induced reinstatement of SA [194]. Infusions of the α_2-AR antagonist yohimbine into the BNST or CeA mimic stress-induced reinstatement [195]. These findings are limited by the non-specific nature of yohimbine as well as data showing that yohimbine drives reinstatement and BNST activity through non-adrenergic mechanisms that might be dependent on orexin and serotonin signaling [164,196,197]. The more selective α_{2A}-AR antagonist BRL 4448 malate also

reinstates cocaine CPP [198]. BNST or CeA infusions of a cocktail of the β_1- and β_2-AR antagonists betaxolol and ICI-118,551, block stress-induced reinstatement of cocaine SA, suggesting a role for β-AR signaling in the effects of stress on extended amygdala activity and reinstatement [165]. Further studies found that pharmacological blockade of β_2-ARs, but not β_1-ARs, in the ventral BNST is capable of blocking stress-induced reinstatement [69]. Conversely, infusions of the β_2-AR selective agonist clenbuterol, but not the β_1-AR selective agonist dobutamine, induces reinternment of cocaine SA [69].

Extended amygdala CRF has also been implicated in stress-induced reinstatement [73,168,188]. Forced swim increases CRF mRNA in cocaine-experienced rodents [188]. Additionally, infusions of CRF into the BNST but not the CEA, reinstates cocaine SA and CPP in a manner similar to stress [168]. CRF-R1 antagonist infusions into the BNST, but not CeA, block stress-induced reinstatement of cocaine SA and CPP [166]. Using a disconnection approach to inhibit the CeA in one hemisphere and CRF-R1s in the contralateral BNST, researchers found that the CeA to BNST CRF pathway is necessary for stress-induce reinstatement, a finding that clarifies the discrepancy between the effects of CRF manipulations between these two brain regions [199]. Other hemispheric disconnection studies show that β_2-ARs drive reinstatement by increasing the activity of CRF neurons in the ventral BNST that project to the VTA [69]. The upregulating effects of β-ARs on excitatory transmission within the extended amygdala are also dependent on CRF-R1 signaling [200–202]. These findings are in accordance with previous reports of a sequential link between CRF and β_2-AR actions on the extended amygdala and reinstatement (**Fig. 4**)[203]. CRF-R1 antagonists pretreatment blocks NE- and β_2-AR-induced reinstatement but suppressing NE signaling does not block CRF-induced reinstatement [69].

Stress-induced activation of the extended amygdala is regulated by NE release through ascending noradrenergic fibers from the NTS to the BNST and CeA [89,202,204,205]. While the entirety of the extended amygdala is regulated by NE, the ventral BNST is one of the most densely innervated NE targets within the brain, providing a promising anatomic locus for targeted pharmacological manipulations [206]. Through release of NE, stress modulates excitatory and inhibitory neurotransmission into the extended amygdala [201,202]. Within the dorsal BNST, NE induces excitatory and inhibitory responses, while in the ventral BNST and CeA the effects of

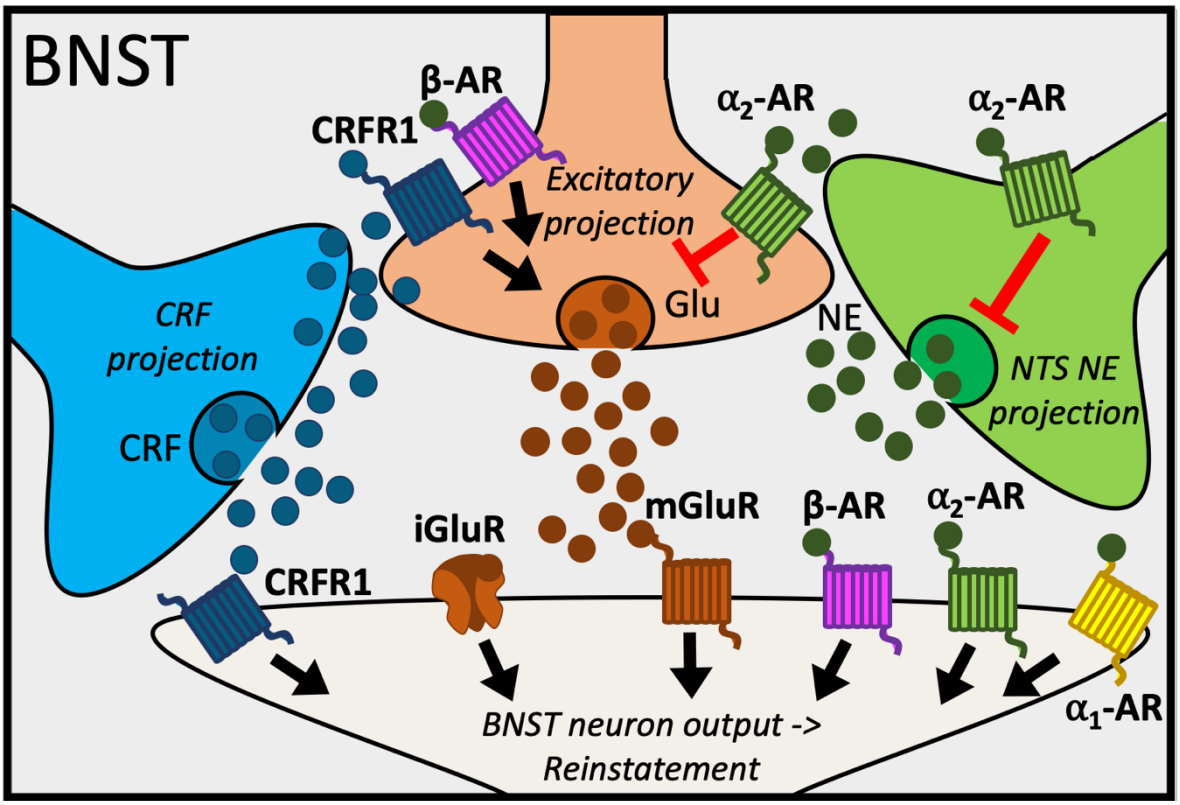

Figure 4. Neurotransmitter signaling pathways in the BNST implicated in stress induced reinstatement. Stress increases the release of glutamate, CRF, and NE into the BNST and acts through pre- and postsynaptic receptors to induce BNST activity and drive reinstatement. CRF = corticotropin releasing factor, CRFR1 = CRF receptor 1, Glu = glutamate, iGluR = ionotropic glutamate receptor, mGluR = metabotropic glutamate receptor, NTS = nucleus tractus solitarii, NE = norepinephrine, α_2-AR = alpha$_2$-adrenergic receptor, α_1-AR = alpha$_1$-adrenergic receptor, β-AR = beta-adrenergic receptor.

adrenergic stimulation are primarily excitatory [202,207]. The diversity in responses is due the large heterogeneity in receptor populations, cell types, and inputs within these regions [208].

In acutely prepared brain slices, β-AR stimulation broadly increases excitatory transmission in the extended amygdala [202,206]. The non-selective β-AR agonist isoproterenol increases spontaneous and evoked excitatory postsynaptic currents (EPSCs) in the BNST and spontaneous EPSCs in the CeA [200,201,206]. Isoproterenol-mediated increases in spontaneous EPSCs within the BNST are β_1-AR-dependent, whereas the potentiation of EPSCs produced by isoproterenol is β_2-AR-dependent [200,206].

Chronic, but not acute applications of NE produce long term depression (LTD) of excitatory transmission within the BNST, which is consistent with $G_{\alpha q}$-mediated mechanisms [209]. Accordingly, NE-mediated LTD in the BNST is blocked by α_1-AR antagonist application [209]. The α_1-AR agonist methoxamine induces LTD in the BNST [209]. α_1-AR-mediated LTD is disrupted in genetic mice models that show increased anxiety- and depression-like phenotypes, suggesting that restoration of BNST α_1-AR-mediated LTD following chronic drug use might be a site of action mediating the anti-reinstatement effects of α_1-AR ligands [209].

The effects of α_{2A}-AR agonists on BNST neuronal activity are complex. α_{2A}-AR are highly expressed in glutamatergic terminals that make axiosommatic connections within the BNST, in intra-BNST cell populations, and NE terminals primarily into the ventral BNST subregion [210,211]. This wide-spread pattern of expression indicated the potential for multi-site regulation of BNST activity (**Fig. 4**). In slice preparations, α_{2A}-AR agonist application decreases BNST excitatory synaptic transmission through input-specific inhibition of excitatory drive from areas such as the PBN, but not the insula [210,212,213]. Chemogenetic mimicry of α_{2A}-AR activation

in the PBN using a $G_{\alpha i}$-coupled designer receptor exclusively activated by designer drugs (G_i-DREADDs) prevents stress-induced increases in BNST cFOS mRNA, particularly in CRF cells [212].

The seemingly paradoxical observation that systemic α_{2A}-AR agonist administration increases cFOS within the BNST suggested that some of the actions of these receptors might be excitatory, but such effects would be masked by the use of electrical stimulation, which presumably induces release from all excitatory terminals, including both guanfacine-inhibited (i.e. PBN) and activated projections [211,214]. The use of the Thy1-COP4 mouse line, which randomly expresses the light gated excitatory tool channelrhodopsin 2 (ChR2), circumvents this limitation because in this line, ChR2s show little co-localization with CGRP, a marker of PBN terminals (Flavin et al., 2014; Harris et al., 2018). In this mouse line, guanfacine potentiates optically-evoked EPCs via a postsynaptic mechanism that is dependent on intra-BNST α_{2A}-AR heteroreceptors and HCN channels [211]. These findings suggest that intra-BNST α_{2A}-ARs play an excitatory role within the BNST. Chemogenetic experiments also provide evidence to this notion, since mimicking intra-BNST α_{2A}-AR heteroreceptor signaling using G_i-DREADDs increases cFOS *ex vivo* and calcium transients *in vivo* [211]. Intra-BNST G_i-DREADD activation also produced anxiety-like responses in the elevated plus maze, consistent with the well-defined role of BNST activity in anxiety [211,215,216].

Given that BNST activity is also critical for stress-induced reinstatement, it would be expected that activation of a $G_{\alpha i}$-coupled signaling in this region, by α_{2A}-AR heteroreceptors or G_i-DREADDs, would be sufficient to drive reinstatement in a manner similar to stress [166]. It is likely that competition between α_{2A}-AR autoreceptors and heteroreceptors within the BNST accounts for some of the divergent responses to α_{2A}-AR agonists reported in the extended

amygdala, as well as the decreased efficacy of these compounds in curving relapse [77,84,85,214,217]. However, the contributions of α_{2A}-AR heteroreceptors to stress-induced reinstatement of cocaine-associated behaviors has not been defined. Overall, the work on this dissertation will address:

Hypothesis: Following stress, elevated levels of NE activate α_{2A}-AR heteroreceptors which increase BNST activity via $G_{\alpha i}$-coupled GPCR signaling to induce reinstatement. Thus, preferentially targeting α_{2A}-AR autoreceptors will decrease NE release, heteroreceptor-dependent activation of the BNST, and stress-induced reinstatement.

Specific Aim 1: Determine the role of α_{2A}-AR heteroreceptors in stress-induced reinstatement of cocaine CPP.

Specific Aim 2: Determine if $G_{\alpha i}$-coupled GPCR signaling in the BNST is sufficient to induce reinstatement of cocaine CPP.

Specific Aim 3: Identify a dose of the α_{2A}-AR agonists guanfacine that does not increase BNST activity and determine if this dose blocks stress-induced reinstatement of cocaine CPP.

Chapter 2

α_{2A}-adrenergic heteroreceptors are required for stress-induced reinstatement of cocaine conditioned place preference

Introduction

Stress is a precipitating factor for craving and relapse in cocaine use disorder (CUD) [162,218,219]; however, there are no FDA-approved medications for the treatment of relapse in CUD. α_{2a}-adrenergic receptor (α_{2a}-AR) agonists inhibit stress-induced reinstatement of operant drug-seeking and conditioned place preference (CPP), animal models useful in the study of stress-induced relapse [71,73,163,194]. In clinical laboratory studies, these compounds have been investigated for stress-induced cocaine craving [58,63], but the application of full α_{2a}-AR agonists for the treatment of CUD has been limited due to adverse effects such as sedation and hypotension [66,220]. Pretreatment with the α_{2a}-AR partial agonist guanfacine reduces stress-induced craving in female and stress-induced sympathetic tone in male CUD patients [79]. Guanfacine also decreases stress-induced craving of nicotine and alcohol in male and female patients, suggesting a potentially broad applicability for the treatment of stress-induced drug use [79,80]. Although guanfacine decreases stress, craving, and withdrawal symptoms in clinical trials, it has not been reported to reduce relapse rates [84,85].

The mechanisms underlying the anti-drug craving effects of guanfacine are unknown, but it has been suggested that guanfacine blunts stress responses through its actions at presynaptic G_i protein-coupled (G_i-coupled) autoreceptors, which decrease norepinephrine (NE) release [221,222]. Pre-clinical studies show that at low doses, guanfacine blocks stress-induced activation of the extended amygdala, a group of brain regions that contains the central amygdala and bed nucleus of the stria terminalis (BNST)[217]. NE-signaling within the BNST is critical for stress-induced reinstatement of

CPP, and previous research suggests that α_{2a}-AR autoreceptors might suppress stress-induced reinstatement by decreasing NE-mediated activation of BNST beta-adrenergic receptors [69,70,165,188].

Guanfacine also activates postsynaptic α_{2a}-AR heteroreceptors [140]. α_{2a}-AR heteroreceptors are expressed in non-adrenergic cells and regulate many of the pharmacological and physiological effects of α_{2a}-AR agonists, including analgesia, sedation, and improvements in cognition [98]. We previously reported that within the dorsal BNST (dBNST), a high dose of guanfacine increased cFOS, a proxy marker of cellular activity, via activation of α_{2a}-AR heteroreceptors and the subsequent blockade of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [94,140,211]. Furthermore, functionally mimicking intra-dBNST α_{2a}-AR heteroreceptor signaling using virally expressed G_i-coupled designer receptors exclusively activated by designer drug (G_i-DREADDs) also increased dBNST cFOS proportionally to high-dose guanfacine, and produced anxiety-like responses [211]. Given the prominent role of BNST activity in stress-induced reinstatement of cocaine seeking behavior, and the interest in guanfacine as a potential treatment for CUD [63], understanding how α_2-AR heteroreceptor signaling may regulate these behaviors is imperative. In this study, we demonstrate that α_2-AR heteroreceptors are necessary for stress-induced reinstatement of CPP, and that mimicking their signaling in the dBNST using G_i-coupled DREADDs is sufficient to induce reinstatement. Finally, we show that a low dose of guanfacine that does not increase dBNST activity blocks stress-induced reinstatement.

Methods and Materials

Reagents

Cocaine hydrochloride was obtained from Sigma Aldrich (#C5776, St. Louis, MO) and RTI international (SWM-2-9041-001-23, Triangle Research Park, NC) and dissolved in 0.9% sterile saline (Hospira, Lake Forest, IL) which was use as vehicle for all *in vivo* injection experiments.

Cocaine was administered at a final volume of 10 ml/g. Clozapine-N-oxide (CNO) was obtained from Sigma Aldrich (#C0832, St. Louis, MO) and diluted in sterile saline. Metacam was obtained from Patterson Veterinary (#07-845-6986; Greeley, CO) and diluted in sterile saline. Guanfacine hydrochloride was obtained from Fisher scientific (#1030; Hampton, NH) and diluted in 0.01 mM ice cold PBS. Primary antibodies included rabbit anti-cfos (Millipore; abe457; RRID: AB_2631318) at 1:2000 dilution and mouse anti-NeuN (Millipore mab377 clone A60; RRID: AB_2298772) at 1:1000 dilution. Secondary antibodies included Cy2 donkey anti-rabbit (711-225-152; RRID: AB_2340612) at 1:500 dilution and Cy3 donkey anti-mouse (715-165-150; RRID: AB_2340813) at 1:500 dilution. Adeno-associated viral (AAV) vectors AAV5-CaMKIIα-hM4Di:mcherry (G_i-DREADD; UNC Viral Vector Core) and AAV5-CaMKIIα-mcherry (mcherry; UNC Viral Vector Core) were used in CNO-induced reinstatement experiments.

Animals

Male and female wild-type (WT), α_{2a}-AR knockout (KO), and KO mice re-expressing α_{2a}-ARs under the DBH promoter (HeteroKO), were bred in house and maintained on a C57BL/6J background were used [102,211]. For chemogenetic studies, male C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were delivered at 6 or 7 weeks of age and acclimated for at least one week before surgical manipulations. For all conditioning experiments, mice were singly-housed at least 2 weeks prior to experiments. Male and female C57BL/6J mice were used for immunohistochemical experiments and were group-housed with 2-5 mice per cage. All procedures were approved by the Vanderbilt University animal care and use committee.

Behavior

Apparatus- Six mouse open field arenas (ENV-510S) with two-sided place preference insets (ENV-512) from Med Associates, Inc. were used in this study. The place preference inserts consisted of a clear polyvinyl chloride box 28. 7 x 28.7 x 20.6 cm partitioned into two sides. One side contained a floor with stainless-steel rods 0.318 x 14. 61 cm, spaced 0.76 cm apart (ENV-3013BR), while the opposite side contained a stainless-steel mesh floor with 0.08 cm rods spaced 0.64 cm apart. In the unmodified apparatus, mice spend more time in the side containing the mesh floor (data not shown). In order to minimize this bias, the light bulb above the un-preferred side was turned off, the side was lined with a stripped black and white wallpaper and the rod floor was covered with a stainless-steel plate.

Conditioning- On day 1, mice were randomly placed in one of the sides and allowed to freely move between the compartments for 20 minutes. To minimize pre-conditioning bias, mice that spent more than 65% of the session time on one side were excluded from the study (n = 7). For each mouse, a side was randomly assigned as the cocaine-paired side. On days 2, 4, 6, and 8, mice were injected with cocaine (15 mg/kg, i.p.) and immediately confined to the paired side for 20 minutes. On days 3, 5, 7, and 9, mice were injected with saline and immediately confined to the unpaired side for 20 minutes. During day 10, mice underwent post-conditioning CPP testing. Mice were placed in the same side that they were originally placed on day 1 and allowed to freely move between the sides for 20 minutes. CPP was defined as an increase in time spent on the cocaine-paired side between the post- and pre-conditioning sessions.

Extinction- Extinction training was conducted daily, starting 24 hours after the post-conditioning CPP test session. During each extinction training session, mice were placed in the apparatus and allowed to move freely between the sides for 20 minutes. Mice underwent extinction training until they met the extinction criterion. This criterion was defined as a decrease in the time

spent on the cocaine-paired side equal or lower to 50% of the difference between the time spent on this side during the post- and pre-conditioning test sessions. Mice that did not reach the extinction criterion within 10 days of extinction training were excluded from experiments (n = 5).

Reinstatement Testing- Mice underwent reinstatement testing 24 hours after reaching the extinction criterion. For stress-induced reinstatement testing, mice underwent forced swim stress in a beaker of warm water (22-26^{o} c) for 6 minutes, towel-dried for 30 seconds, and placed in the CPP apparatus for 20 minutes. For pharmacological blockade studies, mice were injected with guanfacine (0.15 mg/kg, i.p.) 30 minutes prior to forced swim stress. For CNO-induced reinstatement testing, mice received CNO injections (3mg/kg, i.p.) 30 minutes prior to being placed in the CPP apparatus.

Mock saline-saline CPP acquisition, extinction, and stress bias testing- A separate group of WT, KO, and heteroKO mice were subjected to mock saline CPP training and testing. For each mouse, one side of the chamber was randomly selected to be the mock-paired side. On day 1, mice were allowed to explore the apparatus as described above. During days 2 to 9, mice were injected with saline and immediately restricted to one side of the apparatus for 20 minutes. On days 2, 4, 6, and 8 mice were placed on the mock saline-paired side. On days 3, 5, 7, and 9, mice were placed on the mock unpaired side. On day 10, mice underwent CPP testing as described above. Beginning 24 hour following CPP testing, mice underwent extinction training as described above. Since saline-treated mice did not acquired CPP, they did not have an extinction criterion therefore, each saline-treated mouse underwent as many extinction training sessions as a sex-, genotype-, and age-matched cocaine-treated mouse. 24 hours after the last day of extinction training, mice were subjected to stress-induced reinstatement testing as described above.

Stereotaxic Surgery

Mice were anesthetized with isoflurane (initial dose = 3%; maintenance dose = 1.5%) and injected intracranially with either the mcherry or $hM4D_i$ constructs. Bilateral microinjections (300 nL) were made into the dorsolateral BNSTs using previously published coordinates (AP: 0.14, ML: +/- 0.88, DV: -4.24) at a 15.03° angle [211]. Mice were treated with daily 5 mg/kg injections of metacam for 48 hours following surgery. Mice were allowed to recover for at least 3 weeks prior to behavioral testing.

Fluorescent immunohistochemistry

The cFOS upregulation assay was conducted as previously described [211,212]. WT mice received i.p. injections of either vehicle (saline) or guanfacine (0.15 mg/kg or 1 mg/kg). 90 minutes after the injection, mice were transcardially perfused and brains were harvested. Extracted brains were submerged in 4% PFA for 24 hours at 4°C and cryoprotected in 30% sucrose in PBS for a minimum of two days. Coronal sections were cut on a cryostat (Leica, CM3050S) in Optimal Cutting Temperature (OCT) solution (VWR, Radnor, PA) at a thickness of 40 μm and stored in PBS at 4°C until immunological staining and viral assessments.

For cFOS staining, coronal sections were washed with PBS (4x10 min), permeabilized with 0.5% Triton X-100 in PBS (1 hour), and blocked with 5% Normal Donkey Serum (NDS) and 0.5% Bovine Serum Albumin (BSA) for 1 hour at room temperature (RT). Primary antibodies were applied in blocking solution and slices were incubated in primary antibody (rabbit anti-cFOS and mouse anti-NeuN) for 24 hours at RT, washed in PBS (4x10 min), and incubated in combinations of secondary antibodies (Cy2 donkey anti-rabbit and Cy3 donkey anti-mouse) in 0.1% Triton X-100 in PBS for 24 hours at 4°C. Slices were washed in PBS (4x10 min), mounted on Fisher plus slides (Fisher Scientific), and coverslipped with PolyAquamount when dry. All images were obtained with a Zeiss LSM 710 scanning confocal microscope using

either a 20X/0.80 N.A. Plan-Apochromat, 40X/1.30 N.A. C Plan-Apochromat Oil, or 63X/1.40 N.A. Plan-Apochromat Oil objective lens. Excitation/emission wavelengths (nm) for each fluorophore were 448/521.5 (Cy2, cFOS) and 561.0/610.8 (Cy3, mcherry for chemogentic confirmation). The same acquisition parameters and alterations to brightness and contrast in ImageJ were used across all images within an experiment. Cells were manually counted using ImageJ by a blinded researcher. No overt differences were observed between sub-nuclei of the dBNST so all numbers are reported as a single averaged value for each dBNST and then averaged for each animal.

For chemogenetic experiments, injection and viral spread were verified following behavioral testing. Tissue was mounted onto slices with PolyAquamount (Polysciences, Warrington, PA), coverslipped and evaluated by a blinded researcher. Known sites that project to the BNST were also assessed to verify a lack of retrograde expression of the AAV5 construct.

Statistics

Data are represented as means, means ± SEM, or medians. All statistics were run using Prism 8 (GraphPad, La Jolla, CA). Differences between groups were assessed using t-tests, one-way, or 2-way repeated measures ANOVAs. Differences in standard deviation between groups were assessed using the Brown-Forsythe test, when significant differences in standard deviation were found using this test, they are reported in the text. Significance was set at $\alpha=0.05$. When significant main effects were obtained using ANOVA testing, appropriate post-hoc comparisons between groups were performed.

RESULTS

α_{2a}-AR full or heteroreceptor deletion does not impact the acquisition or extinction of cocaine CPP.

Consistent with previous studies [196,197], we determined that conditioning increased the amount of time WT, KO, and HeteroKO mice spent on the cocaine-paired side during the post-conditioning session, with a main effect of conditioning (pre-conditioning test session [PRE] vs post-conditioning test session [POST], ($F_{2, 30}$= 128.7, $p < .0001$; two-way RM ANOVA), and a trend towards a main effect of genotype (WT, KO, or HeteroKO), ($F_{2, 30} = 2.95$, $p = .057$) that is likely due to the HeteroKO mice spending less time in the cocaine-paired side when compared to WTs and KOs (in sec: 819 +/-113 for WT, 821 +/- 138 for KO, and 723 +/- 59 for HeteroKO). The Genotype x Conditioning interaction was not significant ($F_{2, 30} = .99$, $p = .38$) (**Fig 5. C**). We found no between-genotype differences in the increase in time on the paired side following conditioning, represented by the preference score (one-way ANOVA), or differences in locomotion during the post-conditioning session (two-way RM ANOVA) (**Fig 5. D-E**). Next, we determined the impact of full or heteroreceptor deletion on the extinction of cocaine CPP (see Materials and Methods, **Fig 5. F**). We found no differences in the latency to reach the extinction criterion between groups (one-way ANOVA; in days: 3 +/- 1 for WT, KO, and HeteroKO) (**Fig 4. 5**). Taken together, these findings suggest that α_{2a}-AR auto- and heteroreceptors do not play a role in the acquisition or extinction of cocaine CPP.

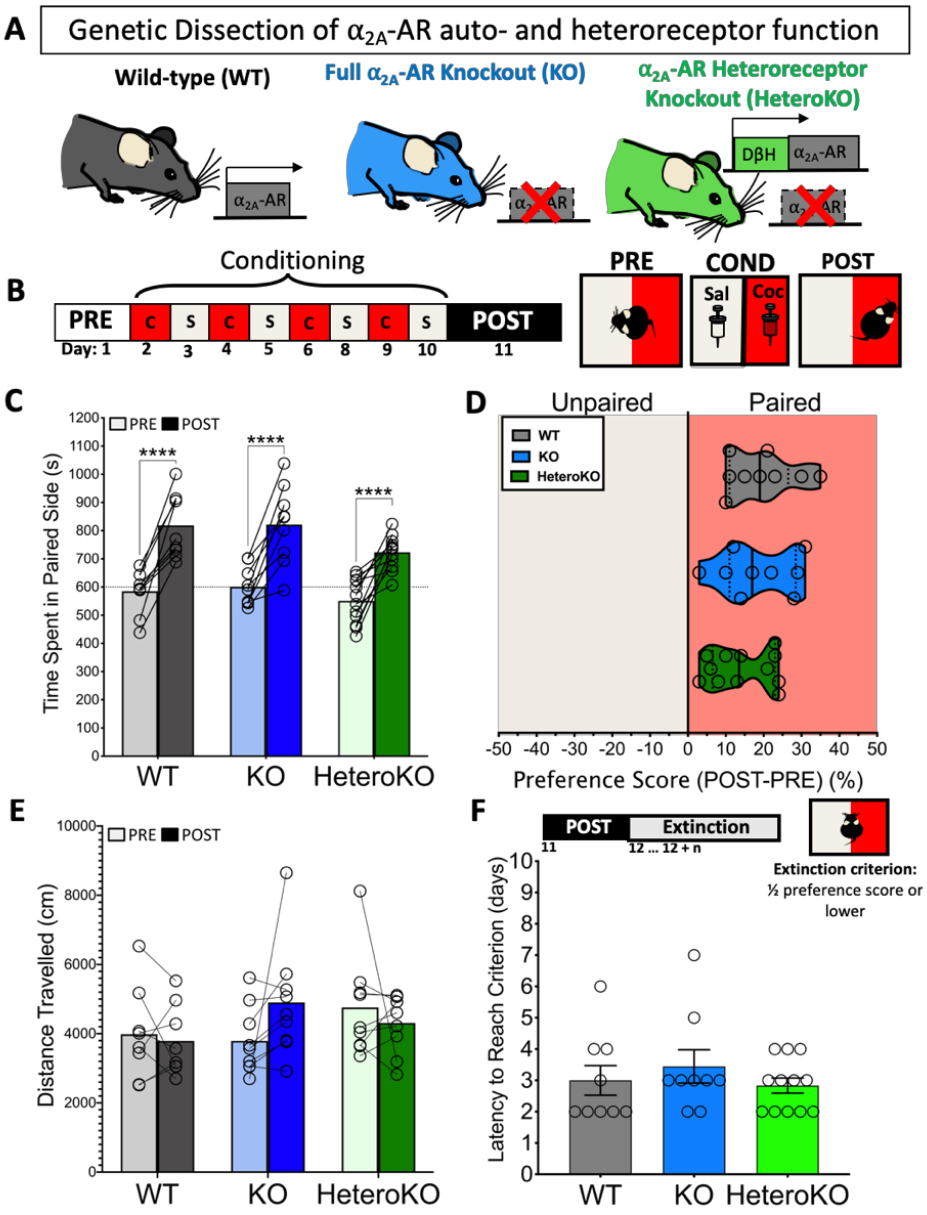

Figure 5. Full or heteroreceptor α_{2A}-AR KO does not disrupt the acquisition or extinction of cocaine CPP. A. Genetic model used to determine relative α_{2A}-AR auto- and heteroreceptor function. **B.** Timeline and schematic of conditioned place preference (CPP) procedure. **C.** Conditioning increased the amount of time mice from all genotypes spent on the paired side when compared to the pre-conditioning session. Data displayed as means with superimposed individual points. **D.** There was no difference between genotypes in the preference scores (% change in time spent on the paired side from the pre- and post-conditioning sessions). Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **E.** There were no intra-genotype differences in the distance travelled in the CPP apparatus during the pre- and post-conditioning sessions. **F.** There were no inter-genotype differences in the latency to reach the extinction criteria. Data displayed as means plus individual points, or means and individual data points +/- SEM (**E**) (WT n = 9, KO n = 8, HeteroKO n = 12) (****p < .0001) (PRE = pre-conditioning session, POST = post-conditioning session).

