# The Role of Limbic Corticotropin Releasing Factor Neural Systems in Motivation and Addiction

by

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## Dedication

This dissertation is dedicated to Dawn Penso for never accepting anything short of my absolute maximum effort.

And to my parents, Mary and Kelan Emery, for their love, support, and understanding.

And lastly, to my high school chemistry teacher Juliana Matz for telling me (in $10^{th}$ grade) not to drop out of a PhD program.

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## Abstract

Corticotropin releasing factor (CRF) is a key regulator of behavioral and physiological responses to stress and is typically associated with anxiety and distress (Gray, 1993). In opponent process theories of addiction, CRF-driven anxiety acts as a negative reinforcer during withdrawal to cause relapse as a means of reducing distress due to hedonic homeostatic dysregulation (George et al., 2012a; G. F. Koob, 2010). However, evidence suggests an alternative role for CRF in positive incentive motivation without distress (Baumgartner et al., 2021, 2022; Lemos et al., 2012; Merali et al., 1998a; Peciña et al., 2006a; Xu et al., 2024). Specifically, optogenetic activation of CRF neurons in central amygdala (CeA) and nucleus accumbens (NAc) generate positive incentive motivation and reward pursuit without distress in *Crh*-cre rats.

However, it is unclear whether CRF itself versus other co-released neurotransmitters from CRF neurons, such as GABA, contributes to the appetitive effects of CRF neuron stimulation. Chapters 2 and 3 investigate whether NAc and CeA CRF neuronal activation requires CRF receptor activation for incentive motivation. Using optogenetics and pharmacology, I activate these neurons while blocking CRF receptors during behavioral tests of motivation. Results show CRF receptor activation is crucial for both regions to enhance reward pursuit. Moreover, blocking CRF receptors diminishes self-stimulation of CRF neurons, implicating CRF signaling in motivation without distress.

Next, the circuitry underlying the incentive effects of CeA CRF neuronal activation is unknown. Chapter 4 presents pilot data on CeA CRF neuron projections to dorsal medial striatum (DMS), mid-anterior lateral hypothalamus (LH), and posterior LH or substantia nigra

(SN). CeA CRF projections to the LH may exhibit a rostrocaudal gradient: those to mid-anterior LH bias rats against laser-paired rewards, while those to posterior LH or SN bias rats toward laser-paired rewards, potentially supporting self-stimulation. However, activation of fibers into the DMS are ineffective in influencing motivation.

Finally, it is possible that extensive drug experience flips the valence of CeA CRF neurons, given opponent process predictions of CRF's role in mediating aversive distress in withdrawal. Chapter 5 explores the impact of long access cocaine self-administration (LgA) on the valence of CeA CRF neuronal activation. Following 14 days of 6-hour daily cocaine self-administration, rats showed altered motivation patterns: males displayed aversion to laser-paired sucrose while females intensified sucrose pursuit. After abstinence, males reverted to preferring laser-paired sucrose, and both sexes increased overall reward pursuit compared to drug-naïve and control rats. Notably, some rats self-stimulated CeA CRF neurons during withdrawal and post-abstinence, suggesting sustained positive incentive motivation. Moreover, CeA CRF neuron activation did not induce aversion post-cocaine exposure. These findings reveal sex-dependent differences in CRF signaling during withdrawal as well as augmented incentive sensitization post-abstinence in both sexes.

This dissertation expands upon conventional views of CRF as a driver of distress by exploring its role in incentive motivation. We provide further evidence for an incentive role for limbic CRF systems, particularly within CeA and NAc. Importantly, CeA CRF neurons can still generate positive incentive motivation, potentially facilitating incentive sensitization, and fail to become aversive following extensive cocaine consumption. Finally, pilot data exploring CeA CRF neuronal projections hint at the specific circuitry underlying CeA CRF driven incentive motivation. This work adds nuance to mechanisms involved in stress-induced relapse and

motivation and provides important insights into stress-associated affective disorders related to motivation.

# Chapter 1 Introduction

After a long day at work, someone, somewhere, is reaching for a glass of wine. Somewhere else, an uncle is celebrating the birth of his niece and lights a cigar, even though he has not smoked in years. On a college campus, a student is on their way to study for their midterm and impulsively spends a little extra cash on a chocolate croissant with their coffee. Why do we reach for these simple pleasures during times of stress, excitement, or pressure? Going further, for people who struggle with addiction, why can relapse interrupt long periods of sobriety after intense emotions? Are we seeking to alleviate stress-induced discomfort or looking to enhance an already positive experience? These questions all speak to an intersection between stress, reward, and motivation where, if something triggers an intense emotional experience, suddenly that resolve to eat healthy or quit smoking is momentarily less important than an irresistible croissant or cigar. Specifically, these examples suggest the existence of brain stress circuitry that regulates and intensifies motivation to seek out rewards.

Primarily released from the paraventricular nucleus of the hypothalamus (PVN), CRF has been studied as a component of the hypothalamic-pituitary-adrenal (HPA) stress response system that initiates the "fight or flight" response first described by Walter Cannon in 1915 and adapted as the "general alarm reaction" by Hans Selye in 1936 (Adamec & McKay, 1993; Binder & Nemeroff, 2010; Cannon, 1915; Chudoba & Dabrowska, 2023; Dunn & Berridge, 1990; Guillemin & Rosenberg, 1955; Harris, 1950; Hauger et al., 2009; Saffran et al., 1955; Selye,

1936; Vale et al., 1981). Beyond the hypothalamus, an extensive network of CRF neurons and receptors also exists in limbic and cortical regions; however, it was initially thought that these CRF systems may be involved in autonomic control or as independent functional systems(Swanson et al., 1983). However, the popularized role for extrahypothalamic CRF populations, has been in the emotional response to stress, including anxiety and distress, and contribute to fear learning (Abiri et al., 2014; Jo et al., 2020; Kong & Zweifel, 2021; Ohmura & Yoshioka, 2009; Pomrenze, Giovanetti, et al., 2019; Radulovic et al., 1999). Because of this, CRF is considered the key regulator of behavioral aversive responses to stress and its canonical role in initiating the endocrine stress response is well characterized (Bale & Vale, 2004; Dedic, Chen, et al., 2018; Gray, 1993; Jiang et al., 2019; Turnbull & Rivier, 1997).

Corticotropin releasing factor (CRF), a neuropeptide involved in initiating the endocrine stress response, has been tied to stress-induced reward seeking primarily through aversive motivation where reward consumption serves as a means of alleviating distress and anxiety (Cottone et al., 2009; Iemolo et al., 2013; G. F. Koob, 2010; G. F. Koob et al., 2014; G. Koob & Kreek, 2007). However, recent evidence suggests that this interface between CRF and motivation may not always require aversion and anxiety and could explain why 'happy stress' events may also cause relapse and reward seeking (Baumgartner et al., 2021, 2022; Ferreira, Zerwes et al., 2016; Hodgins et al., 1995; Hodgins & el-Guebaly, 2004; Lemos et al., 2012; McKay et al., 1995; Peciña et al., 2006a; Shiftman et al., 1985; Walitzer & Dearing, 2006). The role of CRF has been relatively unexplored in mobilizing behavior outside of negatively valenced stress or anxiety-related environments and stimuli. Building evidence demonstrates that CRF systems may play a role to enhance reward seeking via positive incentive motivation without necessitating anxiety and distress (Baumgartner et al., 2021, 2022; Eckenwiler et al.,

2023; Lemos et al., 2012; Peciña et al., 2006a; Xu et al., 2024; Zalachoras et al., 2022). An incentive motivation role for CRF is paradoxical – how can a molecule so tightly linked to distress also be involved in intense reward seeking? This dissertation aims to address that issue, so as to further improve scientific understanding of CRF's roles in behavior.

### 1.1 CRF in the HPA Axis

The existence of a 'first mediator' in response to stress was proposed by Hans Selye around the time that CRF was initially discovered in the ovine paraventricular nucleus of the hypothalamus (PVN) as a hypothalamic releasing factor that triggers release of adrenocorticotropic hormone (ACTH) from the pituitary (Guillemin & Rosenberg, 1955; Selye & Selye, 1956; Vale et al., 1981). Initially, environmental stimuli engage several sensory and limbic regions which provide glutamatergic and noradrenergic input to the hypothalamus to stimulate release of CRF from the PVN (Figure 1-1 A) (Aguilera & Liu, 2012). This action of CRF initiates activation of the hypothalamic-pituitary-adrenal access such that hypothalamic CRF triggers the pituitary to release ACTH into the bloodstream, then ACTH signals at the adrenal glands to release glucocorticoids such as cortisol (in humans) or corticosterone (in rodents) (Figure 1-1 A,B) (Guillemin & Rosenberg, 1955; Harris, 1950; Saffran et al., 1955; Spencer & Deak, 2017). Glucocorticoids act via intracellular glucocorticoid and mineralocorticoid receptors to quickly alter transcription and gene expression and mediate metabolic, immune, cognitive, and behavioral function (Figure 1-1 B) (Spencer & Deak, 2017). Furthermore, glucocorticoids provide negative feedback at both the pituitary and hypothalamic level to temper and adjust stress response via homeostatic regulation (Spencer & Deak, 2017). This cascade is known as the hypothalamic-pituitary-adrenal (HPA) axis and provides the basis

for immediate physiological and behavioral response to stress. However, additional circuitry is involved in the emotional response to stress beyond immediate “fight or flight”.

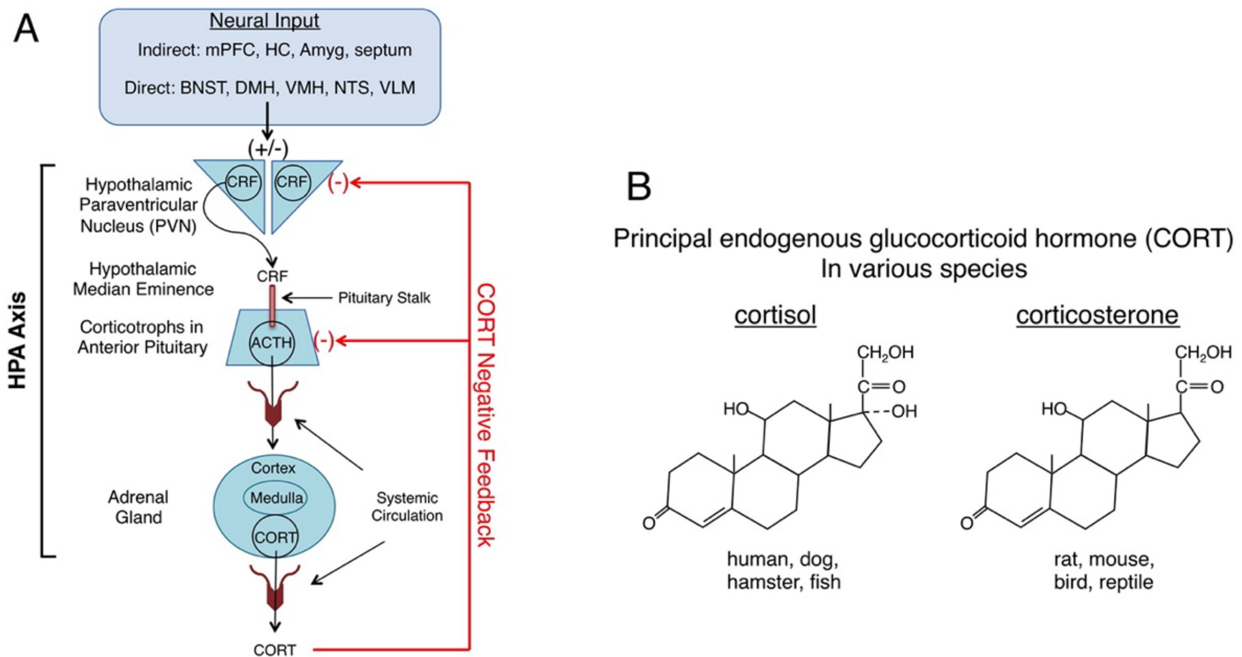



Figure 1-1 CRF in HPA axis signaling

Adapted from (Spencer & Deak, 2017)

## 1.2 CRF in the Limbic System

CRF acts as a neuromodulator to regulate anxiety, learning and memory, arousal, locomotion, sleep, feeding, salience, and sexual reproduction (Dedic, Chen, et al., 2018; Kong & Zweifel, 2021). This is largely due to activity of extrahypothalamic CRF circuitry within the limbic system, an interconnected system of brain regions associated with emotion (Dedic, Chen, et al., 2018). The foundations of the limbic system were characterized by Broca who identified a cortical region including the cingulate and hippocampus that he described as “le grand lobe limbique” based on its elliptical shape, and implicated in emotion (Paul Broca, 1878). Papez then proposed a bidirectional connection between the hypothalamus, amygdala, and the limbic lobe

allowing for both top-down and bottom-up regulation and generation of emotion, a framework which was further refined by Maclean, who named it the limbic system, to tie emotional centers to the autonomic nervous system (Klüver & Bucy, 1938; Maclean, 1949; Papez, 1937). Two anatomical subsystems within this framework are the ventral mesostriatopallidal system, including mesolimbic dopamine projections to the ventral pallidum (VP) and nucleus accumbens (NAc) (Castro et al., 2015; Hooks & Kalivas, 1995; Mogenson et al., 1993; Smith et al., 2009), which are known for their roles in reward-motivated behavior, and the extended amygdala, including the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) (Alheid & Heimer, 1988; Heimer & Van Hoesen, 2006). This circuitry links emotion with motivation and provides output to the orbitofrontal cortex, cingulate cortex, and insular cortex, again providing a basis for both cognitive top-down and physiological bottom-up control of emotion (Heimer & Van Hoesen, 2006).

Since the hypothalamus is central to the original concept of the limbic system, a link between CRF in the PVN and emotional circuitry is unsurprising. While PVN CRF neurons project to the medial eminence to access the hypophysial portal system and activate ACTH release from the pituitary, there are also PVN CRF projections to the amygdala, BNST, and lateral hypothalamus (LH) which integrate physiological stress response with generation of emotional response to stress (McIntyre et al., 2023; Rajamanickam & Justice, 2022). However, beyond the PVN, CRF systems are tightly integrated into extrahypothalamic limbic circuitry and could be mediating emotion and motivation outside of HPA axis activation. Specifically, intrinsic populations of CRF-releasing neurons also are present in the NAc, CeA, and BNST in addition to sparse populations in cortical regions, such as the prefrontal cortex (Dabrowska et al.,

2016; Dedic, Chen, et al., 2018; Hupalo, Bryce, et al., 2019; Hupalo, Martin, et al., 2019; Lemos et al., 2012; Pomrenze et al., 2015; Vale et al., 1981).

NAc CRF neurons have been shown to project both within the NAc, and externally to the VP, while mutual projections allow bidirectional CRF communication between the CeA and BNST (Asok et al., 2018; Dabrowska et al., 2016; Eckenwiler et al., 2023; Erb et al., 2001; Pomrenze et al., 2015). Because of this and other anatomical features, the NAc medial shell has been suggested to serve as a transitional area between extended amygdala regions and striatopallidal circuitry. Additionally, CRF neurons have been found in the ventral tegmental area which serves as a generator for appetitive motivation in the mesolimbic reward pathway and sends significant projections to the NAc (Grieder et al., 2014). CeA and BNST CRF neurons also reach the NAc and prefrontal cortex, in addition to regions associated with pain and fear such as the parabrachial nucleus (Borrego et al., 2022; Pomrenze et al., 2015). Additionally, CeA CRF neurons project to other structures involved in reward, motivation, and goal directed behavior such as the VP, LH, ventral tegmental area (VTA), and dorsal medial striatum (DMS) (Essoh et al., 2022; Pomrenze et al., 2015). Furthermore, limbic regions are equipped to receive CRF signaling via expression of the two CRF receptor types, CRFR1 and CRFR2, in addition to expression of CRF binding protein (CRF-BP) (Behan et al., 1995; Dedic, Chen, et al., 2018; Lemos et al., 2012; Potter et al., 1994). Agonism of each of the two receptors results in distinct behavioral profiles, sometimes opposite in effect such that CRFR1 is more often associated with generating anxiety while CRFR2 may be associated with reducing anxiety, although these effects are region-specific (Bale & Vale, 2004; Dedic, Chen, et al., 2018; Henckens et al., 2016; Radulovic et al., 1999).

Not only does this neurobiological infrastructure allow for stress to influence emotion and motivation, but it also raises the question of whether activation of extrahypothalamic limbic CRF systems can 1) mediate motivation in the absence of aversive stress and 2) regulate and generate HPA axis activation from internal emotional states. The second question is seemingly more straightforward. Notably, CeA and BNST CRF neurons each send projections back to the PVN providing feedback about emotional response to stress or allowing psychological stressors or internal systemic information to preempt and trigger a physiological stress response (Borrego et al., 2022; Chudoba & Dabrowska, 2023; Makino et al., 1999). Additionally, it is possible that HPA axis activation depends on CRF expression and signaling from CeA (Callahan et al., 2013).

The question of whether limbic CRF systems can act independently of aversive stress is less clear. In a prominent neuroscience opponent process theory of addiction, initial drug consumption as a rewarding a-process activates both extended amygdala CRF systems and the HPA axis as an opponent b-process; further, chronic drug use amplifies this b-process response to increase extended amygdala CRF signaling, causing the aversive distress of drug withdrawal (Brady et al., 2009; G. F. Koob, 2010; Majewska, 2002). Additional evidence suggests that psychological stressors may increase CeA CRF neuronal activation without activation of the HPA axis, suggesting that CeA CRF is necessary but not sufficient for HPA axis activation (Callahan et al., 2013; Iwasaki-Sekino et al., 2009).

### 1.3 Traditional Roles for CRF in Emotion

Due to its role in HPA axis activation and the association with stress, both hypothalamic and extrahypothalamic CRF have primarily been studied for their role in anxiety, distress, and other negative emotional states that accompany a stressful experience (Bale & Vale, 2004; Dedic

et al., 2018; Jiang et al., 2019; Smith & Vale, 2006; Turnbull & Rivier, 1997). Intracerebroventricular administration of CRF produces HPA axis activation and anxiety-like responses in rodents which can be blocked by pretreatment with CRF receptor antagonists (Dunn & Berridge, 1990; Erb et al., 2001; Hupalo, Bryce, et al., 2019; Zorrilla et al., 2002). CRF signaling and neuronal populations are also involved in learning, fear memory retrieval, and extinction of fear memories (Abiri et al., 2014; Asok et al., 2018; Jo et al., 2020; Pomrenze, Giovanetti, et al., 2019). Within the extended amygdala, aversive stress increases CRF mRNA expression and GABA release from CRF neurons in both the CeA and the BNST (Daniel et al., 2019; Partridge et al., 2016). Activation of CRF systems in the CeA and BNST is capable of generating distress and has been used to model chronic stress and increase anxiety-like behaviors in the elevated plus maze and open field (Montgomery et al., 2024; Pomrenze, Tovar-Diaz, et al., 2019). While stress and CRF have acute analgesic effects, over-activation of amygdala CRF neurons may block stress-induced analgesia and contribute to the development of chronic pain (Andreoli et al., 2017; Mazzitelli et al., 2022; Yarushkina & Filaretova, 2018; Zhao et al., 2024). Alterations in CRF systems have also been implicated in a range of psychiatric conditions including general anxiety disorder, depression, posttraumatic stress disorder, and addiction (Akil & Nestler, 2023; Bangasser & Kawasumi, 2015; Cottone et al., 2009; G. F. Koob, 2010; Nemeroff et al., 1991; Patriquin & Mathew, 2017; Sanders & Nemeroff, 2016; Sautter et al., 2003; Young & Akil, 1985).

As a contrast to the well-studied role of CRF in aversive distress, NAc and CeA CRF systems are also reported to activate in response to positive experiences like receipt of food or social reward, which are not typically associated with the “fight or flight” response of HPA axis activation; however, food rewards and social interaction have been shown to alter glucocorticoid

levels (Honma et al., 1984; Lim et al., 2007; Merali et al., 1998a; Piazza & Le Moal, 1997). This further begs the question of whether HPA axis activation always generates distress or if HPA activation and CRF systems have multiple modes depending on internal states and environmental contexts.

### 1.4 CRF in Motivation and Addiction: From Drive Reduction to Allostasis

Given CRF's role in anxiety and negative affect, it has been associated with aversive motivation and incorporated into theories of addiction as a driver of distress that people and animals will take drugs to avoid. These perspectives arise from the foundations of homeostatic drive reduction theories. Homeostasis is the process through which an organism maintains a stable internal state by regulating physiology and behavior to achieve a specific physiological set-point (Berridge, 2004; Cannon, 1929). Drive-reduction theories posited that deviation from a homeostatic setpoint created an unpleasant experience, an aversive drive, that increased in intensity the longer it went unaddressed and could be reduced by restoring hemostatic balance (Hull, 1952; Lorenz, 1973). Homeostatic principles have been adopted by opponent process models to describe the relationship between emotions whereby both pleasant and aversive hedonic are part of complementary homeostatic processes, an affective State A and opposing State B, which mediate motivation relative to an affective homeostatic setpoint (Solomon & Corbit, 1974).

A prominent neuroscience opponent process theory of addiction, given names such as *hyperkatifeia* or allostasis theory, describes the euphoric effects of drug use and the subsequent dysphoria of withdrawal, the latter of which is particularly attributed to activation CRF stress circuitry in extended amygdala (George et al., 2012a; G. F. Koob, 2003, 2021; G. F. Koob et al.,

2014; G. F. Koob & Le Moal, 2001; G. Koob & Kreek, 2007). Modeling Solomon and Corbitt's State A and State B (1974), allostasis theory describes an *a-process* of euphoric acute drug effects during intoxication followed by an aversive *b-process* characterized by emotional and physical drug-opposite effects. Here, the *a-process*, onset by drug consumption, is a positive emotional state elevated above the hedonic homeostatic setpoint and the *b-process,* onset by the hedonic effects of the drug, serves as the homeostatic response to divergence from the setpoint (Figure 1-2 A) (George et al., 2012a; G. F. Koob, 2021; G. F. Koob et al., 2014; G. F. Koob & Le Moal, 2001). The *b-process,* generated by activation of extended amygdala CRF systems and the HPA axis, initiates during intoxication and decays slowly to reduce the hedonic effects of the *a-process* while drugs are still onboard, and experienced as withdrawal distress after drug effects end (Figure 1-2 A) (G. F. Koob, 2021). During initial binge/intoxication stages of drug use, the

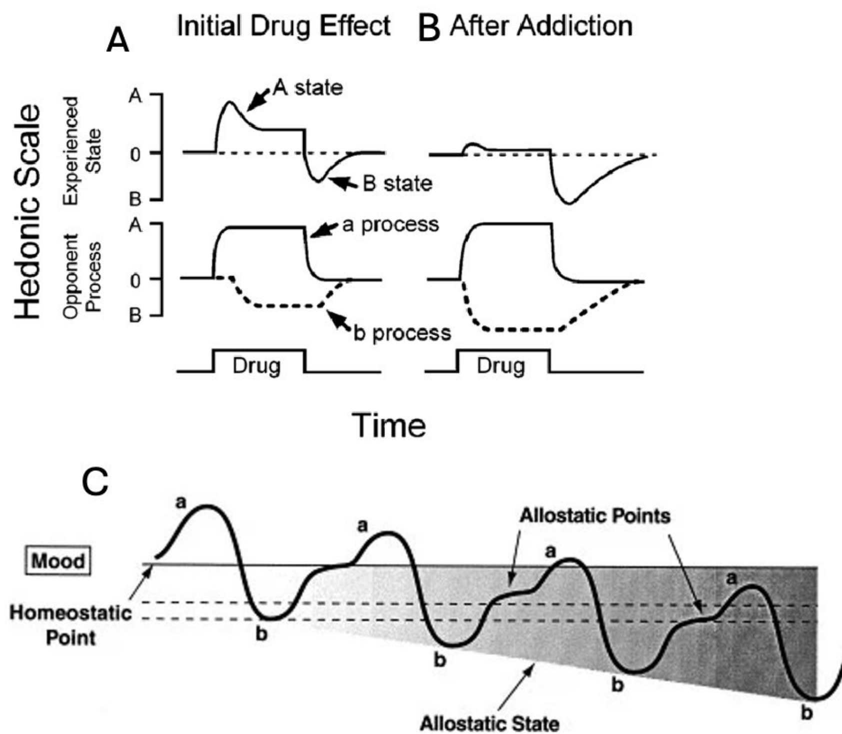



Figure 1-2 Development of allostasis following the development of addiction

Adapted from (G. F. Koob & Le Moal, 2001; Turet, 2015)

euphoric effects of the drug act as positive reinforcers via mesolimbic dopamine signaling to promote further drug consumption (George et al., 2012a; G. F. Koob & Volkow, 2016). However, repeated and chronic drug consumption is posited to specifically amplify and extend the CRF-mediated b-process, which consistently upends affective homeostasis and increases allostatic load (G. F. Koob & Le Moal, 2001).

Allostasis, in contrast with homeostasis, refers to a state of chronic deviation from stable physiological processes and describes a change or adaptation in homeostatic function. As allostatic load increases with consistent drug use, normal homeostatic hedonic setpoints are no longer effective physiological reference points (Figure 1-2 C) (G. F. Koob & Le Moal, 2001). This transition from a homeostatic brain state to an allostatic brain state accompanies within-systems adaptations like drug tolerance, where the brain reward system requires additional drug to create the same euphoria, and between-systems adaptations where chronic activation of reward systems leads to hypersensitization of CRF stress systems in the extended amygdala and magnification of the aversive *b-process* (Figure 1-2 B) (G. F. Koob, 2010; G. F. Koob et al., 2014; G. F. Koob & Le Moal, 2001; G. F. Koob & Volkow, 2016). Magnification of the *b-process* and a shift toward increased extended amygdala CRF signaling are attributed with generating *hyperkatifeia*: intensified distress, malaise, anxiety, and other negative feelings of both withdrawal and other life stressors (G. F. Koob, 2010, 2021). With magnification of the *b-process* comes the withdrawal/negative affect stage of addiction where drug consumption is no longer driven by positive reinforcement by the rewarding effects of the drug, but by negative reinforcement where withdrawal serves as an aversive drive that can be alleviated through hedonic self-medication (G. F. Koob, 2010; G. F. Koob & Le Moal, 2001; G. F. Koob & Volkow, 2016).

Limbic and extended amygdala CRF circuitry are critical in driving aversive motivation and distress during withdrawal. While initial drug consumption triggers both extended amygdala CRF and activation of the HPA axis, neuroadaptations of brain stress systems eventually blunt HPA axis response to drug administration (Armario, 2010; Calogero et al., 1989a; Kershaw et al., 2015; G. F. Koob et al., 2014; G. F. Koob & Volkow, 2016; Manetti et al., 2014; Matta et al., 1998; Piazza & Le Moal, 1996; Schlussman et al., 2002). In contrast, limbic CRF circuitry, including the CeA and BNST in the extended amygdala and the NAc, become hyperactive in response to drugs, drug cues, and withdrawal (Funk et al., 2006; Galesi et al., 2016; George et al., 2007, 2012a; Kasahara et al., 2015; G. F. Koob, 2010; Olive et al., 2002; Richter & Weiss, 1999; Rodríguez de Fonseca et al., 1997; Sarnyai et al., 1995, 2001). In both CeA and BNST, CRF mRNA is elevated following cessation of chronic drug administration and activation of CRF circuitry is necessary for the accompanying withdrawal feelings and heightened stress sensitivity (Connelly & Unterwald, 2020; Erb & Stewart, 1999; George et al., 2007; Valdez et al., 2003).

However, while CRF systems are capable of driving distress during the acute withdrawal period, it is unclear whether CRF systems continue to generate distress beyond withdrawal following an extensive period of abstinence. While changes to CRF systems may last for a matter of weeks (Valdez et al., 2002), relapse in humans can occur after months or even years of abstinence long after the dysphoria of withdrawal has dissipated. Furthermore, this aversive drive hypothesis does not account for incubation of drug craving, a phenomenon whereby cue-triggered cravings become progressively more intense with longer periods of abstinence (Berridge & Robinson, 2016; Li et al., 2015; Pickens et al., 2011). Furthermore, the periods of greatest drug craving do not align with maximal periods of distress. For instance, drug craving

peaks during intoxication when drug-induced increases in dopamine signaling are at their highest (Jaffe et al., 1989; T. E. Robinson & Berridge, 1993; Wise & Bozarth, 1987). Furthermore, it is possible that activation of stress systems contributes to the positive reinforcing effects of drugs and that glucocorticoid or CRF systems are capable of driving reward seeking without generating distress which may be evidence of a role in incentive motivation (Lemos & Alvarez, 2020; Peciña et al., 2006a; Piazza & Le Moal, 1996; Zalachoras et al., 2022).

### 1.5 CRF as a Generator of Positive Incentive Motivation

While CRF has been studied extensively for its role in anxiety and aversive distress, growing evidence has begun to describe a role for CRF in incentive motivation. The phrase 'incentive motivation' describes motivated behavior directed towards obtaining a hedonic reward. In other words, pursuit of a reward usually because previous experience dictates that the reward has an expected hedonic value, although incentive motivation as 'wanting' can also be amplified independently of hedonic 'liking' (Berridge, 2004; Bolles, 1972; T. E. Robinson & Berridge, 1993). Incentive motivation mediated by mesolimbic dopamine-related systems can be triggered by the reward itself, but can also be triggered by cues associated with that reward and is additionally mediated by internal physiological states which can intensify or dampen motivation for a given reward (Berridge, 2004; Bindra, 1974, 1978; Flagel et al., 2008, 2009; T. E. Robinson & Flagel, 2009; Toates, 1986). CRF systems are not only activated by rewards, but are capable of intensifying incentive motivation for both rewards and reward cues (Baumgartner et al., 2021, 2022; Calogero et al., 1989a; Merali et al., 1998a; Peciña et al., 2006a).

Specifically, CRF signaling increases in response to consumption of both food and drug rewards. Cocaine administration stimulates secretion of hypothalamic CRF, allowing the

possibility that CRF could contribute to encoding the rewarding effects of cocaine (Calogero et al., 1989a; Piazza et al., 1993; Piazza & Le Moal, 1997). While CeA CRF increases in response to stressful stimuli, CeA CRF release of the same magnitude is triggered by food consumption (Merali et al., 1998a). These findings suggest that CRF may be triggered by physiologically salient events as opposed to strictly stressful events. CRF release in the PVN and extended amygdala are well-characterized in response to a variety of stressors but limited recordings and measurements have examined CRF in response to rewarding stimuli.

Additional evidence using pharmacological activation of CRF systems further supports a role for CRF in mediating incentive salience. For example, CRF microinjections into the NAc medial shell can intensify cue-triggered ‘wanting’ in a Pavlovian Instrumental Transfer paradigm to the same extent as dopamine-stimulating amphetamine microinjections (Peciña et al., 2006a). Additionally, NAc CRF microinjections are sufficient to induce a conditioned place preference via facilitation of dopamine release into the NAc (Lemos et al., 2012). Finally, CRF microinjections into NAc are also capable of facilitating partner preference formation in monogamous prairie voles without impacting social anxiety-like behavior (Lim et al., 2007). While this evidence cumulatively points to role for CRF in positive incentive motivation, it is limited to the NAc.

Modern tools, such as optogenetics and transgenic rodent lines, have allowed for manipulations of specific populations of CRF neurons both in the NAc and in the CeA and BNST within the extended amygdala. In line with the pharmacological results, optogenetic activation of NAc CRF neurons in *Crh*-cre rats showed amplified incentive motivation for sucrose or cocaine rewards paired with laser stimulation over identical sucrose or cocaine rewards alone, and NAc CRF neuronal stimulation recruited Fos activation of distant reward

circuitry, including the VTA, LH, and VP, in addition to the CeA (Baumgartner et al., 2021, 2022). Additionally, some *Crh*-cre rats would self-stimulate laser excitation of NAc CRF neurons. Perhaps surprisingly considering the well-documented roles of CeA CRF in distress, optogenetic activation of CeA CRF neurons also intensified pursuit of laser-paired sucrose or cocaine rewards, supported self-stimulation, and recruited mesolimbic reward related circuitry in *Crh*-cre rats, which indicates that CRF in the extended amygdala is not solely a circuit for anxiety and distress (Baumgartner et al., 2021, 2022). Additional work has shown that optogenetic activation of PVN CRF neurons in *Crh*-cre mice may support self-stimulation and that CRF projections from PVN to VTA are self-stimulated in both place-based and operant self-stimulation tasks in a CRFR1 and dopamine=dependent manner (Xu et al., 2024). In contrast, activation of BNST CRF neurons in *Crh*-cre rats biased reward pursuit away from laser-paired rewards and led to avoidance of CRF stimulation in a place-based self-stimulation task, supporting previous evidence that BNST CRF neurons generate aversive motivation (Baumgartner et al., 2021, 2022). While not all CRF populations are geared toward incentive motivation, CeA, NAc, and some PVN CRF systems have the capacity to intensify incentive motivation and bias reward decision making, potentially by mediating the salience of rewards or reward cues.

### 1.6 Mechanisms and Mediators for CRF-driven Incentive Motivation

The evidence for an incentive role for CRF signaling continues to grow, however, several major questions arise from the studies using optogenetic activation of CRF neurons in *Crh*-cre rodents. First, it is unclear whether CRF peptide binding and receptor activation are required for the incentive effects elicited by NAc and CeA CRF neuronal activation. CRF neurons co-release other neurotransmitters such as GABA, somatostatin, neuropeptide Y, and dynorphin, which

some may view as more likely candidates for generating incentive effects than CRF itself. Next, while CRF neuronal activation elicits positive incentive motivation in animals with that have not had extensive experience with drugs such as cocaine, it is possible that extended access to cocaine self-administration could flip the valence of CeA CRF neurons from incentive to aversive to fulfil their role in generating anxiety and distress as posited by opponent process theories of addiction (Ahmed et al., 2003; Ahmed & Koob, 1998; Blacktop et al., 2011; Mantsch et al., 2004, 2008). Lastly, the circuitry underlying CRF neuronal incentive motivation is still relatively unknown. While Fos mapping demonstrates that activation of NAc and CeA CRF neurons recruits other reward circuitry, such as the VTA, LH, and VP, the anatomical projections necessary for this functional recruitment are unknown.

*Does CRF neurotransmitter itself mediate the incentive motivation effects of optogenetic CRF neuronal stimulation?* To specifically investigate whether CRF neurotransmitter release and receptor binding mediates the incentive motivation effects of optogenetically stimulating CRF neurons in NAc (Baumgartner et al., 2021, 2022), Chapter 2 examines whether blockade of CRF receptors via intraventricular administration of a global CRF receptor antagonist attenuates the incentive effects of NAc CRF neuronal activation. Here, we show that CRF antagonism prevents development of focused reward seeking otherwise elicited by optogenetic activation of NAc CRF neurons. CRF antagonist administration also attenuates the persistence of incentive motivation for sucrose reward, as the effort requirement to earn rewards increases. Furthermore, blockade of CRF receptor activation reduces optogenetic laser self-stimulation of NAc CRF neurons.

Chapter 3 addresses a similar question to chapter 2, however, now turning attention specifically to CRF neurons of the CeA. While CRF in the NAc has previously been implicated

in incentive motivation, CeA CRF receptor signaling has nearly exclusively been associated with distress and anxiety. Here, we demonstrate that CRF receptor blockade reduces incentive motivation for laser-paired rewards in the two-choice sucrose task (Baumgartner et al., 2021, 2022), and also attenuates laser self-stimulation of CeA CRF neurons. This confirms that CeA CRF neurons signal via CRF receptor activation to amplify and assign incentive motivation, similarly as in NAc.

*Does extended access to cocaine switch the motivational valence of CeA CRF neurons to distress?* In chapter 4, we assess whether extended access cocaine self-administration can flip the valence of CeA CRF neuronal activation from incentive to aversive. Following 14 days of daily 6hr cocaine self-administration sessions, *Crh*-cre rats demonstrated sex-specific alterations in CeA CRF neuronal activation where females pursued more rewards overall but failed to develop either preference or aversion for laser-paired sucrose rewards, while males predominantly pursued sucrose rewards without laser. However, following LgA, both male and female ChR2 rats made a greater number of overall responses in the 2-choice sucrose task compared to eYFP rats who had also administered cocaine or ChR2 rats who were drug naïve, indicating that drug exposure may generalize CeA CRF-driven intensified incentive motivation. Lastly, following LgA, some ChR2 rats began to self-stimulate during withdrawal or following 4 weeks of abstinence, even when they had not self-stimulated during pre-tests. This suggests that CeA CRF neurons still generate positive incentive motivation following extensive cocaine exposure. These results fail to support a role for CeA CRF neuronal signaling in driving distress and provide an incentive role for CeA CRF neurons in modeling addiction.

*What anatomical output projections of CeA CRF neurons are responsible for incentive motivation effects?* Lastly, chapter 5 serves as an exposition of pilot data collected to identify

circuitry underlying the incentive effects of CeA CRF neuronal activation. Here, we present emerging evidence that optogenetic stimulation CeA CRF neurons projecting to the lateral hypothalamus generate aversion in the 2-choice task, are insufficient to support self-stimulation, and is avoided in a place-based self-stimulation task. However, there may be a rostro-caudal gradient to this effect where neurons projecting to the posterior LH generate aversion, but neurons even more posterior, approaching the substantia nigra and rostral VTA, may generate incentive motivation. Furthermore, we demonstrate pilot evidence that neurons projection from CeA to dorsal medial striatum also generate aversive motivation in two-choice and self-stimulation tasks, leaving the chief CeA CRF projections for incentive motivation effects still to be identified.

*Summary*

Overall, this dissertation aims to explore the mechanisms underlying a novel role for CRF signaling in incentive motivation. It confirms that CRF receptor activation contributes to the incentive effects of both NAc CRF and CeA CRF neuronal activation. It also presents evidence regarding whether the valence of CeA CRF neuronal activation is altered by LgA cocaine self-administration and begins to explore the circuitry involved in CRF-driven motivation.

# Chapter 2 Corticotropin Releasing Factor (CRF) Receptor Activation Mediates the Incentive Motivation Effects of Optogenetic CRF Neuronal Excitation in Nucleus Accumbens

## 2.1 Introduction

The nucleus accumbens shell (NAc) is a limbic region involved in generation of positive and negative emotion, pleasure, reward, and motivation (Baumgartner et al., 2020; Castro et al., 2016; Castro & Berridge, 2014; Peciña & Berridge, 2005; Pierce & Kumaresan, 2006; Smith & Berridge, 2007). Drugs of abuse, including psychostimulants, opioids, alcohol, nicotine, and cannabinoids, cause enhanced dopamine release in the NAc which was originally thought to be responsible for the hedonic or reinforcing effects of these drugs (Cheer et al., 2004; J. Chen et al., 1991; Ciano et al., 1995; Di Chiara & Imperato, 1988; Gonzales et al., 2004; Hurd et al., 1989; Nisell et al., 1994; Olds, 1982; Pettit & Justice, 1989; Pierce & Kumaresan, 2006; Tanda et al., 1997; Wise et al., 1995; Yim & Gonzales, 2000), but may more specifically mediate their motivational effects via incentive salience (Berridge & Robinson, 2016; M. J. F. Robinson et al., 2015; T. E. Robinson & Berridge, 1993; Saunders & Robinson, 2010; Yager & Robinson, 2013).

The NAc has been suggested to contribute to compulsive drug seeking which is a hallmark of addiction (Berridge & Robinson, 2016; George et al., 2012a; G. F. Koob et al., 2014; T. E. Robinson & Berridge, 1993; Wise et al., 1995; Wise & Bozarth, 1987; Wise & Morales, 2010). Specifically, neuroscience theories of addiction based on the psychological opponent-process model (Solomon & Corbit, 1974) view the medial shell of the NAc as a transitional area between striatum, which ties motivation to motor output and behavior, and the extended

amygdala, a basal forebrain macrosystem which has been suggested to generate distress during withdrawal (George et al., 2012a; Holmgren & Wills, 2021; G. F. Koob, 2010; G. F. Koob et al., 2014). As such, the NAc is positioned to turn emotional and motivational processing into emotional and motivated behaviors.

Corticotropin releasing factor (CRF) is a neuropeptide known for its role as a key regulator in behavioral response to stress and is released by neurons of the hypothalamus and other limbic structures, including the nucleus accumbens (NAc) (Baumgartner et al., 2021; Dedic, Chen, et al., 2018; Gray, 1993; Pomrenze et al., 2015; Pomrenze, Tovar-Diaz, et al., 2019). Specifically, CRF is traditionally thought to mediate anxiety and the unpleasant emotional response to stressors (Blacktop et al., 2011; Dedic, Chen, et al., 2018; Pomrenze, Tovar-Diaz, et al., 2019). Within the NAc, CRF contributes to behavioral response to pain and other physiological and psychological stressors. For instance, NAc CRF signaling enhances sensitivity to pain, alters sleep/wake behaviors, contributes to dendritic atrophy, and, in socially-stressed mice contributes to social avoidance. (Walsh et al., 2014; T. Wang et al., 2023; Zhao et al., 2024). Following severe stress, microinjections of CRF into the NAc generate conditioned place aversion which persists up to 90 days following stress (Lemos et al., 2012). Furthermore, NAc CRF systems are thought to be involved in drug withdrawal and are posited to contribute to distress and anxiety which may act as negative reinforcers and lead to relapse as a form of hedonic self-medication (G. F. Koob, 2010).

In contrast, other evidence supports an alternative positively-valenced role for NAc CRF systems in incentive motivation. NAc CRF microinjections in rats facilitate mesolimbic dopamine release, establish conditioned place preference, and amplify cue-triggered ‘wanting’ in Pavlovian Instrumental Transfer (PIT) tests similarly to amphetamine induced dopamine-release

(Lemos et al., 2012; Peciña et al., 2006b). Additionally, optogenetic stimulation of NAc CRF neurons of *Crh*-Cre rats drives positive incentive motivation. For example, some *Crh*-Cre rats self-stimulate laser excitation of NAc CRF neurons by itself, without need of other sensory rewards (Baumgartner et al., 2021, 2022). Further, pairing sucrose or i.v. cocaine rewards with optogenetic stimulation of NAc CRF neurons biased reward pursuit and heightened incentive motivation so that rats preferred sucrose or intravenous cocaine rewards paired with CRF laser-stimulation over identical sucrose or cocaine rewards without stimulation, and NAc CRF optogenetic stimulation elevated effort breakpoints to obtain those rewards in progressive ratio tasks (Baumgartner et al., 2021, 2022). NAc CRF neuronal stimulation also increased Fos expression in ventral tegmentum and related limbic structures, suggesting that this positive incentive motivation may be mediated by recruiting mesolimbic reward circuitry to amplify incentive salience attributed to those rewards (Baumgartner et al., 2021, 2022).

However, we cannot yet attribute the incentive effects of optogenetic NAc CRF neuronal stimulation to CRF neurotransmitter since NAc CRF neurons also co-release several other neurotransmitters, including GABA, neurotensin, and somatostatin (Partridge et al., 2016; Pomrenze et al., 2015; Pomrenze, Giovanetti, et al., 2019). It is therefore possible that other neurotransmitters, not CRF, mediate the incentive motivation effects of NAc CRF neuronal stimulation, and that CRF neurotransmitter itself mediates more traditional aversive effects even in NAc.

