SEX-SPECIFIC REGULATION OF PAIN: A NOVEL ROLE FOR DOPAMINE AND CORTICOTROPIN-RELEASING FACTOR SIGNALING IN THE MIDBRAIN AND EXTENDED AMYGDALA

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ABSTRACT

Waylin Yu: SEX-SPECIFIC REGULATION OF PAIN: A NOVEL ROLE FOR DOPAMINE AND CORTICOTROPIN-RELEASING FACTOR SIGNALING IN THE MIDBRAIN AND EXTENDED AMYGDALA
(Under the direction of Thomas L. Kash)

Chronic pain is the leading cause of disability and health care access in the United States. Sex differences in perception, response, and pathological susceptibility are common features of pain, with women being disproportionately affected and inadequately treated for pain across the lifespan. The neural mechanisms that contribute to these outcomes remain poorly understood, as female subjects have historically been underrepresented in pain research. The overarching goal of this dissertation is to identify the neurocircuit mechanisms of pain, with special emphasis on two regions of sex-specific pain modulation: the ventrolateral periaqueductal gray/dorsal raphe (vlPAG/DR) and the bed nucleus of stria terminalis (BNST). We first demonstrate a role for vlPAG/DR dopamine (DA) neurons in adaptive responses to pain, where activation of vlPAG/DR^{DA+} projections to the BNST reduces nociceptive sensitivity in male mice and increases locomotion in female mice. Deletion of the DA rate-limiting enzyme tyrosine hydroxylase (*Th*) in vlPAG/DR mitigates these behaviors, with vlPAG/DR^{DA+}-BNST physiology supporting a key role for DA transmission in sex-specific function. We next characterize the contributions of corticotropin-releasing factor (CRF) in the BNST, as DA and CRF interactions in the BNST have been posited to impact pain. Robust *in vivo* recruitment of BNST^{CRF+} neurons was observed during exposure to a nociceptive stimulus, with male and female mice exhibiting distinct magnitude and synchronization in neuronal responses. We then show that genetic deletion of *Crf* in the BNST reduces nociceptive sensitivity for both sexes and increases paw attending responses in female mice. Finally, we discuss the implications of these

novel pain mechanisms on drug use, citing results that illustrate the consequences of local *Th* and *Crf* deletion on morphine and alcohol treatment. Collectively, these findings establish a role for vlPAG/DR^{DA+} and BNST^{CRF+} neurons in the sex-specific expression of pain, highlighting promising new targets to achieve pain relief and counteract maladaptive drug use with precision medicine approaches.

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(Yuan Guang Yu, Li Rong Wu, Get Ming Chen, Chuan Ying Wu),
parents (Qiu Jian Yu, Qiao Yan Chen), and sister (Lily Yu).
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PREFACE

Overview

Pain is a complex sensory experience that enables the detection of tissue damage and generates unpleasant emotional states to signal danger. Inherent in this experience is the need for behavioral change, as appropriate responses to pain are necessary to counteract the possibility of irrevocable harm or fatal injury. This process of sensing threats and meeting environmental challenges is informed by a combination of innate sensation and experiential learning, demonstrating the interplay of biological mechanisms needed to experience and alleviate pain. In this introduction, we will discuss the multifaceted networks that orchestrate the pain system to ensure survival, as well as the challenges of managing this system when confronted with pathology.

i. Pain as a System of Adaptation

Nearly all vertebrate animals are predisposed to detect noxious stimuli using peripheral sensory cells called nociceptors (Millan, 1999; Julius & Basbaum, 2001). These cells contain temperature-, pressure-, and chemical-sensitive receptors that generate activity in response to tissue damage (Sherrington, 1907; Basbaum et al., 2009). Incoming signals from nociceptors may be processed locally in the spinal cord via reflexive pathways or supraspinally in the brain via transmission to second and third order neurons (Loeser & Melzack, 1999; Millan, 1999). The connections that relay nociceptive information throughout the brain are canonically regarded as ascending tracts (e.g. spinal transmission to the thalamus [spinothalamic], cerebellum [spinocerebellar], reticular formation [spinoreticular], parabrachial [spinoparabrachial], among other regions) or descending tracts (e.g. transmission to the spinal cord from the cortex

[corticospinal], reticular formation [reticulospinal], and hindbrain [descending autonomic], among other regions), where nociceptive information is processed and modulated respectively (Basbaum et al., 2009). The objective of this neuroanatomical organization is to rapidly recognize environmental threats, allowing organisms ample time to engage in “fight-or-flight” behaviors and neutralize any potential for harm (Bandler et al., 2000). Generally, these pain responses are expressed as escape behaviors to increase distance from the source of injury, communicative behaviors to request aid or warn others of the experience, and passive behaviors to conserve energy and prevent further injury. The most advantageous course of action is likely to change depending on the context of pain (Wiech & Tracey, 2013). By consequence, dynamic integration of real-time sensory information and behavior are essential features for the neurobiological basis of pain, possibly explaining why nociceptors in the peripheral nervous system exhibit such diverse circuit engagement in the spinal cord and brain. Understanding the adaptive mechanisms that drive these pain responsive circuits are thus critical for promoting self-preservation and well-being.

ii. Chronic Pain is Maladaptive and Affects Quality of Life

Under standard conditions, pain is an adaptive system of threat detection, response, and safety. In pathological forms, however, pain can be maladaptive. Sensory disorders such as neuropathy and sensitization are commonly reported with physical injury, inflammation, and disease (Millan, 1999; Woller et al., 2017). These etiological factors drive normally innocuous stimuli to become painful, resulting in sustained reductions in pain thresholds and constitutive activation of pain responsive circuits that is disproportionate to the presence of noxious stimuli. The perpetual nature of pathological pain has dire consequences, producing marked effects on the biological, psychological, and social aspects of daily living (Lumley et al., 2011). By impairing work productivity, sleep, interpersonal relationships, and other important contributors to quality of life, chronic pain has become the leading cause of disability and health care access

in the United States (Lumley et al., 2011; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017), with the annual societal cost of pain (an estimated $560 to $636 billion) being greater than that of heart disease ($309 billion), cancer ($243 billion), and diabetes ($188 billion) (Gaskin & Richard, 2012). These statistics indicate that the ability to counteract maladaptive sensory conditions such as chronic pain are essential for maintaining well-being at both the individual and societal level.

iii. The Intersectional Challenges of Pain Treatment

The high prevalence of chronic pain is likely due to the challenging nature of pain management. Effective and accessible pharmacological interventions such as opioids and alcohol promote temporary pain relief while encouraging habitual use, thus increasing the susceptibility for pain exacerbation and other negative consequences of drug dependence (Ballantyne & LaForge, 2007; Hojsted & Sjogren, 2007; Egli et al., 2012). Additional treatment challenges come from reports that there are sex differences in the efficacy of pain intervention. Women are more likely to receive inadequate pain diagnoses and treatment than men, and, by consequence, disproportionately experience severe pain across the lifespan (Paller et al., 2009; Ruau et al., 2012; Bartley & Fillingim, 2013). The need for improved pain treatments that are efficacious for both sexes has been recognized at the federal level, as the National Institute of Health (NIH) has embraced initiatives like Sex as a Biological Variable (SABV) and the Pain Consortium's Helping to End Addiction Long-Term (HEAL) to highlight the importance of these intersectional challenges. These initiatives declare that more research with equal representation of male and female subjects is required to understand, manage, and adequately treat pain, while preventing drug misuse and addiction (Arnegard et al., 2020). The nationwide objective to elucidate novel targets for pain treatment without the negative consequences of addiction is aimed at the emerging public health crisis surrounding opioid use, and more generally, expresses societal demands for safer and more effective pain management.

iv. <u>The Midbrain as a Candidate Region for Sex-Specific Pain Modulation</u>

Evidence for the sex-dependent expression of pain has revealed that divergent neurobiological mechanisms can drive distinct pain responses in male and female subjects (Bodnar & Kest, 2010; Mogil et al., 2020). The periaqueductal gray (PAG) and dorsal raphe (DR), two midbrain structures surrounding the cerebral aqueduct, have been highly implicated in adaptive responses to pain and the reinforcing effects of drug use (Basbaum & Fields, 1979; Van Der Kooy, 1982; Carrive et al., 1993; Bandler & Shipley, 1994; Wang & Nakai, 1994; Bandler et al., 2000; Flores et al., 2006). In humans, viewing images of physical pain and negative affect activated the midbrain structures (Mobbs et al., 2007; Buhle et al., 2013), while electrical stimulation produced endogenous opioid release and sustained analgesia (Hosobuchi, 1977, 1983, 1986; Levy et al., 1987; Roizen et al., 1985; Behbehani, 1995; Sims-Williams et al., 2017). In rodents, the PAG/DR can similarly drive pain relief (Reynolds, 1969; Fields et al., 1976; Basbaum & Fields, 1984) and additionally contribute to locomotor, vocalization, and autonomic functions based on environmental demands (Carrive et al., 1993; Bandler & Shipley, 1994; Bandler et al., 2000), suggesting that these structures are responsible for a range of adaptive behaviors when responding to situations of distress. Notably, the PAG/DR exhibits reciprocal connections between brain regions in the ascending and descending nociceptive tracts **(Figure 1)**, providing these midbrain structures optimal anatomical positioning to integrate nociceptive information with adaptive responses and alter the impact of pain. Sex-specific mechanisms of drug-induced analgesia have been observed in the midbrain (Bobeck et al., 2009; Loyd & Murphy, 2014, Doyle et al., 2017; Schoo et al., 2018), making it likely that these same PAG/DR connections exert significant influence over pain outcomes. Here, we will discuss how PAG/DR and the associated molecular diversity of inputs and outputs can regulate neuronal contributions to pain-related behaviors, with special consideration for potential mechanisms of sex differences in pain.

Preclinical Models of Pain

In order to study the neurobiological mechanisms of pain in animals, it is essential that behavioral models reflect pain phenotypes in humans. Self-report has been regarded as the primary basis of clinical pain evaluation, where patients are given the opportunity to indicate subjective feelings of unpleasantness through rating scales or questionnaires that reflect the extent of pain being suffered (Ong & Seymour, 2004; Fillingim et al., 2016). Since pain is a personal experience that can manifest in ways that are not proportional to nociceptive engagement, and internal distress may not be visible to an observing physician, self-report has remained the standard for contemporary approaches to pain determination in a medical context (Fillingim et al., 2016). Functional measures such as nociceptive sensitivity, aversive learning, and impaired quality of life can additionally inform diagnosis, however, as such measures are commonly used for quantitative assessments of pain in laboratory settings (Staahl & Drewes, 2004; Arendt-Nielsen et al., 2007; Breivik et al., 2008; Taylor et al., 2016). Using behavior as a proximal measure of pain has been strategically applied to standardize pain readouts in patients and enhance the translational relevance of preclinical studies, since pain testing in model organisms entirely relies on non-verbal indications rather than self-report (Brievik et al., 2008; Gregory et al., 2013). The acceptance of functional outcomes as proximal measures for pain in both human and non-human subjects is reflected in a recently updated definition of pain by the International Association for the Study of Pain, which states that the inability to verbally describe pain does not preclude an organism from experiencing it (IASP, 2020). These behavioral indicators have thus served as a necessary link between human and non-human subjects in the study of pain.

To measure pain in a non-verbal manner, preclinical researchers quantify adaptive responses to a noxious stimulus that reflect the aversive nature of the experience and promote self-preservation (Mogil et al., 2009; Gregory et al., 2013). The view that animals can experience pain and express it through functional measures is corroborated in a review by

Walters & Williams (2019), where earlier definitions from Manfred Zimmerman and others are cited to state that pain is “induced by noxious stimulation during injury or imminent injury [and] has a presumed protective function involving overt defensive behavior, internal physiological alterations, and an aversive motivational state that can promote avoidance learning.” The definition outlines three broad categories of functional measures that signify pain in both human and non-human subjects: an increase in (1) nociceptive sensitivity, (2) defensive behaviors, and (3) avoidance learning. To illustrate the implementation and purpose of these pain-related behaviors in preclinical models, we will use examples in the rodent literature to describe how these tests are typically performed and what facets of the human pain experience these models capture.

i. Measuring Nociceptive Sensitivity in Rodents

Nociception, defined as the transduction process from tissue damage to nociceptor activity, is a primary component of pain that is measured by exposing an organism to a noxious stimulus and assessing nociceptive responses (Chapman et al., 1985; Mogil et al., 2009; Zhang et al., 2011; Gregory et al., 2013). Withdrawal of the exposed limb from the source of injury or increased attending of the treated limb are prototypical behaviors associated with nociception, as they indicate an instinctive attempt to mitigate the ongoing tissue damage. Tests to determine nociceptive sensitivity have traditionally used thermal, mechanical, electrical, or chemical stimuli to evoke reflexive or spontaneous behaviors, with unidimensional objective pain assays like the Hargreaves, Von Frey, tail immersion, hot plate, capsaicin, and formalin tests being standard for measuring sensitivity in rodents. These tests have been successfully adapted for use in a variety of animal models and humans (Turk & Melzack, 2011; Gregory et al., 2013), with the recognition that localized and stereotyped motor reflexes in response to pain are well-conserved across species (Sneddon et al., 2014; Mogil, 2019; Walters & Williams, 2019).

ii. Measuring Defensive Behaviors and Avoidance Learning in Rodents

Defensive behaviors are generally expressed as coping mechanisms to diminish the aversive impact of nociceptive engagement (Carrive et al., 1993). These behaviors permit escape from the source of injury, freezing in anticipation of injury, or protective actions that reduce the impact of injury, depending on the demands of the environment (Bandler & Shipley, 1994; Bandler et al., 2000). To evoke these behaviors in the context of pain, rodent models use avoidance paradigms, where subjects can freely explore an environment that is segregated by nociceptive and non-nociceptive compartments. Exposure to the nociceptive side is expected to promote preferential movement towards the compartment that does not drive nociception (for examples, see the thermal escape test, place escape avoidance paradigm, and shuttle box [LaBuda & Fuchs, 2000; Johansen et al., 2001; Moqrich et al., 2005]). Alternatively, subjects may be exposed to tonic-acting irritants such as capsaicin, formalin, or acetic acid in a neutral compartment, eliciting spontaneous behaviors that are motivated by protecting the treated limb. Repeated exposure to these substances results in learned avoidance of the neutral space, where an association between the aversive aspects of nociceptive treatment and the pain-paired compartment is developed through Pavlovian conditioning. By contrast, the promotion of preference learning can be achieved for cues associated with access to analgesia based on the hedonic value of diminished pain (for examples, see conditioned place preference/aversion, real-time place preference, and operant conditioning with analgesic drugs [Navratilova et al., 2013]). Collectively, these tests of defensive behavior and avoidance learning are important metrics for the affective-motivational components of pain. In humans, learned behaviors associated with pain are likely to influence future actions, as the negative emotional aspects of these experiences may be generalized towards impairing daily activities, so the ability to account for pain-related aversion in non-human subjects significantly supports the translational relevance of preclinical models (Li et al., 2008; Turk & Melzack, 2011; Gregory et al., 2013).

To summarize, the behavioral criteria of increased (1) nociceptive sensitivity, (2) defensive behaviors, and (3) avoidance learning have been effective guidelines for establishing pain in preclinical models. Although we primarily focus on rodent applications of testing, these criteria have been used to establish pain in insects, mollusks, crustaceans, fish, birds, cats, primates, and humans (Sneddon et al., 2014; Walters & Williams, 2019), indicating that adaptive responses to pain are evolutionarily conserved, both as innate reflexes to nociceptive threats and learned actions that influence future behaviors.

iii. Modeling Chronic Pain in Rodents

Several preclinical models of chronic pain have been developed to simulate the pathological conditions observed in human patients. The induction of prolonged sensory dysfunction by neuropathy, inflammation, and abdomen constriction are common manipulations in rodents, as these models closely mimic the biopsychosocial consequences of chronic pain observed in humans (Berge, 2011; Lumley et al., 2011; Burma et al.., 2017). Furthermore, these models have proven to be useful for the assessment of analgesic efficacy in pathological conditions, as preclinical determinations of therapeutic potential are an essential part of drug discovery and have historically informed the development of novel pain interventions in humans (Burma et al., 2017). Precise applications of these models are thus needed to accurately characterize the pathological and therapeutic mechanisms behind chronic pain states.

Models of neuropathic pain are generated by traumatic injury to the somatosensory system, resulting in constitutive activation of nociceptive circuits and the development of pain sensitization (Treede et al., 2008). The most common models of neuropathic pain are performed by physically damaging peripheral nerves in the rodent hindlimb (e.g. sciatic nerve injury, chronic constriction injury, and ligation-based methods), with alternative approaches like spinal cord injury, cortical/thalamic excitotoxicity, and systemic neuropathies (via cancer treatment, diabetes, and alcoholism) similarly relying on lesions to key nociceptive circuits. Modeling

neuropathic pain in rodents produces robust phenotypes related to aberrant sensation, as increases in allodynia (i.e. enhanced pain from a non-nociceptive stimulus), hyperalgesia (i.e. enhanced pain from a nociceptive stimulus), and paw guarding are consistent features of the pathological condition (Barrett et al., 2007). Although human patients that suffer from neuropathic pain exhibit similar patterns of pain response, clinical reports of abnormalities in non-painful sensations (e.g. numbness, tingling) and greater distress following episodes of spontaneous pain (i.e. random nociceptive activation, not necessarily caused by a noxious stimulus) have yet to be validated in non-human models (Yarnitsky, 1997; Gregory et al., 2013). Furthermore, the ability to translate treatment outcomes from rodent models to humans has been under scrutiny, with previous studies exhibiting low internal validity for the application of analgesic drugs (Sert & Rice, 2014). Associated impairments in cognitive function, sleep, mobility, and social interaction do not appear to be affected by translational gaps, however, as these phenotypes are consistent across species (Jaggi et al., 2011; Hoke et al., 2012; Jensen et al., 2014; Rice et al., 2018; Sewell, 2018). Overall, models of neuropathic pain are well-suited to drive sensory dysfunction and impair quality of life, but further research is needed to account for how all aspects of the sensory experience in humans translates to rodents.

In contrast to neuropathic models that simulate permanent nerve damage, models of inflammatory pain are generated by immune responses to chemical irritants, resulting in redness, heat, swelling, loss of function, and pain at the site of infection (Muley et al., 2015). Notably, the intensity and duration of these chemical irritants can significantly differ depending on the substance, with short-acting irritants like capsaicin and formalin lasting on the order of minutes, and long-acting irritants like carrageenan and Complete Freund's Adjuvant (CFA) lasting on the order of days (Ren & Dubner, 1999; Wilson et al., 2006). Of these substances, CFA, an antigen emulsion consisting of heat killed *Mycobacterium tuberculosis* in non-metabolizable oils, produces the most extensive inflammatory response (Ren & Dubner, 1999; Wilson et al., 2006). CFA treatment in the hind paw of rodents has been shown to drive

hyperalgesia and enhanced spontaneous pain for up to two weeks, replicating the conditions of persistent inflammatory ailments like rheumatoid arthritis and tendonitis (Stein et al., 1988; Ren & Dubner, 1999; Fehrenbacher et al., 2012). Importantly, these heightened pain effects can be attenuated with non-steroidal anti-inflammatory drugs and opioids in humans and rodents alike (Walker et al., 1999; Wilson et al., 2006), indicating that shared mechanisms may contribute to the pathophysiology of inflammatory pain in both species.

Chronic pain can additionally be modeled through internal nociceptors, as discomfort in the peritoneum results in visceral nociception. Obstruction, distention, and compression of the abdomen and colon causes debilitating internal pain that lacks sufficient treatment options for humans (Sikandar & Dickenson, 2012), necessitating the demand for more preclinical research on visceral nociception. In rodents, a common way to stimulate the nociceptors that line the abdomen and abdominal organs is by injecting acetic acid into the intraperitoneal zone (Ness & Gebhart, 1990). Treatment with acetic acid generates writhing behaviors, a type of spontaneous pain that is expressed by stretching and retracting of the abdomen to indicate discomfort (Muley et al., 2015). Visceromotor nociceptive responses like writhing have been shown to impair home cage behaviors such as nesting and grooming for rodents, with acetic acid driving these behaviors on the order of minutes (Negus et al., 2015). Pain relieving drugs such as opioids can reverse these enhancements in writhing and impairments in home cage behavior, suggesting that acetic acid treatment can simulate the negative impact of pain on quality of life (Negus et al., 2015; Bagdas et al., 2016). Chronic models of visceral nociception have shown similar consequences, where physical mechanisms of colorectal distention are placed in rodents to constrict the abdominal muscles on command (Ness & Gebhart, 1988), leading to spontaneous pain in the viscera and sensitization of other nociceptive modalities. These manipulations of abdominal and colorectal nociception have previously been validated in human subjects (Ness et al., 1990), indicating that the incapacitating effects of sustained abdominal pain and irritable bowel syndrome can be accurately modeled in rodents.

Over the past century, preclinical research has observed great advances in how pain is studied in model organisms. The general acceptance of non-verbal, functional indications of pain has broadened the definition to include protective functions motivated by the aversive experience of nociceptive engagement. These pain-related behaviors are adaptive, susceptible to alteration by pathological conditions, and well-conserved across human and non-human models, indicating high construct validity and translational potential in humans. The assessment of pain through functional outcomes has thus enabled important discoveries to be made based on extensively characterized and validated preclinical models.

Sex Differences in Pain

For over three decades, the NIH has instituted policies to account for sex as a biological variable, with the recognition that disease and treatment outcomes can manifest differently in men and women (Bale & Epperson, 2017; Arnegard et al., 2020). The first major policy, announced in 1990, required the inclusion of women in all NIH-funded clinical investigations. This was followed up in 2014 by a more general policy to factor in sex when designing studies in animal and human subjects, with a new requirement that researchers must justify the exclusion of any sex in grant applications (Clayton & Collins, 2014). Implementation of these policies were a direct response to the unequal representation of male and female subjects. Although nearly identical representation has been established for both sexes in recent studies with human subjects, an investigation by Beery and Zuker (2011) shows that the exclusive use of male subjects was prevalent in at least 80% of animal studies for the fields of general biology, immunology, physiology, pharmacology, endocrinology, and neuroscience. Of a random sampling of 100 neuroscience papers published in top tier journals, 45% lacked information about sex (Berkley et al., 1992). As a result of these practices, the pipeline for identifying important biological mechanisms has remained incomplete, with inadequate female representation potentially leading to an overgeneralization of male findings or missed

mechanistic insight. Examinations of sex as a biological variable are thus critical for the rigorous and transparent conduct of research and will have major implications for the future of personalized health care.

Pain research has been cited as a particularly important area of investigation for sex differences (Hardy & DuBois, 1940; Berkley et al., 1992; Greenspan et al., 2007; Mogil, 2012; Melchior et al., 2016). Women are disproportionately affected by chronic pain, with female patients showing greater prevalence for headache, migraine, low back pain, neck pain, knee pain, chronic fatigue syndrome, fibromyalgia, interstitial cystitis, temporomandibular disorder, and irritable bowel syndrome than their male counterparts (Gerdle et al., 2008; Fillingim et al., 2009; Mogil et al., 2012; Steingrimsdottir et al., 2017). Both clinical (Fillingim et al., 2003; Barnabe et al., 2012; Tang et al., 2012) and experimental (Fillingim & Maixner, 1995; Unruh, 1996; France & Suckowiecki, 1999; Fillingim et al., 2009; Popescu et al., 2010) evaluations of pain additionally demonstrate greater nociceptive sensitivity in women. Although there are examples of comparable pain prevalence and sensitivity by sex (Robinson et al., 1998; Turk & Okifuji, 1999; Racine et al., 2012), the epidemiological consensus is that women have greater susceptibility to pain than men. It remains possible, however, that insufficient accommodation for pain modalities, pain severity, environmental conditions, social expectations, health care access, sample diversity, quantitative methodology, and statistical power confound these conclusions, as such shortcomings are acknowledged in meta-analyses for investigations of clinical pain (Wiesenfeld-Hallin et al., 2005; Fillingim et al., 2009; Hashmi & Davis, 2014; Steingrimsdottir et al., 2017).

Preclinical studies have reinforced this notion that sex is an important determinant of the pain experience. Animal models have corroborated conclusions from human studies of pain, with 85.4% of papers finding that female rodents are more sensitive to pain than males (Mogil et al., 2020). Since biological sex is defined by a spectrum of characteristics, including complex interactions between genetics, anatomical development, and hormones, rather than a binary

difference in a single trait, it has been speculated that multiple biological mechanisms contribute to the differential regulation of pain in males and females. Past investigations into these mechanisms validate that several systems are involved, spanning the peripheral nervous system, immune system, and central nervous system (Mogil et al., 2020). At the genetic level, sex-specific transcriptome changes in immune response and neuronal plasticity have been reported in neuropathic pain patients (North et al., 2019; Ray et al., 2019), while rodent models of neuropathy show a 10% increase in sex-dependent gene modulation compared to uninjured subjects (Stephens et al., 2019). At the protein level, ubiquitous molecular systems such as γ-aminobutyric acid (GABA), dopamine (DA), serotonin (5-HT), calcitonin gene-related peptide, phosphokinases, and toll-like receptors have been implicated in sex-specific contributions to pain (Joseph et al., 2003; Joseph & Levine, 2003; Sorge et al., 2011; Hagiwara et al., 2013; Sorge et al., 2015; Nasir et al., 2016; Tonsfeldt et al., 2016; Araldi et al., 2017; Liu et al., 2017; Megat et al., 2018; Avona et al., 2019; George et al., 2019; Luo et al., 2019; see Mogil et al. [2020] for comprehensive review), with the best known example being the modulatory role of sex hormones on microglia and T-cells in the spinal cord (Sorge et al., 2011; Sorge et al., 2015; Dance, 2019). Additional cases of sex-specific mechanistic drivers are likely to exist, as female subjects are still significantly underrepresented in preclinical pain research. For papers published between 1996 and 2005, 79% of non-human animal experiments exclusively studied male rodents (Mogil, 2012). Although these percentages have marginally improved since 2005 (Mogil, 2020), the exclusion of female subjects in preclinical pain studies persists to this day, thus increasing the likelihood for unexamined mechanisms of pain.

The brain is an important source of sex-specific pain processing and modulation (Cahill, 2006). Although pain has traditionally been thought of as the peripheral engagement of nociceptors, central mediation also plays an important role in the functional expression of nocifensive responses and aversion (Feine et al., 1991; Riley et al., 2001; Sarlani et al., 2004; Kuba et al., 2005; Labus et al., 2008; Kano et al., 2013). Divergence in these pain-related

behaviors have been demonstrated by sex, with the use of passive coping strategies (e.g. quiescence, hyporeactivity) being more common in males and active coping strategies (e.g. escape, paw attending) being more common in females (Archer et al., 1975; Aloisi et al. 1994; Gruene et al., 2015; Keiser et al., 2017; Pellman et al., 2017; Shansky et al., 2018). Much of what we know about the neural mechanisms underlying these discrete pain-related behaviors come from opioid studies, as release of endogenous opioids like enkephalin or treatment with drugs like morphine can induce similar divergences in pain responses (van Ree & de Wied, 1980). In rodents, treatment with μ opioid agonists results in more potent analgesia and sedation in males, and greater reinforcement and locomotor stimulation in females (Klein et al., 1997; Craft, 2008; Bodnar & Kest, 2010; Bobzean et al., 2014; Mavrikaki et al., 2017; for further consideration [e.g. responses to other types of opioids], see Bartok & Craft [1997] and Giles & Walker [2000]). In humans, however, there is greater anti-nociceptive potency in women and more prevalent use in males, with much less known about the sedative and locomotor stimulation side-effects of opioids (Giles & Walker, 2000; Sarton et al., 2000; Chia et al., 2002; Niesters et al., 2010; Jones, 2017; Marsh et al., 2018; for alternative interpretations of clinical data, see Loyd & Murphy, 2014). Although the mechanisms behind these variances between species have yet to be addressed, similar regions of modulation are likely to explain such differences, as robust expression of opioid receptors have been confirmed for the spinal cord, hindbrain, and midbrain – major areas of pain processing and modulation – in both species (Mansour et al., 1995; Moy et al., 2020).

Central Mechanisms of Pain Modulation

Preclinical models have highlighted the contributions of the mesolimbic DA system and the descending pain pathways as key circuits for pain response through endogenous and drug-induced activation of the opioid system (Reichling et al., 1988; Vaughan & Christie, 1997; Kalyuzhny & Wessendorf, 1998; Chiou & Huang, 1999; Becker & Chartoff, 2019; Mogil, 2020).

These midbrain circuits have long been implicated in nociceptive processes, with more recent evidence linking these neural substrates to sex-dependent function.

i. Canonical Pathways of Pain and Pain-Related Drug Use: The Mesolimbic DA System

In the mesolimbic system, activation of presynaptic μ opioid receptors on GABA neurons of the ventral tegmental area (VTA) disinhibits DA release into downstream targets like the nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), and prefrontal cortex (Owstrowski et al., 1982; Matthews & German, 1984; Seutin et al., 1990; Leone et al., 1991; Gardner, 2011). DA projections from the VTA to the NAc form the canonical reward pathway, which has been shown to mediate the motivational (Phillips & LePiane, 1980; van Ree & de Wied, 1980; Bozarth & Wise, 1981a; 1981b; Van der Kooy et al., 1982; Jenck et al., 1987; Di Chiara & Imperato, 1988; Welzl et al., 1989; Koob, 1992; Spanagel et al., 1992; Tanda et al., 1997; Pierce & Kumaresan, 2006) and locomotor sensitization (Joyce et al., 1979; van Ree & de Wied, 1980; Kalivas et al., 1983; Vezina et al., 1987) effects of opioids and other drugs of abuse. These functions may be important for interactions between pain and drug use, as activation of presynaptic μ and δ opioid receptors on GABA neurons of the VTA opposingly regulates alcohol consumption in mice (Job et al., 2007; Margolis et al., 2008). Furthermore, inflammatory pain desensitizes μ opioid receptors in the VTA and DA release in the NAc to promote increased opioid and alcohol consumption in rats (Hipolito et al., 2015; Campos-Jurado et al., 2019). Sex differences in DA signaling additionally show that opioid and alcohol exposure results in greater DA release in the NAc of females (Castner et al., 1993; Blanchard & Glick, 2002; Laasko et al., 2002; Volkow et al., 2004; Walker et al., 2006; Job et al., 2007; Kritzer & Creutz, 2008; Johnson et al., 2010; Bobzean et al., 2014; for evidence to the contrary, see Munro et al., 2006) that correspond to fluctuations in estrogen levels (Shimizu et al., 1993; Xiao & Becker, 1994; Kritzer & Creutz, 2008; Johnson et al., 2010). These mechanisms are suggested to drive sex-specific impairments in sociability, similar to the isolating effects of

chronic pain (Duenas et al., 2006; Trainor, 2011; Campi et al., 2014; Borsook et al., 2016). The mesolimbic DA system may therefore be important for the pursuit and expression of pain relief, providing a possible mechanistic explanation for the sex-specific motivational outcomes observed with drugs of abuse (Taylor et al., 2016; Serafini et al., 2020).

ii. Canonical Pathways of Pain and Pain-Related Drug Use: The Descending Pain Pathways

In contrast to the mesolimbic DA system, more canonical and direct contributions to pain have been established for the descending pain pathways, which integrates signaling from the periaqueductal gray (PAG) to the rostral ventromedial medulla (RVM) and spinal cord (Fields et al., 1976; Fields et al., 1977; Anderson et al., 1977; Fields et al., 1983; Basbaum & Fields, 1978a; 1978b; 1979; 1984; Abols & Basbaum, 1981; Glazer & Basbaum, 1981; Basbaum, 1981; Sankuhler & Gebhart, 1984; Chang et al., 1986; Fang et al., 1989; Heinricher et al., 1989; Jensen & Yaksh, 1989; Potrebic et al., 1995; Ossipov et al., 2010; Lau & Vaughan, 2014; Tobaldini et al., 2018; Bannister & Dickenson, 2020). Seminal work by Howard Fields, Allen Basbaum, and colleagues have established a direct contribution of these structures to pain modulation, where μ and κ opioid agonists disinhibit glutamatergic PAG projections to drive activation of RVM off-cells, resulting in the obstruction of incoming nociceptive information to second order neurons in the spinal cord (Fields et al., 1983; Chang et al., 1986; Fang et al., 1989; Heinricher et al., 1989; Jensen & Yaksh, 1989). Activation of the pathway produces analgesia and has been shown to regulate pain through both endogenous (e.g. stress) and exogenous (e.g. drug use) processes (Butler & Finn, 2009; Ossipov et al., 2010).

Sex differences have been reported for analgesic processes related to the descending pain pathways (Lipa & Kavaliers, 1990; Ge et al., 2004; Bryant et al., 2006; Liu et al., 2007; Juni et al., 2008; Liu & Gintzler, 2013), but a direct evaluation of these circuits has only been performed for the PAG-RVM pathway (Tershner et al., 2000; Loyd et al., 2007; 2008; Loyd & Murphy, 2006; 2009; Bobeck et al., 2009; Doyle et al., 2017). Previous studies have shown that

morphine (μ/δ opioid agonist) produces greater PAG-RVM activation and tolerance in male rats (Krzanowska & Bodnar, 1999; Loyd et al., 2007; 2008), while inflammatory pain preferentially activates the PAG-RVM circuit in males to produce comparable nociceptive sensitivity by sex, suggesting different mechanisms may be driving endogenous pain modulation in females (Loyd & Murphy, 2006). Support for this theory from Anne Murphy and colleagues revealed that morphine interactions with toll-like-receptor 4 on PAG microglia prevents anti-nociception in females (Doyle et al., 2017). These investigations implicate opioid signaling in the PAG as a mechanism for sex-specific expression of pain. Knowing that the structure is important for "fight-or-flight" behaviors, it is also possible that these mechanisms contribute to defensive behaviors and avoidance learning (Di Scala et al., 1987; Vaccarino & Corrigall, 1987; Fanselow, 1991; 1994; Bandler & Shipley, 1994; Bandler et al., 2000; Morgan & Carrive, 2001; Miranda-Paiva et al., 2003; Chieng et al., 2005; Sukikara et al., 2006; Butler et al., 2011; Koutsikou et al., 2014; Twardowschy et al., 2015; Walker et al, 2019), but this has yet to be determined, as midbrain contributions to the functional outcomes of pain have not been extensively studied in both males and females.

iii. Therapeutic Implications of Pain-Related Drug Use by Sex: The Need for Novel Targets

Establishing a more complete understanding of sex-specific mechanisms in pain will have major implications for pain management. Opioids, alcohol, and other accessible drugs of abuse remain a common source of intervention for chronic pain patients. Approximately 25% of pain suffering patients consume alcohol for symptom relief, with men more frequently self-medicating with than women (Riley & King, 2009; Wilsnack et al., 2009; Brennan et al., 2011). By contrast, female patients experience greater severity and poorer outcomes following repeated use of morphine or alcohol than their male counterparts (Rubonis et al., 1994; Brennan et al., 2005; Egli et al., 2012; Kennedy et al., 2013; Hitschfeld et al., 2015; Boissoneault et al., 2019). Women specifically develop compulsivity at a more rapid pace

(Westermeyer & Boedicker, 2000; Greenfield et al., 2010) and suffer greater negative affect in withdrawal than men (Becker & Koob, 2016). These clinical findings show that men are more likely to consume drugs for analgesia, while women are more likely to experience worse outcomes with repeated drug use.

Sex-specific outcomes of pain treatments have been reported for opioids (Craft, 2003; 2008; Bartley & Fillingim, 2013), alcohol (Egli et al., 2012; Boissoneault et al., 2019), Cerebrolysin (Morales-Medina et al., 2019), fluoxetine (Zammataro et al., 2017), and marijuana (Cooper & Haney, 2016), with the directionality of anti-nociceptive bias varying by drug. This suggests that several mechanisms may contribute to differences in pain susceptibility between men and women. For example, discrete treatment outcomes are posited to promote drug relapse in women, as female patients undergoing pain management experience short-lived improvements compared to male patients (Keogh et al., 2005; Pieh et al., 2012). These therapeutic shortcomings are especially problematic since pathological pain not only shares co-morbidity with drug dependence, but depression and anxiety disorders as well (Bair et al., 2003; Ballantyne et al., 2007; Egli et al., 2012; Zale et al., 2015). Drug withdrawal and associated negative emotional states have been shown to worsen pain (Gatch & Lal, 1999; Rhudy & Meagher, 2000; Angst et al., 2003; Ballantyne et al., 2007; Lumley et al., 2011; Egli et al., 2012; Zale et al., 2015), as the interplay between pain and drug use may bidirectionally drive pathology through impairments in mood (Lumley et al., 2011; Egli et al., 2012). More mechanistic evaluations on how these co-morbidities develop are necessary, especially in the context of sex-dependent treatment outcomes, as knowledge on how to target these interactions will be informative for personalized approaches to pain management.

The PAG/DR is a Dynamic Integrator of Pain Circuitry

The midbrain, a phylogenetically conserved portion of the brainstem, consists of a central gray area surrounding the cerebral aqueduct that is comprised of the PAG and dorsal

raphe (DR). These structures exhibit great anatomical and molecular diversity that work in synchrony to control “fight-or-flight” behaviors and is regarded as an essential facilitator of behavioral adaption. The PAG/DR drives alterations in pain modulation, motivated behaviors, and autonomic function in response to the environment. By integrating sensory and emotional information in real-time, these structures can rapidly prepare an organism for physical engagement of threats, which is necessary for mitigating the impact of various contextual challenges. The PAG has been extensively studied for its roles in anti-nociception, escape, freezing, vocalization, heart rate, and vasodilation/vasoconstriction (Bandler & Shipley, 1994; Bandler et al., 2000). These functions are generally segregated by columnar organization within the structure, as active coping strategies are elicited by the dorsolateral PAG (dlPAG; which enables confrontational defensive behaviors, hypertension, tachycardia, extracranial vasodilation, hindlimb and renal vasoconstriction, and non-opioid analgesia) and lateral PAG (lPAG; which enables flight, hypertension, tachycardia, hindlimb vasodilation, extracranial and renal vasoconstriction, and non-opioid analgesia), and passive coping strategies are evoked by the ventrolateral PAG (vlPAG; which enables quiescence, hyporeactivity, hypotension, bradycardia, and opioid analgesia; see Carrive [1993], Bandler & Shipley [1994], and Bandler et al. [2000] for comprehensive reviews). In compliment to the nociceptive and locomotor survival functions of the PAG, the DR exhibits important contributions to affective-motivational behaviors, with roles in pain (Qing-Ping & Nakai, 1994), mood (Cools et al., 2008), reward (Cohen et al., 2015; Li et al., 2016), aversion (Hayashi et al., 2015), social interaction (Dolen et al., 2013; Niederkofler et al., 2016), motor activity (Jacobs & Fornal, 1997), and arousal (Monti, 2010). These multifaceted functions have all been implicated in threat response and are likely to be important contributors to pain outcomes, as a wide range of modulation is necessary for an organism to adapt to the challenges of any given environment.

The dynamic functions of the PAG/DR are enabled by diverse neurochemical compositions and connectivity **(Figures 1-2)**. PAG neurons produce glutamate (Glu) and GABA,

with marked co-expression of DA (Hokfelt et al., 1984; Trulson et al., 1985; Descarries et al., 1986; Arsenault et al., 1988; Stratford & Wirtshafter, 1990; Charara & Parent, 1998; Flores et al., 2004; Flores et al., 2006; Lu et al., 2006), neurotensin (NT) (Beitz et al., 1983; Shipley et al., 1987; Zhong et al., 2019), and vasoactive intestinal polypeptide (VIP) (Loren et al., 1979; Moss & Basbaum, 1983; Smith et al., 1994; Paspalas et al., 2000; Dougalis & Matthews et al., 2012). Other pain-related peptides, including enkephalin, dynorphin, substance P, and 5-HT, have been reported in the PAG as well (Moss et al., 1983; Moss & Basbaum, 1983; Li et al., 1990; Charara & Parent, 1998). Extensive characterization of DR cell types has revealed 30-50% of neurons express 5-HT, with notable presence of Glu, GABA, DA, and neuropeptides (e.g. dynorphin, cholecystokinin) in the region as well (Fu et al., 2010; Okaty et al., 2015; Huang et al., 2019; Ren et al., 2018; 2019). These molecular markers have been shown to anatomically overlap between the PAG and DR, as neurons that co-express Glu with DA or 5-HT transect both structures (Charara & Parent, 1998; Li et al., 2016; Matthews et al., 2016; Taylor et al., 2019; Wang et al., 2019). Whether there is functional divergence between molecularly similar cell types in each structure is unclear, however, and remains to be determined.

The connectivity of PAG and DR exhibits many analogous circuits. The PAG/DR generally integrate sensory input from the spinal cord (Mantyh & Peschanski, 1982; Carrive, 1993; Bandler & Shipley, 1994; Bandler et al., 2000) and exhibit projections to the hindbrain (medulla [Basbaum & Fields, 1984; Ren et al., 2018], locus coeruleus [Ennis et al., 1991], laterodorsal tegmentum [Lu et al., 2006]), limbic system (BNST [Li et al., 2016; Matthews et al., 2016; Ren et al., 2018; Lin et al., 2020], central nucleus of the amygdala [CeA] [Waselus et al., 2012; Li et al., 2016; Matthews et al., 2016; Groessl et al., 2018; Ren et al., 2018; Lin et al., 2020], thalamus [Krout et al., 2000], hypothalamus [Li et al., 2014; Ren et al., 2018]), and cortex (basal forebrain [Lu et al., 2006], prefrontal cortex [Waselus et al., 2012; Ren et al., 2018]) to shape behavior **(Figures 1-2)**. Previous studies have established that divergent functional organization can be segregated by projection targets, with Glu neurons in the vlPAG selectively

contributing to freezing (but not anti-nociceptive) behaviors through projections to the magnocellular nucleus in the medulla (Tovote et al., 2016), and 5-HT neurons in the DR separating contributions to reward/aversive states based on cortical and subcortical projections (Ren et al., 2018). Such delineations suggest that the extensive behavioral repertoire of PAG/DR is facilitated through a combination of cell type and circuit interactions. Additional research to clarify these distinctions is necessary to understand how these structures work as a system. In summary, the PAG/DR may encompass a heterogeneous collection of cell type- and projection-specific functions that contribute to the dynamic nature of pain responses. The behavioral consequences of this circuit-level diversity, however, has yet to be fully characterized.

Rationale for Dissertation

In the present dissertation, we will focus on cell type- and projection-specific interactions between the vlPAG/DR and BNST to evaluate whether these non-canonical systems of pain modulation contribute to sex-dependent outcomes. This is informed by promising evidence in the literature that suggests a role for DA neurons in the vlPAG/DR and downstream interactions with corticotropin releasing factor (CRF) neurons in the BNST in the regulation of pain and pain-related drug use. We predict that vlPAG/DR and BNST will exhibit sex-specific modulation of pain through these DA and CRF signaling systems, with the rationale for this hypothesis supported by the following lines of evidence:

i. Midbrain DA Exhibits Robust Inputs to the Extended Amygdala

DA neurons in the vlPAG/DR were first characterized in a series of neuroanatomical studies, where they were reported to be a dorso-causal extension of the VTA (Hokfelt et al., 1984; Trulson et al., 1985; Descarries et al., 1986; Arsenault et al., 1988; Stratford & Wirtshafter, 1990; Charara & Parent, 1998; Flores et al., 2004; Flores et al., 2006; Lu et al.,

2006). Similar to DA neurons in the VTA, this vlPAG/DR subpopulation is disinhibited by μ opioid agonists and exhibits multifaceted roles for pain, arousal states, and value representation (Flores et al., 2004; Flores et al., 2006; Lu et al., 2006; Li et al., 2016; Matthews et al., 2016; Cho et al., 2017; Groessl et al., 2018; Taylor et al., 2019; Porter-Stransky et al., 2019; Cho et al., 2020; Lin et al., 2020). Notably, the vlPAG/DR and VTA have robust outputs to the extended amygdala, where co-release of DA and Glu activate the BNST and CeA, suggesting that these extended amygdala structures may be important loci of DA signaling for pain modulation.

ii. vlPAG/DR and BNST Form Reciprocal Pathways that are Functionally Uncharacterized

A notable feature of the PAG/DR is that these structures share many downstream targets but only receive reciprocal innervation from a subsection of these targets **(Figures 1A-1B)**. This includes the extended amygdala, where the vlPAG/DR sends excitatory outputs to the BNST and CeA and receives reciprocal inhibitory projections back. Previous studies looking at the function of reciprocal circuits between the vlPAG/DR and CeA have established a critical role for these structures in pain and aversive learning (Rizvi et al., 1991; Avegno et al., 2018; Groessl et al., 2018; Li & Sheets, 2018). However, similar circuit-level investigations have yet to be performed for the vlPAG/DR and BNST. These connections between the midbrain and extended amygdala indicate an exciting prospect for reciprocal inhibition to enhance representations of salient experiences such as pain, since the midbrain has been proposed to incorporate similar motifs of inhibition to hierarchically select incoming information on environmental stimuli (Friesen, 1994; Mysore & Knudsen, 2012). Other reciprocal targets of the PAG/DR, such as the hypothalamus and prefrontal cortex, may also interact with the extended amygdala for an additional layer of modulation (Amat et al., 2005; Marcinkiewcz & Mazonne et al., 2016; Hao et al., 2019; Hwa et al., 2020).

iii. Sex-Specific Mechanisms of Pain: A Possible Role for DA Signaling in vlPAG/DR and BNST

In humans, the functional connectivity of PAG/DR and downstream limbic structures have been reported to differ between men and women when exposed to pain (Linnman et al., 2012), but the mechanisms behind these differences are unclear. Only a few molecular drivers of sex differences have been identified for pain (Mogil et al., 2020), with multiple cases demonstrating a role for DA signaling in the vlPAG/DR and BNST. Systemic knockout of the DA receptor D3 (D3R) generally results in more robust hypoalgesia in female mice, with the greatest sex differences observed with late-phase formalin responses (Liu et al., 2017). A similar reduction of DA receptor D5 (D5R) diminishes acute pain and hyperalgesia in mice, with males exhibiting greater dependence on spinal D5R than females for intact pain sensitivity (Megat et al. 2018). More specific manipulations of BNST show that local antagonism of DA receptor D1 (D1R) results in more frequent formalin responses for female rats (Hagiwara et al., 2013). These data suggest that inactivation of D1-like receptors worsens pain, while inactivation of D2-like receptors increases pain tolerance in a more pronounced manner for female subjects. DA signaling in the brain is thus an important contributor to sex differences in pain.

In the midbrain, greater involvement of the vlPAG-RVM circuit has been observed with morphine anti-nociception and tolerance in male rats (Loyd et al., 2009), while the same outcomes appear to be prevented by morphine-microglia interactions in females (Doyle et al., 2017). Furthermore, persistent inflammatory pain increases presynaptic GABA release but decreases high affinity tonic $GABA_A$ currents exclusively in vlPAG neurons of female rats, resulting in sex-specific morphine anti-nociception (Tonsfeldt et al., 2016). These data suggest that opioids and persistent inflammation can alter GABA signaling in the vlPAG and influence pain sensitivity differently for males and females. Considering that the GABA neurons that gate PAG-RVM are likely to gate activation of DA neurons in the vlPAG/DR as well (Li et al., 2016; Lin et al., 2020), it is possible that $vlPAG^{DA+}$-BNST circuits contribute to sex-specific pain in a similar manner. Previous work from our lab and others have shown that $vlPAG/DR^{DA+}$ activation

reduces pain and sends robust projections to the BNST (Li et al., 2016; Taylor et al., 2019), but these evaluations were limited to male mice and did not take into account the functional role of downstream circuits. Further investigations into the contributions of this DA subpopulation are warranted for both sexes, as the literature has strongly implicated DA signaling in the vlPAG/DR and BNST in a modulatory role for sex-specific expressions of pain.

iv. Evidence for a vlPAG/DR^{DA+}-BNST Circuit

Similar to the vlPAG/DR, the BNST is a center of integration for value representation, motivated behaviors, threat response, and drug use (Kash et al., 2015; Avery et al., 2016; Lebow & Chen, 2016). Although the BNST is not well characterized for pain, the structure has recently been implicated to drive the sensory and affective-motivational components of pain through CRF signaling (Deyama et al., 2009; Tran et al., 2012; Ide et al., 2013; Tran et al., 2014; Minami, 2019; Takahashi et al., 2019; Hara et al., 2020). Evidence for DA-CRF interactions reveal that DA enhancement of Glu in the BNST is gated by CRF receptor 1 (CRF_{R1}) (Kash et al., 2008). Although the source of DA for this modulation has been assumed to be the VTA, the vlPAG/DR remains an intriguing possibility (Silberman et al., 2013). Since vlPAG/DR^{DA+} neurons co-express VIP (Dougalis et al., 2012) and VIP neurons in the vlPAG/DR terminate onto BNST^{CRF+} neurons, vlPAG/DR^{DA+} neurons may directly influence CRF signaling by innervating BNST^{CRF+} neurons (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1997; Kozicz et al., 1998; Silberman et al., 2013). A more direct indication by Meloni et al. (2006) shows that the majority of DA neurons innervating BNST^{CRF+} neurons comes from the vlPAG/DR. Therefore, vlPAG/DR^{DA+} neurons may be interacting with CRF signaling through direct transmission to BNST^{CRF+} neurons. Sex-specific contributions through this system may be possible, since the BNST exhibits male and female differences in anatomy (Allen et al., 1990), parenting behaviors (de Vries, 2008), CRF distribution (Uchida et al., 2019), and DA modulation of pain (Hagiwara et al.., 2013).

To summarize, a critical evaluation of the literature indicates that (i) activation of vlPAG/DR^{DA+} reduces pain (Flores et al., 2004; Li et al., 2016; Taylor et al., 2019), (ii) vlPAG/DR^{DA+} neurons project to the BNST (Li et al., 2016; Matthews et al., 2016; Groessl et al., 2018; Lin et al., 2020), (iii) DA signaling in the BNST drives sex-specific effects on pain (Hagiwara et al., 2013), (iv) DA signaling from vlPAG/DR is likely to interact with BNST^{CRF+} neurons (Meloni et al., 2006; Dougalis et al., 2012), and (v) inhibition of CRF signaling in the BNST reduces pain (Tran et al., 2014; Ide et al., 2016). Based on these converging lines of evidence, we hypothesize that vlPAG/DR^{DA+} and BNST^{CRF+} neurons make mutual and complementary contributions to sex differences in pain.