α_{2a}-AR full or heteroreceptor deletion disrupts stress-induced reinstatement of CPP

We next determined the impact of α_{2a}-AR KO or HeteroKO on stress-induced reinstatement of cocaine CPP. 24 hours after reaching the extinction criterion, WT, KO, and HeteroKO mice underwent stress-induced reinstatement testing (**Fig 6. A**). There was a main effect of stress (extinction session [EXT] vs stress-induced reinstatement session [STRESS]) but no significant genotype x stress interaction (stress $F_{1, 28} = 15.01$, $p = .0006$ interaction $F_{2, 28} = 2.31$, $p = .11$, 2-way RM ANOVA). Sidak's multiple comparisons post-hoc test revealed that stress only significantly increased time in the paired side in WT mice when compared to the last day of extinction (EXT vs STRESS: WT $p < .01$, KO $p = .78$, HeteroKO $p = .15$) (**Fig 6. B**). Stress induced a significant

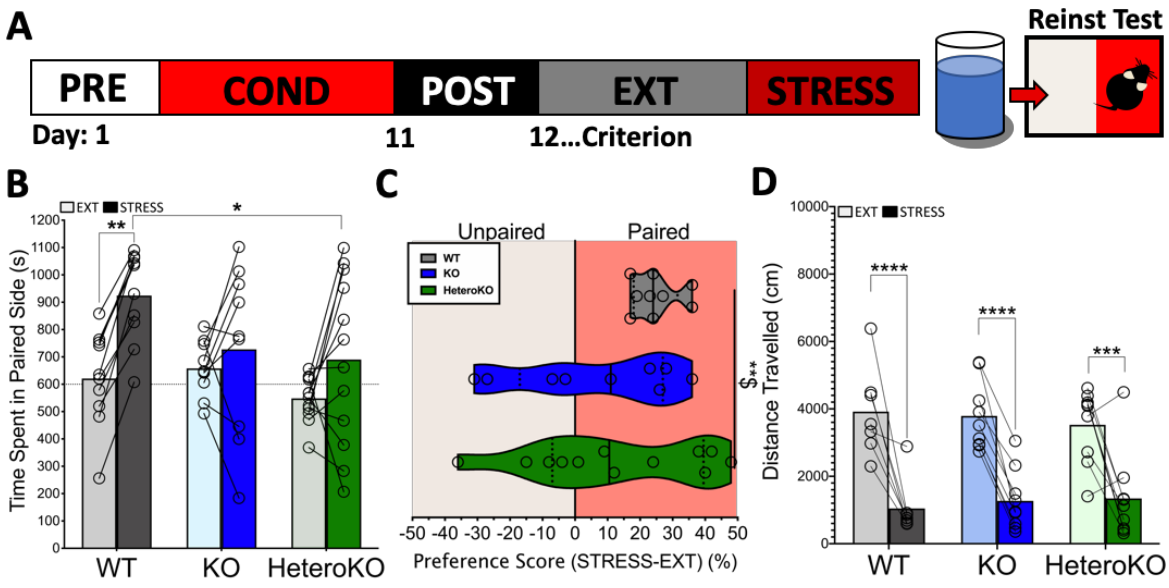

Figure 6. Full or heteroreceptor α_{2A}-AR KO disrupts stress-induced reinstatement of cocaine CPP. A. Timeline and schematic of stress-induced reinstatement of CPP procedure. **B.** during the stress test session (STRESS), 6 minutes of forced swim stress increased the time WT mice spent on the paired side compared to the last day of extinction (EXT) and this increase was different from heteroKO mice. Data displayed as means plus individual points. **C.** There was a difference between genotypes in the variance of preference scores (Brown-Forsythe test for differences in SD, **$ < .01). Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **D.** There was an intra-genotype difference in distance travelled in the CPP apparatus during EXT and STRESS. Data displayed as means plus individual points (WT n = 9, KO n = 8, HeteroKO n = 12) (**p < .001, ****p < .0001) (EXT = last extinction session, STRESS = stress-induced reinstatement session).

difference in the variance of the preference score of WT, KO, and HeteroKO mice (Brown Forsythe test; $F_{2, 27}$= 5.67, p < .001) that was driven by a stress-induced increase in standard deviation (SD) in KO and HeteroKO mice (WT SD = 7.21, KO SD = 24.51, HeteroKO SD = 26.31) (**Fig 5. C**). There was a main effect of stress on locomotion, but no effect of genotype, or the interaction (stress $F_{1, 22}$ = 76.20, p < .0001, genotype $F_{2, 22}$ = .03, p = .97, interaction $F_{2,22}$ = .45, p = .64; 2-way RM ANOVA; Sidak's multiple comparisons post-hoc test, EXT vs STRESS: WT p < .0001 KO p < .0001, HeteroKO p = .0005) (**Fig 6. D**). These findings suggest that stress reinstates cocaine CPP in WT mice, and that this reinstatement might be disrupted in KO mice. Furthermore, this disruption is not ameliorated by re-expression of α_{2a}-AR autoreceptors in heteroKO mice.

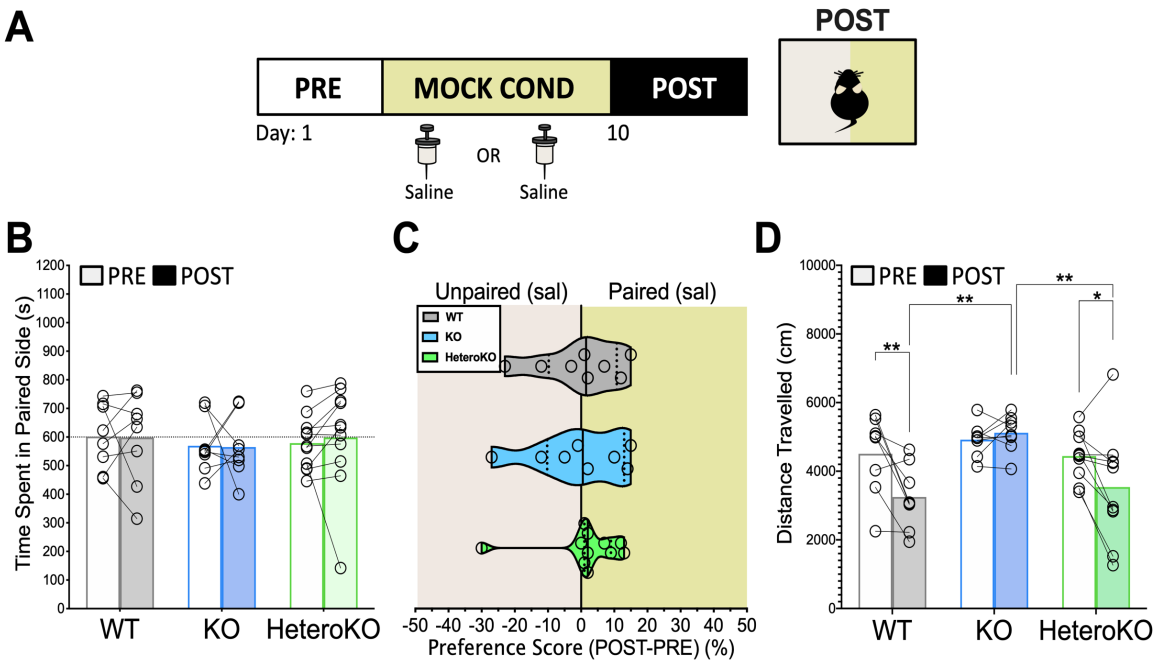

Figure 7. Full or heteroreceptor deletion of α_{2A}-ARs does not induce chamber biases in cocaine-naïve mice but prevents mock conditioning-induced decreases in locomotion in full KOs. A. Timeline and schematic of mock conditioning. Mice were injected with saline before being confined to a side of the chamber. Side placement was alternated daily. **B and C.** Mock conditioning did not change time in a randomly paired side or the preference score across genotypes in cocaine-naïve mice. **D.** WT and heteroKO mice show a mock conditioning-induced decrease in locomotion which do not occur in full KO (WT n = 8, KO n = 8, HeteroKO n = 11). Data displayed as means with individual data points (**B. and D.**) or medians (solid lines) and quartiles (dashed lines with individual data points (**C.**) (*p < .05, **p < .01).

α_{2a}-AR full or heteroreceptor deletion does not produce stress-dependent biases in side occupancy in cocaine-naïve mice

The above findings suggest that, by suppressing locomotion, stress may spuriously increase time on one side of the chamber in a subset of KO and HeteroKO mice, independent of the retrieval of cocaine CPP. We tested this hypothesis by conducting a mock saline CPP experiment followed by stress-induced reinstatement. Mice were subjected to a CPP, extinction, and reinstatement procedure as described above, but only received injections of saline during the conditioning stage (mock conditioning) (**Fig 6. A**). Mock conditioning, genotype, or the mock conditioning x genotype interaction, did not increase time in a randomly-paired side (two-way RM ANOVA), or alter the preference score of WT, KO, or HeteroKO mice (one-way ANOVA) (**Fig 7. B-C**). Interestingly, mock conditioning decreased locomotion in WT and HeteroKO mice, but failed to do so in full KO mice (in cm: 3247 +/-937 for WT, 5115 +/- 537 for KO, and 3537 +/-1618 for HeteroKO). This decrease in locomotion was statistically significant, with main effects for both the genotype and mock conditioning factors as well as the interaction (mock conditioning $F_{1, 27} = 4.12$, $p = .017$, genotype $F_{2, 27} = 11.54$, $p = .029$, interaction $F_{2,27} = 4.88$ $p < .01$; two-way RM ANOVA, Sidak's multiple comparisons test, PRE vs POST: WT $p < .01$, KO $p = .92$, HeteroKO $p < .05$) (**Fig 7. D**). The higher level of locomotion in KO mice following repeated exposure to the chamber is consistent with previous findings suggesting that autoreceptors play a role in suppressing spontaneous hyper-locomotion [102].

24 hours after the last mock extinction training session, mice were subjected to 6 minutes of forced swim stress and placed in the CPP apparatus (**Fig 8. A)**. Cocaine-naïve mice did not show a significant change in preference for a randomly-paired side of the chamber after stress (two-way RM

ANOVA), and we found no inter-genotype differences in preference score of the cocaine-naïve WT, KO, and HeteroKO mice (one-way ANOVA) (**Fig 8. B-C**). Interestingly, stress increased the SD of the preference score across genotypes when compared to the mock post-conditioning test session (26.65 vs 12.56 for WT, 21.76 vs 14.25 in KO, and 19 vs 11.51 for HeteroKO). This increase in SD demonstrates an enhanced variability in how much time mice spent on each side of the chamber. KO mice showed increased locomotion during the last day of mock extinction which was significantly different from WT, with main effects of genotype and stress but no interaction (genotype $F_{2,20} = 3.77$, $p < .05$, stress $F_{1,20} = 68$, $p < .0001$, interaction $F_{2,20} = 1.71$, $p = .205$, two-way ANOVA; Sidak's multiple comparisons test, EXT vs STRESS: WT $p < .05$, KO $p < .0001$, HeteroKO $p < .01$).

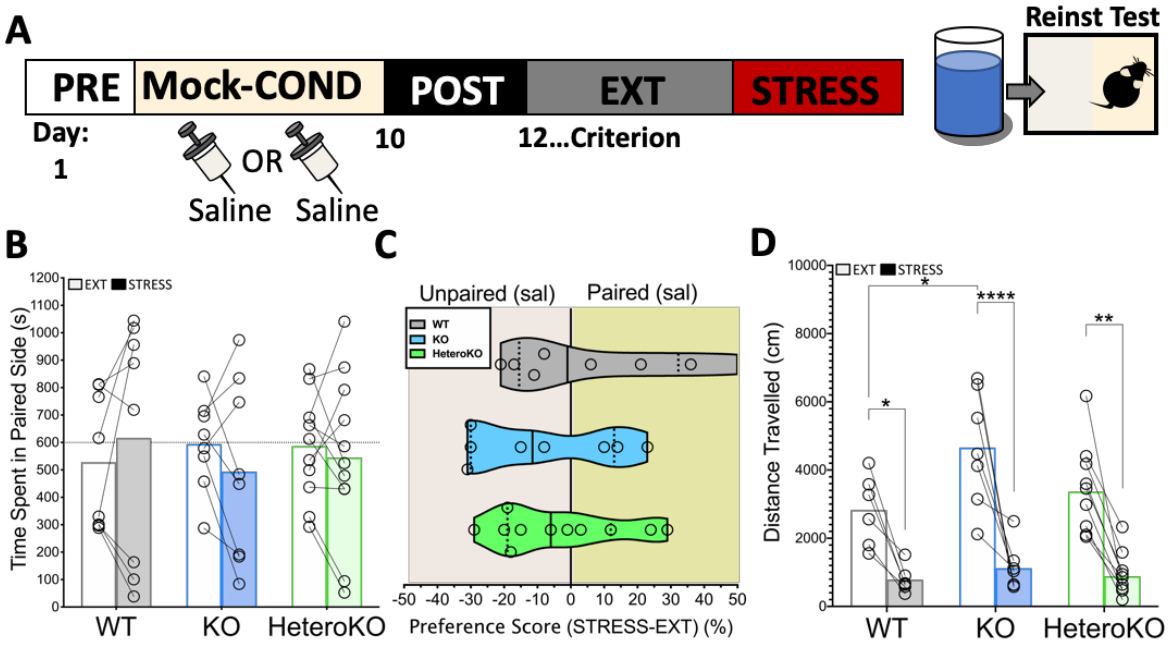

Figure 8. Full or heteroreceptor deletion of α_{2A}-ARs does not induce stress-dependent biases in side occupancy in cocaine-naïve mice. A. Timeline and schematic of mock conditioning, extinction, and stress-induced reinstatement test. **B.** Stress did not change time in a randomly paired side across genotypes in cocaine-naïve mice. **C.** Stress did not produce differences in the preference score between genotypes. **D.** Stress suppressed locomotion across genotypes and there was a difference in locomotion between WT and KO mice during the last day of mock extinction (EXT) (WT n = 8, KO n = 8, HeteroKO n = 11). Data displayed as means with individual data points (B and C) or medians (solid lines) and quartiles (dashed lines with individual data points (C and G) (*$p < .05$, **$p < .01$, ****$p < .0001$) (EXT = last extinction session, STRESS = stress-induced reinstatement session).

However, mice across all genotypes showed a stress-induced suppression in locomotion during the mock reinstatement test with a main effect of stress ($F_{1, 20} = 68$, $p < .0001$; two-way RM ANOVA). (**Fig 8. D**). Taken together, these findings indicate that stress suppresses locomotion and increases variability in side occupancy in cocaine-naïve mice and suggests that variability increases due to decreased movement between sides of the CPP chamber.

Activation of dBNST G_i-coupled GPCR signaling reinstates cocaine CPP

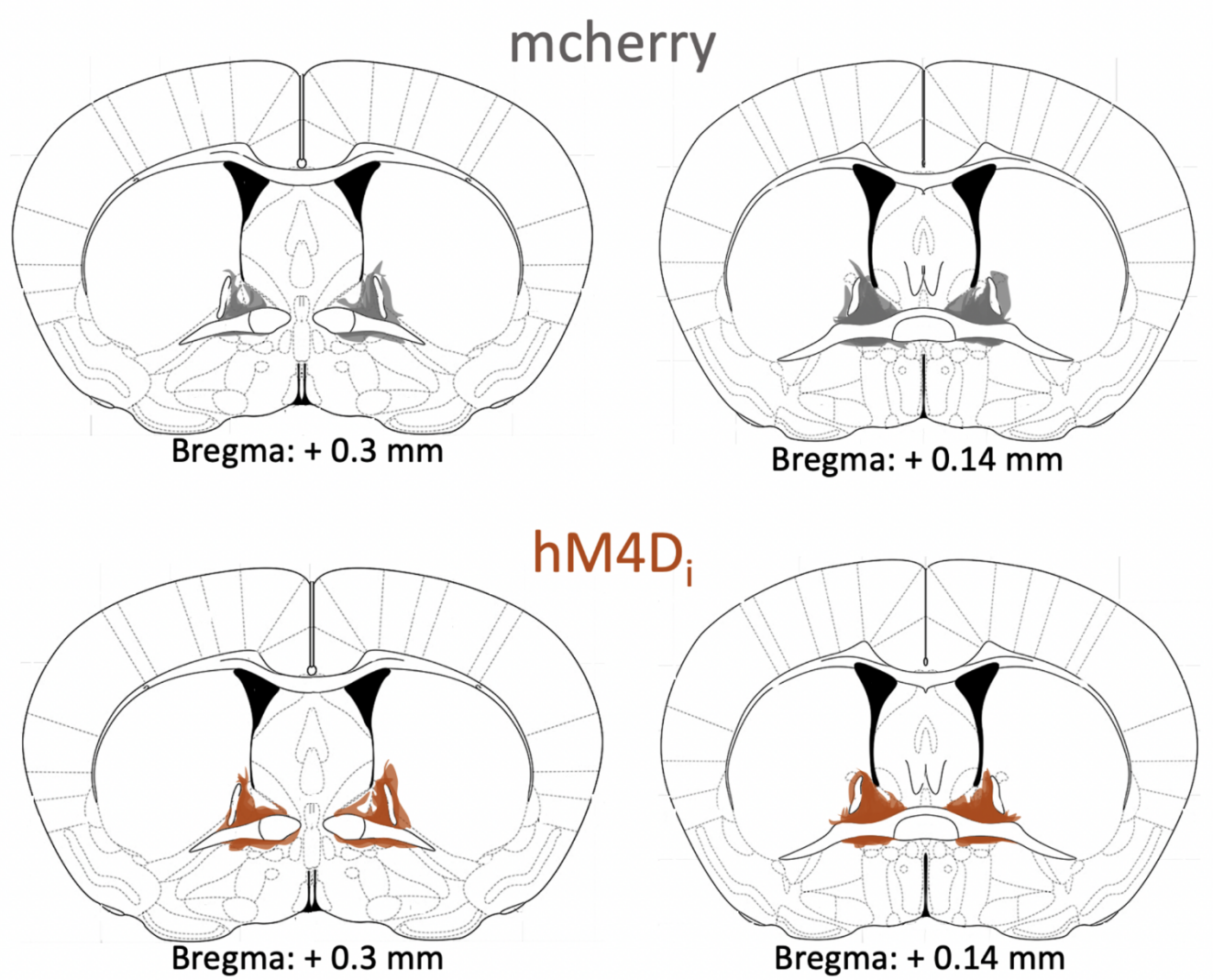

Figure 9. Anatomical localization of virus expression.

We previously reported that α_{2a}-AR heteroreceptors mediate increases in dBNST activity produced by α_{2a}-AR agonists such as guanfacine, as measured by cFOS upregulation [211]. Due to the fact that α_{2a}-AR receptors are G_i-coupled GPCRs, we previously employed a G_i-coupled DREADD strategy to mimic α_{2a}-AR heteroreceptor signaling and found hM4D$_i$, expressed under the CaMKIIα promoter, activation is both sufficient to increase dBNST cFOS in a similar proportion of cells as guanfacine and occlude guanfacine-induced cFOS upregulation, suggesting the recruitment of an overlapping populations of neurons [211]. Due to the prominent role of the BNST in stress-induced reinstatement [166,224], we investigated whether hM4Di enhancement of dBNST activity would be

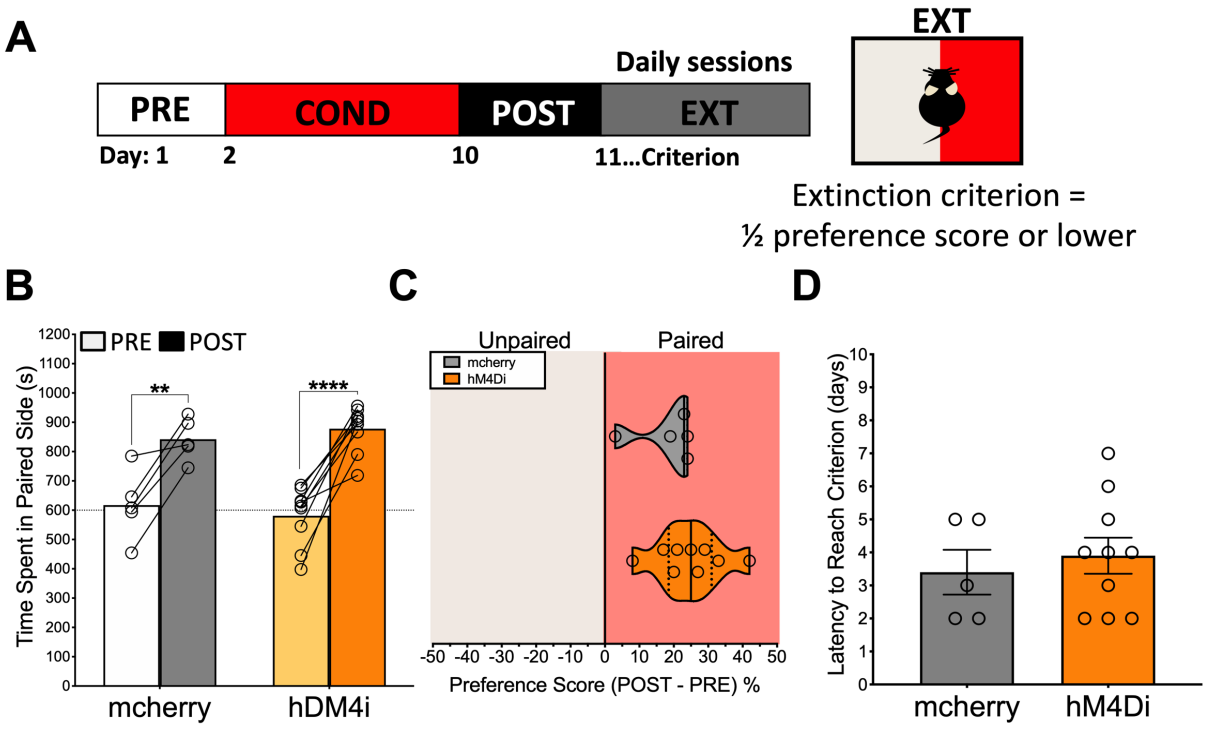

Figure 10. mcherry or mcherry-tagged hM4D$_i$ expression does not impact acquisition or extinction of cocaine CPP. A. Timeline and schematic of CPP and extinction paradigm. **B.** Conditioning increased the time spent in the paired side in mcherry- and hM4D$_i$-expressing mice. Data displayed as means and individual data points. **C.** There was no difference between the preference score of mcherry and hM4D$_i$-expressing mice. Data displayed as medians (solid lines) and quartiles (dashed lines). **D.** There was not difference in the latency to reach the extinction criterion between mcherry and hM4D$_i$-expressing mice. Data displayed as means with individual data points (mcherry n = 5, hM4D$_i$ n = 9) (**p < .01, ****p < .0001).

sufficient to reinstate cocaine CPP. C57BL/6J mice were micro-injected in the dBNST with either AAV5-CaMKIIα-mcherry or AAV5-CaMKIIα-hM4Di:mcherry, which produced robust expression in this region (**Fig 9., Fig 11. B-C**). Following 4 weeks of recovery, mice underwent CPP training, testing, and extinction as described above (**Fig 9. A**).

Conditioning increased time in the paired side for both mcherry- and $hM4D_i$-expressing mice with a main effect of conditioning, but no effects of virus (vs $hM4D_i$) or the interaction (conditioning $F_{1, 12} = 66.28$, $p < .0001$, virus $F_{1,12} = .00$, $p = .996$, interaction $F_{1,12} = 1.26$, $p = .282$; two-way RM ANOVA; Sidak's multiple comparisons test, PRE vs POST: mcherry $p < .01$, $hM4D_i$ $p < .0001$) (**Fig 10. B**). We also found no inter-condition differences in preference score (unpaired t-test, $p = .275$) (**Fig 10. C**), or latency to reach the extinction criterion (unpaired t-test, $P = .593$) (**Fig 10. D**). 24 hours after reaching the extinction criterion, mcherry- and $hM4D_i$-expressing mice were injected with CNO (3 mg/kg, i.p.) as previously described [211] and following a 30-minute period, mice underwent reinstatement testing (**Fig 11. A**). CNO treatment significantly increased the time $hM4D_i$-expressing mice spent on the paired side when compared to the last day of extinction training, but failed to do so in mcherry-expressing mice (**Fig 11. D**). There was a significant main effect for CNO (EXT vs CNO sessions) and a trend towards significance for the interaction, but no effect of virus (CNO $F_{1, 11} = 8$, $p < .05$, interaction $F_{1, 11} = 4.8$, $p = .0506$; virus $F_{1,11} = 1.43$, $p = .25$, two-way RM ANOVA, Sidak's multiple comparisons test, EXT vs CNO: mcherry, $p = .9$, $hM4D_i$ $p < .01$). There was a significant difference between the preference score of $hM4D_i$- and mcherry-expressing mice (unpaired t-test, $t_{2,11} = 2.239$, $p < .05$) (**Fig 11. E**). CNO-treatment did not alter locomotion in $hM4D_i$- or mcherry-expressing mice (two-way RM ANOVA) (**Fig 11. F**). Further, we found that in an additional group of mice in which the location of mcherry or $hM4D_i$ was off target (**Fig 12. A**), CNO did not induce

reinstatement, or alter locomotion (**Fig 12. B-D**). Taken together, these findings suggest that engagement of G_i-GPCR signaling in the dBNST is sufficient to reinstate cocaine CPP .

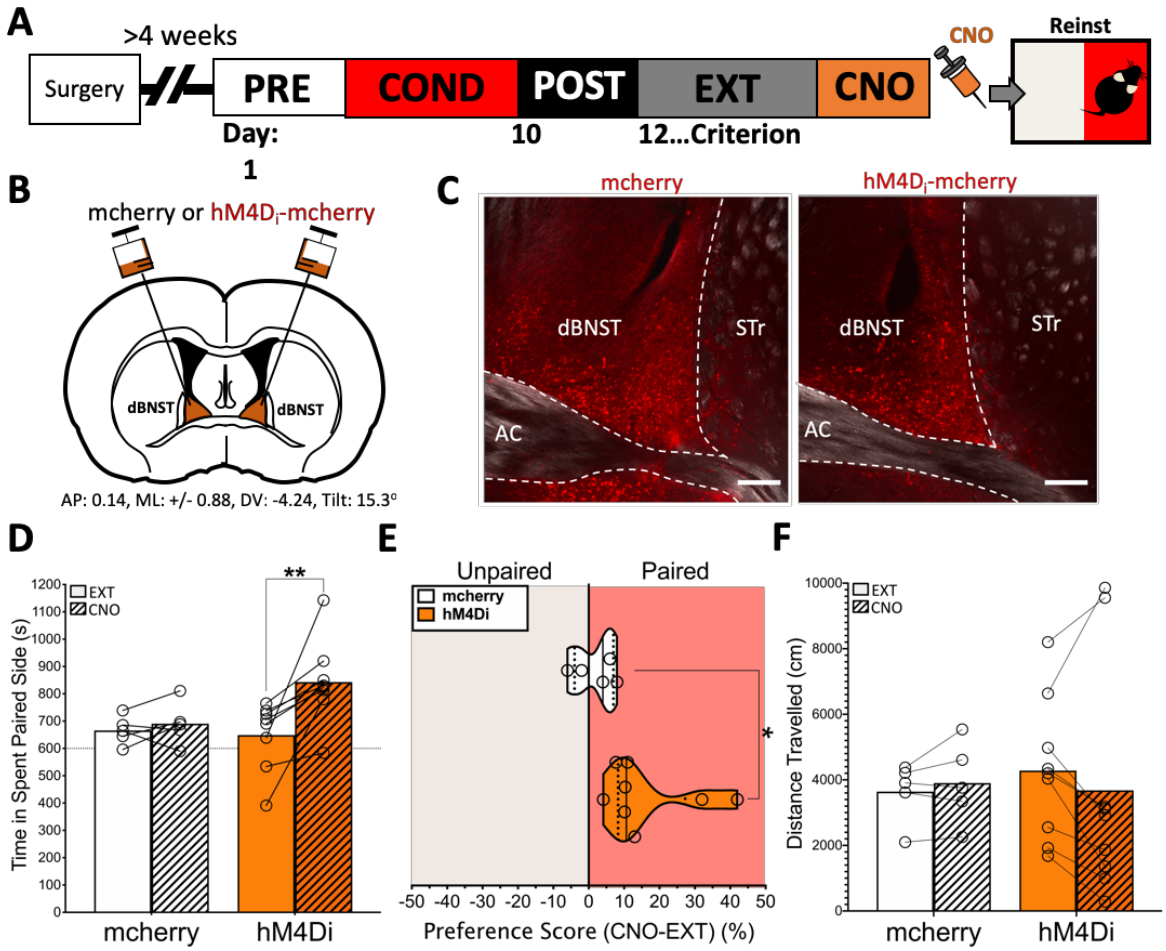

Figure 11. Activation of G_i signaling within the BNST reinstates cocaine CPP. A. Timeline and schematic showing surgery, recovery and CNO-induced reinstatement of CPP procedure. **B.** Schematic and coordinates of injection site for mcherry and mcherry-tagged hM4Di. **C.** Low magnification (10x) images showing spread of mcherry and mcherry-tagged hM4Di in BNST. Scale bar = 200 µm. **D.** CNO treatment (CNO) increased the time hM4Di-expressing mice spent on the paired side when compared to the last day of extinction (EXT) but did not increase the time in mcherry-expressing controls. Data displayed as means plus individual data points. **E.** CNO treatment increased the CPP score of hM4Di-expressing mice compared to mcherry-expressing controls. Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **F.** CNO treatment did not alter locomotion in mcherry- or hM4Di-expressing mice when compared to the EXT session. Data displayed as means with individual data points (mcherry n = 5, hM4Di = 8) ($p < .05$) (EXT = last extinction session, CNO = CNO-induced reinstatement session).