To address whether NAc CRF neurotransmitter in particular contributes to incentive motivation effects from optogenetic CRF neuronal stimulation, or if those effects are due primarily to other co-released neurotransmitters, we tested whether antagonism of CRF-1 and CRF-2 receptors reduces incentive effects produced by NAc CRF neuronal activation. We

administered the CRF antagonist D-Phe-$CRF_{12-41}$ (Basso et al., 1999; Macey et al., 2000; Valdez et al., 2003) prior to optogenetic stimulation of CRF neurons in *Crh*-Cre rats during 1) a two-choice sucrose task where rats could choose between earning a sucrose reward paired with laser stimulation of NAc CRF neurons versus earning an identical sucrose reward without laser stimulation; 2) a progressive ratio (effort breakpoint) task assessing magnitude of incentive motivation for laser-paired sucrose vs sucrose alone; 3) a laser self-stimulation test where rats earn laser pulses to stimulate NAc CRF neurons (Baumgartner et al., 2021, 2022). Our results indicate that blockade of central CRF receptor binding by i.c.v. administration of the CRF antagonist D-Phe-$CRF_{12-41}$ reduced all three incentive motivation effects generated by optogenetic NAc CRF neuronal excitations. These results suggest that activation of CRF receptors by CRF peptide is central to incentive motivation produced by optogenetic stimulation of NAc CRF neurons.

## 2.2 Materials and Methods

*Animals*

*Crh*-cre Wistar rats (n=13 male, n=20 female) were bred and genotyped in-house, using breeders from a transgenic *Crh*-Cre strain originally developed and provided by the Robert Messing lab at the University of Texas (Pomrenze et al., 2015) or obtained from Envigo. Breeding pairs were replaced every 8-10 litters to prevent genetic drift. Prior to surgery, rats were group housed in separate-sex rooms on a 12-hour reverse light/dark cycle at 21℃ with *ad libitum* food and water. Rats were at least 8 weeks old and 250g at the time of surgery. Following surgery, rats were single housed in otherwise identical conditions. All experimental procedures took place during the dark phase of the 24-hr cycle. All experimental procedures were approved

by the University of Michigan Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

*Optogenetic Surgery and Intraventricular Cannula Implantation*

Rats were anesthetized with isoflurane gas (5% induction; 1-3% maintenance) and administered atropine (0.05mg/kg i.p.; Henry Schein), carprofen (5mg/kg, s.c.; Henry Schein), and cefazolin (75mg/kg, s.c.; Henry Schein) prior to placement in the stereotactic apparatus (David Kopf Instruments, Tujunga, CA).

Rats were arbitrarily assigned to either an optogenetic channelrhodopsin stimulation group (NAc ChR2 rats; n=21) or to a control eYFP group (NAc eYFP rats; n=12). Optogenetic ChR2 rats received 1ul bilateral microinjections of a Cre-targeted ChR2 containing virus (AAV-EF1a-DIO-ChR2-eYFP; UNC Vector Core), and control eYFP rats received the optically inactive virus (AAV-EF1a-DIO-eYFP; UNC Vector Core). Bilateral virus microinjections were targeted at the lateral division of NAc (A/P +1.3, M/L ±2.1, D/V -6.67, angle 16°). Microinjections were administered at a rate of 0.1ul/min, and microinjection needles were left in place for 10 additional minutes to ensure diffusion. In the same surgery, optic fibers were bilaterally implanted 0.3mm dorsal to the virus injection site. To allow for pharmacological i.c.v. microinjections, a 22-gauge intraventricular cannula was also implanted into the right lateral ventricle (A/P -0.84, M/L +1.5, D/V -4.5). Cannula and optic fibers were secured with skull screws and acrylic cement. Rats were postoperatively monitored for 7 days and received additional daily carprofen injections 24- and 48-hours following surgery.

*CRF antagonist*

The CRF antagonist (D-Phe$^{12}$,Nle$^{21,38}$,α-Me-Leu$^{37}$)-CRF (12-41) (D-Phe-CRF$_{(12-41)}$; Bachem 4030465) was reconstituted at 5mg/ml and aliquoted in sterile 4% dimethylsulfoxide (DMSO) in isotonic saline and stored at -20°C. Immediately prior to intraventricular injections, D-Phe-CRF$_{(12-41)}$ was diluted to 2mg/ml in 4% DMSO. Rats received i.c.v. microinjections over 30 seconds of either 10ug/5ul of the CRF receptor antagonist D-Phe-CRF$_{(12-41)}$ in 4% DMSO or 5ul of the 4% DMSO vehicle alone 15 minutes prior to behavioral tasks. Microinjections were administered via a 28-gauge microinjector extending 1mm beyond the end of the guide cannula. The microinjector was left in place for a minimum of 30 seconds after the injection to allow for diffusion.

*Two-Choice Sucrose Task*

We adapted the 2-choice sucrose task of Baumgartner et al. (2021) to test the effects of CRF antagonist blockade on incentive preference induced by NAc ChR2 optogenetic pairing. In this task, rats could choose to earn either sucrose pellets accompanied by NAc CRF laser activation (*Laser + Sucrose)* by making nosepokes into a designated porthole or pressing on a designated lever, or to earn equivalent sucrose pellets delivered without laser (*Sucrose Alone*) by making nosepokes into a different porthole or pressing on a different lever. This task was employed here to test whether antagonist blockade of CRF receptors would prevent the development of a preference for the *Laser + Sucrose* option in NAc ChR2 rats that was previously reported by (Baumgartner et al., 2021).

To allow both within-subjects and between-subjects comparisons between vehicle and antagonist conditions, rats went through a pre-training phase, and 3 sequential phases with antagonist or vehicle of 2-choice sucrose tests described below. The pre-training phase consisted

of 4-8 instrumental pre-training sessions in which rats simply learned to nosepoke for sucrose pellets, without any laser present, until they reached a criterion of 50 rewards from each lever or noseport. The final two pre-training sessions also included microinjection habituation in which rats received vehicle i.c.v. microinjections and continued to work for sucrose until they had earned 20 rewards from each lever or noseport. Once the animals finished pre-training, they progressed into 3 laser test phases: 1) Initial 2-choice task where one porthole earned Laser + Sucrose and the other earned Sucrose Alone, in which some rats received antagonist microinjections and other rats received vehicle microinjections for 4 days (between-subjects comparison; fixed ratio 1 (FR1) schedule), 2) Continued 2-choice task but with vehicle/antagonist assignments reversed for 4 days, so that the rats that previously received vehicle now received antagonist, and vice versa (within-subjects comparison; FR1 schedule), and 3) Continued 2-choice task with antagonist/vehicle assignments as in Phase 2, but with an escalation of the effort requirement to earn either option from FR1 to random ratio 6 (RR6) over 5 days, to assess the robustness of any laser-induced preference or avoidance in the 2-choice task (between-subjects comparison).

*Initial instrumental pre-training*

Rats were first pre-trained instrumentally to earn sucrose pellets on a fixed-ratio 1 schedule (FR1) with one of two types of manipulandum. One group of NAc ChR2 and of NAc eYFP rats learned to earn sucrose pellets by making nose pokes into either of two fixed portholes mounted on a wall; the other arbitrarily assigned groups learned to earn sucrose by pressing either of two retractable levers that protruded from a wall. The different nosepoke/lever responses were used to ensure that eventual antagonist results were not dependent on any single type of manipulandum, and the nosepoke/lever press assignment of each rat was kept constant

throughout pre-training and Phase 1 of the 2-choice laser training and test. On the first pre-training day, one lever was extended, or one porthole was illuminated, and a response on it earned a sucrose reward. On the next day, the alternative lever was extended, or the alternative porthole was illuminated, and a response on it earned a sucrose reward. This daily alternation continued over 4 – 5 days until each rat had earned 50 cumulative rewards from each of its two levers or portholes.

*Microinjection habituation days (no laser):* To ensure instrumental behavior would not be disrupted by i.c.v. microinjections and handling, all rats received an i.c.v. microinjection of vehicle prior to two additional days of sucrose pre-training to serve as microinjection habituation sessions. Rats had to earn a minimum of 20 cumulative rewards over the two habituation days from each lever or porthole to move on to 2-choice laser training and testing.

*CRF Antagonist vs. Vehicle Comparisons*

To assess the effects of CRF antagonism, rats underwent 3 phases of 2-choice laser training and tests. Rats were placed in the lap of an experimenter where they received a 30s microinjection of either vehicle or antagonist as described above 15 minutes prior to behavioral testing.

*Phase 1: 2-choice laser preference tests: Laser + Sucrose vs Sucrose Alone*

NAc ChR2 rats and NAc eYFP rats were randomly divided into either CRF Antagonist or Vehicle subgroups, which remained constant throughout Phase 1. Each rat received its assigned antagonist or vehicle microinjection 15 minutes prior to each discriminative *Laser + Sucrose* vs. *Sucrose Alone* choice test in a chamber containing either two portholes or two levers.

For each rat, after receiving a microinjection, one lever or porthole was assigned (counterbalanced across rats) to earn *Laser + Sucrose,* whereas the other lever or porthole earned *Sucrose Alone*. An instrumental response on the *Laser + Sucrose* lever or porthole earned a sucrose pellet accompanied by laser illumination (473nm; 40Hz; 3mW (cycling 10ms on/15ms off for 8-sec bin duration) that began with the instrumental response that earned reward and continued 8-sec while sucrose was consumed. An assigned auditory CS label for each option (8-sec tone or white noise; counterbalanced across rats) also began simultaneously with laser onset and terminated when laser ended (e.g., pure tone label for *Laser + Sucrose* and white noise for *Sucrose Alone;* or vice versa).

In the first few minutes of each 2-choice session, only one lever or porthole was first presented (balanced order across days) until the rat responded and earned its assigned reward (either *Laser + Sucrose* or *Sucrose Alone*). Then the lever retracted, or the porthole dimmed, for an 8-sec time out period. The other lever was next inserted, or porthole illuminated, so the rat could earn the alternative outcome. This alternating presentation of levers or portholes repeated once more, so that the rat earned two assigned rewards from each lever or porthole. These single-choice exposures served to remind a rat each day of both outcomes (typically both completed within 5 min), before they were allowed to choose freely between the two outcomes for the rest of the session.

Subsequently, both levers were always extended or both portholes illuminated simultaneously to allow free choice between *Laser + Sucrose* versus *Sucrose Alone* options. Once a choice was made and its outcome earned, both levers were retracted or both portholes dimmed for an 8-sec time out. Then both levers or both portholes were presented again for another choice. These 2-choice choice presentations continued for the remainder of the 30min

session. Daily sessions were repeated for 4 days to compare laser-induced preferences between the Vehicle and the CRF Antagonist groups on a between-subject basis.

*Phase 2: FR1 Antagonist/Vehicle reversal with New Instrumental Responses.*

To compare CRF antagonist vs vehicle effects in the same rat, on a within-subject basis, previous Antagonist vs Vehicle assignments were reversed for all rats in Phase 2. Rats that received Antagonist in Phase 1 now instead received daily Vehicle microinjections prior to Phase 2 tests. Conversely, all rats that previously received Vehicle now received Antagonist. All rats were also switched to new instrumental manipulanda, to minimize the possibility that any Phase 1 laser-induced preferences would carry over to Phase 2 and confound the effects of Antagonist/Vehicle reversal. That is, rats previously trained on two levers were now switched to two portholes, positioned on the opposite wall from where levers had been (levers were now retracted), and rats previously trained on portholes were now switched to two levers, also placed on the opposite wall from where portholes had been (portholes were removed). For each rat, one new lever or porthole was permanently assigned to earn *Laser + Sucrose,* and the alternative lever or porthole assigned to earn *Sucrose Alone.* Daily 2-choice sessions continued for 4-6 days as in Phase 1 but with pharmacological condition reversed, until each rat again met a response criterion of earning at least 20 rewards per session for 4 consecutive sessions on the new manipulanda. Data from each rat's Antagonist vs Vehicle conditions in Phase 1 and Phase 2 were compared on a within-subject basis.

*Phase 3: Escalation of effort requirement.*

Finally, we assessed whether NAc ChR2 laser-induced preference or avoidance in the 2-choice task was motivated with sufficient robustness to persist even if the effort price of both

rewards was increased. For each rat, its antagonist/vehicle and lever/porthole assignment of Phase 2 was retained in Phase 3. However, the response schedule required to earn either *Laser + Sucrose* or *Sucrose Alon*e was escalated across the next 5 days from FR1 to RR6: FR4 ($1^{st}$ day of escalation), random ratio 4 (RR4, $2^{nd}$ day of escalation), and RR6 ($3^{rd}$-$5^{th}$ days of escalation). To assess the impact of CRF antagonism on preference between *Laser + Sucrose* or *Sucrose Alone*, the last 3 days of Phase 3 testing (RR6) were compared between vehicle and antagonist groups on a between-subject basis.

*Progressive Ratio Test of Effort Breakpoint*

To test whether CRF receptor blockade would prevent the amplification of intensity of incentive motivation for a reward otherwise caused by pairing ChR2 stimulation of NAc CRF neurons, we used a progressive ratio (PR) task (Baumgartner et al., 2021, 2022) to measure effort breakpoint for NAc CRF sugar reward after CRF antagonist versus vehicle on a between-subject basis. Rats were assigned their same antagonist/vehicle status Phases 2/3 of the 2-choice task for both days of PR breakpoint tests, and their same lever/porthole assignments from Phases 2/3. On one PR test day, after receiving their microinjections, only the *Sucrose + Laser* lever or porthole was available, and it earned a sucrose pellet accompanied by 8-sec laser illumination and auditory label as it had previously in the 2-choice task Phases 2 & 3. On the other PR day, after receiving the same microinjection, only the *Sucrose alone* option was available, and it earned a sucrose pellet without laser and different auditory label as it had in the 2-choice task. The order of *Laser + Sucrose* versus *Sucrose Alone* days was counterbalanced across rats. On each day, the number of responses required to earn the next reward increased after each reward was earned (progressive ratio schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178,

219, 268, …) derived from the formula PR = [5e(reward number×0.2)] − 5 and rounded to the nearest integer (Richardson & Roberts, 1996; Saunders & Robinson, 2011). The breakpoint or highest effort reached by the end of the 30-minute session was compared as a measure of the intensity of incentive motivation for reward.

*Laser Self-Stimulation Task*

A laser self-stimulation task was used to assess if brief pulses of NAc ChR2 neuronal excitation carried positive motivational value on their own (without sucrose), and to test whether that value was reduced by antagonist blockade of CRF receptors. Two innocuous metal 0.5 cm diameter metal rods extended 3cm into the self-stimulation chamber, spaced 17cm apart. Touches on one rod (permanently designated as Laser-delivering rod for that rat) triggered a 1s bin of laser stimulation (1 mW; constant illumination). Each touch on the other rod (designated as Inactive for that rat) earned nothing and simply served as a baseline measure of exploratory touches.

Rats were initially diagnosed for laser self-stimulation over three days without any microinjection, classifying them according to 3 levels of self-stimulation performance (Baumgartner et al., 2021, 2022). Rats were classified as High Self-stimulators if they earned >50 laser illuminations in a 30-min session and touched their Laser-delivering rod >2X more often than their Inactive rod. Rats were classified as Low Self-Stimulators if they earned 10 to 49 laser illuminations in a session, and still touched their Laser-delivering rod >2X more often than the Inactive rod. Rats were classified as Failures to self-stimulate if they earned fewer than 10 laser illuminations or failed to touch their Laser-delivering rod at least twice as often as their Inactive rod. Rats that consistently Failed to self-stimulate were discarded from the next phase.

CRF antagonist vs Vehicle effect on Laser Self-Stimulation (Phase 1 – Between-subject comparison): High Self-Stimulators and Low Self-Stimulators were then randomly divided and assigned in equal numbers to either a Vehicle microinjection group or a CRF antagonist microinjection group for the next 4 days for days of laser self-stimulation tests. A between-subject comparison of antagonist/vehicle performance was made by comparing average laser self-stimulations earned across the 4 days.

Laser Self-Stimulation (Phase 2 – Within-subject comparison of antagonist/vehicle effects): Finally, the antagonist/vehicle assignment of each rat was reversed for a further 4 days of laser self-stimulation tests in order to make a within-subject comparison of performance across days. Rats in the Phase 1 Vehicle group were now switched to the Antagonist condition for Phase 2, whereas rats in the Phase 1 Antagonist group were switched to the Vehicle condition. This continued for a final 4 days of laser self-stimulation tests. Number of contacts on each rod was averaged across the 4 vehicle days and 4 antagonist days.

*Histology*

Prior to euthanasia, rats were administered the drug they were assigned in Phase 2 and 3 of the two-choice sucrose task and then delivered 30 minutes of cycling laser stimulation to induce c-fos (3mw 40Hz 8s on, 22s off). Rats were euthanized with a lethal dose of sodium pentobarbital (150-200mg/kg, i.p.; Euthasol) and transcardially perfused with sodium phosphate buffer and 4% paraformaldehyde (PFA). Brains were extracted and postfixed in 4% PFA for 24 hours and then cryoprotected in 25% sucrose for a minimum of 48 hours. Brains were then sectioned into 40um slices using a cryostat (Leica), permeabilized and blocked in % Triton and

% normal donkey serum in sodium phosphate buffer, and stained for GFP (chicken anti-GFP 1:2000; Abcam, ab1397; donkey anti-chicken Alexa 488 1:300; Jackson Immuno, 703-545-155) and Fos (rabbit anti-Fos 1:2500; Synaptic Systems, 226-008; 1:250 donkey anti-rabbit Alexa 594, abcam, ab150076). Brain tissue was mounted and coverslipped with ProlongGold anti-fade mounting medium with DAPI (Cell Signaling Technology, 8961S). Brain tissue was imaged using a digital camera and fluorescence microscope (Leica).

Coronal sections were imaged (10x) magnification. Virus and fiber were considered “on target” if virus was contained within the NAc and the bottom of the fiber tip was located within 0.6mm of fluorescent cells.

*Experimental Design and Statistical Analysis*

Statistical analysis was conducted in R (R Core Team, 2020) using tidyverse (Wickham et al., 2019), tidyr (Wickham, Vaughan, et al., 2024), dplyr (Wickham et al., 2023), lme4 (Bates et al., 2024), lmerTest (Kuznetsova et al., 2020), and emmeans (Lenth et al., 2024). Plot were made using ggplot2 (Wickham, Chang, et al., 2024), ggpubr (Kassambara, 2023), ggbreak (Yu & Xu, 2023), and ggpattern (FC et al., 2022). Tables were made with knitr (Xie et al., 2024), broom.mixed (Bolker et al., 2024), and modelsummary (Arel-Bundock et al., 2024). Linear mixed models were used to analyze experimental data followed by Type III tests with effects coding. Posthoc testing used pairwise t-test comparisons of estimated marginal means with Bonferroni correction.

## 2.3 Results

*Phase 1 and 2: 2-choice laser preference tests: Laser + Sucrose vs Sucrose Alone*

During Phase 1, one group of NAc ChR2 rats received vehicle while the other group received CRF antagonist prior to tests on the first 4 days of the 2-choice *Laser + Sucrose* vs. *Sucrose Alone* task. Following Phase 1, rats in the Vehicle group were reassigned to receive antagonist while the Antagonist group were reassigned to receive vehicle. Since the order in which the drugs were administered did not significantly influence responding (drug order; b=-8.11, df=36.02, t=-0.72, p=0.47), groups were collapsed across drug condition.

Over the 4 vehicle trial days, NAc ChR2 rats receiving vehicle developed a preference for the *Laser + Sucrose* option, increasing their responding for this option with each day (vehicle laser by trial day: b=10.24, df=288.554, t=3.272, p=0.001; Figure 2-1 A). As responding for the *Laser + Sucrose* option increased, responding for the *Sucrose Alone* remained unchanged over trial days (vehicle laser vs vehicle nonlaser by trial day: b=-10.943, df=288.993, t=3.16, p=0.002). By the final trial day, NAc CRF stimulation led NAc ChR2 rats receiving vehicle to respond for the *Laser + Sucrose* option at a ratio of nearly 3:1 over the *Sucrose Alone*, replicating previous results demonstrating that optogenetic NAc CRF neuronal activation can bias motivation for laser-paired rewards (laser: b=-58.26, df=78.67, t=-5.93, p<0.001; Figure 2-1 A).

By contrast, NAc ChR2 rats receiving i.c.v. antagonist failed to develop a preference for either option over the other, and instead only moderately increased their responding similarly for both the *Laser + Sucrose* and the *Sucrose Alone* options across trial days (laser x drug x trial: $F_{1,288.645}$=9.987, p=0.002; posthoc: b=-4.77, df=36.9, t=-0.635, p=1; Figure 2-1 B). These results suggest that CRF antagonist blockade effectively prevented NAc CRF neuronal stimulation from

producing its usual incentive preference effect, and that CRF receptor activation is required for the narrow focusing of incentive motivation produced by paired NAc CRF neuronal activation.

eYFP controls lacking ChR2 chose equally between the *Laser + Sucrose* and the *Sucrose Alone,* similar to the ChR2 rats who received antagonist, and their responding was not changed by CRF antagonist (Laser x drug: $F_{(1,129.21)}=5.39$, $p=0.02$; posthoc vehicle laser vs nonlaser: $p=1$; posthoc antagonist laser x nonlaser: $p = 1$; Figure 2-1 A,B). No sex differences were detected in either ChR2 rats ($b=0.93$, $df=18.416$, $t=0.16$, $p=0.875$) or in eYFP controls ($b=-3.179$, $df=11.921$, $t=-0.717$, $p=0.493$).

*Phase 3: Escalation of effort requirement*

Phase 3 of 2-choice sucrose testing tested the robustness of CRF antagonist effects on incentive motivation generated by NAc CRF activation. Rats continued to receive the same drug they had been assigned in Phase 2; however, the number of responses necessary to earn a sucrose reward progressively increased across trial days 9-13 from FR4 to RR6. NAc ChR2 rats who had received vehicle in Phase 2 developed a preference under vehicle for the *Laser + Sucrose* option and that preference continued to grow under vehicle in Phase 3 as the effort requirement increased (vehicle laser by trial day: $b=42.437$, $df=159.398$, $t=4.628$, $p>0.001$; Figure 2-1 C). While responding for the *Laser + Sucrose* grew each day, responding for *Sucrose Alone* changed minimally and was significantly lower than responding for *Laser + Sucrose* (laser vehicle vs nonlaser vehicle by trial: $b=-41.42$, $df=159.398$, $t=-3.194$, $p=0.002$; Figure 2-1 C). By the final day of RR6, vehicle ChR2 rats worked for *Laser + Sucrose* at a 3:1 ratio over *Sucrose Alone* (laser vehicle vs nonlaser vehicle: $b=-246.051$, $df=35.668$, $t=-4.403$, $p<0.001$; Figure 2-1 C). In contrast, NAc ChR2 rats never developed a preference under antagonist in Phase 2, instead

continuing to choose evenly between *Laser + Sucrose* and *Sucrose Alone* options as the effort requirement increased (laser antagonist x trial: b==9.757, df=160.842, t=-0.705, p=0.482; nonlaser antagonist x trial: b=40.377, df=160.869, t=2.063, p=0.041; Figure 2-1 D). By the final day of RR6, ChR2 antagonist rats were choosing between the *Laser + Sucrose* vs the *Sucrose Alone* at a 1:1 ratio (antagonist laser vs nonlaser: b=51.7, df=23.5, t=0.92, p=1; Figure 2-1 D).

Similarly, to ChR2 rats on antagonist, eYFP rats increased their responding for both options as the effort requirement increased, but continued to choose equally between the two reward options (laser x drug: b=72.99, df=10.39, t=0.31, p=0.77; Figure 2-1 C,D). Furthermore, no sex differences arose in the NAc ChR2 rats (b=7.01, df=18.09, t=0.433, p=0.67); however, eYFP females made more responses than males (sex: b=71.85, df=8.00, t=5.29, p<0.001).

*Breakpoint test of motivation intensity: CRF blockade prevents NAc CRF ChR2 elevation of breakpoint*

Following the final day of the 2-choice sucrose test, we tested whether the increased magnitude of incentive motivation from NAc CRF neuronal stimulation is dependent on CRF receptor activation. NAc ChR2 rats and control eYFP rats were tested in a progressive ratio breakpoint task where one day they could solely respond to earn *Laser + Sucrose* and another day (counterbalanced order) they could solely respond to earn *Sucrose Alone*. On both days of this task, rats received the drug microinjection that had been assigned for Phases 2 and 3 of the 2-choice sucrose task with some rats receiving vehicle on both days and other rats receiving the CRF antagonist.

NAc ChR2 rats who received vehicle reached breakpoints 2x higher on their *Laser + Sucrose* day than on their *Sucrose Alone* day (laser x drug interaction: $F_{1,18}$=4.573, p=0.04; posthoc: b=27.00, df=18, t=3.241, p=0.023; Figure 2-2 A,B). In contrast, NAc ChR2 rats who received antagonist failed to increase breakpoint for *Laser + Sucrose* over *Sucrose Alone* (drug x laser: b=0.44, df=18.0, t=0.048, p=1.0; Figure 2-2 A,B). For eYFP control rats, breakpoint was not altered by either laser or drug (laser: p=0.98, drug: p=0.48, laser x drug p = 0.85, and neither eYFP rats (sex: b=5.81, df=17, t=0.94, p=0.35) nor ChR2 rats (sex: b=3.489, df=17, t=0.67, p=0.51) displayed detectable sex differences.

*CRF receptor blockade attenuates ChR2 laser self-stimulation of CeA CRF neurons*

To assess the incentive value of NAc CRF neuronal stimulation by itself, rats could earn brief 1-sec laser illuminations of NAc CRF neurons by touching one of two metal bars. Rats were initially screened for self-stimulation without microinjections, when none of the 21 ChR2 rats met the criteria for High laser self-stimulation (>50 laser illuminations, plus Laser bar contacts >2X inactive bar contacts). 11 NAc ChR2 rats met criteria for Low self-stimulation (>10 but <50 laser illuminations, plus Laser bar contacts >2X inactive bar contacts), and 10 rats failed to self-stimulate (<10 laser illuminations).

The 11 rats who met Low self-stimulation criteria progressed to 4 additional days of self-stimulation preceded by vehicle microinjections and then 4 final days preceded by CRF antagonist microinjections. On average, NAc ChR2 rats under vehicle made significantly more contacts on their laser-delivering rod, early 3x as many, compared to the inactive rod (laser x drug interaction: $F_{1,11.989}$=10.7672, p=0.007; posthoc: b=42.77, df=23.4, t=4.862, p<0.001; Figure 2-3 A). Of the 11 rats who met Low self-stimulation criteria in the pre-screening, 7 of

them increased their self-stimulation to meet criteria for High self-stimulation while the other 4 continued to self-stimulate at Low levels (Figure 2-3 B).

When NAc ChR2 rats were switched to antagonist, their number of laser bar contacts decreased to the same level as contacts on the nonlaser bar (laser vehicle vs antagonist: b=48.13, df=21.1, t=4.758, p>0.001; antagonist laser vs nonlaser: b=5.44, df=23.4, t=0.619, p=1; Figure 2-3 A). Following antagonist microinjections, no NAc ChR2 rats met criteria for High self-stimulation, only 3 of the rats met criteria for Low self-stimulation, and the remaining 8 rats now failed to meet any self-stimulation criteria (Figure 2-3 B).

NAc eYFP control rats all failed to self-stimulate in the pre-screening and 8/9 remained failures during the vehicle phase while 1 rat met criteria for High self-stimulation. On antagonist, 6/9 failed to self-stimulate while 1 met criteria for low self-stimulation and 2 met criteria for high self-stimulation. Control eYFP rats may have made a slightly higher number of nonlaser contacts than laser contacts while on vehicle (laser x drug interaction: $F_{1,15.913}$=10.057, p=0.006; posthoc: b=-20.9, df=15.9, t=-2.113, p=0.051) and a slightly higher number of laser contacts compared to nonlaser contacts while on antagonist (b=21.3, df=15.9, t=2.155, p=0.0469; Figure 2-3 A). For ChR2 rats, females made marginally fewer contacts than males (sex: b=-12.50, df=11.00, t=-2.91, p=0.01). No sex differences were detected in eYFP rats (sex: b=3.09, df=7.29, t=0.18, p=0.86).

### 2.4 Discussion

These results replicate previous findings that pairing optogenetic activation of NAc CRF neurons with a sensory reward drives a positive preference for laser-paired sucrose over identical alternative sucrose without laser, and increases the intensity of incentive motivation for the laser-paired sucrose, as measured by effort breakpoint in a progressive ratio task (Baumgartner et al., 2021). Our results also replicate reports that some *Crh*-cre rats will at least moderately self-stimulate laser excitation of NAc CRF neurons without additional reward (Baumgartner et al., 2021, 2022).

Critically, our work demonstrates for the first time that these incentive effects require activation of CRF receptors and are blocked by i.c.v. administration of a CRF receptor antagonist. Thus, co-release of other neurotransmitters released by NAc CRF neurons, such as GABA, neurotensin, somatostatin, and dynorphin, were not sufficient on their own to enhance incentive motivation here, unless CRF receptor activation was also allowed (Partridge et al., 2016; Pomrenze et al., 2015; Pomrenze, Giovanetti, et al., 2019).

That is, we demonstrated that, following i.c.v vehicle microinjections, optogenetic activation of NAc CRF neurons is capable of narrowing incentive motivation for a laser-paired sucrose reward over an identical reward without laser when NAc ChR2 rats received vehicle. However, these incentive effects were eliminated in rats who received an i.c.v microinjection of a CRF receptor antagonist prior to testing. Furthermore, NAc CRF neuronal stimulation intensified the magnitude of incentive motivation of NAc ChR2 rats for *Laser + Sucrose* in a progressive ratio task over their motivation for *Sucrose Alone* following vehicle microinjections, but these effects were also blocked by CRF antagonist administration. Lastly, ChR2 self-

stimulated for laser excitation of NAc CRF neurons under vehicle, but this self-stimulation of NAc CRF neurons was attenuated or eliminated by administration of the CRF antagonist.

Where in the brain does CRF receptor antagonism act to block incentive motivation effects of NAc CRF neuronal stimulation? Since the cre-expressing NAc CRF neurons in *Crh*-cre rats may be primarily GABAergic locally projecting interneurons, projecting to nearby sites within NAc, pharmacological studies injecting CRF antagonist directly into the NAc would be valuable, and plausibly might be expected to be sufficient to block incentive motivation effects of NAc CRF neuronal stimulation. Given that previous studies demonstrate that CRF microinjections into the NAc can generate conditioned place preference and enhance cue-triggered motivation, local CRF signaling within the NAc could potentially elicit similar effects (Lemos et al., 2012; Peciña et al., 2006a). NAc CRF microinjections have also been shown to facilitate accumbal dopamine release which is required for generation of a CRF place preference, so if NAc CRF neurons are signaling locally, it is also possible that they are capable of directly modulating accumbal dopamine signaling (Lemos et al., 2012).

However, NAc CRF neurons are also known to project to the ventral tegmental area and the ventral pallidum which are both inextricably involved in motivation and reward pursuit (Castro & Bruchas, 2019; Eckenwiler et al., 2023; Pomrenze et al., 2015). Future studies could explore these additional projection targets of NAc CRF neurons. In addition to identifying projection targets, it would be useful to parse the contribution of the two CRF receptor types in generating incentive motivation. Additionally, recording techniques could provide information about endogenous activity of NAc CRF neurons and CRF release in response to rewards, reward cues, and during motivated behavior to determine what natural behaviors recruit NAc CRF circuitry.

Due to its role in stress and HPA axis activation, central CRF systems are traditionally associated with fear, anxiety, and distress in a broad range of contexts. For instance, microinjections of CRF into the NAc induces anxiety-like behaviors in the elevated plus maze, reduces sucrose preference in a two-bottle choice task, and increases depression-like immobility in the forced swim test (Y.-W. Chen et al., 2012). NAc CRF signaling has also been implicated in modulation of pain, sleep disturbances, and social avoidance following stress (Novoa et al., 2021; Walsh et al., 2014; T. Wang et al., 2023; Zhao et al., 2024). In addiction, activation of CRF systems in the bed nucleus of the stria terminalis, CeA, and NAc have been implicated in the negative affect associated with withdrawal that is thought to cause relapse as a form of hedonic self-medication (Galesi et al., 2016; G. F. Koob, 2010; Marcinkiewcz et al., 2009).

However, in addition to the outlined role for NAc CRF as a generator of distress, there is also evidence that NAc CRF systems are involved in generating positive motivation without distress. Microinjections of CRF into the NAc medial shell amplifies cue-triggered motivation in a Pavlovian Instrumental Transfer (PIT) paradigm similarly to NAc amphetamine microinjection (Peciña et al., 2006a). NAc CRF microinjections also can induce a conditioned place preference and facilitate accumbal dopamine release (Lemos et al., 2012). Additionally, NAc CRF neurons projecting to the ventral pallidum track reward outcomes and mediate acquisition of reward learning (Eckenwiler et al., 2023).

In all, our results demonstrate that NAc CRF neuronal activation generates positive incentive motivation via activation of CRF receptors, attributing CRF receptor activation with a role in incentive motivation without necessitating distress.

## 2.5 Figures

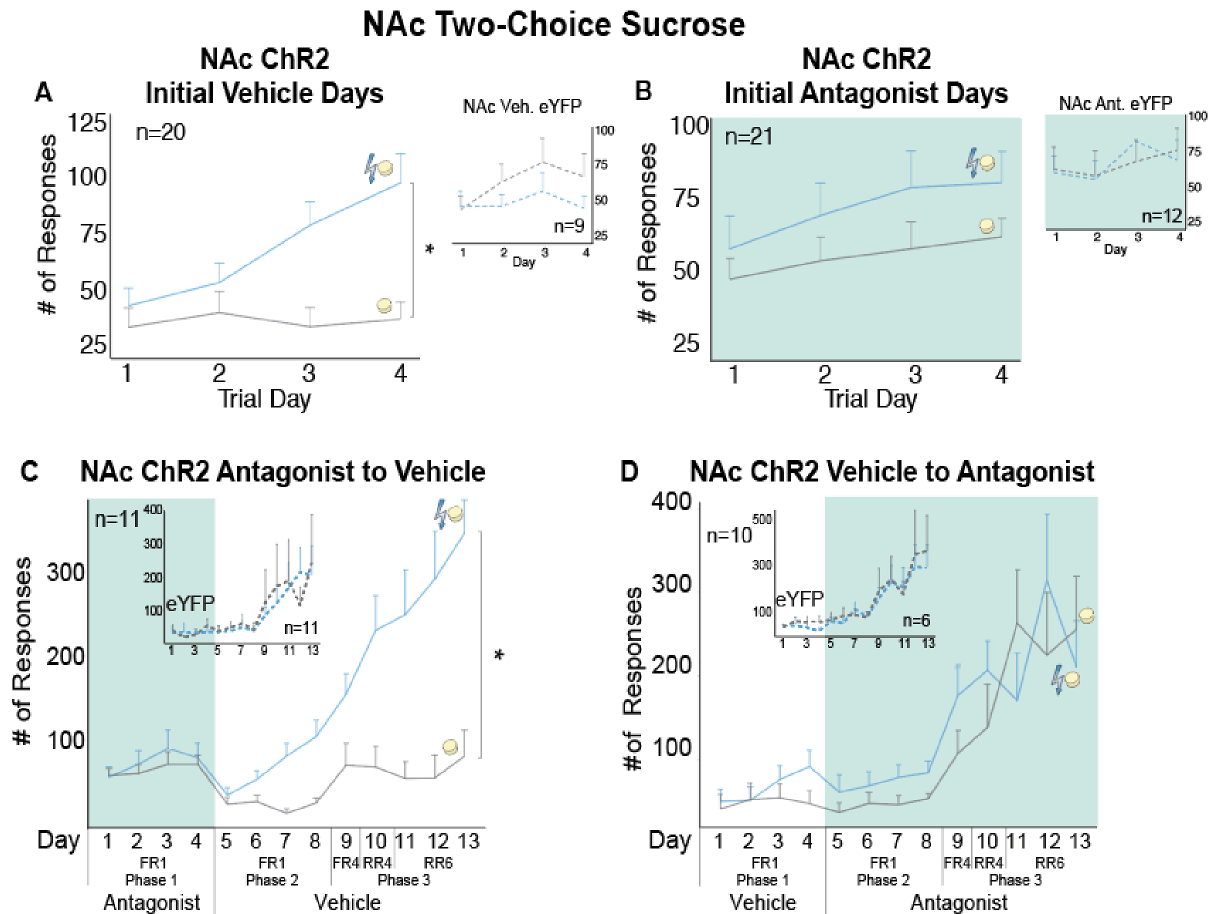



Figure 2-1 CRF antagonism prevents induction of preference for NAc CRF laser-paired sucrose option

A) NAc ChR2 rats develop a >2:1 preference for the *Laser + Sucrose* option while under vehicle. In contrast, eYPF rats on vehicle develop no preference for either option. B) Under CRF antagonist, NAc ChR2 rats fail to develop a significant preference for either the *Laser + Sucrose* or the *Sucrose* Alone option, similar to eYFP controls. C) NAc ChR2 rats maintained a High preference for the *Laser + Sucrose* option when effort requirement increased under vehicle in Phase 3 of the 2-choice task (n=11). By contrast, control NAc eYFP rats (n=11) failed to develop any preference. D) NAc ChR2 rats under CRF antagonist in Phase 3 failed to develop any preference (n=10). Similarly, eYFP rats (n=6) failed to develop a preference under antagonist.

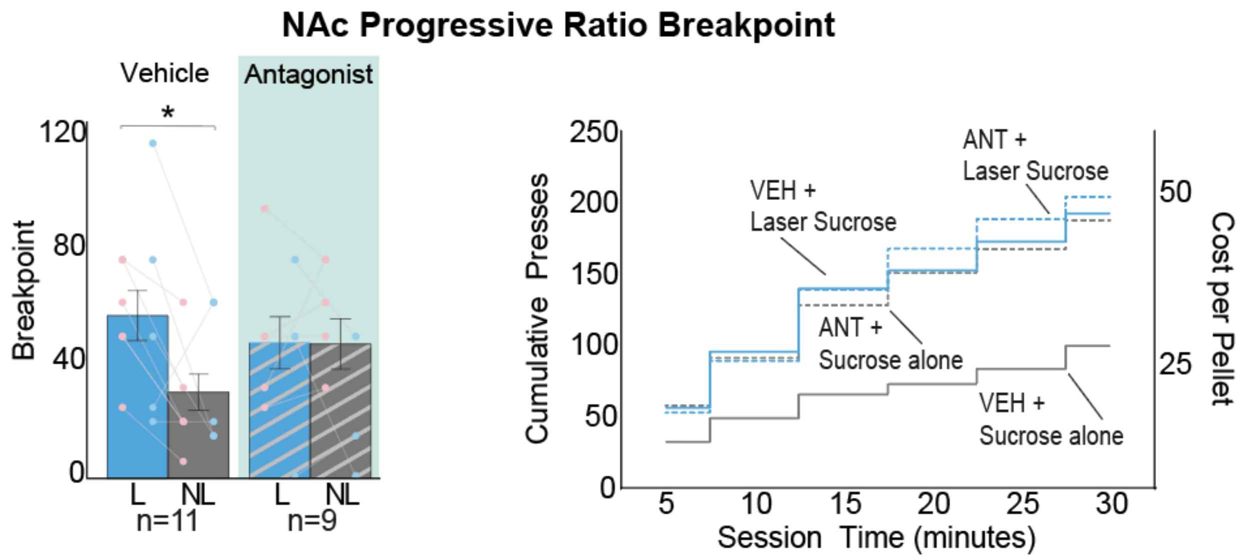



Figure 2-2 CRF antagonism prevents NAc CRF activation-induced breakpoint elevation in progressive ratio task

In the progressive ratio (PR) breakpoint task, NAc ChR2 under vehicle showed increased incentive motivation to obtain the *Laser + Sucrose* option compared to their performance for sucrose alone, reflected as increase in effort breakpoint (n=11) (left). By contrast, NAc ChR2 rats under CRF antagonist (n=9) showed no elevation in *Laser + Sucrose* breakpoint. Pink = Females, Blue = Males, L = Laser, NL = Nonlaser. Means and SEM reported. *p<0.05

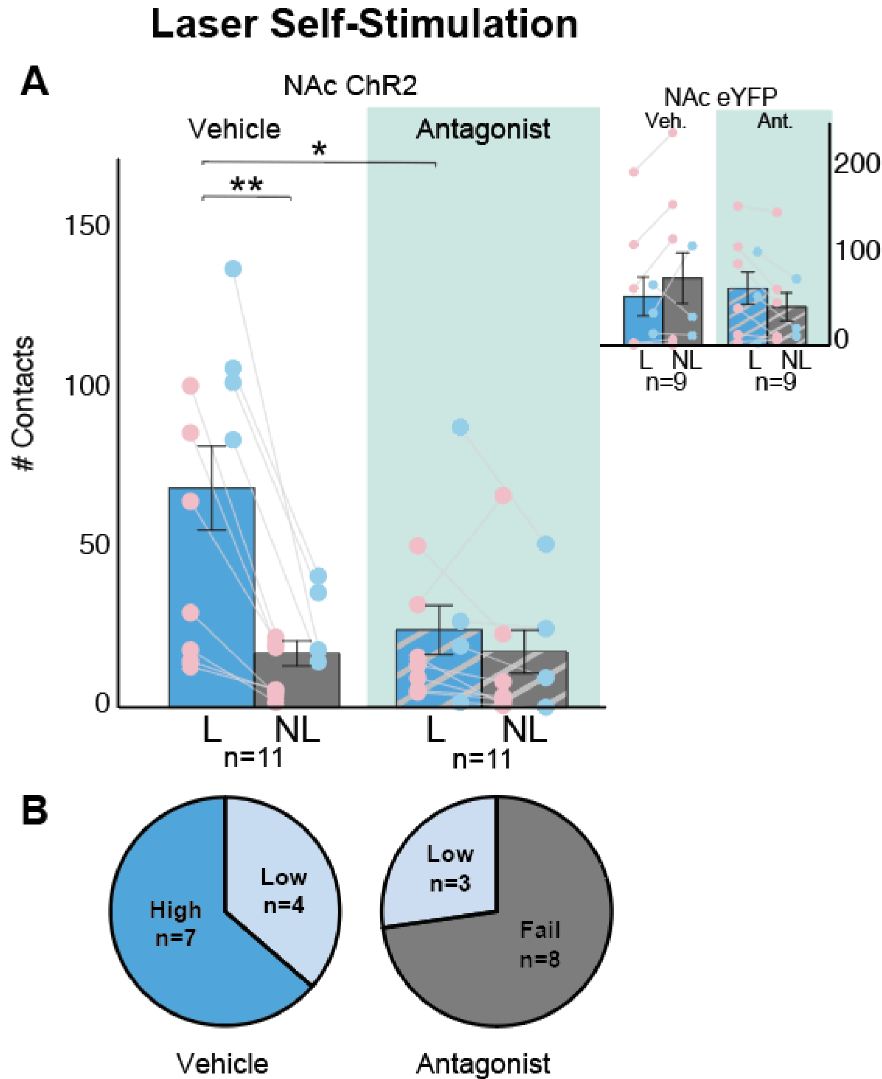



Figure 2-3 NAc CRF laser self-stimulation is attenuated by i.c.v. CRF antagonism

A) Only NAc ChR2 rats that met laser self-stimulation criteria in pre-screening (ether High or Low self-stimulation levels) were used for subsequent tests of Antagonist/Vehicle and are shown here (n=11/20 NAc ChR2 rats). Under vehicle, all 11 of these rats worked to self-stimulate laser excitation of NAc CRF neurons by touching a designated metal rod, earning over 60 illuminations per session on average. By contrast, CRF antagonist administration reduced laser self-stimulation to approximately one-third of vehicle baseline levels on average. B) The 11 individual NAc ChR2 rats from A were classified under vehicle as either High self-stimulators (i.e., earning > 50 illuminations per session) or Low self-stimulators (i.e., earning 10-49 illuminations per session). Under CRF antagonist, the number of both High and Low self-stimulators declined, and 8/11 became Failures to self-stimulate (<10 illuminations per session). Pink = Females, Blue = Males, L = Laser, NL = Nonlaser. Means and SEM reported. *p<0.05, **p<0.01

# Chapter 3 Corticotropin Releasing Factor (CRF) Receptor Activation Mediates the Incentive Motivation Effects of Optogenetic CRF Neuronal Excitation in Central Amygdala

## 3.1 Introduction

Corticotropin releasing factor (CRF) is a peptide neurotransmitter released by neurons of the hypothalamus and several other limbic structures, including extended amygdala components such as the central nucleus of amygdala (CeA) and bed nucleus of the stria terminalis (BNST), and the nucleus accumbens (NAc) (Baumgartner et al., 2021; Dedic, Chen, et al., 2018; Pomrenze et al., 2015; Pomrenze, Tovar-Diaz, et al., 2019). CRF in the brain acts as an integrator of the neural stress response, triggering activation of the hypothalamic-pituitary-adrenal axis and glucocorticoid hormone release (Gray, 1993). Traditionally, CRF activation in extended amygdala structures, including CeA, has been considered to mediate aversive distress, and to motivate behavior to reduce distress via efforts at hedonic self-medication, such as consuming rewards (Adamec & McKay, 1993; Bledsoe et al., 2011; Gray, 1993; Mazzitelli et al., 2022; Pomrenze, Giovanetti, et al., 2019; Zorrilla et al., 2002). For example, neuroscience theories of addiction based on the opponent-process model (Solomon & Corbit, 1974) posit that CRF systems in the CeA trigger aversive feelings of distress, including withdrawal feelings, resulting in increased motivation to consume drugs or binge eating (Cottone et al., 2009; George et al., 2012a, 2012a; G. F. Koob et al., 2014; Moore et al., 2017a, 2017b; Parylak et al., 2011; Valdez et al., 2003; Zorrilla et al., 2014). CeA CRF activation is posited to act as an aversive

negative reinforcer which promotes drug seeking as a method of ameliorating hedonic homeostatic dysregulation and CRF-mediated distress.