Chapter Aims

The overall goal of the work described in this dissertation is to examine the neural mechanisms underlying sex-dependent expressions of pain. We specifically designed a series of experiments that characterize the effects of vlPAG/DR^{DA+} and BNST^{CRF+} neurons on pain-related behaviors in male and female mice, with the intention of identifying molecular targets that may explain the discrete therapeutic outcomes reported for men and women in the clinic.

In Chapter 1, we assessed the function of vlPAG/DR^{DA+} neurons in male and female mice. Using a combination of chemogenetic, optogenetic, and CRISPR/Cas9 approaches, we examined how activation of vlPAG/DR^{DA+} neurons and vlPAG/DR^{DA+} terminals in the BNST regulated the expression of pain-related behaviors. The hypothesis for this initial set of experiments was that vlPAG/DR^{DA+} neurons would make sex-specific contributions to nociceptive sensitivity and locomotion that were dependent on DA signaling to the BNST. This was based on previous evaluations of the vlPAG/DR showing that activation of local excitatory neurons can reduce supraspinal pain (Li et al., 2016; Taylor et al., 2019), as well as more general observations for sex-specific locomotor strategies in threatening contexts (Krzanowska & Bodnar, 2000; Shansky, 2018). Few studies to date have functionally characterized

vlPAG/DR^{DA+} neurons (Flores et al., 2004; Flores et al., 2006; Lu et al., 2006; Li et al., 2016; Matthews et al., 2016; Cho et al., 2017; Groessl et al., 2018; Taylor et al., 2019; Porter-Stransky et al., 2019; Cho et al., 2020; Lin et al., 2020), while no studies have investigated the behavioral consequences of activating vlPAG/DR^{DA+} terminals in the BNST or compared the function of this DA subpopulation in both sexes. Therefore, in this first chapter, we provide a novel assessment of vlPAG/DR^{DA+} function in the context of pain for male and female subjects, with the intention of obtaining more knowledge on how DA signaling contributes to sex differences in pain.

In Chapter 2, we looked downstream to BNST^{CRF+} neurons and examined the role of local CRF expression in pain processing and modulation. We first used *in vivo* calcium imaging to observe single-cell resolution encoding of noxious stimuli by BNST^{CRF+} neurons of male and female mice. These experiments were then followed up by genetic deletion of *Crf* in the BNST to evaluate the necessity of peptide expression for intact pain responses. Although CRF signaling in the BNST has previously been shown to alter the sensory and affective-motivational components of pain, these effects were observed for differing modalities of distress and did not investigate the source of CRF driving these behaviors (Deyama et al., 2009; Tran et al., 2012; Ide et al., 2013; Tran et al., 2014; Minami, 2019; Takahashi et al., 2019; Hara et al., 2020). External sources of CRF from the CeA (among other inputs, like the basolateral amygdala and parabrachial nucleus [Crestani et al., 2013; Miles & Maren, 2019]) have been shown to drive aversive states through the BNST (Asok et al., 2018; de Guglielmo et al., 2019; Pomrenze et al., 2019), but the role of local CRF has been explored to a lesser extent (Walker et al., 2009). Based on the expected results of local CRF receptor engagement, we hypothesized that BNST^{CRF+} neurons would exhibit robust recruitment of activity in response to noxious stimuli and that CRF deletion in the BNST would alter the expression of pain responses in a modality-dependent manner. Considering that CRF signaling has exhibited stark differences in the context of stress for multiple brain regions, with divergences in receptor expression, distribution, trafficking, and signaling leading to increased stress sensitivity in females (Bangasser &

Wiersielis, 2018), we additionally predict that CRF manipulations in the BNST will have greater effects on female subjects. The overall goal of this second chapter is to determine the contributions of CRF in the BNST to pain-related behaviors in male and female subjects, as this well-known but understudied source of CRF has yet to be evaluated in the context of pain.

In Chapter 3, we examine the role of these DA and CRF neurons in pain-induced drug use. Combining a preclinical model of persistent inflammatory pain (CFA) with a voluntary consumption paradigm for alcohol (2-bottle choice), we assessed how male and female mice engage in self-medicating behaviors following injury. This experiment enabled us to develop a novel model for pain-drug interactions and compare outcomes to those reported in clinical investigations. We then validated this model by studying the impact of *Crf* deletion in the BNST on pain-induced drinking and compared how similar methodological approaches with DA in the vlPAG/DR influenced the anti-nociceptive and locomotor effects of morphine. The main hypotheses tested in this chapter was whether (i) inflammatory pain would drive sex-specific increases in alcohol consumption and (ii) local impairments in CRF and DA expression could prevent maladaptive drug use. Considering that vlPAG/DR^{DA+} and BNST^{CRF+} neurons are sensitive to morphine and alcohol (Carboni et al., 2000; Veinante et al. 2003; Flores et al., 2004; Flores et al., 2006; Shalev et al., 2006; Jaferi et al., 2009; Silberman & Winder, 2013; Li et al., 2013; Pliel et al., 2015; Li et al., 2016; Lin et al., 2020) and may directly drive drug use (Flores et al., 2004; Flores et al., 2006; Pliel et al., 2015; Rinker et al., 2017; Lin et al., 2020), our genetic deletion approaches were predicted to attenuate any observed interactions of pain and drug use. The proposed involvement of these DA and CRF mechanisms in sex differences is additionally expected to influence these pain-drug outcomes.

In summary, the experiments in this dissertation were designed to elucidate the role of vlPAG/DR^{DA+} and BNST^{CRF+} neurons in the sex-specific expression of pain. Using a combination of cutting-edge techniques, we were able to highlight the functional contributions of these DA and CRF signaling systems to pain and pain-related drug use. These investigations on

vlPAG/DR^{DA+} and BNST^{CRF+} uncovered critical insight that may explain how pain can manifest in divergent ways for males and females. We believe that the distinct circuit mechanisms described in this dissertation will have a significant impact on our collective understanding of pain modulation by sex.

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
5-HT	serotonin
AA	acetic acid
AAV	adeno-associated virus
ANOVA	analysis of variance
BEC	blood ethanol content
BF	basal forebrain
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CA2BC	continuous access 2-bottle choice
CaMKII	calmodulin-dependent protein kinase II
Cas9	CRISPR associated protein 9
Cb	cerebellum
CeA	central nucleus of the amygdala
CFA	Complete Freund's Adjuvant
ChR2	channelrhodopsin-2
CNO	clozapine-N-oxide
CORT	corticosterone
CRF	corticotropin-releasing factor
CRF_{R1}	corticotropin-releasing factor receptor 1
CRF_{R2}	corticotropin-releasing factor receptor 2
CRISPR	clustered regularly interspaced short palindromic repeats
D1R	dopamine receptor D1
D2R	dopamine receptor D2
D3R	dopamine receptor D3

D5R	dopamine receptor D5
DA	dopamine
DBH	dopamine β-hydroxylase
ddH2O	double-distilled water
DIO	double-floxed inverted orientation / double-floxed inverted open reading frame
dlPAG	dorsolateral periaqueductal gray
DR	dorsal raphe
DREADD	designer receptors exclusively activated by designer drugs
DYN	dynorphin
EF1α	elongation factor 1 alpha
EPM	elevated plus maze
EtOH	ethanol
FOV	field-of-view
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid A receptor
Glu	glutamate
GRIN	gradient-index
H2O	water
hM3Dq	human M3 muscarinic receptor
HPC	hippocampus
hSyn	human synapsin
HYP	hypothalamus
IA2BC	intermittent access 2-bottle choice
IDPS	Inscopix Data Processing Software
IHC	immunohistochemistry
i.p.	intraperitoneal

ISH	in situ hybridization
KA/P	kynurenic acid/picrotoxin
KOR	κ opioid receptor
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
lPAG	lateral periaqueductal gray
MOR	μ opioid receptor
NAc	nucleus accumbens
NDRI	norepinephrine dopamine reuptake inhibitor
NE	norepinephrine
NIH	National Institutes of Health
NT	neurotensin
NTS	nucleus of the solitary tract
oEPSC	optically-evoked excitatory postsynaptic current
oIPSC	optically-evoked inhibitory postsynaptic current
PAG	periaqueductal gray
PB	parabrachial nucleus
PCA/ICA	principle component analysis/independent component analysis
PFC	prefrontal cortex
PKCδ	protein kinase C δ
PVT	paraventricular thalamus
REM	rapid eye movement
ROI	region of interest
RVM	rostral ventromedial medulla
SABV	sex as a biological variable
saCas9	Staphylococcus aureus CRISPR associated protein 9

s.c.	subcutaneous
sg	single guide
SOM	somatostatin
SSRI	selective serotonin reuptake inhibitor
SUDO	simplified up-down method
TH	tyrosine hydroxylase
THA	thalamus
VTA	ventral tegmental area
VIP	vasoactive intestinal peptide
vlPAG	ventrolateral periaqueductal gray

LIST OF SYMBOLS

α	alpha
δ	delta
ΔF/F	delta F / F
κ	kappa
μ	mu

CHAPTER 1. DOPAMINERGIC PROJECTIONS FROM THE PERIAQUEDUCTAL GRAY/DORSAL RAPHE TO THE BED NUCLEUS OF THE STRIA TERMINALIS CONTRIBUTE TO SEX DIFFERENCES IN PAIN-RELATED BEHAVIORS

Introduction

Pain is a complex sensory experience that signals vulnerability to harm and motivates actions for self-preservation. Evidence suggests that men and women respond to pain differently, as variable strategies for coping contribute to the risk and severity of chronic pain (Bartley & Fillingim, 2013). The midbrain plays a well-established role in pain responses, with structures like the periaqueductal gray (PAG) and dorsal raphe (DR) having optimal anatomical positioning to integrate relevant contextual information with fight-or-flight behaviors (Wang & Nakai, 1994; Behbehani, 1995). The ventrolateral column of the PAG (vlPAG) and the neighboring DR are known generators of potent analgesia and enhanced opioid-induced anti-nociception (Hosobuchi et al., 1977; Mayer & Liebeskind, 1974; Fardin et al., 1984; Morgan et al., 1991; Li et al., 1993; Cai et al., 2014; McDevitt et al., 2014; Tovote et al., 2016). Activation of these structures additionally elicits active and passive coping responses to the environment, enabling the use of adaptive anti-nociceptive or locomotor strategies to minimize the impact of pain (Bandler & Shipley, 1994; Maier et al., 1995). Subpopulations of vlPAG/DR have been identified as segregated drivers of these functions, with glutamate (Glu) neurons producing pain relief and escape behaviors in opposition to γ-aminobutyric acid (GABA) neurons (McDevitt et al., 2014; Tovote et al., 2016; Samineni et al., 2017; He et al., 2019; Seo et al., 2019; Vaaga et al., 2020). Notably, these cell types have been implicated in the sex-dependent modulation of pain through opioid signaling, as morphine inhibition of GABA neurons and disinhibition of Glu neurons drives analgesia to a greater extent in male rodents than females (Loyd et al., 2007;

Doyle et al., 2017). Considering that pain disproportionately affects women across the lifespan (Vetvik & MacGregor, 2017), with effective pain management limited by our knowledge on mechanistic drivers of pain in female subjects (Mogil, 2012; Bartley & Fillingim, 2013; Melchior et al., 2016; Gupta et al., 2017; Sorge & Totsch, 2017; Mogil, 2020), more insight on vlPAG/DR cell types and how they regulate the expression of pain in male and female subjects is needed.

Recent studies have implicated vlPAG/DR dopamine (DA) neurons in the modulation of pain sensitivity (Flores et al., 2004; Li et al., 2016). As a subset of local excitatory neurons, vlPAG/DR^{DA+} neurons co-express Glu and play a similar role in anti-nociception (Flores et al., 2004; Li et al., 2016; Tovote et al., 2016; Taylor et al., 2019). Work from our lab and others have shown that vlPAG/DR^{DA+} neurons are disinhibited by μ and κ opioid receptor agonists (Flores et al., 2004; Meyer et al., 2009; Li et al., 2016; Li & Kash, 2019; Lin et al., 2020) and activated by other analgesic drugs (Li et al., 2013) to reduce thermal and mechanical nociceptive sensitivity (Flores et al., 2004; Meyer et al., 2009; Li et al. 2016; Schoo et al., 2018; Taylor et al., 2019). These neurons exhibit additional contributions to arousal, fear learning, memory, reward, and locomotion (Flores et al., 2006; Lu et al., 2006; Cohen et al., 2015; Matthews et al., 2016; Cho et al., 2017; Groessl et al., 2018; Porter-Stransky et al., 2019; Vaaga et al., 2020; Lin et al., 2020), with an overarching effect on incentivized responses to environmental salience (Cho et al., 2017; Lin et al., 2020). Although it remains unclear how vlPAG/DR^{DA+} regulation of these functions come together to influence behavior, there is evidence for functional specificity of vlPAG/DR neurons based on downstream target regions (Tovote et al., 2016; Groessl et al. 2018), suggesting that vlPAG/DR^{DA+} may concurrently recruit circuits to elicit a diverse set of behaviors in response to pain.

Previous characterizations of vlPAG/DR^{DA+} and pain were performed exclusively in male subjects, leaving the function of these neurons undetermined in females. To address this disparity, the present study examines the role of vlPAG/DR^{DA+} neurons in regulating pain responses for male and female mice. We specifically focus on the contributions of vlPAG/DR^{DA+}

projections to the bed nucleus of the stria terminalis (BNST), since multiple studies have reported robust innervation without any functional characterization of the pathway (Li et al., 2016; Matthews et al., 2016; Lin et al., 2020). The BNST is a critical region for allocating emotional value to sensory information and likely plays an important role in pain (Hagiwara et al., 2013; Ide et al., 2013). Data from our lab has shown that the BNST can modulate the nociceptive and aversive aspects of pain differently in male and female mice (Yu et al., 2020), making the vlPAG/DR^{DA+}-BNST pathway a promising candidate for sex-specific regulation of pain-related behaviors. To test this hypothesis, we used a combination of technical approaches, including chemogenetics, optogenetics, slice physiology, and CRSIPR/Cas9-mediated knockout, to assess how vlPAG/DR^{DA+} neurons and vlPAG/DR^{DA+} projections to BNST impact nociceptive sensitivity and related incentive behaviors. We found that vlPAG/DR neurons drive adaptive anti-nociceptive and locomotor behaviors differently in male and female mice via DA transmission to the BNST, with the context of neuronal activation being a key determinant of behavioral outcomes. These findings establish a critical role for vlPAG/DR^{DA+} neurons in the sex-specific expression of pain-related behaviors and may be informative for the future development of precision medicine approaches to mitigate maladaptive responses to pain.

Materials and Methods

Animals

All animals were bred inhouse and maintained on a 12-h light/dark cycle (light on at 7:00, light off at 19:00) with rodent chow and water available *ad libitum*. Male and female TH-Cre mice (aged 6-12 weeks, C57BL/6J background) were group-housed with same-sex littermates until surgery or CFA treatment. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UNC Chapel Hill.

TH-Cre Details

The mouse strain used for this research project, STOCK Tg(Th-cre)FI172Gsat/Mmucd, RRID:MMRRC_029177-UCD, was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis, an NIH-funded strain repository, and was donated to the MMRRC by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT and Charles Gerfen, Ph.D., National Institutes of Health, National Institute of Mental Health.

Number of Animals per Experiment

-Figures 3-4: TH-Cre mice (Male mCherry control [n = 7], Male hM3Dq [n = 7], Female mCherry control [n = 7], Female hM3Dq [n = 7]).

-Figure 5: TH-Cre mice (Female mCherry control in Estrus [n = 7], Female mCherry control in Non-Estrus [n = 7], Female hM3Dq in Estrus [n = 7], Female hM3Dq in Non-Estrus [n = 7]).

-Figure 6: IHC Cohort #1 (S3A) used C57BL/6J mice from TH-Cre breeders (Males [n = 7], Females [n = 7]); IHC Cohort #2 (S3B-S3D) used DBH-eGFP mice (Males [n = 4], Females [n = 4]); ISH Cohort (S3E-S3G) used DBH-eGFP mice (Males [n = 4], Females [n = 2]).

-Figures 7-8: TH-Cre mice (Male eYFP [n = 7], Male ChR2 [n = 6-8], Female eYFP [n = 9-10], Female ChR2 [n = 8]).

-Figure 9: TH-Cre mice (Male eYFP [n = 5], Male ChR2 [n = 5], Female eYFP [n = 5], Female ChR2 [n = 6]).

-Figure 10: RTPP/RTPA (S5A-S5C) used TH-Cre mice (Male eYFP [n = 4], Male ChR2 [n = 5], Female eYFP [n = 5], Female ChR2 [n = 6]); Sociability test with same sex conspecifics (S5D-S5H) used TH-Cre mice (Male eYFP [n = 7], Male ChR2 [n = 8], Female eYFP [n = 5], Female ChR2 [n = 6]); Sociability test with opposite sex conspecifics (S5I-S5M) used TH-Cre mice (Male eYFP [n = 7], Male ChR2 [n = 8], Female eYFP [n = 8], Female ChR2 [n = 8]); Post-isolation sociability test with same sex conspecifics (S5N-S5R) used TH-Cre mice (Male eYFP [n = 4], Male ChR2 [n = 5], Female eYFP [n = 5], Female ChR2 [n = 6]).

-Figures 11 and 13: TH-Cre mice used for E/I transmission experiments (Male ChR2 [n = 5-6], Female ChR2 [n = 3-4]; number of cells per mouse in Figure Captions).

-Figures 12 and 13: TH-Cre mice used for DA transmission experiments (Male ChR2 [n = 4], Female ChR2 [n = 5-6]; number of cells per mouse in Figure Captions).

-Figures 14 and 15: TH-Cre mice (Male hM3Dq + sghTH control [n = 7], Male hM3Dq + TH CRISPR [n = 7], Female hM3Dq + sghTH control [n = 7], Female hM3Dq + TH CRISPR [n = 7]).

Stereotaxic Surgery

Adult mice (> 6 weeks of age) were anesthetized with isoflurane (1-3%) in oxygen (1-2 l/min) and aligned on a stereotaxic frame (Kopf Instruments, Tujunga, CA). All surgeries were conducted using aseptic techniques in a sterile environment. Microinjections were performed with a 1 µl Neuros Hamilton syringe (Hamilton, Reno, NV) and a micro-infusion pump (KD Scientific, Holliston, MA) that infused virus at 100 nl/min. Viruses were administered unilaterally at an angle of 20º in the middle-posterior region of vlPAG/DR (450 nl for all experiments [exception: 500 nl for Figures 7-10 experiments]; relative to bregma: ML 0.00 mm, AP -4.50 mm, DV -3.63 mm). For experiments that required *in vivo* photostimulation, optical fibers were implanted bilaterally at -10º approximately 200 µm over the dorsal BNST (relative to bregma: ML ±0.90 mm, AP 0.23 mm, DV -4.15 mm) and secured with a dental cement headcap. After surgery, mice were given Tylenol water and allowed to recover for 3 weeks or longer before starting experiments.

Behavioral Testing

Chemogenetics

Behavioral testing was initiated 3 weeks after viral injection of Gq-coupled designer receptors exclusively activated by designer drugs (Gq DREADD: a modified human M3 muscarinic receptor [hM3Dq]) or the control fluorophore. 3 mg/kg CNO or vehicle was

administered with an intraperitoneal (i.p.) injection 30 minutes prior to testing to ensure activation of Gq signaling in vlPAG/DR^{DA+} neurons during the behavioral assay. CNO and vehicle administration were counterbalanced by day to account for the order of drug and test exposure, with at least one day between each test session.

Optogenetics

Behavioral testing was initiated 6-8 weeks after viral injection of ChR2 or the control fluorophore. Subjects were acclimated to fiber-optic patch cord (Doric Lenses Inc, Quebec, Canada) tethering three days prior to testing. During behavioral testing, mice with optical fibers implanted over the BNST received 473 nm photostimulation with 5-ms pulses at 20 Hz and 10-15 mW power for varying lengths depending on the assay.

Laser Protocol by Assay:

-Hargreaves: Every two trials alternate by Laser OFF and ON sessions (i.e. OFF: T1-T2,T5-T6, ON: T3-T4,T7-T8). For each “ON” trial, the laser is turned on for one minute prior to heat exposure and remains on until the hind paw withdraws from the heat source. Due to sustained anti-nociceptive effects following the initial Laser ON sessions (T3-T4), analysis was restricted T1-T4.

-Von Frey: The first day serves as a Laser OFF session, where subjects are tethered to the patch cable without any input from the laser. The second day is a Laser ON session, where the laser is on for the duration of the test.

-Acetic Acid + Nesting: The laser is turned on for two 15-minute epochs of a 60-minute test. The first and fourth epochs are Laser OFF sessions, while the second and third epochs are Laser ON sessions.

-Real-Time Place Preference/Aversion: The laser switches on whenever the subject is on the designated stimulation side. Conditional activation of the laser based on subject location is valid

for the duration of the test.

-Tail Immersion: A baseline measure is taken in the Laser OFF session. This is then followed by the Laser ON session an hour later, where the laser is on for the duration of the test.

-Sociability Test: Laser OFF and ON sessions are separated between two test days, where the assignment of laser status is counterbalanced for each day. The laser is on for the duration of the test during Laser ON sessions.

CRISPR/Cas9

Behavioral testing was initiated 3 weeks after viral injection of a 1:3 mix of Cre-inducible hM3Dq and TH CRISPR or CTRL. Viruses associated with TH deletion were provided by Dr. Larry Zweifel, with previous validation experiments detailed in Hunker et al. (2020). The same drug administration and counterbalancing parameters were maintained throughout all chemogenetic experiments (see *Chemogenetics* section).

Inflammatory Pain Model

To model inflammatory pain, subjects were given a 50 nl subcutaneous injection of Complete Freund's Adjuvant (CFA; Sigma, St. Louis, MO), an antigen consisting of heat-killed *Mycobacterium tuberculosum*, in the plantar surface of a single hind paw. Behavioral testing was initiated three days after paw injections, around the time that CFA exhibits maximum inflammatory hyperalgesia.

Hargreaves

The Hargreaves test was used to measure thermal nociceptive sensitivity (Hargreaves et al., 1988). Subjects were placed in Plexiglas boxes on an elevated glass surface and habituated to the behavioral apparatus for a minimum of 30 minutes. The mid-plantar surface of each hind paw was then exposed to a series of heat trials with 10-minute inter-trial intervals. Trials were

conducted with radiant heat exposures sequentially alternating between left and right paws. Six trials were conducted for all experiments except those using optogenetics, which required eight trials to balance light exposure sessions. Beam intensity was set to 25 on the IITC Plantar Analgesia Meter (IITC Life Science, Woodland Hills, CA), producing basal paw withdrawal latencies of approximately 4-6 seconds. A cutoff time of 20 seconds was set to prevent excessive tissue damage.

Von Frey

The Von Frey test was used to measure mechanical nociceptive sensitivity. Subjects were confined to Plexiglas boxes on a custom-made elevated metal wire surface (90 × 20 × 30 cm) and habituated to the behavioral apparatus for a minimum of 30 minutes. Nylon monofilaments of forces ranging from 0.008 to 2 grams (g) were applied to the hind paw using the simplified up-down method (SUDO) described in Bonin et al. (2014). Starting with a mid-range force (0.16 g), the filament was applied to the mid plantar surface of the hind paw for ten trials, then repeated with ascending or descending forces depending on the number of paw withdrawals. Withdrawal thresholds were defined as the minimum force (g) filament that elicits a withdrawal reflex for ≥50% of the trials.

Tail Immersion

The tail immersion test was used to measure the tail flick response following exposure to a water-based thermal nociceptive stimulus. Subjects were restrained in Wypall fold wipers (Kimberly-Clark, Irving, TX) and tails were exposed to 50º C water in the test apparatus (Isotemp 110 Water Bath; Fisher Scientific, Hampton, NH). The tail flick latency was measured in two consecutive trials, where readings were taken 1 cm apart on the tail and averaged together. A cutoff time of 10 seconds was set to minimize tissue damage.

Open Field

Subjects were placed into a white Plexiglas open field (50 × 50 × 25 cm) and allowed to freely explore the arena for 10 minutes. The center of the open field was defined as the central 25% of the arena, where light levels were approximately at 30 lux. Tracking of subject location and activity was achieved with EthoVision (Noldus Information Technologies, Wageningen, Netherlands).

Acetic Acid + Nesting

Nesting procedures were adapted from Negus et al. (2015). The home cages of singly housed mice were transferred from the colony room to a sound attenuated testing room, where subjects habituated for 30 minutes. To model pain-related functional impairments, 10 mg/ml injections (i.p) of ddH2O (Day #1) or 1% acetic acid (AA; Day #2) were administered prior to testing. Nestlets were then divided into six equally sized pieces and distributed into individual zones within the home cage. After 60 minutes, nesting activity was measured by the number of zones cleared. Following treatment with AA, visceral nociceptive sensitivity was determined by hand-scoring writhing behavior (i.e. abdomen contraction with hind limb extension [Bagdas et al. 2016]). Locomotor activity was tracked during ddH2O and AA sessions using EthoVision.

Real-Time Place Preference/Aversion

Subjects were placed in a custom-made black Plexiglas apparatus (52 × 26 × 26 cm) where counterbalanced sides of the arena were assigned for laser stimulation and non-stimulation. Exploration of the stimulation side resulted in 20-Hz laser stimulation, indicative of the rewarding or aversive properties driven by light-sensitive neuronal populations. Mice were able to freely explore the apparatus for 20 minutes, with activity tracking and laser status conducted through EthoVision.

Sociability Test

The three-chamber sociability test was used to compare preferences for interaction with a social target versus an object (Moy et al., 2004). The apparatus consisted of a rectangular Plexiglas box (62 × 37 × 22 cm) containing a central compartment and two end compartments. A 10” diameter wire cup was positioned in the top corner of each end compartment. Subjects were placed in the central compartment and allowed to habituate to the apparatus for 10 minutes. In a subsequent session, a conspecific adult mouse (~6-8 weeks of age) and object (colored labelling tape [Fisher Scientific, Hampton, NH]) were placed in the wire cups to form a social chamber on one end, and a non-social chamber on the other. Subjects were then left to explore the apparatus for 10 minutes. Chamber designations were randomly assigned and counterbalanced. All trials were recorded and tracked with EthoVision to determine the time spent in the social and non-social chambers, as well as the time spent exploring the conspecific mouse versus the object. The ratio of interaction time with each stimulus was then used as a measure of sociability. Subjects were first tested with male conspecific mice, then female conspecific mice, allowing for sociability and locomotion to be measured and categorized for same and opposite sex conspecific mice. After 24 hours of single housing (i.e. social isolation [Matthews et al., 2016]), subjects were tested again with male conspecific mice.

Locomotion

Subjects were tested in a locomotor activity chamber (Accuscan Instruments, Columbus, OH). Following a 30-minute habituation, mice were treated with an i.p. injection of saline or CNO, then returned to the apparatus, where they freely explored the chamber for an additional 90 minutes. Locomotor measures including distance traveled, velocity, and % mobility were tracked with EthoVision.

Physiology

Brain Slice Preparation

Brains were collected 6-12 weeks after infusion of AAV5-EF1α-DIO-ChR2-eYFP in the vlPAG/DR of male and female TH-Cre mice. Subjects were deeply anesthetized with isoflurane for rapid decapitation and tissue retrieval. Brains were immediately sectioned on a Leica 1200S vibratome (Leica Microsystems, Wetzlar, Germany), where coronal slices of the BNST (300 μm) were collected and transferred to a 30 ± 1°C Isotemp 110 Water Bath (Fisher Scientific, Hampton, NH) containing chambers submerged in oxygenated artificial cerebral spinal fluid (ACSF; in mM:124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$). Following an hour of incubation, slices were placed in a recording chamber (Warner Instruments) containing oxygenated ACSF (30°C) that flowed through at rate of 2 ml/min. vlPAG/DR^{DA+} terminals and BNST neurons were visualized using infrared differential interference contrast (DIC) video-enhanced microscopy (Olympus) and a 470 nm fluorescent LED illumination system (CoolLED, Andover, NH).

Slice Whole-Cell Electrophysiology

In TH-Cre mice, fluorescently labeled terminals expressing ChR2 in the BNST were visualized and stimulated with a 470 nm LED. The following stimulation protocols were used to assess optically-evoked excitatory and inhibitory transmission between vlPAG/DR and BNST neurons:

Voltage clamp: (i) Single 1-ms Pulse at -55 mV, (ii) Single 1-ms Pulse at +10 mV, (iii) Paired-Pulse Ratio at -55 mV with inter-sweep intervals (ISI) of 50, 100, 150, 200, and 250 ms, (iv) Paired-Pulse Ratio at +10 mV with ISI of 50, 100, 150, 200, and 250 ms. All experiments were conducted with slices in ACSF. A cesium-methanesulfonate-based internal solution (in mM: 135 cesium methanesulfonate, 10 KCl, 1 $MgCl_2$, 0.2 EGTA, 2 QX-314, 4 MgATP, 0.3 GTP, 20 phosphocreatine, pH 7.3, 285–290 mOsmol) was used for all voltage clamp experiments. In a

subset of these experiments, tetrodotoxin (TTX, 10 μM) and 4-aminopyridine (4-AP) was bath applied for 10 minutes to isolate monosynaptic currents.

Current clamp: 20 Hz (5-ms pulse width, 20 pulses). All experiments were conducted with slices in ACSF, then ACSF + 3 mM Kynurenic Acid + 25 μM Picrotoxin, then ACSF + 3 mM Kynurenic Acid + 25 μM Picrotoxin + 2 μM SCH23390 and/or 2 μM Sulpiride, with a ≥10-minute wash-on period allotted prior to repeating the protocol in a new external solution. A potassium gluconate-based internal solution (in mM: 135 K+ gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP, pH 7.3, 285–290 mOsmol) was used for all current clamp experiments. Recordings acquired with a Multiclamp 700B amplifier were digitized at 10 kHz, filtered at 3 kHz, and analyzed with Clampfit 10.7 (Molecular Devices, Sunnyvale, CA, USA). Borosilicate glass capillaries were pulled using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) to obtain pipette resistances ranging from 2 to 5 MΩ. Input and access resistance were monitored throughout experiments, with ≥20% changes in access resistance excluded for data analysis.

Fast-Scan Cyclic Voltammetry

DA detection was achieved by positioning a carbon fiber microelectrodes (CFME; made in-house) in the dorsal BNST, applying a potential of -0.4 V (vs Ag/AgCl), then rapidly ramping up to 1.3 V (at 400 V/sec) at a rate of 10 Hz (Tarheel CV, Labview; National Instruments, Austin, TX). Fluorescently labeled terminals expressing ChR2 in the BNST were then stimulated with light pulses from a 437 nm LED. Stimulation protocols varied by pulse (1 pulse, 20 Hz with 5, 10, 20, and 40 pulses [5-ms pulse width, 1 mW]) and frequency (2, 5, 10, 20, and 30 Hz with 20 pulses [5-ms pulse width, 1 mW]). Background subtracted cyclic voltammograms (CVs) were then analyzed using HDCV (UNC Chapel Hill). In a subset of these experiments, 2 μM sulpiride was bath applied for 10 minutes to assess the effects of D2R antagonism on DA release.

Histology

Immunohistochemistry

Mice were anesthetized with an injection of Avertin (1 ml, i.p.) and transcardially perfused with chilled 0.01 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in PBS. Brains were extracted and post-fixed in 4% PFA for 24 hours, then placed in PBS for long-term storage at 4° C. Coronal sections (45 µm thick) of vlPAG/DR, BNST, and other structures of interest were collected using a Leica VT1000S vibratome (Leica Microsystems, Nussloch, Germany) and stored in a 50% glycerol, 50% PBS solution at 4° C until immunohistochemistry was performed. Slices were repeatedly washed for 5-minute cycles in PBS, then permeabilized in a 0.5% Triton X-100/PBS solution for 30 minutes. After a 10-minute PBS wash and 1-hour immersion in blocking solution (0.1% Triton X-100/10% Normal Donkey Serum in PBS), the tissue was incubated overnight in primary antibody diluted in the blocking solution (anti-TH [1:1000]; anti-RFP [1:500]) at 4° C. On the following day, slices went through three 10-minute washes in PBS before being incubated for 2 hours in secondary antibodies diluted in PBS (Alexa Fluor 488 Donkey anti-Mouse [1:200]; Alexa Fluor Cy3 Donkey anti-Rabbit [1:200]). The tissue was then washed in PBS for four 10-minute cycles. Processed slices were mounted on slides and coverslipped using Vecta-Shield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) in preparation for imaging.

In Situ Hybridization

Following isoflurane anesthetization and rapid decapitation, the brains of C57BL/6J mice (6-8 weeks; Jackson Laboratory, Bar Harbor, ME) were collected and placed on aluminum foil, where they were immediately frozen on dry ice and stored in a -80° C freezer. Using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany), coronal sections of BNST (12 µM thickness) were obtained and directly mounted onto Superfrost Plus slides (Fisher Scientific, Hampton, NH), then kept at -80° C. In order to fluorescently label Drd1a and Drd2 mRNA in the

BNST, slices were preprocessed with 4% PFA and protease reagent, incubated with target probes for mouse Drd1a and Drd2, then fluorescently labeled with probes targeting the corresponding channels of each receptor (Drd1a in 550, Drd2 in 647; Advanced Cell Diagnostics, Newark, CA). The processed slides were then covered using Vecta-Shield Mounting Medium with DAPI in preparation for imaging.

Confocal Microscopy

All fluorescent images were acquired with the Zeiss 800 Upright confocal microscope and ZenBlue software (Carl Zeiss AG, Oberkochen, Germany), with equipment access granted through the Hooker Imaging Core at UNC Chapel Hill. Validation of virus expression/injection site, optical fiber placement, and immunoreactivity were accomplished with tiled and serial z-stack images obtained through a 20x objective (2 μm optical slice thickness). Images were processed in FIJI (Schindelin et al., 2012) for manual counting and ZenBlue (Carl Zeiss AG, Oberkochen, Germany) for automated counting.

Quantification and Statistical Analysis

Single-variable comparisons were made using paired and unpaired t-tests. Group comparisons were made using one-way ANOVA, two-way ANOVA, or two-way mixed-model ANOVA depending on the number of independent and within-subjects variables in a data set. Following significant interactions or main effects, post-hoc pairwise t-tests were performed and corrected using Sidak's or Tukey's post-hoc tests to control for multiple comparisons. Results of statistical testing are reported in figure legends with significance indicated through markers on figures. Data are expressed as mean ± standard error of the mean (SEM), with significance for p values below 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). All data were analyzed and visualized with standard statistical software packages from GraphPad Prism 8 (GraphPad Software, San Deigo, CA).

Results

vlPAG/DR^{DA+} Neurons Produce Sex-Specific Reductions in Pain Sensitivity

Activation of vlPAG/DR^{DA+} neurons has been shown to reduce pain in male mice (Li et al., 2016; Taylor et al., 2019), but whether these anti-nociceptive effects apply to their female counterparts has yet to be determined. In order to probe vlPAG/DR^{DA+} regulation of pain-related behaviors in both sexes, we first injected an adeno-associated virus carrying Cre-inducible hM3Dq (AAV8-hSyn-DIO-hM3Dq-mCherry; an excitatory designer receptor exclusively activated by designer drugs [DREADD]) or a control virus (AAV8-hSyn-DIO-mCherry) in the vlPAG/DR of adult male and female tyrosine hydroxylase (TH)-Cre mice **(Figure 3A)**. This DREADD approach enabled systemic administration of clozapine-N-oxide (CNO; 3 mg/kg, intraperitoneal [i.p.] injection) to promote hM3Dq signaling in vlPAG/DR neurons that express TH, a rate-limiting enzyme for DA biosynthesis **(Figure 3B)**. Chemogenetic activation of these putative vlPAG/DR^{DA+} neurons was employed during the measurement of pain sensitivity **(Figure 3B)**, where the Hargreaves (heat exposure to the hind paw [6 replicates; Hargreaves et al., 1988; Yu et al., 2018]) and Von Frey (nylon monofilament exposure to the hind paw [SUDO method; Bonin et al., 2014]) tests were used to determine thermal and mechanical nociceptive sensitivity respectively. CNO treatment in hM3Dq mice reduced sensitivity to thermal **(Figure 3C)** and mechanical **(Figure 3E)** nociception compared to mCherry controls for males, while comparable sensitivity was observed in mCherry and hM3Dq mice for females **(Figures 3D and 3F)**. Importantly, CNO-treated mCherry mice exhibited pain thresholds that were analogous to their saline-treated counterparts, suggesting that back-metabolism of CNO is an unlikely contributor to the observed effects in hM3Dq mice (Gomez et al., 2017). Together, these data show that chemogenetic activation of vlPAG/DR^{DA+} neurons contribute to pain sensitivity in a sex-dependent manner, as the anti-nociceptive effects of the dopaminergic subpopulation were limited to male mice.

We next investigated the possibility of vlPAG/DR^{DA+} intervention in pathological pain. Measurements of nociceptive sensitivity were repeated in the same cohort of TH-Cre mice following hind paw exposure to Complete Freund's Adjuvant (CFA; 50 μl, intraplantar [i.pl.] injection), a model of persistent inflammation, in order to determine the impact of vlPAG/DR^{DA+} activation on heightened pain sensitivity. Similar to the effects observed in the naïve condition, CNO treatment in hM3Dq mice attenuated CFA-induced hyperalgesia for thermal and mechanical nociception in males **(Figures 3G and 3I)** but not females **(Figures 3H and 3J)**. vlPAG/DR^{DA+} anti-nociception was primarily active in the CFA-injected paw, with CNO treatment in male hM3Dq mice rescuing pain thresholds for the ipsilateral paw and having no effect on the contralateral paw **(Figures 4A-4D)**. Predictably, engagement of these neurons did not alter tail flick or avoidance behaviors in either sex **(Figures 4E-4P)**, echoing the results of previous studies that have established a specific role for vlPAG/DR^{DA+} neurons in supraspinal pain (Li et al., 2016; Taylor et al., 2019). Consistent with this idea, our results demonstrate that chemogenetic activation of TH neurons in the vlPAG/DR exclusively produces anti-nociception in male mice during naïve and persistent inflammatory pain states, thus highlighting vlPAG/DR^{DA+} as an effective promoter of sex-specific pain relief.

Although chemogenetic activation of vlPAG/DR^{DA+} neurons in female subjects did not produce statistically significant changes in pain sensitivity, a variable range of mechanosensitivity thresholds trending towards anti-nociception was observed in the Von Frey test **(Figure 3F)**. It has been posited that high variations in pain threshold can reflect differences in hormonal status, as fluctuations in testosterone and estrogen levels have been shown to alter DA availability and pain sensitivity (Di Paolo, 1994; Bradshaw et al., 2000; Purves-Tyson et al., 2012; Sorge et al., 2011; 2015). Therefore, it is possible that the functional contributions of vlPAG/DR^{DA+} neurons were overshadowed in female mice by hormonal influences in our initial testing of pain sensitivity. To address this, we tested a separate cohort of female TH-Cre mice (n = 7-8) by tracking the impact of vlPAG/DR^{DA+} activation on nociceptive sensitivity during

different phases of the estrous cycle **(Figure 5A)**. CNO treatment in hM3Dq mice resulted in comparable thermal and mechanical nociceptive sensitivity to mCherry controls regardless of hormonal status, suggesting that vlPAG/DR^{DA+} function is not altered by estrous cycle **(Figures 5B-5C)**. Chemogenetic activation of vlPAG/DR^{DA+} did appear to produce a trend for higher mechanosensitivity thresholds in the Von Frey test, however, where the mean of variable nociceptive responses was suggestive of an anti-nociceptive effect **(Figure 5C**; trend for main effect of Virus: $p = 0.0504$**)**. This outcome is reminiscent of the observations from our initial testing of pain sensitivity, indicating that vlPAG/DR^{DA+} activation in female mice reliably results in variable anti-nociception and that this variability cannot be explained by phase changes in the estrous cycle.

Since hM3Dq expression in TH-positive vlPAG/DR neurons served as a proximal method of targeting vlPAG/DR^{DA+} neurons in our experiments, we next evaluated the histological composition of TH in vlPAG/DR of mice as a means to verify the accuracy of our genetic manipulations to DA neurons. We specifically used immunohistochemistry (IHC) and *in situ* hybridization (ISH) to quantify levels of TH protein and *Th* mRNA in the vlPAG/DR, and found that TH in vlPAG/DR was generally situated in coronal sections ranging from A/P -3.94 mm to A/P -4.84 mm, with peak expression around A/P -4.39 mm in both sexes **(Figures 6B, 6E, and 6H)**. Comparisons by sex revealed that expression of TH and *Th* mRNA in vlPAG/DR neurons did not differ across the anterior/posterior (A/P) axis **(Figures 6A-6B, 6E, and 6H)**. Since TH is a precursor to both DA and norepinephrine (NE), we additionally quantified dopamine beta-hydroxylase (DBH) as a marker for NE in the same tissue using a DBH-eGFP reporter (n = 4) for IHC experiments or *Dbh* mRNA probes in C57BL/6J mice (n = 2-4) for ISH experiments. Contrary to observations in the vlPAG/DR of rats (Suckow et al., 2013), we observed no discernible overlap in TH and DBH expression to suggest the presence of noradrenergic neurons in the vlPAG/DR of mice **(Figures 6B-6G)**. These results demonstrate

that TH expression in vlPAG/DR is a reliable marker of DA in mice, and that our behavioral data accurately reflect the effects of vlPAG/DR^{DA+} activation on nociceptive sensitivity.

vlPAG/DR^{DA+}-BNST Drives Discrete Pain-Related Behaviors in Male and Female Mice

Previously, we demonstrated that vlPAG/DR^{DA+} neurons exhibit robust projections to the BNST (Li et al., 2016). The function of this pathway, however, has yet to be investigated in the context of behavior. Considering the emerging role of BNST DA signaling in pain regulation (Carboni et al., 2000; Kash et al., 2008; Hagiwara et al., 2013; Yu et al., 2020), we sought to test whether vlPAG/DR^{DA+} projections to the BNST can alter pain-related behaviors by selectively expressing channelrhodopsin-2 (AAV5-EF1α-DIO-ChR2-eYFP) or a control fluorophore (AAV5-EF1α-DIO-eYFP) in the vlPAG/DR of adult male and female TH-Cre mice. Optical fibers were bilaterally implanted over the BNST, enabling 473 nm blue light stimulation of vlPAG/DR^{DA+} terminals in the BNST during behavioral testing **(Figures 7A-7B)**. Photostimulation of vlPAG/DR^{DA+}-BNST at 20 Hz in ChR2 mice caused a sustained reduction in thermal nociceptive sensitivity compared to eYFP controls for males but not females **(Figures 7C-7D and 8A-8B)**. This anti-nociceptive effect attenuated CFA-induced hyperalgesia as well, with sensitivity changes primarily exhibited in the ipsilateral paws of males **(Figures 7G-7H and 8C-8H)**. Activation of vlPAG/DR^{DA+}-BNST resulted in similar sex-specific reductions in mechanical nociceptive sensitivity **(Figures 7E-7F and 7I-7J)** but did not affect tail flick responses **(Figure 8I-8J)**. These results suggest that the BNST is an important downstream target of vlPAG/DR^{DA+} neurons for pain modulation, as the sex-specific anti-nociceptive effects of vlPAG/DR^{DA+} neurons were able to be reproduced by activating vlPAG/DR^{DA+} terminals in the BNST.

In addition to the regulation of nociceptive sensitivity, vlPAG/DR^{DA+} neurons have been implicated in a number of other functions, including (but not limited to) locomotion (Flores et al., 2006; Groessl et al., 2018), motivation (Flores et al., 2006; Matthews et al., 2016; Groessl et al.,

2018; Lin et al., 2020), and salience (Lu et al., 2006; Cho et al., 2017; Porter-Stransky et al., 2019). We thus tested the same cohort of eYFP/ChR2 mice in a battery of assays to determine whether vlPAG/DR^{DA+} projections to BNST can modify performance in a variety of behavioral contexts. First, we evaluated the contributions of vlPAG/DR^{DA+}-BNST to pain-related functional impairment by introducing acute visceral nociception (via treatment with 1% acetic acid, i.p. injection) during nesting, an innate behavior in mice that is temporarily impaired by pain and attenuated by analgesic drugs **(Figure 9A;** Negus et al., 2015). By activating vlPAG/DR^{DA+} terminals in BNST during this assay, we were able to measure the projection's contributions to visceral nociceptive (i.e. writhing, as defined by abdominal constriction / hind limb extension), affective-motivational (i.e. attenuation of impaired nesting), and locomotor behaviors related to pain. Although photostimulation of vlPAG/DR^{DA+}-BNST did not attenuate impaired nesting behavior **(Figures 9B-9C)**, marked increases in writhing behavior **(Figures 9D-9G)** and locomotor activity were observed for females but not males **(Figures 9H-9K)**. These data suggest that activation of vlPAG/DR^{DA+}-BNST can promote visceral nociception and locomotion, without any discernible nesting changes, in a sex-specific manner. Given that the expression of writhing relies on bodily movement, however, it was possible that the observed effects on visceral nociception were confounded by increases in locomotion.

To differentiate vlPAG/DR^{DA+}-BNST effects on locomotion from visceral nociception, we performed a series of follow-up assays to measure how pathway contributions to movement-dependent incentive behaviors compare to pain-related phenotypes. Using real-time place preference/aversion (RTPP/RTPA) and sociability with conspecific mice of the same and opposite sex, we were able to measure the rewarding, aversive, and locomotor contributions of the vlPAG/DR^{DA+}-BNST pathway in a variety of contexts **(Figure 10)**. Generally, photoactivation of vlPAG/DR^{DA+}-BNST did not produce side or social preferences for males or females in the RTPP **(Figures 10A-10B)**, sociability test with same sex conspecifics **(Figures 10D-10F)**, sociability test with opposite sex conspecifics **(Figures 10I-10K)**, or post-isolation sociability test

with same sex conspecifics **(Figures 10N-10P)**. However, vlPAG/DR^{DA+}-BNST stimulation did increase locomotion for female subjects in a context-dependent manner, with prototypically salient stimuli like environmental novelty and opposite-sex conspecific mice leading to greater increases in movement compared to more neutral stimuli **(Figures 10C, 10G-10H, 10L-10M, and 10Q-10R)**. These data suggest that vlPAG/DR^{DA+} neurons exclusively drive locomotor responses to environmental salience through the BNST of female mice. Collectively, our results reveal that vlPAG/DR^{DA+} projections to the BNST function in a sex-dependent manner, promoting adaptive behavioral responses to environmental salience by driving anti-nociception in males and context-dependent locomotion in females.

Sex Differences in vlPAG/DR^{DA+}-BNST Transmission and Connectivity

Given the stark differences in behavioral outcomes observed for vlPAG/DR^{DA+}-BNST activation in male and female mice, we next examined the pathway's functional properties using slice physiology. Recording from light-responsive BNST neurons positioned downstream of ChR2-expressing TH neurons in the vlPAG/DR **(Figure 11A)**, we were able to capture two distinct types of physiological responses: (i) optically-evoked excitatory postsynaptic currents (oEPSCs) and (ii) optically-evoked inhibitory postsynaptic currents (oIPSCs) **(Figure 11B)**. oEPSCs were characterized by faster onset latencies **(Figures 11D and 11H)**, larger amplitudes **(Figures 11E and 11I)**, and the ability to persist in the presence of tetrodotoxin (TTX) and 4-aminopyridine (4-AP) compared to oIPSCs **(Figure 13A)**, suggesting that vlPAG/DR^{DA+} neurons form excitatory monosynaptic and inhibitory polysynaptic connections with BNST neurons. When vlPAG/DR^{DA+} terminals in the BNST were exposed to a single 1 ms pulse of blue light, measures of excitation/inhibition (E/I) ratio **(Figure 11C)**, o(E/I)PSCs onset latency **(Figures 11D and 11H)**, and o(E/I)PSCs amplitude did not differ by sex **(Figures 11E and 11I)**. By contrast, measures of paired-pulse ratio (PPR) revealed that females exhibited lower PPR for oIPSCs than males **(Figure 11J)**, with no difference in oEPSC PPR **(Figure**

11F), indicating a higher initial release probability for inhibitory polysynaptic connections in the BNST of female mice. Overall, there was a lower percentage of light-responsive BNST neurons for oIPSCs in female subjects **(Figure 11K)**, but no difference in oEPSC responsivity **(Figure 11G)**, when compared to males. Female mice thus possess less functional connectivity for polysynaptic inhibitory connections between vlPAG/DR^{DA+} terminals and BNST neurons than their male counterparts.

While sex differences in oEPSCs were not observed, it is possible that the fast transmission dynamics of Glu and/or GABA masked more subtle differences in volume transmission by DA, as the timing and strorage mechanisms of co-release have been shown to differ for Glu and DA (Hnasko & Edwards, 2012; Silm et al., 2019). To more directly measure DA signaling in vlPAG/DR^{DA+}-BNST, we first combined optogenetics with fast-scan cyclic voltammetry **(Figure 12A)**, an approach that enabled the *ex vivo* measurement of DA release from ChR2-expressing vlPAG/DR^{DA+} terminals in the BNST. Changes in DA release were observed in a pulse- and frequency-dependent manner. Light stimulation at 20 Hz produced progressive increases of DA following the introduction of more pulses (starting at 1 pulse and ending at 40 pulses), while frequencies ranging from 2 Hz to 30 Hz resulted in parabolic increases of DA, with 10 Hz stimulation yielding the greatest amount of DA release **(Figure 12B)**. Comparisons by sex, however, revealed no differences in DA release, regardless of light stimulation parameters **(Figures 12B)**. Antagonism of dopamine receptor D2 (D2R) similarly showed percentages of optically-evoked DA release in the BNST that were comparable to drug-free conditions for both male and female subjects, despite a trend for D2R-gated DA release in females **(Figure 12C)**. vlPAG/DR neurons thus exhibit similar mechanisms of DA release in the BNST of male and female mice.