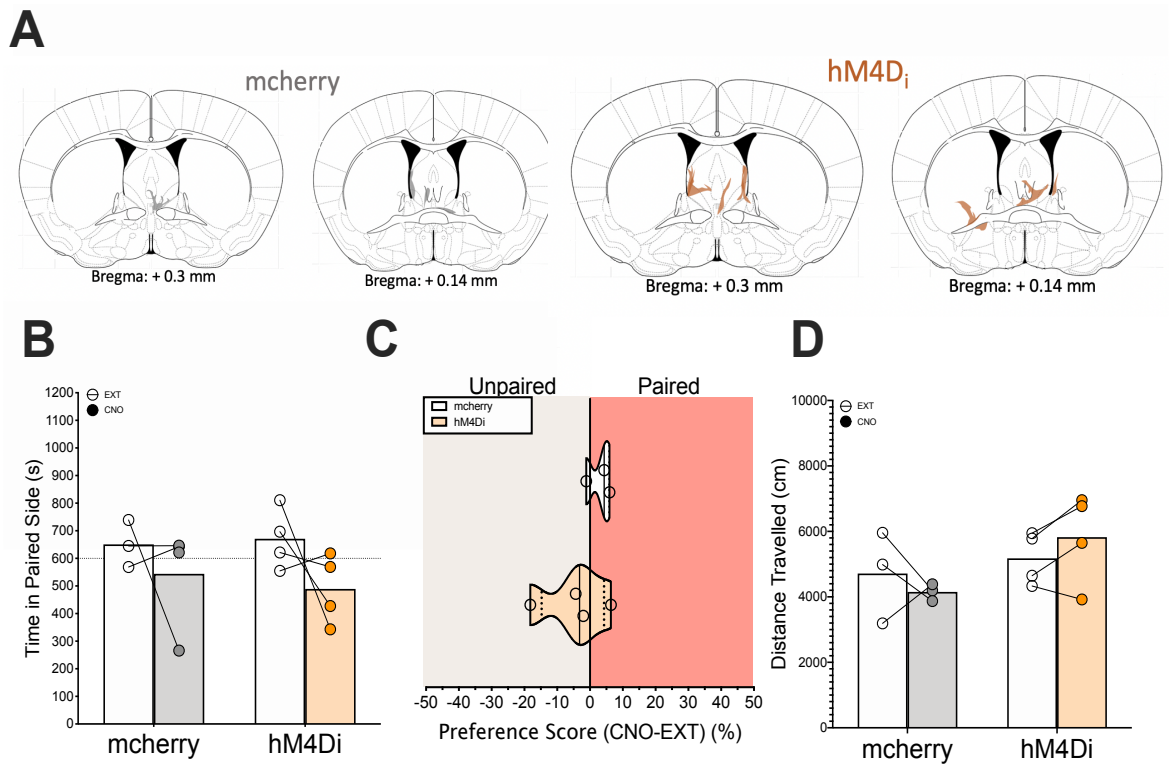

Figure 12. Mice lacking expression of mcherry or mcherry-tagged hM4D$_i$ within the BNST do not express CNO-induced reinstatement. A. anatomical localization of virus expression in the mice. **B.** Time spent in the paired side in non-BNST mcherry- and hM4D$_i$-expressing mice during extinction and following CNO injection. Data displayed as means and individual data points. **C.** Preference score of non-BNST mcherry and hM4D$_i$-expressing mice. Data displayed as medians (solid lines) and quartiles (dashed lines). **D.** Locomotion in non-BNST mcherry and hM4D$_i$-expressing mice during extinction and following CNO injection. Data displayed as means with individual data points (mcherry n = 3, hM4D$_i$ n = 4).

A low dose of guanfacine that does not increase cFOS within the BNST blocks stress-induced reinstatement of cocaine CPP

While non-specific α_2-AR agonism blocks stress-induced reinstatement of drug seeking, high doses of these compounds have negative effects, which limit their clinical applications [220]. We previously reported that a 1 mg/kg dose of the α_{2a}-AR partial agonist guanfacine, which produces a strong sedative effect, increased dBNST activity as measured by cFOS upregulation [211]. Given the prominent role of BNST activity in reinstatement of drug seeking and anxiety-like behaviors [216,225,226], we hypothesized that a lower dose of guanfacine that shows antidepressant- and anxiolytic-like effects (0.15 mg/kg), would not increase cFOS in the dBNST but would block stress induced reinstatement [76,77]. To determine dose-dependent changes in dBNST activity, we assessed cFOS expression within the dBNST following guanfacine administration [211]. Mice were injected with either saline (vehicle, VEH), low (0.15 mg/kg), or high dose (1 mg/kg) guanfacine (**Fig 13. A**). As we previously reported, high dose guanfacine increased the number of cFOS positive cells within the dBNST, however, this effect that was not present in VEH- or low dose-treated mice ($F_{2,14} = .154$, $p < .0001$; one-way ANOVA, Sidak's multiple comparisons test: VEH vs 0.15 mg/kg $p = .88$, VEH vs 1 mg/kg $p < .0001$) (**Fig 13. B-C**).

We next examined the potential anti-reinstatement effects of low dose guanfacine. WT mice that had undergone CPP training and extinction were injected with either VEH or low dose guanfacine, 30 minutes prior to stress (**Fig 13. D**). While we did not find main effects of drug (VEH vs GUAN) or stress, we found a main effect of the treatment x session interaction (Drug $F_{1,12} = .01$, $p = .9$, stress $F_{1,12} = 1.49$, p .24, interaction $F_{1,12} = 5.843$, $p = .032$; two-way RM ANOVA), driven by a stress-induced increase in time on the paired side in VEH-treated mice that was not present in guanfacine-treated mice (**Fig 13. E**). Guanfacine-treated mice also showed a significant decrease in

preference score when compared to VEH-treated mice ($t = 2.417$, $df = 12$, $p < .05$; unpaired t-test) (**Fig 13. F**). While stress significantly suppressed locomotion in both groups, there were no differences in locomotion in VEH- and guanfacine-treated mice following stress, and no main effect of drug, suggesting that low dose guanfacine treatment did not have further sedative effects (interaction $F_{1,12} = 10.34$, $p < .01$, drug $F_{1,12} = .18$, $p = .67$, stress $F_{1,12} = 222.5$, $p < .0001$; two-way RM ANOVA, Sidak's multiple comparisons test, STRESS: VEH vs guanfacine $p = .44$, EXT vs STRESS: VEH $p < .0001$, guanfacine $p < .0001$) (**Fig 13. G**). Taken together, these findings suggest low dose guanfacine does not increase the activity of the BNST but blocks stress-induced reinstatement without impacting locomotion.

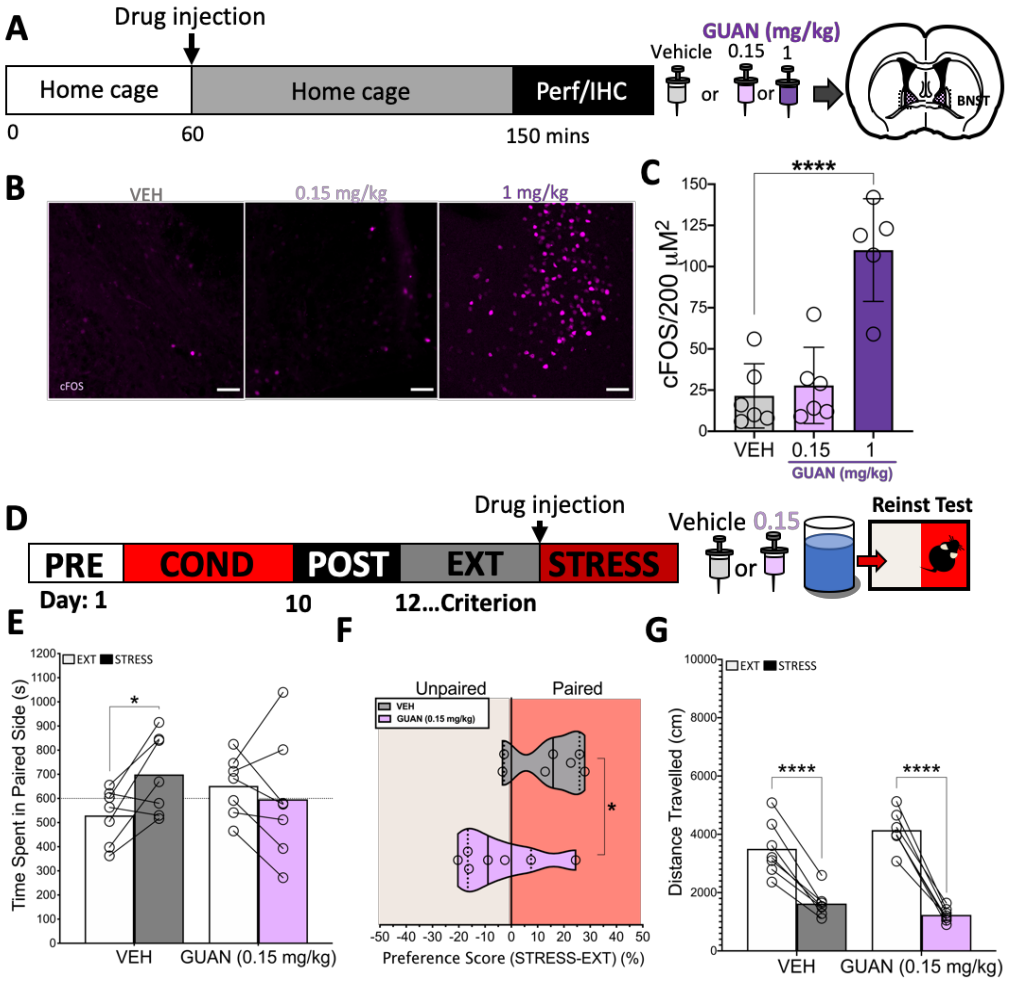

Figure 13. A dose of guanfacine that does not increase BNST cFOS blocks stress-induced reinstatement of cocaine CPP. A. Timeline and schematic of drug injection and immunohistochemistry experiment. Following an hour of acclimation mice were injected with saline (Vehicle, VEH) or guanfacine (GUAN) and sacrificed 90 minutes post-injection. **B.** High magnification (20x) images showing cFOS staining in dorsal BNST. Scale bar = 50 µm. **C.** 1 mg/kg injection of GUAN increased cFOS within the BNST. (VEH n = 6, 0.15 mg/kg n = 6, 1 mg/kg n = 5). **D.** Timeline and schematic of stress-induced reinstatement blockade experiment. Mice were injected with VEH or GUAN (0.15 mg/kg) 30 minutes prior to stress. **E.** Stress increased time in the paired side in VEH-treated mice but failed to do so in GUAN-treated mice. Data displayed as means plus individual data points. **F.** There was a difference in the CPP score of VEH-treated and GUAN-treated mice. Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **G.** Stress suppressed locomotion in VEH- and GUAN-treated mice but there were not inter-treatment differences in locomotion. Data displayed as means plus individual data points (VEH n = 7, GUAN n = 7) (*p < .05, ****p < .0001) (EXT = last extinction session, STRESS = stress-induced reinstatement test session).

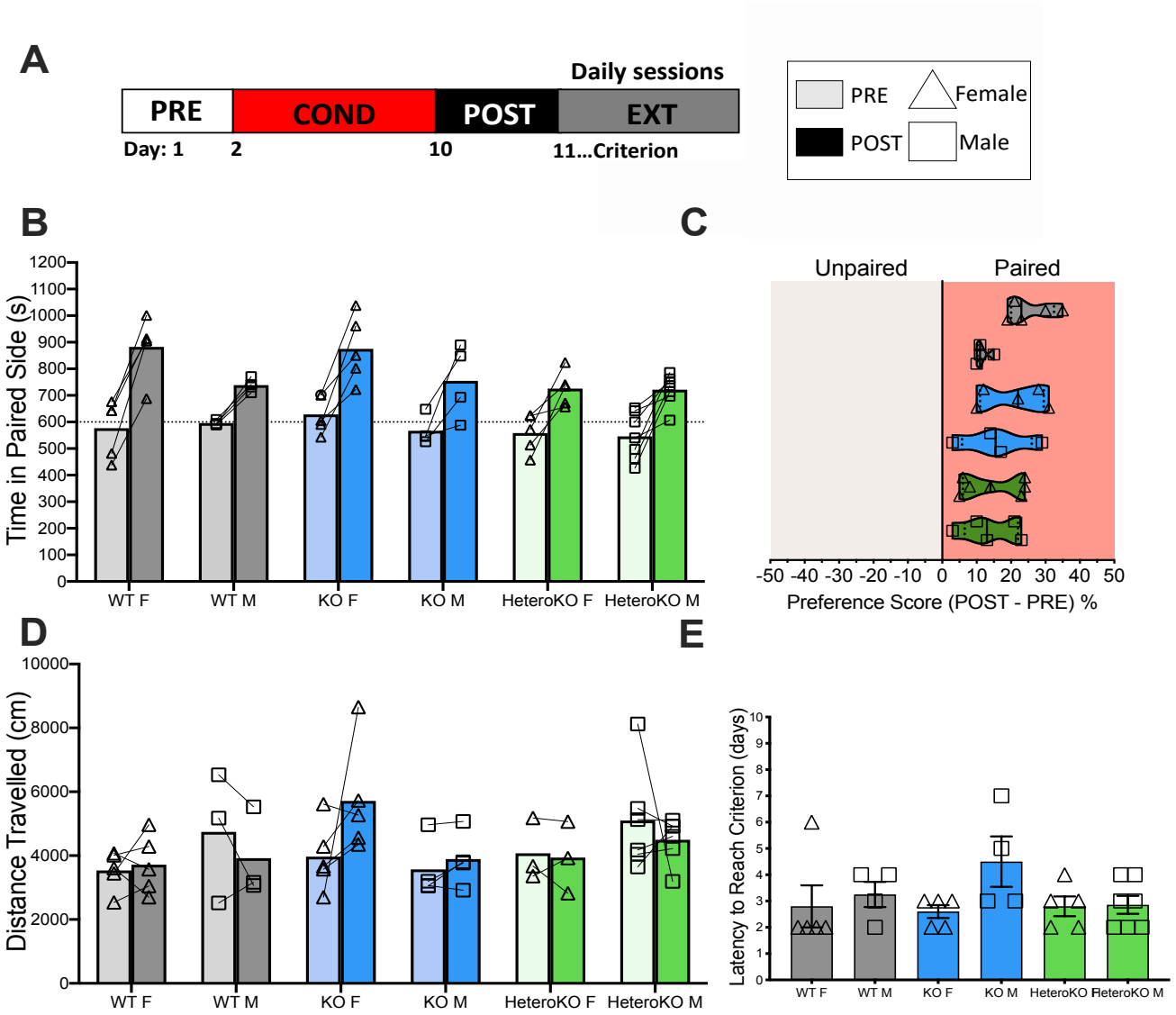

Figure 14. Related to Figure 5, WT, KO, and HeteroKO conditioning and extinction values separated by sex. A. Schematic of experimental paradigm and group key. **B.** Time spent in the paired side during the sessions before (PRE) and after conditioning (POST). Data displayed as means and individual data points. **C.** Preference scores. Data displayed as medians (solid lines) and quartiles (dashed lines). **D.** Locomotion PRE and POST. Data displayed as means with individual data points. **E.** Latency to reach the extinction criterion. Data displayed as means +/- SEM and superimposed individual values (WT female n = 4, WT male n = 4; KO female n = 5, KO male n = 4; HeteroKO female n = 5, heteroKO male n = 7).

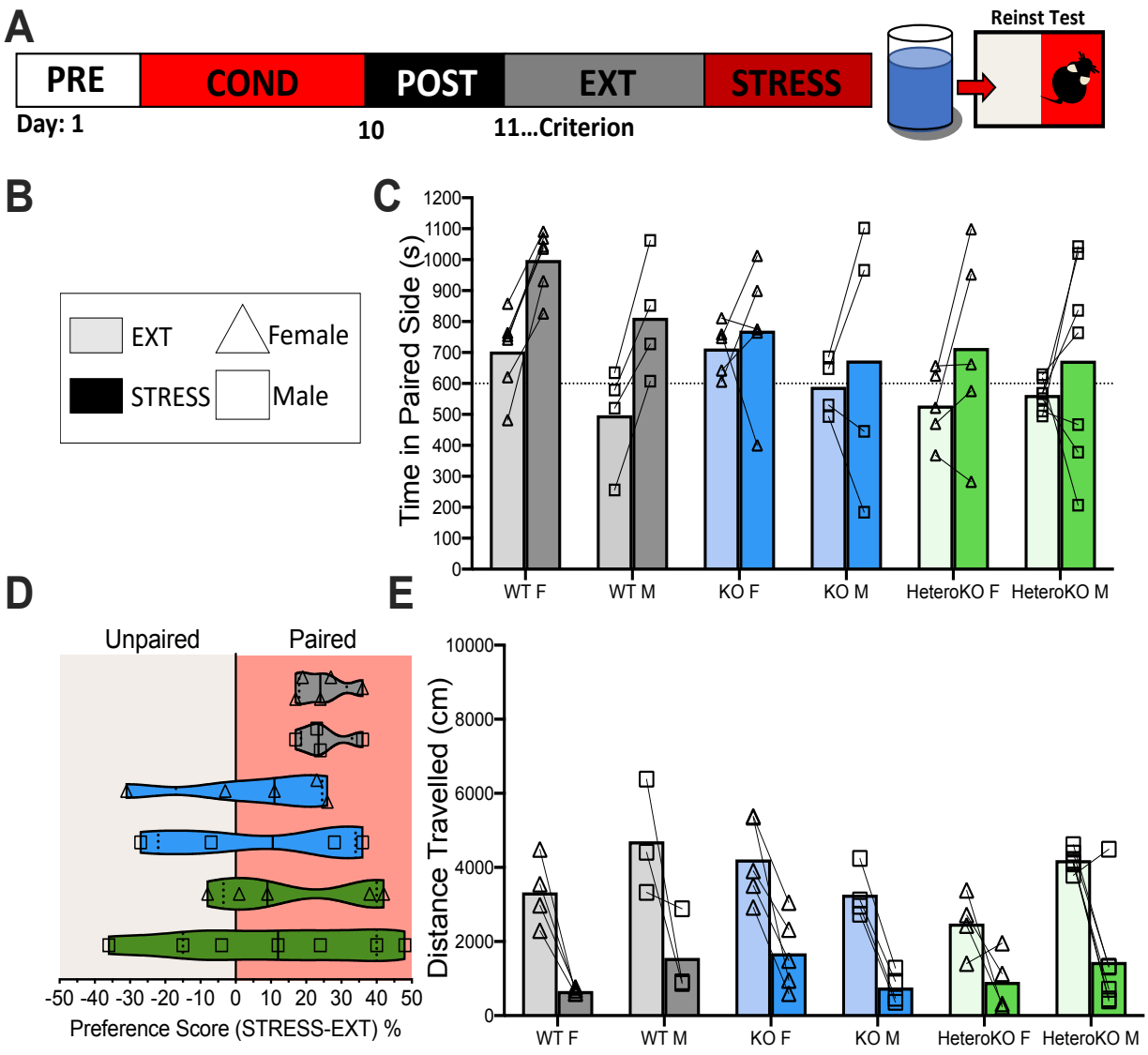

Figure 15. Related to Figure 6, WT, KO, and HeteroKO stress-induced reinstatement test values separated by sex. A. Schematic of experimental paradigm. **B.** Group key. **C.** Time spent in the paired side during the last extinction session (EXT) and after forced swim stress (STRESS). Data displayed as means and individual data points. **D.** Preference scores. Data displayed as medians (solid lines) and quartiles (dashed lines). **E.** Locomotion during the EXT and STRESS sessions. Data displayed as means with individual data points (WT female n = 4, WT male n = 4; KO female n = 5, KO male n = 4; HeteroKO female n = 5, heteroKO male n = 7).

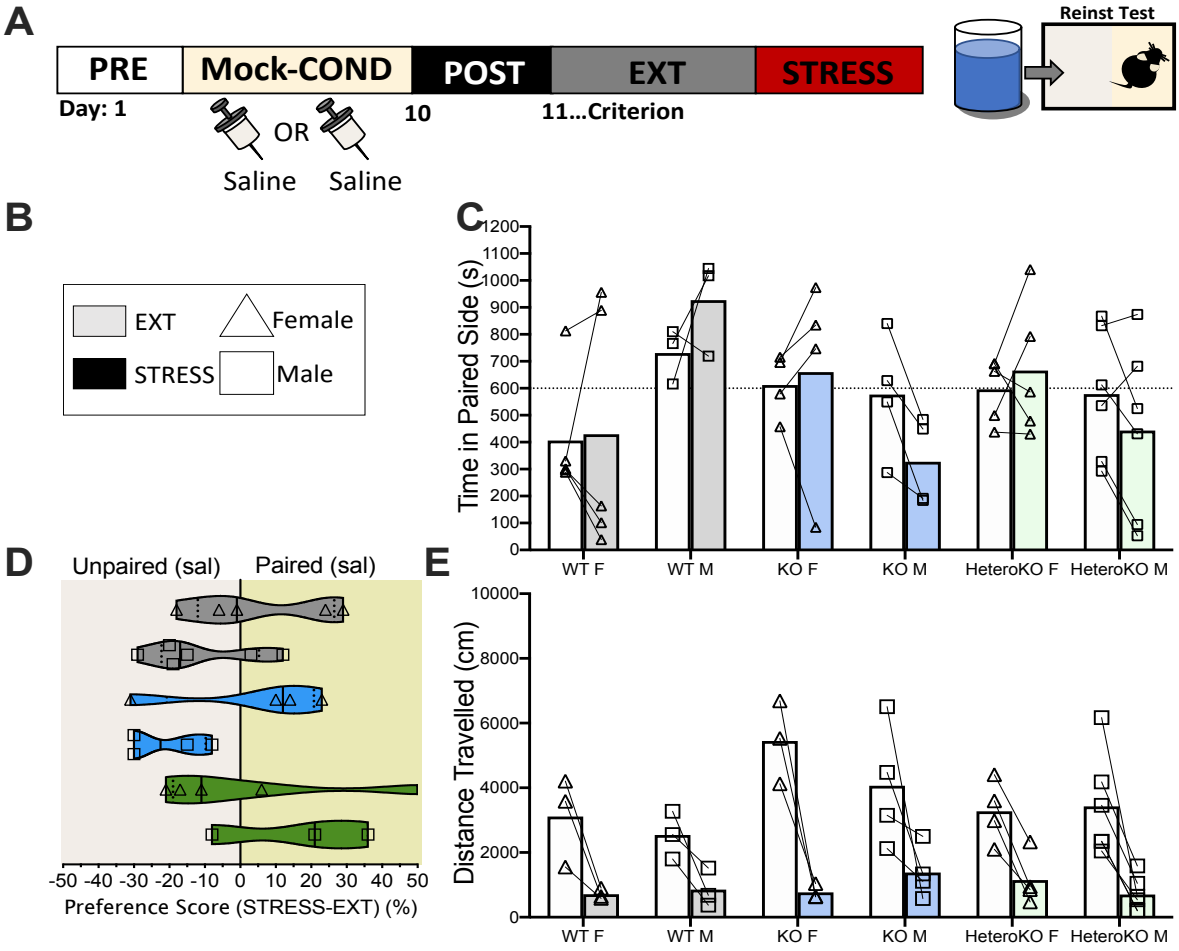

Figure 16. Related to Figure 8, WT, KO, and HeteroKO mock-conditioned stress-induced reinstatement test values separated by sex. A. Schematic of experimental paradigm. **B.** Group key. **C.** Time spent in the paired side during the last mock extinction session (EXT) and after forced swim stress (STRESS). Data displayed as means and individual data points. **D.** Preference scores. Data displayed as medians (solid lines) and quartiles (dashed lines). **E.** Locomotion. Data displayed as means with individual data points (WT female n = 5, WT male n = 3; KO female n = 4, KO male n = 4; HeteroKO female n = 5, heteroKO male n = 6).

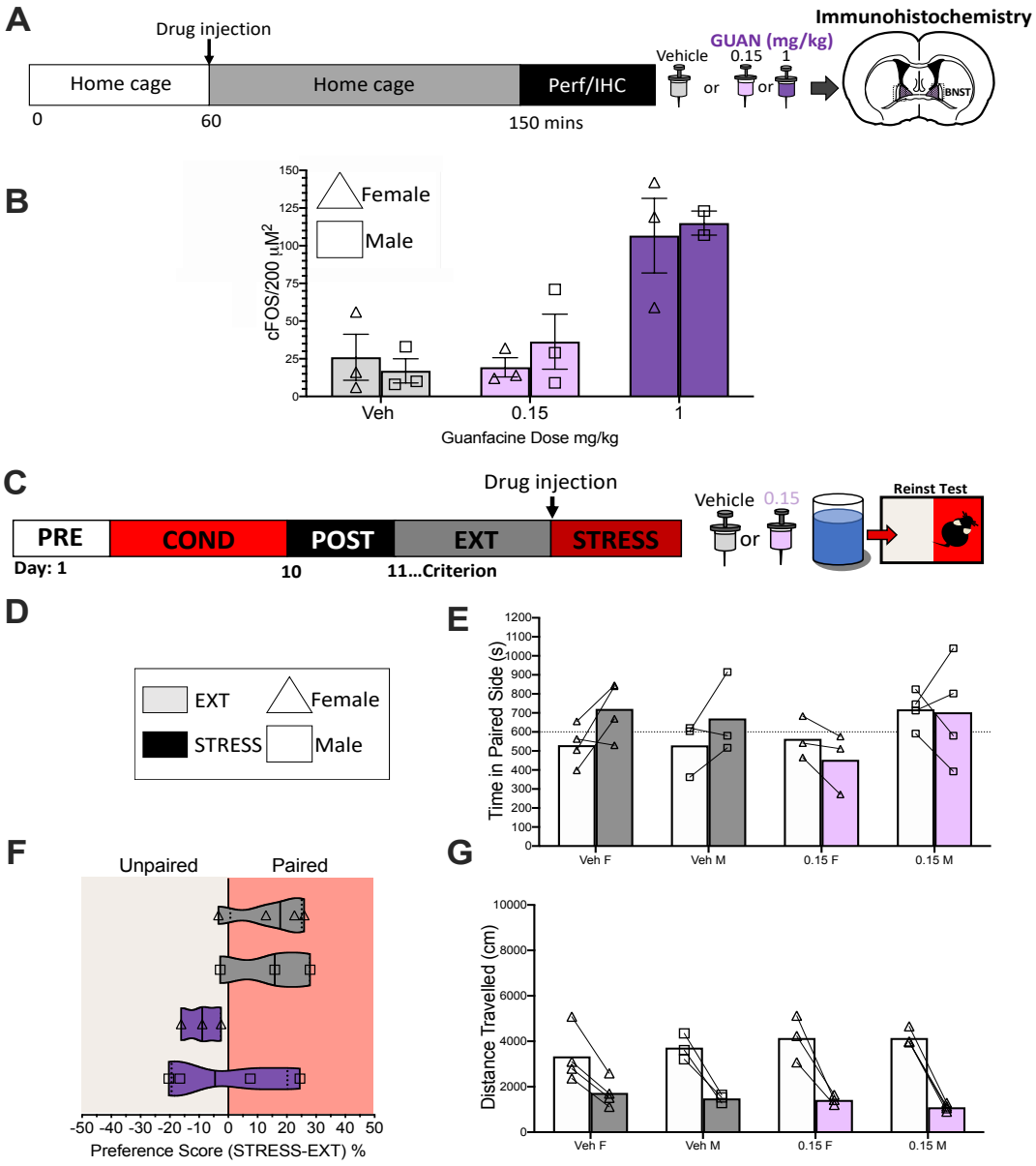

Figure 17. Related to Figure 12, guanfacine regulation of cFOS expression and stress-induced reinstatement test values separated by sex. A. Schematic of experimental paradigm for cFOS upregulation assay. **B.** cFOS expression in mice treated with vehicle (Veh), low-dose (0.15 mg/kg) or high-dose guanfacine (1 mg/kg). Data displayed as means +/- SEM and overlaid individual points. **C.** Experimental paradigm for stress-induced reinstatement assay. **D.** Time spent in the paired side during the last extinction session (EXT) and after forced swim stress (STRESS). Data displayed as means and individual data points. **D.** There were no intergroup differences in the preference score (2-way ANOVA). Data displayed as medians (solid lines) and quartiles (dashed lines). **E.** Locomotion during the EXT and STRESS sessions. Data displayed as means with individual data points (Veh female n = 4, Veh male n = 3, low-dose guanfacine female n = 3, low dose guanfacine male n = 4).