In contrast to the negatively-valenced aversive roles of CeA CRF neurons in distress, other evidence indicates that CRF systems in CeA and nucleus accumbens can also have an alternative positively-valenced role in motivation by directly generating incentive motivation to pursue and consume rewards, even in the absence of any distress. Endogenous CRF release in the central amygdala and paraventricular nucleus of the hypothalamus (PVN) is increased by the consumption of pleasant rewards, as well as by distressing events (Calogero et al., 1989a; Merali et al., 1998a). In terms of causing incentive motivation, CRF microinjections in NAc shell of rats facilitates dopamine release, establishes conditioned place preference, and amplifies cue-triggered incentive salience to pursue rewards in Pavlovian Instrumental Transfer (PIT) tests, similarly to dopamine-stimulation by amphetamine microinjections at the same sites in NAc shell (Lemos et al., 2012; Peciña et al., 2006b). Further, regarding CeA, recent evidence has shown that incentive motivation is also generated by direct optogenetic stimulation of CRF neurons in either CeA or NAc of *Crh*-Cre rats. For example, some *Crh*-Cre rats were willing to work to self-stimulate CeA CRF neurons in absence of any other sensory reward (Baumgartner et al., 2021, 2022). Further, rats preferred to choose sucrose rewards accompanied by CRF laser stimulation over sucrose delivered without CRF neuronal stimulation, and similarly preferred to earn intravenous cocaine infusions accompanied by CRF laser stimulation over cocaine delivered by itself, suggesting that CeA CRF neuronal excitation did not impede, but rather augmented, the reward value of laser-paired sucrose or cocaine (Baumgartner et al., 2021, 2022).Optogenetic CeA CRF neuronal stimulation also amplified the intensity of incentive motivation for laser-paired sucrose or cocaine rewards, measured as increased effort breakpoint in a progressive ratio

task (Baumgartner et al., 2021, 2022). These incentive motivation effects of CeA CRF neuronal stimulation appeared to be mediated by recruiting increased activation of mesolimbic reward circuitry, measured as increased Fos expression in ventral tegmentum, NAc and related limbic structures (Baumgartner et al., 2021, 2022).

However, just as in Chapter 2, it is possible that CRF peptide itself is not responsible for the incentive effects of CeA CRF neuronal stimulation given that CRF neurons in CeA also co-release a number of other neurotransmitters besides CRF, including GABA, neurotensin, somatostatin, and dynorphin (Partridge et al., 2016; Pomrenze et al., 2015; Pomrenze, Giovanetti, et al., 2019). It is therefore possible that those other neurotransmitters, rather than CRF, are responsible for the positively-valenced motivation induced by optogenetic CeA CRF neuron stimulation in the studies described above. In keeping with that possibility, others have reported that chemogenetic activation of CRF neurons in CeA also enhanced aversive fear learning, preventable by shRNA knockdown of CRF peptide (Pomrenze, Giovanetti, et al., 2019), which is consistent with aversive roles of CeA CRF neurotransmitter.

To address whether CRF is the neurotransmitter signal that mediates incentive motivation effects of optogenetic CeA CRF neuronal stimulation, or whether instead those effects are due primarily to other neurotransmitters co-released by the same CRF neurons, we performed the same experiments described in Chapter 2 to test if pharmacological blockade of both CRF receptor types would reduce incentive motivation effects produced by stimulation of CRF neurons in CeA. We tested this by administering the global CRF antagonist D-Phe-$CRF_{12\text{-}41}$ (Basso et al., 1999; Macey et al., 2000; Valdez et al., 2003) prior to optogenetic stimulation of CRF neurons in *Crh*-Cre rats during 1) a two-choice sucrose task where rats could choose between earing a sucrose reward accompanied by laser stimulation of CRF neurons versus

earning an identical sucrose reward without laser stimulation; 2) a progressive ratio (effort breakpoint) task to assess the magnitude of incentive motivation for laser-paired sucrose reward vs sucrose reward without laser; 3) a laser self-stimulation test in which rats could make nose pokes to earn brief laser pulses to stimulate CRF neurons in CeA (Baumgartner et al., 2021, 2022). Our results indicate that blockade of central CRF binding by the CRF antagonist D-Phe-$CRF_{12\text{-}41}$ reduced all three incentive motivation effects otherwise generated by paired optogenetic CRF neuronal excitations. These results suggest that release and binding of CRF neurotransmitter is an important component of incentive motivation produced by optogenetic stimulation of CRF neurons in CeA, providing further evidence for an incentive role of CRF within the extended amygdala.

## 3.2 Materials and Methods

*Animals*

*Crh*-cre Wistar rats (n=20 male, n=16 female) were bred and genotyped in-house, using breeders from a transgenic *Crh*-Cre strain originally developed and provided by the Robert Messing lab at the University of Texas (Pomrenze et al., 2015) or obtained from Envigo. Breeding pairs were replaced every 8-10 litters to prevent genetic drift. Prior to surgery, rats were group housed in separate-sex rooms on a 12-hour reverse light/dark cycle at 21℃ with *ad libitum* food and water. Rats were at least 8 weeks old and 250g at the time of surgery. Following surgery, rats were single housed in otherwise identical conditions. All experimental procedures took place during the dark phase of the 24-hr cycle. All experimental procedures were approved by the University of Michigan Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

*Optogenetic Surgery and Intraventricular Cannula Implantation*

Rats were anesthetized with isoflurane gas (5% induction; 1-3% maintenance) and administered atropine (0.05mg/kg i.p.; Henry Schein), carprofen (5mg/kg, s.c.; Henry Schein), and cefazolin (75mg/kg, s.c.; Henry Schein) prior to placement in the stereotactic apparatus (David Kopf Instruments, Tujunga, CA).

Rats were arbitrarily assigned to either an optogenetic channelrhodopsin stimulation group (CeA ChR2 rats; n=24) or to a control eYFP group (CeA eYFP rats; n=12). Optogenetic ChR2 rats received 1ul bilateral microinjections of a Cre-targeted ChR2 containing virus (AAV-EF1a-DIO-ChR2-eYFP; UNC Vector Core), and control eYFP rats received the optically inactive virus (AAV-EF1a-DIO-eYFP; UNC Vector Core). Bilateral virus microinjections were

targeted at the lateral division of CeA (A/P -2.4, M/L ±4.65, D/V -7.75, angle 4°). Microinjections were administered at a rate of 0.1ul/min, and microinjection needles were left in place for 10 additional minutes to ensure diffusion. In the same surgery, optic fibers were bilaterally implanted 0.3mm dorsal to the virus injection site. To allow for pharmacological i.c.v. microinjections, a 22-gauge intraventricular cannula was also implanted into the right lateral ventricle (A/P -0.7 to -0.84, M/L +1.5 to 2.0, D/V -4.5). Cannula and optic fibers were secured with skull screws and acrylic cement. Rats were postoperatively monitored for 7 days and received additional daily carprofen injections 24- and 48-hours following surgery.

*CRF antagonist*

The CRF antagonist (D-Phe$^{12}$,Nle$^{21,38}$,α-Me-Leu$^{37}$)-CRF (12-41) (D-Phe-CRF$_{(12\text{-}41)}$; Bachem 4030465) was reconstituted at 5mg/ml and aliquoted in sterile 4% dimethylsulfoxide (DMSO) in isotonic saline and stored at -20°C. Immediately prior to intraventricular injections, D-Phe-CRF$_{(12\text{-}41)}$ was diluted to 2mg/ml in 4% DMSO. Rats received i.c.v. microinjections over 30 seconds of either 10ug/5ul of the CRF receptor antagonist D-Phe-CRF$_{(12\text{-}41)}$ in 4% DMSO or 5ul of the 4% DMSO vehicle alone 15 minutes prior to behavioral tasks. Microinjections were administered via a 28-gauge microinjector extending 1mm beyond the end of the guide cannula. The microinjector was left in place for a minimum of 30 seconds after the injection to allow for diffusion.

*Two-Choice Sucrose Task*

We adapted the 2-choice sucrose task of Baumgartner et al. (2021) to test the effects of CRF antagonist blockade on incentive preference induced by CeA ChR2 optogenetic pairing. In

this task, rats could choose to earn either sucrose pellets accompanied by CeA CRF laser activation (*Laser + Sucrose)* by making nosepokes into a designated porthole or pressing on a designated lever, or to earn equivalent sucrose pellets delivered without laser (*Sucrose Alone*) by making nosepokes into a different porthole or pressing on a different lever. This task was employed here to test whether antagonist blockade of CRF receptors would prevent the development of a preference for the *Laser + Sucrose* option in CeA ChR2 rats that was previously reported by (Baumgartner et al., 2021).

To allow both within-subjects and between-subjects comparisons between vehicle and antagonist conditions, rats went through a pre-training phase, and 3 sequential phases with antagonist or vehicle of 2-choice sucrose tests described below. The pre-training phase consisted of 4-8 instrumental pre-training sessions in which rats simply learned to nosepoke for sucrose pellets, without any laser present, until they reached a criterion of 50 rewards from each lever or noseport. The final two pre-training sessions also included microinjection habituation in which rats received vehicle i.c.v. microinjections and continued to work for sucrose until they had earned 20 rewards from each lever or noseport. Once the animals finished pre-training, they progressed into 3 laser test phases: 1) Initial 2-choice task where one porthole earned Laser + Sucrose and the other earned Sucrose Alone, in which some rats received antagonist microinjections and other rats received vehicle microinjections for 4 days (between-subjects comparison; fixed ratio 1 (FR1) schedule), 2) Continued 2-choice task but with vehicle/antagonist assignments reversed for 4 days, so that the rats that previously received vehicle now received antagonist, and vice versa (within-subjects comparison; FR1 schedule), and 3) Continued 2-choice task with antagonist/vehicle assignments as in Phase 2, but with an escalation of the effort requirement to earn either option from FR1 to random ratio 6 (RR6) over

5 days, to assess the robustness of any laser-induced preference or avoidance in the 2-choice task (between-subjects comparison).

*Initial instrumental pre-training*

Rats were first pre-trained instrumentally to earn sucrose pellets on a fixed-ratio 1 schedule (FR1) with one of two types of manipulandum. One group of CeA ChR2 and of CeA eYFP rats learned to earn sucrose pellets by making nose pokes into either of two fixed portholes mounted on a wall; the other arbitrarily assigned groups learned to earn sucrose by pressing either of two retractable levers that protruded from a wall. The different nosepoke/lever responses were used to ensure that eventual antagonist results were not dependent on any single type of manipulandum, and the nosepoke/lever press assignment of each rat was kept constant throughout pre-training and Phase 1 of the 2-choice laser training and test. On the first pre-training day, one lever was extended, or one porthole was illuminated, and a response on it earned a sucrose reward. On the next day, the alternative lever was extended, or the alternative porthole was illuminated, and a response on it earned a sucrose reward. This daily alternation continued over 4 – 5 days until each rat had earned 50 cumulative rewards from each of its two levers or portholes.

*Microinjection habituation days (no laser):* To ensure instrumental behavior would not be disrupted by i.c.v. microinjections and handling, all rats received an i.c.v. microinjection of vehicle prior to two additional days of sucrose pre-training to serve as microinjection habituation sessions. Rats had to earn a minimum of 20 cumulative rewards over the two habituation days from each lever or porthole to move on to 2-choice laser training and testing.

*CRF Antagonist vs. Vehicle Comparisons*

To assess the effects of CRF antagonism, rats underwent 3 phases of 2-choice laser training and tests. Rats were placed in the lap of an experimenter where they received a 30s microinjection of either vehicle or antagonist as described above 15 minutes prior to behavioral testing.

*Phase 1: 2-choice laser preference tests: Laser + Sucrose vs Sucrose Alone*

CeA ChR2 rats and CeA eYFP rats were randomly divided into either CRF Antagonist or Vehicle subgroups, which remained constant throughout Phase 1. Each rat received its assigned antagonist or vehicle microinjection 15 minutes prior to each discriminative *Laser + Sucrose* vs. *Sucrose Alone* choice test in a chamber containing either two portholes or two levers.

For each rat, after receiving a microinjection, one lever or porthole was assigned (counterbalanced across rats) to earn *Laser + Sucrose,* whereas the other lever or porthole earned *Sucrose Alone*. An instrumental response on the *Laser + Sucrose* lever or porthole earned a sucrose pellet accompanied by laser illumination (473nm; 40Hz; 3mW (cycling 10ms on/15ms off for 8-sec bin duration) that began with the instrumental response that earned reward and continued 8-sec while sucrose was consumed. An assigned auditory CS label for each option (8-sec tone or white noise; counterbalanced across rats) also began simultaneously with laser onset and terminated when laser ended (e.g., pure tone label for *Laser + Sucrose* and white noise for *Sucrose Alone;* or vice versa).

In the first few minutes of each 2-choice session, only one lever or porthole was first presented (balanced order across days) until the rat responded and earned its assigned reward (either *Laser + Sucrose* or *Sucrose Alone*). Then the lever retracted, or the porthole dimmed, for an 8-sec time out period. The other lever was next inserted, or porthole illuminated, so the rat

could earn the alternative outcome. This alternating presentation of levers or portholes repeated once more, so that the rat earned two assigned rewards from each lever or porthole. These single-choice exposures served to remind a rat each day of both outcomes (typically both completed within 5 min), before they were allowed to choose freely between the two outcomes for the rest of the session.

Subsequently, both levers were always extended or both portholes illuminated simultaneously to allow free choice between *Laser + Sucrose* versus *Sucrose Alone* options. Once a choice was made and its outcome earned, both levers were retracted or both portholes dimmed for an 8-sec time out. Then both levers or both portholes were presented again for another choice. These 2-choice choice presentations continued for the remainder of the 30min session. Daily sessions were repeated for 4 days to compare laser-induced preferences between the Vehicle and the CRF Antagonist groups on a between-subject basis.

*Phase 2: FR1 Antagonist/Vehicle reversal with New Instrumental Responses.*

To compare CRF antagonist vs vehicle effects in the same rat, on a within-subject basis, previous Antagonist vs Vehicle assignments were reversed for all rats in Phase 2. Rats that received Antagonist in Phase 1 now instead received daily Vehicle microinjections prior to Phase 2 tests. Conversely, all rats that previously received Vehicle now received Antagonist. All rats were also switched to new instrumental manipulanda, to minimize the possibility that any Phase 1 laser-induced preferences would carry over to Phase 2 and confound the effects of Antagonist/Vehicle reversal. That is, rats previously trained on two levers were now switched to two portholes, positioned on the opposite wall from where levers had been (levers were now retracted), and rats previously trained on portholes were now switched to two levers, also placed

on the opposite wall from where portholes had been (portholes were removed). For each rat, one new lever or porthole was permanently assigned to earn *Laser + Sucrose,* and the alternative lever or porthole assigned to earn *Sucrose Alone.* Daily 2-choice sessions continued for 4-6 days as in Phase 1 but with pharmacological condition reversed, until each rat again met a response criterion of earning at least 20 rewards per session for 4 consecutive sessions on the new manipulanda. Data from each rat's Antagonist vs Vehicle conditions in Phase 1 and Phase 2 were compared on a within-subject basis.

*Phase 3: Escalation of effort requirement.*

Finally, we assessed whether CeA ChR2 laser-induced preference or avoidance in the 2-choice task was motivated with sufficient robustness to persist even if the effort price of both rewards was increased. For each rat, its antagonist/vehicle and lever/porthole assignment of Phase 2 was retained in Phase 3. However, the response schedule required to earn either *Laser + Sucrose* or *Sucrose Alon*e was escalated across the next 5 days from FR1 to RR6: FR4 ($1^{st}$ day of escalation), random ratio 4 (RR4, $2^{nd}$ day of escalation), and RR6 ($3^{rd}$-$5^{th}$ days of escalation). To assess the impact of CRF antagonism on preference between *Laser + Sucrose* or *Sucrose Alone*, the last 3 days of Phase 3 testing (RR6) were compared between vehicle and antagonist groups on a between-subject basis.

*Progressive Ratio Test of Effort Breakpoint*

To test whether CRF receptor blockade would prevent the amplification of intensity of incentive motivation for a reward otherwise caused by pairing ChR2 stimulation of CeA CRF neurons, we used a progressive ratio (PR) task (Baumgartner et al., 2021, 2022) to measure effort

breakpoint for CeA CRF sugar reward after CRF antagonist versus vehicle on a between-subject basis. Rats were assigned their same antagonist/vehicle status Phases 2/3 of the 2-choice task for both days of PR breakpoint tests, and their same lever/porthole assignments from Phases 2/3. On one PR test day, after receiving their microinjections, only the *Sucrose + Laser* lever or porthole was available, and it earned a sucrose pellet accompanied by 8-sec laser illumination and auditory label as it had previously in the 2-choice task Phases 2 & 3. On the other PR day, after receiving the same microinjection, only the *Sucrose alone* option was available, and it earned a sucrose pellet without laser and different auditory label as it had in the 2-choice task. The order of *Laser + Sucrose* versus *Sucrose Alone* days was counterbalanced across rats. On each day, the number of responses required to earn the next reward increased after each reward was earned (progressive ratio schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, …) derived from the formula PR = [5e(reward number×0.2)] − 5 and rounded to the nearest integer (Richardson & Roberts, 1996; Saunders & Robinson, 2011). The breakpoint or highest effort reached by the end of the 30-minute session was compared as a measure of the intensity of incentive motivation for reward.

*Laser Self-Stimulation Task*

A laser self-stimulation task was used to assess if brief pulses of CeA ChR2 neuronal excitation carried positive motivational value on their own (without sucrose), and to test whether that value was reduced by antagonist blockade of CRF receptors. Two innocuous metal 0.5 cm diameter metal rods extended 3cm into the self-stimulation chamber, spaced 17cm apart. Touches on one rod (permanently designated as Laser-delivering rod for that rat) triggered a 1s bin of laser stimulation (1 mW; constant illumination). Each touch on the other rod (designated

as Inactive for that rat) earned nothing and simply served as a baseline measure of exploratory touches.

Rats were initially diagnosed for laser self-stimulation over three days without any microinjection, classifying them according to 3 levels of self-stimulation performance (Baumgartner et al., 2021, 2022). Rats were classified as High Self-stimulators if they earned >50 laser illuminations in a 30-min session and touched their Laser-delivering rod >2X more often than their Inactive rod. Rats were classified as Low Self-Stimulators if they earned 10 to 49 laser illuminations in a session, and still touched their Laser-delivering rod >2X more often than the Inactive rod. Rats were classified as Failures to self-stimulate if they earned fewer than 10 laser illuminations or failed to touch their Laser-delivering rod at least twice as often as their Inactive rod. Rats that consistently Failed to self-stimulate were discarded from the next phase.

CRF antagonist vs Vehicle effect on Laser Self-Stimulation (Phase 1 – Between-subject comparison): High Self-Stimulators and Low Self-Stimulators were then randomly divided and assigned in equal numbers to either a Vehicle microinjection group or a CRF antagonist microinjection group for the next 4 days for days of laser self-stimulation tests. A between-subject comparison of antagonist/vehicle performance was made by comparing average laser self-stimulations earned across the 4 days.

Laser Self-Stimulation (Phase 2 – Within-subject comparison of antagonist/vehicle effects): Finally, the antagonist/vehicle assignment of each rat was reversed for a further 4 days of laser self-stimulation tests in order t0 make a within-subject comparison of performance across days. Rats in the Phase 1 Vehicle group were now switched to the Antagonist condition for Phase 2, whereas rats in the Phase 1 Antagonist group were switched to the Vehicle

condition. This continued for a final 4 days of laser self-stimulation tests. Number of contacts on each rod was averaged across the 4 vehicle days and 4 antagonist days.

*Histology*

Prior to euthanasia, rats were administered the drug they were assigned in Phase 2 and 3 of the two-choice sucrose task and then delivered 30 minutes of cycling laser stimulation to induce c-fos (3mw 40Hz 8s on, 22s off). Rats were euthanized with a lethal dose of sodium pentobarbital (150-200mg/kg, i.p.; Euthasol) and transcardially perfused with sodium phosphate buffer and 4% paraformaldehyde (PFA). Brains were extracted and postfixed in 4% PFA for 24 hours and then cryoprotected in 25% sucrose for a minimum of 48 hours. Brains were then sectioned into 40um slices using a cryostat (Leica), permeabilized and blocked in 0.2% Triton and 2.5% normal donkey serum in sodium phosphate buffer, and stained for GFP (chicken anti-GFP 1:2000; Abcam, ab1397; donkey anti-chicken Alexa 488 1:300; Jackson Immuno, 703-545-155) and Fos (rabbit anti-Fos 1:2500; Synaptic Systems, 226-008; 1:250 donkey anti-rabbit Alexa 594, abcam, ab150076). Brain tissue was mounted and coverslipped with ProlongGold anti-fade mounting medium with DAPI (Cell Signaling Technology, 8961S). Brain tissue was imaged using a digital camera and fluorescence microscope (Leica).

Coronal sections were imaged (10x) magnification. Virus and fiber were considered “on target” if virus was contained within the CeA and the bottom of the fiber tip was located within 0.6mm of fluorescent cells.

*Experimental Design and Statistical Analysis*

Statistical analysis was conducted in R (R Core Team, 2020) using tidyverse (Wickham et al., 2019), tidyr (Wickham, Vaughan, et al., 2024), dplyr (Wickham et al., 2023), lme4 (Bates et al., 2024), lmerTest (Kuznetsova et al., 2020), and emmeans (Lenth et al., 2024). Plot were made using ggplot2 (Wickham, Chang, et al., 2024), ggpubr (Kassambara, 2023), ggbreak (Yu & Xu, 2023), and ggpattern (FC et al., 2022). Tables were made with knitr (Xie et al., 2024), broom.mixed (Bolker et al., 2024), and modelsummary (Arel-Bundock et al., 2024). Linear mixed models were used to analyze experimental data followed by Type III tests with effects coding. Posthoc testing used pairwise t-test comparisons of estimated marginal means with Bonferroni correction.

## 3.3 Results

*Phase 1 and 2: 2-choice laser preference tests: Laser + Sucrose vs Sucrose Alone*

During Phase 1, one group of CeA ChR2 rats received vehicle while the other group received CRF antagonist prior to tests on the first 4 days of the 2-choice sucrose task. Following Phase 1, rats in the Vehicle group were reassigned to receive antagonist while the Antagonist group were reassigned to receive vehicle. Since the order in which the drugs were administered did not significantly influence responding (drug order: b=19.08, df=36.86, 1.78, p=0.084), groups were collapsed across drug condition.

Over the 4 trial days, Vehicle ChR2 rats increasingly responded for the *Laser + Sucrose* option (day: b=10.24, df=288.55, t=3.27, p=0.001) while responding for the *Sucrose Alone* option remained at low levels and even slightly decreased across trial days (laser x day: b=-10.943, df=288.99, t= -2.476, p=0.014, Figure 3-1 A). In contrast, Antagonist ChR2 rats increased their responding for *Sucrose + Laser* option similarly to those on vehicle (drug x day: b=-4.88, df=289.99, t=-1.092, p=0.276); however, they also increased their responding for the *Sucrose Alone* option at greater rate (drug x day x laser: b=19.879, df=288.645, t=3.16, p=0.002, Figure 3-1 B). Overall, Vehicle ChR2 rats developed a clear preference for the *Laser + Sucrose* over the *Sucrose Alone* option. ChR2 stimulation of CeA CRF neurons in the Vehicle group (n=24) led to intensified pursuit of the *Laser + Sucrose* option by a 2:1 ratio over the *Sucrose Alone* (posthoc: b=36.29, df=35.2, t=4.88, $p<0.001$; Figure 3-1 A), whereas the Antagonist CeA ChR2 group (n=24) failed to develop a *Laser + Sucrose* preference and pursued both rewards equally (laser x drug x day: $F_{1,164}$=4.465, p=0.036; posthoc: b=-11.2, df=19, t=-0.154, p=1; Figure 6B). These results replicate previous findings that pairing CeA CRF neuronal ChR2 excitation with one sucrose option can focus motivation preferentially upon that laser-paired

option. Our results also indicate for the first time that this preference for the CeA ChR2 laser-paired option is due specifically to release by CeA CRF neurons of CRF neurotransmitter and its receptor activation.

In contrast, eYFP control rats failed to develop a preference regardless of drug ($F_{(1,169.49)}$=7.0405, p=0.008; posthoc: vehicle laser vs nonlaser p=1; posthoc: antagonist laser vs nonlaser: p=0.8; Figure 3-1 A,B). No significant sex differences were identified in ChR2 rats (b=0.93, df=5.875, t=6.011, p=0.875); however, female eYFP rats tended to make a greater number of responses than males (b=13.568, df=11.285, t=2.786, p=0.017).

*Phase 3: Escalation of effort requirement*

In Phase 3 of 2-choice sucrose testing, to assess the robustness of vehicle vs antagonist effects, rats retained the same drug assignment they had in Phase 2 but now the number of responses required to earn each reward increased over 5 days to RR6. CeA ChR2 rats that had received vehicle during Phase 2 and developed a *Laser + Sucrose* preference continued to maintain that strong preference under vehicle as effort requirement increased across trial days (laser vehicle x trial: b=44.391, df=163, t=5.871, p<0.001; nonlaser vehicle x trial: b=-41.773, df=164, t=-3.907, p<0.001). These Vehicle CeA ChR2 rats specifically focused their increased responding on the *Laser + Sucrose* option by a 6:1 ratio over *Sucrose Alone* by the final day of the two-choice sucrose task (laser vehicle vs nonlaser vehicle: b=-277.455, df=22.777, t=-3.83, p<0.001; Figure 3-1 C). In contrast, CeA ChR2 rats that had received antagonist for Phase 2, and chose equally then, increased their responding for both options under continued Antagonist in Phase 3 as the effort requirement increased (laser antagonist x trial: b=-11.881, df=164, t=-1.084, p=0.28; nonlaser antagonist x trial: b=32.743, df=164, t=2.113, p=0.036) and also continued to

choose at an even 1:1 ratio between Laser + *Sucrose* and *Sucrose Alone* (antagonist laser vs nonlaser: $p = 1$; Figure 3-1 D).

By comparison, eYFP rats under Vehicle (n=5) in Phase 3 also increased responses on both options as the effort requirement increased (laser vehicle x trial day: b=55.62, df=90.215, t=3.507, p<0.001; nonlaser vehicle x trial day: b=-24.52, df=90.215, t=-1.093, p=0.277), but never developed a significant preference, and continued to choose equally between options (laser vs nonlaser vehicle: b=-47.8, df=12.502, t=-0.348, p=0.734; Figure 3-1 E). CeA eYFP rats under Antagonist (n=8) also continue to choose equally between the two options (laser vs nonlaser antagonist x trial day: b=251.469, df=12.502, t=1.397, p=0187; Figure 3-1 F). Additionally, CeA eYFP females made a greater number of overall responses than males (sex: b=54.102, df=9.119, t=3.7, p=0.005).

*Breakpoint test of motivation intensity: CRF blockade prevents CeA CRF ChR2 elevation of breakpoint*

After the final sucrose 2-choice test, a progressive ratio breakpoint task was used on the next day to assess whether laser ChR2 stimulation of CeA CRF neurons increased the magnitude of incentive motivation to obtain sucrose rewards, and to test if that increase would be prevented by pharmacological blockade of CRF receptors by i.c.v. antagonist administration. CeA ChR2 rats and control eYFP rats were tested using a progressive ratio schedule on one day working for *Laser* + *Sucrose* and on a different day working for *Sucrose Alone* (balanced order). Some rats in both groups received vehicle on both days, whereas other rats received antagonist on both days. CeA ChR2 rats receiving vehicle achieved higher breakpoints on their *Laser* + *Sucrose* day than on their Sucrose *Alone* day (vehicle laser vs vehicle nonlaser: b=-45.778, df=17, t=-3.717,

p=0.002; Figure 3-2 A). By contrast CeA ChR2 rats receiving antagonist showed no difference in breakpoint over the two days (drug x laser interaction: $F_{1,17}$=7.239, p=0.01; posthoc: p=1). Furthermore, in the *Laser + Sucrose* condition, CeA ChR2 rats receiving Vehicle worked twice as hard as CeA ChR2 rats receiving Antagonist (laser vehicle vs laser antagonist: b=-42.959, df=28.219, t=-2.185, p=0.009; Figure 3-2 A). Thus, overall, CeA ChR2 rats receiving Vehicle differed from CeA ChR2 rats receiving Antagonist in the ability of laser stimulation to enhance the magnitude of incentive motivation (Figure 3-2 A). This pattern of results indicates that antagonist blockade of CRF receptor activation prevented laser ChR2 stimulation of CRF neurons in CeA from enhancing incentive motivation for reward, which it could do successfully in the absence of antagonist.

In contrast, CeA eYFP control rats did not differ in breakpoint between Vehicle vs. Antagonist conditions or between *Laser + Sucrose* vs. *Sucrose Alone* conditions (laser x drug: b=-12.4, df=17, t=-0.59, p=0.56). Instead CeA eYFP rats always remained similar to CeA ChR2 rats in the CRF Antagonist condition. Regarding sex differences, female CeA eYFP rats achieved a higher breakpoint on average than males (sex: b=12.836, df=17, t=2.37, p=0.03) while there was no sex difference in breakpoint for CeA ChR2 rats (sex: b=3.835, df=16, t=0.598, p=0.558).

*CRF receptor blockade attenuates ChR2 laser self-stimulation of CeA CRF neurons*

Rats could earn brief 1-sec CeA laser illuminations by touching one of two metal bars. During initial laser self-stimulation screening days with no pharmacological manipulation, 4 CeA ChR2 rats met criteria for High laser self-stimulation (>50 laser contacts, plus Laser bar contacts >2X inactive bar contacts), 6 CeA ChR2 rats met criteria for Low self-stimulation (>10

but <50 laser contacts, plus Laser bar contacts >2X inactive bar contacts), and 7 CeA ChR2 rats failed to self-stimulate by either criterion.

Rats who met either High or Low laser self-stimulation criteria in prescreening continued for 4 additional days of self-stimulation tests with vehicle microinjection. This was followed by 4 further days with CRF antagonist microinjections. Overall, CeA ChR2 rats on vehicle made 3x as many contacts on the laser rod (75.58±19.36) as on the inactive rod (22.05±6.58) (vehicle laser vs nonlaser: b=-53.525, df=14.082, t=-4.997, $p<0.001$; Figure 3-3 A). However, when switched to Antagonist condition, CeA ChR2 rats reduced their laser self-stimulation to one-half their earlier vehicle level (35.21±7.77) (laser vehicle vs laser antagonist: b=-40.365, df=14.176, t=-3.797, p=0.002; Figure 3-3 A). In the Vehicle condition, 6 CeA ChR2 rats continued to meet criteria for High self-stimulation, but only 2 rats retained this level of self-stimulation in the CRF Antagonist condition (Figure 3-3 B). Half of the group of CeA ChR2 rats who self-stimulated under vehicle entirely ceased self-stimulating under CRF antagonist, and the other 3 showed attenuated self-stimulation, failing to meet criteria for high self-stimulation but still meeting criteria for low self-stimulation (Figure 3-3 B). However, on average under CRF antagonist, CeA ChR2 rats no longer contacted the laser rod more than the nonlaser rod (laser x drug interaction: $F_{1,7}$=0.21, p=0.004; laser vehicle vs nonlaser antagonist: b=21.54, df=13.5, t=2.011, p=0.388; Figure 3-3 A).

By comparison, CeA eYFP control rats overall failed to self-stimulate laser as a group (laser main effect: $F_{1,8}$=0.178, p=0.684, laser x drug interaction: $F_{1,8}$=1.175, p=0.31; Figure 3-3 A). One CeA eYFP control rat met High self-stimulation criteria while the other 8 CeA eYFP rats failed to self-stimulate by either criterion. Control CeA eYFP rats were unaffected by vehicle/antagonist condition (main effect of drug: $F_{1,8}$=2.119, p=0.184).

### 3.4 Discussion

Our results confirm that pairing optogenetic CeA ChR2 activations of CRF neurons with a particular sucrose reward option intensifies and focuses incentive motivation onto that laser-paired option, while an alternative reward becomes neglected. The positive motivational valence of CeA CRF activation was further shown by observation that some *crh*-Cre rats would work to self-stimulate ChR2 laser excitation of CRF neurons in CeA by itself. Most important, our results also demonstrate for the first time that those positive incentive motivation effects of CeA CRF neuronal stimulation require receptor activation by CRF peptide and are not primarily due to co-release of other neurotransmitters produced by CRF neurons. Although CeA CRF neurons do co-release several additional neurotransmitters, such as GABA, neurotensin, somatostatin, and dynorphin (Partridge et al., 2016; Pomrenze et al., 2015; Pomrenze, Giovanetti, et al., 2019), it appears that those other signals are not sufficient to generate robust incentive motivation effects in the absence of CRF neurochemical signaling.

When CeA ChR2 rats received vehicle microinjections, paired optogenetic activation of CeA CRF neurons narrowed their incentive motivation onto the laser-paired sucrose reward in the two-choice task, over an alternative sucrose reward delivered without laser, and magnified the intensity of incentive motivation to obtain laser-paired sucrose in a breakpoint task. However, blockade of CRF receptors by i.c.v. administration of an antagonist drug blocked both the preference induction in the 2-choice task, and the amplification of incentive motivation in the breakpoint task. Further, CRF receptor antagonism eliminated or attenuated laser self-stimulation of CeA CRF neurons in most *crh*-Cre rats that otherwise showed self-stimulation.

Future studies would be needed to identify the precise output projections of CeA CRF neurons, target brain site(s) and CRF receptor subtypes responsible for CeA CRF's ability to enhance incentive motivation. Our i.c.v. route of antagonist administration would have affected multiple structures that receive CRF signals throughout the brain. Beyond CeA itself, CeA CRF neurons also project to the ventral tegmentum (VTA), ventral pallidum (VP), lateral hypothalamus (LH), and bed nucleus of the stria terminalis (BNST) (Pomrenze et al., 2015). Of those, CeA, VTA, VP, or LH might be most the plausible candidates for CeA CRF neuronal stimulation and CRF release to generate positively-valenced incentive motivation, given that multiple studies have indicated that CRF projections from CeA to BNST primarily induce negatively-valenced distress (Beckerman et al., 2013; de Guglielmo et al., 2019; Partridge et al., 2016).

Traditionally, CRF neural systems in CeA have been hypothesized to generate aversive distress, and consequently to motivate consumption of rewards to escape distress (Radulovic et al., 1999; Richter et al., 2000; Valdez et al., 2003; Zorrilla et al., 2002). Inhibition of CRF expression in CeA has been shown to attenuate anxiety-like behavior in the open field and elevated plus maze, and to impair fear learning (Callahan et al., 2013; Pomrenze, Giovanetti, et al., 2019). Distress roles for CRF in CeA are also prominent in opponent process-based theories in addiction neuroscience, which posit increases in CeA CRF release to generate aversive withdrawal feelings and related distress that motivates addicted individuals to take drugs again in a hedonic self-medication attempt to relieve distress (Basso et al., 1999; Cottone et al., 2009; George et al., 2012a; G. F. Koob, 2010; G. F. Koob et al., 2014; Zorrilla et al., 2014).

However, our present results, as well as previous results from our lab and from others, supports an alternative role for CeA CRF in motivating reward pursuit, beyond CeA CRF roles

in mediating distress: namely, that CRF neural systems in CeA can directly amplify and focus incentive motivation to obtain and consume a reward, even in situations that lack distress (Baumgartner et al., 2021, 2022; Calogero et al., 1989a; Lemos et al., 2012; Merali et al., 1998a; Peciña et al., 2006b; Xu et al., 2024; Zalachoras et al., 2022). CRF systems have been suggested to adapt to changing environments and stimuli in modulating motivational salience (Merali et al., 1998a, 2004; Schulkin, 2017).

Our results are consistent with previous studies showing that optogenetic activation of CRF neurons in either CeA or NAc are capable of amplifying incentive motivation for both sucrose and cocaine rewards (Baumgartner et al., 2021, 2022). Those studies also showed that activation of CeA CRF neurons recruited increased activation of mesolimbic reward circuitry, including the VTA and NAc, which presumably mediates the enhanced incentive motivation (Baumgartner et al., 2021, 2022). Neurochemically, microinjections of CRF into the NAc shell establish conditioned place preference, and can amplify cue-triggered incentive motivation for sucrose reward in PIT tests similarly to dopamine stimulation via amphetamine microinjection in NAc (Lemos et al., 2012; Peciña et al., 2006b). Additionally, endogenous CRF is released in CeA and in the hypothalamic PVN in response to receipt of either food or drug rewards (Calogero et al., 1989a; Merali et al., 1998a). Optogenetic stimulation of PVN neurons has also been shown to generate a conditioned place preference and to support self-stimulation (Xu et al., 2024).

In human clinical conditions, CeA CRF neuronal excitation often has been hypothesized to explain how stressful experiences or emotional excitement can trigger bouts of binge eating, relapse in drug addiction, or related forms of excessive pursuit and consumption. Many stressors are aversive, and so such excessive consumption has usually been interpreted as reflecting hedonic self-medication to relieve distress. However, some have suggested that emotional

excitements of even ‘happy stressors’, such as celebrating the birth of a child with family or other events that cause extreme excitement and positive affect, can also elevate risk of relapse in drug addiction and binge eating disorder (Ferreira, Zerwes et al., 2016; Hodgins et al., 1995; Hodgins & el-Guebaly, 2004; McKay et al., 1995; Shiftman et al., 1985; Walitzer & Dearing, 2006). Our results indicate that excitation of CeA CRF neurons which produces increased activation of CRF receptors effectively promotes reward seeking via magnified incentive ‘wanting’ in the absence of distress, potentially providing an explanation of such phenomena.

### 3.5 Figures

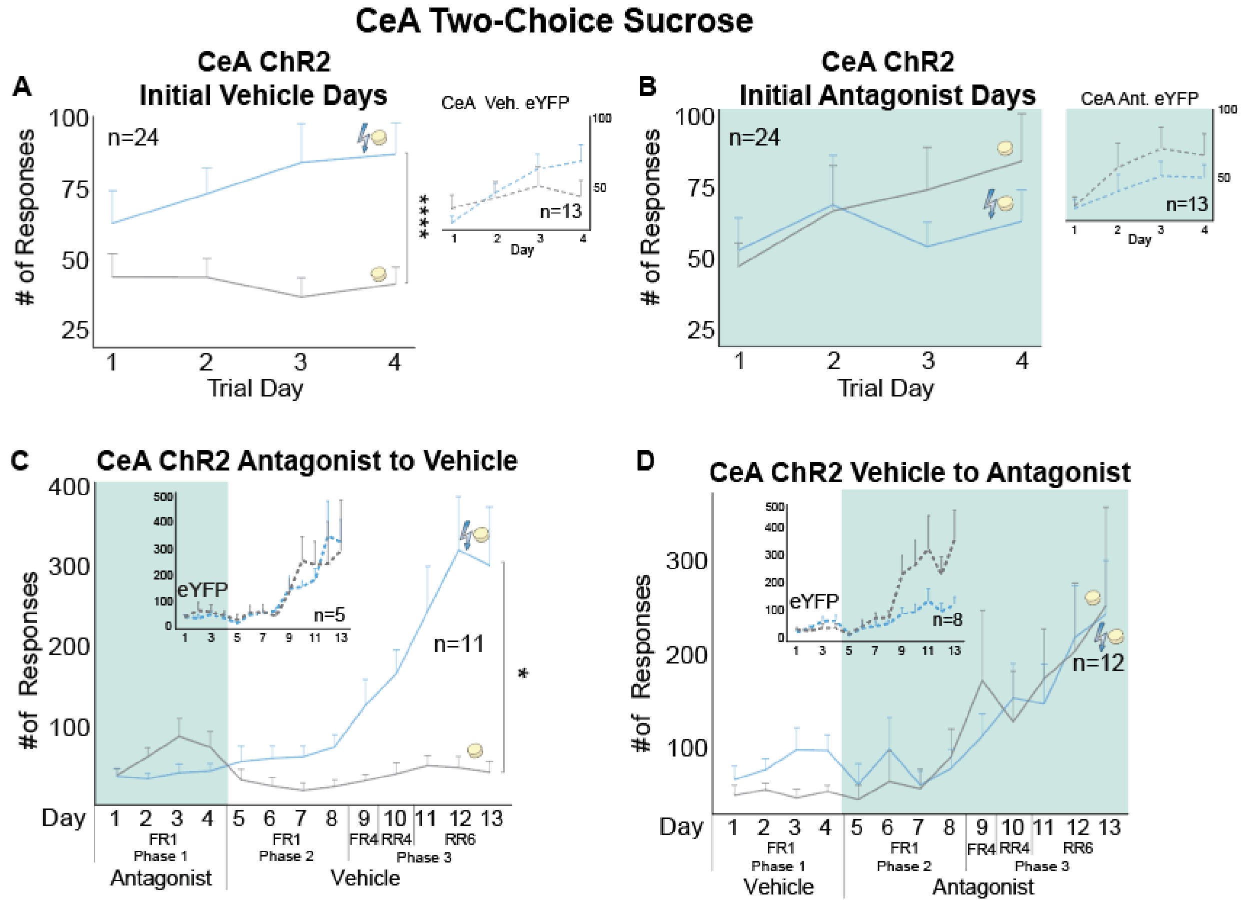



Figure 3-1 CRF antagonism prevents induction of preference for laser-paired sucrose option

A) CeA ChR2 rats develop a 3:2 preference for the *Laser + Sucrose* option while under vehicle. eYFP control rats on vehicle develop no preference for either option. B) Under CRF antagonist, CeA ChR2 rats fail to develop a significant preference for either the *Laser + Sucrose* or the *Sucrose* Alone option, similar to eYFP controls. C) CeA ChR2 rats maintained a High preference for the *Laser + Sucrose* option when effort requirement increased under vehcile in Phase 3 of the 2-choice task (n=11). By contrast, control CeA eYFP rats (n=5) failed to develop any preference. D) CeA ChR2 rats under CRF antagonist in Phase 3 failed to develop any preference (n=12). Similarly, eYFP rats (n=8) failed to develop a preference under antagonist.