Next, we measured the effects of DA transmission from vlPAG/DR to the BNST by repeating our optically-evoked slice physiology experiments with bath application of 3 mM kynurenic acid and 25 μM picrotoxin. This setup allowed us to block Glu and GABA activity and

isolate the effects of DA on downstream BNST neurons **(Figure 12D)**. Following light stimulation at 20 Hz, DA transmission produced three distinct types of physiological responses: depolarization, hyperpolarization, or a mix of the two responses **(Figure 12E)**. In some cases, changes in membrane potential were sustained and able to drive repeated action potentials lasting on the order of minutes **(Figure 13C)**. When responses to DA transmission were quantified, sex differences for depolarization, but not hyperpolarization, were observed, with greater excitatory shifts in membrane potential appearing in male mice **(Figures 12F and 12H)**. DA transmission from vlPAG/DR to the BNST was gated by DA receptors, with depolarization being sensitive to dopamine receptor D1 (D1R) antagonism and hyperpolarization being sensitive to D2R antagonism **(Figures 12G and 12I)**. Despite sex-specific mRNA expression of Drd2 but not Drd1 in the BNST **(Figure 13B)**, D1R antagonism was greater in male mice **(Figures 12G)**, while D2R receptor antagonism did not differ in males and females **(Figures 12I)**. Overall, the percentage of depolarizing, hyperpolarizing, and non-responsive cells did not vary by sex **(Figure 12J)**. Taken together, these results indicate that sexual dimorphism in DA transmission is a notable feature of the vlPAG/DR^{DA+}-BNST pathway, with male mice showing greater D1R-gated depolarization than females. Such discrepancies in physiology would suggest that selective targeting of DA transmission significantly impacts the function of vlPAG/DR^{DA+} neurons.

vlPAG/DR^{DA+} Anti-Nociception and Locomotion is Dopamine-Dependent

To determine the necessity of DA for vlPAG/DR^{DA+} function, we combined chemogenetics with CRSIPR/Cas9-mediated genome editing by co-injecting AAV-hSyn-DIO-hM3Dq-mCherry with either a TH CRISPR (AAV-DIO-saCas9-U6-sgTH) or control (AAV-DIO-saCas9-U6-sghTH) virus in the vlPAG/DR of adult male and female TH-Cre mice **(Figure 14A)**. This approach enabled us to functionally characterize vlPAG/DR^{DA+} neurons following conditional genetic knockout of the DA rate-limiting enzyme TH (Hunker et al., 2020). Validation

of the TH CRISPR virus revealed a 36%-46% reduction of TH expression in the vlPAG/DR of male and female subjects **(Figure 14A)**. Using the Hargreaves and Von Frey tests, we were able to reproduce the anti-nociceptive effects of vlPAG/DR^{DA+} activation, where CNO treatment reduced thermal and mechanical nociceptive sensitivity in hM3Dq+CTRL mice for males but not females **(Figures 14C-14F)**. This effect was attenuated in hM3Dq+TH CRISPR mice, as reductions in TH mitigated the anti-nociceptive effects in males without baseline sensitivity changes in either sex **(Figures 14C-14F)**. These data indicate that the presence of TH in vlPAG/DR is necessary for the sex-specific anti-nociceptive effects of vlPAG/DR^{DA+} activation.

Considering our earlier findings that vlPAG/DR^{DA+} activation in the presence of salient contextual features selectively drives locomotion in females, we next assessed the effect of TH knockout on locomotor activity in a novel environment. We tested the same cohort of hM3Dq+CTRL and hM3Dq+TH CRISPR mice in an open field, where subjects were free to explore the apparatus for an initial 30-minute habituation phase. Subjects were then treated with saline or CNO (3 mg/kg, i.p. injection) before being returned to the apparatus for an additional 90-minute test phase. TH knockout reduced locomotion in a sex-independent manner during the initial 30-minute habituation phase **(Figures 14G-14H and 14K)**. However, no locomotor changes were observed in subsequent habituation **(Figures 14I-14J and 14M)** or test phases **(Figures 14G-14J, 14L, and 14N)** following treatment with saline or CNO. These data provide evidence that TH is necessary for vlPAG/DR neurons to promote novelty-induced locomotion. Taken together, the ability of vlPAG/DR^{DA+} neurons to meet environmental demands such as pain and contextual novelty is evidently dependent on the presence of TH, suggesting that DA in vlPAG/DR is necessary for the sex-specific expression of anti-nociceptive and locomotor behaviors.

Potent analgesic drugs such as morphine can recruit vlPAG/DR mechanisms of pain modulation in a sex-dependent manner (Doyle et al., 2017), making it possible that vlPAG/DR^{DA+} neurons contribute to variable drug effects in males and females. Previous studies

have reported that morphine disinhibits vlPAG/DR^{DA+}, with local reductions in DA mitigating the anti-nociceptive, rewarding, and aversive effects of drug use (Flores et al., 2004; Flores et al., 2006; Li et al., 2016; Lin et al., 2020). To test whether TH knockout in vlPAG/DR can similarly prevent the anti-nociceptive and locomotor effects of systemic morphine administration (3 mg/kg [s.c.]), we repeated behavioral testing in hM3Dq+CTRL and hM3Dq+TH CRISPR mice with pharmacological recruitment of vlPAG/DR^{DA+} and other μ opioid-sensitive circuits as the driver for behavior **(Figure 15A)**. Predictably, morphine produced anti-nociceptive effects for thermal and mechanical nociceptive sensitivity compared to saline treatment for males and females **(Figures 15B-15E)**. In contrast to the specific effects of chemogenetic vlPAG/DR^{DA+} activation, however, the analgesic effect of morphine was unaltered by TH deletion in the vlPAG/DR, suggesting that morphine anti-nociception is not solely dependent on DA in the vlPAG/DR **(Figures 15B-15E)**. Similar results were observed for locomotion, as sex-dependent changes in behavior were not evident for the habituation **(Figures 15F-15G, 15J, and 15L)** or test phases **(Figures 15H-15I, 15K, and 15M)** following treatment with saline or morphine. These results collectively suggest that intact DA signaling is necessary for the sex-specific anti-nociceptive and locomotor effects of vlPAG/DR activation, while systemic morphine recruits extra-vlPAG/DR^{DA+} mechanisms for the expression of these adaptive behaviors.

Discussion

The vlPAG/DR has been implicated in a variety of important pain-related behaviors, including anti-nociception, escape, and other functions necessary for survival (Hosobuchi et al., 1977; Mayer & Liebeskind, 1974; Fardin et al., 1984; Morgan et al., 1991; Li et al., 1993; Bandler & Shipley, 1994; Maier et al., 1995; Loyd & Murphy, 2006; Linnman et al., 2012; Cai et al., 2014; Seo et al., 2019; Wright & McDannald, 2019). Although it is widely accepted that the expression and experience of pain can vary by sex, little is known about the neural mechanisms that contribute to these behaviors (Aloisi et al., 2007; Sorge et al., 2011; Mogil, 2012; Posillico et

al., 2015; Mapplebeck et al., 2016; Doyle et al., 2017; Dance et al., 2019; Inyang et al., 2019). In the present study, we define a midbrain-to-extended amygdala circuit responsible for the generation of divergent pain-related behaviors in male and female mice. Expanding on our findings from Li *et al.* (2016), we report that activation of vlPAG/DR^{DA+} neurons or projections to BNST reduces thermal and mechanical nociceptive sensitivity in male mice. This effect persists after CFA treatment, as activation of this population can additionally attenuate the sensitivity increases associated with long-term inflammation. Surprisingly, vlPAG/DR^{DA+}-BNST activation did not result in anti-nociceptive effects for female mice, instead producing robust locomotor behaviors in a context-dependent manner. Reductions in local TH attenuated the sex-specific behavioral effects of vlPAG/DR^{DA+} activation, possibly reflecting variable DA transmission and polysynaptic inhibition in the BNST. Collectively, these findings provide significant new insight into the functional role of vlPAG/DR^{DA+} neurons and how projections to the BNST contribute to sex differences in pain.

Highlighting the importance of DA transmission in the modulation of pain-related behaviors, we show that activation of vlPAG/DR^{DA+}-BNST reduces pain sensitivity, with TH-dependent anti-nociception occurring exclusively in male mice. In supporting earlier evidence that vlPAG/DR^{DA+} anti-nociception depends on both D1R and D2R (Meyer et al., 2009; Groessl et al., 2018; Taylor et al., 2019), we found that DA transmission to the BNST generates a mix of depolarizing and hyperpolarizing responses that are gated by D1R and D2R respectively. However, more robust D1R-gated depolarization and D1R antagonism were observed in male mice, suggesting that D1R signaling contributes to sex differences in pain sensitivity (Flores et al., 2004; Megat et al., 2018). D1R antagonism in the BNST has previously been shown to exacerbate formalin-induced nociception in a sex-specific manner (Hagiwara et al., 2013). This earlier work, however, reported stronger effects in female rats, contradictory of the DA-dependent anti-nociception we observed in male mice. Considering that our results were limited to DA signaling from a specific input, it is possible that the direction of sex-specific anti-

nociception depends on the source of DA (Glangetas & Georges, 2016). How DA in the BNST influences phasic/escapable (e.g. heat, pressure) and tonic/inescapable (e.g. acetic acid) pain may also differ by sex, as similar biases have been described for other DA and Glu inputs to the BNST (Morgan & Franklin, 1990; Altier & Stewart, 1999; Taylor et al., 2016). Regardless of these distinctions, activation of vlPAG/DR^{DA+} projections to BNST appears to be sufficient to reduce acute and persistent inflammatory pain, providing further evidence that DA can enhance anti-nociception to counteract severe pain (Burrill et al., 1944; Goetzl et al., 1944; Ivy et al., 1944; Dennis and Melzack, 1983; Chudler & Dong, 1995; Altier & Stewert, 1999; Magnusson & Fisher, 2000; Wood et al., 2007; Hagiwara et al., 2013; Megat et al., 2018).

Although activation of vlPAG/DR^{DA+} neurons and its projections to BNST exhibited no effect on nociceptive sensitivity in female mice, the assessment of visceral nociception and valence-specific behaviors revealed an additional role for the projection in sex- and context-dependent locomotion. Consistent with previous reports that vlPAG/DR^{DA+} increases locomotion in a neutral environment depending on the parameters of activation (Correia et al., 2017; Groessl et al., 2018; Taylor et al., 2019), we found that TH in vlPAG/DR is necessary to drive locomotion during the first habituation phase of an open field test for both males and females, despite showing no effects with CNO administration. In the presence of visceral nociception, however, optogenetic activation of vlPAG/DR^{DA+}-BNST selectively increases locomotion in females. A similar phenotype is exhibited in the sociability test, with pathway activation driving locomotor increases in the presence of opposite, but not same, sex mice. Considering that vlPAG/DR^{DA+} activity is modulated by salience (Cho et al., 2017; Porter-Stransky et al., 2019; Cho et al., 2020), it is possible that this sex-specific locomotor phenotype is driven by the synergistic effects of pathway activation and context (Liu & Dan, 2019; Cazettes et al., 2020). Interestingly, the allocation of salience in a novel environment is thought to be modulated by DA signaling in the BNST, with D2R antagonism reducing discriminative learning for safety- and threat-related stimuli (De Bundel et al., 2016). Thus, it remains an intriguing possibility for D2R

in the BNST to regulate salience, as the receptor may act as a mechanism to alter how context impacts vlPAG/DR^{DA+} locomotion in males and females. Here, we report that the BNST exhibits a trend for D2R-gated DA release, with less Drd2 mRNA and fewer polysynaptic inhibitory connections with vlPAG/DR observed in female mice, suggesting a discrepancy in the filtering of incoming excitatory transmission. Evidence for sex-specific modulation of anti-nociceptive (Hagiwara et al., 2013) and locomotor behaviors (Monleon et al., 1998; Schindler & Carmona, 2002) by DA receptor antagonism in the BNST supports this possibility, but a greater understanding of BNST cytoarchitecture in relation to DA signaling is needed to determine how vlPAG/DR^{DA+} inputs contribute to sex-specific responses to pain.

vlPAG/DR^{DA+} neurons likely generate discrete pain responses through distinct third-order neurons, targeting BNST neurons with outputs to structures responsible for locomotion or both anti-nociception and locomotion (e.g. central nucleus of the amygdala [CeA], ventral tegmental area, parabrachial nucleus; Dong et al. ,2001; Lebow & Chen, 2016). Shifts in the strength of these functionally distinct BNST circuits are anticipated as environmental demands differentially modulate the recruitment of vlPAG/DR^{DA+} neurons in males and females. Although a molecular identity for vlPAG/DR^{DA+}-connected BNST neurons has yet to be determined, several markers in the BNST (e.g. GABA, corticotrophin-releasing factor [CRF], protein kinase C delta [PCK-δ]) have exhibited roles in sex-specific responses to stressors and other environmental demands (Janitzky et al., 2014; Klampfl et al., 2016; Smithers et al., 2019) that may be regulated by local DA interactions (Kash et al., 2008; De Bundel et al., 2016). Of particular interest are PCK-δ and somatostatin (SOM) neurons, two segregated subpopulations in the BNST that receive input from nociceptive structures (Ye & Veinante, 2019), respond to peripheral inflammation (Wang et al., 2019), and diverge in colocalization with D2R to modulate salience (De Bundel et al., 2016). vlPAG/DR^{DA+} inputs activate both PCK-δ and SOM neurons in the CeA, which share reciprocal connections with PCK-δ and SOM neurons in the BNST (Ye & Veinante, 2019), suggesting that

these downstream structures may exhibit mutual regulation through specific subsets of cells. Collectively, these sex differences in BNST outputs and cell type are promising candidates to explain the functional divergences observed in this pathway. Similar motifs for a single cell type driving behavior based on outputs have been described in the vlPAG/DR, where activation of Glu neurons produces anti-nociceptive or freezing behaviors based on outputs to the rostral ventromedial medulla or magnocellular nucleus (Liebeskind et al., 1973; Basbaum & Fields, 1984; Tovote et al., 2016). Effects on arousal states also diverge in Glu neurons, as co-expression with DA promotes states of wakefulness such as contextual salience, while co-expression with neurotensin (NTS) paradoxically drives non-REM sleep (Lu et al., 2006; Cho et al., 2017; Porter-Stransky et al., 2019; Zhong et al., 2019). Whether downstream BNST neurons similarly exhibit a specific composition of efferents or molecular identities to determine how vlPAG/DR^{DA+} inputs generate sex-specific anti-nociceptive and locomotor behaviors requires further investigation.

The discovery of sex-specific mechanisms for pain responses has major implications for human health. Considering that pain disproportionately affects women across the lifespan, the identification of a novel mechanism for sex-specific responses to pain may not only explain why disparities in susceptibility exist, but how analgesia can be improved by sex. Having demonstrated that vlPAG/DR^{DA+} neurons are inconsistent drivers of anti-nociception for female mice, with no detectable contributions of the estrous cycle, we posit that vlPAG/DR inputs to the BNST can promote analgesia in females. However, mechanistic differences in DA transmission and polysynaptic inhibitory connections may predispose these neurons to drive more robust locomotor behaviors. This interpretation of vlPAG/DR^{DA+} function is complicated by the fact that increases in locomotion can falsely diminish reflexive response times, resulting in confounded readouts of nociceptive thresholds. It is thus possible that vlPAG/DR^{DA+} neurons concurrently recruit anti-nociceptive and locomotor effects, as increased locomotion would neutralize any measurable changes in pain sensitivity that are happening at the same time. Additional

considerations for these multifaceted mechanisms behind vlPAG/DR^{DA+} function may be of interest for the development of therapeutic interventions, as the ability to selectively tap into anti-nociception and locomotion would be advantageous for reducing the impact of pain in a variety of modalities and contexts.

Current treatment for severe pain requires opioids, but these drugs exhibit sex biases in their efficacy (Kest et al., 2000; Fillingim & Gear, 2004; Bernal et al., 2007; Loyd et al., 2007; Loyd et al., 2008; Craft, 2008; Posillico et al., 2015). Since vlPAG/DR^{DA+} neurons are endogenously recruited by morphine (Flores et al., 2006; Li et al., 2016; Lin et al., 2020) and drive sex-specific responses to pain, vlPAG/DR^{DA+} neurons may be a promising candidate for opioid modulation of pain-related behaviors. In the present study, we report that reducing TH expression by 36%-46% in the vlPAG/DR mitigates the anti-nociceptive and locomotor effects of vlPAG/DR^{DA+} activation, but not systemic morphine, in male and female mice. Since morphine also disinhibits Glu neurons in the vlPAG/DR to produce anti-nociceptive and freezing behaviors, it is possible that these extra-DA mechanisms of μ opioid receptor activation overshadowed the impact of our modest TH knockout in the vlPAG/DR. Previous studies have shown that an approximate 62% loss of local DA neurons was required to attenuate morphine anti-nociception (Flores et al., 2004), while an estimated 50% reduction in TH was needed to block morphine-induced conditioned place preference/aversion (Lin et al., 2020). These data indicate that graded amounts of DA impairment in vlPAG/DR^{DA+} neurons may be necessary to alter the expression of nociceptive and affective-motivational behaviors associated with opioid exposure, since the TH deletion we observed was only able to prevent the sex-specific effects of vlPAG/DR^{DA+} activation and not the more broad effects of morphine. This distinction suggests that selective targeting of vlPAG/DR^{DA+} activation could be a less variable means of impacting the effects of opioid exposure, as opposed to altering DA signaling to prevent these effects.

With these findings in mind, it remains a possibility that vlPAG/DR^{DA+} projections to BNST contribute to opioid modulation of pain responses. Supporting this notion, morphine

increases DA in the BNST (Carboni et al., 2000), with converging DA and Glu release activating pERK via D1R (Valjent et al., 2005). Consistent with these data, we found that activation of vlPAG/DR inputs to BNST produces anti-nociceptive effects in males but not females, a sex-specific effect that may be explained by D1-gated depolarization in the BNST. Notably, the analgesic effects of vlPAG/DR^{DA+} were achieved without promoting reward/aversion (McDevitt et al., 2014). Systemic increases in DA have been shown to reduce the amount of opioids needed for anti-nociception while reducing side effects like sedation, loss of alertness, and cognitive deficits in humans (Forrest et al., 1977; Bruera et al. 1992; Dalal & Melzack, 1998), so vlPAG/DR^{DA+} projections to BNST may be a unique contributor to this effect of DA signaling and opioid anti-nociception in males. Considering that women experience μ-opioid analgesia at the expense of more extreme side effects (Dahan et al., 1998; Sarton et al., 2000; Zacny , 2001; Cepeda & Carr, 2003; Miller & Ernst, 2004), we believe that learning more about the vlPAG/DR^{DA+}-BNST pathway and parallel circuits in the midbrain will help generate novel approaches to achieving comparable analgesia in females and minimize the access gap for effective pain management.

CHAPTER 2. CORTICOTROPIN-RELEASING FACTOR NEURONS IN THE BED NUCLEUS OF THE STRIA TERMINALIS DIFFERENTIALLY INFLUENCE PAIN PROCESSING AND MODULATION IN MALE AND FEMALE MICE

Introduction

Pain is a pervasive and well-conserved source of stress that disrupts homeostasis by driving negative sensory and emotional states (Abdallah & Geha, 2017). Corticotropin-releasing factor (CRF), a peptide known for regulating stress-related behaviors, has been hypothesized to play an impactful, albeit understudied, role in pain modulation (Wei et al., 1986; Lariviere & Melzack, 2000; Sinniger et al., 2004), and the bed nucleus of the stria terminalis (BNST) has emerged as a CRF-enriched structure that can significantly influence the affective-motivational components of pain (Rouwette et al., 2011; Minami & Ide, 2015, Minami, 2019). Bilateral lesions of the BNST suppress the aversive aspects of electrical, visceral, and somatic pain in rats (Crown et al., 2000; Deyama et al., 2007; 2008; 2009). Furthermore, CRF release is increased in the BNST following intraplanar injection of formalin, a tonic-acting inflammatory agent that exacerbates pain sensitivity (Ide et al., 2013), and intra-BNST administration of CRF results in conditioned place aversion in a CRF receptor 1 and 2 ($CRF_{R1/2}$)-dependent manner (Ide et al., 2013). Notably, these studies reported no effect on formalin-induced nociceptive behaviors (Ide et al., 2013; Kaneko et al., 2016). By contrast, intra-BNST antagonism of CRF_{2R} reduces mechanical and colonic nociception, and CRF_{R1} antagonism attenuates stress-induced hyperalgesia (Tran et al., 2012; 2014). Considering the discrepancies on CRF contributions to pain sensitivity reported in these findings, further investigation is required to understand the conditions by which CRF signaling in the BNST can alter the sensory and affective components of pain.

In the present study, we sought to establish the BNST as a possible source of CRF for these $CRF_{R1/2}$-dependent pain-related behaviors. Given the high levels of CRF peptide and receptor expression in the BNST, it is possible that local production of CRF plays an important role in the regulation of pain (Cummings et al., 1983; Sakanaka et al., 1986; Ju et al., 1989; Moga et al., 1989; Morin et al., 1999; Dabrowska et al., 2013; Daniel & Rainnie, 2016). To test this hypothesis, we characterized the contributions of CRF to pain processing and modulation by using genetically targeted manipulations of the peptide in the BNST. First, we used *in vivo* calcium imaging to examine how $BNST^{CRF+}$ neurons encode for acute exposures to noxious heat. We then assessed pain-related behaviors following genetic deletion of CRF in the BNST, allowing us to discern peptide-specific contributions to both acute and sustained nociceptive exposures. In tracking endogenous $BNST^{CRF+}$ activity and manipulating peptide expression, the present study highlights a crucial function of the BNST as a locus of CRF signaling and pain regulation.

Materials and Methods

Animals

Male and female CRF-Cre (Martin et al., 2010) and Floxed-CRF (Zhang et al., 2017) mice (N = 103 total, aged 6-12 weeks, C57BL/6J background) were bred in-house, group-housed with same-sex littermates, and maintained on a 12-h light/dark cycle (light on at 7:00, light off at 19:00) with rodent chow and water available *ad libitum*. Subjects were singly housed for imaging experiments to ensure recovery and prevent post-surgical complications. All procedures were approved by the Institutional Animal Care and Use Committee at UNC Chapel Hill and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Surgeries

Subjects were anesthetized with isoflurane (1-3%) in oxygen (1-2 l/min) and aligned on a stereotaxic frame (Kopf Instruments, Tujunga, CA). All surgeries were conducted using aseptic techniques in a sterile environment. Microinjections were performed with a 1 µl Neuros Hamilton syringe (Hamilton, Reno, NV) and a micro-infusion pump (KD Scientific, Holliston, MA) that infused virus at 100 nl/min. Viruses were administered bilaterally in the dorsal region of BNST (250 nl for all experiments; relative to bregma: ML ±0.90 mm, AP +0.23 mm, DV -4.35 mm). For experiments that required *in vivo* calcium imaging, Gradient-index (GRIN) lenses were implanted unilaterally in the right hemisphere approximately 200 µm over the dorsal BNST (relative to bregma: ML ±0.90 mm, AP 0.23 mm, DV -4.15 mm) and secured with a dental cement headcap. Baseplates were later added to stabilize the attachment of the miniature microscope (see "*Ca^{2+} imaging with miniature microscope*" section for details). After surgery, mice were given *ad libitum* Tylenol water or daily injections of meloxicam (5 mg/kg, subcutaneous [s.c.]) for four consecutive days, then allowed to recover for three weeks or longer before starting experiments.

Behavioral Assays

Hargreaves

Subjects were placed in Plexiglas boxes on an elevated glass surface and habituated to the behavioral apparatus for 30-60 minutes to reduce novelty-induced locomotion and other factors that may confound nociceptive sensitivity measures. The mid-plantar surface of each hind paw was exposed to a series of noxious heat trials that sequentially alternated between left and right paws. After each heat exposure, a 10-minute inter-trial interval was provided before the next trial. Six trials of heat exposure were conducted for all experiments except those using *in vivo* calcium imaging, which we restricted to 4 trials to prevent photobleaching. For Figures 16-17 ($BNST^{CRF+}$ imaging experiments), beam intensity was set to 15 on the IITC Plantar

Analgesia Meter (IITC Life Science, Woodland Hills, CA), producing average basal paw withdrawal latencies of approximately 10 seconds in CRF-Cre mice. For Figures 18-20 (*Crf* deletion experiments), beam intensity was set to 25 on the IITC Plantar Analgesia Meter, producing average basal paw withdrawal latencies of approximately 6 seconds in Floxed-CRF mice. Cutoff times of 20 seconds were set for each trial to prevent excessive tissue damage.

Von Frey

Subjects were held in Plexiglas boxes on a custom-made elevated metal wire surface (90 × 20 × 30 cm) and habituated to the behavioral apparatus for 30-60 minutes to ensure stable readouts of nociceptive sensitivity. Nylon monofilaments of forces ranging from 0.008 to 2 grams (g) were applied to the hind paw using the simplified up-down method (SUDO) described in Bonin *et al.* (2014). Starting with a mid-range force (0.16 g), the filament was applied to the mid-plantar surface of the hind paw for ten trials, then repeated with ascending or descending forces depending on the number of paw withdrawals. Withdrawal thresholds were defined as the minimum force filament that elicits a withdrawal reflex for ≥50% of the trials.

Tail Immersion

Following a 30-minute habituation to the behavioral room, subjects were restrained in Wypall fold wipers (Kimberly-Clark, Irving, TX) and tails were exposed to 50º C water in the test apparatus (Isotemp 110 Water Bath; Fisher Scientific, Hampton, NH). The tail flick latency was measured in two consecutive trials, where readings were taken 1 cm apart on the tail. The two latencies were then averaged together to indicate the reflexive nociceptive threshold of each subject. A cutoff time of 10 seconds was set to minimize tissue damage.

Hot Plate

Subjects were positioned in a custom-made cylindrical Plexiglas container on an IITC

Hot Plate Analgesia Meter (IITC Life Science, Woodland Hills, CA). To elicit sensory-discriminative and affective-motivational behaviors associated with pain, subjects were exposed to a 55º C black anodized aluminum plate (11” x 10.5” x ¾”) for 45 seconds. This sustained version of the hot plate test extends the traditional cutoff time of 20 seconds to 45 seconds to improve readout for behaviors beyond latency to the first paw withdrawal. Behaviors like paw withdrawal (rapid flicking of the limb), paw attending (licking of the limb), paw guarding (intentional lifting of the limb for protection), and escape jumping (propelling into the air in an attempt to leave the apparatus) reflect the extent to which subjects will engage in reflexive responses versus motivated behaviors to reduce the aversive aspects of pain (Corder et al., 2017). Computer-integrated video cameras were used to record each session, allowing for blinded manual scoring of pain-related behaviors elicited by hot plate exposure. The behavioral apparatus was generously provided by the UNC Mouse Behavioral Phenotyping Laboratory.

Open Field

Subjects were given 30 minutes to habituate to the behavioral room before being placed into a white Plexiglas open field (50 × 50 × 25 cm), where they could freely explore the arena for 10 minutes. The center of the open field was defined as the central 25% of the arena, where light levels were approximately 30 lux. Tracking of subject location and activity was collected with EthoVision (Noldus Information Technologies, Wageningen, Netherlands).

Elevated Plus Maze

Following a 30-minute habituation period in the behavioral room, subjects were placed into the center of an elevated plus maze (EPM) and allowed to freely explore the arena for 10 minutes. During testing, subjects were able to explore two open arms (75 × 7 cm) and two closed arms (75 × 7 × 25 cm) that were bounded by a central area (7 × 7 × 25 cm). Light levels were set to approximately 15 lux to promote avoidance behaviors, which were determined by

tracking subject location and activity with EthoVision (Noldus Information Technologies, Wageningen, Netherlands) and measuring time spent in the open and closed arms.

For all experiments, researchers were blinded to genotype/virus treatment conditions of each mouse.

Ca^{2+} Imaging with Miniature Microscope

Surgery

In CRF-Cre mice, a custom-prepared AAVDJ-EF1a-DIO-GCaMP6s virus (UNC Vector Core) was administered unilaterally in the dorsal region of BNST (250 nl in right hemisphere; relative to bregma: ML ±0.90 mm, AP +0.23 mm, DV -4.35 mm). GRIN lenses (0.6 mm diameter/7.3 mm length; Inscopix, Palo Alto, CA) were implanted approximately 200 µm over the virus injection site (relative to bregma: ML ±0.90 mm, AP 0.23 mm, DV -4.15 mm) and secured with a dental cement headcap. To promote a stable recovery following this extensive surgical procedure, subjects were given daily injections of meloxicam (5 mg/kg, s.c.) for four consecutive days. Approximately four weeks after the initial surgery and recovery, baseplates (V2; Inscopix, Palo Alto, CA) were placed above the GRIN lens to stabilize the attachment of the miniature microscope. The baseplate attachment was performed in a visually guided manner, where the miniature microscope was incrementally lowered towards the implanted GRIN lens to identify the optimal distance needed to achieve a field-of-view (FOV) with the maximum number of fluorescent cells. The baseplate was then incorporated into the existing headcap with additional dental cement. Finally, baseplate covers were secured to the top of the headcap in order to protect the miniature microscope attachment site from environmental contaminants that may obscure the imaging FOV.

Behavior

Subjects were tested in the Hargreaves assay 6-8 weeks after the initial surgical procedure and 2-4 weeks after the baseplate procedure. Prior to testing, each subject was anesthetized with isoflurane (1-3%) in oxygen (1-2 l/min) in order to attach the miniature microscope to the baseplate. After two consecutive days of habituation to the miniature microscope, subjects were given a 15-minute tethering session in their respective home cages and a 30-minute tethering session in the Hargreaves apparatus. At the end of the tethering session, a 5-minute imaging session for baseline calcium transients was recorded.

During testing, the mid-plantar surface of each hind paw was exposed to a series of noxious heat trials with 10-minute inter-trial intervals. Each subject was exposed to 4 trials of heat (I15 on the IITC Plantar Analgesia Meter [IITC Life Science, Woodland Hills, CA] respectively), with radiant heat exposures sequentially alternating between left (contralateral) and right (ipsilateral) paws. For each trial, calcium transients were recorded with a minute window prior to onset and following the offset of heat. Specific paw withdrawal latencies for each subject were used instead of a fixed duration to accurately capture individual nociceptive thresholds. Cutoff times of 20 seconds were set to prevent excessive tissue damage.

All recordings of behavior in the Hargreaves assay were performed with Media Recorder and a computer-integrated video camera (#PC212XP, Miniature CCD Camera Series). Simultaneous recordings of $BNST^{CRF+}$ calcium transients were acquired using the nVista data acquisition software (Inscopix, Palo Alto, CA) at 20 fps (Exp Time: 50 ms, Gain: 4-7, Ex. LED Power: 1).

Preprocessing

Using the Inscopix Data Processing Software (IDPS; Inscopix, Palo Alto, CA), recordings were cropped, temporally downsampled to 5 fps, and motion corrected. ΔF/F was then acquired to normalize each pixel value in the recording to a baseline value (as determined by mean

frame). To identify temporally and spatially unique ROIs, we used PCA/ICA to detect putative cell bodies in the imaging FOV. After manually removing ROIs that did not correspond to cells, traces and events were exported as CSV files. Preprocessed ΔF/F recordings were further transported into maximum intensity projection images and movie files for representative depictions of the data.

Data Analysis

Programmatic analysis was applied to extracted traces from IDPS using custom-written code in Python. Briefly, we transformed ΔF/F of baseline and test traces into z-scores for each subject using the formula: $Z = x - \mu / \delta$, where the number of standard deviations (δ) that raw values (x) diverge from baseline values (μ) is determined. We then separated traces by epoch (*Before*, *Heat Start*, *Heat End*, *After*) for each subject and trail to determine averages by sex. Epoch timing was determined by dividing the duration of heat exposure into halves, so that each time block represented an equal amount of time for specific trials and subjects. Analysis was then performed for various combinations of data dimensionality based on these times.

We specifically compared the activity of individual male and female $BNST^{CRF+}$ neurons for the following metrics:

-*Average Z-Score Across Trials by Epoch*: Mean z-score of epochs averaged across trials.

-*Average Z-Score by Trial and Epoch*: Mean z-score of epochs for each trial.

-Average *Neuronal Coactivity by Epoch*: Mean proportion of synchronized cell responses for each epoch. Fractions of responsive cells were calculated at every time frame for individual subjects. The average fraction of responses was then sorted by epoch for comparison.

-*Cumulative Distribution Function Across Trials by Epoch*: Determine the distribution of z-scores averaged across trials by binning the frequency of activity within each epoch.

-*Percent Responsiveness Across Trials*: Determine the proportion of cells that exhibit a statistically significant change in average z-scores during heat exposure by using a Wilcoxon

rank-sum test for each neuron. Two notable events define comparisons of heat responsive neurons with the activity in surrounding epochs: “Heat Onset” (i.e. *Heat On* [*Heat Start* + *Heat End*] vs. *Before*) and “Paw Withdrawal” (i.e. *Heat On* vs. *After*). Positive differences in z-score correspond to activation in response to noxious heat exposure relative to surrounding epochs, while negative differences indicate a relative reduction in activity. Percentage of responsive cells was calculated by factoring in every neuron for each trial. Separate analyses restricted proportions of responsive cells by individual subjects.

-*Percent Responsiveness by Trials*: Determine the change between average z-scores of *Heat On* (i.e. *Heat Start* and *Heat End*) and surrounding epochs for each trial, with positive differences corresponding to activation in response to noxious heat exposure, and negative differences corresponding to inactivation. Percentage of responsive cells was calculated by taking the proportion of neurons exhibiting statistically different *Heat On* activity compared to *Before* and *After* in individual subjects and averaging these values by subject within each trial.

-*Spike Magnitude by Trials*: Identify size of maximum value in *Heat On* (i.e. *Heat Start* and *Heat End*) epoch for each trial.

-*Spike Latency by Trials*: Identify time stamps for maximum value in *Heat On* (i.e. *Heat Start* and *Heat End*) epoch for each trial.

More information on the Python scripts used for analysis can be requested by contacting thomas_kash@med.unc.edu.

Histology

In Situ Hybridization

Following isoflurane anesthetization and rapid decapitation, the brains of Floxed-CRF mice were collected and placed on aluminum foil, where they were immediately frozen on dry ice and stored in a -80° C freezer. Using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany), coronal sections of BNST (12 μm thickness) were obtained and directly

mounted onto Superfrost Plus slides (Fisher Scientific, Hampton, NH), then kept at -80° C. In order to fluorescently label *Crf* mRNA in the BNST, slices were preprocessed with 4% PFA and protease reagent, incubated with target probes for mouse *Crf* (#316091, RNAscope Probe – Mm-Crh; Advanced Cell Diagnostics, Newark, CA), then fluorescently labeled with a probe targeting the corresponding channel for the peptide (Crf in 550; Advanced Cell Diagnostics, Newark, CA). The processed slides were then covered using Vecta-Shield Mounting Medium with DAPI in preparation for imaging.

Confocal Microscopy

All fluorescent images were acquired with the Zeiss 800 Upright confocal microscope and ZenBlue software (Carl Zeiss AG, Oberkochen, Germany), with equipment access granted through the Hooker Imaging Core at UNC Chapel Hill. Validation of virus expression/injection site, GRIN lens placement, and immunoreactivity were accomplished with tiled and serial z-stack images obtained through a 20x objective (3 x 3 tile, 2 μm optical slice thickness, 12 μm total thickness). Images were processed in FIJI (Schindelin et al., 2012) for manual counting and ZenBlue (Carl Zeiss AG, Oberkochen, Germany) for area measurements in each ROI.

Statistical Analysis

Single-variable comparisons were made using unpaired t-tests. Group comparisons were made using two-way ANOVA, two-way repeated measures ANOVA, or two-way mixed-model ANOVA depending on the number of independent and within-subjects variables in a data set. Following significant interactions or main effects, post-hoc pairwise t-tests were performed and corrected using Sidak's or Tukey's post-hoc tests to control for multiple comparisons. Results of statistical testing are reported in figure legends with significance indicated through markers on figures. Data are expressed as mean ± standard error of the mean (SEM), with significance for *p* values below 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). All

data were analyzed and visualized with standard statistical software packages from GraphPad Prism 8 (GraphPad Software, San Diego, CA) and SciPy (Virtanen et al., 2020).

Details on the statistical tests used:

Unpaired t-test used for *Figures 16B, 17G-H*.

Wilcoxon rank-sum test used for *Figures 16D, 17E*.

Kolmogorov–Smirnov test used for *Figure 16H*.

Area under the curve used for *Figures 19D-E*.

Two-way ANOVA with Tukey's post hoc used for *Figures 16E, 18C-G, 19B-C, 20A-D*.

Two-way mixed-model ANOVA with Tukey's post hoc used for *Figures 16G*.

Two-way mixed-model ANOVA with Sidak's post hoc used for *Figures 16B, 16H, 17F-H*.

Two-way repeated measures ANOVA with Sidak's post hoc used for *Figures 17C-D*.

Results

BNST^{CRF+} Neurons are Endogenously Recruited by Noxious Thermal Stimuli

To assess how BNST^{CRF+} neurons are modulated by pain, we used a head-mounted miniature microscope to track *in vivo* somatic calcium activity of individual *Crf+* neurons in the BNST following hind paw exposure to a thermal nociceptive stimulus **(Figure 16)**. By implanting a GRIN lens and injecting an adeno-associated virus carrying Cre-inducible GCaMP6s (AAVDJ-EF1a-DIO-GCaMP6s) in the right hemisphere BNST of adult male and female CRF-Cre mice **(Figure 16A)**, we were able to continuously monitor BNST^{CRF+} activity during repeated trials of noxious heat exposure. Since the length of exposure varied by trial, we analyzed activity across epochs relative to heat exposure: *Before*, *Heat Start*, *Heat End*, and *After* **(Figures 16C and 16E)**. Although paw withdrawal latencies did not differ in male (n = 3) and female (n = 5) mice **(Figure 16B)**, comparisons of BNST^{CRF+} activity averaged across four trials of heat exposure revealed sex-specific recruitment by epoch, with higher fold calcium changes observed for *Heat*

Start and *Heat End* in males (n = 49 cells; 7-30 cells per mouse) and *Heat End* in females (n = 68 cells; 8-19 cells per mouse) **(Figures 16G-1H;** main effect of Epoch: $F_{2.153, 247.7} = 26.31$, $p < 0.0001$**)**. Average z-scores of *Before* and *Heat Start* epochs differed by sex, with males showing a larger magnitude of $BNST^{CRF+}$ activity in the earlier phases of stimulus exposure than females **(Figure 16G;** main effect of Sex: $F_{1, 115} = 13.20$, $p = 0.0004$**)**. Coactivation of neuronal activity, as defined by the average fraction of responsive cells for each frame of an epoch, also differed in the earlier phases of stimulus exposure, with females exhibiting increased $BNST^{CRF+}$ synchrony between the transition from *Before* to *Heat Start* **(Figure 16I;** main effect of Epoch: $F_{1.840, 11.04} = 4.163$, $p = 0.0475$**)**. Notably, while there were sex differences in the magnitude and synchrony of responses when examining average z-scores, the proportion of cells exhibiting time-locked changes to heat exposure did not differ between male and female subjects **(Figure 16C)**. These results indicate that $BNST^{CRF+}$ neurons are recruited by thermal nociception in a manner where the magnitude of activation appears greater in males, while synchronous activation is more prominent in females.

We next evaluated $BNST^{CRF+}$ activity on a trial-by-trial basis to determine how neuronal responses to noxious heat are impacted by repeated exposure **(Figures 17A-17B)**. Higher z-scores were generally observed in the first two trials of *Heat Start* and *Heat End* epochs **(Figures 17C-17D)**. Although male mice exhibited averaged $BNST^{CRF+}$ responses with a greater magnitude of activation than females **(Figures 17G**; $t_{115} = 3.70$, $p = 0.0003$**)**, maximum heat responses were reduced throughout the progression of trials for males **(Figure 17G**; main effect of Sex: $F_{1, 115} = 13.69$, $p = 0.0003$**)**. By contrast, the latency of maximum response became more delayed with additional trials for female mice **(Figure 17H**; Trial x Sex interaction: $F_{3, 345} = 16.90$, $p < 0.0001$**)**, resulting in a trend for later peak times following heat exposure **(Figure 17H**; $t_{115} = 1.789$, $p = 0.0763$**)**. Tracking the percentage of heat responsive $BNST^{CRF+}$ neurons across trials, we observed a combination of positive and negative changes in activity that varied in distribution with repeated exposures **(Figure 17E)**. However, when restricted to cell proportions by subject,

the percentage of heat responsive BNST^{CRF+} neurons for each trial did not significantly differ by trial progression or sex **(Figure 17F)**. These data suggest that multiple exposures to acute thermal nociception can drive distinct changes in the magnitude and timing of cell responses, but not the total proportion of responsive cells among individual subjects, for male and female mice. Together, our *in vivo* imaging results indicate that BNST^{CRF+} neurons are activated by noxious heat in male and female mice.

Genetic Deletion of *Crf* from BNST Reduces Pain Sensitivity

Given the heterogenous nature of peptide expression in the BNST and a fundamental lack of understanding as to what conditions drive CRF release, it was unclear whether the observed BNST^{CRF+} responses to noxious heat in our imaging experiments would translate to a functional role for CRF in the modulation of pain-related behaviors. To address this issue, we genetically deleted CRF in the BNST by bilaterally injecting an adeno-associated virus carrying Cre (AAV5-CaMKIIa-Cre-GFP) or a control virus (AAV5-CaMKIIa-GFP) in the BNST of adult male and female Floxed-CRF mice **(Figure 18A)**. This approach resulted in an approximate 50-60% reduction in *Crf* mRNA-positive neurons in the BNST **(Figures 18B-18D**; Crf mRNA: main effect of Virus: $F_{1, 98} = 66.74$, $p < 0.0001$; Fold Crf mRNA: main effect of Virus: $F_{1, 98} = 60.23$, $p < 0.0001$**)**. Using the Hargreaves and Von Frey tests, we found that CRF deletion reduced nociceptive sensitivity compared to control mice **(Figures 18E-18F**; Hargreaves: main effect of Virus: $F_{1, 99} = 5.677$, $p = 0.0191$; Von Frey: main effect of Virus: $F_{1, 99} = 10.05$, $p = 0.0020$**)**. Notably, the extent of reduction for *Crf* mRNA expression and pain sensitivity did not differ between male and female subjects **(Figures 18C-18F)**. By contrast, reflexive responses in the tail immersion test revealed lower nociceptive thresholds in males, but no anti-nociceptive effect following CRF deletion **(Figure 18G**; Tail Immersion: main effect of Sex: $F_{1, 99} = 4.631$, $p = 0.0338$**)**. These results show that a deficiency of CRF in the BNST increases supraspinal pain

thresholds, suggesting that the presence of CRF may be necessary to preserve the sensory components of pain for both male and female mice.

Genetic Deletion of *Crf* from BNST Differentially Impacts the Sensory and Affective Components of Pain

Considering that CRF signaling in the BNST plays an important role in multiple components of the pain experience, we tested the same CRF deletion and control mice in an extended hot plate test (Corder et al., 2017). Differing from a standard hot plate protocol that terminates after the first paw withdrawal or a relatively brief predetermined cut-off time, this prolonged form of the test lasts 45 seconds to promote the expression of both sensory-discriminative (e.g. paw withdrawal [rapid flicking of the limb]) and affective-motivational (e.g. paw attending [licking of the limb], paw guarding [intentional lifting of the limb for protection], and escape jumping [propelling into the air in an attempt to leave the apparatus]) behaviors **(Figure 19A).** Although genetic deletion of CRF in the BNST did not alter sensory-discriminative responses to the hot plate, as measured by paw withdrawal latency **(Figure 19B)** and cumulative paw withdrawal behaviors **(Figure 19C)**, or affective-motivational responses such as paw guarding and escape jumping **(Figure 19C)**, there was a marked increase in paw attending for Cre-treated female mice **(Figures 19C-19E**; main effect of Virus: $F_{1, 61} = 4.695$, $p = 0.0342$**)**. This sex-specific change in paw attending suggests a divergent role for CRF in the BNST and the sensory-discriminative/affective-motivational components of a prolonged, inescapable thermal nociceptive stimulus. Remarkably, reducing CRF expression in the BNST did not impact other pain-independent affective-motivational behaviors such as avoidance **(Figures 20A and 20C)** and locomotor **(Figures 20B and 20D)** behaviors in the open field **(Figures 20A-20B)** and elevated plus maze **(Figures 20C-20D)**, indicating that the aversive contributions of CRF in the BNST are specific to pain-related contexts like the hot plate.

Discussion

Pain is a multi-faceted experience that can impact negative emotional behaviors, as well as adaptive coping strategies to mitigate harm. In the present study, we identified a subpopulation of BNST neurons that can respond to and modulate these pain-related behaviors through local expression of CRF. Utilizing genetically targeted manipulations of CRF-containing neurons and *Crf* itself in the BNST, we were able to demonstrate the dynamic recruitment of $BNST^{CRF+}$ activity by noxious heat, as well as the modulatory role of CRF on pain in male and female mice. Our results support and expand upon earlier work on intra-BNST CRF signaling and pain (Tran et al., 2014; Minami, 2019) by uncovering previously unknown sex-specific contributions of local CRF. Together, these data enhance our collective knowledge on nociceptive microcircuits in the BNST.

$BNST^{CRF+}$ Neurons Dynamically Respond to Noxious Heat Across Trials in a Sex-Specific Manner

Our imaging experiments determined that $BNST^{CRF+}$ neurons generate greater somatic Ca^{2+} activity in the presence of noxious heat. Since the greatest increases in activity were observed at the *Heat Start* and *Heat End* epochs for males and the *Heat End* epoch for females, we can conclude that $BNST^{CRF+}$ neurons are activated by noxious heat in both sexes. Sex differences at the *Before* and *Heat Start* epochs, where the initial detection of the stimulus elicited greater neuronal responses in males, additionally suggest that neuronal representations of pain differ by sex in the early phases of heat exposure. This difference in early phase processing may indicate a higher probability of detecting the nociceptive stimulus, since males exhibited trends for $BNST^{CRF+}$ neurons with faster maximum heat response times and greater magnitudes of activation. By contrast, females exhibited greater $BNST^{CRF+}$ coactivity changes in the early phases of heat exposure, with the most prominent increases in synchrony happening between the transition from *Before* to *Heat Start*. We posit that these disparities in early phase

activity reflect sex-specific mechanisms of nociceptive processing. Considering that salience is an important predictor of pain sensitivity and analgesic efficacy (Borsook et al., 2013), it is possible that alterations in the timing and synchrony of $BNST^{CRF+}$ responses will change nociceptive detection and contribute to pain sensitivity in a sex-specific manner. This is an intriguing possibility given that BNST neurons expressing *Crf* or the prepronociceptin gene have been reported to be exhibit *in vivo* responses to motivationally salient and aversive stimuli such as predator odor (Giardino et al., 2018; Rodriguez-Romaguera et al., 2020). These observations support prior conclusions that the BNST encodes for salient threats that may cause physical or emotional harm (Grupe et al., 2013; Avery et al., 2016; Minami, 2019).

Since salience can either enhance or diminish the importance of a nociceptive signal with experience (Borsook et al., 2013), we evaluated whether $BNST^{CRF+}$ responses to heat changed with repeated exposure. Comparisons by sex revealed increasingly lower magnitudes of activity for males and slower maximum heat response times for females as trials advanced. Although these subtle reductions in $BNST^{CRF+}$ activity may reflect the diminished salience of pain as stimulus exposures accumulate, it was not reflected in behavior, as thermal nociceptive sensitivity was comparable between male and female mice across trials. This suggests that the observed discrepancies in $BNST^{CRF+}$ encoding between males and females are indicative of a sex-specific means to process pain rather than a mechanism to drive behavioral variation. Quantitative comparisons of pain have shown that female subjects are more sensitive than males in 85.4% of rodent studies (Mogil et al., 2020), so our recordings offer unique insight on how comparable nociceptive sensitivity can be processed in a sex-specific manner. In related structures like the basolateral amygdala (but not the central nucleus of the amygdala [CeA]), somatic Ca^{2+} activity and extracellular single-unit recordings positively scale with increasing pain intensity and repeated nociception (Ji et al., 2010; Grewe et al., 2017; Corder & Ahanonu et al., 2019; Hua et al., 2020), suggesting that BNST responses to pain are distinctly desensitized with experience.

Notably, we did observe variability in BNST^{CRF+} responding on a single cell level for both sexes, where responses to heat were comprised of both increasing and decreasing z-scores compared to activity in the *Before* and *After* epochs. Previous data from our group has suggested that multiple populations of CRF neurons in the BNST differentially contribute to the regulation of affective behaviors (Marcinkiewcz and Mazzone et al., 2016), with BNST^{CRF+} neurons that form local inhibitory synapses playing a greater role in aversive states. Assuming that the magnitude of response is a reliable metric of activation, these data suggest a model where heightened BNST^{CRF+} activation is accompanied by increased inhibitory tone. Suppression of local GABAergic projection neurons has been shown to inhibit dopamine neurons in the ventral tegmental area (Marcinkiewcz and Mazzone et al., 2016, Minami et al., 2019), so it is possible that the rapid decreases in BNST^{CRF+} activity observed prior to paw withdrawal are providing a motivational signal to move away from the noxious stimulus. Although BNST^{CRF+} neurons exhibit local inhibitory connections, it should be noted that they also project to other regions involved in motivated behavior, such as the lateral hypothalamus, where activation of BNST^{CRF+} projections can induce conditioned place aversion (Dabrowska et al., 2016; Giardino et al., 2018). In future experiments, researchers should more rigorously examine the functional contributions of these varied BNST^{CRF+} responses to pain-related behaviors, with special consideration for how the proportion of pain responsive neurons and magnitude of activity interact to inform function.

CRF in the BNST Modulates the Expression of Sensory and Affective Components of Pain

While previous studies have found that pharmacological manipulation of CRF signaling in the BNST can modulate multiple aspects of pain-related behavior (Ide et al., 2013; Tran et al., 2012; 2014; Kaneko et al., 2016), the source of this CRF is unclear, as the BNST contains locally produced CRF and receives CRF input from the CeA (Sakanaka et al., 1986; Dong et al., 2001). In examining how local production of CRF can impact these pain-related behaviors, our

work has found that both thermal and mechanical nociceptive sensitivity are impacted by CRF deletion in the BNST. These results are reminiscent of previously published effects showing that CRF_{R2} antagonism reduces mechanical and colonic nociception in stress-free conditions (Tran et al., 2014). However, similar pharmacological manipulations failed to alter nociceptive behaviors associated with formalin and only blocked conditioned place aversion (Ide et al., 2013; Kaneko et al., 2016). In the context of our results, these studies suggest that CRF signaling in the BNST selectively contributes to the sensory and affective components of pain based on the specific nociceptive stimulus and the context of its presentation. As others have hypothesized, the duration of a nociceptive stimulus and the capacity of an animal to prevent further harm by the stimulus (i.e. escapability) may be important factors in pain processing (Ryan et al., 1985; Bandler et al., 2000; Gandhi et al., 2017). If we categorize these known effects of CRF signaling in the BNST by phasic-acting/escapable nociceptive exposures like the Hargreaves and Von Frey tests versus tonic-acting/inescapable nociceptive exposures like formalin, we can posit that CRF is more likely to alter the sensory components of pain with shorter-acting stimuli and the affective components with longer-acting stimuli.