Table 1. Summary statistics for Figures 5-13.

Figure	Test	Comparison	Stat	P value	Summary
5C	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO	F(2, 27) = 3.17	p = .0577	n.s.
		Conditioning: Pre vs Post	F(1, 27) = 115.1	p = .0001	****
		Genotype x Conditioning	F(2,27) = .99	p = .3819	n.s.
5D	One-way ANOVA	WT vs KO vs heteroKO	F(2, 27) = .09	p = .4007	n. s.
5E	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO	F(2, 23) = .3672	p = .3672	n.s.
		Conditioning: Pre vs Post	F(1, 23) = .6364	p = .6364	n.s.
		Genotype x Conditioning	F(2, 23) = 1290	p =.1290	n.s
5F	Kruskal-Wallis test	WT vs KO vs heteroKO	KW = .84	p = .65	n.s.
6B	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO	F(2, 23) = 2.14	p = .1388	n. s.
		Stress: Pre vs Post	F(1, 27) = 15.01	p = .0006	***
		Genotype x Stress	F(2, 27) = 2.313	p = .1175	n.s.
6C	Brown-Forsythe test	WT vs KO vs heteroKO	F(2, 27) = 5.6	p = .009	**
6D	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO	F(2, 22) = .03	p = .9700	n.s.
		Stress: Pre vs Post	F(1, 22) = 76.2	p = .0001	****
		Genotype x Stress	F(2, 22) = .4	p =.6410	n.s.
7B	2-way repeated measures ANOVA	**Genotype:** WT vs KO vs heteroKO	F(2, 24) = .02	p = .8271	n.s
		Mock conditioning: Pre vs Post	F(1, 24) = .01	p = .8920	n.s
			F(2, 24) = .07	p = 9272	n.s

		Genotype x Mock Conditioning			
Figure	**Test**	**Comparison**	**Stat**	**P value**	**Summary**
7C	One-way ANOVA	WT vs KO vs heteroKO	F(2, 24)	P = .9176	n.s.
7D	2-way repeated measures ANOVA	**Genotype:** WT vs KO vs heteroKO **Mock conditioning:** Pre vs Post **Genotype x Mock Conditioning**	F(2, 24) = 4.1 F(1, 24) = 11.54 F(2, 24) = 4.9	p = .0295 p = .0025 p = .0170	* ** *
8B	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO **Stress:** Pre vs Post **Genotype x Stress**	F(2, 24) = .02 F(1, 24) = .1 F(2, 24) = 1.1	p = .9734 p = .7303 p = .3617	n.s. n.s n.s.
8C	One-way ANOVA	WT vs KO vs heteroKO	F(2, 24) = 1	p = .4	n.s.
8D	2-way repeated Measures ANOVA	**Genotype:** WT vs KO vs heteroKO **Stress:** Pre vs Post **Genotype x Stress**	F(2, 20) = 3.8 F(1, 20) = 68.5 F(2, 20) = .9	p = .0408 p < .0001 p =.5390	* **** n.s.
10B	2-way repeated measures ANOVA	**Virus:** mcherry vs $hM4D_i$ **Conditioning:** Pre vs Post **Virus x Conditioning**	F(1, 12) = .00 F(1, 12) = 66.3 F(1, 12) = 1.3	p = .9961 p < .0001 p = .2823	n.s. **** n.s
10C	t test	mcherry vs $hM4D_i$	t (12) = 1.14	p = .2755	n.s.
10D	t test	mcherry vs $hM4D_i$	t(12) = .55	p = .5931	n.s.
11D	2-way repeated Measures ANOVA	**Virus:** mcherry vs $hM4D_i$ **CNO:** Ext vs CNO **Virus x CNO**	F(1, 11) = 1.4 F(1, 11) = 8 F(1, 11) = 4.8	p = .2560 p = .0163 p = .0506	n.s. * n.s.

Figure	**Test**	**Comparison**	**Stat**	**P value**	**Summary**
11E	t-test	mcherry vs $hM4D_i$	t(11) = 2.234	p = .0468	*
11F	2-way repeated Measures ANOVA	**Virus:** mcherry vs $hM4D_i$	F(1, 11) = .02	p = .8759	n.s.
		CNO: Ext vs CNO	F(1, 11) = .2	p = .6918	n.s.
		Virus x CNO	F(1, 11) = 1	p = .3275	n.s.
13C	One-way ANOVA	Veh vs .15 vs 1 mg/kg	F(2, 24) = 21.34	p < .0001	****
13E	2-way repeated Measures ANOVA	**Drug:** Veh vs GUAN	F(1, 12) = .01	p = .9081	n. s.
		Stress: Ext vs Stress	F(1, 12) = 1.58	p = .2450	n.s.
		Drug x Stress	F(1, 12) = 5.8	p = .0347	*
13F	t-test	Veh vs GUAN	t(12) = 2.417	p = .0325	*
13G	2-way repeated Measures ANOVA	**Drug:** Veh vs GUAN	F(1, 12) = .6725	p = .6725	n.s.
		Stress: Ext vs Stress	F(1, 12) = 222.5	p < .0001	****
		Drug x Stress	F(1, 12) = 10.3	p = .0074	**

DICUSSION

α_{2a}-AR agonists such as guanfacine have risen in popularity for the treatment of several psychiatric conditions [67,227]. α_{2a}-AR agonists target autoreceptors expressed in adrenergic neurons and heteroreceptors expressed in non-adrenergic cells. Determining the relative contributions of these receptor populations to the pharmacological effects of α_{2a}-AR agonists and behavior has been challenging, due to the inability to distinguish these populations using conventional pharmacological or genetic approaches [102]. In the current study, we used selective genetic deletion models, behavior, histology, and chemogenetics to define the role of α_{2a}-AR heteroreceptors on stress-induced reinstatement of cocaine CPP.

In our unbiased two-sided CPP apparatus, stress reinstated cocaine CPP in all wild-type mice, but only a fraction of α_{2a}-AR full and HeteroKO mice. Following stress, we observed a high degree of variability in the amount of time α_{2a}-AR KO and HeteroKO mice spent on the two sides of the apparatus. This increase in variability was also found in cocaine-naïve mice from all genotypes, but not in cocaine-treated WTs. Notably, stress reduces locomotion in the CPP assay independent of genotype or drug exposure history. Therefore, our data suggest that in α_{2a}-AR full and heteroreceptor knockouts, stress-induced reinstatement of CPP is replaced by increased occupancy in an arbitrary side of the chamber, due to an overall reduction in activity rather than side preference. While this is a parsimonious interpretation of the data, currently we cannot unequivocally rule out the possibility that some of the α_{2a}-AR full and HeteroKO mice show stress-induced reinstatement. Regardless of this limitation, it is still clear that α_{2a}-AR heteroreceptors positively regulate reinstatement behavior.

Our previous findings have demonstrated functional recovery of autoreceptors in the dBNST in this model[211], suggesting the surprising result that α_{2a}-AR heteroreceptor deletion disrupts the ability of stress to drive reinstatement of preference for the cocaine-paired side. It is possible that

within the dBNST, α_{2a}-AR auto- and heteroreceptors play opposing roles, where autoreceptors might prevent reinstatement by decreasing NE release, heteroreceptor, and beta-adrenergic signaling [69,165]. Conditions in which large amounts of NE are released into the BNST, such as chronic stress, might override autoreceptor regulation to lead to reinstatement [205,228].

We used a chemogenetic approach to mimic dBNST α_{2a}-AR heteroreceptor signaling to determine the effects of G_i-GPCRs on reinstatement of cocaine CPP. We found that acute activation of BNST $hM4D_i$ was sufficient to induce reinstatement of CPP. This G_i-DREADD-induced reinstatement is consistent with our findings showing that activation of G_i-DREADDs within the BNST increases activity and anxiety-like responses [211]. Previous work has shown that activation of intra-BNST G_i-DREADDs can inhibit drug consumption or the acquisition of CPP [229–232]. Notably, these studies focused on alcohol consumption and CPP acquisition without a focus on reinstatement. Thus, our study provides novel insight into the potential role of G_i-GPCR signaling on stress-induced reinstatement of cocaine CPP. Additionally, we previously reported that direct α_{2a}-AR activation or G_i-DREADD mimicking of α_{2a}-AR signaling in excitatory inputs onto BNST CRF cells decreases stress-induced cFOS [212]. Taken together, these findings highlight the heterogeneous nature of α_{2a}-AR regulation of BNST activity. Future studies will aim to determine the role of different BNST cell populations and projections in the pro-reinstatement effects of G_i-coupled GPCR signaling.

A pro-reinstatement role of heterosynaptic α_{2a}-ARs contrasts with previous reports suggesting that systemic administration of the nonselective α_2-AR antagonist yohimbine and the α_{2a}-AR antagonist BRL-44408 malate reinstate CPP [70]. While yohimbine antagonizes α_{2a}-ARs and increases norepinephrine levels, it is also a 5-HT_{1a} receptor agonist [233,234]. Yohimbine-induced reinstatement of cocaine- and food-seeking, or cocaine CPP is not blocked by application of the α_{2a}-AR agonist clonidine [70,164]. Indeed, yohimbine's effects on drug-associated behaviors requires orexin and

serotonin 5-HT_{1a} receptor signaling [167,196]. Furthermore, we have previously reported that within the BNST, yohimbine produces α_{2a}-AR independent, orexin receptor-1 dependent decreases in excitatory transmission [196]. Therefore, our current findings add to the growing body of evidence suggesting that the pro-reinstatement effects of yohimbine may not be dependent on modulation of adrenergic signaling. BRL-44408 malate also reinstated CPP, presumably through inhibition of α_{2a}-AR autoreceptors [73]. The location of the receptors mediating the pro-reinstatement effects of BRL-44408 malate remain unknown, but would be predicted to involve presynaptic terminal α_{2a}-ARs [212].

As we previously found that a high dose of guanfacine increases BNST activity, we next sought to determine if a low dose of guanfacine, which has been previously reported to reduce anxiety-like behaviors, would lack excitatory effects in the BNST [76,77]. We replicated our previous finding showing that a high dose of guanfacine increases cFOS in the BNST, but found that the low dose did not. This suggests that a low dose of guanfacine does not engage BNST α_{2a}-AR heteroreceptors. We also found that low dose guanfacine blocked stress-induced reinstatement of CPP without impacting locomotion. While previous work has shown that higher doses of the α_{2A}-AR agonist clonidine do not block stress-induced reinstatement [70], the strong hypo-locomotive effects of the 1 mg/kg dose of guanfacine prevented us from testing this dose in the current study. Future studies will aim to determine the effects of intra-dBNST manipulation of α_{2a}-AR signaling. These findings may inform the development of dose-targeted guanfacine for the treatment of substance use disorders and are congruent with recent reports suggesting that the efficacy of guanfacine in some clinical applications is dose-dependent [86,87].

Notably, while α_{2A}-AR agonism shows sex-dependent effects in patients with CUD [79], we did not find any apparent sex differences in the effects of α_{2a}-AR full or heteroreceptor KO, therefore data from male and female mice were combined in our statistical analyses (see **Figures 14-17** for

values separated by sex). However, one limitation of the current study is that it is not sufficiently powered to analyze more subtle sex differences. While a recent report showed that the low dose of guanfacine used in the current study equally prevented forced swim-induced reinstatement of nicotine CPP in male and female mice [217], future investigations will be aimed at determining potential sex differences in α_{2a}-AR regulation of BNST activity as well as low-dose guanfacine on cocaine CPP reinstatement.

Our findings demonstrate a previously unknown role of α_{2a}-AR heteroreceptors in stress-induced reinstatement of cocaine-associated behaviors. Additionally, we also expand on previous reports suggesting that guanfacine should be further explored as a potential treatment for CUD and other drug use disorders.

Chapter 3

Photometric monitoring of BNST activity patterns during acquisition, extinction, and reinstatement of cocaine CPP.

Introduction

The BNST plays a critical role in addiction related behaviors [235]. Previous research using pharmacological and chemogenetic manipulations show that the activity of the BNST is required for the acquisition and stress-induced reinstatement of drug CPP [189,231]. Slice electrophysiology studies suggest that acute and chronic cocaine, and stress, modulate excitatory transmission within the BNST through actions on catecholamine release *ex vivo* [200,236]. However, how the activity of the BNST changes *in vivo* during the acquisition, expression, and stress-induced reinstatement of cocaine CPP remains poorly understood. *In vivo* fiber photometry tools allow for the long-term recording of neuronal activity during behavior, and have been previously integrated into the cocaine CPP procedure to assay changes in the activity of the striatum [237,238]. This chapter presents preliminary data evaluating activity patterns in the BNST throughout the CPP procedure as well as following the application of two reinstating stimuli, stress and intra-BNST $G_{\alpha i}$-GPCR activation.

Methods and Materials

Animals

Male C57BL/6J mice were delivered at 6 or 7 weeks of age and acclimated for at least one week before surgical manipulations. Following surgery, mice were singly-housed and allowed to recover for at least 2 weeks prior to behavioral experiments. All procedures were approved by the Vanderbilt University animal care and use committee.

Stereotaxic Surgery

Mice were anesthetized with isoflurane (initial dose = 3%; maintenance dose = 1.5%), and injected intracranially with recombinant AAV constructs AAV5-CaMKII-hM4Di:mCherry (AAV5-hM4Di; UNC Viral Vector Core) and AAV5-hSyn-GCaMP7f (AAV5- GCaMP7f; Addgene), mixed in equal volumes immediately before injection. Targeted bilateral injections of 750nl virus cocktail were made into the BNST (AP: 0.14, ML: ±0.88, DV: -4.24) as previously described [211,223] at a 15.03° angle at 100nl/min. For fiber photometry experiments, a 400 μm fiber optic cannula (Doric Lenses: MFC_400/430-0.48_6mm_MF1.25_FLT) was planted 0.02mm above the virus injection, and fixed to the skull using a dual-cure resin (Patterson Dental, Inc.).

Behavior

Prior to conditioning experiments, mice were handled and plugged into the patch cable for 5 consecutive days in order to minimize experimenter and cable-induced stress. Cocaine CPP training, extinction, and reinstatement testing was conducted as described on chapter 2[78].

Testing took place in open-field arenas with two-chamber preference inserts, which lacked a top cover to allow for the cable to move with the mice. During conditioning, mice received injections of cocaine (15 mg/kg i.p.) or saline and confined to alternating sides of the CPP apparatus. On the post-conditioning testing day and subsequent days, mice were allowed to move freely between sides. During extinction training, mice were placed in the CPP apparatus daily until they reached the extinction criterion. Mice underwent reinstatement testing 24 hours after reaching the extinction criterion. For stress-induced reinstatement of CPP, mice underwent forced swim stress in a beaker of warm water (22-26^{o} C) for 6 minutes and were placed in the CPP apparatus. For G_i-DREADD-induced reinstatement of CPP, mice were injected with clozapine-N-oxide (CNO) (3 mg/kg i.p.) 30 minutes prior to being placed in the CPP apparatus [78].

Fiber photometry

Fiber photometry data was acquired using a RZ5P fiber photometry workstation (Tucker-Davis Technologies). 470 and 405nm LEDs (ThorLabs) were modulated at distinct carrier frequencies and were passed through a fluorescence minicube (Doric Lenses) coupled to a patch cord (400µm, 0.48 NA) connected to the implanted fiberoptic. Fluorescence was back-projected through the minicube onto a photoreceiver (Newport). Signals were recorded at 1017.3Hz, demodulated in real-time and saved for online analysis. Each channel was low-pass filtered (<2 Hz) and a linear least squared model fit the isosbestic control signal (405 nm) to the calcium signal (470 nm). Change in fluorescence ($\Delta F/F_0$) was calculated as ((470nm signal-fit 405nm signal)/fit 405nm signal). Calcium transients were found by determining locations of local maxima of the $\Delta F/F_0$ trace, thresholded by peak prominence. Traces were either analyzed with a high (0.06) or low (0.03) prominence threshold based on pre-conditioning results, and thresholds were kept consistent for each animal for all subsequent recordings. Spike frequency was defined as the number of transients within the full recording period (1200s) or during time spent in either conditioning chamber. AnyMaze behavioral recordings of location and $\Delta F/F_0$ recordings were down-sampled to 10Hz by averaging with bins of 0.1s, and were then synchronized to correlate animal location with calcium signal. Following the completion of behavioral studies, GcaMP7f, hM4Di, and fiber placement was confirmed using immunobiological approaches [78].

Statistics

Data are represented as means or means ± SEM. All statistics were run using Prism 8 (GraphPad, La Jolla, CA). Differences between sessions were assessed using t-tests and one measures ANOVAs. Significance was set at $\alpha=0.05$. When significant main effects were

obtained using ANOVA testing, Sidak's multiple comparisons tests were used to assess differences between sessions post-hoc.

RESULTS

Acute cocaine administration decreases BNST activity during conditioning

To determine the effect of cocaine on neuronal population activity in the BNST during the acquisition of cocaine CPP, we used an *in vivo* calcium imaging approach to measure transient frequency during conditioning (**Fig. 18A**). Cocaine administration increases locomotion relative to saline administration, as has previously been reported [239] (one way ANOVA; $F_{2, 22} = 15.8$ Sidak's multiple comparisons test S1 vs C1 $p < .001$, S1 vs C4 $p < .01$) (**Fig. 18D**). Cocaine administration decreased the frequency of calcium transients in the dlBNST when compared to saline (one way ANOVA; $F1._{6, 22} = 45.44$, Sidak's multiple comparisons test S1 v C1 $p < .0001$, S1 vs C4 $p < .001$) (**Fig. 18E**).There was a main effect of cocaine administration on amplitude and post-hoc analyses revealed that amplitude was significantly increased during the last day of cocaine administration (C4) (one-way ANOVA; $F1._{6, 22} = 7.05$, $p < .01$, Sidak's multiple comparisons tests, S1 vs C4, $p < .01$). These findings suggest that cocaine suppressed the activity of the dlBNST during conditioning.

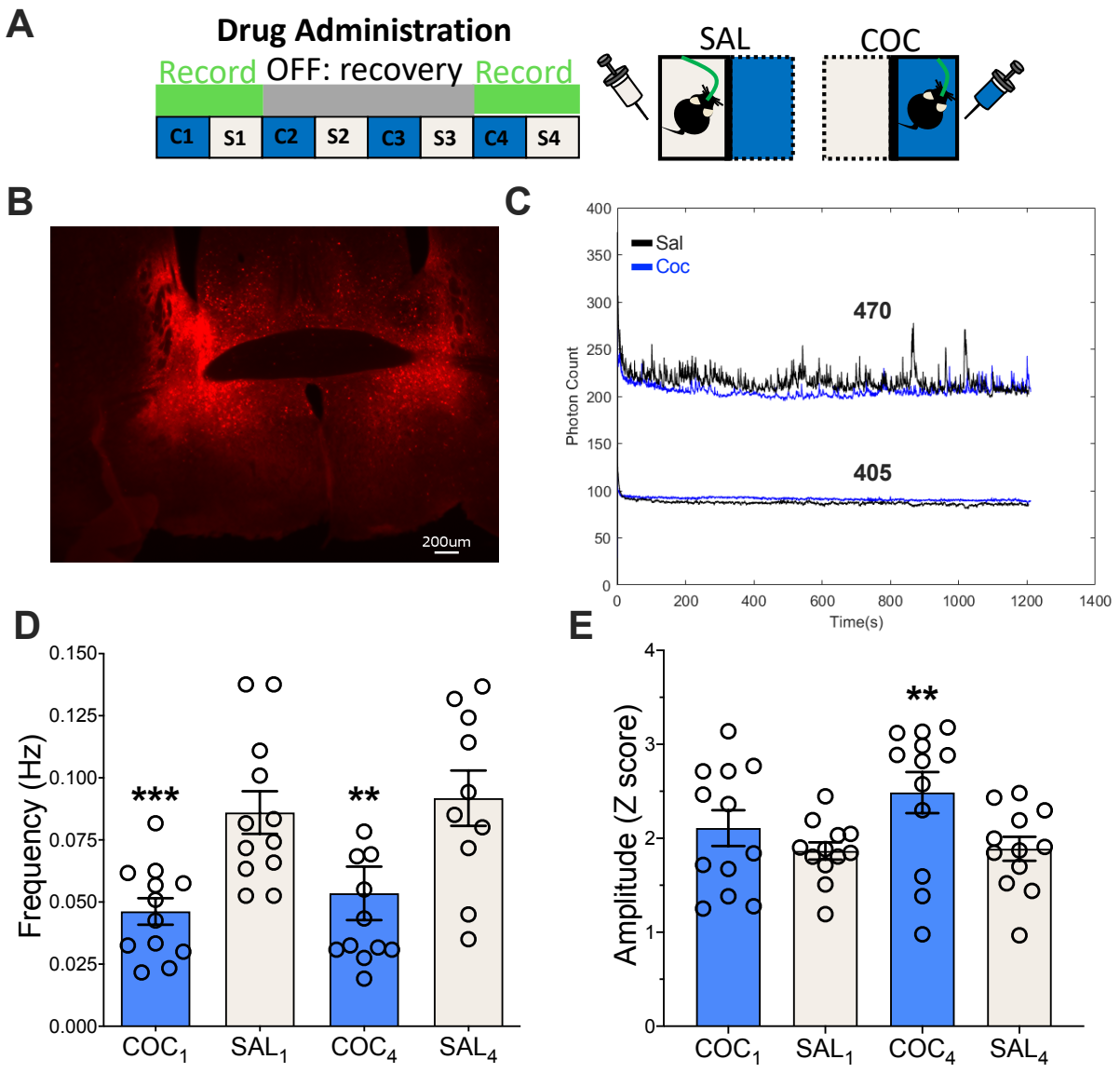

Figure 18. Acute cocaine-administration decreases BNST activity during cocaine conditioning. A. Experimental paradigm. Male mice were injected with either cocaine or saline and restricted to one side of a chamber for a period of 20 minutes with daily alternating drug presentation. **B.** representative low magnification (5X) image showing bilateral expression of GCaMP7f in the BNST. **C.** representative trace showing photon count throughout the duration of the first session following saline (S1) or cocaine (C1) administration for the 470 and 405 (isosbestic control) channels. **D.** Mice show decreased frequency of calcium transients following cocaine administration when compared to the first day of saline administration (One-way ANOVA with Sidak's multiple comparisons test; S1 vs C1, S1 vs C4). **E.** Mice show a significant increase in BNST calcium transient amplitude on the last day of cocaine administration (One-way ANOVA with Sidak's multiple comparisons test; S1 vs C4). Data displayed as means +/- SEM with superimposed individual values (**p < .01, ***p < .001).

Cocaine CPP is associated with a decrease in BNST activity in the unpaired side in the CPP apparatus

We next evaluated changes in BNST activity during the CPP acquisition test (**Fig. 19A)**. Mice spent more time in the cocaine-paired side during the post-conditioning test day (POST) when compared to the pre-conditioning test day (PRE) (paired t-test; $t = 7.58$, $df = 12$, $p < .0001$) (**Fig. 19B**). Mice also showed increased locomotion during POST (paired t-test; $t = 2.48$, $df = 12$, $p < .05$) (**Fig. 19C**). During POST, there was a decrease in the frequency of calcium transient in the dlBNST compared to PRE (paired t-test; $t = 2.32$, $df = 12$, $p < .05$) (**Fig. 19E**). Further analyses revealed that this decrease occurred while the mice were in the unpaired (saline) side (paired t-test; $t = 2.53$, $df = 12$, $p < .05$) (**Fig. 19G),** with no apparent changes in activity during transitions into either side or the amplitude of calcium transients (**Fig. 19H-I, D**).

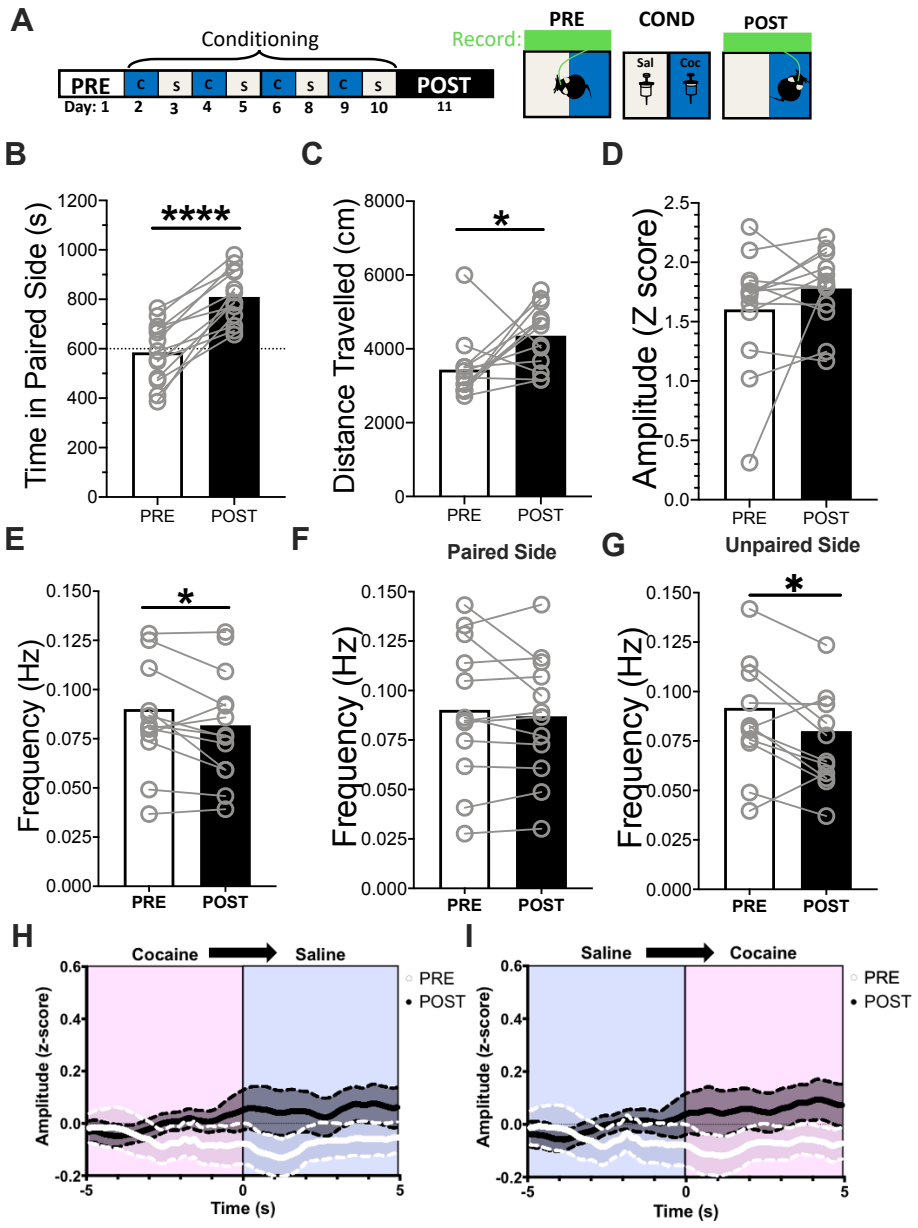

Figure 19. BNST activity is decreased during the CPP test in a chamber-dependent manner. A. timeline and schematic of experimental paradigm. **B.** Conditioning increased the time mice spent on the cocaine-paired side (paired t-test). **C.** Conditioning increased the distance travelled in the chamber (paired t-test). **D.** Conditioning does not alter the amplitude of calcium transients during the pre- conditioning (PRE) and post-conditioning (POST) testing sessions (paired t-test). **E.** Conditioning decreases the overall frequency of calcium transients within the BNST. **F-G.** The effect of cocaine CPP on frequency is driven by decreased frequency in the saline-paired side of the chamber. **H-I.** Calcium signal associated with transition from cocaine to saline or from saline to cocaine paired sides of the chamber is not affected by conditioning. Data displayed as means with superimposed individual values (*p < .05, ****p < .0001).