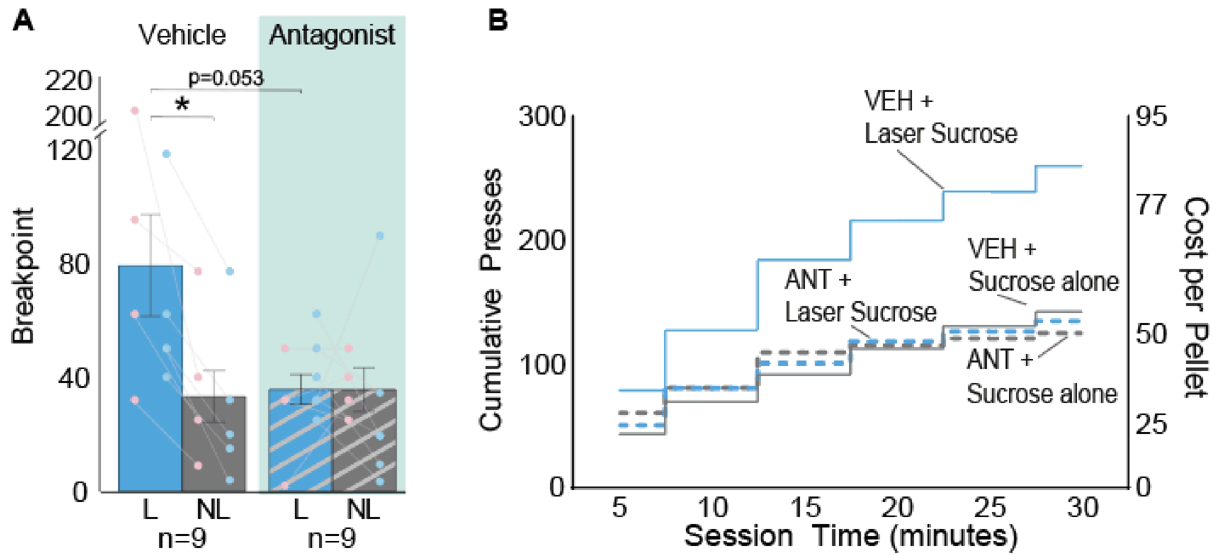



Figure 3-2 CRF antagonism prevents breakpoint elevation in progressive ratio task

In the progressive ratio (PR) breakpoint task, CeA ChR2 under vehicle showed increased incentive motivation to obtain the *Laser + Sucrose* option compared to their performance for sucrose alone, reflected as increase in effort breakpoint (n=9) (left). By contrast, CeA ChR2 rats under CRF antagonist (n=9) showed no elevation in *Laser + Sucrose* breakpoint. Pink = Females, Blue = Males, L = Laser, NL = Nonlaser. Means and SEM reported. *p<0.05

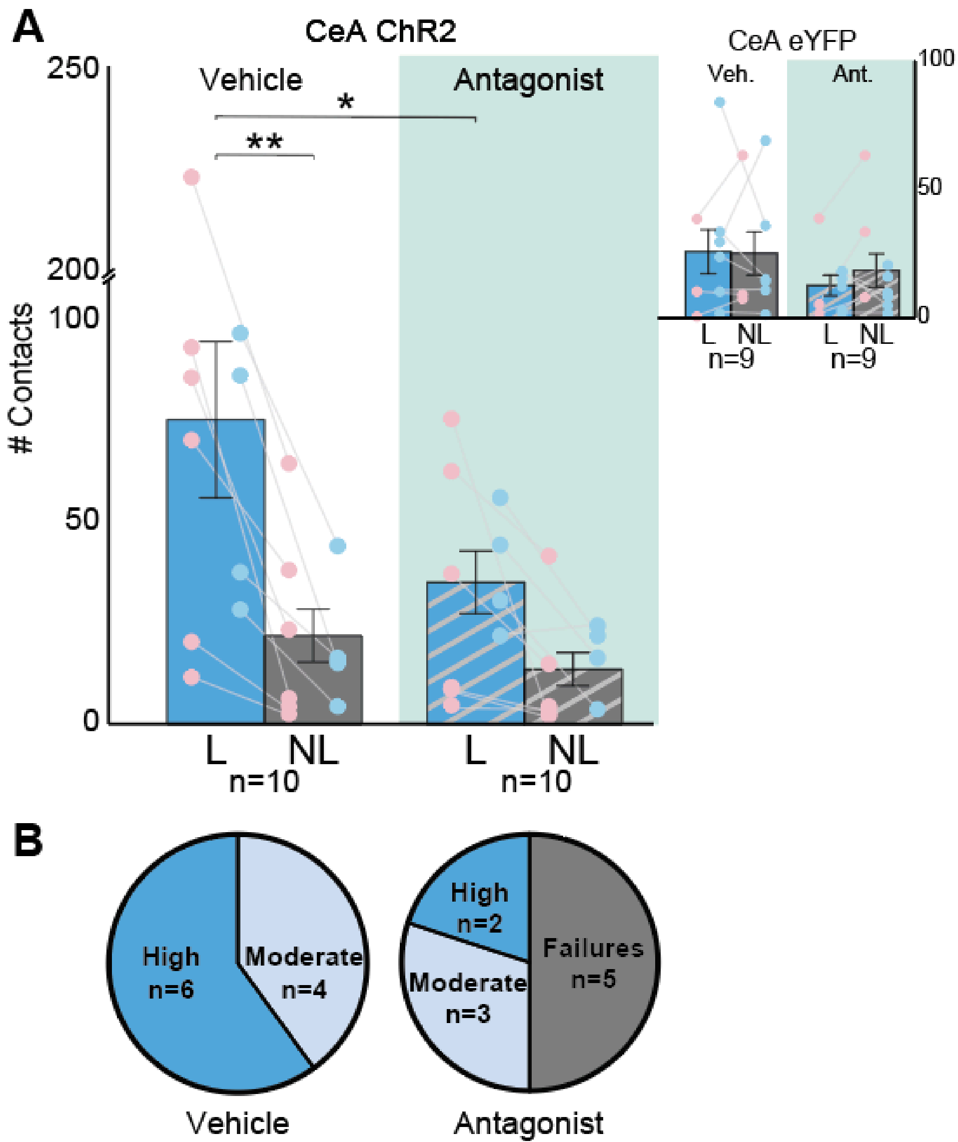



Figure 3-3 CeA CRF laser self-stimulation is attenuated by i.c.v. CRF antagonism

A) Only CeA ChR2 rats that met laser self-stimulation criteria in pre-screening (ether High or Low self-stimulation levels) were used for subsequent tests of Antagonist/Vehicle and are shown here (n=10/17 CeA ChR2 rats). Under vehicle, all 10 of these rats worked to self-stimulate laser excitation of CeA CRF neurons by touching a designated metal rod, earning over 60 illuminations per session on average. By contrast, CRF antagonist administration reduced laser self-stimulation to approximately one-half of vehicle baseline levels on average. B) The 10 individual CeA ChR2 rats from A were classified under vehicle as either High self-stimulators (i.e., earning > 50 illuminations per session) or Low self-stimulators (i.e., earning 10-49 illuminations per session). Under CRF antagonist, the number of both High and Low self-stimulators declined, and half the group became Failures to self-stimulate (<10 illuminations per session). Pink = Females, Blue = Males, L = Laser, NL = Nonlaser. Means and SEM reported. *$p<0.05$

# Chapter 4 Pilot Data: The Role of CeA CRF Projection Targets in Motivation

## 4.1 Introduction

Corticotropin-releasing factor (CRF) is a peptide neurotransmitter traditionally associated with aversive distress and is considered the key regulator of behavioral response to stress (Gray, 1993; Kovács, 2013). CRF released from neurons in the paraventricular nucleus of the hypothalamus travels to the pituitary gland via the hypophysial portal system and then signals the pituitary to release adrenocorticotropic hormone into the bloodstream which, in turn, travels to the adrenal glands and signals the release of glucocorticoids to initiate the "fight or flight" response to stress. Beyond the hypothalamic-pituitary-adrenal (HPA) axis, populations of CRF-containing neurons exist within some structures of the limbic system. Specifically, CRF neurons within the central amygdala (CeA) as well as the bed nucleus of the stria terminalis, both components of the extended amygdala, release CRF as a neurotransmitter. Similarly, the nucleus accumbens also contains neurons that release CRF.

Traditionally, CRF neurons in limbic structures have been viewed to mediate the negative affect and anxiety that accompanies stress, and are implicated in fear and anxiety. For instance, activation of CeA CRF neurons reinstates extinct fear memories in a Pavlovian fear conditioning paradigm, and increases cue-triggered freezing behavior, whereas conversely inhibition or knockdown of CRF expression of CeA CRF neurons leads to fear memory extinction and impaired fear learning (Jo et al., 2020; Pomrenze, Giovanetti, et al., 2019). Furthermore, CeA CRF neurons mediate conditioned flight response to Pavlovian conditioned fear paradigms

(Fadok et al., 2017). CRF mRNA increases in the CeA following acute stress in both males and females (Sterrenburg et al., 2012), and activation of CeA CRF neurons in *Crh*-cre rats can lead to pain- and anxiety-like behavior (Mazzitelli et al., 2022).

However, there is also evidence that CRF systems may be capable of generating positive incentive motivation without distress or anxiety, including in the CeA and CRF. Firstly, receipt of food rewards increases CRF release in the CeA (Merali et al., 2004). Microinjection of CRF into the NAc can amplify cue-triggered 'wanting' for sucrose rewards in the absence of stress, similarly to dopamine-releasing amphetamine microinjections in NAc (Peciña et al., 2006c), and NAc CRF microinjections can also generate a conditioned place preference and facilitate NAc dopamine release (Lemos et al., 2012).

Additionally, some *Crh*-cre rats will optogenetically self-stimulate laser excitation of CRF neurons in both CeA and the nucleus accumbens medial shell (Baumgartner et al., 2021, 2022). Additionally, pairing sucrose or cocaine rewards with optogenetic activation of CeA or NAc CRF neurons in *Crh*-cre rats leads to preference single minded pursuit of these laser-paired rewards over identical sucrose or cocaine rewards without laser in 2-choice tests, and amplifies the intensity of incentive motivation to obtain those rewards in progressive ratio tests of effort breakpoint (Baumgartner et al., 2021, 2022). Furthermore, activation of CeA CRF neurons recruits c-Fos protein expression in a number of reward related regions, including the NAc, ventral tegmental area (VTA), lateral hypothalamus (LH), and ventral pallidum (VP). As discussed in chapters 2 and 3, these incentive effects are mediated via activation of CRF receptors by CRF as a neurotransmitter, as intracerebroventricular (i.c.v.) administration of a CRF receptor antagonist attenuates these incentive effects. However, it is not clear where these CRF receptors are or what circuitry underlies these effects.

CeA CRF neurons project both locally within the CeA, and send long range axons outside the amygdala to more distant brain structures (Pomrenze et al., 2015). While significant work has been done to characterize the circuitry underlying the role of CeA CRF neurons in fear, specifically examining complementary projections between CeA and other stress-associated circuitry (Asok et al., 2018; Borrego et al., 2022; Dabrowska et al., 2016; de Guglielmo et al., 2019), less work has focused on mapping CeA CRF circuitry involved in incentive motivation. CeA CRF neurons are known to send direct projections to mesocorticolimbic regions including the LH, VTA, VP and the dorsal medial striatum which could contribute to the incentive motivation arising from CeA CRF neuronal activation (Dedic, Kühne, et al., 2018; Essoh et al., 2022; Pomrenze et al., 2015; Rodaros et al., 2007).

Here, we sought to characterize the relative roles of projections of CeA CRF neurons to the LH and dorsal medial striatum (DMS) in incentive motivation. First, we injected a cre-dependent channelrhodopsin containing virus in CeA and implanted optic fibers in either LH or DMS of *Crh*-cre rats to activate axon terminals of CeA CRF neurons. Second, we also sought to determine if CRF microinjections into DMS, LH, or CeA were sufficient to generate a conditioned place preference (Lemos et al., 2012).

To assess incentive motivation effects, we used a two-choice sucrose task with laser paired with one of two sucrose reward options (Baumgartner et al., 2021). We also used rod-touch self-stimulation and place-based self-stimulation to determine whether activation of $CeA_{CRF}$→ LH or $CeA_{CRF}$→ DMS projection neurons could bias motivation for laser-paired sucrose rewards or support laser self-stimulation of CRF neurons.

Our results suggest that CRF microinjections into the DMS, CeA, or LH were insufficient to cause either conditioned place preference or avoidance. Additionally, we find that $CeA_{CRF}$→

DMS activation failed to cause rats to specifically pursue or avoid a sucrose reward paired with laser activation over an equal sucrose reward delivered without laser. Thus, $CeA_{CRF}$→ DMS laser stimulation did not support place-based or rod-based self-stimulation. In contrast, we found that optogenetic activation of $CeA_{CRF}$→ LH neurons biased rats to avoid the sucrose reward paired with laser stimulation and prefer an identical laser-paired sucrose reward delivered without laser. Additionally, $CeA_{CRF}$→ LH rats failed to self-stimulate and showed laser-avoidance in the place-based self-stimulation task. However, we find that in a subgroup of rats where optic fiber placement was placed posterior to the LH in substantia nigra (SN), all three $CeA_{CRF}$→ SN rats showed a preference for the laser-paired sucrose and two of the three self-stimulated laser illumination indicating that there may be a rostro-caudal gradient from aversive to incentive effects from LH to the substantia nigra.

## 4.2 Materials and Methods

*Animals*

Separate cohorts of Crh-cre Wistar rats (>250g at surgery) were utilized for all experiments [$CeA_{CRF}$→*LH* (female n= 11, male = 6), $CeA_{CRF}$→*DMS* (female n = 6, male = 1)]. All rats were bred and genotyped in-house. Same-sex groups were housed on a 12-hour reverse light/dark cycle (~21° C) with *ad libitum* food (Purina, St. Louis, MO) and water. All experimental procedures were approved by the University of Michigan Institutional Animal Care & Use Committee in accordance with NIH animal care and use guidelines.

*Surgery*

Rats were anesthetized with isoflurane gas (5% induction; 1-3% maintenance) and administered atropine (0.05mg/kg i.p.; Henry Schein), carprofen (5mg/kg, s.c.; Henry Schein), and cefazolin (75mg/kg, s.c.; Henry Schein) prior to placement in the stereotactic apparatus (David Kopf Instruments, Tujunga, CA).

Following all surgical procedures, rats were postoperatively monitored for 7-10 days and received 5 mg/kg of carprofen 24 and 48 hours after surgery.

*Intracranial Cannulation Surgery*

22-gauge stainless steel guide cannula extending 6mm from the base were fabricated in-house (Kokare et al., 2011), bilaterally implanted into the DMS (A/P: +0.12, M/L: +/-2.6mm, and D/V: -5.0), and secured with screws and dental cement.

*Optogenetic Surgery*

Rats were arbitrarily assigned to either an optogenetic channelrhodopsin stimulation group (CeA ChR2 rats; $CeA_{CRF}$→*LH* n=17, $CeA_{CRF}$→*DMS* n= 7) or to a control eYFP group

(CeA eYFP rats; *CeA*$_{CRF}$→*LH* n=12, *CeA*$_{CRF}$→*DMS* n= 5). Optogenetic ChR2 rats received 1ul bilateral microinjections of a Cre-targeted ChR2 containing virus (AAV-EF1a-DIO-ChR2-eYFP; UNC Vector Core), and control eYFP rats received the optically inactive virus (AAV-EF1a-DIO-eYFP; UNC Vector Core). Bilateral virus microinjections were targeted at the lateral division of CeA (A/P -2.4, M/L ±4.65, D/V -7.75, angle 4°). Microinjections were administered at a rate of 0.1ul/min, and microinjection needles were left in place for 10 additional minutes to ensure diffusion. In the same surgery, optic fibers were bilaterally implanted in the projection target site of either the LH (A/P -4.2, M/L ±2.0, D/V -8.0) or the DMS (A/P: +0.12, M/L: +/-2.6, D/V: -5.0) and secured with screws and dental cement.

*Conditioned Place Preference and DMS CRF Pharmacology*

At least one week after surgery, animals underwent four days of conditioned place preference. On day one, rats were allowed to freely roam a chamber with two compartments for 30 minutes to test their initial place preference. On days two and three, a barrier was installed to close off the gate between the two compartments. Rats were then randomly assigned to receive vehicle microinjections prior to conditioning sessions in one compartment and CRF in the other compartment in a counterbalanced manner. Rats underwent twice daily conditioning sessions where they received either vehicle or CRF injections prior to 30-minute conditioning session. Both vehicle and CRF injections were given on each day in a counterbalanced order with at least four hours in between. On the fourth and final day, no microinjections were given and the barrier between the two chambers was removed, allowing rats to explore both chambers freely

Immediately prior to conditioning sessions, rats received 0.2ul microinjections of either artificial cerebrospinal fluid (aCSF) or CRF (500ng) in aCSF at a rate of 0.2ul/min.

Microinjections were delivered using 33 gauge microinjector tips extending 1mm below the tip of the guide cannula (Kokare et al., 2011).

*Two-Choice Sucrose*

Rats first underwent magazine training (1 day, 30min per day), where they learned to retrieve sucrose from the magazine, and autoshaping (4 days, 45min per day), where rats learned that retraction of the two alternating levers and presentation of the assigned sound cues predicted sucrose delivery to the magazine.

During the subsequent 8 days of two-choice testing, the two levers were introduced in the box: one lever delivered a sucrose reward and laser stimulation (*Laser + Sucrose,* 3 mw, 8 s, 40 Hz or 10Hz), while the other lever delivered a sucrose reward without laser stimulation (*Sucrose Alone)*. Levers were randomly assigned an 8s sound cue of either a tone or white noise counterbalanced across levers. The number of lever presses and quantity of sucrose pellets dispensed were recorded for each lever. Additional sucrose rewards could not be earned during the 8s delivery of sound cue and laser stimulation. During the first day of FR1 testing, rats were able to freely choose either from the beginning of the session; however, on all following days, rats underwent two sessions of forced choice to remind them of the different available outcomes: an initial lever was presented randomly and the rat had to earn one reward from that lever. The alternative lever was then presented and the rat had to earn a reward from that lever. This repeated a second time with the rat earning a total of two rewards from each lever before both levers presented simultaneously for the rat to choose freely for the rest of the session. Following three days of responding on a FR1 schedule, rats progressed through one day of FR4, one day of random ratio (RR) 4, and three days of RR6.

*Progressive Ratio*

Two days of progressive ratio sessions took place following the final day of RR6 Two-Choice Sucrose. Within a single test session, only one of the levers from Two Choice sucrose, laser-paired or not, would appear across counterbalanced days. As the session progressed, the number of lever presses required to receive the reward increased exponentially with each reward earned (progressive ratio schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, …) derived from the formula PR = [5e(reward number×0.2)] − 5 and rounded to the nearest integer (Richardson & Roberts, 1996; Saunders & Robinson, 2011a). The test is designed to assess the "breakpoint" of the animal's motivation to pursue a reward, or the number of lever presses required to earn the reward at which the animal ceases to work for the reward.

*Self-Stimulation*

Rats underwent 3 days of self-stimulation testing (30-minute session). Rats stayed in the same box throughout the testing and the laser pairing stayed on the same side. Within the operant box, the rats were presented with two bars: contact with one bar (labeled "active") earned laser stimulation (1mw, 1s constant or 1mw 1s 10Hz), whereas contact with the other bar (labeled "inactive") earned nothing. Rats were classified as Low self-stimulators if they achieved 10+ laser contacts and 2x as many laser contacts as nonlaser. Rats were classified as High self-stimulators if they achieved 50+ laser contacts and 2x as many laser contacts as nonlaser. For the first 3-day period, rats received either the 10Hz stimulation or the constant stimulation. For the second 3-day period, the rats received the other 1 second constant stimulation in counterbalanced order.

*Real Time Place Preference*

Rats underwent 4 days of real time place preference testing. Rats are placed into a behavioral arena containing two equivalent chambers, with each chamber measuring 14.75” x 14.5”. The two chambers are connected by a 4.5” wide gate. On the first day, rats were free to roam both chambers with no laser stimulation paired to either side. After one day of familiarization, each animal was assigned either Left or Right chamber laser pairing for the remaining three days of testing. For the first 3 of these days, the laser paired chamber is paired with 3 mw, 3s ON 4s OFF, 10Hz stimulation in a counterbalanced manner while the other chamber has no laser stimulation. Total time spent in each chamber is recorded during the 15-minute sessions. Left and Right chamber laser pairing was counterbalanced.

*Statistical Analysis*

To evaluate significance of behavioral tests, we used two-way and three-way repeated measures and mixed-model ANOVAs. These were followed by post-hoc t-test comparisons with Bonferroni corrections.

## 4.3 Results

**Activation of $CeA_{CRF}$→ LH projection neurons generates aversive motivation**

To characterize CeA CRF circuitry that could contribute to the incentive motivation generated by activation of CeA CRF cell bodies, we selectively optogenetically activated CeA CRF projections to the LH.

First, we used a two-choice sucrose task where rats could freely choose throughout a session to lever press for either *Laser + Sucrose* or *Sucrose Alone* in order to determine if activation of $CeA_{CRF}$→ LH projection neurons could bias and intensify incentive motivation for the laser-paired sucrose (Baumgartner et al., 2021). During the two-choice sucrose, $CeA_{CRF}$→ LH ChR2 rats developed an aversion to *Sucrose + Laser* rewards and exclusively preferred the *Sucrose Alone* option at a 6:1 ratio by the $8^{th}$ and final day (time x laser interaction $F_{(7,35)}=3.299$, $p=0.008$, days 8 ChR2 laser:nonlaser $p<0.0001$; Figure 4-1 A). Control eYFP rats did not develop a preference for either *Sucrose + Laser* or *Sucrose Alone* rewards ($p>0.05$; Figure 4-1 B). These results indicate that activation of $CeA_{CRF}$ → LH projections may generate aversive motivation.

However, anatomical analysis of fiber placements within the LH revealed individual differences corresponding to A-P site placements, suggesting a rostral-caudal gradient of sucrose avoidance vs preference on the last day of RR6 (Figure 4-2 A). Specifically, $CeA_{CRF}$→ LH ChR2 rats with anterior-to-middle LH sites (optic fibers -4.20mm to -4.56mm from Bregma) within the LH predominantly pursued the *Sucrose Alone* option and avoided the *Laser + Sucrose* option. However, a subgroup of rats with far posterior sites in LH that merged into substantia nigra (SN; optic fibers >-4.68 mm from Bregma), $CeA_{CRF}$→ posterior LH/SN ChR2 rats maintained a strong >6:1 ratio preference for *Laser + Sucrose* option over *Sucrose Alon*e (Day x

Laser: $F_{(7,28)}$=4.288, p=0.003; posthoc: p<0.0001; Figure 4-2 A, shown in red). Two additional rats with fiber placement in the rostral VTA avoided the *Laser + Sucrose* reward and failed to self-stimulate.

*Sucrose breakpoint test.* Using progressive ratio or breakpoint tests, we sought to determine whether $CeA_{CRF}$ → LH projection activation could change the magnitude or intensity of incentive motivation for sucrose. Here, on one day rats could work to earn rewards on their *Sucrose Alone* lever, and on the other day (balanced order) they could work to earn rewards on their *Laser + Sucrose* lever accompanied by LH laser. On each day, the number of lever presses necessary to earn the next reward successively increased until rats reached their breakpoint, when and the rat gave up. $CeA_{CRF}$ → LH ChR2 rats as a whole reached a breakpoint for *Sucrose Alone* that was 2x as high as their breakpoint for *Laser + Sucrose*; however, this difference was not statistically significant (virus x laser interaction: $F_{(1,9)}$= 3.117, p=0.111; Figure 4-1 C,D). eYFP reached similar breakpoints as ChR2 rats, suggesting that laser activation of $CeA_{CRF}$ → LH projection terminals does not impact the magnitude of motivation for laser-paired sucrose rewards. Lastly, rats with optic fibers in the SN may have shown a slight reduction in breakpoint; however this was not statistically significant (t=1.66, df=4, p=0.17).

*Active touch laser self-stimulation.* Since $CeA_{CRF}$ → anterior LH ChR2 rats preferred the *Sucrose Alone* option in the two-choice test, it is possible that activation of their circuit is aversive. To assess the affective valence of $CeA_{CRF}$ → LH terminal activation, we used both an active touch-based self-stimulation task, where rats could contact a rod to trigger laser stimulation, and a passive place-based self-stimulation task where rats could move freely between two chambers, where entry into one chamber would trigger laser-activation for as long as they remained in that laser-paired chamber. In the touch-based self-stimulation task, $CeA_{CRF}$

→ anterior LH ChR2 rats and eYFP rats performed similarly, making around 20 exploratory contacts on both the active and the inactive rods (laser x virus interaction: $F_{(1,13)}=1.722$, $p>0.05$), indicating that activation of $CeA_{CRF}$ → anterior LH circuit did not have an inherent rewarding effect (Figure 4-2 A).

Notably, two $CeA_{CRF}$→ posterior LH/SN ChR2 rats successfully did meet self-stimulation criteria with one rat qualifying as a Low self-stimulator (>10 illuminations, 2x as many laser as nonlaser contacts) and the other classifying as a High self- (>50 illuminations, >2x as many laser as nonlaser contacts) stimulator who reached up to 70 contacts on the laser rod. These pilot results suggest that the posterior lateral hypothalamic area and/or SN could contain incentive $CeA_{CRF}$ projections.

*Place based self-stimulation.* In the place-based self-stimulation task, $CeA_{CRF}$ → anterior LH activation also failed to generate significant avoidance, and $CeA_{CRF}$ → anterior LH ChR2 rats showed similar behavior to eYFP controls ($t=0.289$, $df=9$, $p=0.78$, unpaired), as well as to their initial no-laser prescreening day ( $t=0.112$, $df = 5$, $p=0.92$, paired; Figure 4-3 B). These results suggest that activation of $CeA_{CRF}$ → LH terminals is not strongly rewarding or aversive or its own, despite its ability to bias pursuit away from the *Laser + Sucrose* option in the two-choice sucrose task. Those with fibers in the posterior LH or SN also did not differ from their baseline day; however, a statistical trend suggests that they spent more time in the laser-paired chamber compared to eYFP controls (baseline: $t=2.08$, $df=4$, $p=0.11$; eYFP: $t=2.19$, $df=8$, $p=0.06$).

**Activation of $CeA_{CRF}$→ DMS projection neurons does not influence motivation**

CeA CRF neurons have also recently been shown to project to the DMS, a region involved in motivation and goal-directed behavior , which could be another potential circuit involved in CeA CRF neuron-generated incentive motivation (Baumgartner et al., 2021; Essoh et al., 2022). However, in the two-choice sucrose task, $CeA_{CRF}$ → DMS ChR2 rats, like eYFP rats, did not show a preference for either the *Laser + Sucrose* or the *Sucrose Alone* options (day x virus x laser: $F_{7(7,35)}$=0.212, p=0.98; Figure 4-4 A,B). In the progressive ratio breakpoint task, $CeA_{CRF}$ → DMS ChR2 rats once again performed similarly to eYFP rats, reaching equal breakpoints for *Sucrose Alone* and *Laser + Sucrose* (laser x virus: $F_{(1,5)}$=1.02, p=0.358; Figure 4-4 C,D). Thus, activation of $CeA_{CRF}$ → DMS projection terminals was insufficient to bias reward preference or to magnify incentive motivation for sucrose rewards.

$CeA_{CRF}$ → DMS terminal activation was similarly ineffective at sustaining touch-based self-stimulation where $CeA_{CRF}$ → DMS ChR2 and eYFP rats once again made ~20 rod contacts on average, regardless of laser activation (laser x virus: $F_{(1,5)}$=3.61, p=0.116; Figure 4-5 A). Consistent with these findings, ChR2 rats also failed to develop either preference or aversion in the place-based self-stimulation task both compared to their own baseline (t=1.364, df=4, p>0.05, paired) and compared to eYFPs (t=0.124, df=5, p>0.05, unpaired; Figure 4-5 B).

**Local CRF Microinjection Pilot: LH, DMS, and CeA**

While effects of $CeA_{CRF}$ → DMS projection activation was ineffective at biasing and magnifying motivation, we sought to test whether CRF microinjections directly into DMS, LH, or CeA could generate appetitive motivation in a conditioned place preference paradigm, as has been shown in the nucleus accumbens (Lemos et al., 2012). Alternatively, CRF microinjections into these limbic structures could cause anxiety and distress in line with CRF's traditional role as

an aversive stress peptide. For the DMS, LH and CeA, bilateral 500ng microinjections of either CRF or vehicle did not generate robust place preference or avoidance (DMS: $t=0.203$, $df=5$, $p=0.847$; LH: $t=1.06$, $df=2$, $p=0.4$; CeA: $t=0.017$, $df=3$, $p=0.99$; Figure 4-6).

### 4.4 Discussion

Our findings suggest that excitation of most $CeA_{CRF}$ → LH projections terminating in anterior through middle LH biases animals against laser-paired sucrose rewards in favor of identical rewards without laser. However, activation of this projection did not alter breakpoint in a progressive ratio task. Additionally, while animals did not self-stimulate $CeA_{CRF}$ → anterior LH terminals in the touch-based self-stimulation task, they also showed neither aversion nor preference for $CeA_{CRF}$ → LH terminal stimulation in the place-based self-stimulation task. That suggests that activation of this circuitry on its own is likely not causing strong aversive motivation such as anxiety or distress. In combination with these self-stimulation results, the two-choice sucrose preference for nonlaser sucrose may be indicative of devaluation of the *Laser + Sucrose* option where $CeA_{CRF}$ → LH projection neurons are encoding relative reward salience.

However, a subgroup $CeA_{CRF}$ → LH rats with far posterior LH sites that also may have intruded into the SN developed a clear preference for laser-paired sucrose rewards. While they did not show an elevated breakpoint for these rewards, two of these rats met self-stimulation criteria in the rod-touch self-stimulation task and the group as a whole trended toward laser preference in the place-based self-stimulation task.

Further, regarding CeA CRF projections to dorsal neostriatum, we found that activation of $CeA_{CRF}$ → DMS projection terminals is insufficient to bias pursuit of either *Laser + Sucrose* or *Sucrose Alone*, does not alter breakpoint, and neither supports self-stimulation nor generates aversion in touch- and place-based self- stimulation tasks. Lastly, we show that while CRF microinjections into LH, DMS, and CeA do not lead to a conditioned place preference, they also

fail to cause conditioned place aversion, suggesting that CRF signaling in these regions will not always generate anxiety and distress.

As a trigger of the HPA axis response to stress, CRF is considered a key regulator of the behavioral response to stress which has traditionally centered around negative emotional distress (Dunn & Berridge, 1990; Gray, 1993; Hauger et al., 2009). Previous studies have shown that CeA CRF neurons are involved in anxiety and depressive like behavior, threat and defensive behaviors, fear learning, and pain modulation (Agoglia et al., 2020; Asok et al., 2018; Callahan et al., 2013; Chudoba & Dabrowska, 2023; Huang et al., 2010; Mazzitelli et al., 2022; Pomrenze, Giovanetti, et al., 2019; Pomrenze, Tovar-Diaz, et al., 2019). In this vein, CRF is associated with anxiety and distress during withdrawal which, in opponent process theories of addiction, is thought to act as a negative reinforcer so that relapse serves to alleviate distress via hedonic self-medication (George et al., 2012b; G. F. Koob, 2010; G. Koob & Kreek, 2007; Valdez et al., 2003; Weiss et al., 2001). To some extent, the results that $CeA_{CRF}$ → anterior LH terminal activation biases pursuit of nonlaser rewards supports previous evidence that CRF systems mediate aversive motivation. However, given that activation of the $CeA_{CRF}$ → anterior LH projection did not generate aversion in place-based self-stimulation suggests that activation of this pathway is not necessarily generating distress but may be encoding relative reward value when multiple rewards are available. Additionally, neither activation of the $CeA_{CRF}$ → DMS projection nor microinjections of CRF into CeA, LH or DMS were sufficient to drive either incentive or aversion motivation, suggesting that CRF may need to act in conjunction with additional stimuli or at different targets to influence affect and motivation.

In support of this, CRF has been demonstrated to enhance incentive salience of rewards and cues within several mesolimbic structures. Within the CeA and hypothalamus, receipt of

food and drug rewards triggers CRF release, suggesting that CRF release is associated with reward seeking behavior (Calogero et al., 1989b; Merali et al., 1998b). Additionally, CRF microinjections within the NAc has been demonstrated to enhance lever pressing for rewards upon cue presentation in a manner similar to amphetamine injections, implicating CRF as a positive motivator for rewards within the NAc (Peciña et al., 2006c). Importantly, CRF's enhancement of motivation via lever presses was shown to be solely dependent on the presentation of a reward-associated cue, ruling out the performance enhancement to be a cause of motor arousal, frustration, or stress from the CRF microinjection. This further corroborates an interaction between DA and CRF within the NAc that drives motivation and reward-seeking behaviors (Baumgartner et al., 2021; Lemos et al., 2012; Peciña et al., 2006c). Within the CeA and NAc, optogenetic stimulation of CRF-releasing neurons causes rats to self-stimulate and develop a preference for activation-paired sucrose and activation-paired cocaine rewards (Baumgartner et al., 2021, 2022).

Future studies are necessary to identify the CRF circuitry underlying the positive incentive motivation that arises from CeA CRF neuronal activation. In addition to the DMS and LH, CeA CRF neurons have been shown to project to the ventral pallidum and the ventral tegmental area, both of which are central to motivation (Pomrenze et al., 2015). Our tentative pilot findings that excitation of $\text{CeA}_{\text{CRF}} \rightarrow$ posterior LH/SN projections in two rats may induce a preference for laser-paired sucrose over sucrose alone, and may support laser self-stimulation on its own, lends support to the possibility that CeA CRF projections to anterior SN may contribute to incentive salience. Additionally, CRF-expressing neurons in the *Crh*-cre rat are GABAergic and could be projecting locally within the central amygdala to generate incentive motivation (Dabrowska et al., 2013; Pomrenze et al., 2015). In addition to specific projection targets, future

work should clarify the contribution of CRF1 and CRF2 receptors, either through intraventricular administration or via local administration intro putative projection targets.

### 4.5 Figures

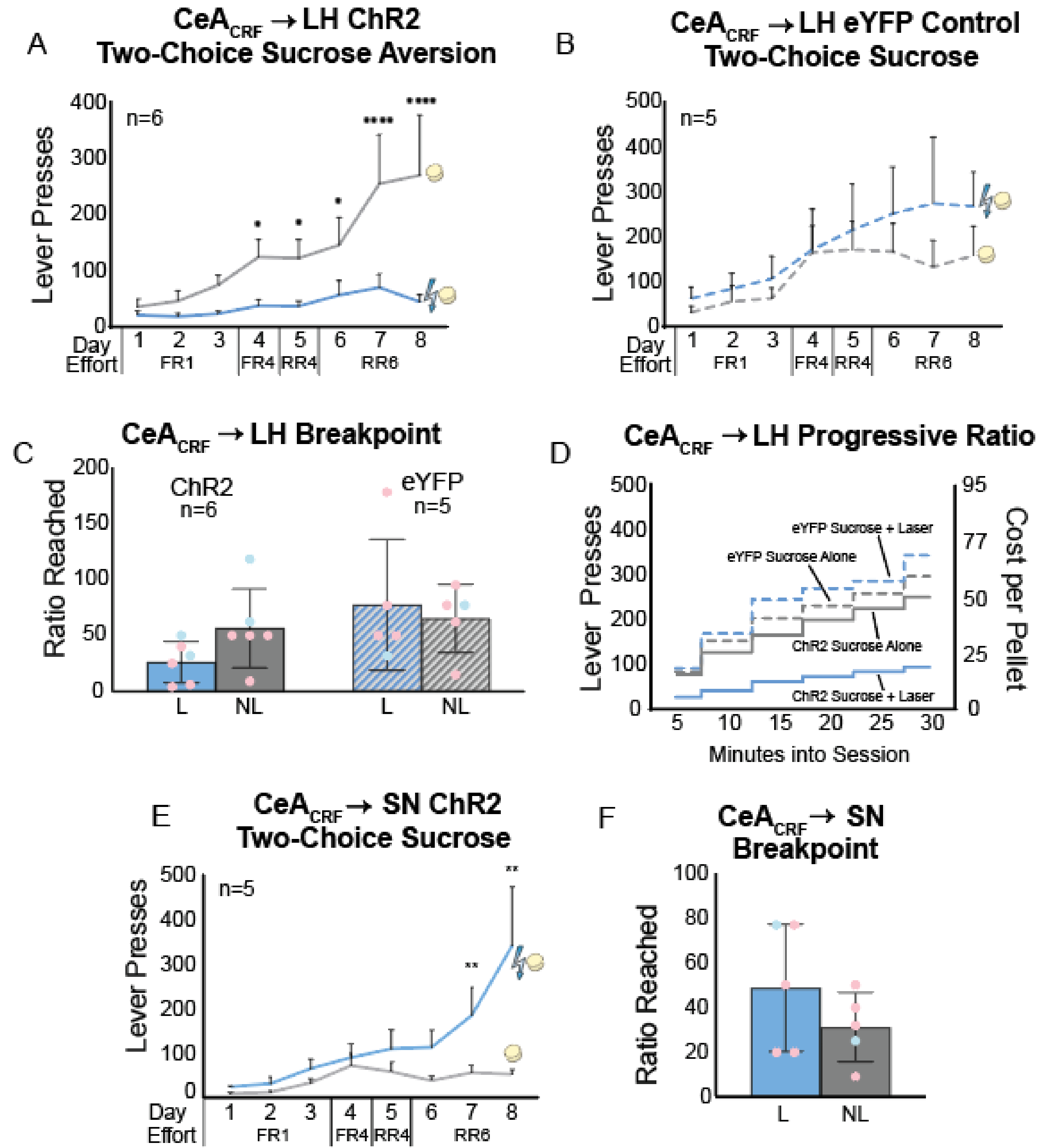


Figure 4-1 Activation of CRF projections from CeA to LH or SN oppositely influence incentive motivation for laser-paired sucrose.

A) ChR2 excitation of CeA CRF terminals in the anterior-medial LH (n=6) bias preference for *Sucrose Alone* over *Laser + Sucrose*. B) inactive eYFP control rats do not develop a preference for either sucrose reward (n=5) C,D) In progressive ratio (PR) breakpoint tests of magnitude of motivation to pursue cocaine, neither $CeA_{CRF}$→LH ChR2 (n=6) or eYFP control (n=5) groups showed any difference in effort breakpoint nor nose poke responses between *Laser+Cocaine* and *Cocaine alone* days. E) $CeA_{CRF}$→SN ChR2 rats develop a preference for the *Laser + Sucrose* option over *Sucrose Alone*, reaching a 6:1 ratio by day 8. F) Activation of $CeA_{CRF}$→ SN terminals does not change breakpoint for laser vs nonlaser rewards. Means and SEM reported. Pink = Female, Blue = Male. **p<0.01

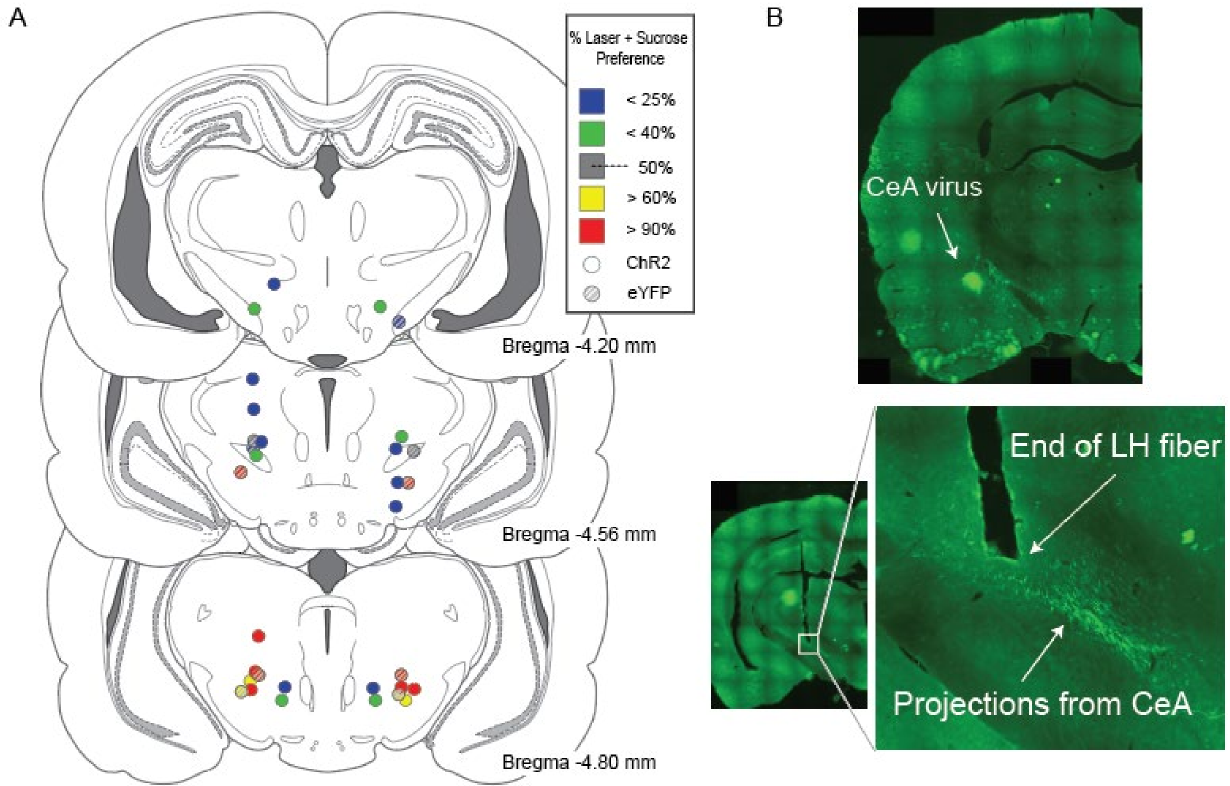



Figure 4-2 Fiber Placements in the anterior-medial LH and posterior LH/SN are functionally distinct

A) Function map of effects of CeA CRF terminal stimulation on % laser preference in the two-choice sucrose task. Yellow or red symbol colors show intensity of enhancement of laser-induced preference for *Laser+Sucrose* option over *Sucrose-alone* while, blue colors show intensity of avoidance of *Laser+Sucrose.* B) Representative images of CeA virus expression (above) and fiber placement over fluorescent CeA CRF terminals in the LH

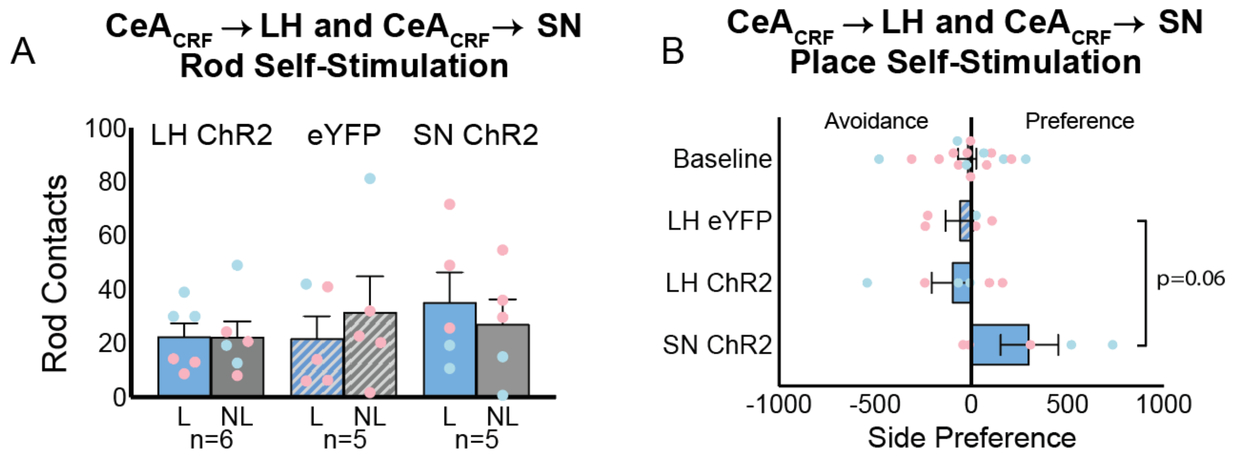



Figure 4-3 Stimulation of CeA CRF terminals in the LH bias rats toward sucrose rewards without laser

A) $CeA_{CRF}$→ LH ChR2 (n=6), eYFP (n=5), and $CeA_{CRF}$→ SN (n=5) rats fail to self-stimulate CRF terminals in the active-touch rod self-stimulation task. B) $CeA_{CRF}$→LH ChR2 and eYFPrats also fail to self-stimulate or avoid the *Laser paired* chamber while $CeA_{CRF}$→ SN rats may prefer the *Laser paired* chamber. Means ± SEM. Pink = Female, Blue = Male.