Contrary to the anti-nociceptive effects for acute nociceptive exposures in the Hargreaves and Von Frey tests, we found that CRF deletion in the BNST did not alter the sensory components of pain in the hot plate when measured by initial latency and cumulative number of paw withdrawals. Of the measures for affective behaviors, only paw attending was altered by CRF deletion, as females exhibited more licks to the site of injury in Cre-treated mice than controls. Unlike the Hargreaves and Von Frey tests, where exposure to the nociceptive stimulus terminates after a paw withdrawal or a fixed number of rapid prodding, the extended hot plate exposes subjects to heat for a duration where the thermal nociceptive stimulus is tonic-acting/inescapable compared to the standard version of the test. This change in the context of nociceptive stimulus presentation may explain why CRF deletion can generate seemingly contradictory phenotypes like sex-independent pain relief and sex-dependent exacerbation of

an adaptive coping behavior. These divergent results possibly indicate a functional distinction in how CRF signaling in the BNST interacts with specific nociceptive stimuli as short- or long-acting stressors. A potential explanation for this discrepancy is that peptide interactions with local CRF receptors differ for males and females, as sex differences in peptide distribution, receptor expression, and stress response are a broad feature of CRF signaling throughout the brain (Bangasser & Wiersielis, 2018; Uchida et al., 2019). Since previous evaluations of CRF signaling in the BNST and pain were performed exclusively in male subjects (Deyama et al., 2007; Tran et al., 2012; 2014; Ide et al., 2013; Kaneko et al., 2016), it remains to be determined if females will exhibit similar trends in coping behavior for other types of nociceptive stressors.

Although there were female-specific changes in the affective-motivational components of pain, no differences in avoidance or locomotors behaviors were observed with CRF deletion in the BNST. Previous studies have shown that CRF infusion in the BNST is anxiogenic and produces conditioned place aversion, while pharmacological inhibition of CRF_{R1} but not CRF_{R2} in the BNST is anxiolytic (Sahuque et al., 2006). Furthermore, work from our lab has shown that chemogenetic inhibition of $BNST^{CRF+}$ neurons reduces avoidance behavior in the open field (Pleil et al., 2015). Our results demonstrate that genetic targeting of CRF production does not reduce avoidance behaviors, similar to the null effects observed in a previous study with CRF overexpression in the BNST (Sink et al., 2013), suggesting that there may be other factors in $BNST^{CRF+}$ neurons driving these changes. Although there has yet to be a comprehensive investigation on $BNST^{CRF+}$ co-expression and its relative contributions to avoidance behaviors, distinct anxiogenic roles have been identified for GABA, CRF, and other modulatory factors in the CeA (Pomrenze et al., 2019). It remains possible, however, that CRF plays a prominent role in avoidance behaviors, and that a greater reduction in *Crf* mRNA beyond 50%-60% is necessary to produce anxiolytic effects. Alternatively, it is feasible that anxiety-like states are more robustly regulated by external CRF contributions than local contributions within the BNST.

Taken together, our work raises important questions on the relationship between the dynamic activity of BNST^{CRF+} neurons and CRF release. Unlike classical neurotransmitters such as GABA, the kinetics of release and activity of neuropeptides can be much longer. Elegant work from the Minami group has demonstrated that CRF can be released in the BNST over the course of an hour following formalin injection (Ide et al., 2013), so it is possible that some of the changes we observed over time in our *in vivo* imaging studies were due to local release of CRF. This is a particularly interesting possibility in the context of chronic pain, since long-term injury has been reported to drive functional upregulations of CRF signaling and putative CRF activity in the BNST (Ide et al., 2013; Takahashi et al., 2019; Hara et al., 2020). Interestingly, treating adult male human subjects with CRF has been shown to relieve pain during persistent inflammatory states such as post-operative pain but lack analgesic properties for healthy individuals (Hargreaves et al., 1987; Lautenbacher et al., 1999), suggesting that lasting pain conditions can change how CRF modulates pain. Future studies examining the relationship between chronic pain and *in vivo* dynamics of BNST^{CRF+} activity in response to noxious stimuli will therefore be essential for a comprehensive understanding of peptide function in pain processing and modulation.

To summarize, our findings have established a previously uncharacterized sex-specific role for CRF in the BNST and pain-related behaviors that will provide important insight into the aversive microcircuitry of the extended amygdala.

CHAPTER 3. IMPLICATIONS FOR DRUG USE

Introduction

Alcohol provides an accessible means of self-medication for pain suffering populations, with approximately 25% of chronic pain patients consuming alcohol for symptom relief (Riley and King, 2009). Although alcohol can act as a transient pharmacological intervention for pain, excessive drinking can lead to increased risk for addiction and related heath complications (Koob and Volkow, 2010). Chronic exposure to alcohol is especially detrimental for pain, with marked increases in sensitivity after drug cessation (Boissoneault et al., 2018; Dhir et al., 2005; Dina et al., 2000, 2007; Dodds et al., 1945; Edwards et al., 2012; Fu et al., 2015; Gatch and Lal, 1999; Jochum et al., 2010; Malec et al., 1987; Riley and King, 2009; Roltsch et al., 2017; Shumilla et al., 2005; Smith et al., 2015; Wolff et al., 1942). Clinical observations have suggested important differences in how men and women consume alcohol and experience the detrimental consequences of alcohol abuse when suffering from pathological pain. Among chronic pain patients, alcohol use is more prevalent in males (Brennan et al., 2011; Egli et al., 2012; Riley and King, 2009; Wilsnack et al., 2009). Females that habitually consume alcohol, however, are more susceptible to pathological pain (Boissoneault et al., 2018). To date, preclinical investigations have failed to model similar sex-specific patterns in drinking and pain, thus providing a major hurdle to studying the mechanisms of pain-related alcohol consumption (Smith et al., 2015; Yezierski and Hansson, 2018).

To address this deficit in preclinical models, the present study examines alcohol consumption in male and female C57BL/6J and Floxed-CRF mice treated with Complete

Freund's Adjuvant (CFA), an antigen emulsion containing heat-dried *Mycobacterium* tuberculosis. CFA was selected to model chronic inflammatory pain, since it closely mimics the time course of persistent injury (Hargreaves et al., 1988; Larson et al., 1986; Ren and Dubner, 1999). Furthermore, postoperative pain, arthritis, and related inflammatory states have exhibited a close relationship with alcohol abuse (King et al., 2012). Therefore, we hypothesized that CFA-treated mice would show increased alcohol consumption compared to uninjured mice. To test this, we exposed saline- and CFA-treated mice of both sexes to a voluntary drinking paradigm (i.e. CA2BC with 20% ethanol [EtOH] for C57BL6/J, CA2BC and IA2BC with 20% EtOH for Floxed-CRF) and measured alcohol consumption across a three-week timespan. Thermal nociceptive thresholds were assessed to ensure that CFA treatment altered pain sensitivity. Blood EtOH concentration (BEC) and plasma corticosterone (CORT) levels were analyzed to assess pharmacologically relevant drinking and alcohol-related stress signaling, two possible contributors to sex differences in alcohol and pain interactions (Edwards et al., 2012; Egli et al., 2012; Fu and Neugebauer, 2008; Heilig and Koob, 2007; Koob, 2013). Taken together, these experiments aim to successfully model sex differences in pain-related alcohol drinking for mice, with the hope that this preclinical model can be applied towards mechanistic investigations of pain and alcohol interactions in the future.

Materials and Methods

Experiment 1: CFA Treatment and Continuous Access 2-Bottle Choice in C57BL6/J Mice

Animals

A total of 32 male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) arrived at 6-weeks of age and were group-housed for one week to habituate to vivarium conditions. Following habituation, mice were individually housed in ventilated Plexiglas cages and given three days of acclimation in a 12-hr reverse light/dark cycle (12am-12pm) prior to the start of the experiment. Subjects were provided ad libitum access to water and Isopro RMH

3000 chow (Prolab, St. Louis, MO) for the duration of the experiment. All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Continuous Access 2-Bottle Choice

Male (N = 16) and female (N = 16) mice were given continuous access to 20% EtOH (w/v) and tap water for three weeks. EtOH solutions were prepared with tap water and 95% EtOH (Pharmaco-AAPER, Brookfield, CT), and delivered via sipper tubes made from 50 mL plastic tubes (Nalgene), rubber stoppers (Fisher Scientific, Agawam, MA), and sipper tubes (Ancare Corp., Bellmore, NY). Prior to EtOH exposure, subjects were given three days to adapt to drinking with sipper tubes. Individually housed mice were then given access to two sipper tubes per cage: one containing 20% EtOH and the other containing tap water. For the span of three weeks, EtOH and water bottles were weighed daily three hours into the dark cycle. Bottle placement (left vs. right) was alternated weekly to control for an inherent or developed side preference. To control for non-consumption-related fluid loss, a dummy cage containing identical sipper tubes was used to subtract weekly dripped fluid values from subject fluid consumption.

Chronic Inflammatory Pain

Prior to EtOH exposure, subjects were given a 50 µl subcutaneous injection of saline or Complete Freund's Adjuvant (CFA; Sigma, St. Louis, MO) in the plantar surface of the right hind paw (n = 8). Drinking experiments started three days after paw injections, around the time that CFA exhibits maximum inflammatory hyperalgesia (Pitzer et al., 2016). To verify that changes in drinking behavior resulted from differences in pain sensitivity, thermal nociceptive thresholds of saline- and CFA-treated mice were assessed one day after the last 24-hr EtOH exposure using

the Hargreaves test. Mice were placed in a plexiglas chamber elevated on a glass plate (IITC Life Science Inc., Woodland Hills, CA) and habituated to the behavioral apparatus for a minimum of 30 minutes. The mid-plantar surface of saline/CFA-treated and untreated hind paws was then exposed to three heat trials each and assessed for paw withdrawal latencies (PWL). The beam intensity was set to produce basal PWLs of approximately 4-6 seconds, with a maximal value of 20 seconds to prevent excessive tissue damage. All testing was conducted with investigators blinded to the experimental conditions.

Blood Ethanol and Corticosterone Concentrations

Following pain sensitivity testing, mice were given four days to recover and re-establish CA2BC drinking behavior in preparation for blood EtOH and corticosterone measurements. On the final drinking day, mice were sacrificed 2 hours into the dark cycle, with trunk blood immediately collected in centrifuge tubes following decapitation. Blood samples were centrifuged at 4°C for 10 min at 3000 RPM, and the plasma was separated for storage at -80°C until analysis. To measure blood EtOH concentration (BEC; mg/dl), 5 µl plasma samples were administered through a Model AM1 Alcohol Analyser (Analox Instruments Ltd., Lunenburg, MA). To measure plasma corticosterone (CORT; ng/ml), 5 µl plasma samples were processed with a commercially available colorimetric ELISA kit (Arbor Assays; Ann Arbor, MI), according to the manufacturer's instructions, with all samples run in duplicate.

Statistical Analysis

All statistical analyses were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA). Analysis of drinking across sessions for saline- and CFA-treated mice (i.e. Treatment x Session for EtOH Intake, EtOH Preference Ratio, Water Intake, and Total Fluid Intake) was performed with a repeated measures analyses of variance (RM-ANOVA). Drinking values for each mouse were averaged across the three-week drinking period to compare Treatment x Sex

with additional two-way ANOVAs. Differences in PWL were determined with a two-way ANOVA comparing Treatment x Paw. Post-hoc analyses with Sidak or Tukey adjustment were performed following significant main group effects. Data are reported as mean plus or minus the standard error of the mean (M ± SEM). Correlational analyses were conducted using linear regressions to assess the predictive relationship of EtOH intake and thermal nociceptive sensitivity, BEC, and CORT levels, with slopes and intercepts assessed for deviation from zero to determine statistical significance. See **Figure 21** for Experimental Timeline.

Experiment 2: CRF Deletion in the BNST with CFA Treatment and Continuous/Intermittent Access 2-Bottle Choice in Floxed-CRF Mice

Animals

A total of male and female Floxed-CRF mice (N = 64 total, aged 6-12 weeks, C57BL/6J background) were bred in-house, initially group-housed with same-sex littermates, and maintained on a 12-h light/dark cycle (light on at 7:00, light off at 19:00) with rodent chow and water available *ad libitum*. Following surgical procedures for the genetic deletion of *Crf* in BNST and a battery of nociceptive sensitivity tests (described in Chapter 2), the mice were transferred to individual housing in new ventilated Plexiglas cages, where they were provided *ad libitum* access to water and Isopro RMH 3000 chow (Prolab, St. Louis, MO). Prior to starting the drinking experiment, subjects were given three days to acclimate to the new home cage and holding room, where a 12-h reverse light/dark cycle (12am-12pm) was implemented to match the conditions of the CA2BC model in Experiment 1. All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Continuous Access 2-Bottle Choice and Intermittent Access 2-Bottle Choice

Male (N = 33) and female (N = 31) mice were treated with a 50 µl subcutaneous injection

of saline or CFA (Sigma, St. Louis, MO) in the plantar surface of the right hind paw. Three days after paw injections, subjects were given continuous access to 20% EtOH (w/v) and tap water for three weeks, then switched to intermittent access to EtOH for five weeks. The drinking setup was prepared with the same methods as Experiment 1, where tap water and 95% EtOH (Pharmaco-AAPER, Brookfield, CT) were delivered via sipper tubes made from 50 mL plastic tubes (Nalgene), rubber stoppers (Fisher Scientific, Agawam, MA), and sipper tubes (Ancare Corp., Bellmore, NY). Individually housed mice were exposed to two sipper tubes per cage: one containing 20% EtOH and the other containing tap water. Subjects were given continuous access to both tubes for the first three weeks, then moved to an intermittent access 2-bottle choice (IA2BC) paradigm to promote escalations in alcohol consumption (as detailed in Hwa et al., 2011) for the next five weeks, where EtOH bottles were available every other day and weighed accordingly. Bottle placement was alternated weekly to control for an inherent or developed side preference and a dummy cage containing identical sipper tubes was used to control for non-consumption-related fluid loss.

Sucrose Drinking

Following CA2BC and IA2BC to 20% EtOH (w/v), subjects were given CA2BC to 1% sucrose (w/v) and tap water for 3 days to assess the hedonic properties of drinking (Pothion et al., 2004). Sucrose solutions were prepared with tap water and 1% D-Sucrose (Fisher Scientific, Hampton, NH). Bottle placement was kept consistent throughout the test given the limited span of the experiment, but a dummy cage was kept as a control for non-consumption-related fluid loss.

Hargreaves

Two days after paw treatment with saline or CFA, subjects were placed in Plexiglas boxes on an elevated glass surface and habituated to the behavioral apparatus for 30-60

minutes. The mid-plantar surface of each hind paw was exposed to a series of 6 noxious heat trials that sequentially alternated between left and right paws. After each heat exposure, a 10-minute inter-trial interval was provided before the next trial. Beam intensity was set to 25 on the IITC Plantar Analgesia Meter, producing average basal paw withdrawal latencies of approximately 6 seconds in Floxed-CRF mice. Cutoff times of 20 seconds were set for each trial to prevent excessive tissue damage.

Plasma Corticosterone

Mice were sacrificed approximately a week after sucrose testing, with trunk blood immediately collected in centrifuge tubes following decapitation. Blood samples were centrifuged at 4°C for 10 min at 3000 RPM, and the plasma was separated for storage at -80°C until analysis. Measurements of plasma corticosterone (CORT; ng/ml) were taken by processing 5 µl plasma samples with a commercially available colorimetric ELISA kit (Arbor Assays; Ann Arbor, MI), according to the manufacturer's instructions, with all samples run in duplicate.

Histology

The procedures described in Chapter 2 were applied to the same tissue samples from Floxed-CRF mice following CA2BC/IA2BC and saline/CFA treatments (see Materials and Methods in Chapter 2: *In Situ Hybridization* and *Confocal Microscopy* sections).

Statistical Analysis

All data were analyzed and visualized with standard statistical software packages from GraphPad Prism 8 (GraphPad Software, San Diego, CA). Single-variable comparisons for cumulative EtOH intake were made using area under the curve analysis. Group comparisons were made using two-way ANOVA, two-way repeated measures ANOVA, or two-way mixed-model ANOVA depending on the number of independent and within-subjects variables in a data

set. Following significant interactions or main effects, post-hoc pairwise t-tests were performed and corrected using Sidak's or Tukey's post-hoc tests to control for multiple comparisons. Data are expressed as mean ± standard error of the mean (SEM), with significance for *p* values below 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

Experiment 3.1

CFA-Treated C57BL6/J Mice Exhibit Greater Alcohol Drinking in a Sex-Dependent Manner

EtOH Intake

Male mice treated with CFA consumed a significantly greater amount of 20% EtOH (w/v) per day (g/kg/24hr) than saline-treated controls, while females did not exhibit any consumption differences across treatments **(Figure 22)**. A RM-ANOVA (Treatment x Session) revealed a main effect for Treatment [$F_{(1, 14)} = 4.768$, $p = 0.0465$] and Session [$F_{(19, 266)} = 1.656$, $p = 0.0437$] in males **(Figure 22A)**, and a main effect for Session [$F_{(19, 266)} = 9.470$, $p < 0.0001$] in females **(Figure 22B)**, but no significant interaction for either sex. Sidak post-hoc analysis revealed no significant difference in EtOH intake between saline- and CFA-treated mice during individual drinking sessions of males or females. Mean drinking values for individual subjects were averaged across three weeks in their respective treatment and sex groupings. A two-way ANOVA (Treatment x Sex) revealed a main effect for Sex [$F_{(1, 28)} = 24.34$, $p < 0.0001$], where female mice exhibit greater EtOH intake than males, regardless of paw treatment **(Figure 22C)**. No main effect for Treatment [$F_{(1, 28)} = 3.524$, $p = 0.0709$] or interaction between Treatment and Sex [$F_{(1, 28)} = 2.229$, $p = 0.1466$] were observed. Tukey post-hoc analysis revealed no significant difference in EtOH intake between saline- and CFA-treated mice for males or females **(Figure 22C)**. Area under the curve analysis for cumulative EtOH intake similarly shows that CFA-treated males had greater cumulative alcohol consumption than saline-treated controls, while females consumed more alcohol regardless of pain state **(Figures 22D-22E)**. A two-way

ANOVA (Treatment x Sex) revealed a main effect for Sex [$F_{(1, 28)} = 13.82$, $p = 0.0009$], where female mice exhibit greater EtOH intake than males **(Figures 22D-22F)**. No main effect for Treatment [$F_{(1, 28)} = 2.209$, $p = 0.1484$] or interaction between Treatment and Sex [$F_{(1, 28)} = 3.054$, $p = 0.0915$] were observed. Tukey post-hoc analysis revealed a significant difference in EtOH intake between saline- and CFA-treated mice, with saline-treated males exhibiting levels of alcohol consumption that were lower than all other experimental groups **(Figure 22F)**.

EtOH Preference Ratio

Preference for EtOH exhibited similar results, with CFA-treated male mice showing a significantly greater EtOH preference ratio than their saline-treated counterparts, while females did not exhibit a preference difference across treatments **(Figure 23)**. A RM-ANOVA (Treatment x Session) revealed a main effect for Treatment [$F_{(1, 14)} = 6.777$, $p = 0.0208$] in males **(Figure 23A)** and Session [$F_{(19, 266)} = 5.297$, $p < 0.0001$] in females **(Figure 23B)**, but no significant interaction for either sex. Sidak post-hoc analysis showed no significant difference in EtOH preference ratio between saline- and CFA-treated mice during individual drinking sessions of males or females. A two-way ANOVA (Treatment x Sex) revealed a main effect for Sex [$F(_{1, 28)} = 14.18$, $p = 0.0008$] but not Treatment [$F_{(1, 28)} = 3.362$, $p = 0.0774$], and a significant interaction between Treatment and Sex [$F_{(1, 28)} = 5.140$, $p = 0.0313$], where EtOH preference ratio was greater in females than males for both treatments **(Figure 23C)**. Tukey post-hoc analysis showed no significant difference in EtOH preference ratio between saline- and CFA-treated mice for males or females **(Figure 23C)**.

Water Intake

CFA-treated mice exhibited water intake that was comparable to saline-treated controls for both sexes **(Figure 24)**. A RM-ANOVA (Treatment x Session) revealed a main effect for Session [$F_{(19, 266)} = 5.661$, $p = < 0.0001$] in males **(Figure 24A)**, no main effect for Treatment or

Session in females **(Figure 24B)**, and no significant interaction for either sex. Sidak post-hoc analysis showed no significant difference in water intake between saline- and CFA-treated mice during individual drinking sessions of males or females. A two-way ANOVA (Treatment x Sex) revealed no main effect for Sex [$F_{(1, 28)} = 2.903$, $p = 0.0995$] or Treatment [$F_{(1, 28)} = 0.06344$, $p = 0.8030$], or a significant interaction between Treatment and Sex [$F_{(1, 28)} = 2.117$, $p = 0.1568$] **(Figure 24C)**. Tukey post-hoc analysis showed no significant difference in water intake between saline- and CFA-treated mice for males or females **(Figure 24C)**.

Total Fluid Intake

CFA-treated mice exhibited total fluid intake that was comparable to saline-treated controls for both sexes **(Figure 25)**. A RM-ANOVA (Treatment x Session) revealed a main effect for Session in males [$F_{(19, 266)} = 13.35$, $p < 0.0001$] **(Figure 25A)** and females [$F_{(19, 266)} = 4.514$, $p < 0.0001$] **(Figure 25B)**, but no significant interaction for either sex. Sidak post-hoc analysis demonstrated no significant difference in total fluid intake between saline- and CFA-treated mice during individual drinking sessions of males or females. A two-way ANOVA (Treatment x Sex) revealed a main effect for Treatment [$F_{(1, 28)} = 1.653$, $p = 0.2091$], but no main effect for Sex [$F_{(1, 28)} = 0.2754$, $p = 0.6039$] or significant interaction between Treatment and Sex [$F_{(1, 28)} = 0.02455$, $p = 0.8766$] **(Figure 25C)**. Tukey post-hoc analysis showed no significant difference in total fluid intake between saline- and CFA-treated mice for males or females **(Figure 25C)**.

<u>CFA-Treated Mice Exhibit Higher Sensitivity to Thermal Nociception in Both Sexes</u>

Male and female C57BL6/J mice treated with CFA exhibit higher sensitivity to thermal nociception, as indicated by lower PWLs, when compared to saline-treated mice **(Figure 26)**. A RM-ANOVA (Treatment x Paw) exhibited a main effect for Treatment [$F_{(1, 14)} = 7.010$, $p = 0.0191$] and Paw [$F_{(1, 14)} = 8.155$, $p = 0.0127$], and a significant Treatment x Paw interaction [$F_{(1,$

$_{14)}$ = 27.46, p = 0.0001] in males **(Figure 26A)**, whereas a RM-ANOVA (Treatment x Paw) exhibited a main effect for Paw [$F_{(1, 14)}$ = 11.00, p = 0.0051], but not Treatment [$F_{(1, 14)}$ = 3.276, p = 0.0918], and a significant Treatment x Paw interaction [$F_{(1, 14)}$ = 10.15, p = 0.0066] in females **(Figure 26B)**. Sidak post-hoc analysis revealed a significant difference in PWLs between saline- and CFA-treated mice for treated paws, but not untreated paws, of both males **(Figure 26A)** and females **(Figure 26B)**. To assess the relationship between pain and drinking, a linear regression was conducted for thermal nociceptive sensitivity of the treated paw as a predictor of EtOH intake. For males, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)}$ = 0.0855, p = 0.7798] with R^2 of 0.0140 **(Figure 26C)**, whereas CFA-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)}$ = 0.1691, p = 0.6952] with R^2 of 0.0274 **(Figure 26C)**. For females, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)}$ = 0.0018, p = 0.9674] with R^2 of 0.0003 **(Figure 26D)**, whereas CFA-treated mice exhibited a significant regression equation of [$F_{(1, 6)}$ = 8.254, p = 0.0283] with R^2 of 0.5791 **(Figure 26D)**.

<u>CFA Does Not Alter Blood Ethanol Concentration and Plasma Corticosterone</u>

Female mice given 2-hr access to EtOH exhibit greater alcohol and total fluid consumption than males **(Figure 27)**. A two-way ANOVA (Treatment x Sex) for 2-hr EtOH intake revealed a main effect for Sex [$F_{(1, 28)}$ = 83.19, p < 0.0001], but not Treatment [$F_{(1, 28)}$ = 0.1574, p = 0.6945], and no significant Treatment x Sex interaction [$F_{(1, 28)}$ = 0.6611, p = 0.4230] **(Figure 27A)**. Tukey post-hoc analysis showed greater 2-hr EtOH intake in female mice, but no significant difference in drinking between saline- and CFA-treated mice **(Figure 27A)**. A two-way ANOVA (Treatment x Sex) for 2-hr EtOH preference ratio revealed no main effect for Treatment [$F_{(1, 28)}$ = 0.2239, p = 0.6397] or Sex [$F_{(1, 28)}$ = 1.673, p = 0.2064], and a significant Treatment x Sex interaction [$F_{(1, 28)}$ = 5.599, p = 0.0251] **(Figure 27B)**. Tukey post-hoc analysis showed no significant difference in EtOH preference ratio between treatments or sex **(Figure**

27B). A two-way ANOVA (Treatment x Sex) for 2-hr water intake revealed no main effect for Treatment [$F_{(1, 28)} = 0.0328$, $p = 0.8574$] or Sex [$F_{(1, 28)} = 0.2640$, $p = 0.6114$], and no significant Treatment x Sex interaction [$F_{(1, 28)} = 3.800$, $p = 0.0613$] **(Figure 27C)**. Tukey post-hoc analysis showed no significant difference in EtOH preference ratio between treatments or sex **(Figure 27C)**. A two-way ANOVA (Treatment x Sex) for 2-hr total fluid intake revealed a main effect for Sex [$F_{(1, 28)} = 8.726$, $p = 0.0063$], but not Treatment [$F_{(1, 28)} = 0.4069$, $p = 0.5288$], and no significant Treatment x Sex interaction [$F_{(1, 28)} = 1.341$, $p = 0.2566$] **(Figure 27D)**. Tukey post-hoc analysis showed higher 2-hr total fluid intake in female mice, but no significant difference in drinking between saline- and CFA-treated mice **(Figure 27D)**.

Following 2-hr access to EtOH, plasma samples were collected to measure BEC and CORT levels. For BEC, a two-way ANOVA (Treatment x Sex) revealed no main effect for Treatment [$F_{(1, 28)} = 0.1365$, $p = 0.7146$] and Sex [$F_{(1, 28)} = 0.8967$, $p = 0.3518$], and no Treatment x Sex interaction [$F_{(1, 28)} = 1.890$, $p = 0.1801$] **(Figure 28A)**. Tukey post-hoc analysis revealed no significant difference in BEC between treatments or sex **(Figure 28A)**. To assess the relationship between drinking and BEC amongst paw treatment groups, a linear regression was conducted for 2-hr EtOH intake as a predictor of BEC. For males, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.0104$, $p = 0.9220$] with R^2 of 0.0017 **(Figure 28B)**, whereas CFA-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 2.334$, $p = 0.1774$] with R^2 of 0.2801 **(Figure 28B)**. For females, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.5927$, $p = 0.4706$] with R^2 of 0.0899 **(Figure 28C)**, whereas CFA-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.6417$, $p = 0.4536$] with R^2 of 0.0966 **(Figure 28C)**.

For CORT, a two-way ANOVA (Treatment x Sex) revealed a main effect for Sex [$F_{(1, 28)} = 7.298$, $p = 0.0116$], but not Treatment [$F_{(1, 28)} = 0.1243$, $p = 0.7270$], and no Treatment x Sex interaction [$F_{(1, 28)} = 0.3202$, $p = 0.5760$] **(Figure 29A)**. Tukey post-hoc analysis revealed greater CORT levels in female mice, but no significant difference between saline- and CFA-treated mice

(Figure 29A). To assess the relationship between drinking and CORT amongst paw treatment groups, a linear regression was conducted for 2-hr EtOH intake as a predictor of CORT. For males, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.3674$, $p = 0.5666$] with R^2 of 0.0577 **(Figure 29B)**, whereas CFA-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.0396$, $p = 0.8487$] with R^2 of 0.0065 **(Figure 29B)**. For females, saline-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.0016$, $p = 0.9691$] with R^2 of 0.0002 **(Figure 29C)**, whereas CFA-treated mice exhibited a non-significant regression equation of [$F_{(1, 6)} = 0.2297$, $p = 0.6487$] with R^2 of 0.0387 **(Figure 29C)**. Taken together, these findings suggest a weak relationship between 2-hr EtOH intake and BEC/CORT.

Experiment 3.2

Genetic Deletion of *Crf* from BNST Influences Pain Interactions with Alcohol Use

CRF relieves pain in peripheral tissues undergoing inflammation, but whether similar conditions impact central mechanisms of pain modulation like CRF in the BNST requires further investigation (Schafer et al., 1997). Here, we hypothesized that genetic deletion of CRF in the BNST would attenuate worsened pain-related behaviors driven by a model of persistent inflammation, since similar reductions in CRF signaling in the BNST have been shown to reduce pain (**Figure 18**; Ide et al., 2013) and alcohol use (Pleil et al., 2015; Rinker et al., 2017). To test this, we first exposed the same CRF deletion and control mice from Chapter 2 to intraplanar treatment of CFA and assessed thermal nociceptive sensitivity. As expected, CFA treatment increased nociceptive sensitivity in the ipsilateral (but not contralateral) paw for all subjects **(Figure 32)**. Contrary to our results in naïve mice, however, CRF deletion did not reduce basal sensitivity for saline-treated mice or attenuate inflammatory pain for CFA-treated mice **(Figures 18 and 32)**.

Considering earlier observations that CFA treatment increases alcohol consumption for males but not females **(Figures 22-23)**, and that CRF may contribute to pain sensitivity in both a

sex-dependent and sex-independent manner for naïve mice, but not intraplanar treated mice **(Figure 18)**, we next investigated how genetic deletion of *Crf* in the BNST affects interactions between pain and alcohol use. Using an eight-week two-bottle choice paradigm, saline- and CFA-treated mice with intact or reduced CRF expression in the BNST were given the choice to voluntarily consume 20% EtOH (w/v) or water (H_2O) **(Figure 30A)**. Subjects had continuous access (CA2BC) to EtOH and H_2O for three weeks, then were switched to an intermittent access (IA2BC) schedule where the availability of EtOH and H_2O was limited to Monday, Wednesday, and Friday to promote escalating alcohol use for five weeks (Hwa et al., 2011).

Comparisons of average alcohol intake and preference ratio for CA2BC and IA2BC revealed that female subjects increase alcohol use across paradigms regardless of pain status and CRF manipulation **(Figures 30B and 30C)**. By contrast, male subjects did not exhibit any increase in alcohol use across paradigms **(Figures 30B and 30C)**. Contrary to our initial predictions where CRF deletion attenuates alcohol use by reducing pain, CRF deletion in the BNST impacted CFA-treated subjects by increasing cumulative alcohol intake compared to CRF-intact (mCherry) control mice **(Figures 30D-30E)**. Although this trend was apparent for both sexes, the greatest increases in cumulative alcohol use were observed for CFA-treated female mice with *Crf* deletion in the BNST **(Figures 30D-30E)**. It is important to note that averaged cumulative alcohol intake only reached significance with area under the curve analysis and not a grouped average by subjects **(Figures 30D-30E, 31A-31B)**, signifying a weak interaction between CRF deletion and pain to drive increases in alcohol use.

Notably, there were no changes in sucrose consumption with CRF deletion or CFA treatment, indicating that the observed effects in drinking were specific to alcohol **(Figures 31C-31D)**. Given the link between CRF signaling and its modulation of pain through stress, we additionally measured plasma corticosterone levels and found no changes with CRF deletion or CFA treatment following the eight-week drinking paradigm **(Figures 31E-31F)**. This suggests CA2BC/IA2BC drinking behaviors were neither modulated nor driven by sex differences in

stress hormones, as was initially hypothesized in Experiment 3.1. Finally, using the genetic deletion validation detailed in Chapter 2 **(Figures 18C-18D)**, we quantified *Crf* mRNA expression in the BNST by pain status and found no differences between saline-treated and CFA-treated mice, revealing no effect of persistent inflammation on CRF expression in the BNST following CA2BC/IA2BC **(Figures 32C-32D)**. Taken together, our results show that deletion of *Crf* in the BNST is not sufficient to reduce inflammatory pain but can work with inflammation to drive increased alcohol use in a sex-independent manner.

Discussion

CFA + CA2BC as a Novel Preclinical Model of Pain-Induced Alcohol Drinking

In Experiment 3.1, we describe sex differences in alcohol drinking following the induction of inflammatory pain. Male CFA-treated mice consumed more alcohol and exhibited higher preference for the drug than saline-treated controls, whereas female CFA-treated mice showed similar alcohol and water intake as controls. Characteristic of C57BL/6J mice with continuous access to high concentrations of alcohol (Jury et al., 2017; Middaugh et al., 1999; Smith et al., 2015), drinking levels differed between sexes, with female mice exhibiting greater EtOH consumption and preference than male mice, regardless of saline or CFA treatment. These findings suggest that chronic inflammatory pain increases alcohol drinking in males, despite higher overall drinking in females. Similar clinical observations have been made in chronic pain patients, with men more commonly self-medicating with alcohol than women (Riley and King, 2009). This pain-related alcohol consumption is greater in males throughout the lifetime, with its peak at early adulthood (Brennan et al., 2011; Riley and King, 2009; Wilsnack et al., 2009). By pairing CFA treatment and CA2BC drinking, our model was able to replicate these human patterns of voluntary drinking in injured young adult male mice, providing evidence that chronic inflammatory pain can potentiate alcohol drinking in a sex-specific manner for rodents.

Although this finding is reflective of clinical reports, it is notably inconsistent with another study on CFA-treated mice that found no effect on pain-related alcohol consumption (Smith et al., 2015). The discrepancy between findings may be due to differences in experimental design. For example, escalating concentrations of alcohol were utilized in Smith *et al.* (2015), while fixed concentrations were used in the present study. Mice given escalating concentrations had CFA administered while being exposed to a lower percentage of alcohol (10%), so consumption may not have produced comparable analgesia to that of a fixed high concentration of alcohol (20%). Theoretically, this would result in a higher dynamic range of alcohol consumption for the fixed concentration paradigm, which could explain why there are more pronounced effects of pain-induced drinking in the present study. Alternatively, the use of differently aged mice (i.e. 30-33 weeks old in Smith *et al.* [2015] versus 7-10 weeks old in the present study) could have altered alcohol consumption as well, since younger rodents drink more than fully developed adults (Holstein et al., 2011; Schramm-Sapyta et al., 2014; Vetter-O'Hagen et al., 2009). Divergences in pain severity following CFA treatment could also explain the inconsistency in findings. Male mice treated with a high volume of CFA (i.e. 50 µl in the present study) exhibited increased alcohol consumption relative to saline-treated controls, while low volume (i.e. 10 µl in Smith *et al.* [2015]) exposure did not alter consumption relative to pre-CFA levels. This suggests that high and low volumes of CFA treatment result in different severities of pain, which can then determine the extent of changes observed with alcohol drinking. If pain severity were to contribute to these consumption differences, a comparison of nociceptive sensitivity should reveal higher pain sensitivity in CFA-treated mice from the present study compared to those in Smith *et al.* (2015). The use of different behavioral assays at non-parallel time points and a lack of parallel control groups (i.e. Pre-CFA mice in Smith *et al.* [2015] versus saline-treated mice in the present study), however, makes it difficult to make a direct comparison. Future studies should examine how the severity of chronic inflammatory pain contributes to sex-specific patterns of drinking with more uniform measures of thermal and mechanical nociception and

control groups. Determining the extent that alcohol concentration and age can alter pain-related drinking may further clarify any remaining drinking differences between the two studies.

We additionally showed that female C57BL/6J mice exhibit greater alcohol consumption than males, an observation that is common among female rodents exposed to voluntary drinking paradigms (Blanchard and Glick, 2002; Cailhol and Mormède, 2001; Chester et al., 2006; Doremus et al., 2005; Jury et al., 2017; Lancaster et al., 1996; Lê et al., 2001; Middaugh et al., 1999; Smith et al., 2015; Truxell et al., 2007). Moreover, our female drinking data matches the maximal average consumption values previously reported in C57BL/6J mice undergoing continuous access to ≥ 10% alcohol (Jury et al., 2017; Middaugh et al., 1999; Smith et al., 2015). With such high baseline alcohol consumption, it is possible that saline-treated females have already reached maximal drinking in our study, so the addition of pain would not increase drinking in females. Although this ceiling effect is possible, it is unlikely, considering that daily EtOH intake in females was around 10-12 g/kg during the first four sessions and escalated to 20-25 g/kg by the last session. If pain were able to drive alcohol drinking in females, early session EtOH intake should have increased up to two times in CFA-treated mice, but that is not the case. Therefore, sex differences in basal alcohol consumption were not likely to prevent pain-related drinking increases for females.

Differences in inflammatory response and alcohol analgesia may provide a better explanation for these sex-specific patterns in drinking. CFA and related inflammatory agents cause female rodents to develop hyperalgesia more rapidly than males, with females being less prone to attenuation of inflammation by alcohol and other analgesic drugs (Alfonso-Loeches et al., 2013; Coleman and Crews, 2018; Cook and Nickerson, 2005; Pascual et al., 2017). Thus, it is possible that CFA-treated females are less susceptible to escalated alcohol consumption relative to saline-treated controls because the drug provides inadequate analgesia to promote further drinking. Although not measured in the present study, ongoing investigations with this

model should assess the potential impact of sex differences in alcohol analgesia and its effects on pain-related drinking.

Three weeks after paw treatment, pain testing revealed that CFA-treated mice had higher thermal nociceptive sensitivity than saline-treated controls for both males and females. Considering the timing of the test, it is unclear whether this difference in sensitivity reflects the contributions of pain to drinking or drinking to pain. This is an important distinction, as cessation of alcohol intake following repeated drug exposure can exacerbate pain sensitivity. This phenomenon, commonly referred to as EtOH withdrawal-induced hyperalgesia (EIH), has been reported in mice (Dhir et al., 2005; Smith et al., 2016), as well as rats (Dina et al., 2000, 2007; Edwards et al., 2012; Fu et al., 2015; Gatch and Lal, 1999; Malec et al., 1987; Roltsch et al., 2017; Shumilla et al., 2005) and humans (Boissoneault et al., 2018; Dodds et al., 1945; Jochum et al., 2010; Riley and King, 2009; Wolff et al., 1942), using a variety of alcohol exposure models. In our CA2BC paradigm, it is unclear whether there were pathological shifts in sensitivity following three weeks of alcohol exposure. The lack of sensitivity differences following increased drinking in CFA-treated males, along with the non-significant regression of Drinking x Pain suggests that the extent of alcohol consumption had little effect on predicting pain sensitivity (and vice versa) in all groups. This result is similar to that of other continuous access experiments in mice, which also failed to produce EIH (Smith et al., 2015). The exception to this finding is CFA-treated females: despite comparable drinking patterns to saline-treated controls, CFA-treated females exhibited a significant negative correlation for Drinking x Pain, where injured females that drink more alcohol experience higher pain sensitivity. This result is reminiscent of clinical data showing that there is higher EIH severity and prevalence in females, with women being more likely to report significant recurrent pain and concurrent chronic pain conditions (Boissoneault et al., 2018). Despite our findings closely following predicted results from the literature, not having nociceptive sensitivity measures prior to EtOH exposure makes it impossible to conclude whether or not these data indicate a causal null effect of alcohol

consumption on pain. Although a limitation in the study, it does not change our interpretation that male-specific increases in alcohol consumption follow the induction of chronic inflammatory pain. However, future experiments should be mindful of establishing baseline nociceptive sensitivity prior to the start of alcohol exposure when studying pain-related alcohol use.

Following 2-hour CA2BC, females consumed more alcohol than males. Contrary to the increased drinking phenotype seen in CFA-treated males during 24-hr alcohol access, no treatment effects were observed. These results suggest that the terminal drinking session did not last long enough to capture peak alcohol consumption (i.e. 4+ hours into the dark cycle) (Smith et al., 2015). Although increases for alcohol drinking in the 2-hr CA2BC were specific to females, no sex effects were found for BEC. By contrast, a previous evaluation of BEC showed no sex differences for alcohol consumption after 30-minute access to 12% EtOH, but higher BECs in females (Middaugh et al., 1999). Although a common result for humans when both sexes are allowed an equal dosing of orally administered EtOH (Ammon et al., 1996; Sutker et al., 1983), such results are less common in rodents (Ho et al., 1989). This difference may be due to feeding manipulations coinciding with BEC measurements after drinking, as suggested in Middaugh et al. (1999). Although caloric mediation of alcohol consumption is well established in the literature (Rodgers et al., 1963; Rodgers, 1966; Thiele et al., 2012), sex differences in prandial metabolism and alcohol consumption are thought to be negligible, as thirst is more strongly motivating for alcohol drinking than caloric need in both sexes (Middaugh et al., 1999). Paradoxically, higher BECs in females are accompanied by enhancements in alcohol metabolism (Baraona et al., 2001; Collins et al., 1975; Mumenthaler et al., 1999), and may explain why female mice that consume a greater amount of alcohol do not produce higher BECs than males.

Similar to alcohol consumption patterns in the 2-hr CA2BC paradigm, CORT was not affected by CFA, but differed by sex. Specifically, female CORT levels were found to be higher than that of males. Higher CORT release following alcohol exposure has previously been

observed in female rodents, with deletion of circulating sex steroids attenuating the difference (Rivier, 1993). However, increased levels of CORT in female mice may not be the result of the drug, as alcohol drinking does not produce differences in CORT relative to consumption of water in males or females (Finn et al., 2004). Although CFA-treated males did not drink more in the 2-hr terminal drinking session, the literature would predict that an increase in drinking after 24-hr access would still not potentiate CORT release in the males, despite showing pain-related increases in alcohol consumption. This suggests that CORT is basally higher in females, and not susceptible to changes following CFA treatment in both sexes. Previous work has found that inflammatory pain increases CORT, but this phenotype has only been observed in male rats and has yet to be investigated in combination with drinking (Butkevich et al., 2013; Harper et al., 2001; Pitcher et al., 2018), suggesting that our findings for CORT are specific to CFA/CA2BC-exposed C57BL/6J mice.

Without pre-treatment CORT levels, it is difficult to determine whether these female-specific elevations in stress are entirely due to sex differences in basal CORT. It is important to consider the alternate possibility that these changes are driven by differences in stress reactivity. It is well-established that maladaptive responses to stress promote alcohol drinking and relapse behavior (Becker et al., 2011; Keyes et al., 2012; Koob, 2001; Koob and Kreek, 2007). Previous studies have linked these behaviors to the sex-dependent expression of stress markers, with females exhibiting higher glucocorticoid levels and alcohol consumption following exposure to various stressors (Cozzoli et al., 2014; Haleem et al., 1988; Heinsbroek et al., 1991; Kudielka and Kirschbaum, 2005; Yoshimura et al., 2003). Considering that female mice exhibit a more sensitive and sustained stress response compared to males (Blanchard and Glick, 2002; Brown and Grunberg, 1995; Hermes et al., 2006; Palanza et al., 2001), it is possible that early experimental stressors such as isolated housing and paw injections increased CORT levels and alcohol consumption for female mice in the present study (Hermes et al., 2006; Lopez et al., 2011; Moriya et al., 2015). This would be contingent on early experimental stressors

upregulating CORT responses for over three weeks in female mice, an improbable but untested concept. Future studies using CFA/CA2BC should more closely investigate this relationship between sex differences in stress reactivity and alcohol drinking.

In conclusion, male and female mice exhibit distinct alcohol drinking behavior when undergoing pain. Compared to saline-treated controls, CFA-treated males show greater alcohol consumption, while CFA-treated females show a closer relationship between alcohol consumption and pain sensitivity. Regardless of pain state, females exhibited higher levels of total alcohol drinking and corticosterone compared to males. These findings reflect sex-specific trends in alcohol use reported by chronic pain populations and may speak to the validity of our CFA/CA2BC model in mice. Mechanistic contributions to sex differences in pain-related alcohol drinking will be required in future applications of the model, with special attention reserved for the role of stress signaling in pain and alcohol interactions.

The Role of DA and CRF Signaling in Pain-Related Drug Use

In Experiment 3.2, we tested the same Floxed-CRF mice from Chapter 2 in a modified version of the CFA/CA2BC paradigm described in Experiment 3.1. Earlier, we reported that CRF deletion in the BNST was able to reduce nociceptive sensitivity for phasic-acting pain exposures in both sexes and selectively increase protective behaviors for tonic-acting/inescapable pain exposures in female mice, suggesting that the impact of impaired CRF expression depends on the duration and context of pain. Based on these functional outcomes, we hypothesized that two opposing results were possible: 1) CRF deletion would counteract the hyperalgesic state elicited by CFA treatment and attenuate the pain-induced escalations in drinking observed for male mice in Experiment 3.1 or 2) CFA treatment, being a sustained driver of heightened sensitivity, would exacerbate the impact of CRF deletion on pain sensitivity and alcohol drinking in female mice, similar to the effect of the prolonged hot plate. Surprisingly, the

results indicate a trend for both CRF deletion and CFA treatment in increased alcohol consumption, with CRF deletion mice generally exhibiting greater alcohol use than control mice and CRF deletion mice with CFA treatment demonstrating the greatest cumulative consumption for male and female subjects. Measurement of sucrose consumption confirmed that these observed outcomes were specific to alcohol rather than a general response to hedonic stimuli.

These results contradict the expected role of CRF in the BNST, as previous studies have shown that chemogenetic inhibition of $BNST^{CRF+}$ neurons reduces binge drinking in mice (Pliel et al., 2015; Rinker et al., 2017). $BNST^{CRF+}$ have specifically been shown to regulate these changes in alcohol use through projections to the VTA (Rinker et al., 2017), while similar circuit mechanisms have been proposed to be involved with pathological pain states. A recent study from the Minami lab showed that neuropathic pain upregulates CRF and CRF_{R1} in the BNST and inhibits VTA-projecting BNST neurons to tonically suppress the mesolimbic DA system in rats (Takahashi et al., 2019). Chronic pain or stress particularly amplifies the contributions of CRF_{R1} to pain-related behaviors, with CRF_{R1} antagonism being more effective at relieving pain in situations that are aversive rather than neutral (Tran et al., 2012; Tran et al., 2014; Takahashi et al., 2019). These convergent circuits for pain and alcohol use suggest that pathological pain can exacerbate tonic suppression of DA signaling in the VTA to increase alcohol use (Takahashi et al., 2019). Although we did not report similar increases in local *Crf* mRNA following CFA treatment or local *Crf* deletion, it is interesting that an absence of CRF produced alcohol use in the same presumed direction as enhanced CRF signaling. It is thus possible that *Crf* mRNA levels do not reflect greater functional changes in CRF signaling, and that inflammatory pain is enhancing suppression of VTA via functional changes such as $BNST^{CRF+}$ excitability or enhanced CRF_{R1} activity. Alternatively, inflammatory pain may be augmenting CRF signaling through external sources like the CeA, which have been shown to be sensitive to CFA treatment (Li & Sheets, 2018). While these possibilities were not assessed in the present study, these factors should be accounted for in future investigations.

Although interactions with pathological pain were essential to the observed drinking phenotypes, *Crf* deletion appeared to have a more robust effect on alcohol use than CFA treatment, as the inflammatory state merely supplemented increases in drinking that were already present with *Crf* deletion alone. We specifically found that CRE-SAL and CRE-CFA mice exhibited the highest alcohol use for both males and females, and that the transition from CA2BC to IA2BC produced greater escalations in alcohol intake and preference exclusively for CRE-CFA females. Overall, CFA-treated females with *Crf* deletion exhibited the greatest consumption increases of all experimental groups. This enhancement of drinking behaviors with CFA treatment in *Crf* deletion mice is reminiscent of the evoked increases in paw attending behaviors for females in the prolonged hot plate. Both outcomes may be explained by interactions between the intensity of pain and *Crf* deletion, where extreme conditions of aversion (i.e. tonic-acting/inescapable nociceptive stimuli [e.g. prolonged hot plate, CFA treatment]) more robustly promote increases in active coping and drinking behaviors for female subjects. These effects are contrary to the sex-independent anti-nociceptive effects seen with acute pain exposures, supporting the theory that lasting pain experiences are a good predictor of female-specific coping behaviors when *Crf* expression in the BNST is impaired. More work is needed, however, to determine how CRF interactions with CFA treatment work in regard to pain and drug use, as uncharacterized roles for CRF signaling and stimulus intensity could possibly explain the paradoxical effects observed in our data.