Stress-induced reinstatement is associated with overall decreases in BNST activity but increased activity during side transitions in the CPP apparatus

Stress-induced reinstatement of cocaine-associated behaviors is blocked by pharmacological inactivation of the BNST [189]. However, how BNST activity may change during stress-induced reinstatement is currently unknown. To address this, we conducted recording of gCAMP7f signals during stress-induced reinstatement of cocaine CPP (**Fig. 20A**) [78]. Stress increased the time mice spent on the paired side during the stress-induced reinstatement test day (STRESS) compared to the last day of extinction (EXT) (paired t-test; $t = 2.618$, $df = 8$, $p < .05$) (**Fig. 20B**). Stress also suppressed locomotion (paired t-test; $t = 7.66$, $df = 8$, $p < .0001$) (**Fig. 20C**). There was a significant decrease in overall calcium transients in the dlBNST during STRESS compared to EXT (paired t-test; $t = 2.79$, $df = 8$, $p < .05$) (**Fig. 20E**). However, there were no changes in frequency when the mice were in the paired or unpaired side, but there was a trend towards a significant decrease in frequency when mice were in the unpaired side (paired t-test; $t = 2.29$, $df = 8$, $p = .053$) (**Fig. 20G**). There was an increase in activity in the BNST during transitions from one side of the chamber to another (**Fig. 20H-I**). These findings suggest that BNST activity is altered during stress-induced reinstatement in a with increases occurring during transitions from one side of the chamber to another.

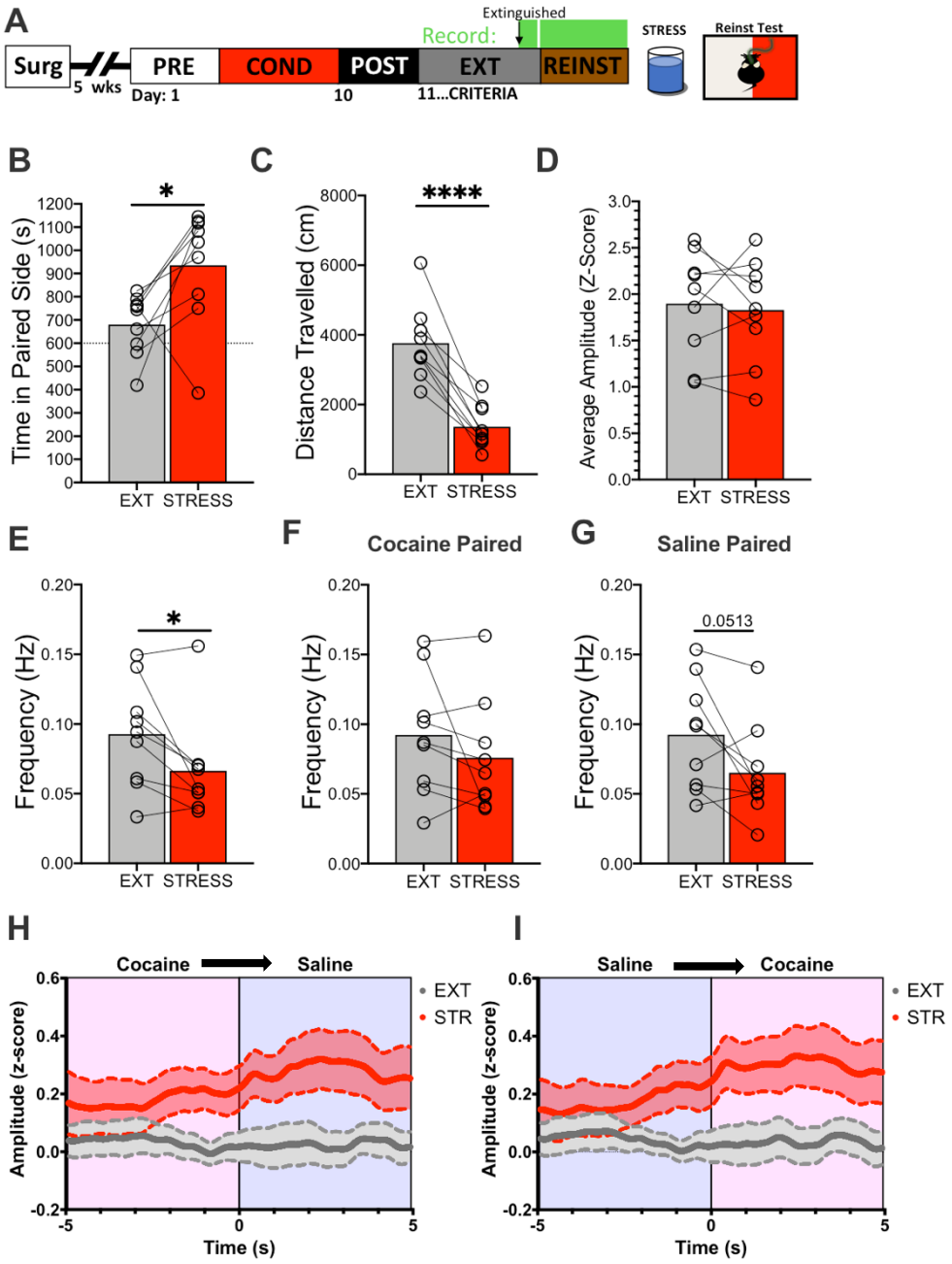

Figure 20. Altered activity patterns in the BNST during stress-induced reinstatement of cocaine CPP. A. timeline and schematic of experimental paradigm. **B.** Stress increased the time mice spent on the cocaine-paired side (paired t-test). **C.** Stress decreased the distance travelled in the chamber (paired t-test). **D.** Stress does not alter the amplitude of calcium transients (paired t-test). **E.** Stress decreases the frequency of calcium transients within the BNST. **F-G.** Effect on frequency may be driven by decreased frequency in the saline-paired side of the chamber. **H-I.** Calcium signal associated with transition from the cocaine- to saline-paired side and from the saline- to cocaine-paired side is increased by stress. Data displayed as means with superimposed individual values (*p < .05, ****p < .0001).

Gi-DREADD activation reinstates cocaine CPP and increases BNST activity independent of side-transitions in the CPP apparatus

We previously reported that intra-BNST activation of Gi-DREADDs induces reinstatement of cocaine CPP in a manner similar to stress and that Gi-DREADDs increase BNST activity in assays of anxiety [78,211]. We investigated if Gi-DREADD-induced reinstatement would also result in BNST activity pattern similar to stress-induced reinstatement (**Fig. 21A**). CNO administration reinstated cocaine CPP (paired t-test; $t = 4.5$, $df = 5$, $p < .05$) without altering locomotion (**Fig. 21B-C**). CNO administration decreased the amplitude of calcium transients in the dlBNST (paired t-test; $t = 5.323$, $df = 5$, $p < .01$) (**Fig. 21D**). CNO increased the frequency of calcium transients in the dlBNST (paired t-test;) (**Fig. 21E**). But further analyses revealed that although there was a trend towards a significant increase in calcium transients in the paired side (paired t-test, $t = 2.27$, $df = 5$, $p = .052$) (**Fig. 21F),** mice did not show significant increases in BNST activity while occupying a particular side or during transitions from one side to the other (**Fig. 21F-I**). Overall, these findings suggest that, as previously reported, activation of $G_{\alpha i}$-GPCR signaling in the BNST increases cellular activity to drive reinstatement. These findings also suggest that Gi-DREADDs induce reinstatement of CPP by upregulating BNST activity in a side-placement independent manner.

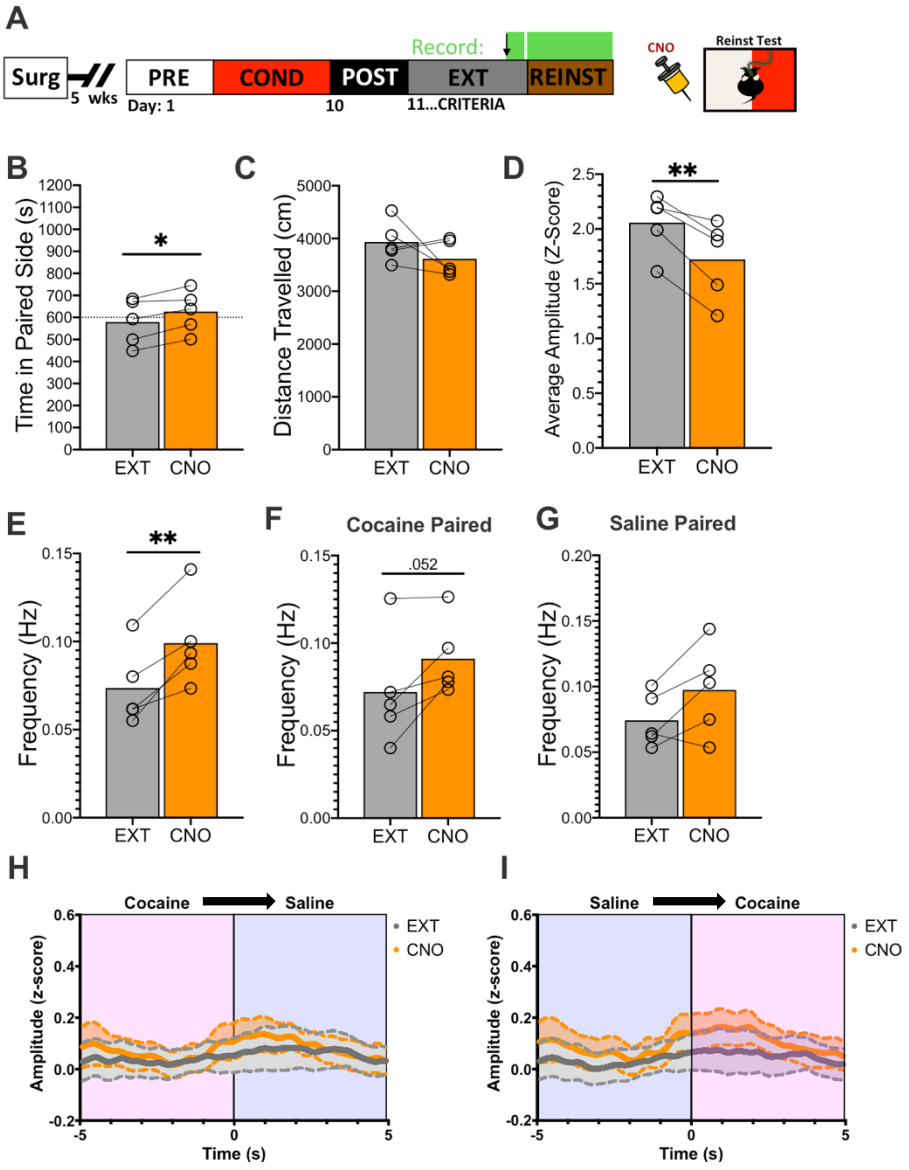

Figure 21. Chemogenetic activation of G_i-GPCR signaling reinstates CPP and increases BNST activity in a side transition-independent manner. A. Timeline and schematic of experimental paradigm. **B.** CNO increased the time mice spent on the cocaine-paired side (paired t-test). **C.** CNO does not affect distance travelled in the chamber (paired t-test). **D.** CNO decreases the amplitude of calcium transients (paired t-test). **E.** CNO increases the frequency of calcium transients within the BNST. **F-G.** Effect on frequency may be driven by decreased frequency in the cocaine-paired side of the chamber. **H-I.** Calcium signal associated with transition from cocaine to saline and from saline to cocaine paired sides of the chamber is not affected by stress. Data displayed as means with superimposed individual values (*p < .05, **p < .01).

DISCUSSION

Using the CPP model of drug-associated behaviors, we assessed changes in patterns of activity in the BNST in response to acute cocaine as well as during the expression and reinstatement of conditioned responses. We found that cocaine administration suppressed activity globally within the BNST, as measured by changes in the frequency of calcium transient pikes. These findings are in accordance with previous studies, which have shown that cocaine administration decreases NAc activity, a process mediated by the downregulation of the activity of dopamine D2 receptor-expressing medium spiny neurons (D2-MSNs)[17,238]. Cocaine, or selective DAT inhibitor administration, also increases dopamine levels in the BNST, so it is likely that cocaine might inhibit BNST activity *in vivo* through a similar D2R-mediated mechanism [240]. Interestingly, BNST activity was also lower during the CPP acquisition test but this effect was not as pronounced as the decrease that occurs following cocaine administration. The decrease in activity observed during the CPP acquisition test in the BNST does not occur in the NAc [238], suggesting a potential divergence between these two regions in their functional responses during the CPP test or to cocaine-associated contexts.

The global suppression of BNST activity observed during the CPP test was driven by a decrease that occurred while mice were in the unpaired side of the chamber. The implications of this finding are currently unknown but it is possible that, given its role as a detector of salient emotional stimuli, the BNST might be more active while the animal is in the paired side due to salient cocaine-induced associations [241].

We were able to recapitulate our previous findings showing that Gi-DREADD activation increases BNST activity *in vivo* and reinstates cocaine CPP, providing further support for an excitatory and pro-reinstatement role of postsynaptic $G_{\alpha i}$-GPCRs in this brain region[78,211].

Surprisingly, stress also decreased BNST activity, a finding that appears to be in contrast with *ex vivo* studies showing that stress increases levels of cFOS within this region, as well as pharmacological studies showing that inactivation of the BNST prevents stress-induced reinstatement [189]. However, preliminary analyses suggest that the activity of the BNST might be elevated during transitions, although this effect was modest. One potential explanation for these discrepancies is that the results from *ex vivo* cFOS analyses and *in vivo* photometry recording might diverge, particularly due to the lack of temporal resolution of immunohistological assessments. Another potential source of the observed differences might be the fact that the BNST is highly heterogenous, and within this region, activation or inhibition of distinct microcircuits can produce opposite responses [208,242,243]. Therefore, future studies will be aimed at determining the response of specific populations to cocaine and stress, particularly CRF-expressing neurons, which have been implicated in stress-driven pathology [203,244]. In summary, these studies provide new insight into the effects of cocaine and stress on BNST activity.

Chapter 4

Discussion and Future Directions

The main hypothesis of this thesis is that following stress, elevated levels of NE activate α_{2A}-AR heteroreceptors which increase BNST activity via $G_{\alpha i}$-coupled GPCR signaling to induce reinstatement and that preferentially targeting α_{2A}-AR autoreceptors will heteroreceptor-dependent activation of the BNST, and stress-induced reinstatement. How the data presented in this thesis provides evidence supporting this hypothesis is discussed in detail below.

Opposing roles of α_{2A}-AR hetero- and autoreceptors in the regulation of BNST activity and stress-induced reinstatement of cocaine CPP.

Since ablation of the ventral noradrenergic bundle (VNB), one of the primary conduits of NE into the brain prevents stress-induced reinstatement, and clinical and preclinical work had established that both chronic drug administration and stress induce a hyper-noradrenergic state correlated with craving and relapse, early studies focused on determining the potential of α_2-AR agonists as anti-reinstatement agents [162,205,245]. Systemic α_2-AR agonist administration prevents stress-induced reinstatement, an effect that was ascribed to actions at inhibitory autoreceptors in NE terminals with the extended amygdala [70,195]. However, furthers studies have expanded our understanding of the complexity of the central α_{2A}-AR adrenergic system, and have defined not only presynaptic autoreceptors, but also both presynaptic and postsynaptic heteroreceptors [98]. While both auto- and heteroreceptors couple to the $G_{\alpha i}$- family of heterotrimeric G proteins, the cellular localization, auxiliary protein coupling, and downstream signaling partners allows for a wide variety of responses [98].

The development of transgenic mouse lines lacking either all α_{2A}-ARs or just heteroreceptors accelerated the investigation of the relative function of auto- and heteroreceptors

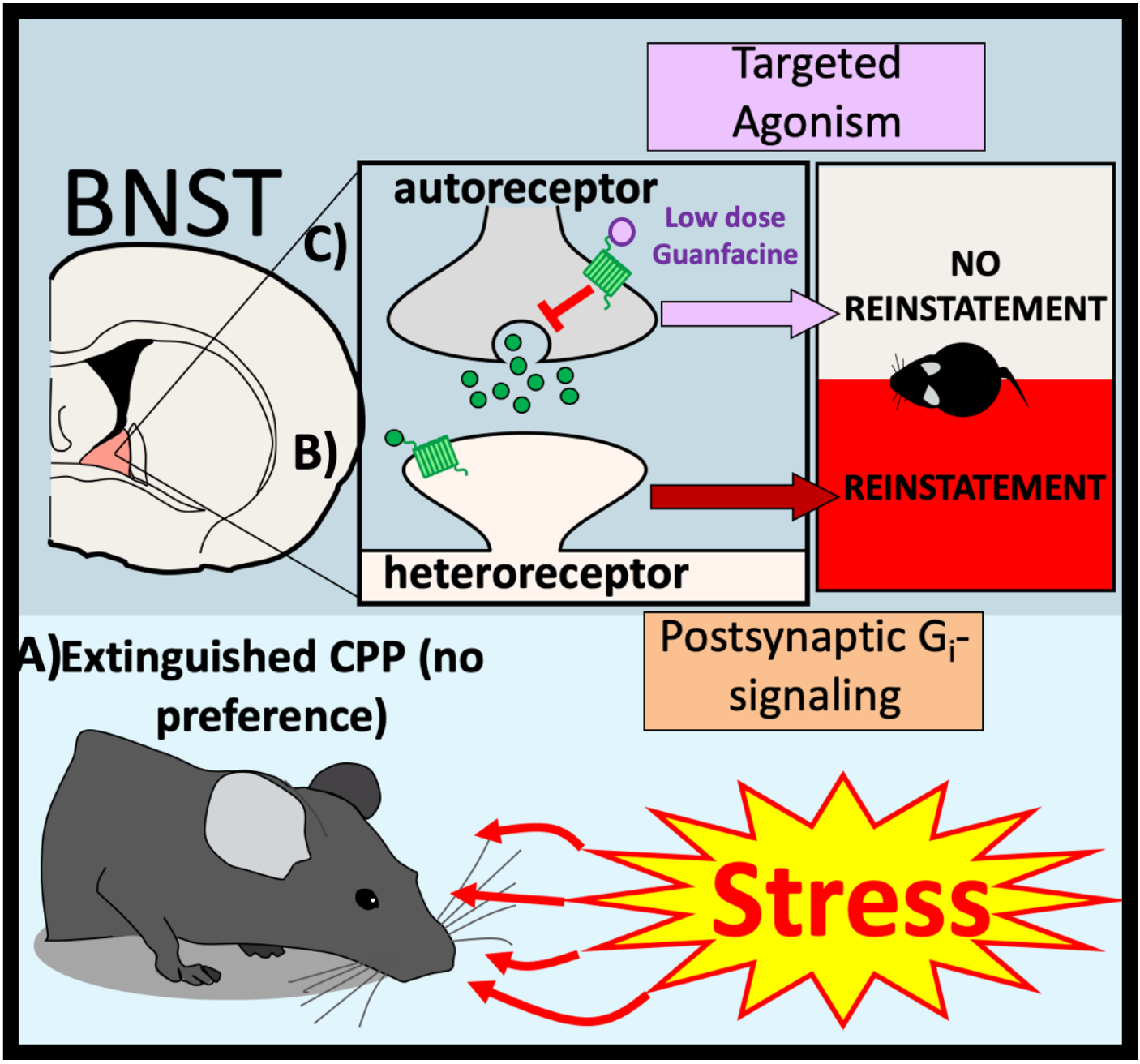

Figure 22. Model of auto- and heteroreceptor regulation of BNST activity and stress-induced reinstatement. A. mice with a history of cocaine, that do not show a preference for the side previously paired with cocaine, stress increases NE release into the BNST. **B.** NE activates postsynaptic $_{2A}$-AR heteroreceptors which increase BNST output to drive reinstatement. **C.** Selectively targeting autoreceptors using a low dose of guanfacine decreases NE release into the BNST and blocks stress-induced reinstatement.

in physiology and pharmacology [102]. Since germline re-introduction of α_2-AR autoreceptors did not rescue many of the deficits that occur in mice lacking all α_{2A}-ARs, researchers deduced that heteroreceptors mediate most of the physiological and pharmacological actions of α_{2A}-ARs [99]. These results and conclusions were surprising, particularly in light of the conventional understanding of α_2-ARs as primarily autoreceptors [103]. Results from studies using DSP toxins which selectively ablate LC neurons, thus functionally eliminating one of the main centers of presynaptic NE and autoreceptor function, in combination with adrenergic receptor ligands, support these conclusions [245,246]. Based on this work, it was expected that some of the anti-reinstatement actions of α_2-AR agonists may have been mediated by α_{2A}-AR heteroreceptors.

The primary finding of this thesis is that α_{2A}-AR heteroreceptors are necessary for stress-induced reinstatement of cocaine CPP. While mice lacking all α_{2A}-ARs show normal acquisition and extinction of cocaine CPP, results that have been previously reported and are in accordance with a minimal role of NE in the binge-intoxication phase of the allostatic cycle of compulsive behaviors, conceptualized by Koob et al., the display disrupted reinstatement [196,197,247–249]. Stress robustly produced reinstatement in all wild-type mice tested in the current studies, but only produced "apparent" reinstatement in a fraction of full α_{2A}-AR knockout mice. A potential reason for these findings includes an elevated baseline of NE in these mice due to the lack of autoreceptor negative feedback loop, which would induce a ceiling effect that would "mask" any effects of forced swim stress on behavior [120]. Results from studies in full α_{2A}-AR knockouts showing that these mice have increase immobility in the forced swim test, as well as higher sensitivity to mild stressors such as saline injections, suggest this possibility is unlikely [120,211].

Another potential explanation for the disrupted reinstatement in full α_{2A}-AR knockouts is that life-long compensatory adaptations to the hyper-noradrenergic environment produced by the

knockout, such postsynaptic α1-, and β-AR, desensitization would counteract the effects of stress [92]. This is an attractive explanation, due to the well-defined pro-reinstatement role of α1-, and β-ARs [70,188]. However, if this explanation was correct, then embryonic re-introduction of α_{2A}-AR autoreceptors would prevent these compensatory mechanisms from occurring. To test this possibility, we employed a differential genetic approach in which we compared the behavioral response of wild-type, full α_{2A}-AR knockouts, and full α_{2A}-AR knockouts re-expressing autoreceptors by targeting expression to the DβH promoter [102].

We have previously validated the α_{2A}-AR heteroreceptor-lacking line using slice voltammetry and pharmacology experiments [211]. While α_{2A}-AR agonists failed to reduce electrically evoked catecholamine release in the BNST, this effect was rescued in α_{2A}-AR heteroreceptor knockouts, demonstrating the functional recovery of autoreceptors [211]. Additionally, heteroreceptor function was evaluated using a slice pharmacology approach in which acutely prepared slices containing the BNST are incubated with compounds but otherwise not stimulated. Since release of NE or other neurotransmitter release is not evoked and slices are allowed to acclimate for a period of time prior to compound wash, this setup allows for the proxy measure of intra-BNST heteroreceptor function [211]. Guanfacine failed to increase cFOS in full knockout mice, a deficit that was not rescued by autoreceptor re-expression [211]. Thus, heteroreceptor-lacking mice show physiological phenotypes that are consistent with intact autoreceptor and disrupted heteroreceptor function within the extended amygdala. We found that mice that express autoreceptors, but lack heteroreceptors, also display aberrant stress-induced reinstatement, suggesting that compensatory adrenergic receptor desensitization is an unlikely mechanism mediating abnormal reinstatement in full knockout mice. However, we currently cannot rule out contributions of postsynaptic adrenergic receptor desensitization to the deficits in

reinstatement found in full α_{2A}-AR knockout mice, particularly given the role of these receptors in the regulation of excitatory transmission within the extended amygdala [202]. Future studies aimed at evaluating changes in α1-, and β-ARs expression and function in the BNST and CeA of α_{2A}-AR knockout mice are warranted. Nevertheless, α_{2A}-AR heteroreceptors play a critical role in stress-induced reinstatement of cocaine CPP.

Since engagement of autoreceptors decreases NE release into the BNST, and the elimination of NE into the BNST via VNB ablation block reinstatement, it appears that the anti-reinstatement actions of α_{2A}-AR agonists may be mediated by autoreceptors [70–72,245]. If this was the case, then a dose of guanfacine that does engage autoreceptors but does not activate heteroreceptors within the BNST would, by avoiding the pro-reinstatement actions of heteroreceptors, decrease stress-induced NE release into the BNST and prevent stress-induced reinstatement. We used an *ex vivo* immunohistological approach that had been previously employed to assess guanfacine-induced increases in BNST cFOS, which were found to be heteroreceptor dependent [211,214]. We recapitulated previous findings showing that an intraperitoneal (i.p.) injection of a relatively high dose of guanfacine (1 mg/kg), increases cFOS in the BNST [211,214]. We also found that a lower dose of guanfacine (0.15 mg/kg) did not increase BNST cFOS, suggesting that this dose does not engage intra-BNST heteroreceptors. As predicted, the 0.15 guanfacine dose blocked stress-induced reinstatement of cocaine CPP. These findings point towards α_{2A}-AR autoreceptors as the key receptor population mediating the anti-reinstatement effects of α_{2A}-AR agonists. Studies using the selective α_{2A}-AR antagonist BRL-4448 malate also support the notion that autoreceptors mediate the anti-reinstatement actions. BRL-4448 malate administration reinstates cocaine CPP [198]. While BRL-4448 malate likely inhibits intra-BNST α_{2A}-AR heteroreceptors, which prevent $G_{\alpha i}$-mediated increases in BNST

activity (discussed below), autoreceptor blockade elevates NE levels which leads to the activation of postsynaptic β_2-ARs increases BNST excitatory drive and reinstatement [198,200].

Currently, a role of α_{2A}-AR heteroreceptors in the anti-reinstatement action of agonists cannot be ruled out. α_{2A}-AR heteroreceptors are also expressed in presynaptic glutamatergic terminals from the parabrachial nucleus into the BNST [212]. Guanfacine, likely acting on this receptor population, inhibits excitatory drive from these terminals onto the BNST (Fetterly et al., 2018; Flavin et al., 2014). Activation of $G_{\alpha i}$-GPCR signaling in the PBN is sufficient to prevent stress-induced cFOS expression, and likely cellular activity, within the BNST [212]. Thus, guanfacine may produce its anti-reinstatement effects through the coordinated actions of BNST presynaptic auto- and heteroreceptor populations.

The studies presented in this thesis suggest that α_{2A}-AR auto- and heteroreceptors play opposing roles in the regulation of BNST activity following stress and stress-induced reinstatement. The evolutionary origins and implications of this oppositional system are currently unknown, but research on α_{2A}-AR regulation of the dorsolateral prefrontal cortex (dlPFC), allows for the formulation of a theoretical framework. α_{2A}-AR auto- and heteroreceptors also play opposing roles in the regulation of dlPFC activity [82,83,250]. Increased NE levels are associated with a weakening of dlPFC networks, which is hypothesized to be a mechanism by which stress and other negative experiences "shuts down" top down control of behavior, which releases deeper older brain structures to direct behavior towards survival quickly [139]. During periods of highly elevated NE, postsynaptic α_{2A}-AR heteroreceptors provide an additional "safeguard," alongside autoreceptors, by bolstering dlPFC network activity against the effects of stress [139]. In contrast to the dlPFC, the BNST plays a critical role in mobilizing the body to respond to stressful or harmful stimuli [208]. Thus, following stress, intra-BNST heteroreceptor

activation may provide an additional mechanism, alongside β-ARs and glutamatergic receptors, ensuring BNST activity and the potential survival of the organism. In substance use disorders and preclinical models, natural appetitive and survival mechanisms are hijacked by chronic drug use [22]. Thus, the multisynaptic α_{2A}-AR regulatory system in the BNST may serve a beneficial evolutionary role, that can be maladaptive in the context of drug addiction.

Further defining the cellular and receptor populations underlying the pro- and anti-reinstatement actions of α_{2A}-ARs

Although the transgenic lines used in this thesis are valuable for the study of the relative contributions of auto- and heteroreceptors to behavior, they present a number of limitations. First, life-long knockout of α_{2A}-ARs could induce compensatory mechanisms that might mask some of the functions of these receptors. Second, full body deletion of receptor population lacks region- and cell-specificity, which is particularly important in the case of α_{2A}-AR heteroreceptors, which can produce varied, often contradictory effects. In future studies use of adeno-associated viral vectors (AAVs) and the cre-recombinase/loxP system will allow for the selective α_{2A}-AR deletion in specific cell populations [251,252]. The role of intra-BNST α_{2A}-AR heteroreceptors on stress-induced reinstatement will be confirmed by AAV delivery into the BNST. Additionally, this approach could be used to establish the presynaptic locus of the anti-reinstatement actions of guanfacine, through the use of retro-viruses in α_{2A}-AR floxed mice in which cre-recombinase would result in the excision of *adra2a* gene in a pathway specific-manner [253]. These specific manipulations will allow the testing of the hypothesis that guanfacine blocks stress-induced reinstatement partially or totally through the inhibition of stress-induced glutamatergic release from PBN terminals onto the BNST, however, it is possible that other excitatory inputs such as the CeA may also play a role in the effects of guanfacine [212,254,255].