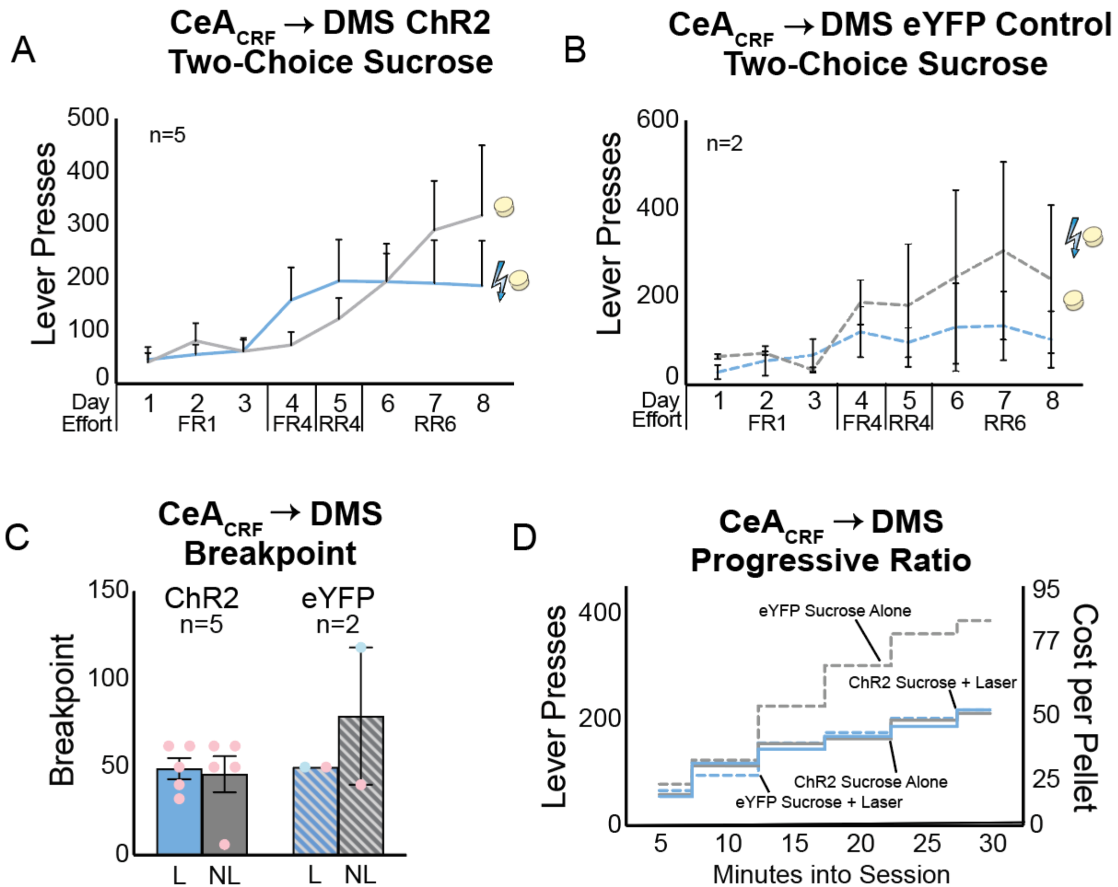



Figure 4-4 Stimulation of CeA CRF terminals in the DMS is insufficient to alter motivation for sucrose rewards

Laser excitation of CeA CRF terminals in the DMS did not direct sucrose preference in the 2-choice task for either A) ChR2 BNST rats (n=5), or B) inactive eYFP control rats (n=2). C) In progressive ratio (PR) breakpoint tests of magnitude of motivation to pursue sucrose, neither ChR2 BNST (n=5) or eYFP control (n=2) groups showed any difference in effort breakpoint nor nose poke responses between *Laser+Sucrose* and *Sucrose alone* days. Means and SEM reported. Pink = Female, Blue = Male.

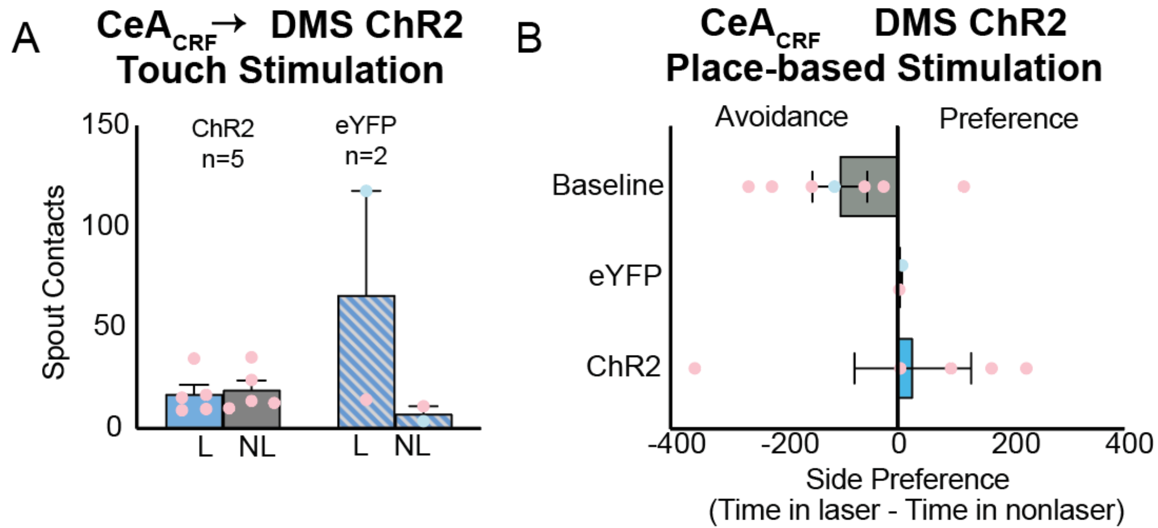



Figure 4-5 *Crh*-cre rats do not self-stimulate CeA CRF projections to the DMS

a) ChR2 $CeA_{CRF}$→ DMS rats failed to self-stimulate for laser and B) ChR2 activation of CeA CRF terminals in DMS (n=5) failed to cause preference for or avoidance of the *Laser-delivering* chamber relative to eYFP controls (n=2). Means and SEM reported. Pink = Female, Blue = Male

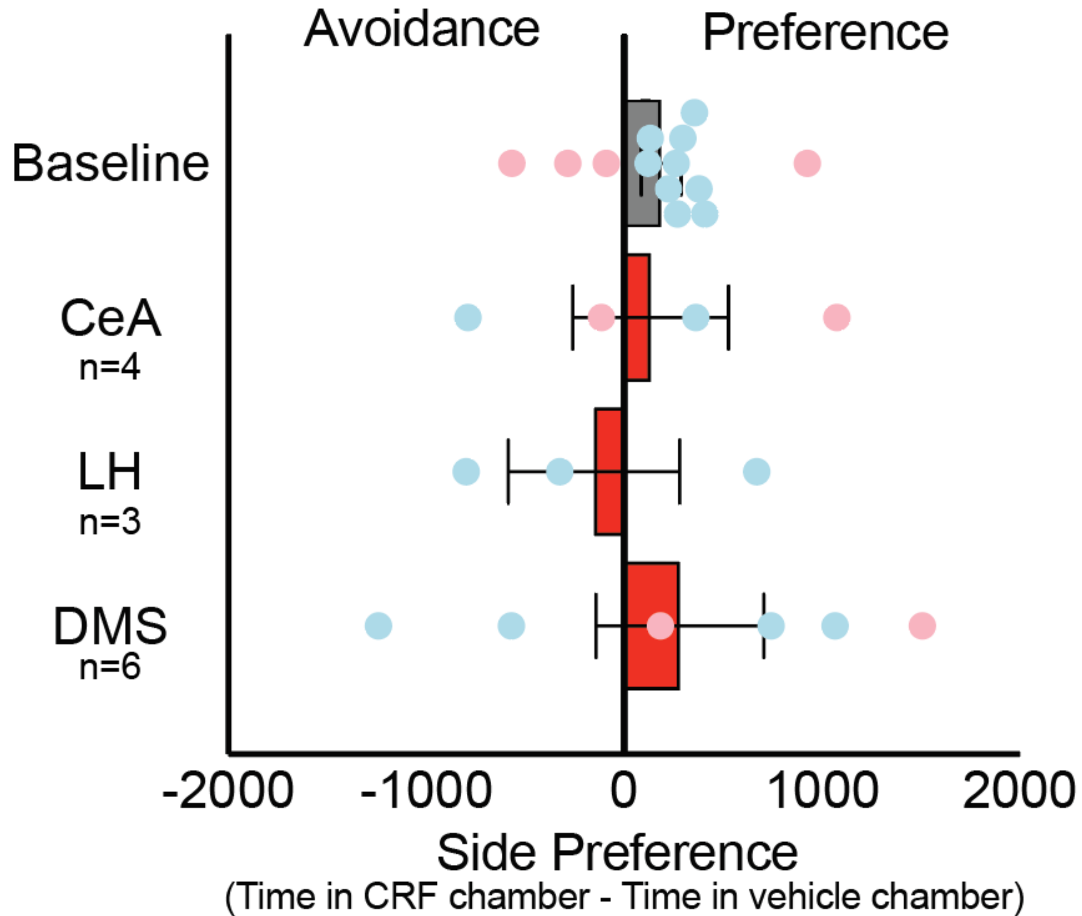



Figure 4-6 Local CRF microinjections into either CeA, LH, or DMS generate neither conditioned place preference nor avoidance

Rats receiving bilateral 500ng microinjections of CRF into CeA (n=4), LH (n=3), or DMS (n=6) fail to develop either conditioned place preference or avoidance. Means ± SEM. Pink = Female, Blue = Male.

# Chapter 5 Extensive Cocaine Consumption Fails to Switch the Valence of Motivation Generated by Optogenetic Excitation of Corticotropin Releasing Factor Neurons in Central Amygdala

## 5.1 Introduction

Corticotropin releasing factor (CRF) is a key regulator of behavioral and physiological responses to stress, triggered by hypothalamic CRF neurons that activate the hypothalamic-pituitary-adrenal axis to elevate glucocorticoid release (Gray, 1993). CRF neurons are also abundant in extended amygdala structures, such as the central nucleus of amygdala (CeA) and bed nucleus of the stria terminalis (BNST), and CRF neurons also appear in other limbic structures such as the nucleus accumbens (NAc) (Baumgartner et al., 2021; Dedic, Chen, et al., 2018; Pomrenze et al., 2015; Pomrenze, Tovar-Diaz, et al., 2019). Activation of CRF neurons in these limbic structures has therefore often been posited to generate anxiety and distress, and to motivate behavior that aims to reduce distress (Adamec & McKay, 1993; Bledsoe et al., 2011; Gray, 1993; Mazzitelli et al., 2022; Pomrenze, Tovar-Diaz, et al., 2019; Zorrilla et al., 2002).

*CeA CRF as withdrawal distress in addiction*. In particular, CRF systems in extended amygdala have featured prominently in some addiction neuroscience theories, such as Hyperkatifeia or Hedonic Dysregulation/Allostasis theory, which view CRF-generated feelings of distress and withdrawal as chief factors underlying pursuit and consumption of addictive drugs (Cottone et al., 2009; George et al., 2012a, 2012a; G. F. Koob et al., 2014; Moore et al., 2017a, 2017b; Parylak et al., 2011; Valdez et al., 2003; Zorrilla et al., 2014). Such theories draw upon the earlier psychological logic of the opponent-process theory of addiction (Solomon &

Corbit, 1974), which posited that drugs induce a positively-valenced hedonic 'a-process' in the brain, which in turn triggers an opposite negatively-valenced 'b-process'. The b-process can subtract from drug a-process effects to contribute to tolerance while drug is on board, and after the a-process ends the longer-lasting b-process may persist to cause unpleasant feelings of withdrawal. Opponent process theories posit that the b-process, specifically, grows with repeated drug taking, incrementally becoming stronger in amplitude and lasting longer in duration, whereas the a-process either shrinks or remains the same (Cottone et al., 2009; George et al., 2012a, 2012a; G. F. Koob et al., 2014; Moore et al., 2017a, 2017b; Parylak et al., 2011; Solomon & Corbit, 1974; Valdez et al., 2003; Zorrilla et al., 2014). Therefore, the experience of drug taking eventually becomes dominated by the strengthened aversive b-process, driving addicted individuals to consume more drug to escape the distress, in attempts that only make the problem worse (Solomon & Corbit, 1974).

Hyperkatifeia and hedonic dysregulation theories suggest specific neural mechanisms to mediate b-process and the a-process roles in the brain (Cottone et al., 2009; George et al., 2012a, 2012a; G. F. Koob et al., 2014; Moore et al., 2017a, 2017b; Parylak et al., 2011; Valdez et al., 2003; Zorrilla et al., 2014). In particular, CRF neural systems in the CeA and BNST are specifically posited to mediate the aversive b-process distress that grows and lengthens with extended drug consumption, causing aversive withdrawal feelings that motivate further drug taking.

The 'aversive CeA CRF' hypothesis has received support from evidence that a range of abused substances, such as stimulants and opioids, can activate CRF systems and the HPA axis (Armario, 2010; Kershaw et al., 2015; Manetti et al., 2014; Matta et al., 1998; Schlussman et al., 2002). Further, following chronic drug experience, CRF signaling in the extended amygdala,

including the CeA and BNST, is argued to become amplified, even if the HPA axis response becomes blunted (G. F. Koob & Le Moal, 2008; G. Koob & Kreek, 2007). CRF is reported to be increased in the CeA and BNST of rats during withdrawal from alcohol, cocaine, opioids, or cannabinoids, consistent with a strengthened b-process (Funk et al., 2006; George et al., 2007; Olive et al., 2002; Richter & Weiss, 1999; Rodríguez de Fonseca et al., 1997). Furthermore, acute withdrawal is often accompanied by intense negative emotional states of anxiety, and distress that can lead to enhanced drug-seeking behavior, and CRF antagonists are reported to block reinstatement/relapse of drug taking in some studies with rats (Baldwin et al., 1991; Basso et al., 1999; George et al., 2007; Huang et al., 2010; Rodríguez de Fonseca et al., 1997; Specio et al., 2008; Valdez et al., 2003; Zorrilla et al., 2002).

*Positive incentive motivation roles of CRF systems*. However, other recent evidence suggests CRF systems can play an alternative and positively-valenced incentive role in motivating reward pursuit and consumption in some situations (Baumgartner et al., 2021, 2022; Lemos et al., 2012; Peciña et al., 2006a; Xu et al., 2024). In these cases, CRF activation in CeA, NAc or hypothalamus can intensify incentive motivation for drug, food or other rewards without inducing distress. For example, NAc CRF microinjections amplify cue-triggered motivation for sucrose rewards similarly to dopamine-stimulating NAc microinjections of amphetamine, and CRF microinjections in NAc can also generate a conditioned place preference for a paired location (Lemos et al., 2012; Peciña et al., 2006a). In optogenetic studies, *Crh*-cre rats have been found to self-stimulate laser excitation of CRF neurons in CeA or NAc (Baumgartner et al., 2021, 2022), and *Crh*-cre mice are reported to self-stimulate laser excitation of CRF neurons in hypothalamic PVN (Xu et al., 2024). Furthermore, optogenetic activation of CeA and NAc CRF neurons of *Crh*-cre rats was found to induce a positive and nearly exclusive preference for an i.v.

cocaine reward paired with optogenetic excitation of CRF neurons in CeA or NAc over an identical cocaine reward received without CRF neuronal excitation in a 2-choice task, and to similarly induce preference for a laser-paired sucrose reward over identical sucrose rewards without laser (Baumgartner et al., 2021, 2022). Finally, optogenetic stimulation of CRF neurons in CeA or NAc also amplified the intensity of incentive motivation for cocaine reward and for sucrose reward, measured as increased breakpoint in progressive ratio tasks (Baumgartner et al., 2021, 2022). That is, simultaneous CRF neuronal activation in CeA or NAc appeared to amplify the incentive value of paired cocaine or sucrose rewards, rather than subtracting value. These demonstrations of CRF roles in amplifying or generating incentive motivation appear consistent with reports that endogenous CRF in CeA and paraventricular nucleus of the hypothalamus (PVN) is not only elevated by unpleasant stresses, but also increased by receipt of pleasant food rewards or drug rewards (Calogero et al., 1989a; Merali et al., 1998a).

*CeA CRF distress may grow as a b-process.* The incentive effects of CRF neuronal stimulation described above appear incongruent with CRF's traditionally hypothesized roles in generating negative distress and subtracting from positive reward states. However, it is important to note that the aversive motivational b-process role of CRF in extended amygdala was not expected to be initially manifest in individuals prior to addiction, but instead was posited to grow only after extensive drug experience (Cottone et al., 2009; George et al., 2012a, 2012a; G. F. Koob et al., 2014; Moore et al., 2017a, 2017b; Parylak et al., 2011; Valdez et al., 2003; Zorrilla et al., 2014). That is, a negatively-valenced CRF b-process role should grow to become more strongly aversive after drugs are repeatedly taken. Consequently, a caveat must be acknowledged regarding CRF roles in incentive motivation described above: all those incentive effects of optogenetic CeA CRF neuronal stimulation were induced in rats or mice that had either

zero or minimal cocaine exposure (Baumgartner et al., 2021, 2022; Lemos et al., 2012; Peciña et al., 2006a; Xu et al., 2024). Conceivably, more extensive drug experience could strengthen CRF as a b-process so that CeA CRF neuronal excitation becomes aversive and subsequently motivates escape and avoidance (George et al., 2012a; G. F. Koob, 2010; G. F. Koob & Le Moal, 2008; Zorrilla et al., 2014).

A cocaine self-administration paradigm often used in addiction neuroscience studies to promote extensive drug consumption is the long access self-administration procedure (LgA), where rodents are given opportunity to self-administer i.v. cocaine in 'long' 6-hour daily sessions (Ahmed & Koob, 1998). The LgA procedure induces escalation of drug intake, and is associated with increased motivation to consume drugs, and persistent drug seeking despite negative outcomes (Ahmed & Koob, 1998; Ben-Shahar et al., 2008; Kippin et al., 2006; Mantsch et al., 2004; Morgan et al., 2006; Paterson & Markou, 2003; Vanderschuren & Everitt, 2004; Wee et al., 2008; Weiss et al., 2001). Specifically, LgA cocaine self-administration has been shown to amplify CRF signaling within the extended amygdala, consistent with a growing b-process that could cause distress during withdrawal (Baldwin et al., 1991; G. F. Koob, 2010; Specio et al., 2008). For example, LgA cocaine self-administration has been shown to increase levels of CRF in amygdala during withdrawal, increase CRF receptor expression in the VTA, and augment CRF-mediated relapse of cocaine seeking (Blacktop et al., 2011; Mantsch et al., 2008, 2016; Richter & Weiss, 1999; Schmeichel et al., 2017; Shaham et al., 2003; Shalev et al., 2010; Vranjkovic et al., 2018).

In this study, we sought to use a LgA procedure to assess whether exposure to extensive cocaine consumption, in two weeks of daily 6-hour long-access self-administration sessions (LgA), would cause optogenetic activation of CeA CRF neurons to flip its motivational valence and

become aversive, so that CeA CRF excitation would be reliably avoided in the 2-choice and laser self-stimulation situations described above. Both before and after 14 days of 6hr/day LgA self-administration of i.v. cocaine that produced elevation in drug consumption, *Crh*-Cre+ rats were tested for CeA CRF laser self-stimulation. Furthermore, following cocaine self-administration, rats were tested in sucrose 2-choice tasks (Ahmed et al., 2003; Mantsch et al., 2004; Specio et al., 2008; Vranjkovic et al., 2018). *Crh*-Cre+ rats were tested both during withdrawal, immediately 1-day after ceasing cocaine self-administration, and again 4 weeks later after a protracted period of drug abstinence to characterize the persistence any LgA cocaine-induced valence switch of CeA CRF neuronal activation to aversive.

## 5.2 Materials and Methods

### Animals

Female (n=17) and male (n=18) *Crh*-Cre+ Wistar rats were bred and genotyped in house from breeders obtained from the Messing Laboratory at the University of Texas (Pomrenze et al., 2015). Rats were housed in same-sex pairs at 21°C under reverse light cycle (lights-off 7am; testing began 1-3 hours after lights-off) with ad libitum access to food and water for all behavioral tasks. After catheter surgery (>3 months old; >250g), rats were single housed in otherwise identical conditions. During cocaine self-administration training, rats were food restricted (85-90% previous body weight) and then returned to ad libitum access to food for LgA self-administration and all subsequent behavioral tasks.

### Fiber and Virus Implantation

Rats were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance). Prior to surgery, rats received atropine (0.05 mg/kg, IP, Henry Schein), cefazolin (75 mg/kg, SC, Henry Schein), and carprofen (5 mg/kg, SC, Henry Schein). Rats received bilateral infusions of either optically active AAV-DIO-ChR2-eYFP (n=26) or optically inactive control virus AAV-DIO-eYFP (n=9) in the CeA (A/P: -2.4, M/L: ± 4.65, D/V: -7.75 virus, -7.45 fiber at 4° angle). A 1.0 µl volume of virus per hemisphere was microinjected at each bilateral site over a 10-min period (0.1 µl / min), and the microinjector was left in place for an additional 10-min to allow diffusion. Optic fibers (200 µm) were bilaterally implanted in the same surgery and placed so that each fiber tip was aimed 0.3 mm dorsal to the virus microinjection site and secured with skull screws and dental cement. Rats were given 7-10 days for recovery from surgery before behavioral testing. Carprofen (5 mg/kg, SC) was given 24 and 48 hours after surgery.

**Pre-Cocaine Behavioral Tests of Incentive Motivation vs Avoidance and Pre-Training**

*Active-touch Laser Self-Stimulation Task*

At least one month following optogenetic surgery but prior to any cocaine self-administration, the incentive value of CeA CRF neuronal excitation by laser stimulation alone was tested using a 30-min self-stimulation task for 6 days (1s 1mw 10Hz for the first 3 days, 1s 1mw constant stimulation for the last 3 days). Each laser self-stimulation chamber had two empty two metal rods (0.5cm diameter, 3cm long, spaced 17cm apart). One rod was designated as active, and each touch on it earned 1 second of 10Hz or constant laser stimulation. The other rod was inactive and touches on it earned nothing and served as a control measure of exploration. On day 1 rats were assessed for laser self-stimulation, classifying them according to 3 levels of performance (Baumgartner et al., 2021, 2022). Rats were classified as Robust Self-stimulators if they earned >50 laser illuminations in a 30-min session and touched their Laser-delivering rod >2X more often than their Inactive rod on average over their three test days. Rats were classified as Moderate Self-Stimulators if they earned 10 to 49 laser illuminations on average per session, and still touched their Laser-delivering rod >2X more often than the Inactive rod. Rats were classified as Failures to self-stimulate if they earned fewer than 10 laser illuminations or failed to touch their Laser-delivering rod at least twice as often as their Inactive rod.

This same 6-day self-stimulation task was repeated starting 24hr after the final self-administration session and again after 4 weeks (28 days) of abstinence.

*Place-based laser self-stimulation task*

In a different place-based self-stimulation vs. avoidance test, rats could earn laser self-stimulations by entering or remaining in a designated *Laser-delivering* chamber within a 2-chamber apparatus, each measuring 14.75” x 14.5” and connected by a 4.5" wide gate. Both sides of the chamber were identical, consisting of clear, plexiglass walls and black floors. On an initial preference screening day, rats were placed into the middle of the gate were allowed to move freely between the two chambers without laser on either side to assess baseline side preference. For Days 2-4, one side of the chamber was randomly assigned laser-paired while the other had no laser pairing. This assignment was counterbalanced among rats. Entering the laser delivering chamber triggered a laser cycle of 3 seconds ON (10 Hz; 3 mW) followed by 4 seconds OFF, which repeated continually as long as the rat remained in the laser delivering chamber. Laser was terminated as soon as the rat left the laser delivering chamber and remained off as long as the rat remained in the center chamber or nonlaser chamber. Place preference or avoidance was determined by comparing time spent in the laser delivering vs nonlaser chamber on the baseline habituation day and on Day 4. This same 4-day self-stimulation task was repeated starting 24hr after the final self-administration session and again after 4 weeks (28 days) of abstinence.

*Sucrose Training and Autoshaping*

Prior to the self-administration period, rats were trained for 1 day to retrieve sucrose pellets from a pellet dispenser: a pellet was delivered to the dispenser dish once every minute for 25 minutes. Next, rats had 4 days of 45-min autoshaping training where one of two levers appeared in alteration every minute for 8 seconds, simultaneously with a distinctive 8-sec tone or

white noise assigned to each lever and followed immediately by a sucrose pellet delivery to the dish. This served to prepare rats for the Two-Choice Sucrose operant task they would complete after finishing self-administration.

**Intrajugular Catheter Implantation**

In a separate surgery immediately following the end of the preliminary behavioral tasks (~5 weeks following optogenetic surgery), rats intended for cocaine self-administration tests were anesthetized again as above and were implanted with an intravenous catheter in the jugular vein. Rats were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance). Prior to surgery, rats received atropine (0.05 mg/kg, IP, Henry Schein), cefazolin (75 mg/kg, SC, Henry Schein), and carprofen (5 mg/kg, SC, Henry Schein). Silastic intrajugular catheters (0.28 mm internal diameter) were threaded into the right jugular vein, then passed subcutaneously along the dorsal neck and secured to an anchor exiting from the dorsal mid-scapular region. Rats were allowed 10 days recovery before beginning any behavioral tests. Intrajugular catheters were flushed daily with 0.1 ml heparinized saline and 0.2 ml gentamicin sulfate (Sparhawk, KS) for 10 days, and with either sterile saline alone or heparinized saline thereafter, to prevent infections or clogs. Catheter patency was tested once before behavioral testing by intravenous injection of 0.2 ml methohexital sodium to induce ataxia (Brevital; 20 mg/ml in sterile water, JHP, MI). Rats that became ataxic within 10s used for cocaine self-administration while those who failed to become ataxic (n=5) were used as drug naïve controls who "self-administered" saline. 1 rat died during surgery and 2 were removed due to broken optic fibers, leaving 27 rats to progress to self-administration.

**Acquisition of Cocaine Self-Administration**

Each box had two static noseports. One noseport was illuminated and designated as active for earning cocaine: each nosepoke in it led to an intravenous 50µl infusion of 0.5 mg/kg cocaine over 2.4 seconds. The other noseport was not illuminated and was designated as inactive: nosepokes in it earned nothing and simply served as a measure of baseline exploratory behavior. A 20 second timeout period followed each cocaine infusion from the active noseport, when its light was turned off and further nosepokes earned nothing until the end of the timeout. Each session lasted 1 hour or until 20 infusions were reached, whichever came first. To move on from training to 6hr self-administration, each rat had to have 3 consecutive sessions of 6 or more infusions (typically 3-10 days). Of the 27 rats who began training, 24 went on to 6hr self-administration sessions.

**Long-access (6hr) Cocaine Self-Administration Sessions**

Extended access self-administration sessions were identical to self-administration training sessions, except that session duration was extended to 6 hours. Rats were placed into operant boxes for 6 hours a day for 14 days to allow for escalation of intake. For each rat, the same noseport that had been illuminated and paired to cocaine led to an intravenous 50µl infusion of 0.5 mg/kg cocaine over 2.4 seconds. The other noseport was not illuminated and was designated as inactive: nosepokes in it earned nothing and simply served as a measure of baseline exploratory behavior. A 20 second timeout period followed each cocaine infusion from the active noseport, when its light was turned off and further nosepokes earned nothing until the end of the timeout. Of the 24 rats who began LgA 6hr administration, 13 successfully completed 14 trial day and went on to complete post-drug behavioral assessments. Rats who failed to complete 14

days of self-administration with intact catheters (n=9) were removed from the LgA Cocaine ChR2 experimental group, but still underwent all behavioral testing to assess effects of intermediate cocaine intake. 2 rats contracted infections and were removed from the study entirely. Saline control rats whose catheters were not patent (n=5) were also allowed to self-administer for 6 hours each day but self-administered isotonic saline rather than cocaine.

**Behavioral Tests of Incentive Motivation vs Avoidance**

Rats began acute withdrawal behavioral testing for incentive effects starting exactly 24 hours after the start of the last session (referred to as Day 1). The rats were tested again 4 weeks following cessation of cocaine self-administration to model a period of abstinence. After this period, the motivational valence of CeA CRF neurons was assessed again (starting on day 28).

*Active-touch Laser Self-Stimulation Task*

The rod-based self-stimulation task was repeated as described prior to LgA cocaine self-administration. The incentive value of CeA CRF neuronal excitation by laser stimulation alone following chronic cocaine self-administration was once again tested using a 30-min self-stimulation task for 6 days (1s 1mw 10Hz for the first 3 days, 1s 1mw constant stimulation for the last 3 days). One rod was designated as active, and each touch on it earned 1 second of 10Hz or constant laser stimulation. The other rod was inactive and touches on it earned nothing and served as a control measure of exploration. For each individual rat, the same rod remained consistently laser-paired across trial days and also across pre-drug, 24hr withdrawal, and 4-week abstinence timepoints. Rats were reclassified as High stimulators (>50 illuminations and >2X more laser contacts than inactive contacts), Low stimulators (>10 illuminations and >2X more

laser contacts than inactive contacts), or Failures (<10 illuminations or <2x as many laser contacts as inactive contacts).

*Place-based laser self-administration task*

The place-based self-stimulation task was repeated as described prior to LgA cocaine self-administration. Rats could earn laser self-stimulations (3s on, 4s off; 10 Hz; 3 mW) by entering or remaining in a designated *Laser-delivering* chamber within a 2-chamber apparatus. On an initial preference screening day, rats were placed into the middle of the gate were allowed to move freely between the two chambers without laser on either side to assess baseline side preference. For Days 2-4, one side of the chamber was randomly assigned as laser-paired while the other had no laser pairing. For each individual rat, the same chamber remained consistently laser-paired across trial days and also across pre-drug, 24hr withdrawal, and 4-week abstinence timepoints. Place preference or avoidance was determined by comparing time spent in the laser delivering vs nonlaser chamber on the baseline habituation day and on Day 4.

*Two-Choice Sucrose task*

The two-choice sucrose task measured whether adding CRF-expressing neuronal stimulation in the CeA to a sucrose reward (*Laser + Sucrose*) made that reward more or less desirable than an identical sucrose reward without laser (*Sucrose Alone*).

Discriminative 2-option training & choice tests: One lever was then permanently assigned to earn *Laser + Sucrose* for each rat (laser assignment was counter-balanced across rats) and the other lever was assigned to earn *Sucrose Alone*. In the first few minutes of each 2-choice session, only one lever was first presented (balanced order across days) until the rat

responded and earned its assigned reward (either *Laser + Sucrose* or *Sucrose Alone*). Then the lever retracted for an 8-sec time out period. The alternative lever was next inserted, or porthole illuminated, so the rat could earn its alternative outcome. Presentation of each lever and its earned outcome repeated once more, so that the rat earned two assigned rewards from each lever or porthole. These single-choice exposures served to remind a rat each day of both outcomes (typically both completed within 5 min), before they were allowed to choose freely between the two outcomes for the rest of the session.  Subsequently, both levers were always extended simultaneously to allow free choice between *Laser + Sucrose* versus *Sucrose Alone* options. Once a choice was made and its outcome earned, both levers were retracted for an 8-sec time out. Then both levers were presented again for another choice. These 2-choice choice presentations continued for the remainder of the 30min session.

Days 1 to 3 of discriminative testing offered both outcomes on a fixed ratio 1 (FR1) schedule, where each *Sucrose + Laser* lever press earned a sucrose pellet with 8-sec laser stimulation (10Hz; 3 mW) plus its auditory label (tone or white noise), and each press on the *Sucrose Alone* lever press earned a sucrose pellet plus its own auditory label (auditory label assignments balanced across rats). Following completion of the 3 FR1 days, the required effort to earn a reward escalated to FR4 responding on Day 4, random ratio 4 (RR4) on Day 5, and random ratio 6 (RR6) on Days 6 to 8.

*Progressive Ratio*

To determine whether CeA CRF neuronal excitation changed the magnitude of incentive motivation to earn sucrose reward, we used a progressive ratio (PR) or breakpoint task. Rats were tested one day using the same parameters as the two-choice task with only the *Laser +*

*Sucrose* lever available for 30 minutes, and it earned a sucrose pellet accompanied by 8-sec laser illumination and usual auditory label as it had previously in the 2-choice task. A second test on another day was run in an identical manner but with only the *Sucrose Alone* lever available, and it earned a sucrose pellet without laser and its own auditory label as it had in the 2-choice task. The order of *Laser + Sucrose* versus *Sucrose Alone* days was counterbalanced across rats. On each day, the number of responses required to earn the next reward increased after each reward was earned (progressive ratio schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, …) derived from the formula PR = [5e(reward number×0.2)] − 5 and rounded to the nearest integer (Richardson & Roberts, 1996; Saunders & Robinson, 2011). The breakpoint or highest effort reached by the end of each 30-minute session was compared as a measure of the intensity of incentive motivation for reward.

**Fos Induction and Brain Harvest**

Rats underwent a final 30-min laser stimulation session (3mw 10 Hz 8sec on, 22sec off) for Fos induction that ended 45-min before euthanasia and transcardial perfusions, and were euthanized using 200 mg/kg of sodium pentobarbital (i.p.; Euthasol, Covetrus). Sodium phosphate buffer rinse and 4% paraformaldehyde (PFA) were used to perfuse the heart. Brains were extracted, postfixed overnight in 4% PFA, cryoprotected for 48 hours in a 25% sucrose solution, sectioned into 40μm slices (Leica, Wetzlar, Germany), and stained for Fos protein and GFP expression before being mounted onto slides.

**Immunohistochemistry**

Tissue was rinsed for 10 minutes in 0.1 M sodium phosphate buffer (NaPB) three times and blocked with 5% normal donkey serum (60 min). Tissue was incubated overnight at room temperature in rabbit anti-cFos (1:2500; Catalog#: 226 008, Synaptic Systems, Göttingen, Germany) and chicken anti-GFP (1:2000; Catalog#: AB13970; Abcam, Cambridge, MA). Brain slices were then rinsed 3 times for 10 minutes in 0.1 M NaPB. Slices were then incubated with donkey anti-rabbit Alexa Fluor 594 secondary (1:250; Catalog #: AB150064; Abcam, Cambridge, MA) and donkey anti-chicken Alexa Fluor 488 (1:300; Catalog#: AB2340375; Jackson Immunoresearch, West Grove, PA) for 120 minutes. Tissue was then rinsed 3 times for 10 minutes in 0.1M NaPB. Tissue was mounted onto slides using mounting medium containing DAPI. Images were taken using a digital camera (Qimaging, Surrey, BC, Canada) and a fluorescent microscope (Leica, Wetzlar, Germany). Immunoreactivity was visualized with filters with excitation bands 515-545 for Fos protein and 490-510 for virus.

**Statistical Analysis**

To assess escalation of cocaine intake, a pairwise t-test was used to compare the final day cocaine intake (day 14) intake to the first day of intake. Statistical analysis was conducted in R (R Core Team, 2020) using tidyverse (Wickham et al., 2019), tidyr (Wickham, Vaughan, et al., 2024), dplyr (Wickham et al., 2023), lme4 (Bates et al., 2024), lmerTest (Kuznetsova et al., 2020), and emmeans (Lenth et al., 2024). Plot were made using ggplot2 (Wickham, Chang, et al., 2024), ggpubr (Kassambara, 2023), ggbreak (Yu & Xu, 2023), and ggpattern (FC et al., 2022). Tables were made with knitr (Xie et al., 2024), broom.mixed (Bolker et al., 2024), and modelsummary (Arel-Bundock et al., 2024). Linear mixed models were used to analyze

experimental data followed by Type III tests with effects coding. Posthoc testing used pairwaise t-test comparisons of estimated marginal means with Bonferroni correction.

## 5.3 Results

### Escalation of Cocaine Self-Administration

Of the 28 *crh*-cre rats trained to self-administer i.v. cocaine, 23 CeA ChR2 rats met the training criteria of at least 6 cocaine infusions per day for 3 consecutive days. Those who failed to meet this criterion after one week of training underwent another test of catheter patency and failed to become ataxic. Of the 5 who failed to self-administer, 2 failed catheter checks after the first day and were added to the saline self-administration drug naïve control group. Of the 25 rats who went on to self-administer cocaine and meet criteria, 14 made it through all 14 days of self-administration but of these, 2 were confirmed to have off-target virus expression and fiber placement and were removed from the CeA ChR2 group and considered separately as anatomical controls, leaving 9 rats in the CeA ChR2 group and 3 rats in the eYFP control group.

A hallmark of LgA cocaine self-administration as a model of addiction is an increase in cocaine intake across trial days (Ahmed & Koob, 1998, 1999; Ferrario et al., 2005). For 12 on-target *Crh*-cre rats who completed 14 days of LgA cocaine self-administration, cocaine intake increased significantly across days, taking 1.5 times as many infusions on day 14 as they did on day 1 ($F_{2.895,39.86}$=3.585, p=0.0231; df=11, t=3.115, p=0.0098) (Figure 5-1 A). Consistent with previous LgA cocaine self-administration studies (Ahmed et al., 2003; Ahmed & Koob, 1999; Ferrario et al., 2005), rats self-administered cocaine at around 150 infusions per session while those who self-administered saline self-administered fewer than 10 infusions on average by the final day (drug: b=-141.744, df=13.611, t=-9.696, p<0.001; Figure 5-1 A). Furthermore, *Crh*-cre rats escalated their cocaine self-administration each day for a final average intake of over 30 infusions per session by the final day (day: b=2.29, df=206.004, t=4.195, p<0.001). There were

no detectable sex differences in total infusions during cocaine self-administration (sex: b=3.294, df=6.6849, t=0.553, p=0.59).

Another characteristic of LgA cocaine self-administration as a model of addiction is escalation of cocaine infusions during the first hour of the six hour session (Ahmed & Koob, 1998, 1999; Ferrario et al., 2005). *Crh*-cre rats who self-administered cocaine slightly increased their first hour infusions over the 14 trial days (day: b=0.778, df=196.333, t=3.823, p<0.001). However, there was not a significant change in first hour infusions from the first trial day to the last, despite a 20% increase in the average number of first hour infusions (day 1 vs day 14: b=8.48, df=12m t=1.633, p=0.129; Figure 5-1 B).

**CeA CRF neuronal stimulation generates incentive sensitization following a period of abstinence**

Following the final day of 6h LgA cocaine self-administration, or saline self-administration for drug-naïve control ChR2 rats, we tested whether optogenetic activation CeA CRF neurons could induce a preference or avoidance for laser-paired sucrose rewards compared to equivalent sucrose rewards delivered without laser excitation, and change the magnitude of incentive motivation for sucrose rewards (Baumgartner et al., 2021).

In the two-choice sucrose task starting 24hrs after the end of LgA cocaine/saline access, control ChR2 rats who had only self-administered saline developed a greater preference for the *Laser + Sucrose* option over the *Sucrose Alone* option, leading to a 3:1 preference for the *Laser + Sucrose* by the 8$^{th}$ and final trial day (laser by day: b=45.486, , SE= 7.473, df=161.388, t=6.087, p<0.001; nonlaser by day: b=-35.37, SE=10.556, df=161.208, t=-3.351, p=0.001; Table 1; Figure 5-2 A). Four weeks later when these same control rats were retested after a month of

"abstinence", they once again preferred the *Laser + Sucrose* to the *Sucrose Alone,* this time choosing the *Laser + Sucrose* by a nearly 5:1 ratio, and lever pressed more for the *Laser + Sucrose* than they had during the earlier timepoint (timepoint x laser: b=-175.894, SE=64.923, df=161.208, t=-2.709, p=0.007; timepoint: x=134.452, SE=46.013, df=161.539, t=2.922, p=0.004; Table 1; Figure 5-2 B). Saline control ChR2 rats also moderately increased their overall responding for sucrose rewards from the 24-hr timepoint to the 1-month timepoint (b=-76.381, SE=37.479, df=245.656, -2.038, p=0.043; Figure 5-3 C), due specifically to an increase in pressing for *Laser + Sucrose* but not for *Sucrose Alone* (Laser withdrawal vs abstinence: p<0.001; nonlaser withdrawal vs nonlaser abstinence: p=1; Table 1). There were no detectable sex differences in saline control ChR2 rats (Table 1). This confirms previous findings that optogenetic activation of CeA CRF neurons consistently generates preference and incentive motivation for laser-paired sucrose in drug naïve *Crh*-cre rats (Baumgartner et al., 2021).

Cocaine ChR2 rats tested 24 hr after the $14^{th}$ and final day of 6hr daily LgA cocaine self-administration failed to show a preference and chose essentially equally between *Laser + Sucrose* and *Sucrose Alone* options (laser x day: b=29.186, SE=8.30, df=249.518, t=3.517, p=0.001, nonlaser x day: b=22.633, SE=11.713, df=248.24, t=1.932, p=0.054; Table 2; Figure 5-2 C). Cocaine ChR2 rats approximately made the same number of total lever presses during 24-hr withdrawal as Drug Naïve ChR2 rats and Cocaine eYFP controls (Drug naïve: p=0.184; eYFP: p=0.207; Table 3; Figure 5-3 C).

A sex difference was detected in Cocaine CeA ChR2 rats on the sucrose 2-choice task after 24 hrs of cocaine withdrawal. *Laser + Sucrose* option on average during withdrawal, males and females exhibited differences in their reward pursuit. Firstly, Cocaine ChR2 females made a 1.5-2x as many lever presses for sucrose (213.722±19.2) than males (120.54±16.49) on the

whole, a sex difference that was not seen in the Drug Naïve ChR2 rats or eYFP controls, indicating that LgA cocaine self-administration enhances sucrose reward pursuit in withdrawal in females relative to males (p=0.017; Table 2; Figure 5-4). Additionally, focusing specifically on female groups, Cocaine ChR2 females made over 1.5x as many lever presses for sucrose (692.00±67.007) as Saline control CeA ChR2 females at 24 hrs after self-administration day 14 (433.00±147.00), suggesting that LgA cocaine experience may have sensitized incentive motivation for sucrose in females f (p=0.045; Table 2; Figure 5-4 A). Only 3 cocaine CeA ChR2 males completed the sucrose 2-choice task during the 24 hr withdrawal day and tended to prefer the *Sucrose Alone* option over the *Laser + Sucrose* option, by a 2:1 ratio (p=0.02; Table 2; Figure 5-4 C). These sex difference suggests that LgA cocaine self-administration may be able to flip the motivational valence of CeA CRF neurons during withdrawal, but only in males. Females, on the other hand, show no preference for *Laser + Sucrose* or *Sucrose Alone* but demonstrate intense and generalized reward pursuit during withdrawal that indicates incentive sensitization.