Evidence for female-specific escalations in alcohol consumption from CA2BC to IA2BC refutes earlier speculation that CFA treatment could be driving ceiling effects in alcohol drinking for female C57BL6/J mice in the CA2BC paradigm. Given this dynamic range of alcohol consumption, it is interesting that our CA2BC paradigm in CFA-treated Floxed-CRF mice failed to reproduce the male-specific increases in drinking seen in Experiment 3.1 or escalate consumption in females. The inability to replicate our initial findings in males and females may be explained by strain or cohort differences, as the average alcohol consumption in Floxed-CRF

mice appeared to be lower than C57BL6/J mice (Yoneyama et al., 2008). Measures of plasma CORT levels support this notion, as C57BL6/J mice exhibit sex-specific baseline levels of stress, while these levels are comparable for male and female Floxed-CRF mice. In Experiment 3.1, we specifically show that female C57BL6/J mice, regardless of pain status, have higher levels of CORT than males following a 2-hour terminal drinking session, with averages ranging from 44.02-55.54 ng/mL in males and 82.32-85.00 ng/mL in females. By contrast, Experiment 3.2 shows comparable CORT levels in both sexes of Floxed-CRF mice following approximately 10 days of abstinence from alcohol, with averages ranging from 52.07-95.27 ng/mL in males and 41.56-63.47 ng/mL in females. When directly comparing C57BL/6 mice with Floxed-CRF control mice, only saline-treated males (55.54 ng/mL vs. 56.58 ng/mL) exhibited similar levels of CORT across strains, while CFA-treated males (44.02 ng/mL vs. 95.27 ng/mL), saline-treated females (82.32 ng/mL vs. 63.47 ng/mL), and CFA-treated females (85.00 ng/mL vs. 41.56 ng/mL) showed strain differences. Although the varying circumstances prior to sample collection make interpretation of this comparison difficult, it is an interesting possibility for strain differences in basal and evoked levels of stress hormones to preclude the expression of sex differences in pain-induced drinking. Hyperactive CRF signaling in females have been posited to drive sex differences in stress and alcohol use, so it is possible that small alterations in the *Crf* gene of the Floxed-CRF mouse strain inherently modifies basal drinking and CORT compared to C57BL6/J mice (Pleil & Skelly, 2018). As with all novel preclinical models, further investigation is required to determine the validity and reproducibility of these early results.

Similar to our approach with *Crf* in the BNST, we showed in Chapter 1 that CRSIPR/Cas9-mediated deletion of *Th* in the vlPAG/DR mitigated the sex-specific effects of vlPAG/DR^{DA+} activation, but failed to do so for systemic morphine administration. These data suggest that greater mechanistic complexity underlies sex differences in pain and drug use beyond the DA and CRF signaling mechanisms we have tested. The vlPAG/DR and BNST are both heterogeneous structures in their molecular composition, connectivity, and function **(Figure**

1). vlPAG/DR^{DA+} and BNST^{CRF+} neurons exhibit co-expression with various neurotransmitters, neuromodulators, and peptides (e.g. Glu, GABA, VIP, NT, oxytocin; Dabrowska et al., 2013), and these factors likely contribute to the interactions between pain and drug use in conjunction with DA and CRF **(Figure 2)**. A possible shortcoming of our experiments is that the techniques we used do not properly control for the contributions of other co-expressed factors. Mutant knockout and CRISPR/Cas9 approaches can promote loss of function in targeted genes but changes in other RNA or protein can compensate for this deficiency (El-Brolosy & Stainier, 2017; Stasyshyn et al., 2019). Transcriptional adaption through damage or mutation to DNA have been reported and may increase expression of off-target genes within the same neuron (Stasyshyn et al., 2019). Evaluations of transcriptional changes following deletion of *Th* from vlPAG/DR or *Crf* from BNST were not performed, so it is possible that other molecular drivers of pain and/or drug use that are co-expressed with DA and CRF may confound the current outcomes and interpretation of these data. It is also important to note that parallel circuits in the vlPAG/DR and BNST are likely to provide redundant support for the effects of systemic drug administration, since morphine and alcohol recruit various regions containing μ opioid and GABA receptors to reduce pain and promote drug use (Mansour et al., 1995; Davies, 2003; Moy et al., 2020). This widespread engagement may explain why deletion of *Th* and *Crf* in a single brain region is not sufficient to prevent the effects of drug use. These genetic deletion studies thus signify the need for greater mechanistic insight on the relative contributions of co-expressing factors and projections associated with DA and CRF to understand how these signaling systems work together to modulate pain.

CHAPTER 4: DISCUSSION

Summary

In this dissertation, we aimed to expand the limited knowledge on DA and CRF systems in the vlPAG/DR and BNST and their contributions to sex differences in pain. Using a series of experiments to evaluate the impact of cell type- and projection-specificity in these systems, we determined that vlPAG/DR^{DA+} and BNST^{CRF+} neurons can modulate pain responses based on interactions between sex and behavioral context. This work has enabled us to characterize a novel sex-specific ascending circuit where vlPAG/DR^{DA+} projections to BNST link these DA and CRF systems and how they influence the pain experience. The major findings from each chapter are as follows:

Chapter 1: First, we demonstrated that vlPAG/DR^{DA+} neurons produce discrete pain-related behaviors in male and female mice through DA signaling to the BNST. We specifically showed that vlPAG/DR^{DA+}-BNST activation reduces nociceptive sensitivity in males, while increasing locomotion in females, and that these sex-dependent behaviors can be selectively evoked in different contexts using similar stimulation parameters. Genetic deletion of *Th* in vlPAG/DR was able to block these behaviors, while DA transmission and downstream inhibitory connectivity between vlPAG/DR and BNST differed in male and female mice, suggesting that DA interactions with local BNST circuitry plays a critical role in pain.

Chapter 2: We next revealed that BNST^{CRF+} neurons, which have been implicated in DA signaling interactions (Meloni et al., 2006; Kash et al., 2008; Dougalis et al., 2012), exhibit dynamic *in vivo* recruitment by noxious heat. Sex-specific activation of BNST^{CRF+} neurons was observed with larger and faster magnitudes in males and higher synchrony in females. Genetic

deletion of *Crf* in the BNST produced reductions in nociceptive sensitivity for acute exposures to pain in both sexes, while increasing protective behaviors for prolonged exposures exclusively in females. These results indicate that CRF in the BNST may modulate pain-related behaviors based on the duration and context of exposure.

Chapter 3: Finally, we developed and tested CFA/CA2BC, a novel preclinical model of pain-induced alcohol drinking in C57BL6/J mice, where persistent inflammation produced increases in alcohol consumption for males but not females. We then used a modified version of this model to show that *Crf* deletion in the BNST enhances pain-induced alcohol drinking in both sexes, while greater escalations between CA2BC and IA2BC were observed in female mice. More generally, we discuss the consequences of impairing local DA and CRF expression on measures of pain-related drug use.

Collectively, these chapters illustrate the many contributions of DA and CRF signaling to pain, revealing novel targets for the regulation of pain processing and modulation that exhibit divergent function in male and female mice.

Classifying Novel Sex-Specific Mechanisms of Pain

The rapid emergence of SABV research has revealed disparate descriptions of mechanistic differences between male and female expressions of pain, but few studies have illustrated how these molecular drivers may work together. Here, we propose a singular model of vlPAG/DR^{DA+} and BNST^{CRF+} contributions to pain by first considering how the endpoints of pain-related behaviors relate in male and female subjects. Three broad categories of sex-specific function have been described by McCarthy et al. (2012), where model organisms can exhibit 1) sexual dimorphism (forms of the endpoint are distinct in males and females), 2) sex differences (an endpoint is expressed on a continuum but the averages differ in males and females), or 3) sex convergence and divergence (similar endpoints are driven by dissimilar mechanisms or evoked by a challenge such as injury or stress in males and females). In our

own findings, we report that vlPAG/DR^{DA+} projections to BNST exhibit a combination of sexual dimorphism and sex divergence in anti-nociceptive and locomotor responses, as 20 Hz stimulation of this pathway elicited different outcomes based on sex and contextual salience of the environment. This suggests that vlPAG/DR^{DA+} activation exclusively drives changes in nociceptive sensitivity in males and locomotion in females. Remember, however, that vlPAG/DR^{DA+} activation was able to produce trends for reduced sensitivity to mechanical nociception in two cohorts of female mice, while *Th* deletion in the vlPAG/DR suppressed novelty-induced locomotor activity in the open field for males and females. These findings indicate that vlPAG/DR^{DA+} contributions to anti-nociceptive and locomotor functions exist in both sexes and manifest differently according to the parameters and context in which neuronal activation is taking place. So rather than sexual dimorphism, the function of DA in the vlPAG/DR is more likely to constitute a sex difference.

In contrast to this DA population, BNST^{CRF+} neurons exhibited convergences in nociceptive encoding, as the magnitude and timing of neuronal activity differed in males and females without any variation in nociceptive sensitivity. Genetic deletion of *Crf* in the BNST, however, resulted in a conditional sex difference, as the manipulation produced comparable reductions in nociceptive sensitivity for males and females, but increased protective behaviors exclusively in females. Such provisional terms for pain outcomes may reflect an element of sex divergence, since the prolonged hot plate, a longer acting and less escapable noxious stimulus, was required to evoke female-specific indications of spontaneous pain. Reconciling these findings, we propose that convergences in pain processing and divergences in pain modulation translate to differences in the functional significance of BNST^{CRF+} activity size and timing in male and females. How these mechanisms change with sustained pain and modulate function are likely to differ by sex, as repeated trials of nociceptive exposure resulted in progressive decreases in the magnitude of activation and increases in the spike latency for male and female subjects respectively. The ability of temporal correlations to transmit information in accordance

with the magnitude of neuronal ensemble activity has recently been characterized (Frost et al., 2020), so it remains an intriguing possibility for BNST^{CRF+} magnitude/synchrony to drive modulation of responses to sustained pain. Such a mechanism could explain the female-specific protective behaviors observed with *Crf* deletion.

When applied to pain-induced drug use in C57BL6/J mice, CFA treatment produced escalations in alcohol consumption for males and higher overall drinking and plasma CORT levels in females, suggesting that these pain-drug interactions are susceptible to sex differences. However, these effects were strain-dependent, as Floxed-CRF mice did not exhibit the same trends for CFA-induced escalations or CORT. Instead, genetic deletion of *Crf* in the BNST interacted with CFA treatment to increase alcohol consumption in both males and females. Discrepancies in alcohol use between male and female subjects were restricted to CA2BC-IA2BC escalations for CFA-treated females with *Crf* deletion. Since this phenotype relied on a tonic-acting/inescapable nociceptive stimulus (i.e. CFA) to evoke distinct iterations by sex, we classified it as a sex difference and divergence, similar to the conditionality described with female-specific protective behaviors in the prolonged hot plate.

Taken together, vlPAG/DR^{DA+} and BNST^{CRF+} neurons appear to promote sex differences or convergences in acute pain and sex divergences in prolonged pain. These outcomes show that both DA and CRF subpopulations can modulate reflexive, spontaneous, and locomotor responses to pain based on the context of the noxious treatment and environment, with female-specific behaviors being driven to a greater extent when these populations were manipulated in the presence of tonic-acting/inescapable nociceptive stimuli (e.g. locomotion with visceral nociception, protective behaviors with prolonged hot plate, CA2BC to IA2BC escalations with CFA) or contextual salience (e.g. locomotion with opposite sex conspecifics and environmental novelty). Our interpretation asserts that the repertoire of pain responses following manipulation of vlPAG/DR^{DA+} and BNST^{CRF+} neurons has the potential to be elicited in both sexes, but the

context in which these behaviors are evoked can drive discrete functional outcomes for male and female mice.

This delineation of mechanistic similarities and differences is necessary for precision approaches to combating the pathophysiology of pain, but it is important to note that observed differences by sex do not inherently signify that one sex is better suited for survival or more deserving of care than the other. Careful interpretation is required to obtain biological insight that is not biased towards societal attitudes on male and female behavior. In reference to our findings, we observed that vlPAG/DR^{DA+}-BNST anti-nociception could be advantageous in male mice when confronted with acute exposures to noxious stimuli. By contrast, vlPAG/DR^{DA+}-BNST locomotion in female mice may be beneficial for pain sources with fixed locations or sustained durations, enabling active coping strategies in response to discomfort. The anti-nociceptive and locomotor responses elicited by this pathway are not inherently adaptive or maladaptive without knowing the specific context in which these behaviors are engaged. Interestingly, we report that pathway activation did not indiscriminately drive sex-specific behaviors: the anti-nociceptive effects in males were not preserved in environments where females exhibited locomotion (e.g. visceral nociception), nor were locomotor behaviors in females present in environments where males exhibited anti-nociception (e.g. Hargreaves, Von Frey). The mechanisms of sex-specific pain responses may thus selectively contribute to function based on environmental context to highlight the respective strengths of male and female coping strategies. Similar selectivity with *Crf* deletion in the BNST has been established based on temporal characteristics of nociceptive stimuli. We conclude that vlPAG/DR^{DA+} and BNST^{CRF+} neurons are important for the regulation of sex-specific responses to pain in relation to the environment.

The Role of Salience in Pain Modulation

Our unifying theory for the functional role of vlPAG/DR^{DA+} and BNST^{CRF+} neurons contends that salience may be encoded by and influencing these neurons to orchestrate

adaptive responses to pain. The concept of salience “refers to the physical distinctiveness or conspicuity of a stimulus, a relative property that depends on its relationship to the other surrounding stimuli” (Legrain et al., 2011). Hierarchies of environmental stimuli are ordered in the brain by their potential for reward or harm, as these cues provide the greatest value in physical and emotional payoff. Pain, being an event that is defined by its potential for harm, is thus a uniquely salient sensory experience that demands immediate attention for informed responses to the environment (Borsook et al., 2013). By contrast, the assignment of salience to non-nociceptive tactile stimuli requires voluntary attention or behavioral relevance to motivate action (Downar et al., 2003). In humans, neuroimaging has been used to parse out this distinction between nociceptive and non-nociceptive stimuli and identify structures related to pain. Several studies have made the link between nociception and salience as essential components of the pain experience (Legrain et al., 2011; Mouraux et al., 2011; Linnman et al., 2012a; Borsook et al., 2013), showing that the intensity and unpleasantness of noxious stimuli scale with activity in responsive regions.

This network of brain regions showing correlated activity with nociceptive exposure, referred to as the “pain matrix,” has revealed important contributions from the PAG in the intersection of pain and salience (Peyron et al., 2000; Apkarian et al., 2006; Linnman et al., 2012a). By integrating information from peripheral afferents and higher centers of the brain, the PAG is well-situated to gate stimulus representations, organize relevant information in the environment, and modify behaviors based on contextual salience (Linnman et al., 2012a). Recent preclinical findings have established a role for vlPAG/DR^{DA+} neurons in the functional modulation of salience. In one of the first behavioral characterizations of these DA neurons, Clifford Saper and colleagues demonstrated that vlPAG/DR^{DA+} neurons are necessary for wakefulness in rats (Lu et al., 2006). This seminal study found that approximately 50% of TH cells in vlPAG/DR were activated during natural wakefulness or environmental stimulation, while chemical lesion of DA neurons in vlPAG/DR resulted in a 20% increase in sleep (Lu et al.,

2006). As established by the Weinshenker lab, noradrenergic signaling from the locus coeruleus regulates these vlPAG/DR^{DA+} contributions to wakefulness through astrocytes (Porter-Stransky et al., 2019). Elegant work from the Gradinaru lab further expanded on these wake/sleep findings by showing that *in vivo* vlPAG/DR^{DA+} activity increases with wakefulness and other prototypically salient experiences such as pain (footshock), social contact (unfamiliar mouse of the opposite sex), and novelty (unfamiliar object) in mice (Cho et al., 2017). Since wakefulness and related salient experiences all constitute heightened arousal states, these findings are compelling indications that vlPAG/DR^{DA+} neurons contribute to and are modulated by experiences that necessitate attention.

As discussed in Chapter 1, several of our findings support a role for vlPAG/DR^{DA+} in salience. This was particularly evident in experiments with optogenetic activation of vlPAG/DR^{DA+} terminals in the BNST, as the intensity and unpleasantness of nociceptive stimuli appeared to modulate pathway function. With a phasic-acting/escapable nociceptive exposure, pathway activation produced anti-nociception in male mice. With a tonic-acting/inescapable nociceptive exposure, however, the same manipulation resulted in increased writhing and locomotor behaviors in female mice. These data suggest that changing contextual salience with nociceptive stimuli of varying duration/escapability may prime the vlPAG/DR^{DA+}-BNST pathway to function in a sex-specific manner. Supporting this role for salience, we showed that vlPAG/DR^{DA+}-BNST activation in the presence of significant non-painful experiences (e.g. socializing with an opposite sex conspecific, environmental novelty) was able to drive female-specific locomotor increases as well. This indicates that contextual salience can modulate vlPAG/DR^{DA+}-BNST contributions to adaptive behaviors in the absence of nociceptive stimuli. Clinical evidence shows that multimodal response networks associated with pain (i.e. regions that respond to nociceptive stimuli, as well as non-nociceptive/tactile, auditory, and visual stimuli) are scaled with perceived salience and novelty of the presented stimulus (Mouraux & Plaghki, 2007; Iannetti et al., 2008; Lee et al., 2009; Legrain et al., 2011; Mouraux et al., 2011).

This functional modulation by salience is reminiscent of the data we describe, implying that some mechanistic contributions to pain reflect broader stimulus gating processes that can happen in the presence or absence of tissue damage. Together, these findings indicate that vlPAG/DR^{DA+} function is not strictly limited to pain and may instead apply more broadly to states of vigilance, where contextual salience and threat response work in tandem to promote survival.

Our findings additionally implicate the BNST in functional modulation by salience, as DA input relied on this structure to drive context-specific behaviors. Previous investigations suggest that the region is involved in value representation, particularly when it comes to how the BNST processes threatening or aversive stimuli (Avery et al., 2016). Increased functional connectivity between the BNST and downstream regions regulating stimulus detection (e.g. anterior insula, caudate nucleus) has been observed in patients with post-traumatic stress disorder (Rabellino et al., 2017). These salience networks have been linked to maladaptive anxiety following threat exposure, with the BNST and associated regions exhibiting longer lasting responses to hostile cues in patients with anxiety disorders compared to healthy controls (Herrmann et al., 2016; Brinkmann et al., 2017; Jenks et al., 2020). By contrast, regions like the amygdala, anterior cingulate cortex, and ventrolateral prefrontal cortex exhibit phasic responses that correspond to the onset of threat (Herrmann et al., 2016). The BNST is thus is a key region for sustained responses to stimuli that exhibit the potential for harm and may contribute to the intersection of pain and salience through hypervigilant monitoring of threats (Sommerville et al., 2010).

This sustained attention for aversive stimuli is likely to involve CRF signaling, as antagonism of CRF receptors in the BNST mitigates persistent threat monitoring and other forms of adaptive anxiety (Davis et al., 2000; Heinrichs & Koob, 2004; Hummel et al., 2010). Noting that similar manipulations can block the sensory and affective-motivational components of pain (Ide et al., 2013; Tran et al., 2014), it is possible that the same effects on threat detection and response are taking place through CRF signaling in the BNST to modulate pain and affect. As detailed in Chapter 2, we demonstrate that BNST^{CRF+} neurons exhibit greater increases in

activity during a noxious heat exposure compared to the epochs surrounding the exposure, with the largest magnitude and highest fraction of synchrony happening at the start of heat for males and female subjects respectively. This evidence for *in vivo* engagement of BNST^{CRF+} neurons shows that activation happens in response to detection of the stimulus (i.e. at onset of heat) rather than motivated withdrawal from the stimulus (i.e. at the offset of heat). Progressive changes across trials in the magnitude and latency of maximum heat response additionally suggest that there is an element of novelty to stimulus encoding, where the extent of response decreases and becomes more delayed with habituation. These data corroborate findings in clinical studies that have shown that repeated exposure to a nociceptive stimulus leads to dissociations in how the stimulus is represented by the brain (Becerra et al.,1999).

Biases towards the detection of unexpected nociceptive stimuli reinforces the idea that the BNST is modulated by salience, as the dynamics of responsivity in BNST^{CRF+} neurons may reflect the association between the valence of a stimulus with its surroundings. Potentially supporting this notion, genetic deletion of *Crf* in the BNST may be impairing the detection of nociceptive stimuli to reduce reflexive sensitivity in both sexes, while enhancing salience for more demanding (i.e. longer acting/less escapable) nociceptive exposures in females. Although the contributions of BNST^{CRF+} neurons to threat salience have not been explicitly investigated, activation of GABA neurons in the BNST can drive immediate transitions from non-REM sleep to wakefulness in mice (Kodani et al., 2017), making it a possibility for co-expression of CRF to influence arousal states and alter the perception of an environment or experience. This reciprocal modulation between the BNST and contextual salience would allow arousal states to either influence or be influenced by the structure. The potential link between BNST^{CRF+} neurons and environment has major implications for threat response, as they can change the represented value of a stimulus relative to its surroundings and/or inform safe navigation of an environment. This proposed function thus supports a role for BNST^{CRF+} neurons in states of threat vigilance and response.

These findings mutually conclude that the ability of salience to modulate vlPAG/DR^{DA+} and BNST^{CRF+} function depends on several factors that can change the distinctive value of a stimulus relative to other factors in the environment. In the case of nociceptive stimuli, these neuronal populations modulate responses to pain based on features like modality, duration, and escapability, with the greater net intensity and unpleasantness of the stimulus resulting in the more painful and salient experiences. This trend for vlPAG/DR^{DA+} and BNST^{CRF+} functions differing when a stimulus is phasic-acting/escapable versus tonic-acting/inescapable may be reflective of how pain primes neuronal function based on the intensity/salience of the experience. Neuroimaging has revealed that a defining factor of distinguishing pain from inert experiences is prolonged salience, since pain produces longer lasting representations of brain activity than non-painful stimuli (Downar et al., 2003). This is especially true of the PAG and BNST, which have been reported to exhibit particularly sustained responses to the potential of harm (Davis et al., 2000; Herrmann et al., 2016). Similarly sustained physiological and behavioral phenotypes for these regions were observed in our own experiments with mice, where optogenetic stimulation of vlPAG/DR^{DA+} terminals in the BNST at 20 Hz produced firing in downstream BNST neurons lasting on the order of minutes, as well as anti-nociceptive effects that overlapped with subsequent Laser OFF sessions despite ten minute intervals between trials. These sustained properties of vlPAG/DR^{DA+} transmission to BNST are likely to be a product of DA signaling, as phasic stimulation (≥ 20 Hz) of DA release has been shown to similarly drive sustained activity on the order of minutes in the prefrontal cortex (Lohani et al., 2019; Yagishita et al., 2019). Behavioral states that are prototypically salient can modulate the impact of this phasic DA release on the activity of brain networks and influence functional outputs, thus revealing the cyclical impact of DA on processes like attention, behavioral flexibility, and sustained affective-motivational states (Lohani et al., 2019).

Sex differences in the consequences of phasic DA activation were evident in our investigation of the vlPAG/DR^{DA+}-BNST pathway as well. We demonstrated that 20 Hz

stimulation of vlPAG/DR^{DA+}-BNST drives sex differences in DA transmission, as males exhibited more robust D1R-gated depolarizations in the BNST than females. These firing conditions were also modulated by behavioral states, which changed pathway function in a sex-specific manner. Salient characteristics such as stimulus intensity selectively produced anti-nociceptive and locomotor behaviors through the vlPAG/DR^{DA+}-BNST pathway for male and female mice. These properties of sustained DA signaling extended to BNST^{CRF+} neurons, as more tonic-acting/inescapable nociceptive contexts evoked female-specific behaviors as well. We posit that sustained activation may be important for driving behaviors of proportionate duration to the extended activity signatures of pain. By sustaining analgesic or defensive behaviors that last as long as the representation of the nociceptive experience itself, an organism is afforded adequate time to maintain vigilance in an environment and inform future behaviors following the initial suite of protective actions. Since inhibition of vlPAG/DR^{DA+} neurons increases delta (0.5-4 Hz) frequency oscillations while decreasing theta (8-12 Hz) and gamma (40-100 Hz) to reduce environmental salience (Cho et al., 2017), and higher frequency oscillations are likely to correspond to increased salience, it is possible that the sustained effects of 20 Hz stimulation in vlPAG/DR^{DA+}-BNST are influencing pain-related behaviors by emulating heightened arousal states. More direct evaluations of this hypothesis in male and female subjects, as well as similar investigations for BNST^{CRF+} neurons, are required to test this possibility.

Although we primarily studied the role of vlPAG/DR^{DA+} neurons in the context of pain, these neurons have been implicated in salience for both rewarding and aversive stimuli through associative learning tasks (Cho et al., 2017; Lin et al., 2020; Cho et al., 2020). Inhibition of vlPAG/DR^{DA+} neurons reduces time spent in a compartment with either a rewarding (opposite sex conspecific, high fat food, morphine) or aversive (2,5-dihydro-2,4,5-trimethylthiazoline, footshock, morphine withdrawal) stimulus (Cho et al., 2017; Lin et al., 2020), while activation increases the valence association of these cues (Cho et al., 2020). Both internal and external factors that alter salience can affect how these neurons process rewarding and aversive stimuli,

with satiety diminishing the ability of these neurons to respond to appetitive reward, while the extinction of novelty in a behavioral context can diminish responses to pain (Cho et al., 2020). Using the same CRISPR/Cas9-mediated knockout of *Th* in vlPAG/DR as Chapter 1, Lin et al. (2020) demonstrated the importance of DA signaling to associative learning, with TH deletion impairing incentive memory for high fat food, morphine reward, and morphine withdrawal. The study further shows that *in vivo* DA release in the BNST and CeA corresponds to appetitive reward and pain stimuli, with conditioning transiently shifting activity to the predictive auditory cue over five days of training (Lin et al., 2020), providing evidence that vlPAG/DR^{DA+} neurons communicates with the extended amygdala through DA signaling to modulate the motivational salience of the rewarding or aversive aspects of a stimulus relative to other cues in the environment. This form of associative learning has been shown to involve vlPAG/DR^{DA+} projections to the CeA (Groessl et al., 2018), but a similar assessment for the role of BNST and motivational salience has yet to be performed.

Evidence supporting a role for the BNST in motivational salience shows that DA release increases with a palatable reward (sucrose) and decreases with appetitive punishment (quinine), suggesting that DA release in the BNST is attuned to the rewarding and aversive components of salient stimuli (Park et al., 2012). While this is likely due to the influence of the VTA, as similar biases for DA release in the BNST were not reported in the vlPAG/DR (Lin et al., 2020), this concept introduces an intriguing possibility for vlPAG/DR neurons to modify DA signaling to BNST based on the positive or negative valence of a stimulus. In the present dissertation, we generally report that this DA system contributes to pain, a prototypically aversive experience, but the ability of vlPAG/DR^{DA+}-BNST to change functional outputs for both rewarding and aversive behavioral contexts corroborates a broader role in salient experiences. Describing the observed effects on pain as a facet of motivational salience may thus be more accurate in representing the overarching function of vlPAG/DR^{DA+} neurons and their projections to the BNST. Similar conclusions have been made for the mesolimbic DA system, which has

exhibited multifaceted roles in anti-nociceptive, associative learning, and salience (Berridge & Robinson, 1998; Taylor et al., 2016). This suggests that the dopaminergic systems we associate with pain response, including the vlPAG/DR and VTA, may more broadly apply as regions for motivational salience (Woodward et al., 1999; Legrain et al., 2011; Linnman et al., 2012a; Taylor et al., 2016).

Motivational Salience as a Driver of Pain-Related Drug Use

Considering the evidence for vlPAG/DR^{DA+} and BNST^{CRF+} contributions to pain and motivational salience, there remains an intriguing possibility that these signaling systems can influence the rewarding and aversive aspects of analgesic drug use. In this dissertation, we report that genetic deletion of *Th* in vlPAG/DR specifically mitigates the anti-nociceptive and locomotor effects of the neuronal population, while failing to prevent the same effects following systemic morphine treatment. This suggests that DA in vlPAG/DR is not solely responsible for the expression of morphine anti-nociception or locomotion, as parallel circuits that are sensitive to μ opioid agonists (e.g. descending pain pathway, mesolimbic DA system) drive intact expression of these behaviors. As demonstrated by the Luo lab, however, *Th* deletion in vlPAG/DR impairs associative learning for the rewarding and aversive effects of morphine use, with the absence of DA producing weaker conditioned place preference and avoidance for morphine treatment and withdrawal respectively (Lin et al., 2020). From this perspective, it appears that DA signaling in vlPAG/DR is essential for the expression of associative learning with morphine, but formation of this association does not require the anti-nociceptive or locomotor effects of the drug, as we show that expression of these behaviors are not impaired. This suggests that vlPAG/DR^{DA+} neurons primarily contribute to morphine use by modulating the motivational salience of rewarding and aversive components and strengthening associations between the valence of the drug and the environment. Interestingly, the circuits behind these reinforcing and punishing properties may be segregated, as glutamatergic input from lateral

parabrachial nucleus to vlPAG/DR^{DA+} neurons exclusively alters associative learning for the rewarding aspects of morphine and more generally disrupts the encoding of appetitive rewards like sucrose (Lin et al., 2020). Whether vlPAG/DR^{DA+} projections to the BNST and parallel circuits in this DA system exhibit similar biases for positive or negative valence remains to be seen, as circuit level investigations with vlPAG/DR^{DA+} have been limited for morphine, alcohol, and other analgesic drugs of abuse.

By contrast, BNST^{CRF+} neurons have been widely implicated in drug use through DA signaling. Several drugs of abuse, including morphine, alcohol, nicotine, and cocaine increase extracellular DA in the BNST (Carboni et al., 2000). Along with vlPAG/DR, the VTA shares reciprocal projections with the BNST (Kudo et al., 2012; Jennings et al., 2013; Silberman et al., 2013), which includes DA input to BNST^{CRF+} neurons (Meloni et al., 2006) and BNST^{CRF+} projections to VTA (Rodaros et al., 2007). It has specifically been proposed that DA input from vlPAG/DR and VTA enhances activation of BNST^{CRF+} neurons, thus triggering Glu projections to activate the mesolimbic DA system and promote drug use (Meloni et al., 2006; Dumont et al., 2008; Silberman et al., 2013). These DA and CRF signaling interactions in the BNST have been posited to drive cue-induced reinstatement, where predictive cues of drug environments act as stressors to promote relapse (Mahler & Aston-Jones, 2012; Silberman & Winder., 2013; Goode & Maren, 2018). This link between drug and cue is a form of associative learning that is established through repeated pairings of rewarding (i.e. acute effects of drugs: hedonic value, positive mood, pain relief) or aversive (i.e. chronic effects of drugs: withdrawal, negative mood, hyperalgesia) drug effects with the environments these effects take place in (Mantsch et al., 2015). The involvement of BNST^{CRF+} neurons in cue-induced reinstatement through DA signaling suggests that the role of CRF in associative learning may be akin to that of vlPAG/DR^{DA+} neurons, with both regulating the motivational salience of rewarding and aversive stimuli.

Although we were not able to test this hypothesis directly, we showed that genetic deletion of *Crf* in the BNST interacts with persistent inflammatory pain to increase alcohol consumption in male and female mice. This effect suggests that sustained increases in pain intensity, a more salient experience than that of uninjured control mice, may paradoxically be enhanced by CRF deletion to promote greater alcohol use. Contrary to the expected result of CRF deletion reducing links between alcohol and its reinforcing effects in the context of inflammatory pain, the manipulation may instead have impaired the association between alcohol and its aversive consequences, leading mice to consume more alcohol. Though an obvious explanation for this outcome is not apparent, conceptualizing how DA signaling interacts with $BNST^{CRF+}$ neurons to encode for reward/aversion may provide mechanistic insight into these findings. Previous studies have shown that DA release is opposingly modulated by rewarding and aversive stimuli (Park et al., 2012) and can promote $BNST^{CRF+}$ activation (Silberman et al., 2013). DA input to BNST specifically signals for reward predictive cues rather than the absence of reward (Park et al., 2013), implicating $BNST^{CRF+}$ neurons in responses to stimuli with positive valence. In pathological pain conditions, however, CRF signaling in the BNST is enhanced through local upregulation of CRF and CRF_{R1}, resulting in the tonic suppression of VTA-projecting BNST neurons (Takahashi et al., 2019; Hara et al., 2020). This mechanism is believed to impair affective-motivational contributions of the mesolimbic DA system and promote aversion (Minami, 2019), as CRF_{R1} antagonism in the BNST increases extracellular DA release in the nucleus accumbens and produces conditioned place preference in a rat model of neuropathic pain (Takahashi et al., 2019). Thus, it is conceivable for pathological pain states to enhance $BNST^{CRF+}$ responsivity to aversive stimuli by altering the motivational salience of these negative experiences. This shift in attentional bias from rewarding stimuli to aversive stimuli could potentially explain the paradoxical effects of *Crf* deletion in the BNST on alcohol use.

DA and CRF Signaling Systems as Potential Therapeutic Targets for Pain

Work from our lab and others have established that vlPAG/DR^{DA+} and BNST^{CRF+} neurons are sensitive to morphine and alcohol (Carboni et al., 2000; Veinante et al. 2003; Flores et al., 2004; Flores et al., 2006; Shalev et al., 2006; Jaferi et al., 2009; Silberman & Winder, 2013; Li et al., 2013; Pliel et al., 2015; Li et al., 2016; Lin et al., 2020) and can modulate pain (Ide et al., 2013; Tran et al., 2014; Li et al., 2016; Taylor et al., 2019), but how these DA and CRF signaling systems are engaged by contemporary methods of pain management is unclear. Current pharmacological approaches to treatment generally include non-steroidal anti-inflammatory drugs, topical anesthetics, corticosteroids, anticonvulsants, antidepressants, and opioids. These analgesic drug classes employ an extensive range of mechanisms that aim to reduce nociceptive signaling, enhance immune system efficiency, or improve associated mood and autonomic symptoms in both the peripheral and central nervous system, demonstrating that pain constitutes a system-wide experience with multiple etiological factors.

Among chronic pain patients, the use of opioids and antidepressants have been common routes of intervention for pain management (Watson, 2000; Verdu et al., 2008; Busse et al., 2018). These drug classes exhibit unique treatment properties in efficacy and applicability, with antidepressants being recommended as a first line medication for severe pain and opioids being reserved for more refractory conditions (Watson, 2000). What unifies opioids and antidepressants is their ability to provide symptom relief through the recruitment of the catecholaminergic system. The majority of known analgesic mechanisms are based on endogenous opioid signaling (Holden et al., 2005), where μ, κ, and δ opioid interactions have been shown to promote NE and DA release (Van Loon et al., 1981; Jenson & Smith, 1982; Basbaum et al., 1984; Baron et al., 1985; Pang & Vasko, 1986; Bouaziz et al., 1996; Furst, 1999; Zubieta et al., 2003; Narita et al., 2005; Wood, 2006; Niikura et al., 2010; Massaly et al., 2016). Antidepressants similarly promote catecholaminergic signaling, as tricyclic antidepressants drive sustained extracellular levels of 5-HT, NE, and acetylcholine in the dorsal

horn of the spinal cord to produce pain relief (Sansone & Sansone, 2008; Verdu et al., 2008; Obata et al., 2017). Notably, 5-HT and NE reuptake inhibitors (e.g. amitriptyline, nortrypyline, imipramine) and related classes that increase catecholamine availability (e.g. venlafaxine, bupropion) have been reported to be more effective for pain treatment than selective 5-HT reuptake inhibitors (SSRI; e.g. paroxetine, citalopram, fluoxetine) (Max et al., 1992; Richeimer et al., 1997; Sansone & Sansone, 2008). The lower doses needed to drive pain relief but not mood changes further suggest that this specific drug class may be especially effective for influencing pain (Sullivan & Robinson, 2006; Sansone & Sansone, 2008). However, tricyclic antidepressants have also been reported to produce adverse side-effects like dry mouth, dizziness, nausea, heart palpitations, drowsiness (Riediger et al., 2017).

Comparisons of tricyclic subclasses reveal that atypical antidepressants may be a good alternative for providing pain relief with less severe side-effects than traditional tricyclics (Semenchuk & Davis, 2001; Sansone & Sansone, 2008; Riediger et al., 2017). Bupropion, a NE and DA reuptake inhibitor (NDRI), has been reported to mitigate pain and associated disturbances in quality of life for 73% of neuropathic pain patients in a double-blind randomized trial (Semenchuk & Davis, 2001). The drug specifically enhances catecholaminergic signaling in the midbrain and hindbrain and produces analgesia with better tolerated side-effects than other antidepressants (Ascher et al., 1995; Wolfe & Trevedi, 2004). Similar functional outcomes have been observed with opioid use, as stimulants like dextroamphetamine can promote the release of NE and DA to reduce the minimal amount of morphine needed for anti-nociception (Forrest et al., 1977). This net enhancement of catecholaminergic signaling can additionally reduce the side-effects of morphine, with notable improvements for sedation, loss of alertness, and cognitive deficits in humans (Bruera et al. 1992; Dalal & Melzack, 1998). Considering that presynaptic μ and κ opioid receptors located on GABA neurons in the vlPAG/DR disinhibit local DA neurons to drive analgesia and wakefulness, we speculate that NDRI antidepressants like bupropion similarly drive sustained NE and DA signaling through vlPAG/DR^{DA+} neurons to

relieve pain and eliminate side-effects (Li et al., 2016; Li & Kash, 2019). In support of this theory, we observed that activation of vlPAG/DR^{DA+} projections to the BNST can produce analgesia without promoting reward or aversion in mice. Furthermore, we know that NE and DA work together to enhance arousal states, with noradrenergic signaling from the locus coeruleus regulating vlPAG/DR^{DA+} contributions to sleep-wake transitions (Porter-Stransky et al., 2019). Opioids and antidepressants may thus be activating vlPAG/DR^{DA+} circuits to help regulate the expression of pain, arousal, and the associated effects of drug use/environment to contribute to the overall outcomes of these pharmacological interventions.

The promotion of DA release by these two classes of pain medications is expected to affect CRF signaling in the BNST as well (Carboni et al., 2000; Meloni et al., 2006). Having previously established the possibility of reciprocal modulation between the midbrain and BNST, we posit that DA input to the BNST can modulate pain and motivational salience of drugs through CRF signaling. Early pharmacology experiments implicate CRF in analgesia, with intravenous, intracerebroventricular, intracisternal, and intrathecal application of CRF reducing nociceptive sensitivity in rodents and humans (Guillemin et al., 1977; Wei et al., 1986; Hargreaves et al., 1987; 1989a; 1989b; Dunn & Berridge, 1990; Bianchi et al., 1991; Owens & Nemeroff, 1991; Song & Takemori, 1991; Kita et al., 1993; Schafer et al., 1994; Bianchi & Panerai, 1995; Mousa et al., 1996; Schafer et al., 1996; Lariviere & Melzack, 2000; Cabot et al., 2005; Nijsen et al., 2005; Vit et al., 2006; Mousa et al., 2007). These anti-nociceptive effects were especially reliable in inflamed peripheral tissues, with CRF triggering the release of opioid peptides to relieve pain (Schafer et al., 1997). Although fewer descriptions of CRF contributions to pain exist for central tissues (Valentino & Foote, 1987; Bianchi & Panerai, 1995; Lariviere & Melzack, 2000; Cui et al., 2004; Sinniger et al., 2004; Mousa et al., 2007; Ji and Neugebauer., 2007; 2008; Bourbia et al., 2010; Ji et al., 2013; Zhang et al., 2016; Zhang & Xu, 2017; Song et al., 2020), we were able gain some insight on this by demonstrating that activation of vlPAG/DR^{DA+} projections to the BNST reduces inflammatory pain through possible innervation

of CRF neurons (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1997; Kozicz et al., 1998; Meloni et al., 2006; Silberman et al., 2013). We further show that *Crf* deletion in the BNST interacts with inflammatory pain to increase the voluntary consumption of alcohol, a drug that activates vlPAG/DR^{DA+} neurons (Li et al., 2013), promotes increases in extracellular DA in the BNST (Carboni et al., 2000), and likely interacts with DA to reduce pain (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1997; Kozicz et al., 1998; Meloni et al., 2006; Egli et al., 2012; Silberman et al., 2013). Since chronic pain enhances CRF signaling in the BNST, with multiple studies suggesting that enhancements in local CRF and CRF$_{R1}$ activity drive the transition to pathology (Sink et al., 2012; Tran et al., 2014; Takahashi et al., 2020), CRF is thus well positioned to contribute to the analgesic function of opioids (Schafer et al., 1997; Mousa et al., 2007; Jaferi et al., 2009; 2011), antidepressants (Brady et al., 1992; Holmes et al., 2003; Nielsen, 2006; Marcinkiewcz and Mazzone et al., 2016), and alcohol (Olive et al., 2002; Helig et al., 2007; Silberman et al., 2013; Pleil et al., 2015; Rinker et al., 2017) through interactions with DA signaling.

Sex differences in treatment efficacy remain one of the greatest issues of inequality in pain management. Indeed, responses to pain interventions have been shown to differ for men and women (Fillingam & Gear, 2004; Keogh, 2008), with multiple classes of analgesic drugs driving poorer long-term outcomes for women (Ciccone & Holdcroft, 1999; Cepeda & Carr, 2003; Craft, 2003; LeGates et al., 2018). We theorize that vlPAG/DR^{DA+} neurons contribute to these drug-induced disparities in pain outcomes, as DA signaling to the BNST produces analgesia for males but not females. We additionally propose that BNST^{CRF+} neurons mediate these downstream processes by altering representations of reward and aversion associated with analgesic drug use. Throughout our investigation, we have observed that female-specific function of these neurons is more prominent during salient contexts. In the presence of tonic-acting/inescapable nociceptive stimuli, we found that vlPAG/DR^{DA+}-BNST activation increases locomotor behaviors in females (with acetic acid) and exhibits more divergent anti-nociceptive

effects between sexes (with CFA treatment, where the trend for reduced mechanical nociceptive sensitivity in females is suppressed), while *Crf* deletion in BNST drives greater protective behaviors (with prolonged hot plate) and escalation from CA2BC to IA2BC in females (with CFA treatment). The ability for more aversively salient stimuli to elicit these behaviors indicates that vlPAG/DR^{DA+} and BNST^{CRF+} neurons may selectively reserve active coping behaviors for more demanding environmental challenges in females but not males. This female-specific susceptibility for behavioral recruitment in more aversive contexts may reflect greater biases in how these DA and CRF signaling systems encode for negative valence in female subjects.

We presume that this proposed mechanism is due to sex-dependent thresholds of nociceptive salience. In support of this theory, men report less pain when focusing attention on the ongoing nociceptive event, but women do not exhibit threshold changes when employing the same strategy (Keogh et al., 2000). Furthermore, women cope better with pain when focusing on the sensory components of the experience, and not the emotional components (Keogh & Herdenfeldt, 2002; Zubieta et al., 2002). Psychosocial coping mechanisms such as rumination over pain-related information also disproportionately affect women (Bartley & Fillingim, 2013). These differences in attention – where male focus may be biased towards reducing the sensory components of pain and female focus may be fixated on the aversive aspects of the experience – could more broadly explain why the outcomes of analgesic drug use differ for men and women. Knowing that vlPAG/DR^{DA+} neurons are important for associating the rewarding and aversive aspects of morphine use with an environment (Lin et al., 2020), sex differences in the salience of the drug experience likely explain discrepancies in long-term efficacy. Although the evidence strongly suggests that women disproportionately experience poorer therapeutic outcomes due to greater attending of the aversive components of pain and drug use, whether this theory translates to the involvement of vlPAG/DR^{DA+} and BNST^{CRF+} neurons in contexts related to treatment remains to be determined.

Proposed Model

In this dissertation, we established a novel sex-specific role for DA and CRF signaling in pain. The series of experiments described in Chapters 1-3 establish that vlPAG/DR^{DA+} and BNST^{CRF+} neurons contribute to pain with potentially important mechanistic differences for DA signaling and local inhibitory connectivity in males and females. Here, we propose a comprehensive circuit encompassing the functional properties of the midbrain and extended amygdala, where dopaminergic projections from vlPAG/DR and VTA terminate on BNST^{CRF+} neurons and either work in synchrony or compete to influence behaviors during salient experiences such as pain and drug use (Eiden et al., 1985; Petit et al., 1995; Kozicz et al., 1997; Kozicz et al., 1998; Meloni et al., 2006; Dougalis et al., 2012; Park et al., 2012; Silberman et al., 2013). DA signaling in the BNST enhances and is modulated by contextual salience, which may drive reciprocal feedback to the vlPAG/DR and VTA and sustain the actions of these midbrain DA systems (Gray & Magnusen, 1992; Kudo et al., 2012; Jennings et al., 2013; Silberman et al., 2013; Hao et al., 2019). Longer-term insults such as neuropathic and inflammatory pain are posited to robustly increase CRF and CRF$_{R1}$ activity in a sex-specific manner, possibly leading to the constitutive activation of aversive salience. Considerations for male and female circuitry and function are detailed in **Figure 33**.

Future Directions

Despite achieving important insight on sex differences in pain through our experiments, we recognize the need for more conclusive evidence on the functional role of DA and CRF signaling by sex. Here, we offer several lines of investigation to achieve a better understanding of vlPAG/DR^{DA+} and BNST^{CRF+} neurons and their contributions to pain and drug use. The following questions serve to guide future experimenters in their ongoing pursuit of knowledge, with the intention of exposing the basic principles necessary to develop more effective and equitable intervention strategies of pain treatment in men and women.

i. Selectively Activating vlPAG/DR^{DA+} and BNST^{CRF+} Function in Males and Females

Having characterized sex-specific contributions of vlPAG/DR^{DA+} and BNST^{CRF+} neurons to pain, the next logical question is whether we take can advantage of this new mechanistic knowledge to selectively manipulate behaviors for males and females. For example, if there was a scenario where it would be advantageous to relieve pain, increasing D1R activity and/or the polysynaptic connectivity in the BNST for female mice would presumably drive comparable anti-nociceptive to males. If it were more advantageous to move, however, we speculate that reducing D1R activity and/or polysynaptic engagement in male mice would produce comparable locomotion to females. Given our knowledge on BNST^{CRF+} encoding dynamics in response to noxious stimuli, we may also be able to reduce the pro-nociceptive effects of tonic-acting/inescapable nociception or pro-drinking effects of CFA treatment in females with *Crf* deletion by altering the magnitude, timing, and synchrony of BNST^{CRF+} neurons in relation to pain exposure. These proof-of-concept experiments would test important hypotheses on how vlPAG/DR^{DA+} and BNST^{CRF+} neurons are recruited by and regulate pain for both sexes and ultimately inform us on the utility of these neuronal populations as targets for behavioral modulation.

ii. Pathological Pain as a Consequence or Driver of vlPAG/DR^{DA+} and BNST^{CRF+} Function

Having demonstrated that vlPAG/DR^{DA+}-BNST activation can attenuate heightened nociceptive sensitivity in CFA-treated male mice, we know that these neurons can preserve anti-nociceptive function in the presence of persistent inflammatory pain. It remains unclear, however, whether these inflammatory conditions affect basal function of vlPAG/DR^{DA+} neurons to drive pathological pain sensitivity. Preliminary data suggests that vlPAG/DR^{DA+} neurons are not changed by inflammation, as saline- and CFA-treated males show comparable excitatory, inhibitory, and DA transmission between vlPAG/DR^{DA+} terminals and BNST neurons (data not

shown). These data do not preclude changes from happening in vlPAG/DR^{DA+} neurons of female mice, however, as we have observed a higher prevalence for female-specific behaviors in conditions of more severe pain. It is thus possible for a prolonged pain state to alter transmission from vlPAG/DR^{DA+} neurons to the BNST in female mice. CFA reduces the efficacy of morphine anti-nociception in females through a reduction in tonic $GABA_A$ currents of vlPAG/DR neurons (Tonsfeldt et al., 2016), presumably reducing disinhibition of vlPAG/DR^{DA+} neurons and DA transmission to the BNST of female mice. However, it is also feasible for the prolonged presence of pain to drive sustained increases in basal excitability, as neural representations of nociceptive stimuli are less sensitive to habituation in chronic pain patients (Malinen et al., 2011). Instead of reducing pathway function then, CFA may act as a motivationally salient stimulus akin to visceral nociception, opposite sex conspecific mice, or environmental novelty, where vlPAG/DR^{DA+} transmission to BNST is continually primed to drive locomotion in females. Future studies should test these dueling hypotheses, as it will provide important mechanistic insight on how vlPAG/DR^{DA+} neurons influence the experience of pathological pain.

Similar questions remain for BNST^{CRF+} neurons, since increased paw attending behaviors were observed with *Crf* deletion in female mice. Whether CFA treatment produces tonic-acting/inescapable effects similar to that of prolonged hot plate exposure was not been assessed, but we did observe female-specific increases in alcohol consumption between CA2BC and IA2BC, suggesting that the exacerbation of pain and drug seeking behaviors of *Crf* deletion in the BNST can explain similar effects observed with CFA treatment. Although there was no interaction between *Crf* deletion and CFA treatment in our own measures of nociceptive sensitivity, future investigations should monitor how spontaneous pain and avoidance learning behaviors are affected by these manipulations, as these outcomes more accurately reflect the increases in protective behaviors observed in females. Follow-up studies should also track how the induction of pathological pain affects the processing of nociceptive information, since

upregulation of CRF and/or CRF_{R1} in the BNST has been observed with chronic pain and stress (Tran et al., 2014; Takahashi et al., 2019). Under the assumption that prolonged pain conditions enhance CRF signaling in the BNST, it may also be possible for the transition from acute to pathological pain to be blocked by deleting CRF_{R1} from BNST. In data not shown, genetic deletion of *Crfr1* from the BNST did not alter nociceptive sensitivity or alcohol use in male or female mice. However, the same measures were not tested after CFA treatment, so it may be possible that that genetic deletion of *Crfr1* in the BNST prior to the induction of pathological pain is needed to prevent the development of dysfunction (Tran et al., 2014; Takahashi et al., 2019). Collectively, these experiments would be valuable indications for the therapeutic potential of vlPAG/DR^{DA+} and $BNST^{CRF+}$ neurons pertaining to whether they can accelerate or impede the induction of pathological pain.

iii. <u>Cell Type Diversity and the Contributions of Parallel Circuits</u>

Revealing the behavioral contributions of vlPAG/DR^{DA+} and $BNST^{CRF+}$ neurons has opened the possibility for other cell types and circuits in these regions to play similar functional roles, since these structures exhibit great molecular diversity. For example, it is well known that Glu neurons in the vlPAG/DR project to the RVM as a primary component of the descending pain pathway, but whether these circuits exhibit sex-specific effects on pain is unclear. Given that DA neurons are a subset of these Glu neurons, but do not project to the RVM, it is possible for these ascending and descending pain circuits to either exhibit functional distinction or redundancy. Evidence suggests that RVM-projecting Glu neurons in the vlPAG/DR exhibit sex differences in the endogenous opioid system, with male rats showing fewer Glu projections, greater μ opioid receptor expression, and more robust morphine anti-nociception than females (Loyd et al., 2008; Bobeck et al., 2009; Doyle et al., 2017). However, a definitive experiment has yet to show that Glu neurons in the vlPAG/DR produce these sex-specific effects on nociceptive sensitivity using the same stimulation parameters and in the absence of μ opioid signaling. This

prospective divergence of μ opioid activation and anti-nociceptive potential could determine the relative importance of the vlPAG^{GLU+}-RVM and vlPAG/DR^{DA+}-BNST pathways for pain, as it would show whether the sex-specific functions of our novel ascending pain pathway are conserved in the canonical descending pain pathway.