Additionally, the role of CRF signaling within the BNST on stress-induced and NE-mediated reinstatement of cocaine associated behaviors is well established [158,166,168]. Yet, how α_{2A}-AR heteroreceptors expressed in BNST CRF-producing neurons regulated BNST activity and output in response to agonists or stress remains unknown. *in situ* hybridization analyses aimed at classifying the BNST cell population activated by guanfacine (e.i cFOS-expressing), revealed a cell-autonomous pattern of activation within this region [211]. Activation by guanfacine was spread across cells expressing many peptides, with the only apparent common feature among the cells being the co-expression of α_{2A}-AR mRNAs [211]. The systematic deletion of α_{2A}-AR from discreet cell populations within the BNST will allow for a more causal exploration of the actions of these receptors in BNST physiology and responses.

Potential sex differences in α_{2A}-AR heteroreceptor regulation of BNST activity and stress-induced reinstatement

Stress and negative emotional experiences are more often cited as reasons for relapse in female, when compared to male patients with CUD [43,153,162]. The reasons underlying sex differences in relapse causes are complex, including different adrenergic and hormonal responses to stress as well as reported incidence of traumatic events leading to PTSD and comorbid substance use [79,256]. In clinical laboratory studies, female subjects with CUD show higher elevations in craving compared to male subjects, as well as activation of amygdala, hippocampal, and insular circuits in response to a stressful imagery exercise [158,161]. In stressful imagery exercise studies, guanfacine was statistically more effective at curbing craving in female subjects, a finding that was proposed to be mediated by the higher baseline of craving in female subject increasing the therapeutic window of the compound [79]. In addition to different responses

to α_{2A}-ARs, sex differences in the response to CRF, a downstream target of stress- and NE-, have been reported in patients with CUD [161].

Preclinical studies also show evidence of sex differences in stress-induced cocaine-associated behaviors. Female rodents show increased CPP acquisition in relationship to a relatively low dose of cocaine, an effect that is estrous cycle-dependent [257]. Female rodents also show more pronounce stress- and CRF-induced reinstatement of cocaine SA [168,258]. In addition to these findings, stress increases the co-expression of CRF and PRKδ in the BNST of females, but not male mice, suggesting that stress differentially regulates BNST cell populations that have been implicated in stress-induced reinstatement [212]. While it is possible that α_{2A}-AR heteroreceptors regulate BNST activity and reinstatement differently in male and female mice, we did not observe any apparent sex differences in the current studies, therefore, the results presented show data from mixed-sex data sets. However, these studies were not sufficiently powered to assess more subtle differences in behavior. Future studies will aim to determine sex-specific effects of α_{2A}-AR deletions and guanfacine treatments on stress-induced reinstatement.

$G_{\alpha i}$-GPCR activation within the BNST reinstates cocaine CPP: implications for the use of G_i-DREADDs as inhibitory tools

$G_{\alpha i}$-GPCR signaling has been canonically associated with inhibition of neuronal activity. $G_{\alpha i}$- suppresses adenylyl cyclase (AC) activity, cAMP production, and intracellular signaling [92]. In addition to direct actions at AC, $G_{\alpha i}$-GPCR can inhibit neuronal activity via $G_{\beta\gamma}$ -mediated G protein-coupled inwardly-rectifying potassium channel (GIRK) opening, which hyperpolarizes neurons, and through $G_{\beta\gamma}$ interactions with vesicular release machinery, which prevents neurotransmitter exocytosis [92,259]. Due to their inhibitory effects, many $G_{\alpha i}$-GPCR ligands are used within research and clinical settings, particularly in the treatment of conditions

characterized by imbalances in excitatory and inhibitory balance such as epilepsy and attention deficit/hyperactivity disorder (ADHD) [36,67,260]. $G_{\alpha i}$-GPCR also shows promise as a potential treatment for drug use disorders, particularly drug-withdrawal induced anxiety, negative affect, and relapse [63,261].

In light of these findings, it would be expected that $G_{\alpha i}$-GPCR activation within the BNST could decrease stress-induced activation of the BNST and subsequently, reinstatement. However, we found that activation of $G_{\alpha i}$- signaling in mice we extinguished CPP was sufficient to induce reinstatement, even in the absence of stress. We used a chemogenetic approach in which a $G_{\alpha i}$-coupled designer receptor exclusively activated by designer drug (G_i-DREADD) was bi-laterally expressed into the BNST. These G_i-DREADDs were activated via systemic injections of the DREADD ligand clozapine-n-oxide (CNO). The ability of CNO to induce DREADD activation and $G_{\alpha i}$-GPCR signaling has been extensively validated *in vitro* and *in vivo* [262]. While potential DREADD-independent psychotropic effects of CNO have been raised as a concern for *in vivo* chemogenetic studies, we did not observe any effects of CNO on locomotion or reinstatement behaviors in mice either expressing control proteins in the BNST or with off-target expression G_i-DREADDs. These observations suggest that the dose of CNO used in the current studies (3 mg/kg) did not produce confounding DREADD-independent effects.

We previously reported that within the BNST, G_i-DREADDs induce the activation of about 30 percent of cells, a proportion that is similar to that of high-dose guanfacine [211]. G_i-DREADDs also increased calcium *in vivo* transients, another measure of neuronal activity. Taken together, these findings suggested that G_i-DREADDs may act through a similar mechanism as α_{2A}-AR heteroreceptors, namely HCN channel gating, to increase BNST activity and behavioral output [211]. These findings also suggest that in other brain regions where α_{2A}-AR

heteroreceptors increase neuronal activity via HCN channel gating, such as the prefrontal cortex, G_i-DREADDs may also produce excitatory effects [140]. Although G_i-DREADD actions will vary based on cellular expression, the current findings challenge the general assumption that these receptors are putative inhibitory switches that can be readily employed in *in vivo* investigations of activity without ex vivo validation.

While some studies have reported slice electrophysiology validation of G_i-DREADD inhibitory actions alongside behavioral results, the practice has become less common as the use of chemogenetic technology has become more commonplace [263–265]. The use of chemogenetic systems that do not rely on $G_{\alpha i}$-GPCR signaling to produce inhibition, such as the Ivecmectin-gated chloride channel GluCαβ, or optogenetic silencers, may provide an alternative in HCN-channel-sensitive neuronal populations, but these tools have their own set of limitations [266–268]. Therefore, tools should always be validated to ensure that no confounding effects are introduced impede the proper interpretation of results.

Dose-targeted approaches for the treatment of CUD and stress-driven neuropathology with α_2-AR agonists

α_{2A}-AR agonists, such as clonidine and guanfacine, have been investigated as potential pharmacotherapies against relapse, while these compounds have been effective at decreasing stress-induced craving and sympathetic activation in patients with cocaine use disorder, they have not been efficacious at preventing relapse [84,85]. There are many factors underlying the lack of efficacy of α_{2A}-AR agonists in clinical studies, including attrition due to negative side effects [85]. Due to binding to other α_2-AR subtypes, particularly α_{2C}-ARs as well as imidazole receptors, clonidine produces strong sedative and hypotensive effects [66,269]. Lofexidine, another α_2-AR

agonist, has been reported to produce cognitive deficits, leading to high dropout rates in clinical trials [72].

The α_{2A}-AR agonist guanfacine has a more favorable therapeutic profile when compared to non-subtype selective compounds such as clonidine and lofexidine [66]. Additionally, the development of extended release guanfacine formulations has allowed for the administration of similar doses over longer periods of time, further minimizing adverse side-effects [87]. In a meta-analysis of phase 3 blind, placebo-controlled, studies of guanfacine extended release for the treatment of ADHD, a dose-dependent increase in adverse side effects was found for doses ranging from 1-4 mg [270]. The single doses used in clinical laboratory studies have been smaller than the ones used in extended-release formulations with ranges of 0.1-2 mg. Within this dose range, the primary adverse effect reported is dry mouth, with little or no sedation. However, the lower dose range also shows less efficacy in many laboratory tasks and conditions, including decreasing stress effects on cognitive flexibility in subjects with CUD [86,271]. Taken together, these findings underscore the importance of dose in the design and implementation of the α_2-AR agonist treatments.

Dose is also a critical factor in stress-induced reinstatement studies employing α_2-AR agonists. Systemic administration of clonidine blocks foot-shock stress-induced reinstatement of SA and forced swim-induced reinstatement of CPP [70,71]. Yet the anti-reinstatement actions of clonidine are dose dependent, a 0.03 mg/kg dose blocks reinstatement but a 0.3 mg/kg dose does not [70]. The lack of efficacy of clonidine at higher doses was ascribed to α_{2A}-AR-independent mechanisms, which also might explain the sedative and hyperlocomotive effects of higher doses [70]. However, guanfacine also showed a narrow efficacy window in the forced swim test, which is useful in the study of antidepressant-like actions, and is used as stressor to reinstate CPP [77]. Only

one out of five doses assayed in these studies (0.15 mg/kg) was able to decrease immobility, the primary measure of antidepressant-like actions [77]. Taken together, these studies show that the efficacy of α_{2A}-AR agonists to regulate stress-associated maladaptive behaviors is dose-dependent.

Since higher doses of guanfacine increase the activity of the BNST via the activation of α_{2A}-AR heteroreceptor and $G_{\alpha i}$-coupled signaling, it is likely that the likely that some of guanfacine's lack of clinical efficacy may be mediated by α_{2A}-AR heteroreceptor and extended amygdala activation. Future studies using functional imaging modalities should aim to determine if guanfacine produces dose-dependent changes in the activity of the BNST in healthy populations and populations with CUD.

Overall conclusions

Stress is often cited as a precipitating factor for relapse of cocaine use. Therefore, α_{2a}-AR agonists such as guanfacine, which decreases stress-driven responses such as craving, has been investigated as a potential treatment for relapse in CUD. However, guanfacine has not been shown to be efficacious at preventing relapse. The mechanism underlying the lack of efficacy of guanfacine is poorly understood, but the work presented in this thesis provides insight into how α_{2a}-AR auto- and heteroreceptor populations regulate guanfacine's actions. The work on this thesis also shows that α_{2a}-AR heteroreceptors are required for stress-induced reinstatement of cocaine CPP, a finding that broadens our understanding of how α_{2a}-ARs regulate behavior. Our findings also expand our understanding of the functional consequences of $G_{\alpha i}$-GPCR signaling within the extended amygdala.

Appendix

SNAP23Δ mice display altered cocaine CPP behaviors but normal responses to stress and guanfacine

Due to the necessity of α_{2A}-AR heteroreceptors for stress-induced reinstatement as well as the anti-reinstatement effects of a dose of guanfacine that does not increase BNST activity, it is proposed that loci of the anti-reinstatement actions of guanfacine might be presynaptic. $G_{\alpha i}$-GPCRs expressed in presynaptic terminals can inhibit neurotransmitter release in a variety of ways including the regulation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) and the direct inhibition of the vesicle release complex via $G_{\beta\gamma}$ interactions with soluble NSF-attachment protein receptors (SNAREs) [8,96].

To begin defining the possible role of $G_{\beta\gamma}$ – SNARE interactions on the anti-reinstatement effects of guanfacine, we utilized the SNAP25Δ3 mouse model in which the 3 terminal amino acids of the SNAP25 protein are truncated, resulting in a loss of receptor-specific inhibitory actions on neurotransmission [259]. SNAP25Δ3 mice display a wide range of physiological deficits, but alterations in their response to stress and a guanfacine inhibition of PBN excitatory input in the BNST suggest that $G_{\beta\gamma}$ – SNARE interactions may be important for the regulation of stress-induced reinstatement and BNST activity by α_{2A}-AR agonists [259].

To test this hypothesis, 6-12 week old male wild-type and SNAP25Δ3 mice underwent cocaine CPP acquisition, extinction and forced swim stress-induced reinstatement as described in chapter 2[78]. To access differences in stress-induced neuronal activity in brain regions that play a role in stress processing, mice were subjected to 6 minutes of forced swim stress before brain extraction and immunohistological staining for cFOS, as described in chapter 2 [78,212].

We found that SNAP25Δ3 mice did not show differences in cocaine-induced increases in locomotion during conditioning, with a main effect of treatment (saline vs cocaine) (two-way RM ANOVA; treatment $F_{3,257} = 45.04$, $p < .0001$) (**Fig. 23B**). WT and SNAP25Δ3 mice spent more time on the paired side following conditioning and there was a main effect of conditioning (PRE vs POST) and genotype (two-way repeated measures ANOVA, conditioning $F_{1,31} = 23$, $p < .0001$; Genotype $F_{1,31} = 1.58$, $p < .05$) (**Fig. 23C**). Sidak's multiple comparisons tests revealed both intra- and inter-genotype differences. However, there were no significant differences in the magnitude of conditioning, represented by the preference score, between SNAP25Δ3 and WT mice (**Fig. 23D**). These findings suggest that the SNAP25Δ3 mutation alters cocaine CPP expression but that the disruption does not inhibit the locomotor effects of cocaine or CPP acquisition.

Next, we determined the effects of the SNAP25Δ3 mutation of the extinction and stress-induced reinstatement of cocaine CPP (**Fig. 24A**). By day 2 of extinction training, SNAP25Δ3 mice spent less time in paired side when compared to WTs. Two-way ANOVAs revealed significant main effects of day and genotype (day $F_{3, 88} = 3.88$, $p < .05$, genotype $F_{1, 88} = 5.24$, $p < .05$). Sidak's multiple comparisons test revealed a significant difference between WT and SNAP25Δ3 mice on day 2 of extinction training (**Fig. 24B**). WT and SNAP25Δ3 mice spent more time in the paired side during the reinstatement test session, with a main effect of stress (two-way ANOVA, stress $F_{1,16} = 27.37$, $p < .0001$) (**Fig. 24C-D**). These findings suggest that the SNAP25Δ3 mutation decreases CPP retention during extinction without impacting reinstatement. The deficit in CPP retention is likely driven by the altered hippocampal activity reported in SNAP25Δ3 mice, since hippocampal function is critical for the regulation of cocaine CPP extinction and cocaine-associated memories [259,272].

Next, we assessed potential changes in stress-induced neuronal activation in regions that have been implicated in stress processing and drug-associated behaviors [73,247] (**Fig. 25A**). We found no significant differences in forced swim stress-induced cFOS expression across the brain, including the BNST (**Fig. 25B-C**). Additionally, in preliminary studies, guanfacine appears to be equally effective at preventing stress-induced reinstatement of cocaine CPP in WT and SNAP25Δ3 mice (**Fig. 26**).

Overall, these findings suggest that $G_{\beta\gamma}$-SNARE interactions may not play a significant role in the anti-reinstatement effects of guanfacine. However, several caveats need to be taken into consideration while interpreting these results. First, these studies are currently underpowered. Second, the SNAP25Δ3 truncation is a life-long mutation that spans the whole body, therefore compensatory mechanisms may mask the potential effects of $G_{\beta\gamma}$-SNARE decoupling on the pharmacological actions of α_{2A}-AR agonists. Thus, further studies using pathway and region-specific manipulations of $G_{\beta\gamma}$-SNARE are warranted to uncover potential effects.

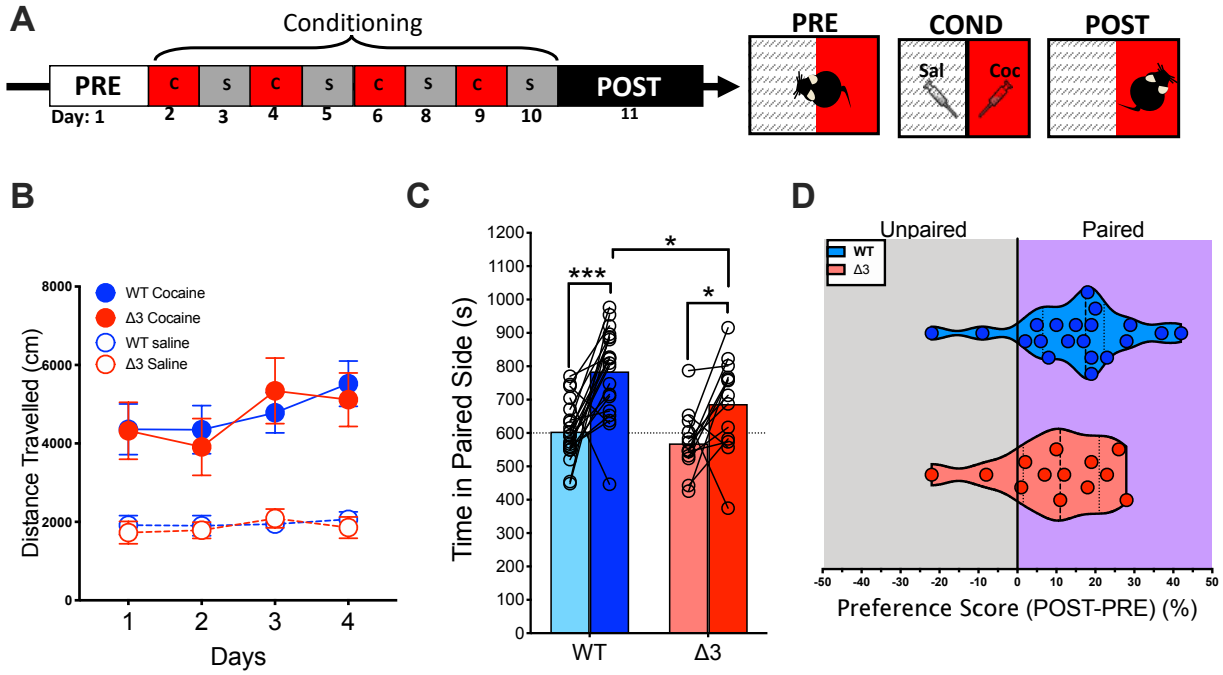

Figure 23. SNAP25Δ3 mice show normal locomotor responses to cocaine and reduced cocaine CPP. A. Experimental design and timeline. **B.** Distance travelled during conditioning in WT and SNAP23Δ mice. **C.** WT and SNAP23Δ mice spend more time in the paired side following conditioning, but there was inter-group difference in the amount of time mice from both groups spend in the paired side during the post-conditioning test session. **D.** Magnitude of CPP, represented by the preference score for WT and SNAP23Δ mice. Data displayed as means and superimposed individual data points, or means plus quartiles (*p < .05, ***p < .001).

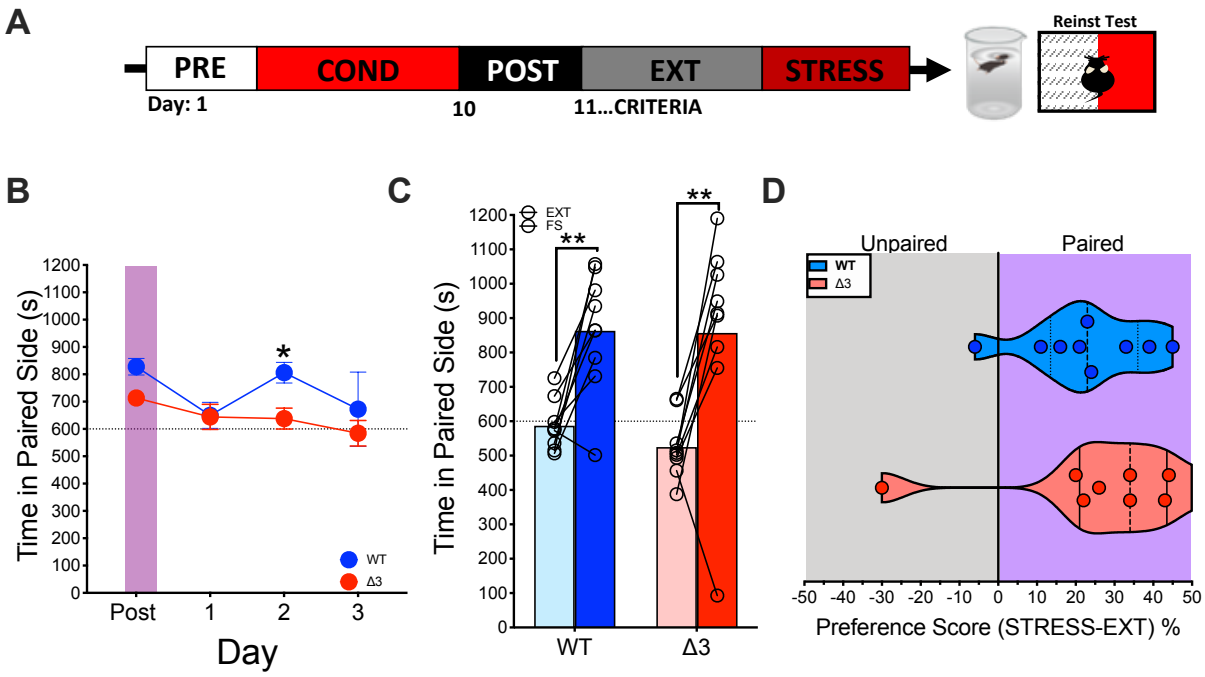

Figure 24. SNAP25Δ3 mice show accelerated extinction of cocaine CPP and no differences in stress-induced reinstatement. A. Experimental design and timeline. **B.** SNAP23Δ mice show significant extinction of cocaine CPP by extinction day 2 when compared to WT mice. **C.** WT and SNAP23Δ mice spend more time in the paired side during the forced swim stress-induced reinstatement test session (FS) when compared to the last day of extinction (EXT). **D.** Magnitude of reinstatement, represented by the preference score for WT and SNAP23Δ mice. Data displayed as means and superimposed individual data points, or means plus quartiles (**p < .01).

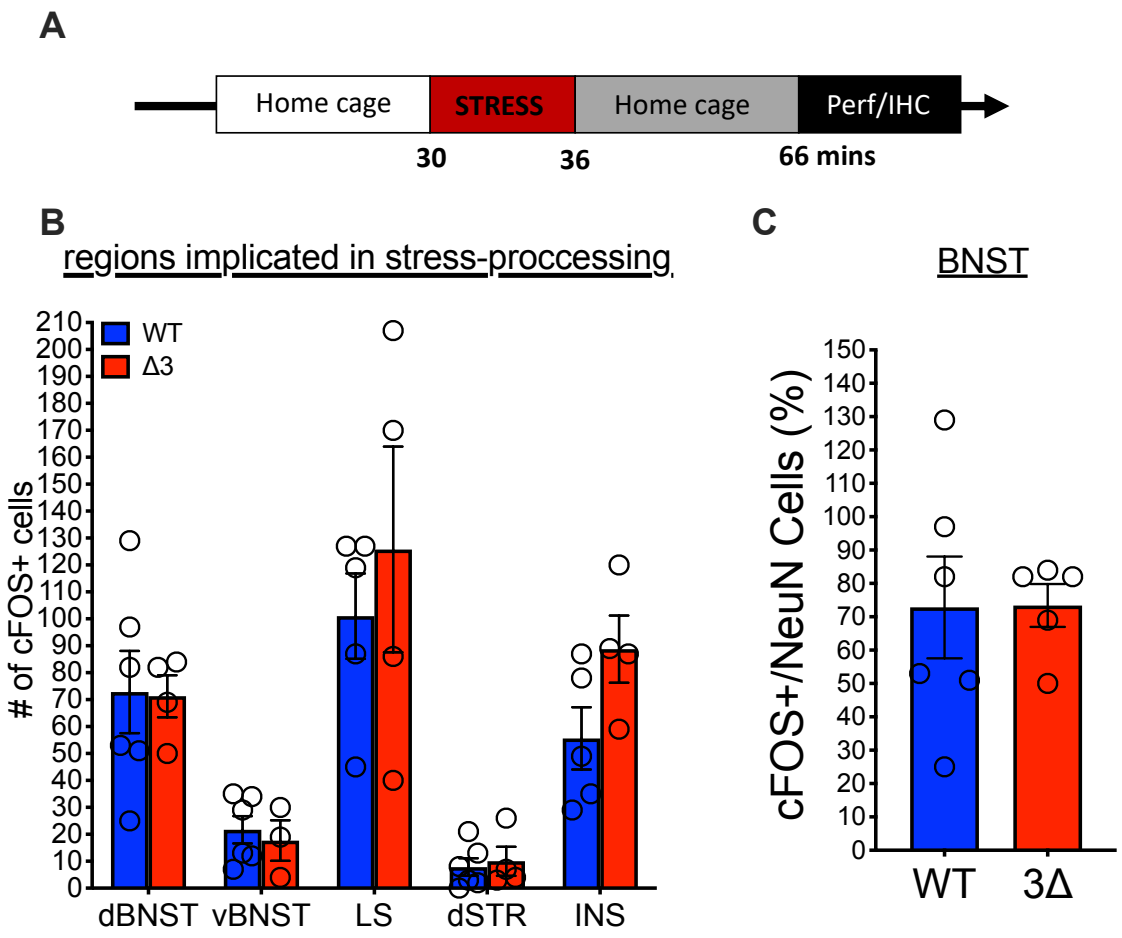

Figure 25. Lack of stress-induced differences in neuronal activation in SNAP25Δ3 and WT mice. A. Experimental design of stress-induced neuronal activation assay. **B.** Quantification of cFOS in brain regions implicated in stress-induced responses following forced swim stress. **C.** quantification of cFOS following stress in the BNST of SNAP23Δ and WT mice. Data displayed as means +/- SEM and superimposed individual data points) (perf = perfusion, IHC = Immunohistochemistry) (dBNST = dorsal BNST, vBNST = ventral BNST, LS = lateral septum, dSTR = dorsal striatum, INS = insula.

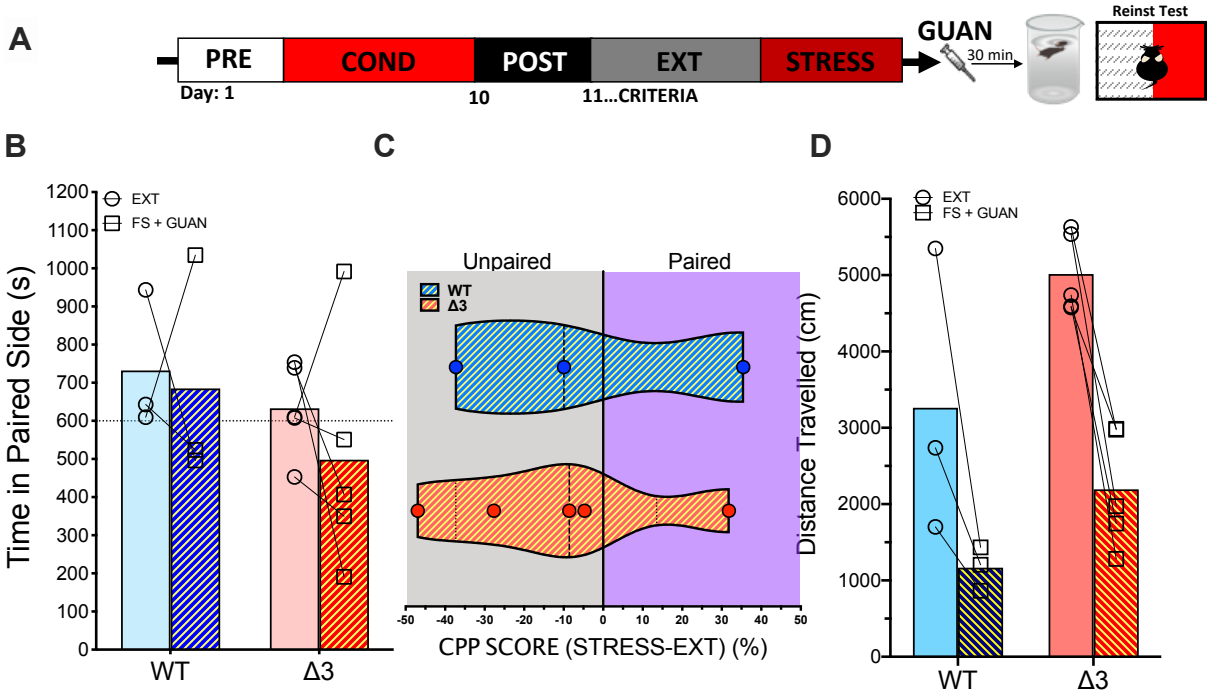

Figure 26. preliminary findings showing guanfacine blockade of stress-induced reinstatement of cocaine CPP in SNAP23Δ mice. A. Experimental design and timeline. **B.** Guanfacine prevents stress-induced increases in spent on the time on the paired side in WT and SNAP23Δ mice. **C.** preference score for WT and SNAP23Δ mice. **D.** Distance travelled by WT and SNAP23Δ during the last day of extinction (EXT) and during the forced swim stress-induced reinstatement test (FS). Data displayed as means plus superimposed individual data points.