Four weeks later, following a month of abstinence from cocaine self-administration, Cocaine CeA ChR2 rats as a group once again equally preferred the *Laser + Sucrose* and the *Sucrose Alone* evenly (b=14.428, SE=21.646, df=145.154, t=0.667, p=0.506; Figure 5-2 D). Notably, Cocaine ChR2 rats increased their total lever pressing by 50% (p<0.001; Figure 5-3 C), now pressing more than they did during 24-hr withdrawal. Further, cocaine CeA ChR2 rats now made over 2x as many lever presses for sucrose rewards as either the Drug Naïve ChR2 or the Cocaine eYFP rats (drug naïve: p=0.028; eYFP: p=0.018; Table 4; Figure 5-3 C). This suggests that potential sensitization induced by extensive cocaine consumption, when combined with CeA CRF neuronal excitation, produced an increase in overall incentive motivation to obtain and

consume sucrose rewards that applied both to *Laser + Sucrose* and *Sucrose Alone* options. There was no longer a sex difference in sucrose pursuit after 1-month abstinence, indicating this motivation potentiation applied similarly to females and males. ($p=0.724$; Table 2). This was due specifically due to males increasing their lever pressing for the *Laser + Sucrose* option, which they had previously avoided during 24 hr withdrawal (laser withdrawal vs abstinence: $b=-255.213$, $SE=53.292$, $df=253.946$, $t=-4.789$, $p<0.001$; Figure 5-4 D). Further, males now preferred the *Laser + Sucrose* option at a >10:1 ratio over their *Sucrose Alone* option after 1-month abstinence, similarly to the control saline CeA ChR2 group and similar to previous reports for drug-naïve rats (abstinence laser vs nonlaser: $b=261.596$, $SE=56.259$, $df=245.154$, $t=4.650$, $p<0.001$; Figure 5-4 D). By comparison, female Cocaine ChR2 rats after 1 month abstinence were similar to their 24-hr withdrawal timepoint, equally preferring both *Laser + Sucrose* and *Sucrose Alone* options ($b=-50.067$, $SE=35.583$, $df=245.154$, $t=-1.407$, $p=0.161$; Figure 5-4 B), and pressing for both sucrose rewards overall more than Saline control ChR2 rats ($p=0.033$; Table 2). This seems consistent with general incentive sensitization of motivation for sucrose after extended access to cocaine in females, which applied equally to *Laser + Sucrose* and *Sucrose Alone*.

Cocaine self-administering eYFP control rats showed a slight preference for the *Laser + Sucrose option* (laser vs nonlaser: $b=-120.306$, $SE=48.372$, $df=86.00$, $t=-2.487$, $p=0.015$); however, while their lever-pressing for the *Laser +Sucrose* increased across trial days, their lever-pressing for the *Sucrose Alone* increased at a similar rate, indicating that this baseline preference did not significantly change over time (laser x day: $b=28.111$, $SE=8.176$, $df=86.00$, $t=3.438$, $p<0.001$; nonlaser x day: $-15.66$, $SE=11.563$, $df=86.00$, $t=-1.363$, $p=0.176$; Figure 5-2 E). Following the one-month abstinence period, eYFP rats failed to show a significant preference

for either the *Laser + Sucrose* or the *Sucrose Alone* option (p=0.745) and chose at a 1:1 ratio between the two rewards at the final trail day (p=0.541; Table 3; Figure 5-2 F). Sex differences in eYFP rats could not be assessed as the group only had one female due to attrition during self-administration.

Overall, these results suggest that LgA cocaine self-administration causes incentive sensitization in females that generalizes and intensifies sucrose reward seeking both during 24 hr withdrawal and after a month of abstinence. On the other hand, in males LgA cocaine self-administration may temporarily flip the valence of CeA CRF neuronal stimulation to aversive, causing *Crh*-cre males to pursue the nonlaser reward at 24 hr withdrawal timepoint. However, this effect reverses following one month of drug abstinence, such that optogenetic CeA CRF neuronal activation in males now generates a preference and intensifies incentive motivation and single-minded pursuit of *Laser + Sucrose* over *Sucrose Alone*.

**Amount of cocaine consumed is uncorrelated with laser preference in two choice sucrose**

To assess whether the total amount of cocaine consumption influenced the valence generated by stimulation of CeA CRF neurons, we measured correlations between total amount of cocaine consumed and the percent preference for the *Laser* + Sucrose reward. During withdrawal and during abstinence, total quantity of cocaine consumed was not correlated with preference for the *Laser + Sucrose* option (withdrawal: r=-0.43, df=12, t=-1.692, p=0.1164; abstinence: r=-0.539, df=9, t=-1.921, p=0.087; Figure 5-5 A). Additionally, the change in the number of first hour cocaine infusions from the first day of LgA to the final day is thought to signal the development of dependence, so it is possible that a greater increase in first hour infusions may influence CeA CRF valence. However, the percent change in first hour infusions

was uncorrelated with preference for the *Laser + Sucrose* option (withdrawal: r=0.244, t=0.62, df=6, p=0.56; abstinence: r=0.64, t=1.45, df=3, p=0.24). Sex differences in correlations were unable to be assessed due to the low number of males.

**CeA CRF neuronal activation does not alter breakpoint in drug naïve or cocaine ChR2 rats**

A progressive ratio breakpoint task was used to determine whether CeA CRF neuronal activation influences the magnitude of motivation for the *Laser + Sucrose* option compared to the *Sucrose Alone*. There were no differences is the intensity of motivation for the *Laser +* Sucrose compared to the *Sucrose Alone* either during 24-hr withdrawal or following a month of abstinence for the Saline Control ChR2 rats, the Cocaine ChR2 rats, or the Cocaine eYFP rats (Table 5; Figure 5-6). There were also no differences in breakpoint between any of the three groups (Table 5; Figure 5-6). Neither drug self-administration nor laser activation of CRF neurons appeared to have any effect on motivation to pursue sucrose rewards

**CeA CRF neuronal activation does not generate aversive motivation in CeA ChR2 rats**

To assess the valence of CeA CRF neuronal self-stimulation by itself, in the absence of any reward, we used a place-based laser self-stimulation task and an active-touch laser self-stimulation task.

In the place-based task, rats could move freely between two identical chambers. On the first day, rats could freely explore the chamber without laser activation to screen for any initial preference. Subsequently, on trial days 2-4, entry into one designated chamber would always trigger laser stimulation of CeA CRF neurons, which continued to cycle On/Off as long as the rat remained in that chamber, while entry into the other chamber would terminate stimulation. *Crh-*

cre rats were assessed for place-based self-stimulation relative to their nonlaser prescreening baseline prior to any self-administration. Self-stimulation was assessed prior to LgA, and after a 24-hr withdrawal, and again following one month of drug abstinence to track any changes in CeA CRF valence over time or due to drug experience.

In place-based laser self-stimulation tests prior to any cocaine exposure, CeA CRF activation did not cause development of either a place-preference or place avoidance in CeA ChR2 rats who would go on to self-administer cocaine ($p=0.242$), or ChR2 rats who would never self-administer cocaine ($p=0.730$), or in eYFP controls who would go on to self-administer cocaine ($p=0.499$; Table 6; Figure 5-7 A). Our initial place-based test thus revealed no clear valence of CeA CRF neuronal activation.

Following 14 days of LgA self-administration, after 24hrs withdrawal, Cocaine CeA ChR2 rats continued to spend time evenly in both chambers, changing minimally from their exploration during the initial no-laser habituation day and their preference score during withdrawal remained similar to their pre-drug preference score (withdrawal cocaine ChR2 vs baseline: $b=160.493$, $SE=143.255$, $df=76.422$, $t=1.12$, $p=1.000$; cocaine ChR2 pre vs withdrawal: $b=312.037$, $SE=144.481$, $df=79.138$, $t=2.16$, $p=0.10$; Table 6; Figure 5-7 B). Thus, 14 days of LgA cocaine self-administration failed to cause CeA CRF neuronal activation to become aversive during acute withdrawal.

Similarly, following one month of abstinence, CeA CRF neuronal activation once again failed to produce either attraction or avoidance as rats continued to explore the chambers roughly equally (abstinence cocaine chR2 vs baseline: $b=222.750$, $SE=126.321$, $df=78.919$, $t=1.763$, $p=0.49$; Table 6; Figure 5-8 C). These results suggest that LgA cocaine self-administration does

not cause CeA CRF neuronal activation to generate aversive motivation or clear distress, as animals did not passively avoid stimulation of CeA CRF neurons.

Just as Drug Naïve ChR2 rats failed to show an initial preference at the "pre-drug" timepoint, during "withdrawal" they explored similarly to baseline and to the Cocaine ChR2 and Cocaine eYFP groups (Table 6; Figure 5-7 B). Additionally, there was no significant difference in place-based laser preference between pre-"drug" and "24 hr withdrawal" timepoints in Drug Naïve ChR2 rats (b=196.533, SE=205.837, df=78.07, t=0.955, p=1.00; Table 6; Figure 5-7 B). However, after an additional month of "abstinence", Drug Naïve ChR2 rats significantly increased the time they spent exploring the laser-paired chamber (b=-555.954, SE=217.543, df=74.64, t=-2.556, p=0.038; Table 6; Figure 5-7 B,C).

eYFP rats showed no differences in exploration of the laser and nonlaser chambers compared to any other groups at any timepoint (Table 6). They also equally explored both chambers at all timepoints (Table 6; Figure 5-7 A,B,C). Sex was not included in analyses for any groups due to low and inconsistent numbers in groups across timepoints.

**LgA cocaine self-administration alters self-stimulation of CeA CRF neurons during withdrawal and after a period of abstinence**

In a second active-touch test of laser self-stimulation, rats could earn brief 1 second CeA stimulations by touching one of two innocuous metal rods. Prior to cocaine LgA experience, the group of CeA ChR2 rats who would go on to self-administer cocaine showed a greater number of laser contacts than nonlaser contacts at baseline prior to self-administration as a whole (cocaine ChR2 laser: b=-8.23, SE=3.737, df=161.546, t=-2.202, p=0.029; Table 8, Figure 5-8 A). Despite this group effect, none met criteria to be High self-stimulators (>50 illuminations, plus laser rod

contacts >2X inactive rod contacts), and only 2 of 9 rats met criteria as Low self-stimulators (>10 illuminations, plus laser rod contacts >2X inactive rod contacts) while the remaining 7 failed to meet self-stimulation criteria (Figure 5-9 A). Interestingly, Cocaine ChR2 rats overall contacted the laser rod more than the nonlaser rod regardless of timepoint, suggesting that laser activation of CeA CRF neurons was generally positive in this group (laser main effect: $F_{1,54.381}=7.839$, $p=0.007$; pairwise laser vs nonlaser: b=7.483, SE=2.747, df=11.801, t=2.725, $p=0.019$). In contrast, of the ChR2 rats who would remain drug naïve, one rat met criteria for High self-stimulation (>50 illuminations, plus laser bar contacts >2X inactive bar contacts) while the other 4 animals failed to meet self-stimulation criteria (Figure 5-9 B) and the group on the whole made the same number of laser and nonlaser contacts (Drug Naïve ChR2 Laser: $p=0.107$; Table 7; Figure 5-8 B). Lastly, the eYFP controls rats showed no difference between laser and nonlaser rod contacts on average; however,2 of 3 rats met criteria for Low self-stimulation (eYFP Laser: $p=0.884$; Table 9; Figure 5-8 A, Figure 5-9 C).

In the withdrawal period 24hrs following cessation of LgA cocaine self-administration, Cocaine ChR2 rats no longer contacted the laser rod significantly more than the nonlaser rod, (cocaine ChR2 withdrawal x laser: b=2.941, SE= 6.025, df=314.694, t=0.488, $p=0.626$; Table 8). Yet notably, three rats who failed to self-stimulate during pre-LgA testing now met criteria for Low levels of self-stimulation (Figure 5-9 A). On the other hand, the 2 cocaine ChR2 rats who self-stimulated prior to drug intake did not self-stimulate during 24 withdrawal. Either self-stimulation of CeA CRF neurons is not consistent across timepoints, or it is possible that the emergence of several Low self-stimulators suggests that LgA experience did not make CeA ChR2 excitation strongly aversive.

Drug Naïve ChR2 rats once again failed to self-stimulate on average as 3 rats failed to meet self-stimulation criteria and 1 met criteria for Low self-stimulation (drug naïve ChR2 withdrawal x laser: b=0.477, SE=6.650, df=166.401, t=0.072, p=0.934; Table 7; Figure 5-8 B, Figure 5-9 B). This seems to support the possibility that CeA CRF stimulation is not consistent across time periods, but suggests that the likelihood of self-stimulation reduces over time as opposed to increases over time. This could explain the reduction in stimulation seen in the two Cocaine ChR2 rats who failed to stimulate during withdrawal; however, this does not rule out the effects of relative drug intake or other factors. For Cocaine eYFP rats during withdrawal, the 2 rats who initially met Low self-stimulation criteria now failed to meet criteria (Figure 5-9 C). Once again, during withdrawal, eYFP controls showed no difference in number of contacts for the laser or nonlaser rod (cocaine eYFP withdrawal x laser: b=-0.709, SE = 7.66, df=184.258, t=-0.093, p=0.926; Table 9; Figure 5-8 C).

Following a one-month period of drug abstinence, a statistical trend suggests that Cocaine CeA ChR2 rats may have once again contacted the laser rod more than the nonlaser rod (cocaine ChR2 4wk laser vs nonlaser: b=8.932, SE=4.913, df=52.701, t=1.818, p=0.075; Table 8). This indicates that some positive incentive value of CeA CRF neuronal activation may return following withdrawal. Further, two of the three CeA ChR2 rats who self-stimulated during 24 hr withdrawal continued to meet Low self-stimulation criteria after a month of abstinence, while one rat who had self-stimulated during withdrawal now failed to do so. A third CeA ChR2 rat, who had self-stimulated prior to LgA but not during withdrawal, once again began to self-stimulate at low levels following a month of abstinence (Figure 5-9 A). Again, this mixed but persisting self-stimulation pattern suggests that LgA experience does not leave CeA ChR2 excitation strongly aversive at 1 month after cocaine taking ends.

At one month following the end of LgA saline or cocaine self-administration, neither Drug Naive ChR2 rats (Figure 5-8 B) nor Cocaine eYFP rats (Figure 5-8 C) make more laser contacts than nonlaser contacts (drug naive chR2: abstinence x laser : b=0.91, SE=6.389, df= 147.929, t=0.143, p=0.887; Table 7; cocaine eYFP: abstinence x laser: b=2.416, SE=7.660, df=184.258, t=0.315, p-0.753; Table 9). No rats from either group met self-stimulation criteria at this timepoint.

## 5.4 Discussion

### Overview

Our results demonstrate that exposure to LgA cocaine self-administration reduces the ability of optogenetic CeA CRF neuronal activation to focus incentive motivation on a laser-paired sucrose reward as has been shown in drug naïve *Crh*-cre rats (Baumgartner et al., 2021). This shift in CeA CRF-driven incentive motivation was defined by unique sex differences where, following cocaine self-administration, females increased their overall sucrose pursuit but pursued the *Laser + Sucrose* and *Sucrose Alone* options equally both during withdrawal and following a period of abstinence. In contrast, males preferred the *Sucrose Alone* in 24-hr withdrawal, a valence flip compared to drug naïve controls. However, they then reversed into preference for *Laser + Sucrose* following a one-month period of abstinence, indicating the reemergence of positively-valenced incentive salience attribution at least in males. In the progressive ratio breakpoint task, ChR2 activation was insufficient to alter the magnitude of motivation for either *Laser + Sucrose* or *Sucrose Alone*, unlike previous work from our lab showing that CeA CRF neuronal activation in rats without extensive cocaine exposure could increase breakpoint for laser-paired rewards (Baumgartner et al., 2021).

In our place-based self-stimulation task activation of CeA CRF neurons was not sufficient to drive either laser-place-avoidance or preference at any timepoint. While CeA CRF neuronal activation may have biased males away from laser-paired sucrose, it does not seem to cause intense enough negative affect in either males or females to cause them to avoid a place where laser is delivered.

Perhaps most important regarding the question of whether LgA cocaine experience switches the valence of CeA CRF neuronal excitation's motivational effects from incentive to

aversive, at least a few CeA ChR2 rats that received LgA cocaine experience continued to self-stimulate brief laser illuminations afterwards in the active-touch task. Three CeA ChR2 rats met criteria for Low self-stimulation at the 24hr withdrawal timepoint (10 to 50 illuminations; twice as many contacts on laser rod as on inactive rod), and three CeA ChR2 rats also met Low self-stimulation criteria after a subsequent month of drug abstinence. The absence of any High self-stimulators may imply that LgA experience somewhat diminishes the incentive value of CeA ChR2 laser excitation by itself, but does not abolish it. That seems inconsistent with the hypothesis that CeA CRF neuronal excitation becomes highly aversive, due to its strengthening as a b-process in response to extensive cocaine experience. Further, it is important to recognize that CeA ChR2 laser self-stimulation is consistently weak in our lab's previous studies, and that many CeA ChR2 rats simply fail to meet self-stimulation CeA laser, even with little or no cocaine experience. Thus, the persistence of a few Low self-stimulators after extensive LgA experience may not be much reduced below non-drug levels of CeA ChR2 self-stimulation. This is bolstered by our findings that some rats will self-stimulate CeA CRF neurons both during withdrawal and after a period of abstinence.

This work further supports previous work from our lab and others demonstrating that ChR2 activation of CeA CRF neurons generates incentive motivation without distress, implicating CRF systems in reward pursuit (Baumgartner et al., 2021, 2022; Lemos et al., 2012; Merali et al., 1998; Peciña et al., 2006; Warlow et al., 2020). Our results provide evidence of flexibility in the valence of CeA CRF neuronal excitation following chronic cocaine self-administration; however, our results fail to support an aversive role for CeA CRF neurons both during withdrawal and after a period of extended abstinence despite previous work suggesting that CeA and CRF neurons are both capable of valence flips (Lemos et al., 2012; Warlow et al.,

2020). This stands in contrast to traditional opponent process views which implicate CeA CRF to be integral in generating distress and anxiety during withdrawal, leading to relapse as a mechanism of hedonic self-medication (Koob & Le Moal, 2008b; Zorrilla et al., 2014). Although valence flips in CRF signaling may occur following exposure to severe life events, such as extreme stress, a valence flip did not reliably occur in our experiment following extended drug exposure (Lemos et al., 2012; Warlow et al., 2020).

Given that CeA CRF activation increased general sucrose pursuit in Cocaine ChR2 rats following abstinence, and that this effect was specifically due to an increase in pursuit of the *Laser + sucrose* reward compared to the *Sucrose Alone,* our findings may highlight the need for even longer-term studies, as CeA CRF neurons may facilitate focused incentive motivation again beyond one month of abstinence. Alternatively, it is also possible that CeA CRF activation continues to facilitate incentive sensitization where any reward could become hyper-attractive, as for example is consistent with female high pursuit of both *Laser + Sucrose* and *Sucrose Alone* in the 2-choice task. If CRF CeA neurons are able to generate incentive motivation in the future, this finding would support incentive sensitization theories of addiction by providing a role for CRF, and potentially stress, can facilitate relapse via intensified positive incentive motivation rather than as drivers of distress and hedonic self-medication. Previous work has shown that 90 days after exposure to severe stress, CRF microinjections in the NAc were unable to facilitate dopamine signaling and generate a conditioned place preference as seen in drug naïve rats (Lemos et al., 2012). In males, we saw possible temporary reversal of the effects of drug exposure on the valence of CeA CRF activation after 24 hr; however this effect did not last following the one month abstinence period where CeA CRF activation once again became appetitive and total reward pursuit increased. Thus, long-term studies examining changes in

CRF-mediated reward seeking are necessary to determine how long these effects persist, fade away, or even continue to intensify.

*Individual Differences*

Although our results overall support diminished incentive motivation in withdrawal, there was 1 cocaine ChR2 rat who developed a laser preference and 3 who remained neutral in the two-choice task in withdrawal. However, after abstinence, 4 rats developed a laser preference while 1 remained neutral after abstinence, and 1 showed aversion, suggesting the possibility of individual differences in CRF signaling pathways. For example, individual differences in CRF-R1 receptors in the VTA have opposite effects on motivation, where low anxiety rats had higher CRF-R1 expression and DA firing compared to high anxiety rats (Zalachoras et al., 2022). Analyzing differences in receptor availability in animals who flipped compared to those who did not may give us more insight into receptor-based differences in these animals. Similarly, assessing baseline anxiety through behavioral tasks such as an open-field task in each rat prior to behavior and self-administration may also give us insight into whether or not high or low anxiety individuals are at a greater risk for developing a CeA CRF valence flip. Similarly, analyzing which rats are sign-trackers and which are goal-trackers may help us see which rats are more likely to have affective flip and which are not. Sign-trackers have been found to show greater sensitization following cocaine treatment, are particularly susceptible to cocaine cues, and are more likely to relapse in the presence of such cues; similarly, they have been found to be resistant to Pavlovian extinction (Flagel et al., 2008; Saunders & Robinson, 2011b) . Sign-trackers also show greater dopamine D1 receptor expression in comparison to goal-trackers(Flagel et al., 2007). Overall, since sign-trackers show greater propensity for addiction

and relapse, combined with evidence of molecular differences between sign- and goal-trackers, these phenotypes may also play a role in CeA CRF neuron valence.

**Limitations**

Even though ChR2 rats escalated their intake over the 14 LgA trials, we failed to see escalation in the number of first-hour infusions. A higher dose of cocaine could facilitate self-administration and a more intensive self-administration experience may be more likely to cause a valence flip. Additionally, our Cocaine ChR2, Cocaine eYFP, and Drug Naive ChR2 groups were unbalanced across sexes which prevented analysis of sex differences between groups.

**Future Research**

Given the changes in CeA CRF neuronal activation, it would be interesting to determine whether NAc CRF neuronal activation is also altered by LgA cocaine self-administration, especially considering that a CRF neuron valence flip has already been found following stress exposure (Lemos et al., 2012). It is also possible that CRF peptide signaling is not solely responsible for the changes seen in valence in CeA CRF neurons, as other neurotransmitters co-released by CRF neurons may mediate these effects. Additionally, further studies using global CeA activation, not specific to any particular cell-type, would help us understand if this effect is specific to CRF neurons or the CeA as a whole. Further assessing the molecular mechanisms to this valence flip would be beneficial as well to understanding what exactly is changing within the CeA to produce this affective flip, such as assessing any differences within CRF-R1 and R2 receptors.

Similarly, repeating this experiment with different drugs may help us look at valence changes more thoroughly. For example, our study focused on cocaine since much of the foundational opponent process theory work uses cocaine; however, opioids produce much more severe and longer lasting withdrawal symptoms than stimulants, so using drugs like opioids may also give interesting or different results.

## 5.5 Figures

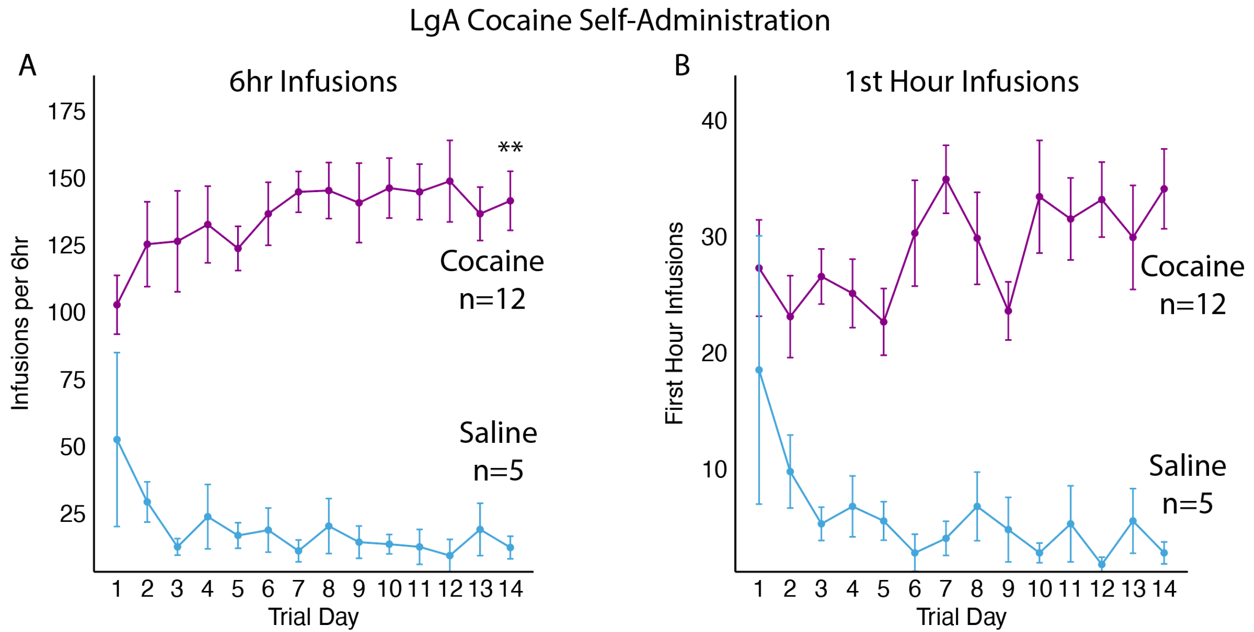
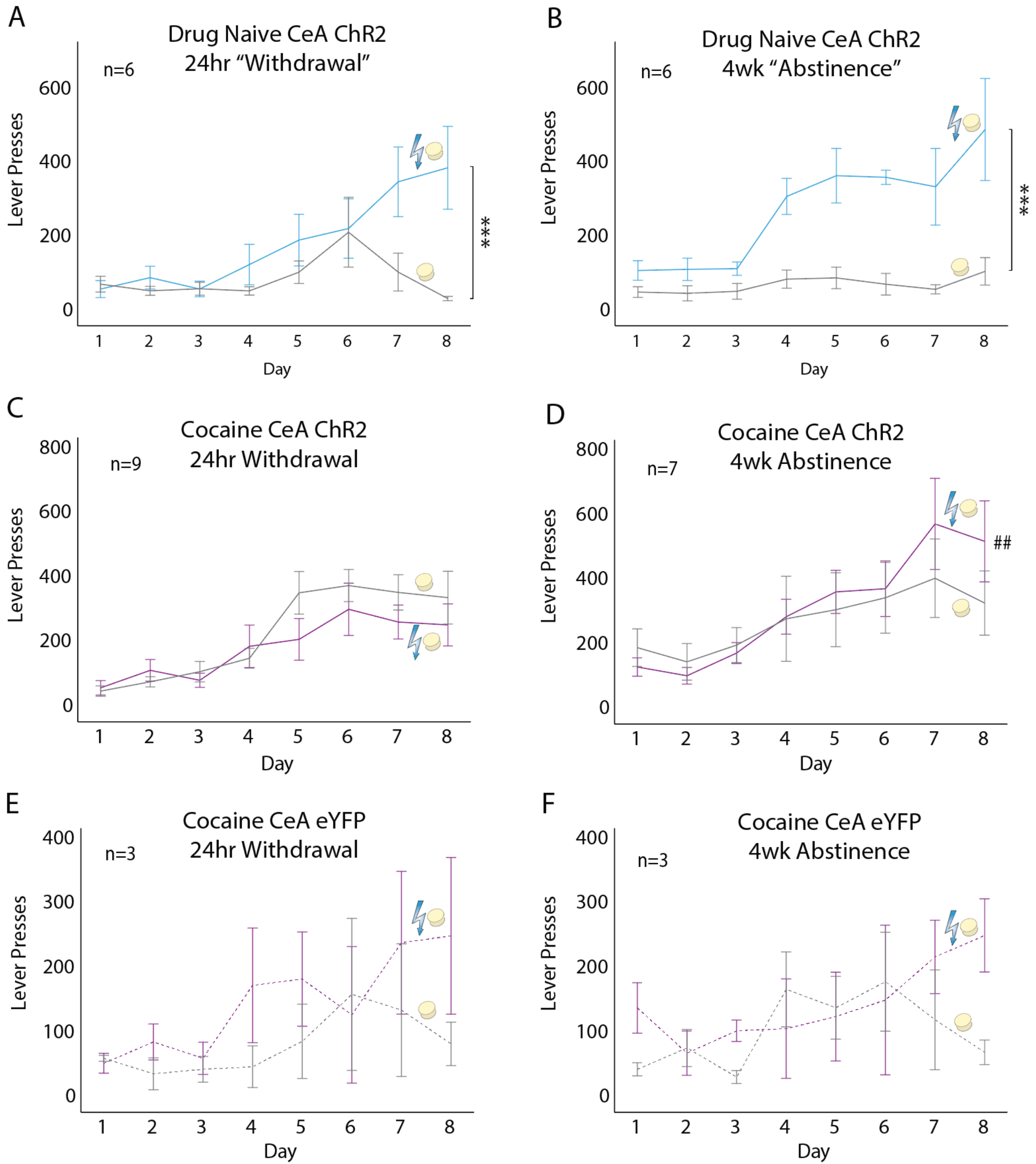



Figure 5-1 *Crh*-cre rats escalate cocaine intake over 14 days of 6hr LgA self-administration.

A) Mean (±SEM) number of cocaine infusions during each day of 6h LgA cocaine administration increases through day 14, where rats took significantly more cocaine than they did on the first day ($p<0.01$). In contrast, rats self-administering saline earn a similar or decreasing number of infusions over trial days as they did during day 1 ($p>0.05$). B) Cocaine infusions earned during the first hour of each day of 6-h LgA cocaine administration did not escalate across days of LgA sessions in rats self-administering cocaine ($p>0.05$). or saline ($p>0.05$).



Two-Choice Sucrose
A
Drug Naive CeA ChR2
24hr "Withdrawal"
n=6
Lever Presses
600
400
200
0
***
1 2 3 4 5 6 7 8
Day
B
Drug Naive CeA ChR2
4wk "Abstinence"
n=6
Lever Presses
600
400
200
0
***
1 2 3 4 5 6 7 8
Day
C
Cocaine CeA ChR2
24hr Withdrawal
n=9
Lever Presses
800
600
400
200
0
1 2 3 4 5 6 7 8
Day
D
Cocaine CeA ChR2
4wk Abstinence
n=7
Lever Presses
800
600
400
200
0
##
1 2 3 4 5 6 7 8
Day
E
Cocaine CeA eYFP
24hr Withdrawal
n=3
Lever Presses
400
300
200
100
0
1 2 3 4 5 6 7 8
Day
F
Cocaine CeA eYFP
4wk Abstinence
n=3
Lever Presses
400
300
200
100
0
1 2 3 4 5 6 7 8
Day

Figure 5-2 LgA cocaine self-administration prevents development of preference for laser-paired sucrose rewards

Shows instrumental 2-choie task where rats lever press on two different levers. Responses to one lever earn a sucrose reward (Sucrose Alone; FR1-RR6 Schedule) accompanied by a discrete 8s CS+ tone. Lever pressing into a second port located on the opposite side of the same wall earned an identical sucrose pellet paired with a different 8-s CS+ tone and additional blue laser stimulation (Sucrose + Laser; 3 mW; 10 Hz; FR1- RR6 Schedule). A-B) Drug-naïve CEA CRF ChR2 rats prefer Sucrose + laser (solid blue lines) over sucrose alone (solid grey lines) following an acute 24-h withdrawal period ($p < 0.001$; n = 6), and after a protracted 4-wk period of cocaine abstinence ($p < 0.001$; n = 6). C-D) Cocaine CeA ChR2 rats choose equally between sucrose alone (solid grey lines) and sucrose + laser (solid purple lines) when tested during the 24-h withdrawal period ($p > 0.05$; n = 9) and after 4 weeks of abstinence ($p > 0.05$; n = 7). Cocaine CeA ChR2 rats made more sucrose + laser responses after 4 weeks of abstinence relative to the number of sucrose + laser responses made during 24-h withdrawal (## $p < 0.01$). E-F) Cocaine eYFP control virus rats lacking the ChR2 gene respond equally between sucrose alone (dotted grey line) and sucrose + laser (dotted purple line) at both time points. All data presented as mean and SEM.

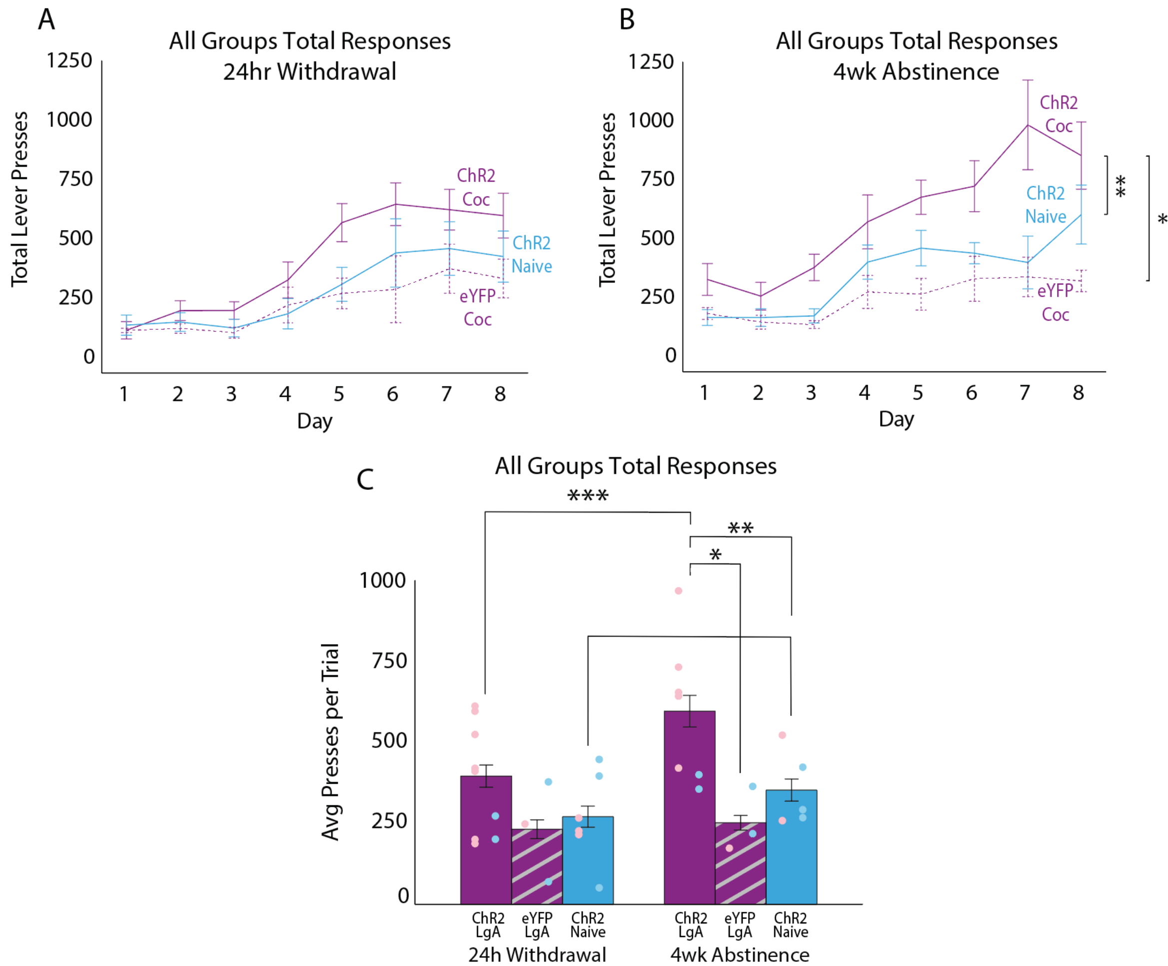



Figure 5-3 CRF neuronal activation generates incentive sensitization following LgA cocaine self-administration

Shows sum of responses made for Sucrose Alone and Sucrose + Laser options during instrumental sucrose 2-choice test. A) No differences in total number of responses made between drug naïve CeA ChR2 rats (blue solid line), cocaine ChR2 Rats (purple solid line), and cocaine eYFP control rats (dotted purple line) during the 24-h withdrawal period. B-C) When rats were retested in the instrumental sucrose 2-choice test, cocaine CeA ChR2 rats made significantly more total responses relative to drug naïve ChR2 rats ($p< 0.01$) and cocaine eYFP controls ($p < 0.5$). Cocaine ChR2 rats also made more total responses in the instrumental task after 4 weeks of abstinence relative to the 24-h withdrawal time point (*** $p < 0.001$). All data presented as means and SEM

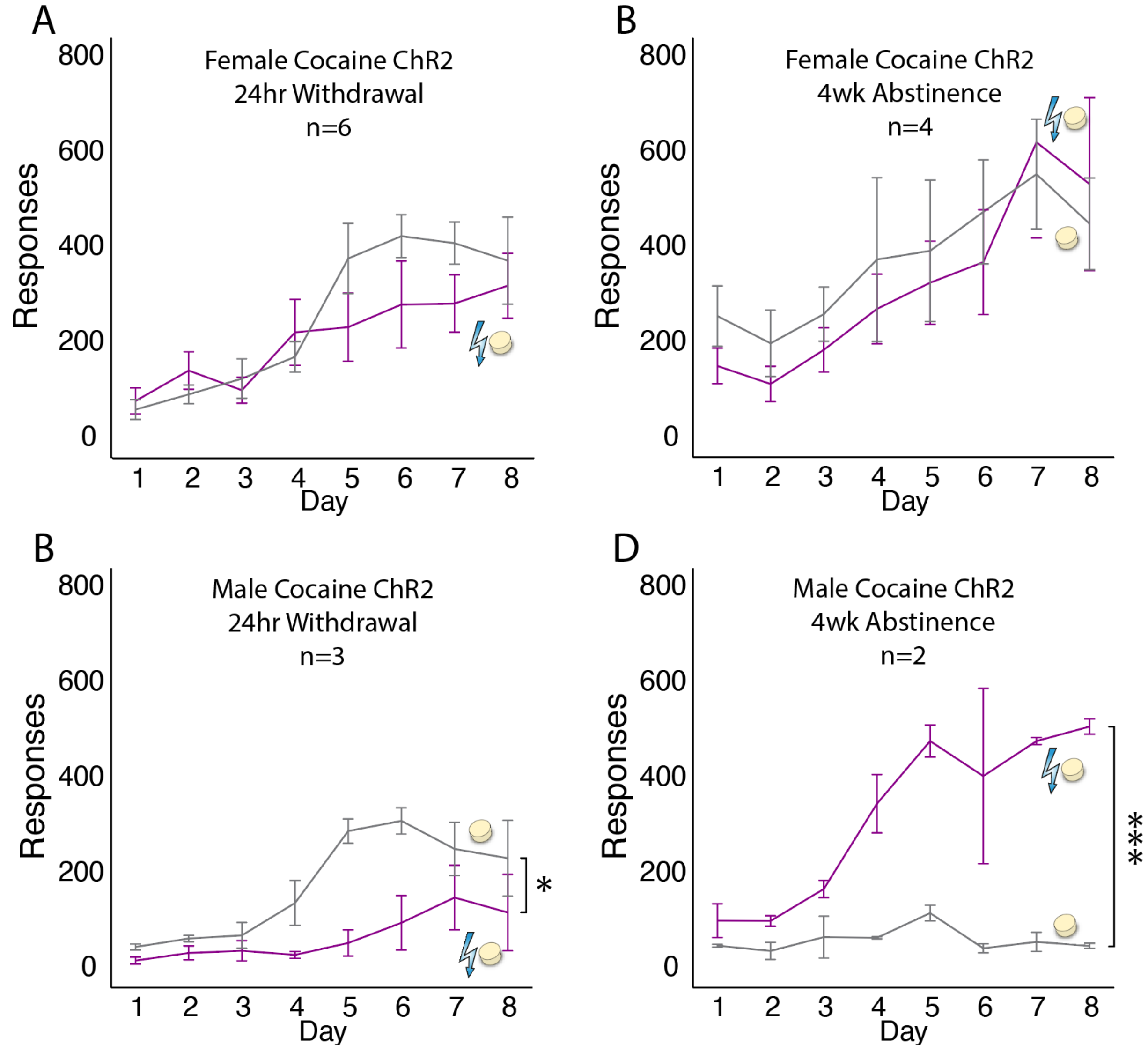



Figure 5-4 Males and females are differentially affected by activation of CeA CRF neurons following LgA cocaine self-administration.

Shows sex differences between male and female cocaine ChR2 rats during instrumental 2-choice task. A-B) Female cocaine ChR2 rats choose equally between sucrose alone (grey lines) and sucrose + laser (purple lines) at both time points tested. C) Male cocaine CeA ChR2 rats avoid sucrose + laser when tested during 24-h withdrawal period. D) When re-tested in the instrumental 2-Choice task after 4-weeks of cocaine abstinence, the motivational valence of activating CeA CRF neurons flips from positive to incentive. Male cocaine ChR2 rats prefer sucrose + laser over sucrose alone (*** $p < 0.001$).

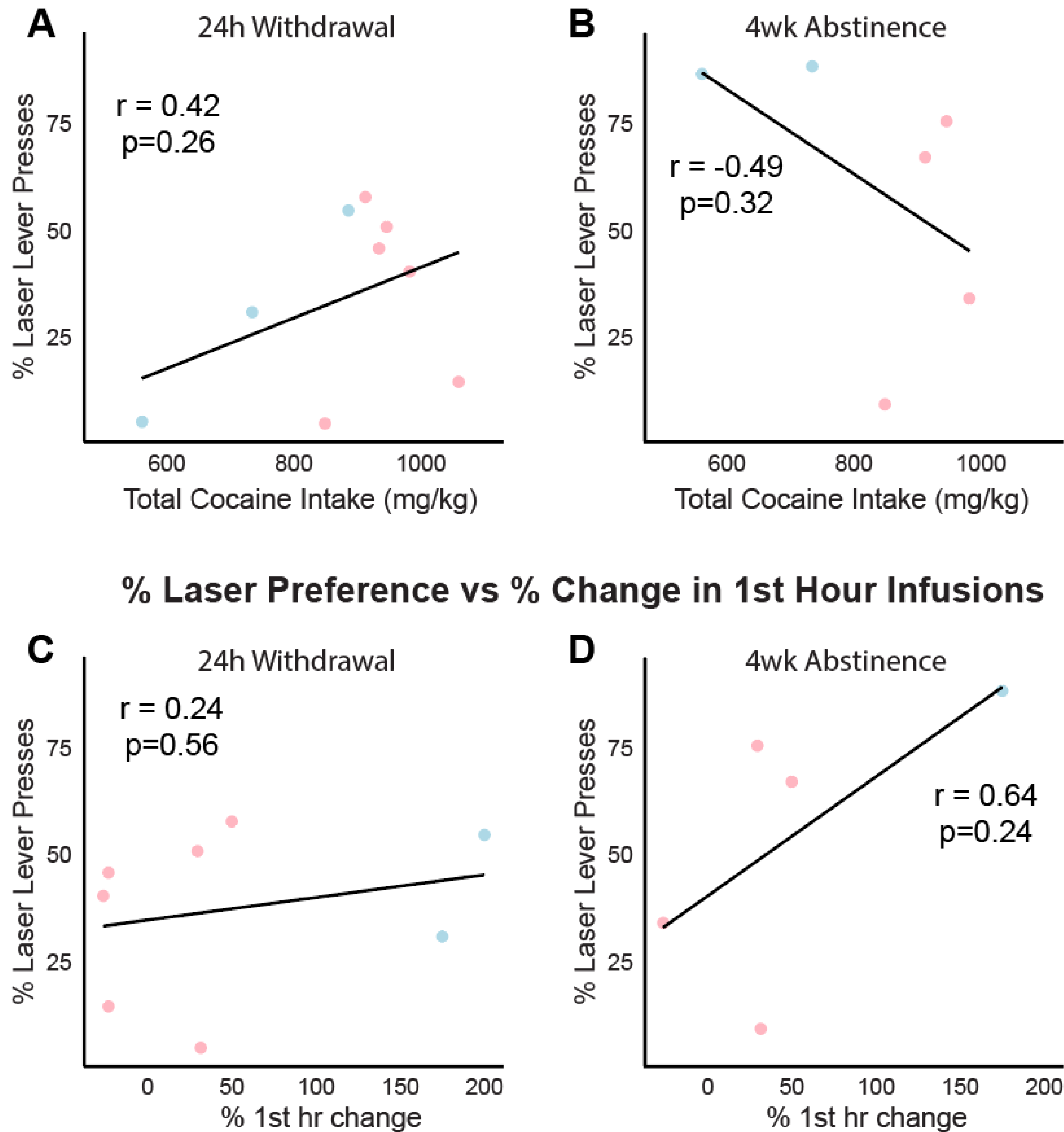
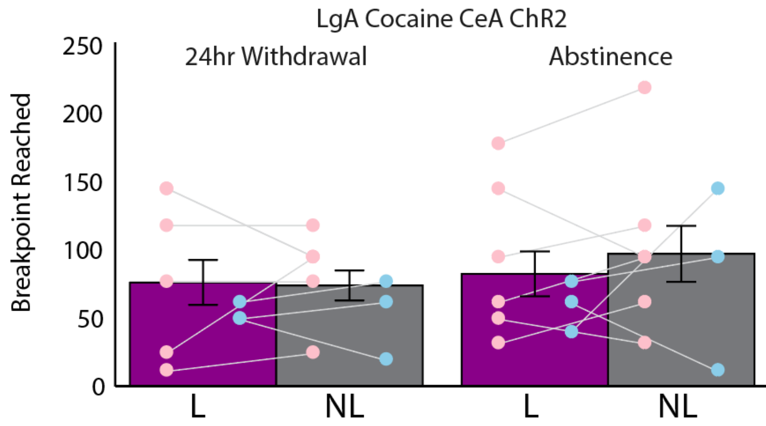
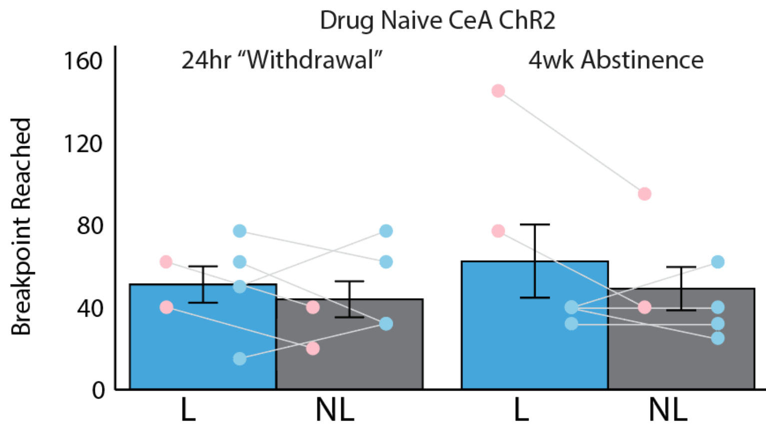



Figure 5-5 Neither total cocaine intake nor change in first hour infusions from self-administration day 1 to day 14 correlate with *Laser + Sucrose* preference in the two-choice task

A) There is no correlation between total cocaine intake during LgA self-administration and % preference for *Laser + Sucrose* during the 24hr withdrawal timepoint or B) after one month of abstinence. C) There is no correlation between escalation of first hour intake between day 1 and day 14 of LgA self-administration and *Laser + Sucrose* preference during withdrawal or D) following one month of abstinence. Mean±SEM. Pink = Female, Blue = Male.