Co-expression in these vlPAG/DR neurons is likely to be essential for function. Within the vlPAG/DR, neurons that express DA also produce Glu (Li et al., 2016) and VIP (Dougalis et al., 2012). Interestingly, Glu and DA neurons in the vlPAG/DR produce comparable reductions in pain sensitivity but make distinct contributions to anxiety-like behavior (Taylor et al., 2019), while similar observations have been made for the function of Glu and DA co-release in the VTA as well (Zell et al., 2020). This DA subpopulation has also been shown to promote wakefulness, while vlPAG/DR neurons that co-express Glu and NT opposingly drive non-REM sleep (Zhong et al., 2019), indicating that functional divergence exists within Glu neurons. Functional specificity is additionally determined by downstream target regions, as these same Glu neurons can selectively drive anti-nociceptive or freezing behaviors based on outputs to the RVM and magnocellular nucleus (Tovote et al., 2016). These examples demonstrate the complexity of cell type- and projection-specific contributions to function in the vlPAG/DR. How these molecular and anatomical interactions contribute to the role of vlPAG/DR^{DA+} neurons in pain-related behavior requires further investigation.

Co-expression of VIP in DA neurons may have particularly interesting implications for vlPAG/DR^{DA+} projections to the BNST. Since VIP neurons in vlPAG/DR project to the BNST (Eiden et al., 1985; Petit et al., 1995) and form synapses on BNST^{CRF+} neurons (Kozicz et al., 1997; Kozicz et al., 1998), similar connectivity may occur between vlPAG/DR^{DA+} and BNST^{CRF+} neurons (Meloni et al., 2006; Dougalis et al., 2012). Although there has been ample cytoarchitectural evidence supporting the existence of a vlPAG/DR^{DA+}-BNST^{CRF+} circuit (Meloni et al., 2006; Dougalis et al., 2012), future studies will need to utilize more direct functional approaches to examine this possibility (e.g. establishing connectivity with optically-evoked

transmission from vlPAG/DR^{DA+} terminals to BNST^{CRF+} neurons using TH-Cre x CRF-Flp mice). Comparisons of DA and VIP release in the BNST may also be informative, as VIP expression in the vlPAG/DR is enhanced by chronic pain (Castorina et al., 2019) and VIP receptors in the BNST are increased by stress (Hammack et al., 2009). Whether VIP transmission produces similar downstream activation as DA signaling in the BNST is unknown, but VIP innervation of BNST^{CRF+} neurons suggests that the peptide could play a role in pain processing and modulation.

Assuming vlPAG/DR^{DA+} projections terminate on BNST^{CRF+} neurons, forthcoming investigations of BNST^{CRF+} neurons should explore how DA inputs from vlPAG/DR contribute to functional outcomes relative to the VTA. Determining whether mesolimbic inputs innervate the same downstream neurons as vlPAG/DR and then deciphering how these inputs work in combination or competition to drive behavior will be critical to our understanding of DA signaling in the BNST, since both DA sources appear to contribute to pain through the modulation of nociceptive salience (Taylor et al., 2016; Cho et al., 2020; Lin et al., 2020). The possibility of DA-responsive BNST neurons directly contributing to reciprocal projections back to the PAG and VTA is also noteworthy, since similar mechanisms of inhibitory feedback have been implicated in enhancing the salience of environmental cues (Friesen, 1994; Mysore & Knudsen, 2012). RNA sequencing of the BNST neurons that share connections with vlPAG/DR and VTA will need to be performed in the future as well. Considering the heterogeneity of the BNST, where several diverse subpopulations of GABA (86%) and Glu (14%) neurons are present (Nguyen et al., 2016), these downstream neurons will likely consist of BNST^{CRF+} subpopulations that co-express various combinations of neuropeptides (e.g. neuropeptide Y, dynorphin, somatostatin, protein kinase C δ, pituitary adenylate cyclase-activating polypeptide, oxytocin, neurotensin, nociception, orexin, vasoactive intestinal peptide) that differentially contribute to the processing of rewarding and aversive stimuli (Kash et al., 2015; Ye & Veinante, 2020). Greater efforts to investigate the functional divergence of these vlPAG/DR and BNST populations are

needed to understand how such molecularly and anatomically diverse structures compute sensory information and inform behavior.

iv. The Role of Environmental Salience and Circuit Priming

Our finding that vlPAG/DR^{DA+}-BNST function is regulated by context has widespread implications on how internal and external states can influence behavior. Having determined that the same parameters of stimulation were able to produce sex-specific outcomes based on behavioral context, we posit that highly salient experiences can prime neuronal networks to have greater basal excitation in a sex-specific manner, thus increasing the potential for activity and altering the weights of relative connections (Lee & Lee, 2013; Kuchibhotla et al., 2016; Pakan et al., 2016). Network-level analysis that factors in multiple behavioral contexts may thus be necessary to fully appreciate how the vlPAG/DR^{DA+}-BNST circuit functions, since pain and other stimuli that necessitate attention appear to prime the projection to produce discrete function outcomes for males and females. A promising approach for monitoring how neuronal dynamics change with context is by simultaneously tracking *in vivo* activity of vlPAG/DR^{DA+} and BNST^{CRF+} neurons at the single cell type and network level. As demonstrated in Cho et al. (2017), it is possible to record a single population of neurons using fiber photometry and track broader changes in arousal with electroencephalography and electromyography. Applying similar methods with variable levels of nociceptive salience would allow us to correlate oscillatory changes with a single population of cells for several modalities and intensities of pain. Using this experimental design to monitor vlPAG/DR^{DA+} and BNST^{CRF+} activity could be a promising approach to attain a more holistic picture of the relationship between pain and salience.

Based on clinical evidence, we would expect stimuli with greater nociceptive salience to engage in more prolonged network level activity and correlate with particularly sustained responses in the PAG and BNST (Downar et al., 2003; Herrmann et al., 2016). In our initial

attempts at tracking pain processing, we showed that BNST^{CRF+} responses to phasic-acting/escapable nociception were not sustained (i.e. spikes in activity were primarily restricted to the duration of the heat exposure). However, it remains unclear how more salient nociceptive experiences would be encoded by BNST^{CRF+} neurons, as one would predict upregulations in CRF signaling to drive longer representations of a tonic-acting/inescapable stimulus. In support of this hypothesis, clinical studies have observed that the sustained activation seen with nociceptive exposure can be diminished with habituation in health adults but not chronic pain patients (Malinen et al., 2011; Borsook et al., 2013), indicating that sustained attention may contribute to the experience of prolonged pain. Greater attention for aversive information may further drive the sex-specific expression of pain, as hyperfixations over pain-related information have been posited to contribute to poorer outcomes in pain intervention for women (Keogh et al., 2005; Bartley & Fillingim, 2013). Future investigations should consider testing whether dysfunction in motivational salience contributes to pathological pain by monitoring vlPAG/DR^{DA+} and BNST^{CRF+} neurons in CFA-treated mice. Since sustained attention is believed to amplify the aversive aspects of chronic pain and drive higher instances of pathology in women, we predict that CFA treatment will result in sustained encoding for nociceptive stimuli across repeated exposure trials when compared to controls, with representations lasting longer in female subjects. Monitoring sex differences will be important, as it will determine if we can model these links between pain and motivational salience in vlPAG/DR^{DA+} and BNST^{CRF+} neurons.

v. <u>Changing the Perception of Pain Interventions</u>

To address more immediate concerns about how to leverage our knowledge on vlPAG/DR^{DA+} and BNST^{CRF+} neurons and pain, we propose a straightforward evaluation determining whether vlPAG/DR^{DA+} and/or BNST^{CRF+} activation can enhance the anti-nociceptive, hedonic, and adverse effects of analgesic drugs. This could be achieved by pairing optogenetic activation of DA and CRF neurons with morphine or alcohol and testing nociceptive

sensitivity to see how endogenous and artificial recruitment of these neurons interact to influence pain responses. Alternatively, manipulating neuronal activity during operant conditioning for sucrose- or quinine-infused morphine/alcohol could illuminate the role of these neurons during rewarding and aversive drug experiences. Highlighting how these circuits encode for the motivational salience of rewarding and aversive stimuli may help us understand how vlPAG/DR^{DA+} and BNST^{CRF+} neurons relatively process the analgesic and punishing components of drug use. Understanding how these DA and CRF signaling systems can be manipulated to change the experience of drug use may lead to better treatment outcomes for pain in the future.

vi. Conclusions

Through these proposed experiments, we hope to achieve greater equity in pain management. The field of pain has historically excluded women in research and, by consequence, obstructed access to thoroughly informed pain relief for half of the world's population. The effects of untreated pain are vast, as vital measures of quality of life such as income, education, and deaths of despair – notably by drug abuse and suicide – are correlated with higher pain (Lumley et al., 2011; Gaskin & Richard, 2012; Case & Deaton, 2017). Factoring in female subjects into the design and implementation of research is a simple act but these practices are necessary to correct for the past exclusionary practices of the field and, most importantly, improve health outcomes. My contribution to this broader goal is this characterization of sex-specific functions in vlPAG/DR^{DA+} and BNST^{CRF+} neurons. The hope for this work going forward is that it will be informative for the field and translate to more equitable therapeutic outcomes by sex.

FIGURES & CAPTIONS

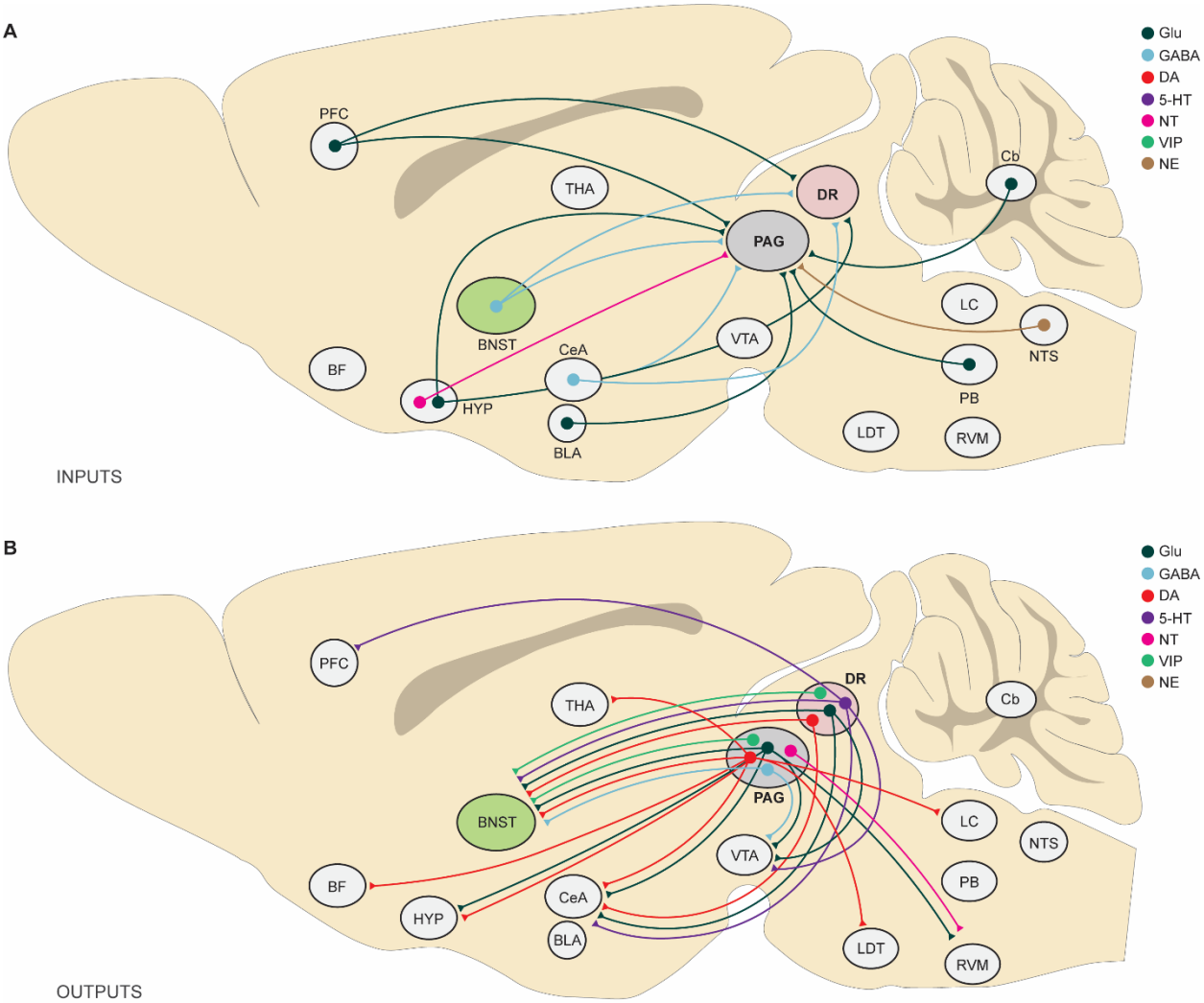

Figure 1. Whole Brain Input-Output Map of PAG/DR Circuitry

(A) Inputs to the PAG/DR. The PAG/DR receives inputs from BNST, BLA, Cb, CeA, HYP, NTS, PB, and PFC.

(B) Outputs from the PAG/DR. The PAG/DR sends outputs to BF, BNST, CeA, HYP, LC, LDT, THA, PFC, RVM, and VTA.

Abbreviations: BF: basal forebrain, BLA: basolateral amygdala, BNST: bed nucleus of the stria terminalis, Cb: cerebellum, CeA: central nucleus of the amygdala, DR: dorsal raphe, HYP: hypothalamus, LC: locus coeruleus, LDT: laterodorsal tegmental nucleus, NTS: nucleus of the solitary tract, PAG: periaqueductal gray, PB: parabrachial nucleus, PFC: prefrontal cortex, RVM: rostral ventromedial medulla, THA: thalamus, VTA: ventral tegmental area; Glu: glutamate, GABA: γ-aminobutyric acid, DA: dopamine, 5-HT: serotonin, NT: neurotensin, VIP: vasoactive intestinal peptide, NE: norepinephrine.

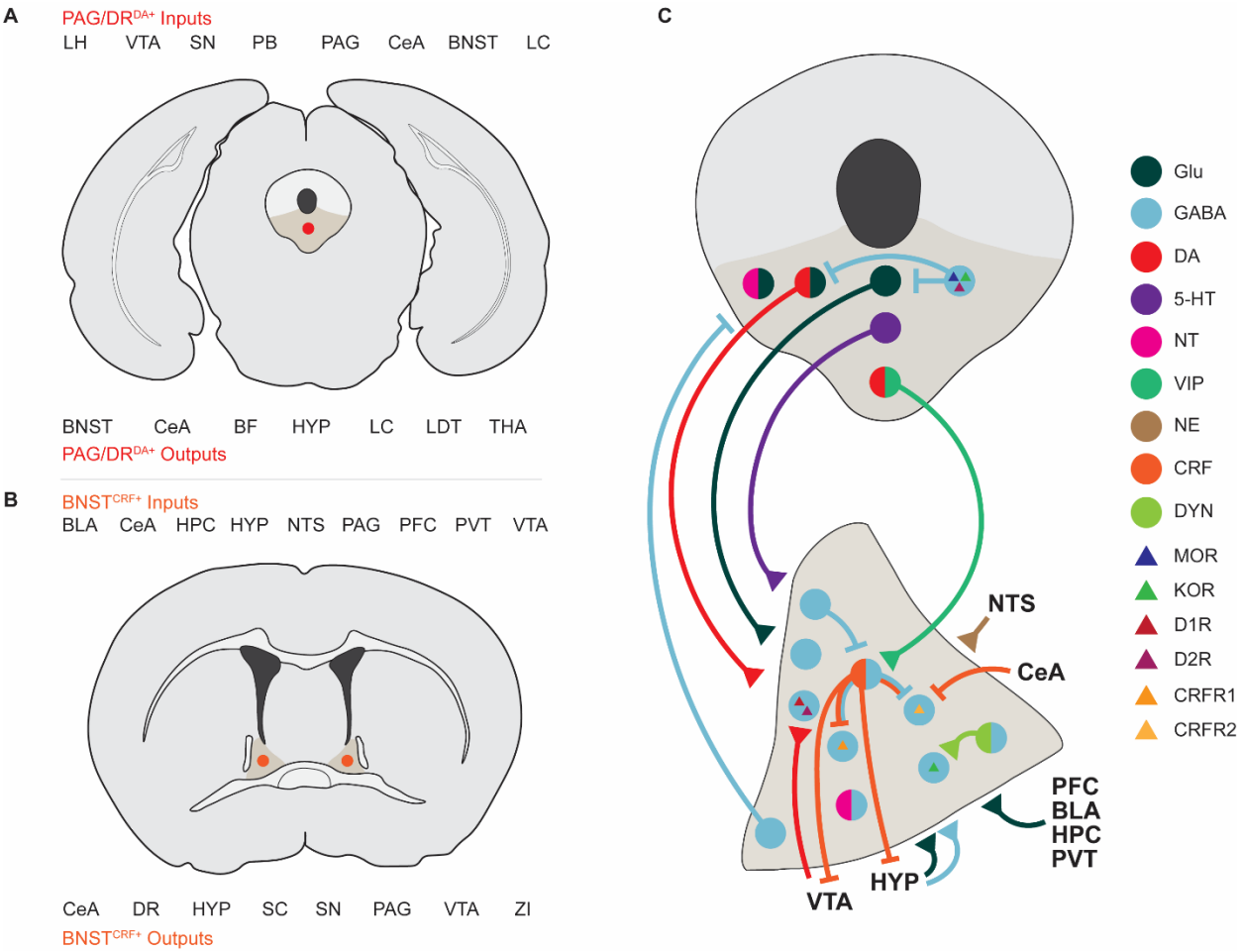

Figure 2. Cell Type and Input-Output Map of PAG/DR and BNST Microcircuitry

(A) Inputs and outputs of vlPAG/DR^{DA+} neurons.

(B) Inputs and outputs of BNST^{CRF+} neurons.

(C) Input-output map of PAG/DR and BNST microcircuitry. Cell types and connectivity is indicated for the structures, with an emphasis on DA and CRF signaling systems.

Abbreviations: BLA: basolateral amygdala, CeA: central nucleus of the amygdala, HPC: hippocampus, HYP: hypothalamus, NTS: nucleus of the solitary tract, PB: parabrachial nucleus, PFC: prefrontal cortex, PVT: paraventricular thalamus, VTA: ventral tegmental area; Glu: glutamate, GABA: γ-aminobutyric acid, DA: dopamine, 5-HT: serotonin, NT: neurotensin, VIP: vasoactive intestinal peptide, NE: norepinephrine, CRF: corticotropin-releasing factor, DYN: dynorphin, MOR: μ opioid receptor, KOR: κ opioid receptor, D1R: dopamine receptor D1, D2R: dopamine receptor D2, CRFR1: corticotropin-releasing factor receptor 1, CRFR2: corticotropin-releasing factor receptor 2.

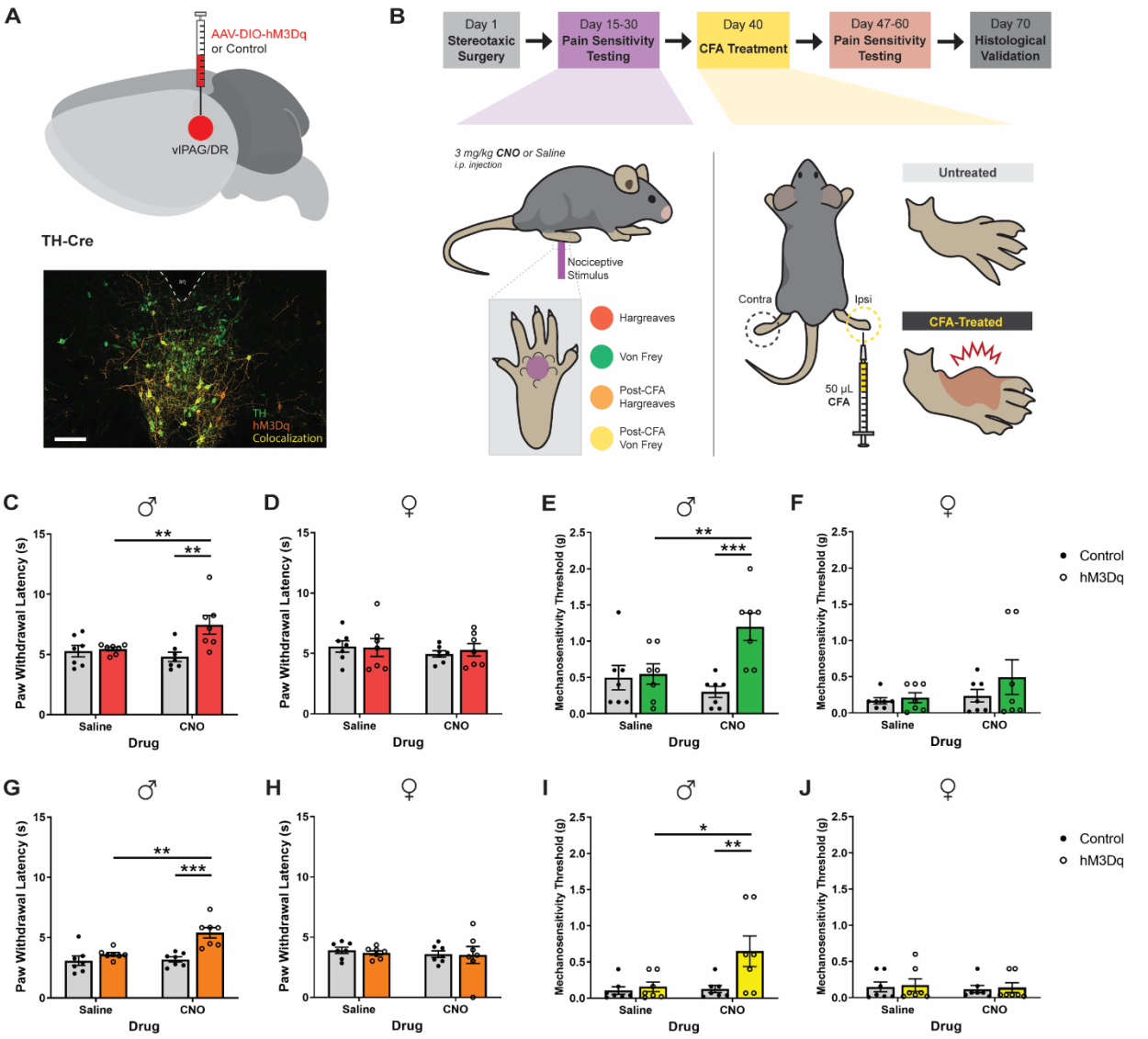

Figure 3. Activation of vlPAG/DR^{DA+} Produces Sex-Specific Reductions in Pain Sensitivity

(A) Chemogenetic approach for vlPAG/DR^{DA+} activation. (Top) Diagram of virus infusion. (Bottom) Representative image of hM3Dq (orange), TH (green), and colocalization (yellow) in vlPAG/DR. Scale bar, 100 µm.

(B) Timeline with schematic of pain sensitivity testing and CFA treatment.

(C-D) Thermal nociceptive sensitivity of **(C)** male (n = 7) and **(D)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: Drug x Virus interaction [$F_{(1,12)} = 9.955$, $p = 0.0083$], no main effect of Drug [$F_{(1,12)} = 3.811$, $p = 0.0746$], main effect of Virus [$F_{(1,12)} = 5.813$, $p = 0.0329$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.2139$, $p = 0.6520$] or main effect of Drug [$F_{(1,12)} = 0.7924$, $p = 0.3909$] and Virus [$F_{(1,12)} = 0.04543$, $p = 0.8348$] for females).

(E-F) Mechanical nociceptive sensitivity of **(E)** male (n = 7) and **(F)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: Drug x Virus interaction [$F_{(1,12)} = 11.21$, $p = 0.0058$], no main effect of Drug [$F_{(1,12)} = 3.222$, $p = 0.0979$], main effect of Virus [$F_{(1,12)} = 7.853$, $p = 0.0160$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.6219$, $p = 0.4456$] or main effect of Drug [$F_{(1,12)} = 1.671$, $p = 0.2205$] and Virus [$F_{(1,12)} = 1.316$, $p = 0.2736$] for females).

(G-H) Post-CFA thermal nociceptive sensitivity in **(G)** male (n = 7) and **(H)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: Drug x Virus interaction [$F_{(1,12)} = 8.089$, $p = 0.0148$], main effect of Drug [$F_{(1,12)} = 10.23$, $p = 0.0077$] and Virus [$F_{(1,12)} = 16.21$, $p = 0.0017$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.03255$, $p = 0.8598$] or main effect of Drug [$F_{(1,12)} = 0.3740$, $p = 0.5522$] and Virus [$F_{(1,12)} = 0.1154$, $p = 0.7400$] for females).

(I-J) Post-CFA mechanical nociceptive sensitivity in **(I)** male (n = 7) and **(J)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 4.642$, $p = 0.0522$], main effect of Drug [$F_{(1,12)} = 5.540$, $p = 0.0365$] and Virus [$F_{(1,12)} = 5.430$, $p = 0.0381$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.001187$, $p = 0.9731$] or main effect of Drug [$F_{(1,12)} = 0.5143$, $p = 0.4870$] and Virus [$F_{(1,12)} = 0.07060$, $p = 0.7950$] for females).

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 3C-3J.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that CNO treatment results in male hM3Dq mice with greater paw withdrawal latencies and mechanosensitivity thresholds than male mCherry control and female hM3Dq mice.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

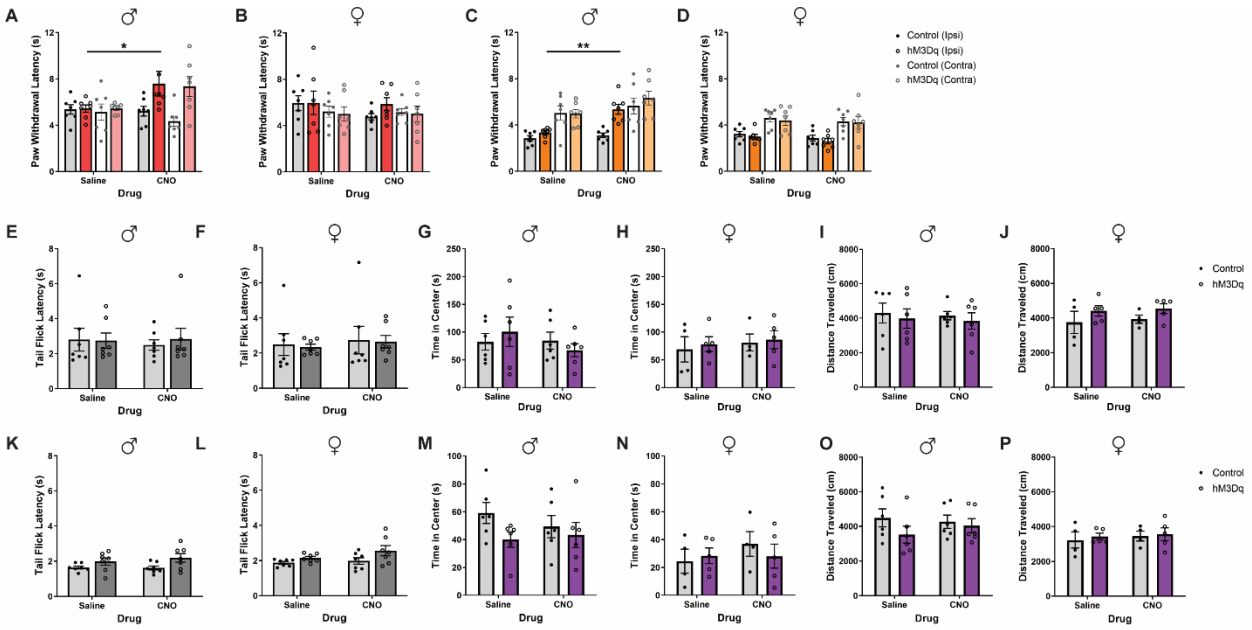

Figure 4. vlPAG/DR^{DA+} Attenuates Pain Sensitivity Without Affecting Tail Flick or Anxiety-Like Behaviors in Naïve and CFA-Treated Mice

(A-B) Thermal nociceptive sensitivity by ipsilateral and contralateral paw for **(A)** male (n = 7) and **(B)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: Drug x Virus/Paw interaction [$F_{(3,24)} = 3.979$, $p = 0.0196$], main effect of Drug [$F_{(1,24)} = 4.478$, $p = 0.0449$], no main effect of Virus/Paw [$F_{(3,24)} = 3.004$, $p = 0.0502$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus/Paw interaction [$F_{(3,24)} = 0.6055$, $p = 0.6178$] or main effect of Drug [$F_{(1,24)} = 0.8648$, $p = 0.3617$] and Virus/Paw [$F_{(3,24)} = 0.6215$, $p = 0.6079$] for females).

(C-D) Post-CFA thermal nociceptive sensitivity by ipsilateral and contralateral paw for **(C)** male (n = 7) and **(D)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus/Paw interaction [$F_{(3,24)} = 1.746$, $p = 0.1844$], main effect of Drug [$F_{(1,24)} = 12.89$, $p = 0.0015$] and Virus/Paw [$F_{(3,24)} = 12.75$, $p < 0.0001$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus/Paw interaction [$F_{(3,24)} = 0.0460$, $p = 0.9866$] or main effect of Drug [$F_{(1,24)} = 1.775$, $p = 0.1953$], main effect of Virus/Paw [$F_{(3,24)} = 12.17$, $p < 0.0001$] for females).

(E-F) Tail flick response in **(E)** male (n = 7) and **(F)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.01733$, $p = 0.8974$] or main effect of Drug [$F_{(1,12)} = 1.536$, $p = 0.2388$] and Virus [$F_{(1,12)} = 0.02716$, $p = 0.8719$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.01733$, $p = 0.8974$] or main effect of Drug [$F_{(1,12)} = 1.536$, $p = 0.2388$], main effect of Virus [$F_{(1,12)} = 0.02716$, $p = 0.8719$] for females).

(G-J) Open field test. Measures for **(G-H)** center time (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.3255$, $p = 0.5788$] or main effect of Drug [$F_{(1,12)} = 0.6795$, $p = 0.4258$] and Virus [$F_{(1,12)} = 0.01891$, $p = 0.8929$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.09438$, $p = 0.7639$] or main effect of Drug [$F_{(1,12)} = 0.4115$, $p = 0.5333$] and Virus [$F_{(1,12)} = 0.2079$, $p = 0.6566$] for females) and **(I-J)** distance traveled (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.001836$, $p = 0.9665$] or main effect of Drug [$F_{(1,12)} = 0.2168$, $p = 0.6498$] and Virus [$F_{(1,12)} = 0.01907$, $p = 0.8925$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.03190$, $p = 0.8612$] or main effect of Drug [$F_{(1,12)} = 0.06748$, $p = 0.7994$] and Virus [$F_{(1,12)} = 2.921$, $p = 0.1131$] for females) in **(G, I)** male (n = 7) and **(H, J)** female (n = 7) TH-Cre mice following saline or CNO injection.

(K-L) Post-CFA tail flick response in **(K)** male (n = 7) and **(L)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.6959$, $p = 0.4205$] or main effect of Drug [$F_{(1,12)} = 0.4143$, $p = 0.5319$], main effect of Virus [$F_{(1,12)} = 4.967$, $p = 0.0457$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.8544$, $p = 0.3735$] or main effect of Drug [$F_{(1,12)} = 2.431$, $p = 0.1449$], main effect of Virus [$F_{(1,12)} = 4.800$, $p = 0.0489$] for females).

(M-P) Post-CFA open field test. Measures for **(M-N)** center time (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.5665$, $p = 0.4662$] or main effect of Drug [$F_{(1,12)} = 0.9871$, $p = 0.3401$] and Virus [$F_{(1,12)} = 1.283$, $p = 0.2795$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 1.358$, $p = 0.2665$] or main effect of Drug [$F_{(1,12)} = 0.1128$, $p = 0.7428$] and Virus [$F_{(1,12)} = 1.024$, $p = 0.3315$] for females) and **(O-P)** distance traveled (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 1.151$, $p = 0.3045$] or main effect of Drug [$F_{(1,12)} = 0.1132$, $p = 0.7424$] and Virus [$F_{(1,12)} = 0.3576$, $p = 0.5609$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.4247$, $p = 0.5269$] or main effect of Drug [$F_{(1,12)} = 0.07608$, $p = 0.7874$] and Virus [$F_{(1,12)} = 0.1648$, $p = 0.6919$] for females) in **(M, O)** male (n = 7) and **(N, P)** female (n = 7) TH-Cre mice following saline or CNO injection.

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 4A-4P.

Comparisons of Sex x Virus/Paw (Two-way ANOVA with Tukey's post hoc) reveal that CNO treatment results in male hM3Dq mice with greater paw withdrawal latencies for the ipsilateral and contralateral paws than females in the CFA (but not the Naïve) condition, suggesting that sex differences in anti-nociceptive effects were more pronounced after CFA treatment.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

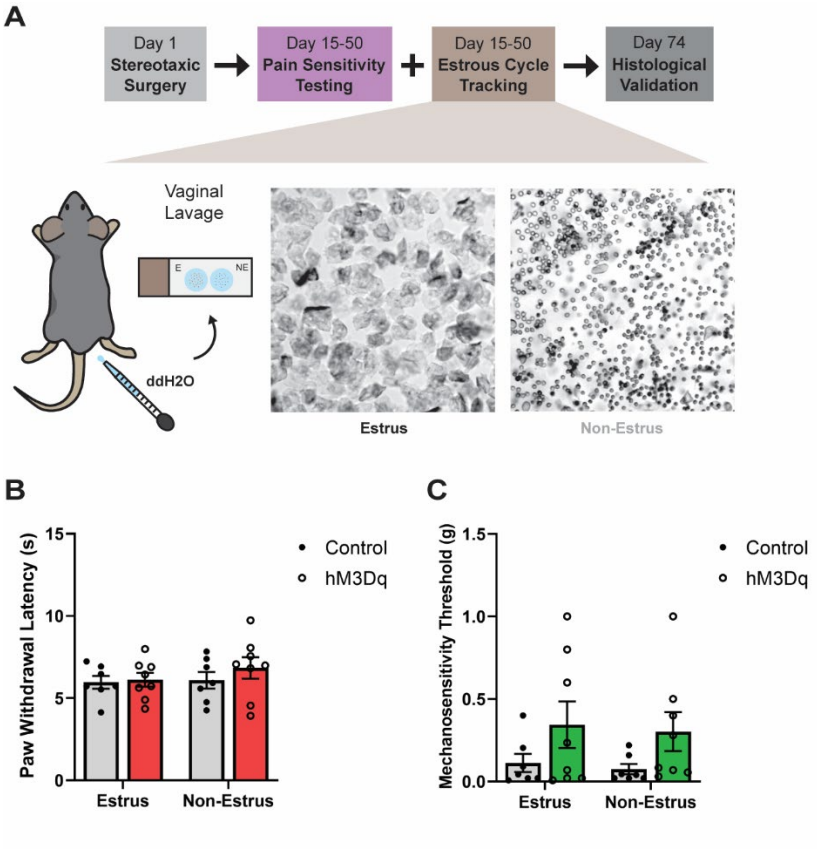

Figure 5. Estrous Cycle Does Not Alter vlPAG/DR^{DA+} Contributions to Nociceptive Sensitivity in Female Mice

(A) Timeline with schematic of pain sensitivity testing and estrous cycle tracking in female TH-Cre mice.

(B) Thermal nociceptive sensitivity of female (n = 7-8) TH-Cre mice following CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Estrus Cycle x Virus interaction [$F_{(1,13)} = 0.2458$, $p = 0.6283$] or main effect of Estrus Cycle [$F_{(1,13)} = 0.4980$, $p = 0.4929$] and Virus [$F_{(1,13)} = 1.227$, $p = 0.2881$]).

(C) Mechanical nociceptive sensitivity of female (n = 7-8) TH-Cre mice following CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Estrus Cycle x Virus interaction [$F_{(1,13)} = 0.0005$, $p = 0.9816$] or main effect of Estrus Cycle [$F_{(1,13)} = 0.1595$, $p = 0.6961$] and Virus [$F_{(1,13)} = 4.650$, $p = 0.0504$]).

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 5B-5C.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

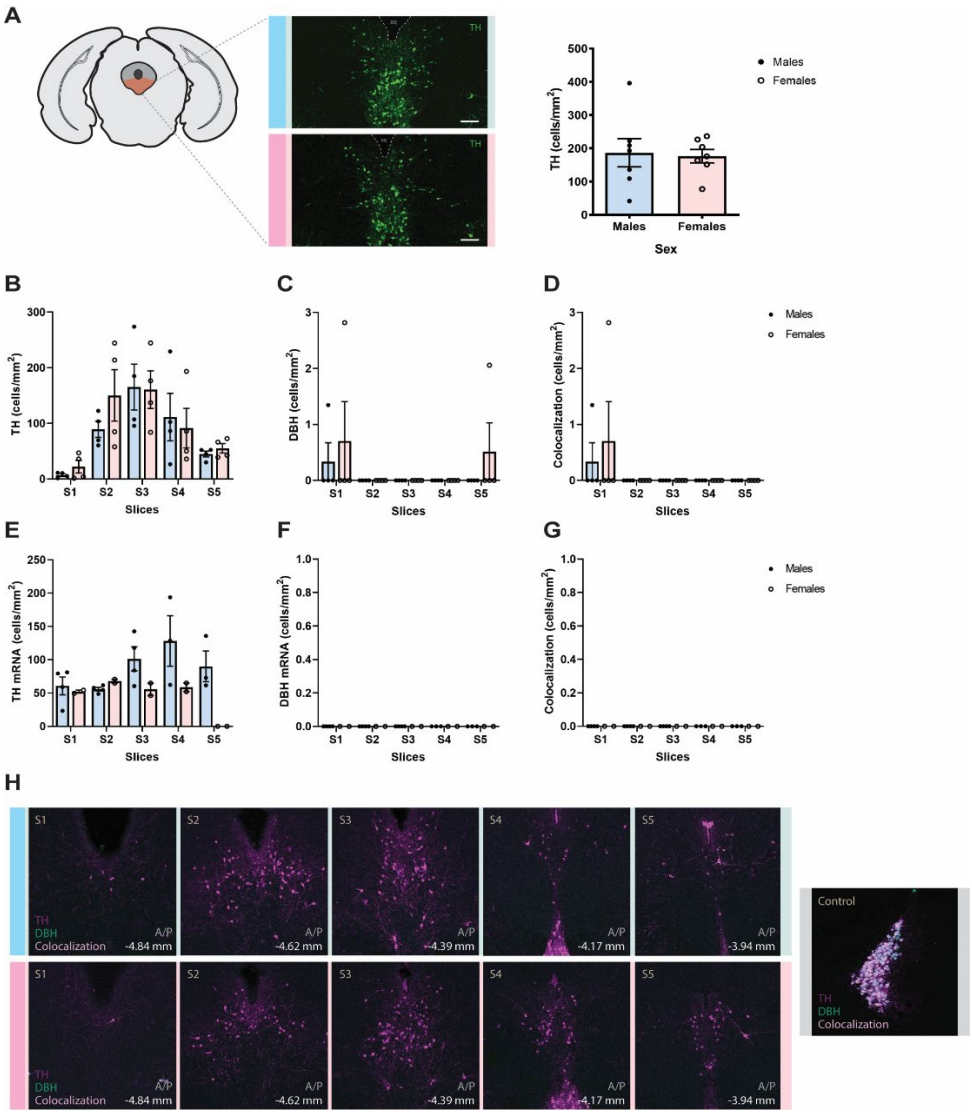

Figure 6. TH and DBH Expression Across the A/P Axis in vlPAG/DR

(A) (Left) Representative images of TH immunoreactivity (green) in vlPAG/DR of male (top/blue row) and female (bottom/pink row) mice. Scale bars, 100 µm. (Right) TH immunoreactivity in vlPAG/DR of male and female mice (n = 7; unpaired t-test, $t_{(12)} = 0.2148$, $p = 0.8335$).

(B-D) Expression of **(B)** TH, **(C)** DBH, and **(D)** TH/DBH colocalization in the vlPAG/DR of male (n = 5 slices per mouse / 4 mice total) and female (n = 5 slices per mouse / 4 mice total) mice. No sex differences were observed across the A/P axis. (2-way ANOVA with Tukey's post hoc: no A/P x Sex interaction [$F_{(4,30)} = 0.5462$, $p = 0.7031$], main effect of A/P [$F_{(4,30)} = 7.987$, $p = 0.0002$], no main effect of Sex [$F_{(1,30)} = 0.4503$, $p = 0.5073$] for TH; 2-way ANOVA with Tukey's post hoc: no A/P x Sex interaction [$F_{(4,30)} = 0.3490$, $p = 0.8426$] or main effect of A/P [$F_{(4,30)} = 1.237$, $p = 0.3165$] and Sex [$F_{(1,30)} = 0.8896$, $p = 0.3531$] for DBH; 2-way ANOVA with Tukey's post hoc: no A/P x Sex

interaction [$F_{(4,30)} = 0.2216$, $p = 0.9243$] or main effect of A/P [$F_{(4,30)} = 1.778$, $p = 0.1592$] and Sex [$F_{(1,30)} = 0.2216$, $p = 0.6413$] for TH/DBH colocalization).

(E-G) Expression of **(E)** TH mRNA, **(F)** DBH mRNA, and **(G)** TH/DBH mRNA colocalization in the vlPAG/DR of male (n = 5 slices per mouse / 4 mice total) and female (n = 5 slices per mouse / 2 mice total) mice. No sex differences were observed across the A/P axis. (2-way ANOVA with Tukey's post hoc: no A/P x Sex interaction [$F_{(4,18)} = 2.219$, $p = 0.1077$], no main effect of A/P [$F_{(4,18)} = 1.747$, $p = 0.1837$], main effect of Sex [$F_{(1,18)} = 10.24$, $p = 0.0050$] for TH mRNA; N/A for DBH mRNA; N/A for TH/DBH mRNA colocalization [N/A due to no variation among replicates]).

(H) Representative images of TH immunoreactivity (purple), DBH-eGFP (cyan), and colocalization (pink) in vlPAG/DR of male (top/blue row) and female (bottom/pink row) mice across the A/P axis. TH and DBH colocalization in the locus coeruleus (right/gray) is shown as a positive control.

Unpaired (two-tailed) t-test used for Figure 6A.

Two-way ANOVA with Tukey's post hoc used for Figures 6B-6G.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

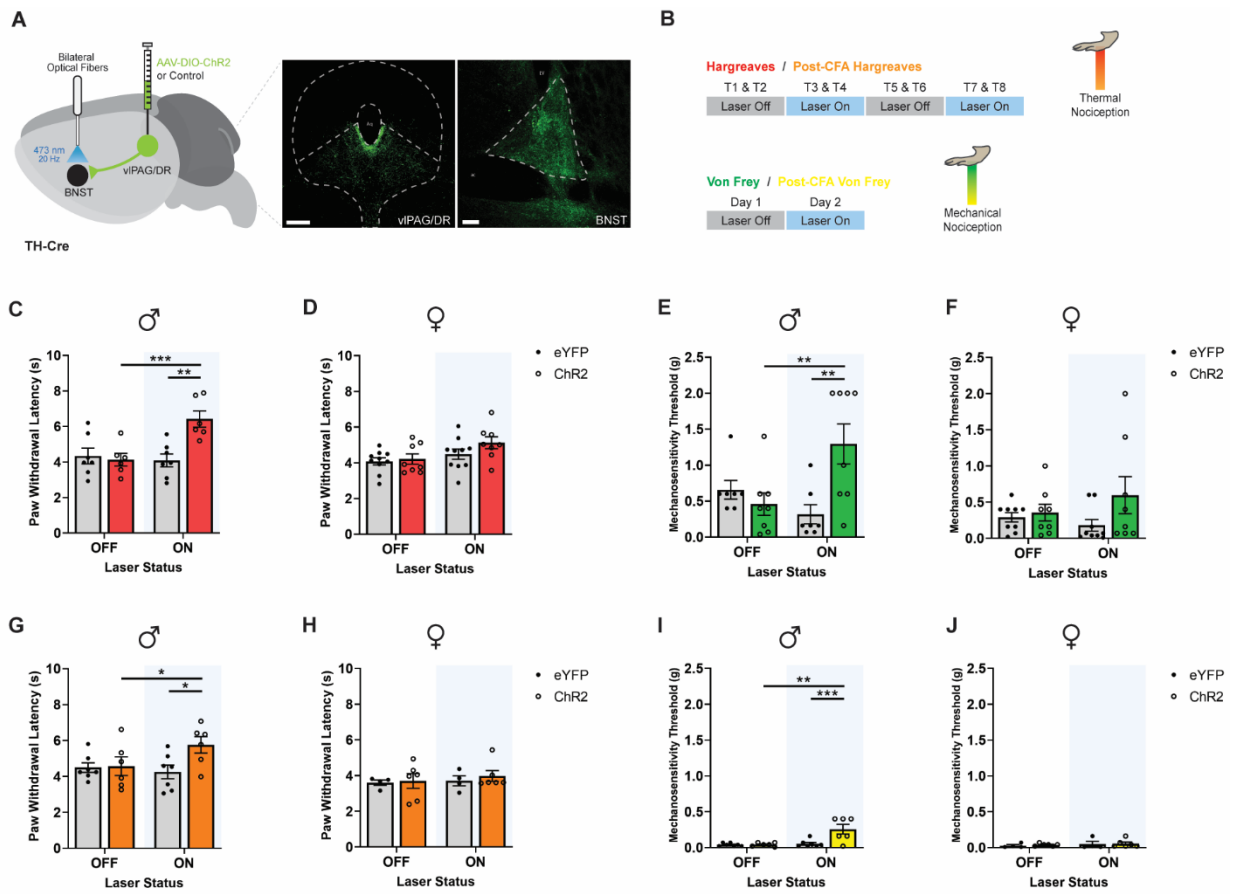

Figure 7. vlPAG/DR^{DA+}-BNST Drives Anti-Nociceptive Behaviors in Male, but not Female, Mice

(A) Optogenetic approach for vlPAG/DR^{DA+} terminal activation in the BNST. (Left) Diagram of virus infusion and optical fiber implantation. (Right) AAV-DIO-ChR2 (green) expression in vlPAG/DR and BNST. Scale bar, 200 µm for vlPAG/DR; 100 µm for BNST.

(B) Schematic of photostimulation during thermal and mechanical nociceptive sensitivity testing. Epochs of laser exposure (i.e. trials [T1-8] for Hargreaves, days for Von Frey) are indicated for each assay.

(C-D) Thermal nociceptive sensitivity averaged by laser status in **(C)** male (n = 6-7) and **(D)** female (n = 8-10) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,11)}$ = 16.72, p = 0.0018], main effect of Laser [$F_{(1,11)}$ = 10.89, p = 0.0071], no main effect of Virus [$F_{(1,11)}$ = 4.657, p = 0.0539] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,16)}$ = 0.8441, p = 0.3719], main effect of Laser [$F_{(1,16)}$ = 5.513, p = 0.0321], no main effect of Virus [$F_{(1,16)}$ = 1.948, p = 0.1819] for females).

(E-F) Mechanical nociceptive sensitivity averaged by laser status in **(E)** male (n = 7-8) and **(F)** female (n = 8-9) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,13)}$ = 17.99, p = 0.0010], no main effect of Laser [$F_{(1,13)}$ = 3.202, p = 0.0969] and Virus [$F_{(1,13)}$ = 2.808, p = 0.1177] for males; 2-way mixed-model

ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,15)} = 2.428$, $p = 0.1400$] or main effect of Laser [$F_{(1,15)} = 0.3336$, $p = 0.5721$] and Virus [$F_{(1,15)} = 2.075$, $p = 0.1702$] for females).

(G-H) Post-CFA thermal nociceptive sensitivity averaged by laser status in **(G)** male (n = 6-7) and **(H)** female (n = 4-6) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,11)} = 5.587$, $p = 0.0376$], no main effect of Laser [$F_{(1,11)} = 2.458$, $p = 0.1453$] and Virus [$F_{(1,11)} = 2.655$, $p = 0.1315$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,8)} = 0.07627$, $p = 0.7894$] or main effect of Laser [$F_{(1,8)} = 0.3549$, $p = 0.5678$] and Virus [$F_{(1,8)} = 0.2628$, $p = 0.6220$] for females).

(I-J) Post-CFA mechanical nociceptive sensitivity averaged by laser status in **(I)** male (n = 6-7) and **(J)** female (n = 4-6) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,11)} = 8.950$, $p = 0.0123$], main effect of Laser [$F_{(1,11)} = 11.37$, $p = 0.0062$] and Virus [$F_{(1,11)} = 9.776$, $p = 0.0096$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,8)} = 0.02840$, $p = 0.8704$] or main effect of Laser [$F_{(1,8)} = 1.280$, $p = 0.2907$] and Virus [$F_{(1,8)} = 0.1248$, $p = 0.7331$] for females).