Bibliography

1. Stolberg, V. B. The use of coca: Prehistory, history, and ethnography. *Journal of Ethnicity in Substance Abuse* **10**, 126–146 (2011).
2. Rivera, M. A., Aufderheide, A. C., Cartmell, L. W., Torres, C. M. & Langsjoen, O. Antiquity of coca-leaf chewing in the south central andes: A 3,000 Year archaeological record of coca-leaf chewing from Northern Chile. *J. Psychoactive Drugs* **37**, 455–458 (2005).
3. Goldstein, R. A., DesLauriers, C. & Burda, A. M. Cocaine: History, Social Implications, and Toxicity-A Review. *Disease-a-Month* **55**, 6–38 (2009).
4. Satel, S. L., Southwick, S. M. & Gawin, F. H. Clinical features of cocaine-induced paranoia. *Am. J. Psychiatry* **148**, 495–498 (1991).
5. Johanson, C.-E. & Fischman, M. The Pharmacology of Cocaine Related to Its Abuse*. *Pharmacol. Rev.* **41**, 3–52 (1989).
6. Uhl, G. R., Hall, F. S. & Sora, I. Cocaine, reward, movement and monoamine transporters. *Mol. Psychiatry* **7**, 21–26 (2002).
7. O'Leary, M. E. & Chahine, M. Cocaine binds to a common site on open and inactivated human heart (Nav 1.5) sodium channels. *J. Physiol.* **541**, 701–716 (2002).
8. O'Leary, M. E. & Hancox, J. C. Role of voltage-gated sodium, potassium and calcium channels in the development of cocaine-associated cardiac arrhythmias. *Br. J. Clin. Pharmacol.* **69**, 427–442 (2010).
9. Xiao, Y. F. *et al.* Electrophysiologic properties of lidocaine, cocaine, and n-3 fatty-acids block of cardiac Na+ channels. *Eur. J. Pharmacol.* **485**, 31–41 (2004).
10. Schwartz, B. G., Rezkalla, S. & Kloner, R. A. Cardiovascular effects of cocaine. *Circulation* **122**, 2558–2569 (2010).
11. Dela Peña, I., Gevorkiana, R. & Shi, W. X. Psychostimulants affect dopamine transmission through both dopamine transporter-dependent and independent mechanisms. *Eur. J. Pharmacol.* **764**, 562–570 (2015).
12. Simmler, L. D. *et al.* Blockade of the 5-HT transporter contributes to the behavioural, neuronal and molecular effects of cocaine. *Br. J. Pharmacol.* **174**, 2716–2738 (2017).
13. Jones, J. D., Hall, F. S., Uhl, G. R., Rice, K. & Riley, A. L. Differential involvement of the norepinephrine, serotonin and dopamine reuptake transporter proteins in cocaine-induced taste aversion. *Pharmacol. Biochem. Behav.* **93**, 75–81 (2009).
14. Lüscher, C. & Ungless, M. A. The mechanistic classification of addictive drugs. *PLoS Med.* **3**, 2005–2010 (2006).
15. De Wit, H. & Wise, R. A. Blockade of cocaine reinforcement in rats with the dopamine receptor blocker pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. *Can. J. Psychol.* **31**, 195–203 (1977).
16. Volkow, N. D., Fowler, J. S. & Wang, G. J. Role of dopamine in drug reinforcement and addiction in humans: Results from imaging studies. *Behav. Pharmacol.* **13**, 355–366 (2002).
17. Ikegami, A. & Duvauchelle, C. L. Dopamine Mechanisms and Cocaine Reward. *Int. Rev. Neurobiol.* **62**, 45–94 (2004).
18. Ritz, M. C., Lamb, R. J., Goldberg, S. R. & Kuhar, M. J. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science (80-.).* **237**, 1219–1223 (1987).

19. Roberts, D. C. S., Corcoran, M. E. & Fibiger, H. C. On the role of ascending catecholaminergic systems in intravenous self-administration of cocaine. *Pharmacol. Biochem. Behav.* **6**, 615–620 (1977).
20. Andrews, C. M. & Lucki, I. Effects of cocaine on extracellular dopamine and serotonin levels in the nucleus accumbens. *Psychopharmacology (Berl).* **155**, 221–229 (2001).
21. Ferraro, T. N. *et al.* Cocaine intake by rats correlates with cocaine-induced dopamine changes in the nucleus accumbens shell. *Pharmacol. Biochem. Behav.* **66**, 397–401 (2000).
22. Kalivas, P. W. & Volkow, N. D. The neural basis of addiction: A pathology of motivation and choice. *Am. J. Psychiatry* **162**, 1403–1413 (2005).
23. Di Chiara, G. & Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5274–5278 (1988).
24. Rocha, B. A. Stimulant and reinforcing effects of cocaine in monoamine transporter knockout mice. *Eur. J. Pharmacol.* **479**, 107–115 (2003).
25. Hall, F. S. *et al.* Cocaine-conditioned locomotion in dopamine transporter, norepinephrine transporter and 5-HT transporter knockout mice. *Neuroscience* **162**, 870–880 (2009).
26. Rocha, B. A. *et al.* Cocaine self-administration in dopamine-transporter knockout mice. *Nat. Neurosci.* **1**, 132–137 (1998).
27. Giros, B., Jaber, M., Jones, S. R., Wightman, R. M. & Caron, M. G. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606–612 (1996).
28. Jones, S. R. *et al.* Profound neuronal plasticity in response to inactivation of the dopamine transporter. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4029–4034 (1998).
29. Mahler, S. V. *et al.* Chemogenetic manipulations of ventral tegmental area dopamine neurons reveal multifaceted roles in cocaine abuse. *J. Neurosci.* **39**, 503–518 (2019).
30. Sora, I. *et al.* Molecular mechanisms of cocaine reward: Combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5300–5305 (2001).
31. Hall, F. S. *et al.* Cocaine mechanisms: Enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. *Neuroscience* **115**, 153–161 (2002).
32. Chen, R. *et al.* Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 9333–9338 (2006).
33. Proctor, S. L., Kopak, A. M. & Hoffmann, N. G. Compatibility of current DSM-IV and proposed DSM-5 diagnostic criteria for cocaine use disorders. *Addict. Behav.* **37**, 722–728 (2012).
34. Bose, J., Hedden, S. L., Lipari, R. N. & Park-Lee, E. Key substance use and mental health indicators in the United States: Results from the 2015 National Survey on Drug Use and Health. Publication No. SMA 16-4984, NSDUH Series H-51. *Subst. Abus. Ment. Heal. Serv. Adm.* 1–97 (2018). doi:10.1016/j.drugalcdep.2016.10.042
35. John, W. S. & Wu, L. T. Trends and correlates of cocaine use and cocaine use disorder in the United States from 2011 to 2015. *Drug Alcohol Depend.* **180**, 376–384 (2017).
36. Kampman, K. M. The treatment of cocaine use disorder. *Sci. Adv.* **5**, 1–9 (2019).
37. Hedegaard, H., Bastian, B. A., Trinidad, J. P., Spencer, M. & Warner, M. Drugs most frequently involved in drug overdose deaths: United states, 2011–2016. *Natl. Vital Stat.*

Reports **67**, (2018).
38. Kim, S. T. & Park, T. Acute and chronic effects of cocaine on cardiovascular health. *Int. J. Mol. Sci.* **20**, 1–22 (2019).
39. Frazer, K. M., Richards, Q. & Keith, D. R. The long-term effects of cocaine use on cognitive functioning: A systematic critical review. *Behav. Brain Res.* **348**, 241–262 (2018).
40. Blanco-Presas, L. *et al.* Cognitive impairment associated with cocaine use: The role of co-existent alcohol abuse/dependence. *Drug Alcohol Depend.* **189**, 70–75 (2018).
41. Pérez-Arce, P., Carr, K. D. & Sorensen, J. L. Cultural issues in an outpatient program for stimulant abusers†. *J. Psychoactive Drugs* **25**, 35–44 (1993).
42. Lappan, S. N., Brown, A. W. & Hendricks, P. S. Dropout rates of in-person psychosocial substance use disorder treatments: a systematic review and meta-analysis. *Addiction* **115**, 201–217 (2020).
43. Sayre, S. L. *et al.* Determining predictors of attrition in an outpatient substance abuse program. *Am. J. Drug Alcohol Abuse* **28**, 55–72 (2002).
44. Alterman, A. I., McKay, J. R., Mulvaney, F. D. & McLellan, A. T. Prediction of attrition from day hospital treatment in lower socioeconomic cocaine-dependent men. *Drug Alcohol Depend.* **40**, 227–233 (1996).
45. White, J. M., Winn, K. I. & Young, W. Predictors of attrition from an outpatient chemical dependency program. *Subst. Abus.* **19**, 49–59 (1998).
46. Pomara, C. *et al.* Data Available on the Extent of Cocaine Use and Dependence: Biochemistry, Pharmacologic Effects and Global Burden of Disease of Cocaine Abusers. *Curr. Med. Chem.* **19**, 5647–5657 (2012).
47. Hedegaard, H., Miniño, A. M. & Warner, M. Drug Overdose Deaths in the United States, 1999-2017. *NCHS Data Brief* 1–8 (2018).
48. Grabowski, J. *et al.* Dextroamphetamine for cocaine-dependence treatment: A double-blind randomized clinical trial. *J. Clin. Psychopharmacol.* **21**, 522–526 (2001).
49. Berro, L. F., Frussa-Filho, R., Tufik, S. & Andersen, M. L. Treatment of cocaine addiction with amphetamine, a sleep-suppressant drug: Associative learning, sleep patterns and clinical perspectives. *Psychopharmacology (Berl).* **231**, 457–458 (2014).
50. Nuijten, M. *et al.* Sustained-release dexamfetamine in the treatment of chronic cocaine-dependent patients on heroin-assisted treatment: A randomised, double-blind, placebo-controlled trial. *Lancet* **387**, 2226–2234 (2016).
51. Rush, C. R., Stoops, W. W., Sevak, R. J. & Hays, L. R. Cocaine choice in humans during D-amphetamine maintenance. *J. Clin. Psychopharmacol.* **30**, 152–159 (2010).
52. Greenwald, M. K., Lundahl, L. H. & Steinmiller, C. L. Sustained release d-amphetamine reduces cocaine but not speedball-seeking in buprenorphine-maintained volunteers: A test of dual-agonist pharmacotherapy for cocaine/heroin polydrug abusers. *Neuropsychopharmacology* **35**, 2624–2637 (2010).
53. Hart, C. L., Haney, M., Vosburg, S. K., Rubin, E. & Foltin, R. W. Smoked cocaine self-administration is decreased by modafinil. *Neuropsychopharmacology* **33**, 761–768 (2008).
54. Dackis, C. A., Kampman, K. M., Lynch, K. G., Pettinati, H. M. & O'Brien, C. P. A double-blind, placebo-controlled trial of modafinil for cocaine dependence. *Neuropsychopharmacology* **30**, 205–211 (2005).
55. Dackis, C. A. *et al.* A double-blind, placebo-controlled trial of modafinil for cocaine dependence. *J. Subst. Abuse Treat.* **43**, 303–312 (2012).

56. Kampman, K. M. *et al.* A double-blind, placebo-controlled trial of amantadine, propranolol, and their combination for the treatment of cocaine dependence in patients with severe cocaine withdrawal symptoms. *Drug Alcohol Depend.* **85**, 129–137 (2006).
57. Kampman, K. M. *et al.* Effectiveness of propranolol for cocaine dependence treatment may depend on cocaine withdrawal symptom severity. *Drug Alcohol Depend.* **63**, 69–78 (2001).
58. Fox, H. C. *et al.* Guanfacine effects on stress, drug craving and prefrontal activation in cocaine dependent individuals: Preliminary findings. *J. Psychopharmacol.* **26**, 958–972 (2012).
59. Shorter, D., Lindsay, J. A. & Kosten, T. R. The alpha-1 adrenergic antagonist doxazosin for treatment of cocaine dependence: A pilot study. *Drug Alcohol Depend.* **131**, 66–70 (2013).
60. Newton, T. F. *et al.* Noradrenergic α 1 receptor antagonist treatment attenuates positive subjective effects of cocaine in humans: A randomized trial. *PLoS One* **7**, 1–9 (2012).
61. Shorter, D. I. *et al.* Doxazosin treatment in cocaine use disorder: pharmacogenetic response based on an alpha-1 adrenoreceptor subtype D genetic variant. *Am. J. Drug Alcohol Abuse* **2990**, (2020).
62. Sofuoglu, M. *et al.* Carvedilol does not reduce cocaine use in methadone-maintained cocaine users. *J. Subst. Abuse Treat.* **73**, 63–69 (2017).
63. Fox, H. & Sinha, R. *The role of guanfacine as a therapeutic agent to address stress-related pathophysiology in cocaine-dependent individuals*. *Advances in Pharmacology* **69**, (Elsevier Inc., 2014).
64. Gowing, L., Farrell, M., Ali, R. & Jm, W. Alpha 2 -adrenergic agonists for the management of opioid withdrawal (Review). *Library (Lond).* 10–13 (2009). doi:10.1002/14651858.CD002024.pub5.www.cochranelibrary.com
65. Jobes, M. L. *et al.* Clonidine blocks stress-induced craving in cocaine users. *Psychopharmacology (Berl).* **218**, 83–88 (2011).
66. Seedat, Y. K. Clonidine and guanfacine - Comparison of their effects on haemodynamics in hypertension. *South African Med. J.* **67**, 557–559 (1985).
67. Fiks, A. G. *et al.* Changing patterns of alpha agonist medication use in children and adolescents 2009-2011. *J. Child Adolesc. Psychopharmacol.* **25**, 362–367 (2015).
68. Platt, D. M., Rowlett, J. K. & Spealman, R. D. Noradrenergic mechanisms in cocaine-induced reinstatement of drug seeking in squirrel monkeys. *J. Pharmacol. Exp. Ther.* **322**, 894–902 (2007).
69. Vranjkovic, O., Gasser, P. J., Gerndt, C. H., Baker, D. A. & Mantsch, J. R. Stress-Induced Cocaine Seeking Requires a Beta-2 Adrenergic Receptor-Regulated Pathway from the Ventral Bed Nucleus of the Stria Terminalis That Regulates CRF Actions in the Ventral Tegmental Area. *J. Neurosci.* **34**, 12504 –12514 (2014).
70. Mantsch, J. R. *et al.* Involvement of noradrenergic neurotransmission in the stress- but not cocaine-induced reinstatement of extinguished cocaine-induced conditioned place preference in mice: role for β-2 adrenergic receptors. *Neuropsychopharmacology* **35**, 2165–2178 (2010).
71. Erb, S. *et al.* Alpha-2 adrenergic receptor agonists block stress-induced reinstatement of cocaine seeking. *Neuropsychopharmacology* **23**, 138–150 (2000).
72. Highfield, D., Yap, J., Grimm, J. W., Shalev, U. & Shaham, Y. Repeated lofexidine treatment attenuates stress-induced, but not drug cues-induced reinstatement of a heroin-

cocaine mixture (speedball) seeking in rats. *Neuropsychopharmacology* **25**, 320–331 (2001).
73. Mantsch, J. R., Baker, D. A., Funk, D., Lê, A. D. & Shaham, Y. Stress-induced reinstatement of drug seeking: 20 years of progress. *Neuropsychopharmacology* **41**, 335–356 (2016).
74. Feltenstein, M. W., Henderson, A. R. & See, R. E. Enhancement of cue-induced reinstatement of cocaine-seeking in rats by yohimbine: Sex differences and the role of the estrous cycle. *Psychopharmacology (Berl).* **216**, 53–62 (2011).
75. Buffalari, D. M., Baldwin, C. K. & See, R. E. Treatment of cocaine withdrawal anxiety with guanfacine: Relationships to cocaine intake and reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl).* **223**, 179–190 (2012).
76. Mineur, Y. S. *et al.* Interaction between noradrenergic and cholinergic signaling in amygdala regulates anxiety- and depression-related behaviors in mice. *Neuropsychopharmacology* **43**, 2118–2125 (2018).
77. Mineur, Y. S. *et al.* Antidepressant-like effects of guanfacine and sex-specific differences in effects on c-fos immunoreactivity and paired-pulse ratio in male and female mice. *Psychopharmacology (Berl).* **232**, 3539–3549 (2015).
78. Perez, R. E. *et al.* α 2A-adrenergic heteroreceptors are required for stress-induced reinstatement of cocaine conditioned place preference. *Neuropsychopharmacology* **2**, 1–9 (2020).
79. Fox, H. C., Morgan, P. T. & Sinha, R. Sex differences in guanfacine effects on drug craving and stress arousal in cocaine-dependent individuals. *Neuropsychopharmacology* **39**, 1527–1537 (2014).
80. McKee, S. A. *et al.* A translational investigation targeting stress-reactivity and prefrontal cognitive control with guanfacine for smoking cessation. *J. Psychopharmacol.* **29**, 300–311 (2015).
81. Hains, A. B., Yabe, Y. & Arnsten, A. F. T. Chronic stimulation of alpha-2A-adrenoceptors with guanfacine protects rodent prefrontal cortex dendritic spines and cognition from the effects of chronic stress. *Neurobiol. Stress* **2**, 1–9 (2015).
82. Ramos, B. P., Stark, D., Verduzco, L., Van Dyck, C. H. & Arnsten, A. F. T. α2A-adrenoceptor stimulation improves prefrontal cortical regulation of behavior through inhibition of cAMP signaling in aging animals. *Learn. Mem.* **13**, 770–776 (2006).
83. Wang, M. *et al.* a2A-Adrenoceptors Strengthen Working Memory Networks by Inhibiting cAMP-HCN Channel Signaling in Prefrontal Cortex. *Cell* **129**, 397–410 (2007).
84. Haney, M. *et al.* Guanfacine decreases symptoms of cannabis withdrawal in daily cannabis smokers. *Addict. Biol.* **24**, 707–716 (2019).
85. Krupitsky, E. *et al.* Naltrexone with or without guanfacine for preventing relapse to opiate addiction in St.-Petersburg, Russia. *Drug Alcohol Depend.* **132**, 674–680 (2013).
86. Barcelos, N. M. *et al.* Guanfacine treatment for prefrontal cognitive dysfunction in older participants: a randomized clinical trial. *Neurobiol. Aging* **70**, 117–124 (2018).
87. Verplaetse, T. L. *et al.* Pharmacokinetics and pharmacodynamics of immediate-release versus extended-release guanfacine in adult daily smokers. *J. Clin. Psychopharmacol.* **39**, 124–128 (2019).
88. Berridge, C. W. & Waterhouse, B. D. The locus coeruleus-noradrenergic system: Modulation of behavioral state and state-dependent cognitive processes. *Brain Research Reviews* **42**, 33–84 (2003).

89. Fox, M. E., Bucher, E. S., Johnson, J. A. & Wightman, R. M. Medullary Norepinephrine Projections Release Norepinephrine into the Contralateral Bed Nucleus of the Stria Terminalis. *ACS Chem. Neurosci.* **7**, 1681–1689 (2016).
90. Bucher, E. S. *et al.* Medullary norepinephrine neurons modulate local oxygen concentrations in the bed nucleus of the stria terminalis. *J. Cereb. Blood Flow Metab.* **34**, 1128–1137 (2014).
91. Szabadi, E. Functional neuroanatomy of the central noradrenergic system. *Journal of Psychopharmacology* **27**, 659–693 (2013).
92. Limbird, L. E. Historical Perspective for Understanding of Adrenergic Receptors. *Curr. Top. Membr.* **67**, 1–17 (2011).
93. Ahlquist, R. P. A study of the adrenotropic receptors. *Am. J. Physiol.* **153**, 586–600 (1948).
94. Alamo, C., López-Muñoz, F. & Sánchez-García, J. Mechanism of action of guanfacine: a postsynaptic differential approach to the treatment of attention deficit hyperactivity disorder (adhd). *Actas Esp. Psiquiatr.* **44**, 107–12 (2016).
95. Dolphin, A. C. Mechanisms of modulation of voltage-dependent calcium channels by G proteins. *J. Physiol.* **506**, 3–11 (1998).
96. Yoon, E. J., Gerachshenko, T., Spiegelberg, B. D., Alford, S. & Hamm, H. E. Gβγ interferes with Ca2+-dependent binding of synaptotagmin to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. *Mol. Pharmacol.* **72**, 1210–1219 (2007).
97. Mcelligott, Z. A. & Winder, D. G. Modulation of glutamatergic synaptic transmission in the bed nucleus of the stria terminalis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **33**, 1329–1335 (2009).
98. Gilsbach, R., Albarrán-Juárez, J. & Hein, L. Pre- versus Postsynaptic Signaling by α 2-Adrenoceptors. *Curr. Top. Membr.* **67**, 139–160 (2011).
99. Gilsbach, R. & Hein, L. Are the pharmacology and physiology of α 2adrenoceptors determined by α 2-heteroreceptors and autoreceptors respectively? *British Journal of Pharmacology* **165**, 90–102 (2012).
100. Brede, M., Philipp, M., Knaus, A., Muthig, V. & Hein, L. alpha2-adrenergic receptor subtypes - novel functions uncovered in gene-targeted mouse models. *Biol. cell* **96**, 343–8 (2004).
101. Jahan, K., Pillai, K. K. & Vohora, D. DSP-4 induced depletion of brain noradrenaline and increased 6-hertz psychomotor seizure susceptibility in mice is prevented by sodium valproate. *Brain Res. Bull.* **142**, 263–269 (2018).
102. Gilsbach, R. *et al.* Genetic dissection of α2-adrenoceptor functions in adrenergic versus nonadrenergic cells. *Mol. Pharmacol.* **75**, 1160–1170 (2009).
103. Starke, K. Presynaptic autoreceptors in the third decade: focus on α2-adrenoceptors. *J. Neurochem.* **78**, 685–693 (2001).
104. Westfall, T. C. Evidence that noradrenergic transmitter release is regulated by presynaptic receptors. *Fed. Proc.* **43**, 1352–7 (1984).
105. Lewczuk, B. Presynaptic autoregulation of norepinephrine release from the sympathetic nerve fibers in the pineal gland of the domestic pig. Pharmacological characterization of alpha(2)adrenoceptors mediating this process. *Neuro Endocrinol. Lett.* **23 Suppl 1**, 111–7 (2002).
106. Trendelenburg, A. U. *et al.* All three α2-adrenoceptor types serve as autoreceptors in

postganglionic sympathetic neurons. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **368**, 504–512 (2003).
107. Bücheler, M. M., Hadamek, K. & Hein, L. Two α2-adrenergic receptor subtypes, α2A and α2C, inhibit transmitter release in the brain of gene-targeted mice. *Neuroscience* **109**, 819–826 (2002).
108. Boehm, S. Presynaptic α2-adrenoceptors control excitatory, but not inhibitory, transmission at rat hippocampal synapses. *J. Physiol.* **519**, 439–449 (1999).
109. Gilsbach, R. *et al.* Sympathetic α2-adrenoceptors prevent cardiac hypertrophy and fibrosis in mice at baseline but not after chronic pressure overload. *Cardiovasc. Res.* **86**, 432–442 (2010).
110. Kobinger, W. Central blood pressure regulation. Involvement of presynaptic or postsynaptic, alpha 1- or alpha 2-adrenoceptors? *Chest* **83**, 296–9 (1983).
111. Kobinger, W. & Walland, A. Facilitation of vagal reflex bradycardia by an action of clonidine on central α-receptors. *Eur. J. Pharmacol.* **19**, 210–217 (1972).
112. Walland, A., Kobinger, W. & Csongrady, A. Action of clonidine on baroreceptor reflexes in conscious dogs. *Eur. J. Pharmacol.* **26**, 184–190 (1974).
113. Hoefke, W., Kobinger, W. & Walland, A. Relationship between activity and structure in derivatives of clonidine. *Arzneimittelforschung.* **25**, 786–93 (1975).
114. Cohn, J. N. *et al.* Plasma Norepinephrine as a Guide to Prognosis in Patients with Chronic Congestive Heart Failure. *N. Engl. J. Med.* **311**, 819–823 (1984).
115. Cottingham, C. & Wang, Q. α2 adrenergic receptor dysregulation in depressive disorders: Implications for the neurobiology of depression and antidepressant therapy. *Neurosci. Biobehav. Rev.* **36**, 2214–2225 (2012).
116. Callado, L. F. *et al.* Selective Increase of α2A-Adrenoceptor Agonist Binding Sites in Brains of Depressed Suicide Victims. *J. Neurochem.* **70**, 1114–1123 (2002).
117. Escribá, P. V., Ozaita, A. & García-Sevilla, J. A. Increased mRNA expression of α2A-adrenoceptors, serotonin and receptors and μ-opioid receptors in the brains of suicide victims. *Neuropsychopharmacology* **29**, 1512–1521 (2004).
118. García-Sevilla, J. A. *et al.* Up-regulation of immunolabeled α(2A)-adrenoceptors, G(i) coupling proteins, and regulatory receptor kinases in the prefrontal cortex of depressed suicides. *J. Neurochem.* **72**, 282–291 (1999).
119. Wang, B., Wang, Y., Wu, Q., Huang, H. P. & Li, S. Effects of α2A adrenoceptors on norepinephrine secretion from the locus coeruleus during chronic stress-induced depression. *Front. Neurosci.* **11**, 1–8 (2017).
120. Schramm, N. L., McDonald, M. P. & Limbird, L. E. The α2A-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. *J. Neurosci.* **21**, 4875–4882 (2001).
121. Kanno, O. & Clarenbach, P. Effect of clonidine and yohimbine on sleep in man: Polygraphic study and EEG analysis by normalized slope descriptors. *Electroencephalogr. Clin. Neurophysiol.* **60**, 478–484 (1985).
122. Takahashi, K., Kayama, Y., Lin, J. S. & Sakai, K. Locus coeruleus neuronal activity during the sleep-waking cycle in mice. *Neuroscience* **169**, 1115–1126 (2010).
123. Swift, K. M. *et al.* Abnormal Locus Coeruleus Sleep Activity Alters Sleep Signatures of Memory Consolidation and Impairs Place Cell Stability and Spatial Memory. *Curr. Biol.* **28**, 3599-3609.e4 (2018).
124. Aston-Jones, G., Chiang, C. & Alexinsky, T. *Discharge of noradrenergic locus coeruleus*

neurons in behaving rats and monkeys suggests a role in vigilance. *Progress in Brain Research* **88**, (Elsevier B.V., 1991).
125. Porter-Stransky, K. A. *et al.* Noradrenergic Transmission at Alpha1-Adrenergic Receptors in the Ventral Periaqueductal Gray Modulates Arousal. *Biol. Psychiatry* **85**, 237–247 (2019).
126. Yu, X., Franks, N. P. & Wisden, W. Sleep and sedative states induced by targeting the histamine and noradrenergic systems. *Frontiers in Neural Circuits* **12**, 4 (2018).
127. Ramesh, V. & Kumar, V. M. The role of alpha-2 receptors in the medial preoptic area in the regulation of sleep-wakefulness and body temperature. *Neuroscience* **85**, 807–17 (1998).
128. Zhang, Z. *et al.* Neuronal ensembles sufficient for recovery sleep and the sedative actions of α 2 adrenergic agonists. *Nat. Neurosci.* **18**, 553–561 (2015).
129. Szot, P. *et al.* The anticonvulsant and proconvulsant effects of α2- adrenoreceptor agonists are mediated by distinct populations of α2a-adrenoreceptors. *Neuroscience* **126**, 795–803 (2004).
130. Weinshenker, D. & Szot, P. The role of catecholamines in seizure susceptibility: New results using genetically engineered mice. *Pharmacol. Ther.* **94**, 213–233 (2002).
131. Suenaga, N. The Role of the Locus Coeruleus in Regulation of Seizure Susceptibility in Rats. *Jpn. J. Pharmacol.* **32**, 1075–1081 (1982).
132. Philbin, K. E., Bateman, R. J. & Mendelowitz, D. Clonidine, an α 2-receptor agonist, diminishes GABAergic neurotransmission to cardiac vagal neurons in the nucleus ambiguus. *Brain Res.* **1347**, 65–70 (2010).
133. Shi, T. J. S., Winzer-Serhan, U., Leslie, F. & Hökfelt, T. Distribution and regulation of α2-adrenoceptors in rat dorsal root ganglia. *Pain* **84**, 319–330 (2000).
134. Howe, J. R., Yaksh, T. L. & Go, V. L. W. The effect of unilateral dorsal root ganglionectomies or ventral rhizotomies on α2-adrenoceptor binding to, and the substance P, enkephalin, and neurotensin content of, the cat lumbar spinal cord. *Neuroscience* **21**, 385–394 (1987).
135. Pan, Y. Z., Li, D. P. & Pan, H. L. Inhibition of glutamatergic synaptic input to spinal lamina IIo neurons by presynaptic α2-adrenergic receptors. *J. Neurophysiol.* **87**, 1938–1947 (2002).
136. Ogon, I. *et al.* Sympathectomy and sympathetic blockade reduce pain behavior via alpha-2 adrenoceptor of the dorsal root ganglion neurons in a lumbar radiculopathy model. *Spine (Phila. Pa. 1976).* **40**, E1269–E1275 (2015).
137. Ogon, I. *et al.* Attenuation of pain behaviour by local administration of alpha-2 adrenoceptor antagonists to dorsal root ganglia in a rat radiculopathy model. *Eur. J. Pain (United Kingdom)* **20**, 790–799 (2016).
138. Filippi, A. *et al.* Catecholamines reduce transient receptor potential vanilloid type 1 desensitization in cultured dorsal root ganglia neurons. *J. Physiol. Pharmacol.* **67**, 843–850 (2016).
139. Arnsten, A. F. T. & Jin, L. E. Guanfacine for the treatment of cognitive disorders: A century of discoveries at Yale. *Yale J. Biol. Med.* **85**, 45–58 (2012).
140. Wang, M. *et al.* α2A-adrenoceptors strengthen working memory networks by inhibiting cAMP-HCN channel signaling in prefrontal cortex. *Cell* **129**, 397–410 (2007).
141. Terry, A. V., Callahan, P. M., Schade, R., Kille, N. J. & Plagenhoef, M. Alpha 2A adrenergic receptor agonist, guanfacine, attenuates cocaine-related impairments of

inhibitory response control and working memory in animal models. *Pharmacol. Biochem. Behav.* **126**, 63–72 (2014).
142. Schulz, K. P., Clerkin, S. M., Fan, J., Halperin, J. M. & Newcorn, J. H. Guanfacine modulates the influence of emotional cues on prefrontal cortex activation for cognitive control. *Psychopharmacology (Berl).* **226**, 261–271 (2013).
143. Fox, H., Sofuoglu, M. & Sinha, R. Guanfacine enhances inhibitory control and attentional shifting in early abstinent cocaine-dependent individuals. *J. Psychopharmacol.* **29**, 312–323 (2015).
144. Milivojevic, V., Fox, H. C., Jayaram-Lindstrom, N., Hermes, G. & Sinha, R. Sex differences in guanfacine effects on stress-induced stroop performance in cocaine dependence. *Drug Alcohol Depend.* **179**, 275–279 (2017).
145. Kawaura, K., Karasawa, J. ichi, Chaki, S. & Hikichi, H. Stimulation of postsynapse adrenergic α2A receptor improves attention/cognition performance in an animal model of attention deficit hyperactivity disorder. *Behav. Brain Res.* **270**, 349–356 (2014).
146. Kauser, H., Sahu, S. & Panjwani, U. Guanfacine promotes neuronal survival in medial prefrontal cortex under hypobaric hypoxia. *Brain Res.* **1636**, 152–160 (2016).
147. Selye, H. A syndrome produced by diverse nocuous agents [13]. *Nature* **138**, 32 (1936).
148. McEwen, B. S., Gray, J. D. & Nasca, C. Redefining neuroendocrinology: Stress, sex and cognitive and emotional regulation. *J. Endocrinol.* **226**, T67–T83 (2015).
149. Javidi, H. & Yasollahie, M. Post-traumatic stress dissorder. *Int. J. Occup. Enviromental Med.* **3**, 2–29 (2012).
150. Kilpatrick, D. *et al.* National Estimates of Exposure to Traumatic Events and PTSD Prevalence Using DSM-IV and DSM-5 Criteria Dean. *J. Trauma. Stress* **26**, 537–547 (2013).
151. Najavits, L. M. *et al.* Cocaine dependence with and without PTSD among subjects in the national institute on drug abuse collaborative cocaine treatment study. *Am. J. Psychiatry* **155**, 214–219 (1998).
152. Back, Bonnie S. Dansky, Scott F. Co, S. Cocaine Dependence with and without Post-traumatic Stress Disorder: A Comparison of Substance Use, Trauma History and Psychiatric Comorbidity. *Am. J. Addict.* **9**, 51–62 (2000).
153. Waldrop, A. E., Back, S. E., Verduin, M. L. & Brady, K. T. Triggers for cocaine and alcohol use in the presence and absence of posttraumatic stress disorder. *Addict. Behav.* **32**, 634–639 (2007).
154. Khoury, L., Tang, Y. L., Bradley, B., Cubells, J. F. & Ressler, K. J. Substance use, childhood traumatic experience, and Posttraumatic Stress Disorder in an urban civilian population. *Depress. Anxiety* **27**, 1077–1086 (2010).
155. Lawson, K. M., Back, S. E., Hartwell, K. J., Maria, M. M. S. & Brady, K. T. A comparison of trauma profiles among individuals with prescription opioid, nicotine, or cocaine dependence. *Am. J. Addict.* **22**, 127–131 (2013).
156. Back, S. E. *et al.* Early life trauma and sensitivity to current life stressors in individuals with and without cocaine dependence. *Am. J. Drug Alcohol Abuse* **34**, 389–396 (2008).
157. Scheidell, J. D. *et al.* Childhood traumatic experiences and the association with marijuana and cocaine use in adolescence through adulthood. *Addiction* **113**, 44–56 (2018).
158. McRae-Clark, A. L. *et al.* Impact of gender on corticotropin-releasing factor and noradrenergic sensitivity in cocaine use disorder. *Journal of Neuroscience Research* **95**, 320–327 (2017).