## Progressive Ratio



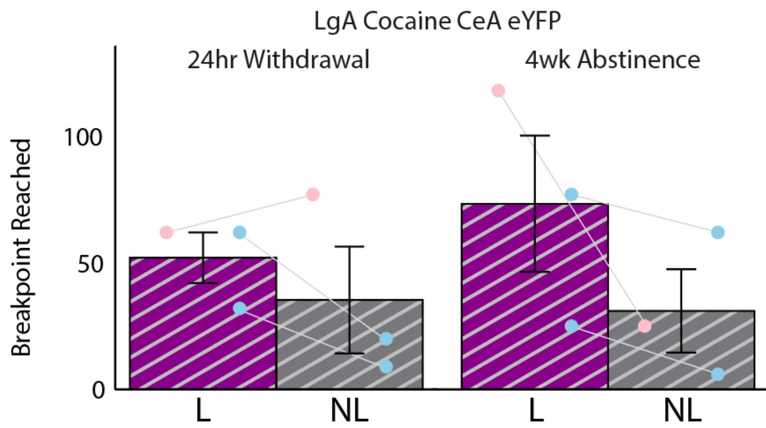



Figure 5-6 CeA CRF neuronal stimulation fails to alter breakpoint in all groups

We used a progressive ratio test of motivation on two consecutive days. On one day, rats responded for sucrose + laser and on the other day, rats responded for sucrose alone on a progressive ratio schedule of reinforcement so that the effort required to obtain the next sucrose reward increased exponentially. Cocaine ChR2 (top) rats reached similar breakpoints ($p > 0.05$) for sucrose + laser (purple bars) and sucrose alone (grey bars) during withdrawal and abstinence time periods. Similarly, drug naïve ChR2 rats (middle) and cocaine eYFP control rats (bottom) work equally for sucrose + laser (drug naïve ChR2: blue bars; cocaine eYFP; striped purple bars) and sucrose alone (drug naïve ChR2 rats: grey bars; cocaine eYFP controls: striped grey bars). Data presented as means and SEM.

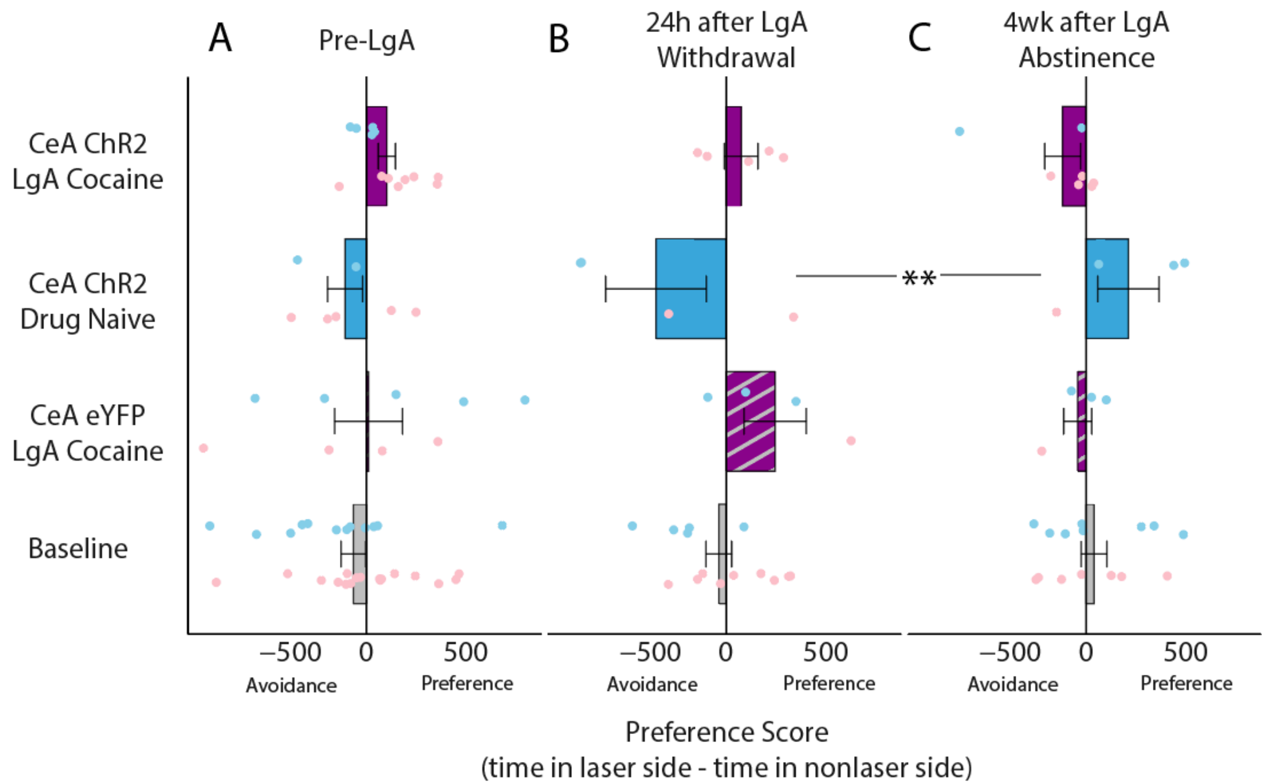



Figure 5-7 Activation of CeA CRF neurons fails to generate aversion in a place-based self-stimulation task following LgA cocaine self-administration

No self-stimulation in the passive place-based laser self-administration task where rats could earn laser stimulations (3 mW;10 Hz; 3-s ON/ 4-s OFF) by spending time in the laser-paired chamber while spending time in another chamber earned nothing. A) Neither cocaine CeA ChR2 rats, drug naïve ChR2 rats, nor cocaine eYFP controls self-stimulated in the place-based task prior when tested prior to LgA self-administration sessions. B) CeA ChR2 rats, drug naïve ChR2 rats, and cocaine eYFP controls fail to self-stimulate after 14 days of LgA sessions and C) after 4 weeks of abstinence. Drug naïve CeA ChR2 rats spend more time in laser-paired chamber after 4-weeks of abstinence compared to time spent in the laser-delivering chamber during the acute withdrawal period (** $p < 0.01$).

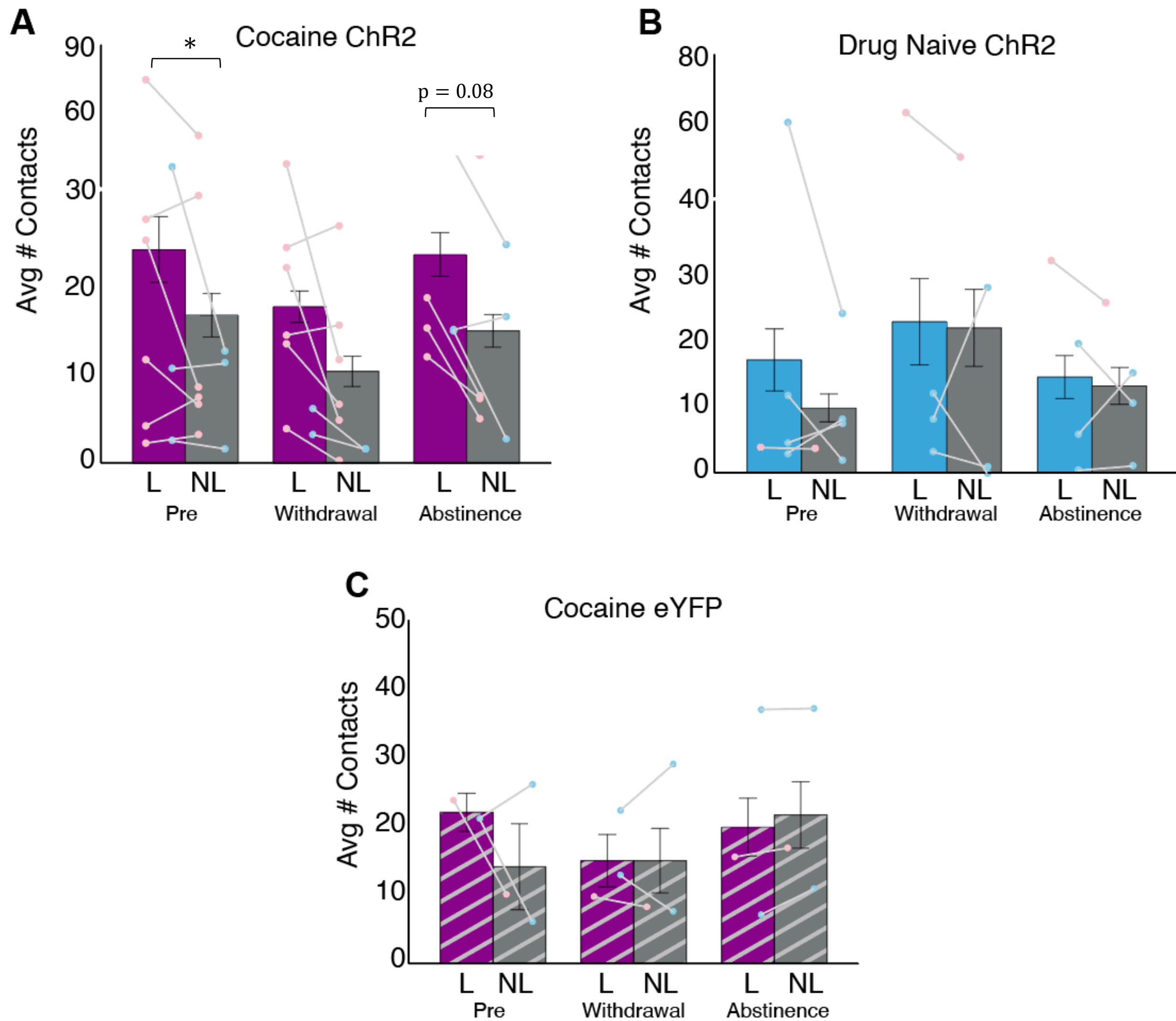



Figure 5-8 Cocaine ChR2 rats consistently make more contacts for laser self-stimulation than for the inactive nonlaser rod

Active rod-based laser self-administration task where touching a laser-paired rod earned laser stimulation (3 mW; 1-s constant illumination and 1-s 10 Hz) and touching a separate inactive rod earned nothing. A) Cocaine CeA ChR2 rats self-stimulated in the rod task (laser main effect: $F_{1,54.381}$=7.839, p = 0.02). Prior to cocaine access, cocaine CeA ChR2 rats made laser rod contacts relative to the control rod (*p < 0.05). Cocaine ChR2 rats failed to self-stimulated when tested during 24-h withdrawal period, and trended for self-stimulation when tested again after 4 weeks of abstinence. B) No rod self-stimulation in drug naïve ChR2 rats at any point tested C) cocaine eYFP control rats fail to self-stimulate for laser in the rod task at any time point tested.

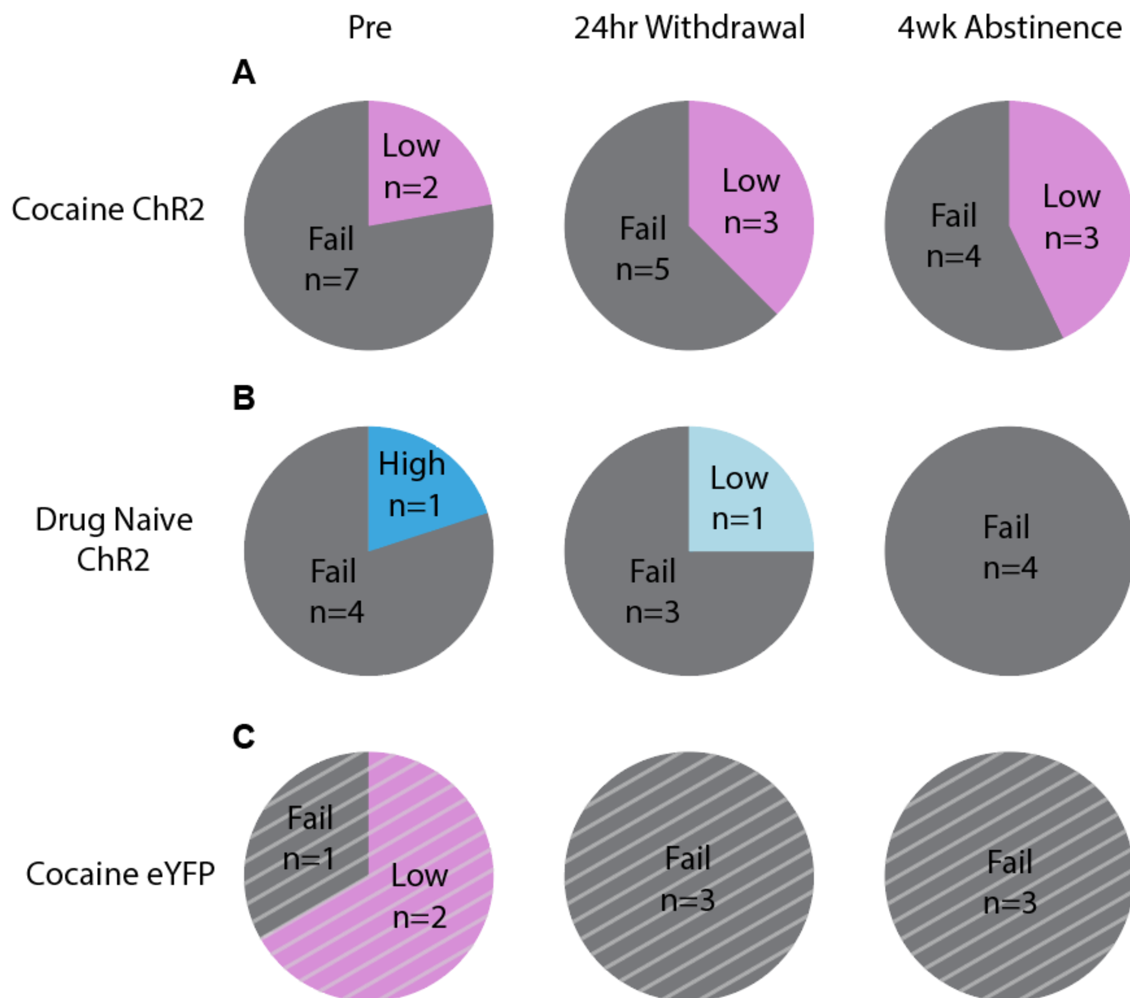



Figure 5-9 More Cocaine ChR2 rats meet self-stimulation criteria following LgA cocaine

In the rod-touch task, rats were categorized for their propensity to self-stimulate into one of three groups: high self-stimulators made > 50 laser-rod contacts and at least 2x as many laser contacts compared to non-laser rod contacts; low self-stimulators made >10 laser-rod contacts and at least 2x as many laser-rod contacts vs non-laser rod contacts. A) A subset of cocaine ChR2 rats self-stimulate in the rod task across the three time points tested. B) No consistent rod self-stimulation in drug naïve ChR2 rats or in C) eYFP controls at any time point tested.

## 5.6 Statistical Tables

Table 1. Drug Naive CeA ChR2 Two-Choice Sucrose Linear Mixed Model Summary

*Drug Naive CeA ChR2 Two-Choice Sucrose*

| Term | Estimate | Std. Error | t-value | df | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 339.979 | 38.189 | 8.903 | 27.987 | <0.001 | 261.752 | 418.207 |
| Sex | 3.631 | 23.327 | 0.156 | 4.184 | 0.884 | -60.026 | 67.288 |
| Timepoint | 134.452 | 46.013 | 2.922 | 161.539 | 0.004 | 43.587 | 225.317 |
| Laser | -199.090 | 44.624 | -4.461 | 161.208 | <0.001 | -287.213 | -110.966 |
| Day | 45.486 | 7.473 | 6.087 | 161.388 | <0.001 | 30.729 | 60.244 |
| Timepoint x Laser | -175.894 | 64.923 | -2.709 | 161.208 | 0.007 | -304.103 | -47.685 |
| Timepoint x Day | 9.197 | 10.926 | 0.842 | 161.293 | 0.401 | -12.380 | 30.774 |
| Laser x Day | -35.370 | 10.556 | -3.351 | 161.208 | 0.001 | -56.215 | -14.525 |
| Timepoint x Laser x Day | -13.261 | 15.443 | -0.859 | 161.208 | 0.392 | -43.757 | 17.236 |
| SD (Intercept) Animal | 52.493 | | | | | | |
| SD Observations | 115.507 | | | | | | |

*Drug Naive CeA ChR2 Two Choice Sucrose Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| Laser 24h - Nonlaser 24h | 73.871 | 23.830 | 161.011 | 3.100 | 0.014 |
| Laser 24h - Laser 4wk | -101.893 | 25.331 | 163.056 | -4.023 | <0.001 |
| Laser 24h - Nonlaser 4wk | 100.926 | 25.331 | 163.056 | 3.984 | <0.001 |
| Nonlaser 24h - Laser 4wk | -175.763 | 25.331 | 163.056 | -6.939 | <0.001 |
| Nonlaser 24h - Nonlaser 4wk | 27.055 | 25.331 | 163.056 | 1.068 | 1 |
| Laser 4wk - Nonlaser 4wk | 202.819 | 25.832 | 161.011 | 7.851 | <0.001 |

*Drug Naive CeA ChR2 Two-Choice Sucrose Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Sex | 323.259 | 323.259 | 1 | 4.184 | 0.024 | 0.884 |
| Timepoint | 27132.799 | 27132.799 | 1 | 161.853 | 2.034 | 0.156 |
| Laser | 1043173.154 | 1043173.154 | 1 | 161.208 | 78.188 | 0.000 |
| Day | 755435.532 | 755435.532 | 1 | 161.376 | 56.621 | 0.000 |
| Timepoint:Laser | 97931.702 | 97931.702 | 1 | 161.208 | 7.340 | 0.007 |
| Timepoint:Day | 1470.773 | 1470.773 | 1 | 161.376 | 0.110 | 0.740 |
| Laser:Day | 394754.911 | 394754.911 | 1 | 161.208 | 29.588 | 0.000 |
| Timepoint:Laser:Day | 9837.539 | 9837.539 | 1 | 161.208 | 0.737 | 0.392 |

Table 2. Cocaine CeA ChR2 Two-Choice Sucrose Linear Mixed Model Summary

*Cocaine CeA ChR2 Two-Choice Sucrose*

| Term | Estimate | Std. Error | t-value | df | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 240.831 | 38.580 | 6.242 | 84.233 | <0.001 | 164.113 | 317.549 |
| Sex | 71.919 | 26.224 | 2.742 | 21.667 | 0.012 | 17.485 | 126.353 |
| Timepoint | 310.910 | 53.465 | 5.815 | 250.945 | <0.001 | 205.613 | 416.207 |
| Laser | 156.133 | 48.574 | 3.214 | 245.220 | 0.001 | 60.457 | 251.810 |
| Day | 29.272 | 7.984 | 3.667 | 246.453 | <0.001 | 13.548 | 44.997 |
| Sex x Timepoint | -82.765 | 31.802 | -2.602 | 253.303 | 0.01 | -145.396 | -20.134 |
| Sex x Laser | -30.500 | 27.687 | -1.102 | 245.220 | 0.272 | -85.034 | 24.035 |
| Timepoint x Laser | -388.966 | 74.680 | -5.208 | 245.220 | <0.001 | -536.062 | -241.870 |
| Timepoint x Day | 39.182 | 12.242 | 3.201 | 245.755 | 0.002 | 15.070 | 63.293 |
| Laser x Day | 22.419 | 11.267 | 1.990 | 245.220 | 0.048 | 0.226 | 44.612 |
| Sex x Timepoint x Laser | 186.331 | 43.293 | 4.304 | 245.220 | <0.001 | 101.058 | 271.604 |
| Timepoint x Laser x Day | -58.376 | 17.297 | -3.375 | 245.220 | 0.001 | -92.446 | -24.306 |
| SD (Intercept) Animal | 51.117 | | | | | | |
| SD Observations | 159.119 | | | | | | |

*Cocaine CeA ChR2 Two-Choice Sucrose Estimated Marginal Means Pairwise Comparisons*

| Contrast | Sex | Laser | Timepoint | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|---|---|---|
| 24h - 4wk | | | | -81.112 | 23.678 | 237.615 | -3.426 | <0.001 |
| F - M | | | | 123.739 | 41.712 | 8.367 | 2.967 | 0.017 |
| Laser - Nonlaser | | | | 14.428 | 21.646 | 245.154 | 0.667 | 0.506 |
| Laser - Nonlaser | | | 24h | -76.909 | 27.683 | 245.154 | -2.778 | 0.006 |
| Laser - Nonlaser | | | 4wk | 105.765 | 33.285 | 245.154 | 3.178 | 0.002 |
| 24h - 4wk | F | Laser | | -89.683 | 35.729 | 243.802 | -2.510 | 0.013 |
| 24h - 4wk | M | Laser | | -255.213 | 53.292 | 253.946 | -4.789 | <0.001 |
| 24h - 4wk | F | Nonlaser | | -93.341 | 35.729 | 243.802 | -2.612 | 0.01 |
| 24h - 4wk | M | Nonlaser | | 113.791 | 53.292 | 253.946 | 2.135 | 0.034 |
| F - M | | Laser | 24h | 143.838 | 52.493 | 21.512 | 2.740 | 0.012 |
| F - M | | Laser | 4wk | -21.692 | 60.988 | 32.314 | -0.356 | 0.724 |
| F - M | | Nonlaser | 24h | 82.839 | 52.493 | 21.512 | 1.578 | 0.129 |
| F - M | | Nonlaser | 4wk | 289.971 | 60.988 | 32.314 | 4.755 | <0.001 |
| Laser - Nonlaser | F | NA | 24h | -46.409 | 30.915 | 245.154 | -1.501 | 0.135 |
| Laser - Nonlaser | F | NA | 4wk | -50.067 | 35.583 | 245.154 | -1.407 | 0.161 |
| Laser - Nonlaser | M | NA | 24h | -107.408 | 45.935 | 245.154 | -2.338 | 0.02 |
| Laser - Nonlaser | M | NA | 4wk | 261.596 | 56.259 | 245.154 | 4.650 | <0.001 |

*Cocaine CeA ChR2 Two-Choice Sucrose Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Sex | 224600.237 | 224600.237 | 1 | 8.431 | 8.871 | 0.017 |
| Timepoint | 234391.710 | 234391.710 | 1 | 253.686 | 9.258 | 0.003 |
| Laser | 26706.544 | 26706.544 | 1 | 245.220 | 1.055 | 0.305 |
| Day | 2790717.011 | 2790717.011 | 1 | 246.272 | 110.223 | 0.000 |
| Sex:Timepoint | 5045.282 | 5045.282 | 1 | 237.791 | 0.199 | 0.656 |
| Sex:Laser | 212195.367 | 212195.367 | 1 | 245.220 | 8.381 | 0.004 |
| Timepoint:Laser | 686847.759 | 686847.759 | 1 | 245.220 | 27.128 | 0.000 |
| Timepoint:Day | 33687.510 | 33687.510 | 1 | 246.272 | 1.331 | 0.250 |
| Laser:Day | 15511.044 | 15511.044 | 1 | 245.220 | 0.613 | 0.435 |
| Sex:Timepoint:Laser | 469010.843 | 469010.843 | 1 | 245.220 | 18.524 | 0.000 |
| Timepoint:Laser:Day | 288383.678 | 288383.678 | 1 | 245.220 | 11.390 | 0.001 |

Table 3 Cocaine eYFP Two Choice Sucrose Linear Mixed Model Summary

*Cocaine CeA eYFP Two-Choice Sucrose*

| Term | Estimate | Std. Error | t-value | df | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 232.483 | 87.576 | 2.655 | 1.355 | 0.173 | -381.035 | 846.001 |
| Sex | -29.389 | 48.372 | -0.608 | 86.000 | 0.545 | -125.549 | 66.771 |
| Timepoint | 23.609 | 99.403 | 0.238 | 1.000 | 0.852 | -1239.432 | 1286.651 |
| Laser | -120.306 | 48.372 | -2.487 | 86.000 | 0.015 | -216.465 | -24.146 |
| Day | 28.111 | 8.176 | 3.438 | 86.000 | 0.001 | 11.857 | 44.365 |
| Timepoint x Laser | 42.028 | 68.408 | 0.614 | 86.000 | 0.541 | -93.963 | 178.018 |
| Timepoint x Day | -7.956 | 11.563 | -0.688 | 86.000 | 0.493 | -30.943 | 15.030 |
| Laser x Day | -15.766 | 11.563 | -1.363 | 86.000 | 0.176 | -38.752 | 7.221 |
| Timepoint x Laser x Day | 5.341 | 16.353 | 0.327 | 86.000 | 0.745 | -27.167 | 37.849 |
| SD (Intercept) Animal | 79.524 | | | | | | |
| SD Observations | 91.779 | | | | | | |

*Cocaine CeA eYFP Two Choice Sucrose Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| Laser - Nonlaser | 53.458 | 18.734 | 86 | 2.854 | 0.005 |

*Cocaine CeA eYFP Two-Choice Sucrose Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Timepoint | 505.012 | 505.012 | 1 | 86 | 0.060 | 0.807 |
| Sex | 475.171 | 475.171 | 1 | 1 | 0.056 | 0.852 |
| Laser | 70983.612 | 70983.612 | 1 | 86 | 8.427 | 0.005 |
| Day | 155858.669 | 155858.669 | 1 | 86 | 18.503 | 0.000 |
| Timepoint:Laser | 3179.401 | 3179.401 | 1 | 86 | 0.377 | 0.541 |
| Timepoint:Day | 3520.286 | 3520.286 | 1 | 86 | 0.418 | 0.520 |
| Laser:Day | 21607.143 | 21607.143 | 1 | 86 | 2.565 | 0.113 |
| Timepoint:Laser:Day | 898.669 | 898.669 | 1 | 86 | 0.107 | 0.745 |

Table 4 Combined Groups Two Choice Sucrose Total Responses Linear Mixed Model Summary

*All Groups Total Responses Two-Choice Sucrose*

| Term | Estimate | Std. Error | t-value | df | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 632.994 | 52.212 | 12.124 | 21.822 | <0.001 | 524.663 | 741.326 |
| Sex | 52.189 | 31.494 | 1.657 | 14.732 | 0.119 | -15.045 | 119.423 |
| Timepoint | 203.153 | 48.495 | 4.189 | 249.972 | <0.001 | 107.642 | 298.664 |
| Group | -239.459 | 113.216 | -2.115 | 23.283 | 0.045 | -473.506 | -5.412 |
| Drug | -96.140 | 68.957 | -1.394 | 14.075 | 0.185 | -243.964 | 51.684 |
| Day | 71.169 | 6.681 | 10.652 | 242.438 | <0.001 | 58.008 | 84.329 |
| Timepoint x Drug | -80.794 | 50.108 | -1.612 | 253.290 | 0.108 | -179.474 | 17.887 |
| Timepoint x Group | -219.903 | 100.973 | -2.178 | 243.899 | 0.03 | -418.793 | -21.013 |
| Timepoint x Day | 13.026 | 10.033 | 1.298 | 241.873 | 0.195 | -6.738 | 32.789 |
| Group x Day | -30.712 | 16.393 | -1.873 | 241.589 | 0.062 | -63.005 | 1.580 |
| Timepoint x Group x Day | -23.597 | 23.428 | -1.007 | 241.501 | 0.315 | -69.747 | 22.552 |
| SD (Intercept) Animal | 4.264 | | | | | | |
| | 131.535 | | | | | | |
| Cor (Intercept) Drug, Animal | -0.885 | | | | | | |
| SD Drug | 26.226 | | | | | | |
| SD Observations | 168.040 | | | | | | |

*Total Responses Estimated Marginal Means Pairwise Comparisons*

| Contrast | Timepoint | Group | Estimate | SE | df | t-value | p-value | Drug |
|---|---|---|---|---|---|---|---|---|
| Cocaine - Saline | 24h | ChR2 | 96.140 | 69.443 | 13.756 | 1.384 | 0.188 | NA |
| Cocaine - Saline | 4wk | ChR2 | 176.934 | 73.419 | 16.697 | 2.410 | 0.028 | NA |
| 24h - 4wk | NA | ChR2 | -157.175 | 33.808 | 251.583 | -4.649 | <0.001 | Cocaine |
| 24h - 4wk | NA | ChR2 | -76.381 | 37.479 | 245.656 | -2.038 | 0.043 | Saline |
| 24h - 4wk | NA | eYFP | -20.566 | 48.513 | 241.046 | -0.424 | 0.672 | Cocaine |
| ChR2 - eYFP | 24h | NA | 131.049 | 98.581 | 13.029 | 1.329 | 0.207 | Cocaine |
| ChR2 - eYFP | 4wk | NA | 267.658 | 100.154 | 13.766 | 2.672 | 0.018 | Cocaine |

*Total Responses Two-Choice Sucrose Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Sex | 77542.114 | 77542.114 | 1 | 14.732 | 2.746 | 0.119 |
| Timepoint | 98619.040 | 98619.040 | 1 | 242.480 | 3.493 | 0.063 |
| Group | 328852.806 | 328852.806 | 1 | 15.689 | 11.646 | 0.004 |
| Drug | 119769.589 | 119769.589 | 1 | 12.002 | 4.242 | 0.062 |
| Day | 2620781.923 | 2620781.923 | 1 | 241.501 | 92.813 | 0.000 |
| Timepoint:Drug | 73412.397 | 73412.397 | 1 | 253.290 | 2.600 | 0.108 |
| Timepoint:Group | 133930.081 | 133930.081 | 1 | 243.899 | 4.743 | 0.030 |

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Timepoint:Day | 309.858 | 309.858 | 1 | 241.501 | 0.011 | 0.917 |
| Group:Day | 371888.727 | 371888.727 | 1 | 241.501 | 13.170 | 0.000 |
| Timepoint:Group:Day | 28646.073 | 28646.073 | 1 | 241.501 | 1.014 | 0.315 |
| Group:Drug | | | | | | |
| Timepoint:Group:Drug | | | | | | |

*Progressive Ratio*

Table 5 Progressive Ratio Breakpoint Linear Mixed Model Summary

| Term | Estimate | Std. Error | t-value | df | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 66.990 | 12.639 | 5.300 | 36.011 | <0.001 | 41.358 | 92.622 |
| Sex | 14.550 | 7.368 | 1.975 | 15.323 | 0.067 | -1.127 | 30.227 |
| Timepoint | 9.420 | 13.497 | 0.698 | 46.914 | 0.489 | -17.733 | 36.573 |
| Laser | 2.700 | 13.024 | 0.207 | 46.242 | 0.837 | -23.513 | 28.913 |
| Drug | -11.140 | 20.872 | -0.534 | 35.316 | 0.597 | -53.498 | 31.218 |
| Group | -10.140 | 26.424 | -0.384 | 35.739 | 0.703 | -63.744 | 43.464 |
| Laser x Drug Naive | -9.867 | 21.268 | -0.464 | 46.242 | 0.645 | -52.672 | 32.939 |
| Laser x Drug Cocaine eYFP | -19.367 | 27.112 | -0.714 | 46.242 | 0.479 | -73.933 | 35.199 |
| Drug Naive ChR2 x Timepoint | 1.913 | 21.561 | 0.089 | 46.506 | 0.93 | -41.474 | 45.301 |
| Cocaine eYFP x Timepoint | 11.913 | 27.342 | 0.436 | 46.406 | 0.665 | -43.111 | 66.937 |
| Laser x Timepoint | 11.967 | 18.924 | 0.632 | 46.242 | 0.53 | -26.120 | 50.053 |
| Timepoint x Laser x Drug Naive | -18.133 | 30.390 | -0.597 | 46.242 | 0.554 | -79.296 | 43.030 |
| Timepoint x Laser x eYFP | -37.633 | 38.587 | -0.975 | 46.242 | 0.334 | -115.295 | 40.028 |
| SD (Intercept) Animal | 26.973 | | | | | | |
| SD Observations | 29.123 | | | | | | |

*Progressive Ratio Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| F - M | 29.1 | 14.743 | 15.105 | 1.974 | 0.067 |

*Progressive Ratio Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Sex | 3307.164 | 3307.164 | 1 | 15.323 | 3.899 | 0.067 |
| Timepoint | 1696.991 | 1696.991 | 1 | 46.479 | 2.001 | 0.164 |
| Laser | 1593.806 | 1593.806 | 1 | 46.242 | 1.879 | 0.177 |
| Drug | 241.621 | 241.621 | 1 | 35.316 | 0.285 | 0.597 |
| Group | 1078.657 | 1078.657 | 1 | 15.115 | 1.272 | 0.277 |
| Laser x Group | 3707.075 | 1853.538 | 2 | 46.242 | 2.185 | 0.124 |
| Timepoint x Group | 226.992 | 113.496 | 2 | 46.569 | 0.134 | 0.875 |
| Timepoint x Laser | 162.943 | 162.943 | 1 | 46.242 | 0.192 | 0.663 |
| Timepoint x Laser:Group | 890.280 | 445.140 | 2 | 46.242 | 0.525 | 0.595 |

Table 6 Place-based Self Stimulation Linear Mixed Model Summary

*Place-based Self Stimulation*

| Term | Estimate | Std. Error | df | t-value | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | -19.180 | 65.836 | 76.512 | -0.291 | 0.772 | -150.290 | 111.930 |
| Withdrawal | -35.116 | 98.141 | 76.330 | -0.358 | 0.721 | -230.567 | 160.334 |
| Abstinence | 55.755 | 94.612 | 87.153 | 0.589 | 0.557 | -132.292 | 243.802 |
| Cocaine eYFP | 78.083 | 114.882 | 83.499 | 0.680 | 0.499 | -150.393 | 306.559 |
| Drug Naive ChR2 | -47.384 | 136.832 | 86.171 | -0.346 | 0.730 | -319.391 | 224.622 |
| Cocaine ChR2 | 116.428 | 98.684 | 79.034 | 1.180 | 0.242 | -79.997 | 312.853 |
| Withdrawal x eYFP | 312.069 | 196.446 | 70.501 | 1.589 | 0.117 | -79.680 | 703.819 |
| Abstinence x eYFP | -94.677 | 195.196 | 72.890 | -0.485 | 0.629 | -483.711 | 294.357 |
| Withdrawal x Drug Naive ChR2 | -161.417 | 223.871 | 71.087 | -0.721 | 0.473 | -607.793 | 284.960 |
| Abstinence x Drug Naive ChR2 | 303.666 | 207.917 | 75.153 | 1.461 | 0.148 | -110.511 | 717.843 |
| Withdrawal x Cocaine ChR2 | -276.920 | 169.214 | 69.280 | -1.637 | 0.106 | -614.469 | 60.628 |
| Abstinence x Cocaine ChR2 | -339.178 | 156.869 | 72.508 | -2.162 | 0.034 | -651.853 | -26.502 |
| SD (Intercept) Animal | 215.509 | NA | NA | NA | NA | NA | NA |
| SD Observations | 279.326 | NA | NA | NA | NA | NA | NA |

*Place-based Self Stimulation Estimated Marginal Means Pairwise Comparisons*

| Contrast | Bar | Timepoint | Estimate | SE | df | p-value | t-value |
|---|---|---|---|---|---|---|---|
| Baseline - eYFP | | | -150.547 | 94.899 | 90.505 | 0.697 | -1.586 |
| Baseline - Sal | | | -0.032 | 104.601 | 92.880 | 1.000 | 0.000 |
| eYFP - Sal | | | 150.515 | 137.090 | 98.473 | 1.000 | 1.098 |
| Pre - Acute | Baseline | | 35.116 | 98.614 | 80.219 | 1.000 | 0.356 |
| Pre - Prolonged | Baseline | | -55.755 | 95.353 | 89.667 | 1.000 | -0.585 |
| Acute - Prolonged | Baseline | | -90.872 | 108.161 | 78.333 | 1.000 | -0.840 |
| Pre - Acute | eYFP | | -276.953 | 176.058 | 79.048 | 0.359 | -1.573 |
| Pre - Prolonged | eYFP | | 38.922 | 176.058 | 79.048 | 1.000 | 0.221 |
| Acute - Prolonged | eYFP | | 315.875 | 197.513 | 71.167 | 0.343 | 1.599 |
| Pre - Acute | Sal | | 196.533 | 205.837 | 78.070 | 1.000 | 0.955 |
| Pre - Prolonged | Sal | | -359.421 | 191.838 | 83.346 | 0.193 | -1.874 |
| Acute - Prolonged | Sal | | -555.954 | 217.543 | 74.640 | 0.038 | -2.556 |
| Pre - Acute | Coc | | 312.037 | 144.481 | 79.138 | 0.101 | 2.160 |
| Pre - Prolonged | Coc | | 283.422 | 139.051 | 92.334 | 0.133 | 2.038 |
| Acute - Prolonged | Coc | | -28.615 | 160.264 | 82.481 | 1.000 | -0.179 |
| Baseline - eYFP | | Pre | -78.083 | 115.724 | 86.522 | 1.000 | -0.675 |
| Baseline - Sal | | Pre | 47.384 | 138.016 | 88.826 | 1.000 | 0.343 |
| Baseline - Coc | | Pre | -116.428 | 99.219 | 82.617 | 1.000 | -1.173 |
| eYFP - Sal | | Pre | 125.467 | 169.229 | 95.305 | 1.000 | 0.741 |
| eYFP - Coc | | Pre | -38.345 | 139.739 | 96.112 | 1.000 | -0.274 |

| Contrast | Bar | Timepoint | Estimate | SE | df | p-value | t-value |
|---|---|---|---|---|---|---|---|
| Sal - Coc | | Pre | -163.812 | 158.433 | 95.268 | 1.000 | -1.034 |
| Baseline - eYFP | | Acute | -390.152 | 168.530 | 78.304 | 0.139 | -2.315 |
| Baseline - Sal | | Acute | 208.801 | 190.293 | 79.239 | 1.000 | 1.097 |
| Baseline - Coc | | Acute | 160.493 | 143.255 | 76.422 | 1.000 | 1.120 |
| eYFP - Sal | | Acute | 598.953 | 234.675 | 83.175 | 0.075 | 2.552 |
| eYFP - Coc | | Acute | 550.645 | 198.872 | 83.819 | 0.042 | 2.769 |
| Sal - Coc | | Acute | -48.308 | 217.464 | 83.519 | 1.000 | -0.222 |
| Baseline - eYFP | | Prolonged | 16.594 | 166.817 | 81.076 | 1.000 | 0.099 |
| Baseline - Sal | | Prolonged | -256.282 | 168.045 | 83.349 | 0.786 | -1.525 |
| Baseline - Coc | | Prolonged | 222.750 | 126.321 | 78.919 | 0.490 | 1.763 |
| eYFP - Sal | | Prolonged | -272.876 | 219.786 | 86.509 | 1.000 | -1.242 |
| eYFP - Coc | | Prolonged | 206.156 | 191.004 | 87.843 | 1.000 | 1.079 |
| Sal - Coc | | Prolonged | 479.031 | 193.143 | 90.859 | 0.090 | 2.480 |

*Place-based Self Stimulation Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Timepoint | 86097.71 | 43048.85 | 2 | 82.123 | 0.552 | 0.578 |
| Group | 346440.26 | 115480.09 | 3 | 88.595 | 1.480 | 0.225 |
| Timepoint x Group | 1294378.18 | 215729.70 | 6 | 72.125 | 2.765 | 0.018 |

Table 7 Drug Naive CeA ChR2 Touch-based Self Stimulation Linear Mixed Model Summary

*Drug Naive ChR2 Touch-based Self Stimulation*

| Term | Estimate | Std. Error | df | t-value | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 20.943 | 4.819 | 14.916 | 4.346 | 0.001 | 10.667 | 31.219 |
| Laser | -5.978 | 3.520 | 18.008 | -1.698 | 0.107 | -13.373 | 1.418 |
| Withdrawal | 15.981 | 4.990 | 243.090 | 3.202 | 0.002 | 6.151 | 25.810 |
| Abstinence | 7.898 | 4.835 | 237.618 | 1.633 | 0.104 | -1.628 | 17.423 |
| 1s Constant Stim | -2.681 | 1.044 | 238.926 | -2.568 | 0.011 | -4.737 | -0.625 |
| Sex | 1.203 | 4.108 | 13.667 | 0.293 | 0.774 | -7.628 | 10.034 |
| Withdrawal x Laser | 0.477 | 6.650 | 166.401 | 0.072 | 0.943 | -12.653 | 13.607 |
| Abstinence x Laser | 0.910 | 6.389 | 147.929 | 0.143 | 0.887 | -11.714 | 13.535 |
| SD (Intercept) Animal | 0.797 | | | | | | |
| SD (Intercept) Laser | 17.773 | | | | | | |
| Cor. (Intercept) Laser | -0.502 | | | | | | |
| SD Laser | 9.838 | | | | | | |
| SD Observations | 17.126 | | | | | | |

*Drug Naive ChR2 Touch-based Self Stimulation Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| Laser - Nonlaser | 7.483 | 2.747 | 11.801 | 2.725 | 0.019 |

*Drug Naive ChR2 Touch-based Self Stimulation Type III Tests*

| Source | Sum.Sq. | Mean.Sq. | ndf | ddf | F.value | P.value |
|---|---|---|---|---|---|---|
| Laser | 537.930 | 537.930 | 1 | 17.575 | 1.834 | 0.193 |
| Timepoint | 5743.853 | 2871.926 | 2 | 245.495 | 9.792 | 0.000 |
| Parameter | 1934.689 | 1934.689 | 1 | 238.926 | 6.596 | 0.011 |
| Sex | 25.134 | 25.134 | 1 | 13.667 | 0.086 | 0.774 |
| Laser x Timepoint | 6.064 | 3.032 | 2 | 142.008 | 0.010 | 0.990 |

Table 8 Cocaine CeA ChR2 Touch-based Self Stimulation Linear Mixed Model Summary

*Cocaine ChR2 Touch-based Self Stimulation*

| Term | Estimate | Std. Error | df | t-value | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 28.152 | 5.693 | 30.635 | 4.945 | <0.001 | 16.536 | 39.767 |
| Laser | -8.230 | 3.737 | 161.546 | -2.202 | 0.029 | -15.609 | -0.850 |
| Withdrawal | -7.730 | 4.508 | 321.990 | -1.715 | 0.087 | -16.599 | 1.139 |
| Abstinence | 0.757 | 4.601 | 303.108 | 0.164 | 0.869 | -8.298 | 9.811 |
| 1s Constant Stim | -0.951 | 6.962 | 206.735 | -0.137 | 0.891 | -14.676 | 12.774 |
| 1s 10Hz Stim | 1.417 | 3.846 | 220.883 | 0.368 | 0.713 | -6.162 | 8.996 |
| Sex | -2.469 | 3.899 | 14.136 | -0.633 | 0.537 | -10.824 | 5.887 |
| Withdrawal x Laser | 2.941 | 6.025 | 314.694 | 0.488 | 0.626 | -8.913 | 14.796 |
| Abstinence x Laser | -0.703 | 5.933 | 318.717 | -0.118 | 0.906 | -12.376 | 10.970 |
| SD (Intercept) Animal | 0.001 | | | | | | |
| SD (Intercept) Laser | 16.309 | | | | | | |
| Cor. (Intercept) Laser | -1.000 | | | | | | |
| SD Laser | 3.073 | | | | | | |
| SD Observations | 22.815 | | | | | | |

*Cocaine ChR2 Touch-based Self Stimulation Type III Tests*

| Source | Sum Sq. | Mean Sq. | ndf | ddf | F-value | P-value |
|---|---|---|---|---|---|---|
| Laser | 4108.278 | 4108.278 | 1 | 54.381 | 7.893 | 0.007 |
| Timepoint | 2404.181 | 1202.090 | 2 | 331.581 | 2.309 | 0.101 |
| Parameter | 271.711 | 135.855 | 2 | 252.750 | 0.261 | 0.770 |
| Sex | 208.638 | 208.638 | 1 | 14.136 | 0.401 | 0.537 |
| Laser x Timepoint | 179.654 | 89.827 | 2 | 317.780 | 0.173 | 0.842 |

Table 9 Cocaine CeA eYFP Touch-based Self Stimulation Linear Mixed Model Summary

*Cocaine ChR2 Touch-based Self Stimulation Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| Laser - Nonlaser | 7.483 | 2.747 | 11.801 | 2.725 | 0.019 |

*Cocaine eYFP Touch-based Self Stimulation*

| Term | Estimate | Std. Error | df | t-value | p-value | CI Lower | CI Upper |
|---|---|---|---|---|---|---|---|
| Intercept | 20.350 | 3.966 | 10.613 | 5.131 | <0.001 | 11.581 | 29.119 |
| Laser | -0.633 | 4.299 | 34.437 | -0.147 | 0.884 | -9.365 | 8.098 |
| Withdrawal | -6.123 | 5.676 | 171.721 | -1.079 | 0.282 | -17.327 | 5.080 |
| Abstinence | -4.373 | 5.676 | 171.721 | -0.770 | 0.442 | -15.577 | 6.830 |
| 1s Constant Stim | 0.394 | 1.512 | 198.956 | 0.260 | 0.795 | -2.589 | 3.376 |
| Sex | -3.009 | 3.595 | 6.856 | -0.837 | 0.431 | -11.545 | 5.528 |
| Withdrawal x Laser | -0.709 | 7.660 | 184.258 | -0.093 | 0.926 | -15.821 | 14.403 |
| Abstinence x Laser | 2.416 | 7.660 | 184.258 | 0.315 | 0.753 | -12.696 | 17.528 |
| SD (Intercept) Animal | 0.000 | | | | | | |
| SD (Intercept) Laser | 8.660 | | | | | | |
| Cor. (Intercept) Laser | 1.000 | | | | | | |
| SD Laser | 4.484 | | | | | | |
| SD Observations | 22.227 | | | | | | |

*Cocaine eYFP Touch-based Self Stimulation Estimated Marginal Means Pairwise Comparisons*

| Contrast | Estimate | SE | df | t-value | p-value |
|---|---|---|---|---|---|
| Laser - Nonlaser | 7.483 | 2.747 | 11.801 | 2.725 | 0.019 |

*Cocaine eYFP Touch-based Self Stimulation Type III Tests*

| Source | Sum.Sq. | Mean.Sq. | ndf | ddf | F.value | P.value |
|---|---|---|---|---|---|---|
| Laser | 0.152 | 0.152 | 1 | 13.680 | 0.000 | 0.986 |
| Timepoint | 1085.862 | 542.931 | 2 | 190.653 | 1.099 | 0.335 |
| Parameter | 33.449 | 33.449 | 1 | 198.956 | 0.068 | 0.795 |
| Sex | 346.049 | 346.049 | 1 | 6.856 | 0.700 | 0.431 |
| Laser x Timepoint | 68.039 | 34.020 | 2 | 179.556 | 0.069 | 0.933 |

# Chapter 6 General Discussion

*Summary*

Despite their conventional role in stress, distress, and anxiety, limbic CRF systems are also able to trigger reward seeking by generating and focusing positive incentive motivation without anxiety. Understanding the paradoxical role for CRF systems in both stress and incentive motivation will provide important perspective on addiction where both negative stressors, such as pain, or happy stressors, such as celebrating the birth of a child, can trigger relapse even after extensive periods of abstinence (Ferreira, Zerwes et al., 2016; Hodgins et al., 1995; Hodgins & el-Guebaly, 2004; McKay et al., 1995; Shiftman et al., 1985; Walitzer & Dearing, 2006). Additionally, dysregulation of both positive incentive and traditional stress CRF systems may contribute to stress associated affective disorders like depression, which is characterized by loss of motivation. This dissertation provides further evidence for an incentive role for limbic CRF systems in addiction and stress-related volitional disorders.