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 7C-7J.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that Laser ON treatment results in male ChR2 mice with greater paw withdrawal latencies than male eYFP mice in the Naïve condition, and male ChR2 mice with greater paw withdrawal latencies and mechanosensitivity thresholds than male eYFP and female hM3Dq mice in the CFA condition.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

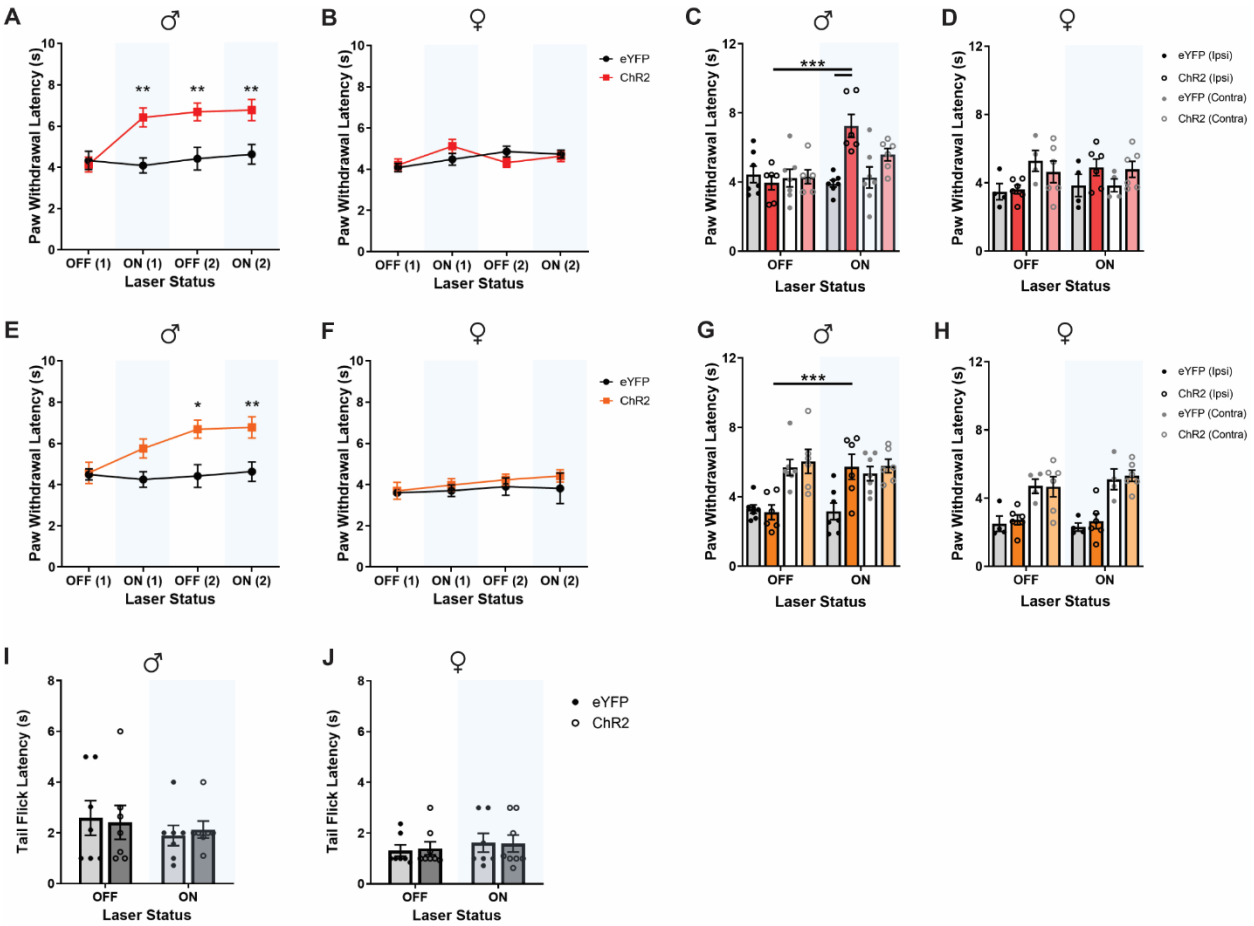

Figure 8. vlPAG/DR^{DA+}-BNST Produces Sustained Supraspinal Analgesia in Male, but not Female, Mice Without Affecting Tail Flick Responses

(A-B) Thermal nociceptive sensitivity by laser status and session. **(A-B)** Light ON and OFF sessions in **(A)** male (n = 6-7) and **(B)** female (n = 8-10) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(3,33)} = 6.827$, $p = 0.0011$], main effect of Laser [$F_{(3,33)} = 7.966$, $p = 0.0004$] and Virus [$F_{(1,11)} = 10.45$, $p = 0.0080$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(3,24)} = 1.601$, $p = 0.2015$], no main effect of Laser [$F_{(3,33)} = 2.185$, $p = 0.1020$] and Virus [$F_{(1,8)} = 0.04641$, $p = 0.8322$] for females). **(C-D)** Thermal nociceptive sensitivity by ipsilateral and contralateral paw for **(C)** male (n = 6-7) and **(D)** female (n = 4-6) TH-Cre mice for Light OFF and ON sessions (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus/Paw interaction [$F_{(3,22)} = 7.800$, $p = 0.0010$], main effect of Laser [$F_{(1,22)} = 11.56$, $p = 0.0026$] and Virus/Paw [$F_{(3,22)} = 3.276$, $p = 0.0402$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus/Paw interaction [$F_{(3,16)} = 3.106$, $p = 0.0561$] or main effect of Laser [$F_{(1,16)} = 0.08316$, $p = 0.7768$] and Virus/Paw [$F_{(3,16)} = 1.184$, $p = 0.3470$] for females).

(E-F) Post-CFA thermal nociceptive sensitivity by Light ON and OFF sessions in **(E)** male (n = 6-7 for Naïve and CFA) and **(F)** female (n = 8-10 for Naïve, 4-6 for CFA) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(3,33)} = 2.349$, $p = 0.0904$], no main effect of Laser [$F_{(3,33)} = 2.710$, $p = 0.0609$], main effect of Virus [$F_{(1,11)} = 23.43$, $p = 0.0005$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(3,24)} = 0.1462$, $p = 0.9311$] or main effect of Laser [$F_{(3,33)} = 0.6225$, $p = 0.6074$] and Virus [$F_{(1,8)} = 0.9130$, $p = 0.2479$] for females).

(G-H) Post-CFA thermal nociceptive sensitivity by ipsilateral and contralateral paw for **(G)** male (n = 6-7) and **(H)** female (n = 4-6) TH-Cre mice for Light OFF and ON sessions (2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus/Paw interaction [$F_{(3,22)} = 6.343$, $p = 0.0029$], no main effect of Laser [$F_{(1,22)} = 2.858$, $p = 0.1050$], main effect of Virus/Paw [$F_{(3,22)} = 9.234$, $p = 0.0004$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus/Paw interaction [$F_{(3,16)} = 0.4414$, $p = 0.7266$] or main effect of Laser [$F_{(1,16)} = 0.3821$, $p = 0.5452$], main effect of Virus/Paw [$F_{(3,16)} = 18.00$, $p < 0.0001$] for females).

(I-J) Tail flick responses by laser status in **(I)** male (n = 7) and **(J)** female (n = 7-8) TH-Cre mice.

(2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,12)} = 0.1901$, $p = 0.6706$] or main effect of Laser [$F_{(1,12)} = 1.051$, $p = 0.3254$] and Virus [$F_{(1,12)} = 0.002219$, $p = 0.9632$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,13)} = 0.03543$, $p = 0.8536$] or main effect of Laser [$F_{(1,13)} = 0.6576$, $p = 0.4320$] and Virus [$F_{(1,13)} = 0.008466$, $p = 0.9281$] for females).

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 8A-8B, 8E-8F, 8I-8J.

Two-way ANOVA with Tukey's post hoc used for Figures 8C-8D, 8G-8H.

Comparisons of Sex x Virus/Paw (Two-way ANOVA with Tukey's post hoc) reveal that CNO treatment results in male hM3Dq mice with greater paw withdrawal latencies for the ipsilateral paw than females in both the Naïve and CFA condition, suggesting that sex differences in anti-nociceptive effects were more pronounced in a specific paw.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

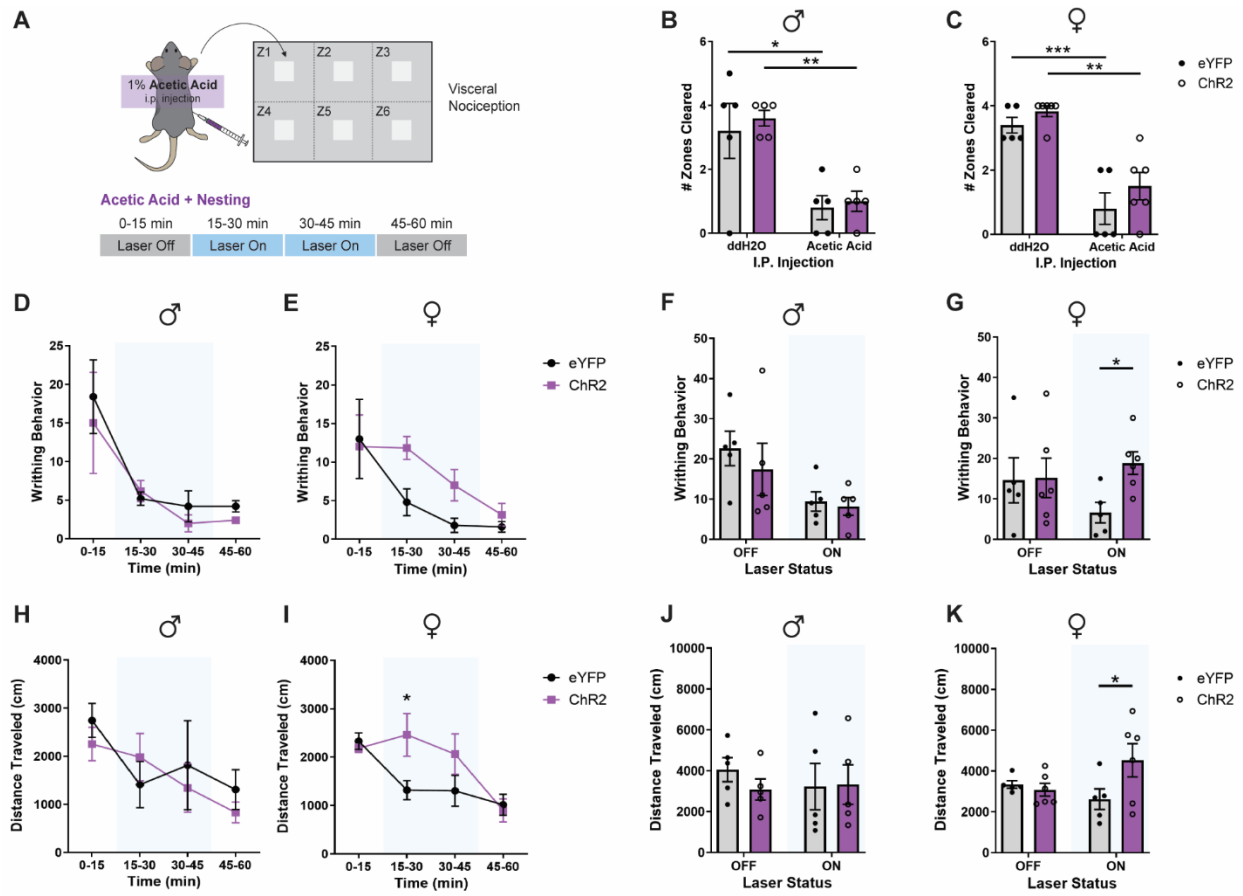

Figure 9. vlPAG/DR^{DA+}-BNST Drives Pain-Related Locomotor Behaviors in Female, but not Male, Mice

(A) Schematic of photostimulation during visceral nociceptive sensitivity testing.

(B-C) Cleared nesting zones with ddH2O/Acetic Acid treatment following optogenetic activation of vlPAG/DR^{DA+}-BNST in **(B)** male (n = 5) and **(C)** female (n = 5-6) TH-Cre mice (2-way mixed-model ANOVA with Sidak's post hoc: no Pain Treatment x Virus interaction [$F_{(1,8)}$ = 0.04878, p = 0.8307], main effect of Pain Treatment [$F_{(1,8)}$ = 30.49, p = 0.0006], no main effect of Virus [$F_{(1,8)}$ = 0.2857, p = 0.6075] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Pain Treatment x Virus interaction [$F_{(1,9)}$ = 0.1657, p = 0.6935], main effect of Pain Treatment [$F_{(1,9)}$ = 56.71, p < 0.0001], no main effect of Virus [$F_{(1,9)}$ = 2.231, p = 0.1695] for females).

(D-E) Writhing by Light ON and OFF sessions in **(D)** male (n = 5) and **(E)** female (n = 5-6) subjects (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(3,24)}$ = 0.1835, p = 0.9066], main effect of Time [$F_{(3,24)}$ = 8.786, p = 0.0004], no main effect of Virus [$F_{(1,8)}$ = 0.5922, p = 0.4637] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(3,27)}$ = 1.063, p = 0.3812], main effect of Time [$F_{(3,27)}$ = 6.542, p = 0.0018], no main effect of Virus [$F_{(1,9)}$ = 2.122, p = 0.1722] for females).

(F-G) Writhing averaged by laser status in **(F)** male (n = 5) and **(G)** female (n = 5-6) subjects (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,8)}$ = 0.2177, p = 0.6532], main effect of Laser [$F_{(1,8)}$ =

6.829, p = 0.0310], no main effect of Virus [$F_{(1,8)}$ = 0.5922, p = 0.4637] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,9)}$ = 2.219, p = 0.1705] or main effect of Laser [$F_{(1,9)}$ = 0.3062, p = 0.5935] and Virus [$F_{(1,9)}$ = 2.122, p = 0.1791] for females; however, unpaired t-test for Laser ON only revealed statistical significance exclusively for females [t(9) = 3.185, p = 0.0111]).

(H-I) Distance traveled by Light ON and OFF sessions in **(H)** male (n = 5) and **(I)** female (n = 5-6) subjects (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(3,24)}$ = 0.9763, p = 0.4203], main effect of Time [$F_{(3,24)}$ = 4.961, p = 0.0081], no main effect of Virus [$F_{(1,8)}$ = 0.1569, p = 0.7024] for males; 2-way mixed-model ANOVA with Sidak's post hoc: Time x Virus interaction [$F_{(3,27)}$ = 3.772, p = 0.0221], main effect of Time [$F_{(3,27)}$ = 10.88, p < 0.0001], no main effect of Virus [$F_{(1,9)}$ = 1.793, p = 0.2134] for females).

(J-K) Distance traveled by laser status in **(J)** male (n = 5) and **(K)** female (n = 5-6) subjects (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,8)}$ = 1.310, p = 0.2854] or main effect of Laser [$F_{(1,8)}$ = 0.3975, p = 0.5460] and Virus [$F_{(1,8)}$ = 0.1569, p = 0.7024] for males; 2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,9)}$ = 5.603, p = 0.0421], no main effect of Laser [$F_{(1,9)}$ = 0.6172, p = 0.4523] and Virus [$F_{(1,9)}$ = 1.793, p = 0.2134] for females).

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 9B-9K.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that Laser ON treatment results in female ChR2 mice with greater writhing behaviors than female eYFP and male ChR2 mice. By contrast, distance traveled does not differ by sex when compared within laser status, signifying that the female-specific locomotor differences observed in Figure 3K are restricted to comparisons across laser status and within sex.

Data are shown as mean ±SEM. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.

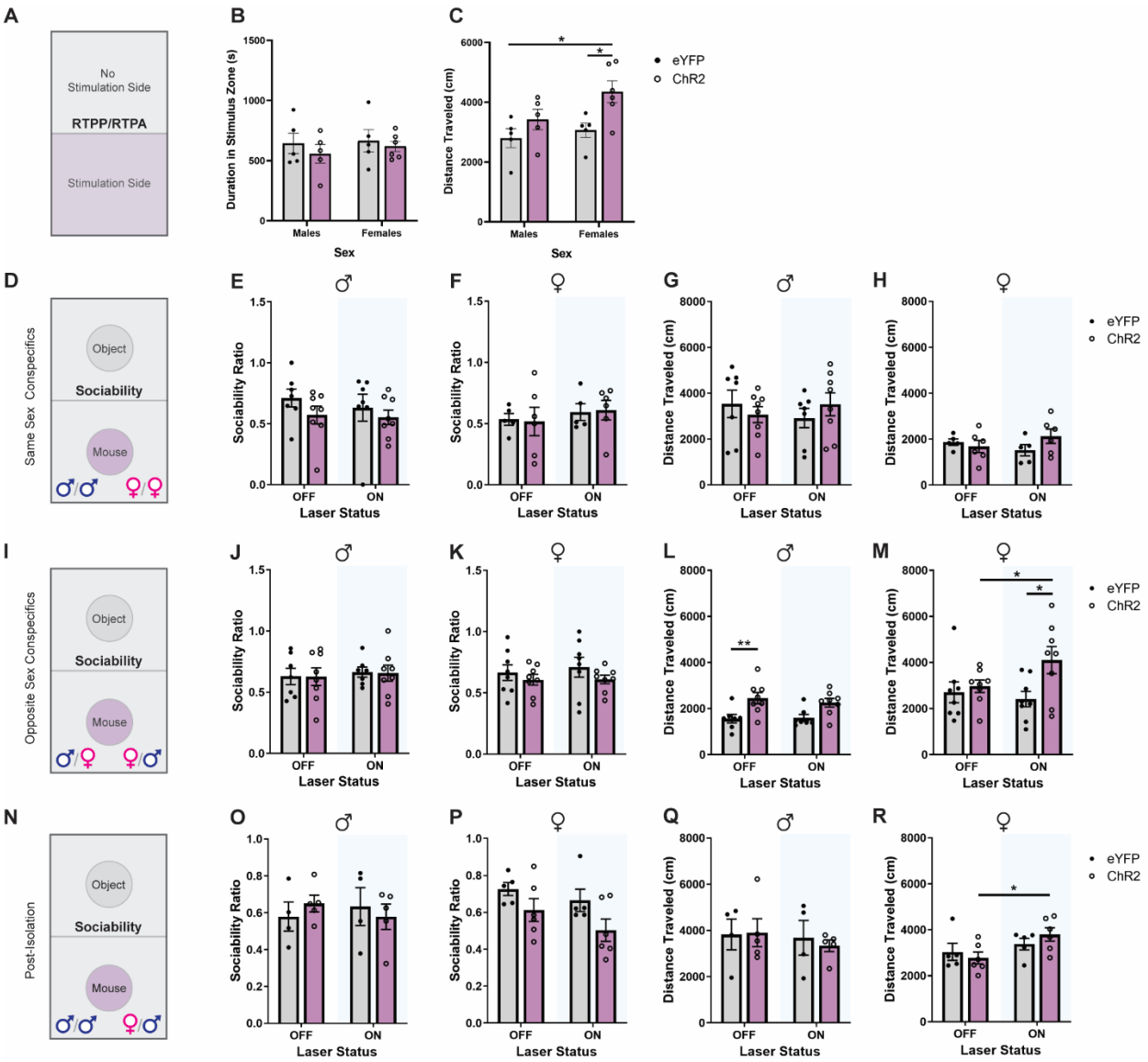

Figure 10. vlPAG/DR^{DA+}-BNST Increases Context-Dependent Locomotor Behaviors in Female, but not Male, Mice

(A-C) Real-time place preference/aversion with optogenetic activation of vlPAG/DR^{DA+}-BNST. **(A)** Schematic of RTPP/RTPA with designated sides for 20 Hz light stimulation (purple) and no stimulation (gray). Comparisons by sex are shown for **(B)** duration in stimulus zone (n = 5-6; 2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,17)}$ = 0.07355, p = 0.7895] or main effect of Sex [$F_{(1,17)}$ = 0.3119, p = 0.5838] and Virus [$F_{(1,17)}$ = 0.7953, p = 0.3879]) and **(C)** distance traveled (n = 5-6; 2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,17)}$ = 1.036, p = 0.3230] or main effect of Sex [$F_{(1,17)}$ = 3.325, p = 0.0859, main effect of Virus [$F_{(1,17)}$ = 8.512, p = 0.0096]). **(D-H)** Sociability test with a same sex conspecific mouse and novel object. **(D)** Schematic of sociability test. **(E-F)** Ratio of mouse and object exploration by laser status (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x

Virus interaction [$F_{(1,13)}$ = 0.1482, p = 0.7065] or main effect of Laser [$F_{(1,13)}$ = 0.3906, p = 0.5480] and Virus [$F_{(1,13)}$ = 1.867, p = 0.1950] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,9)}$ = 0.1463, p = 0.7110] and main effect of Laser [$F_{(1,9)}$ = 3.141, p = 0.1101] or Virus [$F_{(1,9)}$ = 9.159e-5, p = 0.9926] for females) and **(G-H)** distance traveled (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,13)}$ = 4.011, p = 0.0665] or main effect of Laser [$F_{(1,13)}$ = 0.1065, p = 0.7493] and Virus [$F_{(1,13)}$ = 0.01096, p = 0.9182] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,9)}$ = 4.764, p = 0.0569] or main effect of Laser [$F(_{1,9)}$ = 0.06317, p = 0.8072] and Virus [$F_{(1,9)}$ = 0.4294, p = 0.5287] for females) in **(E, G)** male (n = 7-8) and **(F, H)** female (n = 5-6) TH-Cre mice.

(I-M) Sociability test with an opposite sex conspecific mouse and novel object. **(I)** Schematic of sociability test. **(J-K)** Ratio of mouse and object exploration by laser status (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,13)}$ = 0.003895, p = 0.9512] or main effect of Laser [$F_{(1,13)}$ = 0.4360, p = 0.5206] and Virus [$F_{(1,13)}$ = 0.006109, p = 0.9389] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,14)}$ = 0.2141, p = 0.6507] or main effect of Laser [$F_{(1,14)}$ = 0.2706, p = 0.6111] and Virus [$F_{(1,14)}$ = 1.323, p = 0.2694] for females) and **(L-M)** distance traveled (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,13)}$ = 0.6009, p = 0.4521] or main effect of Laser [$F_{(1,13)}$ = 0.2998, p = 0.5933, main effect of Virus [$F_{(1,13)}$ = 10.92, p = 0.0057] for males; 2-way mixed-model ANOVA with Sidak's post hoc: Laser x Virus interaction [$F_{(1,14)}$ = 7.305, p = 0.0172], no main effect of Laser [$F_{(1,14)}$ = 2.511, p = 0.1354] and Virus [$F_{(1,14)}$ = 3.232, p = 0.0938] for females) in **(J, L)** male (n = 7-8) and **(K, M)** female (n = 8) TH-Cre mice.

(N-R) Sociability test with a male conspecific mouse and novel object following social isolation. **(N)** Schematic of post-isolation sociability test. **(O-P)** Ratio of mouse and object exploration by laser status (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,7)}$ = 0.8180, p = 0.3958] or main effect of Laser [$F_{(1,7)}$ = 0.6009, p = 0.4521] and Virus [$F_{(1,7)}$ = 0.009865, p = 0.9237]; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,9)}$ = 0.2679, p = 0.6172] or main effect of Laser [$F_{(1,9)}$ = 3.466, p = 0.0956] and Virus [$F_{(1,9)}$ = 4.238, p = 0.0696] for females) and **(Q-R)** distance traveled (2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,7)}$ = 0.2124, p = 0.6589] or main effect of Laser [$F_{(1,7)}$ = 0.5946, p = 0.4659] and Virus [$F_{(1,7)}$ = 0.03920, p = 0.8487] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Laser x Virus interaction [$F_{(1,9)}$ = 2.459, p = 0.1513], main effect of Laser [$F_{(1,9)}$ = 9.767, p = 0.0122], no main effect of Virus [$F_{(1,9)}$ = 0.04573, p = 0.8354] for females) for **(O, Q)** male (n = 4-5) and **(P, R)** female (n = 5-6) TH-Cre mice.

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures S5E-S5H, S5J-S5M, S5O-S5R.

Two-way ANOVA with Tukey's post hoc used for Figures S5B-S5C.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that Laser ON treatment generally results in ChR2 mice with greater distance traveled than eYFP mice when not segregated by same and opposite sex conspecifics. The only retained post hoc effect showed that male ChR2 mice exhibit greater distance traveled than male eYFP mice with Laser OFF treatment, signifying that the female-specific locomotor differences observed in Figures 10M and 10R are restricted to comparisons across laser status and within sex.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

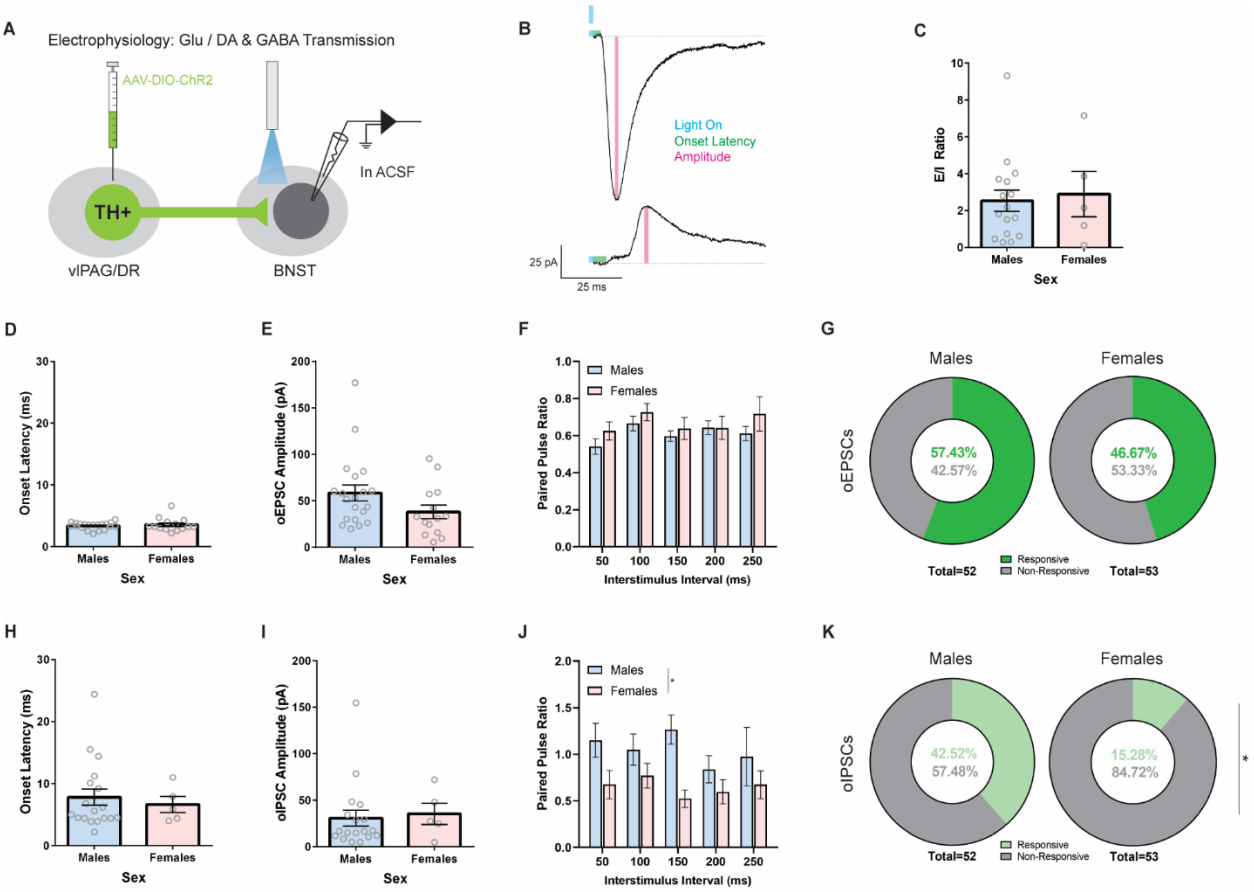

Figure 11. Sex Differences in vlPAG/DR^{DA+}-BNST Transmission and Connectivity

(A) Experimental schematic illustrating whole cell patch clamp in ACSF. Recordings were performed in the BNST following optogenetic activation of vlPAG/DR^{DA+} terminals.

(B) Representative o(E/I)PSC traces from BNST neurons following activation of vlPAG/DR^{DA+} terminals with a single 1 ms pulse of 473 nm light (blue). Onset latency (green) and amplitude (pink) are indicated to highlight the distinct properties of excitatory and inhibitory transmission between vlPAG/DR^{DA+} and BNST.

(C) E/I ratio comparison in male mice (n = 16 cells / 1-7 cells per mouse / 5 mice total) and female (n = 5 cells / 1-3 cells per mouse / 3 mice total) TH-Cre mice (unpaired t-test: $t_{(19)} = 0.3007$, $p = 0.7669$).

(D) Onset latency comparison for oEPSCs in male (n = 20 cells / 1-7 cells per mouse / 6 mice total) and female (n = 14 cells / 1-5 cells per mouse / 4 mice total) subjects (unpaired t-test: $t_{(32)} = 0.6429$, $p = 0.5249$).

(E) Amplitude comparison for oEPSCs in male (n = 20 cells / 1-7 cells per mouse / 6 mice total) and female (n = 14 cells / 1-5 cells per mouse / 4 mice total) subjects (unpaired t-test: $t_{(32)} = 1.700$, $p = 0.0989$).

(F) Paired-pulse ratio of oEPSCs in male (n = 19-20 cells at each ISI / 1-7 cells per mouse / 6 mice total) and female (n = 12-14 cells at each ISI / 1-5 cells per mouse / 4 mice total) subjects (2-way ANOVA with Tukey's post hoc: no ISI

x Sex interaction [$F_{(4,153)} = 0.3571$, $p = 0.8388$] or main effect of ISI [$F_{(4,153)} = 1.584$, $p = 0.1813$] and Sex [$F_{(1,153)} = 3.570$, $p = 0.0607$]).

(G) Percentage of responsive and non-responsive cells for oEPSCs in male (n = 52 cells) and female (n = 53 cells) subjects (Fisher's exact test: $p = 0.3314$).

(H) Onset latency comparison for oIPSCs in male (n = 18 cells / 1-7 cells per mouse / 6 mice total) and female (n = 5 cells / 1-3 cells per mouse / 3 mice total) subjects (unpaired t-test: $t_{(21)} = 0.4467$, $p = 0.6597$).

(I) Amplitude comparison for oIPSCs in male (n = 18 cells / 1-7 cells per mouse / 6 mice total) and female (n = 5 cells / 1-3 cells per mouse / 3 mice total) subjects (unpaired t-test: $t_{(21)} = 0.2712$, $p = 0.7889$).

(J) Paired-pulse ratio of oIPSCs (n = 3-15 cells) in male (n = 12-15 cells at each ISI / 1-4 cells per mouse / 6 mice total) and female (n = 3-4 cells at each ISI / 1-3 cells per mouse / 3 mice total) subjects (2-way ANOVA with Tukey's post hoc: no ISI x Sex interaction [$F_{(4,78)} = 0.2449$, $p = 0.9119$] or main effect of ISI [$F_{(4,78)} = 0.1636$, $p = 0.9562$], main effect of Sex [$F_{(1,78)} = 5.212$, $p = 0.0252$]).

(K) Percentage of responsive and non-responsive cells for oIPSCs in male (n = 52 cells) and female (n = 53 cells) subjects (Fisher's exact test: $p = 0.0015$).

Unpaired (two-tailed) t-test used for Figures 11C-11E, 11H-11I.

Fisher's exact test used for Figures 11G, 11K.

Two-way ANOVA with Tukey's post hoc used for Figures 11F, 11J.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

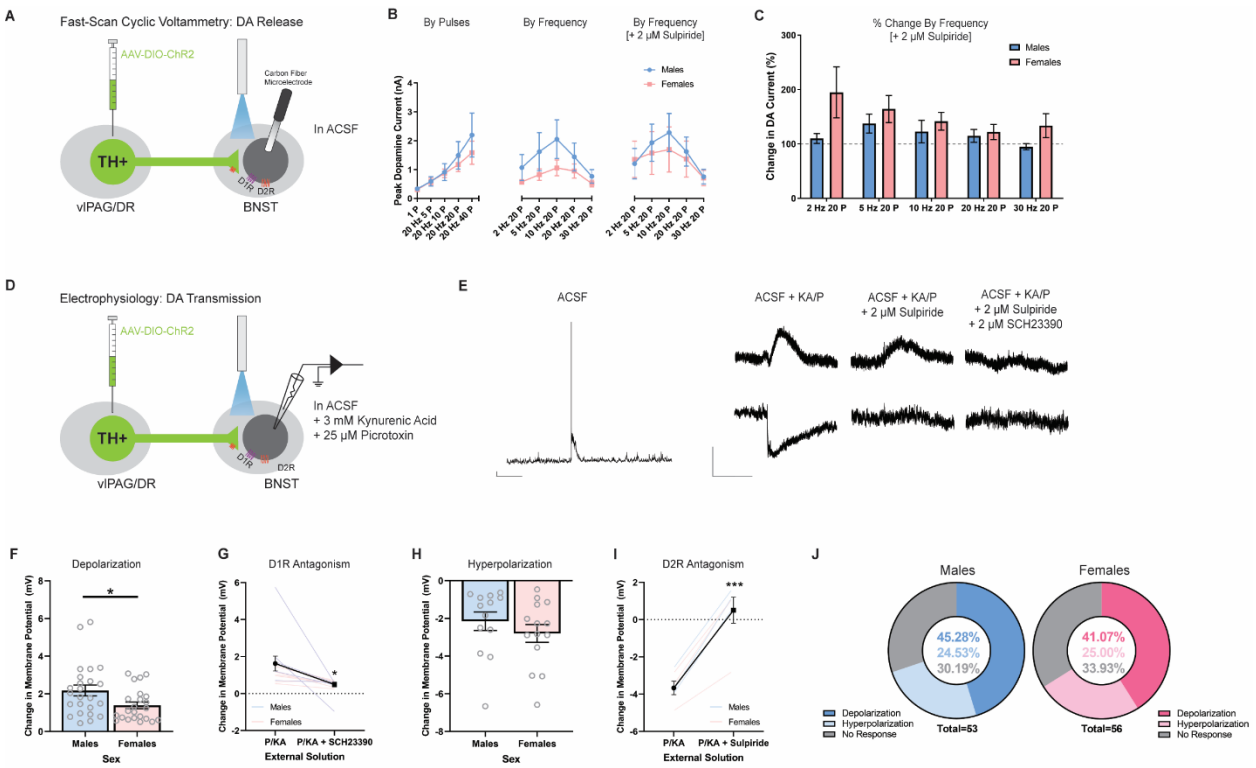

Figure 12. Sex Differences in vlPAG/DR^{DA+}-BNST Dopaminergic Transmission

(A) Experimental schematic illustrating fast-scan cyclic voltammetry in ACSF. Recordings were performed in the BNST following optogenetic activation of vlPAG/DR^{DA+} terminals.

(B-C) Peak DA current following photostimulation of vlPAG/DR^{DA+} terminals in the BNST in male (n = 6-7 slices / 1-4 slices per mouse / 4 mice total in ACSF; n = 6 slices / 1-3 slices per mouse / 4 mice total in ACSF + Sulpiride) and female (n = 8 slices / 1-2 slices per mouse / 6 mice total; n = 6 slices / 1-2 slices per mouse / 5 mice total in ACSF + Sulpiride) TH-Cre mice. Recordings with **(B)** varying light pulses (2-way mixed-model ANOVA with Sidak's post hoc: no Pulse x Sex interaction [$F_{(4,52)} = 0.7132$, $p = 0.5867$], main effect of Pulse [$F_{(4,52)} = 15.84$, $p < 0.0001$], no main effect of Sex [$F_{(1,13)} = 0.2812$, $p = 0.6049$]) and frequencies (2-way mixed-model ANOVA with Sidak's post hoc: no Frequency x Sex interaction [$F_{(4,48)} = 1.542$, $p = 0.2052$], main effect of Frequency [$F_{(4,48)} = 9.436$, $p < 0.0001$], no main effect of Sex [$F_{(1,12)} = 1.733$, $p = 0.2127$]) were performed in ACSF, then **(C)** repeated with varying frequencies in ACSF + 2 μM sulpiride (2-way mixed-model ANOVA with Sidak's post hoc: no Frequency x Sex interaction [$F_{(4,40)} = 0.7303$, $p = 0.5766$], main effect of Frequency [$F_{(4,40)} = 8.330$, $p < 0.0001$], no main effect of Sex [$F_{(1,10)} = 0.08074$, $p = 0.7821$]). Drug effects were quantified using the percentage change in peak DA current following D2R antagonism (2-way mixed-model ANOVA with Sidak's post hoc: no Frequency x Sex interaction [$F_{(4,40)} = 1.940$, $p = 0.1225$], main effect of Frequency [$F_{(4,40)} = 2.736$, $p = 0.0420$], no main effect of Sex [$F_{(1,10)} = 2.138$, $p = 0.1744$]).

(D) Experimental schematic illustrating whole cell patch clamp in ACSF + 3 mM kynurenic acid + 25 μM picrotoxin. Recordings were performed in the BNST following optogenetic activation of vlPAG/DR^{DA+} terminals.

(E) Representative traces of BNST neurons responding to optically-evoked DA transmission from vlPAG/DR^{DA+} terminals. (Left) Depolarization following 20 Hz (5 ms width, 20 pulses) stimulation in ACSF. (Left Middle) Same stimulation parameters in ACSF + kynurenic acid (KA) / picrotoxin (P) produces a mix of depolarization and hyperpolarization. (Right Middle-Right) These responses are blocked by the addition of SCH23390 or sulpiride to ACSF + KA/P. Scale bars, x = time (4 sec), y = membrane potential (2 mV).

(F-I) Change in membrane potential of BNST neurons following optically-evoked DA transmission from vlPAG/DR. Comparison of **(F)** depolarization and **(H)** hyperpolarization size between male (depolarization: n = 26 slices / 1-10 slices per mouse / 7 mice total; hyperpolarization: n = 13 slices / 2-6 slices per mouse / 4 mice total) and female (depolarization: 23 slices / 1-6 slices per mouse / 8 mice total; hyperpolarization: 14 slices / 1-5 slices per mouse / 6 mice total) TH-Cre mice (unpaired t-test, depolarization: $t_{(47)} = 2.028$, $p = 0.0242$; hyperpolarization: $t_{(25)} = 0.9501$, $p = 0.3512$). DA receptor antagonism of **(G)** depolarization (n = 14 cells; 7 cells per sex / 1-3 cells per mouse / 6 mice total) and **(I)** hyperpolarization (n = 6 cells; 3 cells per sex / 1 cell per mouse / 6 mice total) is shown with individual cells from male (blue) and female (pink) subjects (paired t-test, D1R antagonism: $t_{(13)} = 2.523$, $p = 0.0222$; D2R antagonism: $t_{(5)} = 9.708$, $p = 0.0002$; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Sex interaction [$F_{(1,12)} = 1.444$, $p = 0.2526$], main effect of Drug [$F_{(1,12)} = 6.585$, $p = 0.0247$], no main effect of Sex [$F_{(1,12)} = 0.7438$, $p = 0.4054$] for D1R antagonism; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Sex interaction [$F_{(1,4)} = 1.665$, $p = 0.2664$], main effect of Drug [$F_{(1,4)} = 106.8$, $p = 0.0005$], no main effect of Sex [$F_{(1,4)} = 0.6425$, $p = 0.4677$] for D2R antagonism; with males exhibiting greater D1R antagonism than females, $p = 0.0408$).

(J) Percentage of BNST neurons showing depolarization, hyperpolarization, and no response following light-evoked DA transmission from vlPAG/DR^{DA+} terminals in male (n = 53 cells) and female (n = 56 cells) subjects (Chi-square test, $p = 0.8900$).

Unpaired (two-tailed) t-test used for Figures 12F, 12H.

Paired (two-tailed) t-test used for Figures 12G, 12I.

Chi-square test used for Figures 12J.

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 12B-12C, 12G, 12I.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

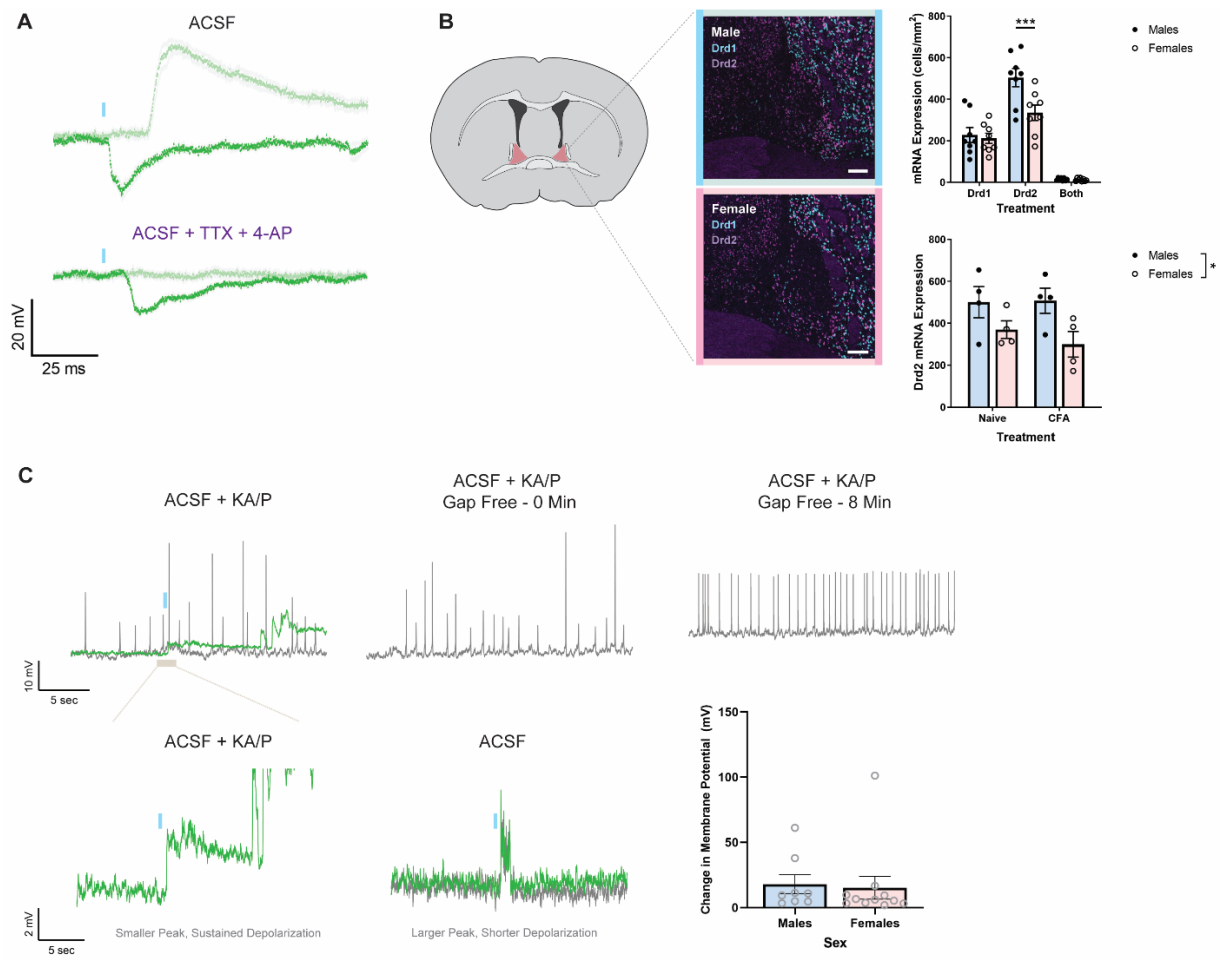

Figure 13. Excitatory and Inhibitory Signaling in vlPAG/DR^{DA+}-BNST

(A) oEPSCs (green) and oIPSCs (light green) in ACSF and ACSF + 5 mM TTX + 200 mM 4-AP to assess monosynaptic and polysynaptic connectivity between vlPAG/DR^{DA+} and BNST neurons.

(B) (Left) Expression of Drd1 (blue) and Drd2 (purple) mRNA in the BNST of male and female mice. Scale Bar, 100 µm. (Right-Top) Average number of BNST neurons expressing Drd1, Drd2, and Drd1-Drd2 colocalization in male and female mice (n = 4; 2-way ANOVA with Tukey's post hoc: DA-R x Sex interaction [$F_{(2,42)} = 4.927$, $p = 0.0120$], main effect of DA-R [$F_{(2,42)} = 94.68$, $p < 0.0001$] and Sex [$F(_{1,42)} = 6.815$, $p = 0.0125$]). (Right-Bottom) Average number of BNST neurons expressing Drd2 in male and female C57BL/6J mice following CFA treatment (n = 4; 2-way ANOVA with Tukey's post hoc: no interaction [$F_{(1,12)} = 0.4050$, $p = 0.5365$] or main effect of Paw Treatment [$F_{(1,12)} = 0.2744$, $p = 0.6099$], main effect of Sex [$F_{(1,12)} = 7.866$, $p = 0.0159$]).

(C) Example trace of light-evoked DA transmission from vlPAG/DR and sustained depolarization in the BNST. (Upper-Left) Light activation (blue) of first sweep (green) produced sustained depolarization, where membrane potential increased over time and led to repeated firing in the second sweep (gray). (Upper-Middle and Upper-Right) Firing is sustained for several minutes. (Lower-Left) Close up of the first sweep shows light-evoked depolarization

following DA transmission has a smaller and more sustained peak than (Lower-Middle) the same cell responding to light activation in ACSF. (Lower-Right) Comparison of 20 Hz (5 ms, 20 pulses) stimulation of BNST in ACSF by sex (n = 10 cells / 1-2 cells per mouse / 9 mice total for males; n = 11 cells / 1-3 cells per mouse / 7 mice total for females; unpaired t-test: $t_{(19)} = 0.06774$, $p = 0.9467$).

Unpaired (two-tailed) t-test used for Figures 13C

Two-way ANOVA with Tukey's post hoc used for Figure 13B.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

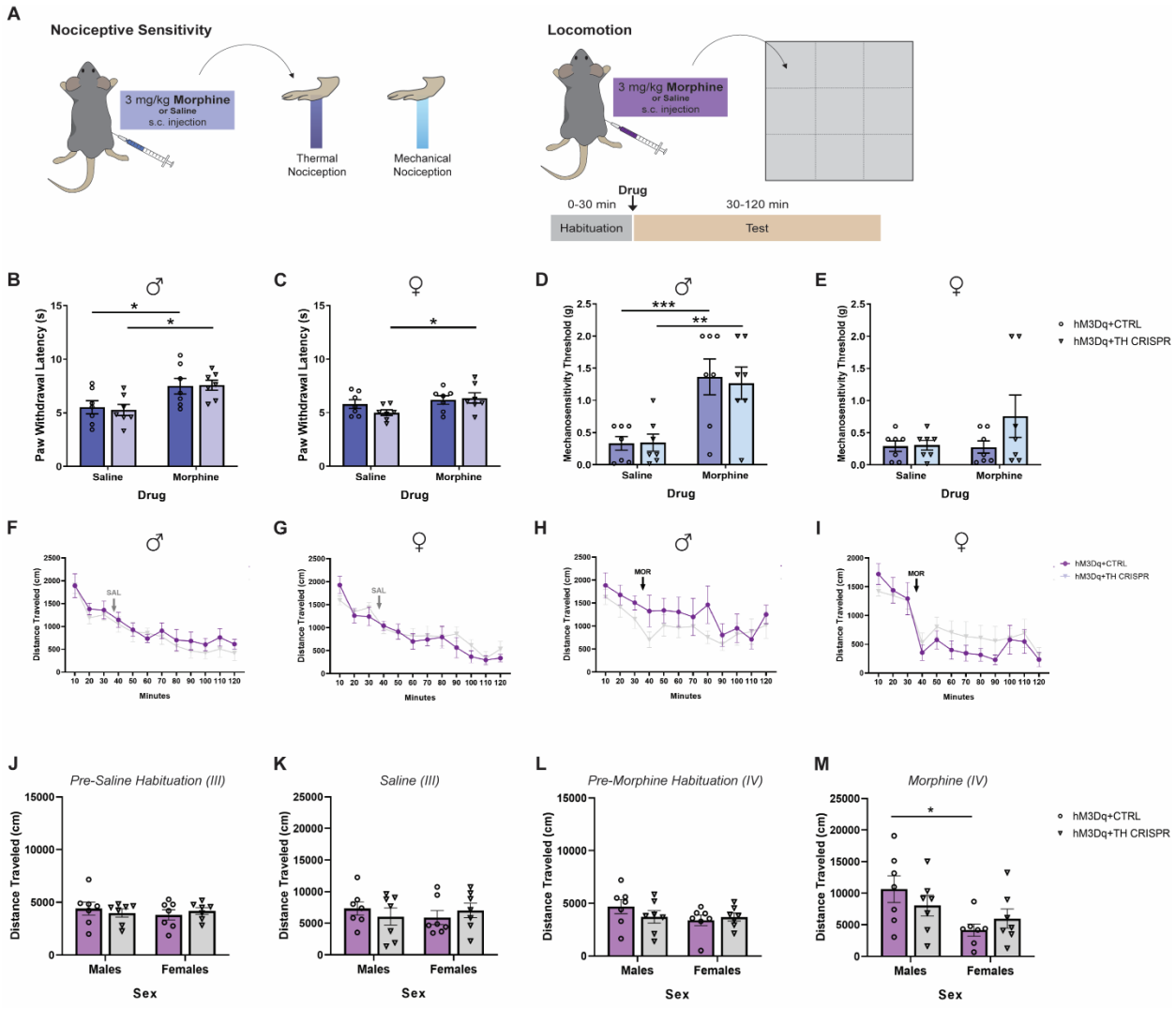

Figure 14. vlPAG/DR^{DA+} Anti-Nociception and Locomotion is Dopamine-Dependent

(A) Chemogenetic approach for vlPAG/DR^{DA+} activation with local genetic deletion of TH. (Top-Left) Diagram of virus infusion. (Top-Right) Detailed schematic of TH CRISPR provided by Zweifel lab. (Bottom-Left) Validation of TH deletion in vlPAG/DR (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.08343$, $p = 0.7752$], main effect of Sex [$F_{(1,24)} = 5.063$, $p = 0.0339$] and Virus [$F_{(1,24)} = 15.44$, $p = 0.0006$]) for male (n = 7; unpaired t-test: $t_{(12)} = 2.389$, $p = 0.0342$) and female (n = 7; unpaired t-test: $t_{(12)} = 3.257$, $p = 0.0069$) TH-Cre mice. (Bottom-Right) hM3Dq expression (orange), TH immunoreactivity (green), and colocalization (yellow) in vlPAG/DR. Scale bar, 100 µm.

(B) Schematic illustrating the measurement of thermal and mechanical nociceptive sensitivity and locomotion following treatment with saline or CNO.

(C-D) Thermal nociceptive sensitivity of **(C)** male (n = 7) and **(D)** female (n = 7) TH-Cre mice following saline or CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 4.719$, $p = 0.0506$], main effect of Drug [$F_{(1,12)} = 6.501$, $p = 0.0255$], no main effect of Virus [$F_{(1,12)} = 1.384$, $p = 0.2622$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 1.092$, $p = 0.3166$] or main effect of Drug [$F_{(1,12)} = 0.5589$, $p = 0.4691$] and Virus [$F_{(1,12)} = 0.08844$, $p = 0.7713$] for females).