159. Francke, I. D. avil., Viola, T. W., Tractenberg, S. G. & Grassi-Oliveira, R. Childhood neglect and increased withdrawal and depressive severity in crack cocaine users during early abstinence. *Child Abus. Negl.* **37**, 883–889 (2013).
160. Waldrop, A. E. *et al.* Community-dwelling cocaine-dependent men and women respond differently to social stressors versus cocaine cues. *Psychoneuroendocrinology* **35**, 798–806 (2010).
161. Brady, K. T. *et al.* Response to corticotropin-releasing hormone infusion in cocaine-dependent individuals. *Arch. Gen. Psychiatry* **66**, 422–430 (2009).
162. Sinha, R, Catapano, D, O'Malley, S. Stress-induced craving and stress response in cocaine dependent individuals. *Psychoneuroendocrinology* 343–351 (1999).
163. Lê, A. D., Harding, S., Juzytsch, W., Funk, D. & Shaham, Y. Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats. *Psychopharmacology (Berl).* **179**, 366–373 (2005).
164. Nair, S. G. *et al.* Role of dorsal medial prefrontal cortex dopamine D1-family receptors in relapse to high-fat food seeking induced by the anxiogenic drug Yohimbine. *Neuropsychopharmacology* **36**, 497–510 (2011).
165. Leri, F., Flores, J., Rodaros, D. & Stewart, J. Blockade of stress-induced but not cocaine-induced reinstatement by infusion of noradrenergic antagonists into the bed nucleus of the stria terminalis or the central nucleus of the amygdala. *J. Neurosci.* **22**, 5713–5718 (2002).
166. Erb, S. & Stewart, J. A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *J. Neurosci.* **19**, 1–6 (1999).
167. Dzung Lê, A., Funk, D., Harding, S., Juzytsch, W. & Fletcher, P. J. The role of noradrenaline and 5-hydroxytryptamine in yohimbine-induced increases in alcohol-seeking in rats. *Psychopharmacology (Berl).* **204**, 477–488 (2009).
168. Buffalari, D. M., Baldwin, C. K., Feltenstein, M. W. & See, R. E. Corticotrophin releasing factor (CRF) induced reinstatement of cocaine seeking in male and female rats. *Physiol. Behav.* **105**, 209–214 (2012).
169. Azizbeigi, R. & Haghparast, A. Involvement of orexin-2 receptor in the ventral tegmental area in stress- and drug priming-induced reinstatement of conditioned place preference in rats. *Neurosci. Lett.* **696**, 121–126 (2019).
170. Rinker, J. A. *et al.* Extended Amygdala to Ventral Tegmental Area Corticotropin-Releasing Factor Circuit Controls Binge Ethanol Intake. *Biol. Psychiatry* **81**, 930–940 (2017).
171. Vranjkovic, O. *et al.* Enhanced CRFR1-dependent regulation of a ventral tegmental area to prelimbic cortex projection establishes susceptibility to stress-induced cocaine seeking. *J. Neurosci.* **38**, 10657–10671 (2018).
172. Yamauchi, N. *et al.* Activation of the neural pathway from the dorsolateral bed nucleus of the stria terminalis to the central amygdala induces anxiety-like behaviors. *Eur. J. Neurosci.* **48**, 3052–3061 (2018).
173. Polter, A. M. & Kauer, J. A. Stress and VTA synapses: Implications for addiction and depression. *Eur. J. Neurosci.* **39**, 1179–1188 (2014).
174. Holly, E. N. & Miczek, K. A. Ventral tegmental area dopamine revisited: Effects of acute and repeated stress. *Psychopharmacology* **233**, 163–186 (2016).
175. You, Z. B., Wang, B., Zitzman, D., Azari, S. & Wise, R. A. A role for conditioned ventral tegmental glutamate release in cocaine seeking. *J. Neurosci.* **27**, 10546–10555 (2007).

176. Wang, B. *et al.* Cocaine Experience Establishes Control of Midbrain Glutamate and Dopamine by Corticotropin-Releasing Factor: A Role in Stress-Induced Relapse to Drug Seeking. (2005). doi:10.1523/JNEUROSCI.0955-05.2005
177. McFarland, K., Davidge, S. B., Lapish, C. C. & Kalivas, P. W. Limbic and Motor Circuitry Underlying Footshock-Induced Reinstatement of Cocaine-Seeking Behavior. *J. Neurosci.* **24**, 1551–1560 (2004).
178. Blacktop, J. M. *et al.* Antagonism of GABA-B but not GABA-A receptors in the VTA prevents stress- and intra-VTA CRF-induced reinstatement of extinguished cocaine seeking in rats. *Neuropharmacology* **102**, 197–206 (2016).
179. Williams, C. L., Buchta, W. C. & Riegel, A. C. CRF-R2 and the heterosynaptic regulation of VTA glutamate during reinstatement of cocaine seeking. *J. Neurosci.* **34**, 10402–10414 (2014).
180. Wang, B., You, Z. B., Rice, K. C. & Wise, R. A. Stress-induced relapse to cocaine seeking: Roles for the CRF2 receptor and CRF-binding protein in the ventral tegmental area of the rat. *Psychopharmacology (Berl).* **193**, 283–294 (2007).
181. Chen, N. A. *et al.* Knockdown of CRF1 receptors in the ventral tegmental area attenuates cue- and acute food deprivation stress-induced cocaine seeking in mice. *J. Neurosci.* **34**, 11560–11570 (2014).
182. Graziane, N. M., Polter, A. M., Briand, L. A., Pierce, R. C. & Kauer, J. A. Kappa opioid receptors regulate stress-induced cocaine seeking and synaptic plasticity. *Neuron* **77**, 942–954 (2013).
183. Tung, L. W. *et al.* Orexins contribute to restraint stress-induced cocaine relapse by endocannabinoid-mediated disinhibition of dopaminergic neurons. *Nat. Commun.* **7**, 12199 (2016).
184. Alheid, G. F. Extended Amygdala and Basal Forebrain. *Ann. N. Y. Acad. Sci.* **985**, 185–205 (2006).
185. Fox, A. S. & Shackman, A. J. The central extended amygdala in fear and anxiety: Closing the gap between mechanistic and neuroimaging research. *Neuroscience Letters* **693**, 58–67 (2019).
186. Pedersen, W. S. *et al.* Higher resting-state BNST-CeA connectivity is associated with greater corrugator supercilii reactivity to negatively valenced images. *Neuroimage* **207**, 116428 (2020).
187. Gorka, A. X., Torrisi, S., Shackman, A. J., Grillon, C. & Ernst, M. Intrinsic functional connectivity of the central nucleus of the amygdala and bed nucleus of the stria terminalis. *Neuroimage* **168**, 392–402 (2018).
188. McReynolds, J. R. *et al.* Beta-2 adrenergic receptors mediate stress-evoked reinstatement of cocaine-induced conditioned place preference and increases in CRF mRNA in the bed nucleus of the stria terminalis in mice. *Psychopharmacology (Berl).* **231**, 3953–3963 (2014).
189. Buffalari, D. M. & See, R. E. Inactivation of the bed nucleus of the stria terminalis in an animal model of relapse: Effects on conditioned cue-induced reinstatement and its enhancement by yohimbine. *Psychopharmacology (Berl).* **213**, 19–27 (2011).
190. Kovács, L. Á., Schiessl, J. A., Nafz, A. E., Csernus, V. & Gaszner, B. Both basal and acute restraint stress-induced c-Fos expression is influenced by age in the extended amygdala and brainstem stress centers in male rats. *Front. Aging Neurosci.* **10**, 248 (2018).

191. Makino, S. *et al. Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat*. *Brain Research* **850**, (1999).
192. McReynolds, J. R., Christianson, J. P., Blacktop, J. M. & Mantsch, J. R. What does the Fos say? Using Fos-based approaches to understand the contribution of stress to substance use disorders. *Neurobiol. Stress* **9**, 271–285 (2018).
193. Kreibich, A. S. & Blendy, J. A. cAMP response element-binding protein is required for stress but not cocaine-induced reinstatement. *J. Neurosci.* **24**, 6686–6692 (2004).
194. Shaham, Y., Highfield, D., Delfs, J., Leung, S. & Stewart, J. Clonidine blocks stress-induced reinstatement of heroin seeking in rats: An effect independent of locus coeruleus noradrenergic neurons. *Eur. J. Neurosci.* **12**, 292–302 (2000).
195. Lee, B., Tiefenbacher, S., Platt, D. M. & Spealman, R. D. Pharmacological blockade of α2-adrenoceptors induces reinstatement of cocaine-seeking behavior in squirrel monkeys. *Neuropsychopharmacology* **29**, 686–693 (2004).
196. Conrad, K. L. *et al.* Yohimbine depresses excitatory transmission in BNST and impairs extinction of cocaine place preference through orexin-dependent, norepinephrine-independent processes. *Neuropsychopharmacology* **37**, 2253–2266 (2012).
197. Davis, A. R. *et al.* Yohimbine impairs extinction of cocaine-conditioned place preference in an α2-adrenergic receptor independent process. *Learn. Mem.* **15**, 667–676 (2008).
198. Vranjkovic, O., Hang, S., Baker, D. A. & Mantsch, J. R. β-adrenergic receptor mediation of stress-induced reinstatement of extinguished cocaine-induced conditioned place preference in mice: Roles for β1 and β2 adrenergic receptors. *J. Pharmacol. Exp. Ther.* **342**, 541–551 (2012).
199. Erb, S., Salmaso, N., Rodaros, D. & Stewart, J. A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl).* **158**, 360–365 (2001).
200. Nobis, W. P., Kash, T. L., Silberman, Y. & Winder, D. G. β-Adrenergic receptors enhance excitatory transmission in the bed nucleus of the stria terminalis through a corticotrophin-releasing factor receptor dependent and cocaine-regulated mechanism. *Biol. Psychiatry* **69**, 1083–1090 (2011).
201. Silberman, Y. & Winder, D. G. Corticotropin releasing factor and catecholamines enhance glutamatergic neurotransmission in the lateral subdivision of the central amygdala. *Neuropharmacol.* **70**, 316–323 (2013).
202. Flavin, S. A. & Winder, D. G. Noradrenergic control of the bed nucleus of the stria terminalis in stress and reward. *Neuropharmacol.* **70**, 324–330 (2013).
203. Brown, Z. J., Tribe, E., D'Souza, N. A. & Erb, S. Interaction between noradrenaline and corticotrophin-releasing factor in the reinstatement of cocaine seeking in the rat. *Psychopharmacology (Berl).* **203**, 121–130 (2009).
204. Aston-Jones, G., Delfs, J. M., Druhan, J. & Zhu, Y. The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. in *Annals of the New York Academy of Sciences* **877**, 486–498 (New York Academy of Sciences, 1999).
205. Schmidt, K. T. *et al.* Stress-Induced Alterations of Norepinephrine Release in the Bed Nucleus of the Stria Terminalis of Mice. *ACS Chem. Neurosci.* **10**, 1908–1914 (2019).
206. Egli, R. E. *et al.* Norepinephrine modulates glutamatergic transmission in the bed nucleus of the stria terminalis. *Neuropsychopharmacology* **30**, 657–668 (2005).

207. Gungor, N. Z., Yamamoto, R. & Pare, D. Glutamatergic and gabaergic ventral BNST neurons differ in their physiological properties and responsiveness to noradrenaline. *Neuropsychopharmacology* **43**, 2126–2133 (2018).
208. Lebow, M. A. & Chen, A. Overshadowed by the amygdala: The bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatry* **21**, 450–463 (2016).
209. McElligott, Z. A. & Winder, D. G. α1-Adrenergic receptor-induced heterosynaptic long-term depression in the bed nucleus of the stria terminalis is disrupted in mouse models of affective disorders. *Neuropsychopharmacology* **33**, 2313–2323 (2008).
210. Flavin, S., Matthews, R. T., Wang, Q., Muly, E. C. & Winder, D. G. α2A-Adrenergic Receptors Filter Parabrachial Inputs to the Bed Nucleus of the Stria Terminalis. *J. Neurosci.* **34**, 9319–31 (2014).
211. Harris, N. A. *et al.* Dorsal BNST α2A -adrenergic receptors produce HCN-dependent excitatory actions that initiate anxiogenic behaviors. *J. Neurosci.* **38**, 8922–8942 (2018).
212. Fetterly, T. L. *et al.* α 2A-adrenergic receptor activation decreases parabrachial nucleus excitatory drive onto BNST CRF neurons and reduces their activity In vivo. *J. Neurosci.* **39**, 472–484 (2018).
213. Shields, A. D., Wang, Q. & Winder, D. G. α2A-adrenergic receptors heterosynaptically regulate glutamatergic transmission in the bed nucleus of the stria terminalis. *Neuroscience* **163**, 339–351 (2009).
214. Savchenko, V. L. & Boughter, J. D. Regulation of neuronal activation by alpha2A adrenergic receptor agonist. *Neurotox. Res.* **20**, 226–239 (2011).
215. Stamatakis, A. M. *et al.* Amygdala and bed nucleus of the stria terminalis circuitry: Implications for addiction-related behaviors. *Neuropharmacology* **76**, 320–328 (2014).
216. Avery, S. N., Clauss, J. A. & Blackford, J. U. The Human BNST: Functional Role in Anxiety and Addiction. *Neuropsychopharmacology* **41**, 126–141 (2016).
217. Lee, A. M., Calarco, C. A., McKee, S. A., Mineur, Y. S. & Picciotto, M. R. Variability in nicotine conditioned place preference, stress-induced reinstatement, and effects of guanfacine in male and female mice. *Genes, Brain Behav.* 1–17 (2019). doi:10.1111/gbb.12601
218. Karlsgodt, K. H., Lukas, S. E. & Elman, I. Psychosocial stress and the duration of cocaine use in non-treatment seeking individuals with cocaine dependence. *Am. J. Drug Alcohol Abuse* **29**, 539–551 (2003).
219. Levandowski, M. L. *et al.* Crack cocaine addiction, early life stress and accelerated cellular aging among women. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **71**, 83–89 (2016).
220. Khan, Z. P., Ferguson, C. N. & Jones, R. M. Alpha-2 and imidazoline receptor agonists. *Anaesthesia* **54**, 146–165 (1999).
221. Brown, J., Doxey, J. C. & Handley, S. Effects of α-adrenoceptor agonists and antagonists and of antidepressant drugs on pre- and postsynaptic a-adrenoceptors. *Eur. J. Pharmacol.* **67**, 33–40 (1980).
222. Okada, M. *et al.* Effects of acute and sub-chronic administrations of guanfacine on catecholaminergic transmissions in the orbitofrontal cortex. *Neuropharmacology* **156**, 107547 (2019).
223. Paxinos, G. & Franklin, B. *The Mouse Brain in Stereotaxic Coordinates.* (2004).
224. Vranjkovic, O., Pina, M., Kash, T. L. & Winder, D. G. The bed nucleus of the stria terminalis in drug-associated behavior and affect : A circuit-based perspective.

Neuropharmacology **122**, 100–106 (2017).
225. Crowley, N. A. *et al.* Dynorphin Controls the Gain of an Amygdalar Anxiety Circuit. *Cell Rep.* **14**, 2774–2783 (2016).
226. Clauss, J. A., Avery, S. N., Benningfield, M. M. & Blackford, J. U. Social anxiety is associated with BNST response to unpredictability. *Depress. Anxiety* 666–675 (2019). doi:10.1002/da.22891
227. Gowing, L., Farrell, M., Ali, R. & Jm, W. Alpha2-adrenergic agonists for the management of opioid withdrawal (Review). *Library (Lond).* 10–13 (2009). doi:10.1002/14651858.CD002024.pub5.www.cochranelibrary.com
228. Cecchi, M., Khoshbouei, H., Javors, M. & Morilak, D. A. Modulatory effects of norepinephrine in the lateral bed nucleus of the stria terminalis on behavioral and neuroendocrine responses to acute stress. *Neuroscience* **112**, 13–21 (2002).
229. Pleil, K. E. *et al.* NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. *Nat. Neurosci.* **18**, 545–552 (2015).
230. Rinker, J. A. *et al.* Extended Amygdala to Ventral Tegmental Area Corticotropin-Releasing Factor Circuit Controls Binge Ethanol Intake. *Biol. Psychiatry* **81**, 930–940 (2017).
231. Pina, M. M., Young, E. A., Ryabinin, A. E. & Cunningham, C. L. The bed nucleus of the stria terminalis regulates ethanol-seeking behavior in mice. *Neuropharmacology* **99**, 627–638 (2015).
232. Companion, M. A. & Thiele, T. E. Assessment of ventral tegmental area-projecting GABAergic neurons from the bed nucleus of the stria terminalis in modulating binge-like ethanol intake. *Eur. J. Neurosci.* **48**, 3335–3343 (2018).
233. Abercrombie, E. D., Keller, R. W. & Zigmond, M. J. Characterization of hippocampal norepinephrine release as measured by microdialysis perfusion: Pharmacological and behavioral studies. *Neuroscience* **27**, 897–904 (1988).
234. Winter, J. C. & Rabin, R. A. Yohimbine as a serotonergic agent: Evidence from receptor binding and drug discrimination. *J. Pharmacol. Exp. Ther.* **263**, 682–689 (1992).
235. Ch'ng, S., Fu, J., Brown, R. M., Mcdougall, S. J. & Lawrence, A. J. The intersection of stress and reward: BNST modulation of aversive and appetitive states. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **87**, 108–125 (2018).
236. Kash, T. L., Nobis, W. P., Matthews, R. T. & Winder, D. G. Dopamine enhances fast excitatory synaptic transmission in the extended amygdala by a CRF-R1-dependent process. *J. Neurosci.* **28**, 13856–13865 (2008).
237. Siciliano, C. A. & Tye, K. M. Leveraging calcium imaging to illuminate circuit dysfunction in addiction. *Alcohol* **74**, 47–63 (2019).
238. Calipari, E. S. *et al.* In vivo imaging identifies temporal signature of D1 and D2 medium spiny neurons in cocaine reward. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 2726–2731 (2016).
239. Tilley, M. R. *et al.* Cocaine reward and locomotion stimulation in mice with reduced dopamine transporter expression. *BMC Neurosci.* **8**, (2007).
240. Carboni, E., Silvagni, A., Rolando, M. T. & Di Chiara, G. Stimulation of in vivo dopamine transmission in the bed nucleus of stria terminalis by reinforcing drugs. *J. Neurosci.* **20**, (2000).
241. Avery, S. N., Clauss, J. A. & Blackford, J. U. The Human BNST: Functional Role in Anxiety and Addiction. *Neuropsychopharmacol. Rev.* **41**, 126–141 (2016).
242. Pati, D. *et al.* Chronic intermittent ethanol exposure dysregulates a GABAergic

microcircuit in the bed nucleus of the stria terminalis. *Neuropharmacology* 107759 (2019). doi:10.1016/j.neuropharm.2019.107759
243. Wang, Y. *et al.* A bed nucleus of stria terminalis microcircuit regulating inflammation-associated modulation of feeding. *Nat. Commun.* **10**, 2769 (2019).
244. Sinha, R., Garcia, M., Paliwal, P., Kreek, M. J. & Rounsaville, B. J. Stress-induced cocaine craving and hypothalamic-pituitary-adrenal responses are predictive of cocaine relapse outcomes. *Arch. Gen. Psychiatry* **63**, 324–331 (2006).
245. Wang, X., Cen, X. & Lu, L. Noradrenaline in the bed nucleus of the stria terminalis is critical for stress-induced reactivation of morphine-conditioned place preference in rats. *Eur. J. Pharmacol.* **432**, 153–61 (2001).
246. Fritschy, J. M. & Grzanna, R. Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? *Prog. Brain Res.* **88**, 257–68 (1991).
247. Koob, G. F. & Volkow, N. D. *Neurobiology of addiction: a neurocircuitry analysis*. **3**, (2016).
248. Koob, G. F. & Le Moal, M. Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* **24**, 97–129 (2001).
249. Koob, G. F. & Le Moal, M. Drug abuse: Hedonic homeostatic dysregulation. *Science (80-.).* **278**, 52–58 (1997).
250. Gamo, N. J., Wang, M. & Arnsten, A. F. T. Methylphenidate and atomoxetine enhance prefrontal function through α2-adrenergic and dopamine D1 receptors. *J. Am. Acad. Child Adolesc. Psychiatry* **49**, 1011–1023 (2010).
251. Ahmed, B. Y. *et al.* Efficient delivery of Cre-recombinase to neurons in vivo and stable transduction of neurons using adeno-associated and lentiviral vectors. *BMC Neurosci.* **5**, 4 (2004).
252. Sauer, B. Inducible gene targeting in mice using the Cre/lox system. *Methods A Companion to Methods Enzymol.* **14**, 381–392 (1998).
253. Saunders, A. & Sabatini, B. L. Cre activated and inactivated recombinant adeno-associated viral vectors for neuronal anatomical tracing or activity manipulation. *Curr. Protoc. Neurosci.* **2015**, 1.24.1-1.24.15 (2015).
254. Beckerman, M. A., Van Kempen, T. A., Justice, N. J., Milner, T. A. & Glass, M. J. Corticotropin-releasing factor in the mouse central nucleus of the amygdala: Ultrastructural distribution in NMDA-NR1 receptor subunit expressing neurons as well as projection neurons to the bed nucleus of the stria terminalis. *Exp. Neurol.* **239**, 120–132 (2013).
255. Ahrens, S. *et al.* A central extended amygdala circuit that modulates anxiety. *J. Neurosci.* **38**, 5567–5583 (2018).
256. Hyman, S. M. *et al.* Severity of childhood trauma is predictive of cocaine relapse outcomes in women but not men. *Drug Alcohol Depend.* **92**, 208–216 (2008).
257. Calipari, E. S. *et al.* Dopaminergic dynamics underlying sex-specific cocaine reward. *Nat. Commun.* **8**, 13877 (2017).
258. Anker Justin J., J. J. & Carroll, M. E. Sex differences in the effects of allopregnanolone on yohimbine-induced reinstatement of cocaine seeking in rats. *Drug Alcohol Depend.* **107**, 264–267 (2010).
259. Zurawski, Z. *et al.* Disabling the Gβγ-SNARE interaction disrupts GPCR-mediated presynaptic inhibition, leading to physiological and behavioral phenotypes. *Sci. Signal.*

12, (2019).
260. Bozzi, Y., Provenzano, G. & Casarosa, S. Neurobiological bases of autism–epilepsy comorbidity: a focus on excitation/inhibition imbalance. *Eur. J. Neurosci.* **47**, 534–548 (2018).
261. Bedse, G., Centanni, S. W., Winder, D. G. & Patel, S. Endocannabinoid Signaling in the Central Amygdala and Bed Nucleus of the Stria Terminalis: Implications for the Pathophysiology and Treatment of Alcohol Use Disorder. *Alcoholism: Clinical and Experimental Research* **43**, 2014–2027 (2019).
262. Roth, B. L. DREADDs for Neuroscientists. *Neuron* **89**, 683–694 (2016).
263. Robinson, S. *et al.* Chemogenetic silencing of neurons in retrosplenial cortex disrupts sensory preconditioning. *J. Neurosci.* **34**, 10982–10988 (2014).
264. Fucich, E. A., Paredes, D., Saunders, M. O., David, X. & Morilak, A. Activity in the Ventral Medial Prefrontal Cortex Is Necessary for the Therapeutic Effects of Extinction in Rats. **38**, 1408–1417 (2018).
265. Tuscher, J. J., Taxier, L. R., Fortress, A. M. & Frick, K. M. Chemogenetic inactivation of the dorsal hippocampus and medial prefrontal cortex, individually and concurrently, impairs object recognition and spatial memory consolidation in female mice. (2018). doi:10.1016/j.nlm.2018.11.002
266. McEown, K. *et al.* Chemogenetic inhibition of the medial prefrontal cortex reverses the effects of REM sleep loss on sucrose consumption. *Elife* **5**, (2016).
267. Lerchner, W. *et al.* Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl- channel. *Neuron* **54**, 35–49 (2007).
268. Adamantidis, A. R., Zhang, F., de Lecea, L. & Deisseroth, K. Optogenetics: Opsins and optical interfaces in neuroscience. *Cold Spring Harb. Protoc.* **2014**, 815–822 (2014).
269. Ernsberger, P., Giuliano, R., Willette, R. N. & Reis, D. J. Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. *J. Pharmacol. Exp. Ther.* **253**, 408–18 (1990).
270. Huss, M. *et al.* Distinguishing the efficacy and sedative effects of guanfacine extended release in children and adolescents with attention-deficit/hyperactivity disorder. *Eur. Neuropsychopharmacol.* **29**, 432–443 (2019).
271. Moran-Santa Maria, M. M., Baker, N. L., Ramakrishnan, V., Brady, K. T. & McRae-Clark, A. Impact of acute guanfacine administration on stress and cue reactivity in cocaine-dependent individuals. *Am. J. Drug Alcohol Abuse* **41**, 146–52 (2015).
272. Hitchcock, L. N. & Lattal, K. M. Involvement of the dorsal hippocampus in expression and extinction of cocaine-induced conditioned place preference. *Hippocampus* **28**, 226–238 (2018).