<u>CRF Receptor Activation in CRF neuron generated incentive motivation</u>

Given the traditional role for CRF as a trigger of the HPA axis, anxiety, and distress, CRF is has been posited by opponent process theories of addiction as the cause of malaise and anxiety during withdrawal that acts as a negative reinforcer to drive drug consumption as a means of hedonic self-medication. However, recent evidence has shown that optogenetic activation of CeA and NAc CRF neurons generate incentive motivation without distress. Since CRF neurons do not singularly express CRF, it is possible that co-release of other

neurotransmitters from these neurons are responsible for generating appetitive motivation. By blocking CRF receptors globally in the brain via intracerebroventricular administration of a CRF antagonist, D-Phe-CRF$_{(12\text{-}41)}$, prior to behavioral assessments of motivation. We show, for the first time, that optogenetic activation of CeA and NAc CRF neurons requires activation of CRF receptors to generate incentive motivation.

These findings replicate previous work by our lab which found that optogenetic laser stimulation of CRF neurons in the CeA and NAc of *crh*-Cre rats intensifies and focuses pursuit of a laser-paired sucrose or cocaine reward over an equal reward without laser stimulation, and also supports laser self-stimulation of CRF neurons indicating positive valence of CRF neuronal excitation in CeA and NAc (Baumgartner et al., 2021, 2022). This contributes to additional studies which demonstrated that CRF microinjections into the NAc medial shell could intensify cue-triggered motivation in a Pavlovian instrumental transfer paradigm similarly to microinjections of amphetamine (Peciña et al., 2006a). Additionally, NAc microinjections have been shown to facilitate accumbal dopamine release to facilitate development of a CRF conditioned place preference (Lemos et al., 2012).

In contrast, the majority of previous literature exploring limbic CRF reinforces a role in generating anxiety, fear, and distress. CRF signaling has been shown to regulate pain signaling, alter sleep architecture, and lead to dendritic atrophy as a result of stress (Andreoli et al., 2017; T. Wang et al., 2023; Zhao et al., 2024). Furthermore, CRF and CRF receptor expression (both mRNA and protein) have been shown to increase in the extended amygdala following social stress and during withdrawal – both of which are typically associated with negative affect and anxiety (Boutros et al., 2018; Connelly & Unterwald, 2020; Lunden & Kirby, 2013). Additionally, shRNA knockdown of CRF from CeA CRF neurons impairs fear learning and

activation of CeA CRF projections to the BNST have been shown to generate anxiety (Pomrenze, Giovanetti, et al., 2019; Pomrenze, Tovar-Diaz, et al., 2019).

However, we cannot unilaterally and explicitly attribute these incentive effects to CRF. Since CRF receptors are not solely activated by CRF, it is possible that CRF receptor blockade interfered with the binding of the urocortin family of peptides which may have influenced behavior. Urocortins are related to CRF and are known to suppress food intake and regulate anxiety, learning and physiological processes (Spiga et al., 2006; Spina et al., 1996; Telegdy et al., 2005). Notably, urocortins have a higher affinity for CRFR2 receptors than CRF does (Vaughan et al., 1995). While it is possible that blockade of CRF receptors interfered with baseline CRF signaling, eYFP control rats showed no differences in behavior following administration of antagonist compared to vehicle. It may be possible that activation of CRF neurons also causes urocortin signaling at CRF receptors, however this would have to be an indirect mechanism as urocortins are not highly expressed in CeA or NAc CRF neurons and would not be co-released due to optogenetic activation (Deussing et al., 2010; Henckens et al., 2016; Lewis et al., 2001; Merchenthaler et al., 1982; Reyes et al., 2001). Thus, while it seems unlikely that urocortins are involved in our incentive effects, we did not have the necessary controls to rule out this possibility.

Furthermore, future work should aim to identify the contributions of which specific CRF receptors are involved in generating incentive motivation. Moreover, genetic knockouts or pharmacological antagonism of CRFR1 and CRFR2 have been shown to have opposite effects in behavioral and endocrine response to stress where CRFR1 is typically associated with anxiety and CRFR2 is implicated in reduction of anxiety, though these effects are brain region specific (Bale & Vale, 2004; Dedic, Chen, et al., 2018; Henckens et al., 2016; Radulovic et al., 1999;

Refojo et al., 2011; Tian et al., 2024; Uribe et al., 2020). Both CRFR1 and CRFR2 have been shown to be necessary for facilitation of dopamine release and generation of conditioned place preference by CRF microinjections in the NAc (Lemos et al., 2012). Furthermore, individual differences in CRF receptor expression could contribute to different magnitudes of incentive motivation driven by CeA and NAc CRF neuronal activation, potentially explaining why some rats may self-stimulate while others do not. For instance, work has shown that different alleles of the CRFR1 gene determine whether CRF microinjections into the VTA enhance firing frequency of VTA DA neurons and enhance breakpoint for sucrose rewards (Zalachoras et al., 2022). Thus, specific blockade of CRFR1 and CRFR2, ideally at local projection targets, will provide further insight into the cellular and molecular mechanisms that contribute to CRF-driven incentive motivation.

Together, findings from Chapters 2 and 3 highlight the ability of extended amygdala CeA and limbic NAc CRF populations to specifically act via CRF receptors to cause focused and intensified incentive motivation.

CRF circuitry underlying incentive motivation

Due to the predominant interest in CRF as a stress molecule, CRF circuitry involved in stress and anxiety have had significant focus relative to CRF circuitry that could mediate appetitive motivation. Since CeA CRF neuronal activation has been shown to generate incentive motivation and recruit fos expression in reward related brain regions, we sought to functionally characterize CeA CRF projections that could mediate the appetitive effects arising from optogenetic stimulation of CeA CRF neurons by injection a cre-dependent channelrhodopsin into the CeA and implanting bilateral optic fibers above projection targets in the lateral hypothalamus

(LH) and dorsomedial striatum (DMS). We demonstrate that activation of CeA CRF terminal in the lateral hypothalamus biases *Crh*-cre rats away from laser-paired rewards, fails to shift breakpoint for laser-paired sucrose rewards, and does not support self-stimulation in either active-touch rod self-stimulation tasks or place-based self-stimulation tasks. However, in a subgroup of rats whose optic fibers were in the posterior LH or substantia nigra (SN), activation of CeA CRF terminals enhanced incentive motivation for laser-paired sucrose rewards, was self-stimulated in a few rats, and looks to have supported self-stimulation in a place-based self-stimulation task. In contrast, a third group of rats with optic fibers in the DMS chose evenly between laser-paired and nonlaser sucrose and failed to self-stimulate, suggesting that activation of this projection did not influence motivation. Lastly, microinjections of CRF directly into LH, DMS, and CeA itself were insufficient to develop conditioned place preference or aversion.

This work consists of preliminary pilot data but suggests distinct roles for CeA CRF circuitry that contribute to mediation of motivated behavior. In the context of the LgA cocaine findings from chapter 5, these results provide potential target sites for cocaine-induced alterations in CRF signaling.

CeA CRF terminal activation in the LH caused rats to prefer nonlaser sucrose rewards over identical rewards paired with laser. This effect may be mediated by CRFR1 receptors which are highly expressed in the LH and inhibition of which have been shown to reduce anxiety and increase exploration in response to stress (Eghtesad et al., 2022). This data fits with the traditional role for CRF in generating aversive motivation to a mild extent; however lack of avoidance in place-based self-stimulation could indicate that CeA CRF terminal activation in the LH is only aversive when paired with a food reward which could be due to an association

between the food reward and mismatched autonomic response caused by CRF terminal activation, making the animal wary of that particular reward without necessarily causing anxiety.

Interestingly, stimulation of CeA CRF projection fibers in the SN biased incentive motivation for sucrose rewards. CeA CRF neurons have been shown to project to the SN pars compacta (SNc) and have been implicated in potential generation of salience processing (Kong & Zweifel, 2021; Steinberg et al., 2020). Specifically, CeA CRF projections to the SNc are activated by both appetitive and aversive stimuli and predictive cues and optogenetic inhibition of these projections impairs learning about both appetitive and aversive cues and rewards (Steinberg et al., 2020). SNc neurons also express CRFR1 in a subpopulation of DA neurons which are tightly physically intermingled with DA neurons in the VTA (Refojo et al., 2011). In fact, this subpopulation of CRFR1 expression VTA/SNc neurons have been shown to play an anxiolytic role in behavior (Refojo et al., 2011). CeA CRF projections to SNc are a promising circuit for appetitive motivation, particularly given the role of SNc neurons in mediating both reward and aversion (Berridge & Robinson, 1998; Ilango et al., 2014).

Lastly, while activation of CeA CRF projections to the DMS and direct microinjections of CRF into CeA, LH and DMs failed to generate incentive motivation, they also failed to cause anxiety or distress. While all of these regions have been implicated in CRF-mediated behaviors to one extent or another, our microinjection results may indicate that CRF alone is not sufficient to alter behavior without additional environmental or physiological mediators such as stress, drug use, hunger, or other mediators of motivation which CRF signaling could potentially modulate.

Thus, Chapter 4 characterized 3 projections of CeA CRF neurons: the DMS which failed to bias motivation, the LH which appeared to generate mild aversion, and the SN which appears

to have generated incentive effects. This work is still preliminary but has opened the door for exciting future studies the explore specific mechanisms through which CRF can mediate incentive motivation.

CeA CRF neurons enhance incentive sensitization after extensive drug consumption

While CeA CRF neuronal incentive motivation has been demonstrated in drug naïve rats previously in our lab and in Chapters 2, 3, and 4, the relevance of these appetitive effects to addiction remains unclear. In opponent process theories of addiction, CeA CRF circuitry is strongly implicated in driving distress during withdrawal which serves as a negative reinforcer to drive relapse as an act of hedonic self-medication (George et al., 2012a; G. F. Koob, 2010; G. F. Koob et al., 2014). Since CRF systems have been shown to sensitize and magnify as an aversive b-process following the development of physical substance dependence, it is possible that the affective valence of CRF could flip from our incentive effects to mediate aversive motivation and distress in line with opponent process theories. Global CeA optogenetic activation and microinjections of CRF into the NAc have been shown to flip valence from appetitive to aversive following stress (Lemos et al., 2012; Warlow et al., 2020), so it seems plausible that CeA CRF systems may also be capable of such a reversal following extensive drug experience.

Much of the work which has previously shown sensitization of extended amygdala CRF systems used long access (LgA) cocaine self-administration to model addiction, characterized by increasing escalation of drug consumption and the development of physical dependence (Ahmed et al., 2003; Ahmed & Koob, 1998, 1999; Ferrario et al., 2005). Thus, we used the same 14 day self-administration task where rats could freely self-administer cocaine for 6hr/day. Following LgA self-administration, *Crh*-cre rats underwent behavioral tests of motivation to determine

whether activation of CeA CRF neurons could still bias pursuit of laser-paired rewards and support self-stimulation. During withdrawal, females increased their overall sucrose pursuit but did not seem to distinguish between the *Laser + Sucrose* and *Sucrose Alone* options either during withdrawal or following a period of abstinence. In contrast, males in withdrawal exhibited a valence flip where they preferred the *Sucrose Alone* over the *Laser + Sucrose*. However, after a one-month period of abstinence, they then reverted into preference for *Laser + Sucrose*, similar to drug naive controls, suggesting that positively-valenced incentive salience attribution is flexible and can reemerge some time after drug consumption stops. However, the small number of males makes these sex-specific findings preliminary.

Most importantly, CeA CRF neuronal activation generated neither laser-place-avoidance nor place-preference during with withdrawal or after the one-month abstinence period. In conjunction with the active-touch self-stimulation task where a subset of rats self-stimulated during withdrawal and after abstinence, it appears that LgA cocaine self-administration did not cause CeA CRF activation to become intensely aversive which stands in contrast to the opponent process role for CeA CRF as a magnified and aversive b-process that drives withdrawal.

These findings complicate previous work that has relied on CeA CRF systems simply as drivers of anxiety and distress. While a wide variety of stressors activate CeA CRF neurons as measured by increased c-fos, our findings call into question whether activation of these neurons is sufficient to cause anxiety and distress, and if so, under what conditions and contexts (Porter & Hayward, 2011; Sterrenburg et al., 2012; Walker et al., 2019).

Additionally, our work highlights the importance of examining sex differences in both stress and addiction related research. While our male and female rats self-administered cocaine similarly, they had divergent responses during withdrawal where females became hyper-

motivated for both laser and nonlaser rewards while males potentially showed a flip from incentive to aversive motivation arising from CeA CRF neuronal activation. This could suggest that males and females experience different types of compulsion for reward pursuit where females are driven by incentive sensitization during withdrawal but males are not to the same extent. Further, evidence that both sexes show incentive sensitization that is intensified by CeA CRF stimulation after abstinence could provide an additional psychological mechanism to explain how stress causes relapse or compulsive reward seeking even after significant time away from drugs.

Future work should determine what signaling mechanisms are involved in the behavioral changes we demonstrate following extensive cocaine consumption. For instance, in Chapters 2 and 3, we demonstrate that CRF receptor activation is responsible for the incentive effects arising from CeA CRF neuronal activation; however, the balance of neurotransmitter release from these neurons could shift due to drug exposure such that molecules like dynorphin are preferentially released (G. F. Koob, 2021; G. F. Koob et al., 2014; G. F. Koob & Volkow, 2016; Mantsch et al., 2004, 2016; Pomrenze et al., 2015; Pomrenze, Giovanetti, et al., 2019; Shalev et al., 2010). Furthermore, CRF receptor and binding protein have been shown to have altered expression following extensive drug consumption. It is possible that upregulation of CRF itself, either CRF receptor, changes in the ratio of CRFR1 to CRFR2, or changes in cleavage of CRF binding protein contribute to the changes we see in reward pursuit during CeA CRF neuronal activation (Blacktop et al., 2011; Cottone et al., 2009; Daniel et al., 2019; Lunden & Kirby, 2013; Uribe et al., 2020; Vranjkovic et al., 2018).

In sum, Chapter 5 demonstrates that CeA CRF neuronal activation retains effects of incentive motivation following LgA cocaine self-administration and fails to become aversive as

would be predicted by opponent process theories of addiction which implicate CRF hypersensitization as a magnified aversive b-process that drives distress and reward pursuit as a means of alleviating distress. As such, CeA CRF neurons alternatively serve to intensify reward pursuit via enhanced incentive motivation which persists even after an extended period of abstinence.

<u>Facing the monster under the bed: What if it isn’t always a monster?</u>

Research in psychology and biomedical science has predominantly focused on CRF and stress in terms of preventing stress-related diseases. From this perspective, stress becomes something “bad” which needs to be alleviated, mitigated, or pharmacologically interrupted in order to manage depression, addiction, generalized anxiety, PTSD, etc. (Beehner & Bergman, 2017). However, research based in this perspective falls victim to tunnel vision where studies upon studies are designed to explore the role of stress and CRF in negative emotion, in fear, in reduced social interaction, and in an abundant array of other proxies for the human discomfort associated with the detrimental aspects of stress (Huang et al., 2010). This leads to bias in research where the types of stress or the roles of CRF that humans understand as negative garner attention while the aspects of stress or CRF function that exist beyond distress are either overlooked or forced to fit into an unnecessarily limited definition. For example, in this dissertation focused on thoroughly exploring the incentive roles for CRF and stress, less than 20 citations specifically pertain to these roles. On the other hand, >70 primarily explore the role of CRF in anxiety, negative reinforcement, pain, and fear. That is not to say that these studies are not important in describing a very real role of CRF systems and stress, but to point out the specific framework within which these systems have predominantly been studied. While many of

these works clearly demonstrate CRF's role in distress, which is not in question, many others force the frame of anxiety and distress onto CRF when alternative explanations could also explain enhanced drug seeking, increases or decreases in feeding, and even fear learning (Beehner & Bergman, 2017; Kong & Zweifel, 2021; Nisell et al., 1994; Pomrenze, Giovanetti, et al., 2019)

Further, stress is not a particularly well-defined phenomenon. Does stress always require activation of the HPA axis and glucocorticoid release? Does stress always require generation of anxiety and, conversely, does anxiety always signify stress? What is the autonomic output of stress and how does one differentiate that from, say, excitement or general arousal? These subtle differences are often overlooked as unnecessary added complexity, but grappling with this added complexity is unavoidable moving forward in research into stress, motivation, and addiction if we, as a field, desire to see beyond the current tunnel where stress and CRF are the root of distress. "Bad" stress, as well as "good" stress, also conceptualized as distress and eustress, have been invoked in immune function, cancer prognosis, athletic performance, and college achievement where stress allows for dynamic adaptation as opposed to specifically as an alarm system (Bienertova-Vasku et al., 2020; Dhabhar, 2014; McEwen & Akil, 2020; Natsir et al., 2021; O'Sullivan, 2011; Rodríguez et al., 2013; Wu et al., 2022). Many studies have used autonomic markers, such as heartrate, pupil dilation, skin conductance, etc. as measures of CRF- or stress-generated anxiety; however, these changes are hallmarks of arousal *generally*, including positive excitement, surprise, and aggression (Bradley et al., 2008; Hachenberger et al., 2023; Hilton & Zbrożyna, 1963).

In addiction research specifically, many studies examining the role of CRF in relapse assume this framework where CRF increases anxiety and acts as a negative reinforcer. One of

the primary measures in these studies is propensity to relapse, which is frequently attributed to the negative affective impacts of CRF without specifically examining distress or anxiety, or, in some cases, finding little evidence of anxiety but fitting the framework anyway (Blacktop et al., 2011; Bolton et al., 2018; Galesi et al., 2016; Mantsch et al., 2004). Further studies have used hypothalamic self-stimulation reward thresholds as evidence that stress and CRF signaling contribute to negative emotional states, but this assumes that lack of reward pursuit equates to negative affect as opposed to reduced motivation (Bruijnzeel et al., 2007, 2009; Holtz et al., 2015; Marcinkiewcz et al., 2009). Notably, CRF receptor blockade diminishes stress-induced reinstatement (Blacktop et al., 2011; Bruijnzeel et al., 2009; Vranjkovic et al., 2018; B. Wang et al., 2007) which can be interpreted as reduction of distress or, in light of our current findings, could be viewed as reduction of stress-induced CRF-driven incentive motivation.

Moving forward, research centering around CRF and stress must be interpreted with open-mindedness to roles beyond anxiety and distress. Even further, studies should be designed with the intent to directly assess both incentive and aversive effects of CRF system manipulations, particularly when examining reward seeking behaviors, in order to parse distinct roles for CRF as a negative reinforcer or as a driver of incentive motivation. After all, if CRF is only considered through a framework of anxiety and distress, then that may be all we ever find.

CRF in motivation and salience

There is no debate that CRF systems are deeply connected to stress and anxiety, but our understanding of their role in mediating behavior should not be so limited. A common thread between studies that fit the framework of negative reinforcement/aversive motivation and those that provide evidence for appetitive motivation is clear: CRF is mediating motivated behavior.

CRF is also mediating affective behavior by both increasing and decreasing anxiety, depending on which parts of the system are involved (Dedic, Kühne, et al., 2018; Refojo et al., 2011; Zalachoras et al., 2022). Given the ability for CRF to improve memory consolidation and evidence that CeA CRF is necessary for fear learning, some proportion of CRF signaling is likely encoding salience. In other words, CRF systems respond to important information in the environment, whether it be threats, rewards, mates or other relevant stimuli (Ell et al., 2011; Lim et al., 2007; Merali et al., 1998a, 2004; Sanford et al., 2017). During withdrawal and even following long periods of abstinence, drugs and drug cues become hyper-salient – an effect to which sensitized CRF systems in addiction could contribute (Berridge & Robinson, 2016; George et al., 2012a; G. F. Koob, 2010; T. E. Robinson & Berridge, 1993). In fact, recent work posits that CeA CRF neurons, specifically, may be the most likely neuronal population in the CeA to encode salience since they are activated in response to both unexpected neutral stimuli, aversive events, appetitive events, and predictive cues (Jo et al., 2020; Kong & Zweifel, 2021; Merali et al., 1998a, 2003, 2004).

Work from our lab supports this view, as CRF microinjections into NAc were able to enhance cue-triggered motivation and optogenetic activation of NAc and CeA CRF neurons biased pursuit toward laser-paired rewards (Baumgartner et al., 2021, 2022; Peciña et al., 2006a). Further, evidence that CRF neurons modulate mesolimbic dopamine signaling suggests an interface between salience encoding and the output of both appetitive and aversive motivation (Dedic, Kühne, et al., 2018; Refojo et al., 2011; Ungless et al., 2003; Zalachoras et al., 2022). Further, previous work has also shown that glucocorticoids are able to enhance the salience of palatable foods, providing a mechanism for stress to enhance reward seeking by making food or other rewards hyper-attractive (Dallman et al., 2003, 2005; Tomiyama et al., 2011).

Modes vs modules: experience dependent roles for CRF populations

It is common in neuroscience research to isolate and manipulate defined brain regions to understand the relationship between structure and function, but what happens when a single brain region can mediate multiple or opposing functional outputs? The shift in valence of CeA CRF neuronal activation seen in males and the incentive sensitization triggered in females during withdrawal following LgA cocaine self-administration provides evidence that valence and motivation generated by the same structure and cell populations can mediate different functions and behaviors according to life experience, such as drug use, and physiological state, such as that seen in withdrawal versus in abstinence. These findings invoke the debate surrounding brain frameworks of 'modules,' where distinct units control distinct functions, versus 'modes' where brain regions, circuits, etc. are capable of mediating a spectrum of behavioral responses depending on internal and external contexts (Berridge, 2019).

It is easy to think about CRF neuronal populations within distinct brain regions as individual 'modules' that generate consistent behavioral effects. In this case, we have repeatedly shown that activation of CeA and NAc CRF neurons can focus and intensify incentive motivation and, in examining specific projection targets, attempt to further identify more specific modules that contribute to this behavior (Baumgartner et al., 2021, 2022). However, evidence from the LgA self-administration experiments suggest that incentive motivation arising from CRF neuronal activation does not always generate the same behavioral effects. The impact of CRF neuronal activation, at least in the CeA, is dependent on previous drug experience. Thus, it is possible that this CRF 'module' has distinct effects on reward salience, autonomic response, or motivated behavior depending on drug history. There is precedent for these effects in CRF

systems as well as in the CeA. In one example, CRF microinjections into the NAc could generate conditioned place preference in drug naïve animals; however, following stress, the valence of CRF signaling flips to becomes aversive such that CRF microinjections now generate conditioned place aversion (Lemos et al., 2012). In CeA, global optogenetic activation drives intense pursuit of laser paired stimuli ranging from sucrose rewards to a painful electrified rod; however, these motivational effects are reversed when activation of CeA CRF takes place in a Pavlovian fear-learning paradigm (Warlow et al., 2020).

The findings of this dissertation contribute additional evidence of a novel 'mode' for CeA CRF neurons where LgA cocaine self-administration sex-dependently influences both the intensity and the valence of motivation elicited by optogenetic activation. In females, LgA self-administration triggers a persistent 'mode' where CeA CRF activation facilitates incentive sensitization. In males, CeA CRF activation becomes somewhat aversive before transitioning to mediate similar incentive sensitization effects as seen in females. While 'modes' of function in other brain regions can be dependent on the immediate environment, it seems possible that these CeA CRF 'modes' may persist for some time, potentially in line with ideas about long-lasting incentive sensitization following drug use (Baumgartner et al., 2020; Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993)

<u>CRF mechanisms interfaces with mesolimbic dopamine circuitry to influence motivation</u>

The mesolimbic dopamine pathway is central to motivation and reward. CRF has been shown to mediate function of the mesolimbic dopamine pathway, though predominantly via mediation of hypodopaminergic states that are thought to contribute to negative affect in depression and reduced motivation (Akil & Nestler, 2023; Binder & Nemeroff, 2010; Y.-W.

Chen et al., 2012). However, there is evidence that CeA and NAc CRF neuronal activation recruits c-fos protein expression in reward related circuitry, including the LH, VTA, and VP (Baumgartner et al., 2021, 2022). Both BNST and CeA CRF neurons project to VTA (Dedic, Kühne, et al., 2018). Specifically, these VTA projection neurons are a subpopulation of GABAergic long range projection neurons that also co express calcium/calmodulin-dependent protein kinase 2α and are negative for somatostatin and protein kinase delta, suggesting that these neurons exist within less than 10% of the population of CeA cells (Dedic, Kühne, et al., 2018; Kong & Zweifel, 2021; Pomrenze et al., 2015).Genetic knockout of CRF from these neurons increases anxiety and defensive behaviors and leads to reduced dopamine release in the PFC, presumable by modulating VTA DA signaling (Dedic, Kühne, et al., 2018). Within the VTA, activation of CRFR2 via CRF and CRF binding protein (CRFBP) have been shown to facilitate NMDA excitation of dopamine neurons (Ungless et al., 2003). Further, CRFR2 activation with CRFBP binding mimics foot shock induced reinstatement of drug seeking, suggesting a potential mechanism for stress induced relapse (Grieder et al., 2014; B. Wang et al., 2007). These are all potential mechanisms through which CRF can potentially mediate either incentive or aversive motivation via modulation of midbrain dopamine release.

Nearby the VTA and sharing physically intermingled DAergic neuronal populations, the SNc is also a major DA output region to control motivation and motor behavior (Berridge & Robinson, 1998; Rossi et al., 2013). The SNc is rich in CRFR1 expressing dopamine neurons but is distinct from the VTA in that it has minimal expression of CRFBP (Refojo et al., 2011). CeA CRF neurons have been shown to project to the SNc which supports our findings that activation of this projection can generate incentive motivation (Kong & Zweifel, 2021; Steinberg et al.,

2020). CRF neurons could be a distinct population of CeA to SN projection neurons involved in encoding salience of rewards, punishments, and related cues (Steinberg et al., 2020).

Upstream of the SNc and VTA, CRFR1 and R2 receptors are expressed on DAergic fiber terminals and have been functionally shown to facilitate DA release in the NAc (Lemos et al., 2012). The vast majority of NAc CRF neurons have been shown to be D1-, D2-, or D1/D2-contanining spiny projection neurons that project to the VP and the ventral midbrain (Eckenwiler et al., 2023). While not specific to CRF neurons, D1 spiny projection neurons from NAc to VP have been shown to be necessary for reinstatement of cocaine seeking (Pardo-Garcia et al., 2019). Further cell-type specific analysis of NAc CRF neurons could yield insight into promising mechanisms of incentive motivation.

In sum, CRF infrastructure is well-positioned withing the midbrain to specifically modulate mesolimbic dopamine signaling as a mechanism for driving incentive motivation. Changes in the relationship between CRF and dopamine following chronic drug use could provide a neurobiological basis for the enhanced incentive sensitization generated by CeA CRF neuronal activation following LgA cocaine self-administration.

A role for CRF in incentive sensitization theory of addiction

CRF is central to opponent process theories of addiction where initial drug consumption triggers a hedonic a-process that is then homeostatically countered by an aversive drug-opposite b-process: anxiety and distress due to extended amygdala CRF signaling (George et al., 2012a). As physical dependence develops, this aversive CRF-driven b-process is thought to magnify and become increasingly more intense to the effect that individuals consume drugs in order to alleviate worsening distress (G. F. Koob, 2010). However, evidence presented in this dissertation

suggests that CRF signaling within the CeA, a component of the extended amygdala, does not generate intense aversive motivation following dependence-inducing LgA cocaine self-administration (Ahmed et al., 2003; Ahmed & Koob, 1998, 1999).

Instead, optogenetic CRF neuronal activation differentially influenced motivation to pursue laser-paired sucrose rewards in males and females during withdrawal where males showed mild laser aversion and females showed intensified reward seeking overall. After a one-month period of abstinence, both males and females showed increased reward seeking but no preference for sucrose with laser over sucrose without. Further, CRF activation never became aversive in self-stimulation tasks and some rats even self-stimulated CeA CRF neurons. This evidence requires that the role of CeA CRF neurons be re-evaluated in terms of incentive motivation rather than negative reinforcement.

These findings of heightened reward ‘wanting’ fit better with incentive sensitization frameworks of addiction. Here, relapse is driven by sensitized dopaminergic ‘wanting’ systems that drive craving triggered by drugs and drug cues (Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993). Importantly, this work presents a mechanisms by which stress could potentially generate intense ‘wanting’ which become stronger over periods of abstinence, consistent with an incubation of craving (T. E. Robinson & Berridge, 1993). Given the evidence that CeA CRF neurons may mediate salience, sensitization of CRF systems after cocaine use could also cause sensitization of the attribution of salience by these neurons, and potentially NAc CRF neurons, which could explained the heightened reward seeking seen following abstinence and the ability of CRF microinjections to generate cue-triggered motivation (Kong & Zweifel, 2021; Merali et al., 1998a; Peciña et al., 2006a). Future work should examine the neurobiological underpinnings of the hypersensitization of CeA CRF neurons in generating reward seeking.

Future directions:

We can hypothesize that since CRF activation can drive reward pursuit that CRF neuronal activation contributes to motivation, however, we need to show that these CRF systems are engaged during reward seeking without artificial activations. Pharmacological antagonism can get at this to some extend but modern recording via in vivo electrophysiology, fiber photometry, and two-photon imaging provide more direct ways of measuring CRF neuronal activation and CRF release, especially with new sensors such as GRAB-CRF (H. Wang et al., 2023). future work should seek to identify CRF signaling targets during both reward receipt, presentation of reward cues, and following stress. Evidence for an incentive role for CRF is convincing, however, there is minimal evidence that the optogenetic manipulations described in this dissertation are actually generating a realistic brain state. This necessitates functional recording to show the typical involvement of CeA and NAc CRF neurons in motivation.

Additionally, with the advent of fos-TRAP systems, it may be possible to fos-TRAP CRF neurons during withdrawal in order to selectively reactivate withdrawal relevant circuitry during assessments of motivated behavior such as our two-choice sucrose task. This could allow for functional comparison in functional circuitry during reward seeking between drug naïve rats and those used to model addiction. Not only would this allow researchers to assess the valence of CRF withdrawal circuits, but it would also allow for tracking changes in these circuits at different timepoints.

Further characterization of the dynamic relationship between CRF and dopamine systems will be necessary to define a mechanism for CRF enhanced incentive sensitization. This is particularly interesting given the changes seen in Chapter 4 where LgA cocaine self-

administration differentially altered the motivational effects of CeA CRF neuronal activation in males and females. This speaks to the potential for an interaction between CRF, sex hormones, and potentially dopamine in mediating motivation and reward seeking.

While stress and CRF are tightly linked, it would be interesting to try to gather evidence as to whether the two systems can be dissociated. It may be that extrahypothalamic CRF systems could regulate behavior without triggering HPA axis response, and even that activation of specific hypothalamic CRF circuitry, such as projections from the PVN to the VTA, can generate positive incentive motivation (Xu et al., 2024). In the future, it will be necessary to more closely assess HPA axis activation and blood glucocorticoid levels to determine whether activation of these CRF systems are triggering a canonical stress response and, if so, whether glucocorticoid release is also contributing to incentive motivation (Dallman et al., 2003, 2005; Honma et al., 1984; Merali et al., 1998a; Piazza et al., 1993; Piazza & Le Moal, 1996; Tomiyama et al., 2011).

Lastly, it makes sense to explore individual differences that influence the effects of CRF on motivation. This could explain why some *Crh*-cre rats will self-stimulate CRF neurons but others will not. Individual differences, such as propensity for “sign tracking” or “goal tracking” may be mediated by glucocorticoids in some conditions, despite there being no clear differences in glucocorticoid systems in these two groups at baseline (Lopez et al., 2021; Rice et al., 2018). Additionally, trait anxiety, genetic variation in CRF receptors, and genetic variation in dopaminergic signaling could all influence the effects of CRF system activation or stress (Crum et al., 2018; Zalachoras et al., 2022).

<u>Clinical Implications of CRF-driven incentive motivation</u>

Pharmacological interventions targeting CRF systems to treat addiction and depression have been largely ineffective at mitigating cravings, even if they mitigate HPA axis response (Kwako et al., 2015; Schwandt et al., 2016; Shaham & de Wit, 2016). While discussions of the disconnect between preclinical work in rodent models and lack of effective treatment in humans can be ascribed to neurobiological differences, it is also possible that CRF signaling mechanisms are not the main driver of drug craving (Berridge & Robinson, 2016; George et al., 2012a; G. F. Koob, 2010; T. E. Robinson & Berridge, 1993). While individuals certainly use drugs as a method of hedonic self-medication to alleviate distress and anxiety, underlying incentive sensitization may cause cravings to persist even if the direct causes or biological underpinnings of stress are addressed. In other words, while stress and anxiety may contribute to drug seeking via negative reinforcement and potentially via sensitized stress mechanisms of incentive motivation, alleviating distress may not be sufficient to halt cravings and relapse (Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993; Shaham & de Wit, 2016).

If relapse and craving are mediated by an underlying effect of enhanced dopamine signaling, it is possible that dopamine mediating drugs, such as dopamine receptor partial agonists, antagonists, or allosteric modulators could be effective (Martinez, 2020; Moreira & Dalley, 2015; Pulvirenti & Koob, 1994; Yuan et al., 2024). However, while development of drugs targeting dopamine systems took off between the 1950s and 1970s, progress has since stalled such that new drugs are marginally, if at all, better than those that already exist (Shad, 2023). Thus, it may be appropriate to look outside of pharmacological treatment in order to effectively help individuals struggling with stress-related motivational disorders like depression and addiction.

As of the submission of this dissertation, the current fad in bio-hacking motivation and controlling cravings is the notion of "dopamine detox," popularized by Dr. Anna Lembke and based in opponent process notions that consistent exposure to highly rewarding experiences, such as drugs or potentially social media cause dysregulation of the balance between pleasure and distress. A "dopamine detox" calls for abstinence from anything that causes pleasure in order to allow reward systems to regain homeostasis and break the cycle of pleasure-bingeing and then, of course, the opponent process of dissatisfaction, anxiety, and distress (Lembke, 2021). While this has gained pop-culture popularity, it harkens back to defunct ideas about dopamine as a pleasure molecule and, similar to issues with CRF receptor antagonist in treating addiction, assumes that once the anxiety and distress of the aversive opponent b-process have passed, the pain-pleasure balance will be restored and cravings will cease (Lembke, 2021). While this approach may be helpful in the short term in helping individuals cease their immediate bingeing behaviors, whether doom scrolling, smoking, or otherwise, the promise of reduced cravings after a period of abstinence is likely misleading given what we know about cue-triggered relapse which does not necessitate pain or distress to cause cravings (Fraser et al., 2023; Fraser & Janak, 2019; Perry et al., 2014; Saunders et al., 2013; Saunders & Robinson, 2010; Vafaie & Kober, 2022; Yager & Robinson, 2013).

Clinical approaches to treating stress-associated motivational disorders like addiction and depression must account for the psychological underpinnings of behavior, not just the pharmacological mechanisms. Patients and clinicians must be aware of the long term effects of incentive sensitization and, as described in this dissertation, the role of stress and CRF mechanisms that mediate incentive motivation and are responsive to both positive and negative events (Merali et al., 1998a, 2004; Refojo et al., 2011; Steinberg et al., 2020; Zalachoras et al.,

2022). By informing patients of the potential for long term craving and the various cues, contexts, and emotional states that can serve as triggers, clinicians can more effectively work with patients to develop coping mechanisms to prevent relapse.

Lastly, the role of CRF and stress in incentive sensitization, in addition to anxiety and negative reinforcement, make the relationship between stress and addiction very prominent. As the United States struggles with growing opioid addiction, one strategy for addressing drug abuse could be to provide support and resources that mitigate relapse-triggering sources of stress such as financial, housing, or food insecurity (Basile, 2022; Betancourt et al., 2023; Glei & Weinstein, 2019; Goldman-Hasbun et al., 2019; Marcinkiewcz et al., 2009; Sinha, 2008). The current approach to managing addiction in the U.S. criminalizes substance use with imprisonment which exacerbates risk of overdose and increases drug use following release (Binswanger Ingrid A. et al., 2007; Volkow, 2021). Given what we know about stress-triggered drug seeking, criminalization of substance use actively increases relapse and drug seeking (G. F. Koob & Volkow, 2016). Thus, the final clinical implication of the work discussed in this dissertation is that treatment for compulsive drug seeking will likely be ineffective if patients are constantly facing inescapable and uncontrollable stressors. While addiction treatment on an individual level may attempt to prepare people to cope with stressors, treating substance use disorders will require social change in order to reduce potential stress driven sensitization of drug craving.

## Conclusions

While CRF systems are traditionally tied to stress response, distress, and anxiety, some CRF systems appear to play a role in appetitive motivation. This dissertation demonstrated that limbic CRF systems in the NAc and CeA generate incentive motivation via activation of CRF

receptors, providing confirmation that CRF signaling from extended amygdala related structures can increase reward seeking without distress. Further, we show that CeA CRF neuron activation does not generate aversive distress during withdrawal following extensive cocaine self-administration and may enhance incentive sensitization for sucrose rewards after a period of abstinence. Our data suggests that circuitry underlying the effects of CeA CRF neuron driven incentive motivation may include projections to the SN or posterior hypothalamus.

These findings shed light on how many types of stress, including happy life events like a marriage or a new job, could trigger relapse in addiction or drive incentive motivation for other rewards without necessitating distress. Dysregulation of incentive CRF systems could lead to a hypo-motivated state which could lead to the avolition characteristic of depression. In the other direction, dysregulation of incentive CRF systems could contribute to hyper-compulsive reward seeking in addiction and binge-eating disorders, even after a period of abstinence.

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