(E-F) Mechanical nociceptive sensitivity of **(E)** male (n = 7) and (F) female (n = 7) TH-Cre mice following CNO or saline injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 4.657$, $p = 0.0519$], main effect of Drug [$F_{(1,12)} = 9.057$, $p = 0.0109$] and Virus [$F_{(1,12)} = 5.552$, $p = 0.0363$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: Drug x Virus interaction [$F_{(1,12)} = 7.305$, $p = 0.0192$], no main effect of Drug [$F_{(1,12)} = 0.05274$, $p = 0.8222$] and Virus [$F_{(1,12)} = 5.642e\text{-}5$, $p = 0.9941$] for females).

(G-H) Locomotor activity of **(G)** male (n = 7) and **(H)** female (n = 7) TH-Cre mice following a saline injection (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 1.090$, $p = 0.3743$], main effect of Time [$F_{(4.638,55.66)} = 36.95$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.5790$, $p = 0.4614$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 1.497$, $p = 0.1397$], main effect of Time [$F_{(4.80,57.60)} = 29.09$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.4847$, $p = 0.4996$] for females).

(I-J) Locomotor activity of **(I)** male (n = 7) and **(J)** female (n = 7) TH-Cre mice following a CNO injection (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 1.843$, $p = 0.0529$], main effect of Time [$F_{(5.062,60.75)} = 41.17$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.1730$, $p = 0.6848$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 0.5433$, $p = 0.8706$], main effect of Time [$F_{(4.410,52.92)} = 39.16$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.8127$, $p = 0.3851$] for females).

(K) Pre-Saline Habituation (I). Averaged locomotor activity prior to saline treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.04664$, $p = 0.8308$] or main effect of Sex [$F_{(1,24)} = 0.2023$, $p = 0.6569$], main effect of Virus [$F_{(1,24)} = 5.770$, $p = 0.0244$]).

(L) Saline (I). Averaged locomotor activity after saline treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.03254$, $p = 0.8584$] or main effect of Sex [$F_{(1,24)} = 0.02904$, $p = 0.8661$] and Virus [$F_{(1,24)} = 0.2752$, $p = 0.6046$]).

(M) Pre-Saline Habituation (II). Averaged locomotor activity prior to CNO treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.4548$, $p = 0.5065$] or main effect of Sex [$F_{(1,24)} = 0.7035$, $p = 0.4099$] and Virus [$F_{(1,24)} = 0.4513$, $p = 0.5081$]).

(N) CNO (II). Averaged locomotor activity after CNO treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.02032$, $p = 0.8878$] or main effect of Sex [$F_{(1,24)} =$

0.3066, $p = 0.5849$] and Virus [$F_{(1,24)} = 2.797$, $p = 0.1075$]).

Unpaired (two-tailed) t-test used for Figure 14A.

Two-way ANOVA with Tukey's post hoc used for Figures 14A, 14K-14N.

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 14C-14J.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that CNO treatment results in a Sex x Virus interaction and main effect of Sex for the Hargreaves test, as well as main effects of Sex and Virus for the Von Frey test, with post hoc tests showing that male hM3Dq + CTRL mice exhibit greater mechanosensitivity thresholds than male hM3Dq + TH CRISPR mice.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

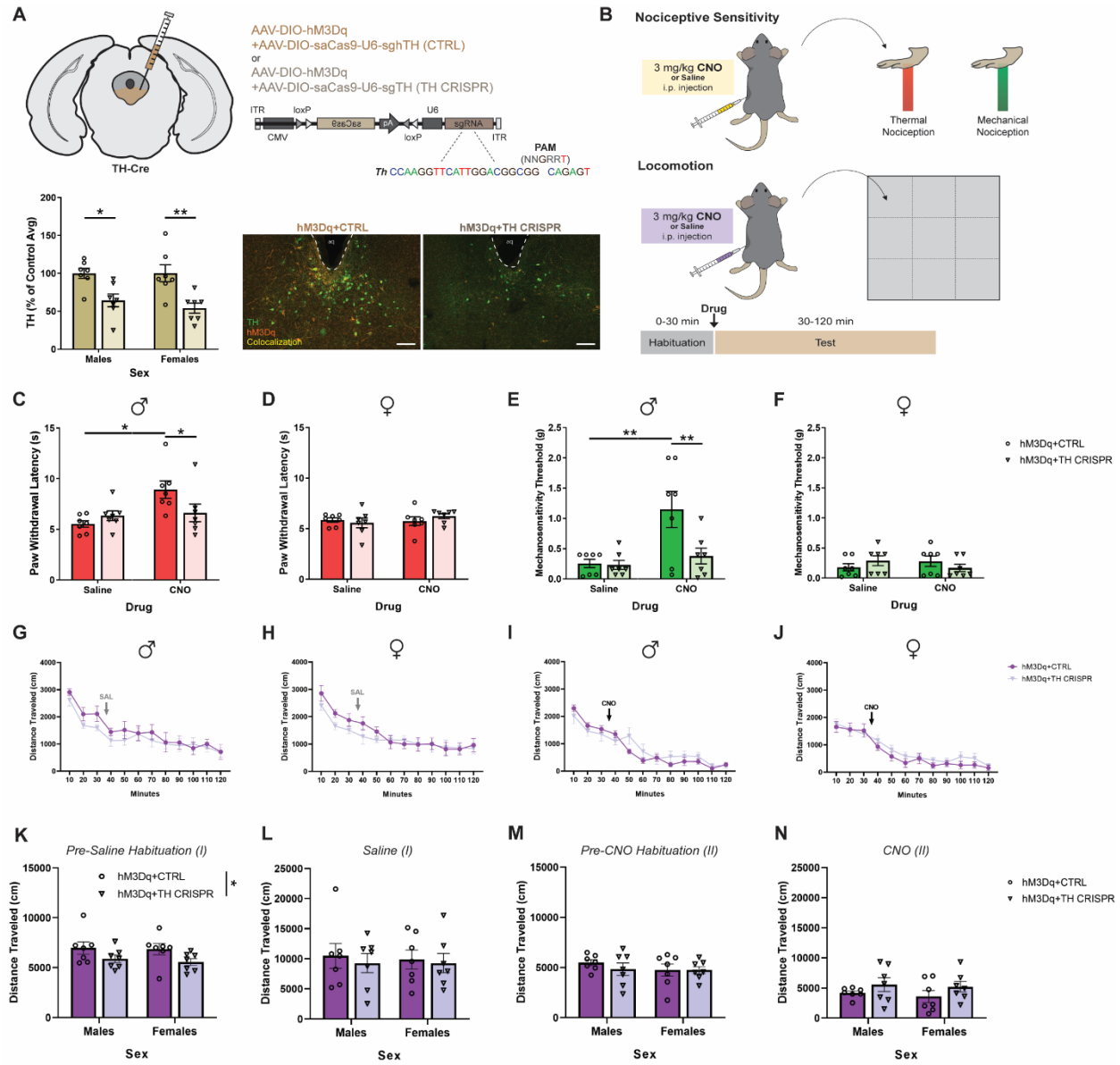

Figure 15. Morphine Anti-Nociception and Locomotion is Not Dependent on vlPAG/DR Dopamine

(A) Pharmacological approach for opiate-induced vlPAG/DR^{DA+} activation with local genetic deletion of TH. Schematic illustrating measurement of thermal and mechanical nociceptive sensitivity and locomotion following systemic treatment with saline or morphine.

(B-C) Thermal nociceptive sensitivity of **(B)** male (n = 7) and **(C)** female (n = 7) TH-Cre mice following saline or morphine injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.1093$, $p = 0.7467$], main effect of Drug [$F_{(1,12)} = 18.70$, $p = 0.0010$], no main effect of Virus [$F_{(1,12)} = 0.01576$, $p = 0.9022$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 1.788$, $p = 0.2060$], main effect of Drug [$F_{(1,12)} = 5.647$, $p = 0.0350$], no main effect of Virus [$F_{(1,12)} = 0.5461$, $p = 0.4741$] for females).

(D-E) Mechanical nociceptive sensitivity of **(D)** male (n = 7) and **(E)** female (n = 7) TH-Cre mice following saline or morphine injection (2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 0.1383$, $p = 0.7165$], main effect of Drug [$F_{(1,12)} = 43.71$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.0300$, $p = 0.8654$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Drug x Virus interaction [$F_{(1,12)} = 1.757$, $p = 0.2097$] or main effect of Drug [$F_{(1,12)} = 1.548$, $p = 0.2372$] and Virus [$F_{(1,12)} = 1.806$, $p = 0.2038$] for females).

(F-G) Locomotor activity of **(F)** male (n = 7) and **(G)** female (n = 7) TH-Cre mice following a saline injection (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 0.4578$, $p = 0.9258$], main effect of Time [$F_{(3.177,38.13)} = 27.86$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.4267$, $p = 0.5259$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 1.475$, $p = 0.1482$], main effect of Time [$F_{(4.396,52.75)} = 36.34$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.1568$, $p = 0.6991$] for females).

(H-I) Locomotor activity of **(H)** male (n = 7) and **(I)** female (n = 7) TH-Cre mice following a morphine injection (2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 0.9981$, $p = 0.4516$], main effect of Time [$F_{(4.032,48.39)} = 7.084$, $p = 0.0001$], no main effect of Virus [$F_{(1,12)} = 1.015$, $p = 0.3336$] for males; 2-way mixed-model ANOVA with Sidak's post hoc: no Time x Virus interaction [$F_{(11,132)} = 1.033$, $p = 0.4214$], main effect of Time [$F_{(3.130,37.56)} = 20.89$, $p < 0.0001$], no main effect of Virus [$F_{(1,12)} = 0.4201$, $p = 0.5291$] for females).

(J) Pre-Saline Habituation (III). Averaged locomotor activity prior to saline treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 0.7391$, $p = 0.3985$] or main effect of Sex [$F_{(1,24)} = 0.1799$, $p = 0.6753$] and Virus [$F_{(1,24)} = 0.003611$, $p = 0.9526$]).

(K) Saline (III). Averaged locomotor activity after saline treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 1.080$, $p = 0.3091$] or main effect of Sex [$F_{(1,24)} = 0.03754$, $p = 0.8480$] and Virus [$F_{(1,24)} = 0.005701$, $p = 0.9404$]).

(L) Pre-Morphine Habituation (IV). Averaged locomotor activity prior to morphine treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 1.379$, $p = 0.2518$] or main effect of Sex [$F_{(1,24)} = 1.411$, $p = 0.2465$] and Virus [$F_{(1,24)} = 0.3702$, $p = 0.5486$]).

(M) Morphine (IV). Averaged locomotor activity after morphine treatment in male (n = 7) and female (n = 7) TH-Cre mice (2-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F_{(1,24)} = 1.880$, $p = 0.1831$], main effect of Sex [$F_{(1,24)} = 7.039$, $p = 0.0139$], no main effect of Virus [$F_{(1,24)} = 0.05683$, $p = 0.8136$]).

Two-way ANOVA with Tukey's post hoc used for Figures 15J-15M.

Two-way mixed-model ANOVA with Sidak's post hoc used for Figures 15B-15I.

Comparisons of Sex x Virus (Two-way ANOVA with Tukey's post hoc) reveal that morphine treatment results in a main effect of Sex for the Hargreaves and Von Frey tests, with no sex difference in how TH knockout impacts the effects of morphine.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

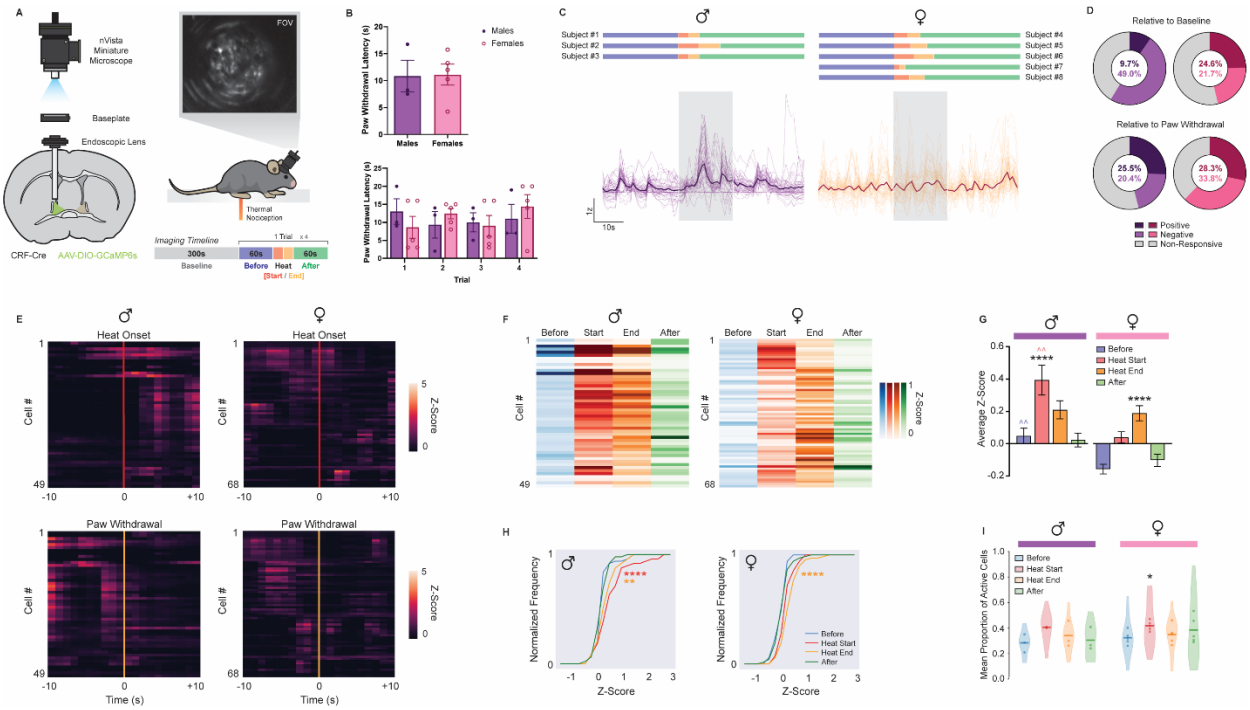

Figure 16. Measurement of Single-Cell Ca^{2+} Activity in BNSTCRF+ Neurons During Pain

(A) Miniature microscope imaging in $BNST^{CRF+}$ neurons. (Left) Diagram of GCaMP6s infusion and endoscopic lens / baseplate implantation in the BNST of CRF-Cre mice. (Upper-Right) Imaging field-of-view (FOV) depicting GCaMP6s expression in $BNST^{CRF+}$ neurons. (Lower-Right) Experimental timeline with schematic of $BNST^{CRF+}$ imaging during thermal nociceptive sensitivity testing. Epochs relative to heat exposure are color-coded: Before (blue), Heat Start (red), Heat End (orange), and After (green).

(B) (Left) Thermal nociceptive sensitivity of male (n = 3) and female (n = 5) CRF-Cre mice, as measured by the average paw withdrawal latency (PWL) following four trials of the Hargreaves test (unpaired t-test: $t(6) = 0.07921$, $p = 0.9394$). (Right) Average PWL of male and female subjects by trial (Two-way mixed-model ANOVA with Sidak's post hoc: no Trial x Sex interaction [$F(3, 18) = 1.159$, $p = 0.3526$] or main effect of Trial [$F(2.498, 14.99) = 0.5867$, $p = 0.6041$]; main effect of Sex [$F(1, 6) = 0.0062$, $p < 0.0001$]).

(C) Relative fluorescence change (ΔF/F) of $BNST^{CRF+}$ activity during noxious heat exposure. Z-scores of male (n = 49 cells / 7-30 cells per mouse) and female (n = 68 cells / 8-19 cells per mouse) subjects are represented over time by aligning traces that were averaged across trials to the onset of the heat stimulus. Individual cell activity is indicated in lighter colors, with total average cell activity in darker colors, and maximum heat duration (20 seconds) in the transparent blue block. Scale bars, x = time (10 sec), y = z-score based on relative ΔF/F (1 z-score). Imaging timelines for individual subjects are located above to represent epochs via the color-coding system described in **(A)**.

(D) Total percentage of pain responsive cells for male (purple) and female (magenta) subjects across four trials. Cells

that respond to noxious heat exposure with positive (darker), negative (lighter), and no (gray) changes in z-score were determined relative to the *Before* and *After* epochs, as demarcated by the onset of heat exposure and paw withdrawal (Wilcoxon rank-sum, $p < 0.05$).

(E) Peri-event heatmap of average $BNST^{CRF+}$ activity surrounding heat onset (red line) and paw withdrawal (orange line).

(F) Average z-score of each neuron across epochs for male and female subjects.

(G) Average z-score by epoch for male and female subjects (Two-way mixed-model ANOVA with Tukey's post hoc: Sex x Epoch interaction [$F(3, 345) = 5.996$, $p = 0.0005$], main effect of Sex [$F(1, 115) = 13.20$, $p = 0.0004$] and Epoch [$F(2.153, 247.7) = 26.31$, $p < 0.0001$]). * denotes comparison of epochs for each sex, ^ denotes comparison of the same epoch between sexes.

(H) Cumulative distribution function comparing z-score frequency for each epoch in male and female subjects (KS test: By Sex – Before [$D(117) = 0.3466$, p value = 0.0014], Heat Start [$D(117) = 0.3850$, p value = 0.0002], Heat End [$D(117) = 0.1815$, p value = 0.2759], After [$D(117) = 0.2611$, p value = 0.0334]; Comparing each epoch within males results in significance with Heat Start and Heat End over Before and After [Before vs. Heat Start, $D(98) = 0.4694$, p value = 2.183e-05; Before vs. Heat End, $D(98) = 0.4286$, p value = 0.0001; Heat Start vs. After, $D(98) = 0.4082$, p value = 0.0003; Heat End vs. After, $D(98) = 0.3265$, p value = 0.0079], whereas comparing each epoch within females results in significance with Heat End over Before, Heat Start, and After [Before vs. Heat Start, $D(136) = 0.2647$, p value = 0.0135; Before vs. Heat End, $D(136) = 0.4558$, p value = 7.372e-07; Heat Start vs. Heat End, $D(136) = 0.2352$, p value = 0.0386; Heat Start vs. After, $D(136) = 0.2941$, p value = 0.0041; Heat End vs. After, $D(136) = 0.3970$, p value = 2.634e-05]).

(I) Neuronal coactivity as measured by mean proportion of active cells (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Epoch interaction [$F(3, 18) = 0.5414$, $p = 0.6601$] or main effect of Sex [$F(1, 6) = 0.9203$, $p = 0.3744$]; main effect of Epoch [$F(1.840, 11.04) = 4.163$, $p = 0.0475$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

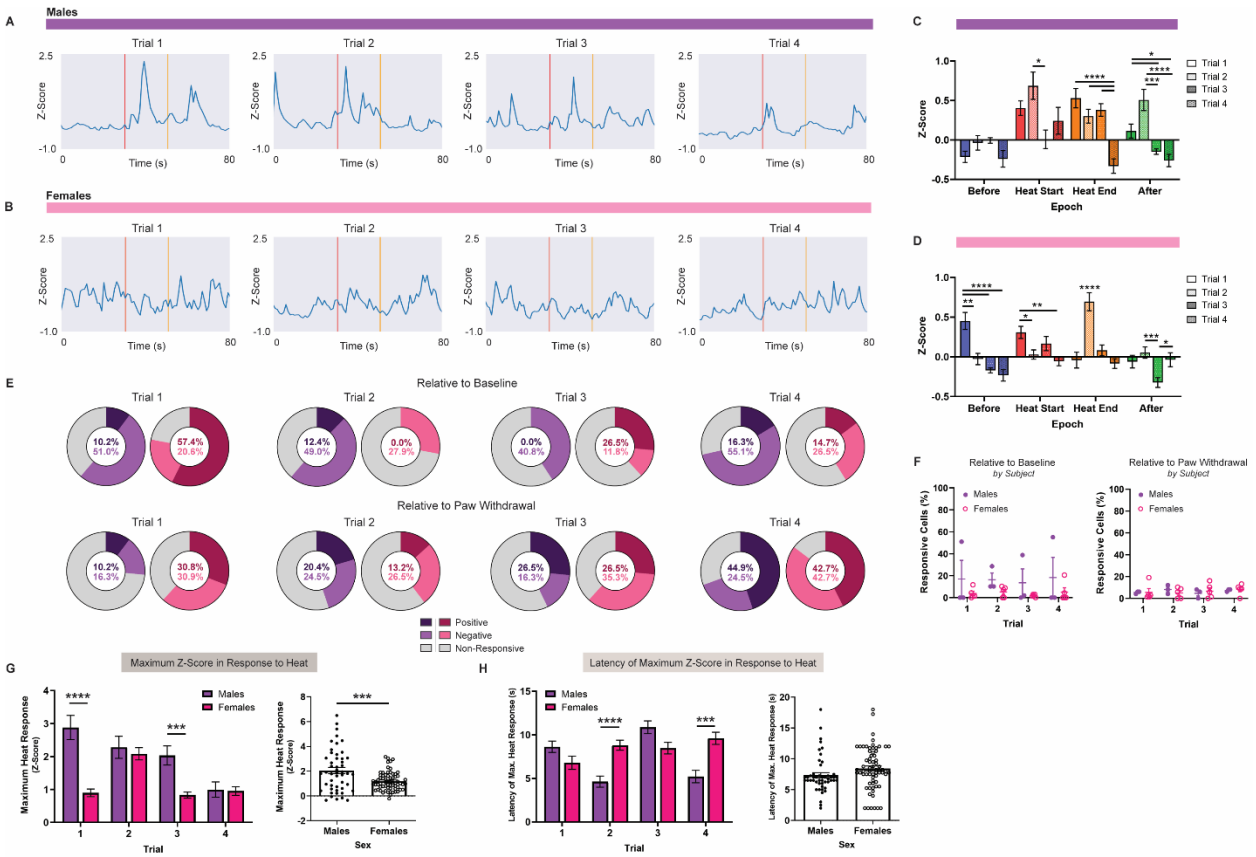

Figure 17. Progression of BNST^{CRF+} Activity Across Pain Exposure Trials

(A-B) Average BNST^{CRF+} traces of **(A)** male (n = 49 cells / 7-30 cells per mouse / 3 mice total) and **(B)** female (n = 68 cells / 8-19 cells per mouse / 5 mice total) subjects by trial. Within the representative time window, heat exposure starts at 30 seconds (first line [red]) with the maximum possible heat duration indicated at 50 seconds (second line [orange]).

(C-D) Average z-score by epoch and trials for **(C)** male and **(D)** female subjects (Males = Two-way repeated measures [RM] ANOVA with Sidak's post hoc: Epoch x Trial interaction [$F(9, 576) = 7.373$, $p < 0.0001$], main effect of Epoch [$F(2.477, 475.5) = 21.42$, $p < 0.0001$] and Trial [$F(3, 192) = 8.695$, $p < 0.0001$]; Females = Two-way RM ANOVA with Sidak's post hoc: Epoch x Trial interaction [$F(9, 804) = 17.04$, $p < 0.0001$], main effect of Epoch [$F(2.203, 590.4) = 13.95$, $p < 0.0001$] and Trial [$F(3, 268) = 6.913$, $p = 0.0002$]).

(E) Percentage of pain responsive cells for male (purple) and female (magenta) subjects were determined relative to epochs surrounding the onset of heat exposure and paw withdrawal across trials 1-4. Positive, negative, and non-responsive cells are indicated by darker purple/magenta, lighter purple/magenta, and gray respectively (Wilcoxon rank-sum, $p < 0.05$).

(F) Proportion of pain responsive cells in individual subjects by trial and sex relative to the epochs surrounding (Left)

heat onset (Two-way mixed-model ANOVA with Sidak's post hoc: no Trial x Sex interaction [$F(3, 18) = 0.0466$, $p = 0.9862$] or main effect of Trial [$F(1.092, 6.553) = 0.4473$, $p = 0.5435$] and Sex [$F(1, 6) = 1.510$, $p = 0.2651$]) and (Right) paw withdrawal (Two-way mixed-model ANOVA with Sidak's post hoc: no Trial x Sex interaction [$F(3, 18) = 0.3670$, $p = 0.7777$] or main effect of Trial [$F(2.740, 16.44) = 0.3368$, $p = 0.7818$] and Sex [$F(1, 6) = 0.0008$, $p = 0.9776$]).

(G) (Left) Maximum z-score in response to heat by trial and sex (Two-way mixed-model ANOVA with Sidak's post hoc: Trial x Sex interaction [$F(3, 345) = 14.96$, $p < 0.0001$], main effect of Trial [$F(2.609, 300) = 20.28$, $p < 0.0001$] and Sex [$F(1, 115) = 13.69$, $p = 0.0003$]). (Right) Average maximum z-score in response to heat by trial and sex (unpaired t-test: $t(115) = 3.70$, $p = 0.0003$).

(H) (Left) Latency of maximum z-score in response to heat by trial and sex (Two-way mixed-model ANOVA with Sidak's post hoc: Trial x Sex interaction [$F(3, 345) = 16.90$, $p < 0.0001$], main effect of Trial [$F(2.717, 312.5) = 7.890$, $p < 0.0001$], no main effect of Sex [$F(1, 115) = 3.20$, $p = 0.0763$]). (Right) Average latency of maximum z-score in response to heat stimulus by trial and sex (unpaired t-test: $t(115) = 1.789$, $p = 0.0763$).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

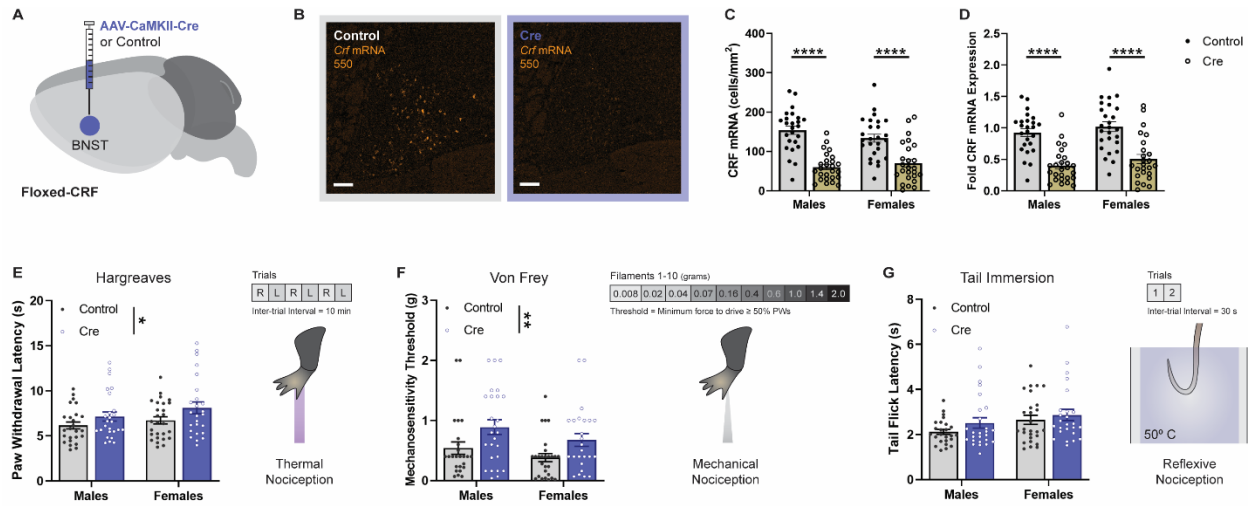

Figure 18. CRF Deletion in BNST Reduces Nociceptive Sensitivity

(A) Diagram of Cre-dependent approach for CRF deletion in BNST.

(B) Representative histology of CRF mRNA expression in BNST of Floxed-CRF mice following virus infusion.

(C-D) Quantification of CRF mRNA expression in BNST of male (n = 25-26) and female (n = 25-26) subjects by (C) cells/mm^2 (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 98) = 2.548, p =0.1136] or main effect of Sex [F(1, 98) = 0.2667, p = 0.6067]; main effect of Virus [F(1, 98) = 66.74, p < 0.0001]) and (D) Fold change compared to control (Two-way ANOVA with Tukey's post hoc: Two-way ANOVA: no Sex x Virus interaction [F(1, 98) = 0.007260, p =0.9323] or main effect of Sex [F(1, 98) = 2.239, p = 0.1378]; main effect of Virus [F(1, 98) = 60.23, p < 0.0001]).

(E-G) Pain sensitivity of male (n = 25-26) and female (n = 25-27) subjects with schematic of behavioral test for **(E)** thermal (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 99) = 0.1585, p =0.6914] or main effect of Sex [F(1, 99) = 2.385, p = 0.1257]; main effect of Virus [F(1, 99) = 5.677, p = 0.0191]), **(F)** mechanical (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 99) = 0.07088, p =0.7906] or main effect of Sex [F(1, 99) = 3.297, p = 0.0724]; main effect of Virus [F(1, 99) = 10.05, p = 0.0020]), and **(G)** reflexive (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 99) = 0.1933, p =0.6611] or main effect of Virus [F(1, 99) = 2.224, p = 0.1391]; main effect of Sex [F(1, 99) = 4.631, p = 0.0338]) nociception.

Data are shown as mean ±SEM. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.

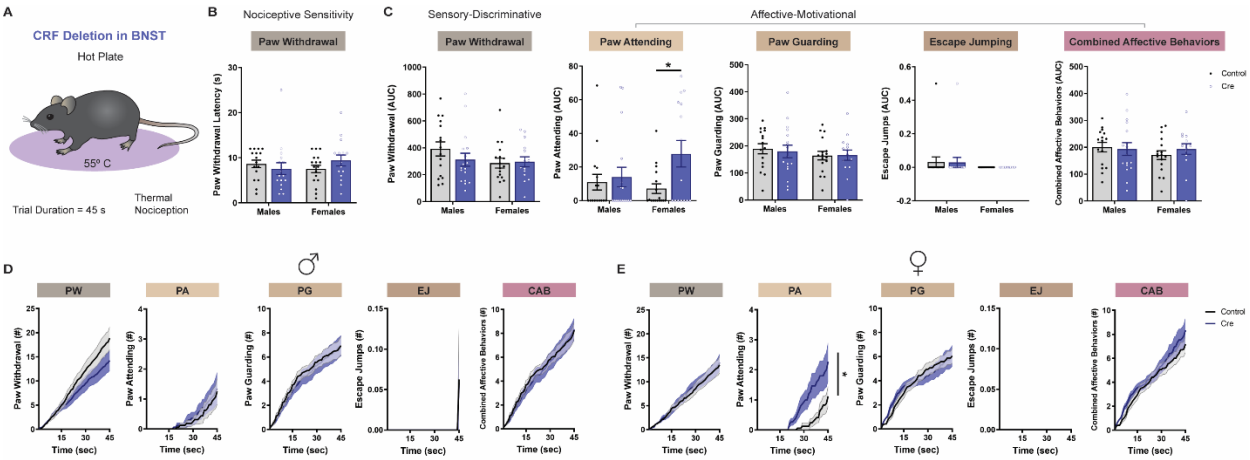

Figure 19. CRF Deletion in BNST Differentially Alters Sensory-Discriminative and Affective-Motivational Behaviors

(A) Schematic of hot plate test.

(B) Thermal nociceptive sensitivity of male (n = 16-17) and female (n = 15-17) Floxed-CRF mice, as defined by latency to first paw withdrawal for each mouse (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 1.853, p =0.1785] or main effect of Sex [F(1, 61) = 0.1124, p = 0.7386] and Virus [F(1, 61) = 0.1524, p = 0.6976]).

(C) Quantification of sensory-discriminative and affective-motivational behaviors throughout the 45-second exposure to hot plate. Averaged AUC of paw withdrawal (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 1.072, p =0.3047] or main effect of Sex [F(1, 61) = 1.885, p = 0.1747] and Virus [F(1, 61) = 0.6208, p = 0.4338]), paw attending (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 2.605, p =0.1117] or main effect of Sex [F(1, 61) = 0.8298, p = 0.3659]; main effect of Virus [F(1, 61) = 4.695, p = 0.0342]), paw guarding (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 0.09333, p =0.7610] or main effect of Sex [F(1, 61) = 1.026, p = 0.3152] and Virus [F(1, 61) = 0.04448, p = 0.8337]), escape jumping (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 0.001778, p =0.9665] or main effect of Sex [F(1, 61) = 1.936, p = 0.1691] and Virus [F(1, 61) = 0.001778, p = 0.9665]), and combined affective behaviors (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [F(1, 61) = 0.5696, p =0.4533] or main effect of Sex [F(1, 61) = 0.5631, p = 0.4559] and Virus [F(1, 61) = 0.1580, p = 0.6924]) are shown, with corresponding cumulative distribution functions displayed in **(D-E)**.

(D-E) Sensory-discriminative and affective-motivational behaviors are exhibited across time with cumulative distribution functions for **(D)** male and **(E)** female subjects (paw withdrawal: CON (M): 391.9 [341.0-442.9], CRE (M): 311.7 [263.2-360.2], CON (F): 285.9 [249.1-322.7], CRE (F): 296.8 [261.1-332.5]; paw attending: CON (M): 11.00 [4.672-17.33], CRE (M): 14.03 [6.087-21.97], CON (F): 7.147 [2.442-11.85], CRE (F): 27.87 [17.95-37.78]; paw

guarding: CON (M): 189.3 [171.7-207.0], CRE (M): 179.3 [156.7-201.9], CON (F): 163.8 [147.6-180.0], CRE (F): 165.6 [147.8-183.4]; escape jumping: CON (M): 0.03125 [0-0.2762], CRE (M): 0.2941 [0-0.2671], CON (F): 0 [0-0], CRE (F): 0 [0-0]; combined affective behaviors: CON (M): 200.4 [183.4-217.3], CRE (M): 193.4 [170.1-216.7], CON (F): 170.9 [154.3-187.5], CRE (F): 193.5 [173.8-213.1]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

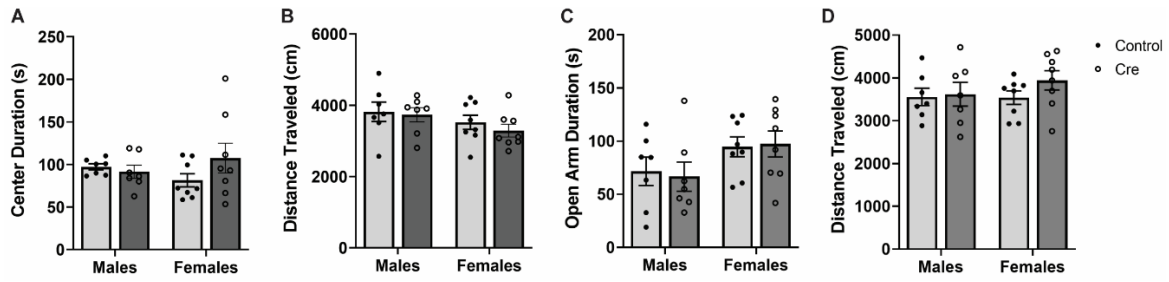

Figure 20. CRF Deletion in BNST Does Not Alter Avoidance Behaviors

(A) Avoidance behaviors in male (n = 7) and female (n = 8) Floxed-CRF mice, as measured by duration in the center (seconds) of the open field (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F(1, 26) = 2.098$, $p = 0.1594$], no main effect of Sex [$F(1, 26) = 0.0001$, $p = 0.9911$] and Virus [$F(1, 26) = 0.8511$, $p = 0.3647$]).

(B) Locomotor behaviors in male (n = 7) and female (n = 8) subjects, as measured by distance traveled (cm) in the open field (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F(1, 26) = 0.1251$, $p = 0.7265$], no main effect of Sex [$F(1, 26) = 3.015$, $p = 0.0943$] and Virus [$F(1, 26) = 0.5665$, $p = 0.4584$]).

(C) Avoidance behaviors in male (n = 7) and female (n = 8) subjects, as measured by duration in the open arms (seconds) of the elevated plus maze (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F(1, 26) = 0.0995$, $p = 0.7549$], main effect of Sex [$F(1, 26) = 4.908$, $p = 0.0357$], no main effect of Virus [$F(1, 26) = 0.0083$, $p = 0.9280$]).

(D) Locomotor behaviors in male (n = 7) and female (n = 8) subjects, as measured by distance traveled (cm) in the elevated plus maze (Two-way ANOVA with Tukey's post hoc: no Sex x Virus interaction [$F(1, 26) = 0.6221$, $p = 0.4374$], no main effect of Sex [$F(1, 26) = 0.4944$, $p = 0.4882$] and Virus [$F(1, 26) = 1.161$, $p = 0.2911$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

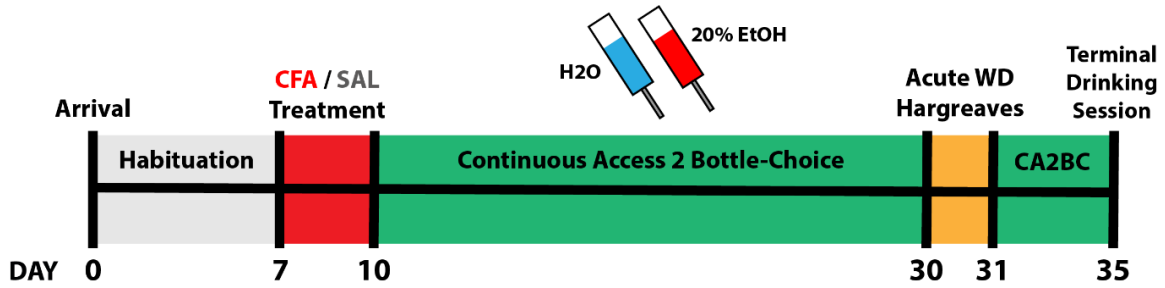

Figure 21. Experimental Timeline of CFA/CA2BC.

Days are marked relative to arrival of subjects. Following a week-long habituation, adult male (n = 8) and female (n = 8) C57BL6/J mice were treated with a 50 µL paw injection of saline or CFA. Subjects were tested in the CA2BC paradigm for three weeks and then assessed for thermal nociceptive sensitivity during acute withdrawal. Following a 2-hr terminal drinking session on Day 35, brain tissue and blood samples were collected.

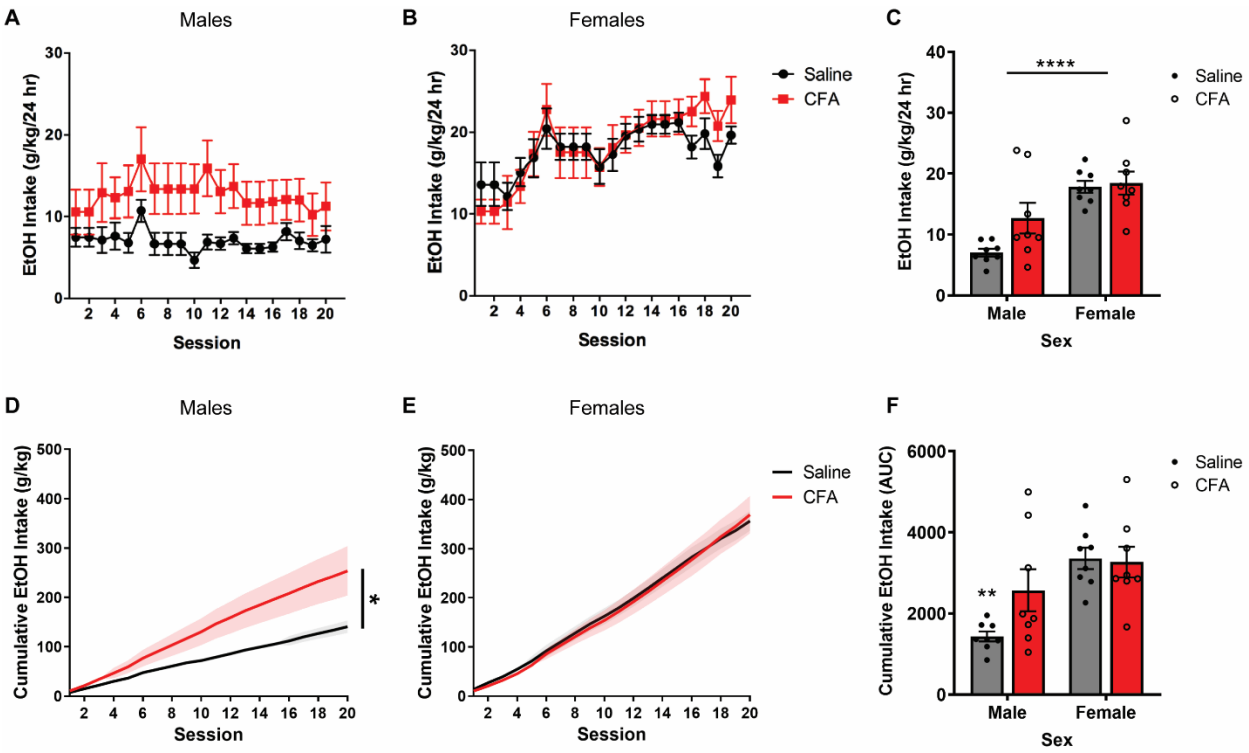

Figure 22. Daily Alcohol Intake (g/kg/24 hr) of Saline- or CFA-Treated C57BL/6J Mice Over a 3-Week Continuous Access Regimen.

(A-B) Pain-related consumption of 20% EtOH (w/v) was assessed for (A) males (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [F(19, 266) = 0.7802, p = 0.7301], main effect of Session [F(19, 266) = 1.656, p = 0.0437] and Inflammatory Pain [F(1, 14) = 4.768, p = 0.0465]) and (B) females (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [F(19, 266) = 1.149, p = 0.3023], main effect of Session [F(19, 266) = 9.470, p < 0.0001], no main effect of Inflammatory Pain [F(1, 14) = 0.09215, p = 0.7659]) in 20 consecutive sessions.

(C) Averages of daily intake were compared between saline- and CFA-treated males and females (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [F(1, 28) = 2.229, p = 0.1466], main effect of Sex [F(1, 28) = 24.34, p < 0.0001], no main effect of Inflammatory Pain [F(1, 28) = 3.524, p = 0.0709]).

(D-E) Cumulative distribution functions of ethanol intake for (D) male and (E) female subjects (SAL (M): 1432 [1301-1564], CFA (M): 2571 [2045-3098]; SAL (F): 3357 [3094-3620], CON-CFA (F): 3265 [2863-3667]).

(F) Averaged AUC of cumulative ethanol intake for male (n = 8-9) and female (n = 8) Floxed-CRF mice (Two-way ANOVA: no Sex x Inflammatory Pain interaction [F(1, 28) = 3.054, p =0.0915], main effect of Sex [F(1, 28) = 13.82, p = 0.0009], no main effect of Inflammatory Pain [F(1, 28) = 2.209, p = 0.1484]), with corresponding cumulative distribution functions displayed in (D-E).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

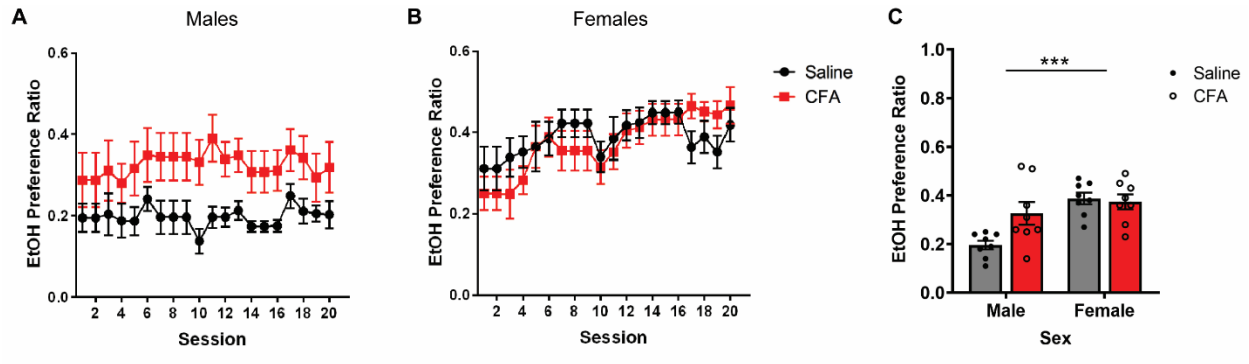

Figure 23. Daily Alcohol Preference Ratio of Saline- or CFA-Treated C57BL/6J Mice Over a 3-Week Continuous Access Regimen.

(A-B) Pain-related alcohol preference was assessed for (A) males (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 0.4569$, $p = 0.9765$] or main effect of Session [$F(19, 266) = 0.9432$, $p = 0.5298$], main effect of Inflammatory Pain [$F(1, 14) = 6.777$, $p = 0.0208$]) and (B) females (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 1.374$, $p = 0.1392$], main effect of Session [$F(19, 266) = 5.297$, $p < 0.0001$], no main effect of Inflammatory Pain [$F(1, 14) = 0.1650$, $p = 0.6907$]) in 20 consecutive sessions.

(C) Averages of daily intake were compared between saline- and CFA-treated males and females (Two-way mixed-model ANOVA with Sidak's post hoc: Sex x Inflammatory Pain interaction [$F(1, 28) = 5.140$, $p = 0.0313$], main effect of Sex [$F(1, 28) = 14.18$, $p = 0.0008$], no main effect of Inflammatory Pain [$F(1, 28) = 3.362$, $p = 0.0774$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

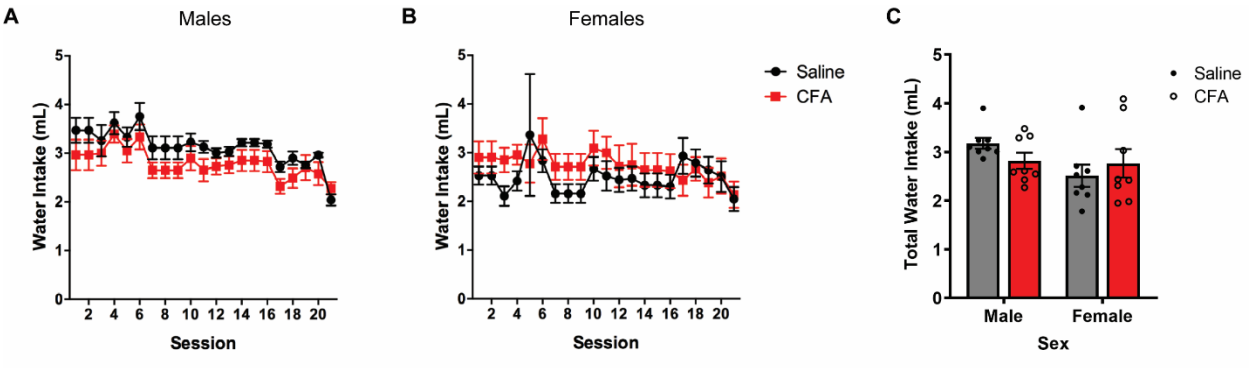

Figure 24. Daily Water Intake (mL) of Saline- or CFA-Treated C57BL/6J Mice Over a 3-Week Continuous Access Regimen.

(A-B) Pain-related consumption of water was assessed for (A) males (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 0.2718$, $p = 0.9992$] or main effect of Session [$F(19, 266) = 5.661$, $p < 0.0001$] and Inflammatory Pain [$F(1, 14) = 3.264$, $p = 0.0923$]) and (B) females (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 1.129$, $p = 0.3211$] or main effect of Session [$F(19, 266) = 1.320$, $p = 0.1698$] and Inflammatory Pain [$F(1, 14) = 0.4658$, $p = 0.5060$]) in 20 consecutive sessions.

(C) Averages of daily intake were compared between saline- and CFA-treated males and females (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 2.117$, $p = 0.1568$] or main effect of Sex [$F(1, 28) = 2.903$, $p = 0.0995$] and Inflammatory Pain [$F(1, 28) = 0.06344$, $p = 0.8030$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

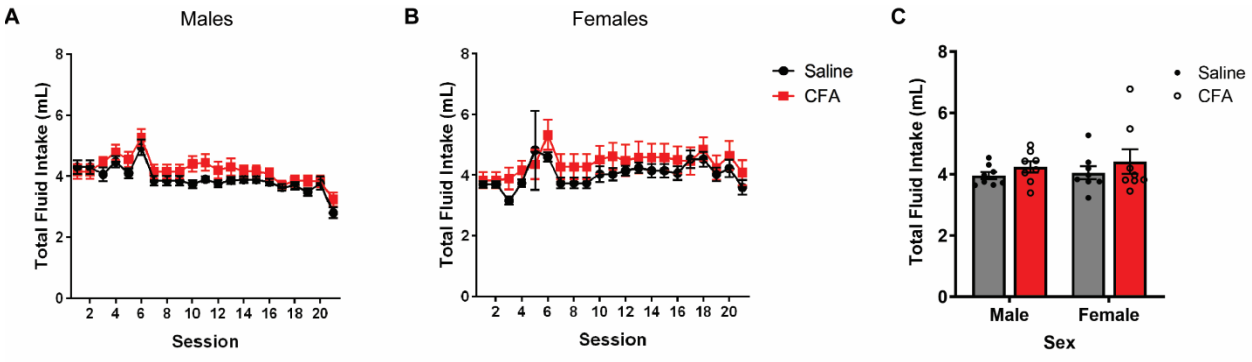

Figure 25. Daily Alcohol and Water Intake (mL) of Saline- or CFA-Treated C57BL/6J Mice Over a 3-Week Continuous Access Regimen.

(A-B) Pain-related consumption of fluids was assessed for (A) males (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 1.295$, $p = 0.1862$], main effect of Session [$F(19, 266) = 13.35$, $p < 0.0001$], no main effect of Inflammatory Pain [$F(1, 14) = 1.727$, $p = 0.2100$]) and (B) females (n = 8) (Two-way mixed-model ANOVA with Sidak's post hoc: no Session x Inflammatory Pain interaction [$F(19, 266) = 0.7379$, $p = 0.7782$], main effect of Session [$F(19, 266) = 4.514$, $p < 0.0001$], no main effect of Inflammatory Pain [$F(1, 14) = 0.6436$, $p = 0.4358$]) in 20 consecutive sessions.

(C) Averages of daily intake were compared between saline- and CFA-treated males and females (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 0.02455$, $p = 0.8766$] or main effect of Sex [$F(1, 28) = 0.2754$, $p = 0.6039$] and Inflammatory Pain [$F(1, 28) = 1.653$, $p = 0.2091$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

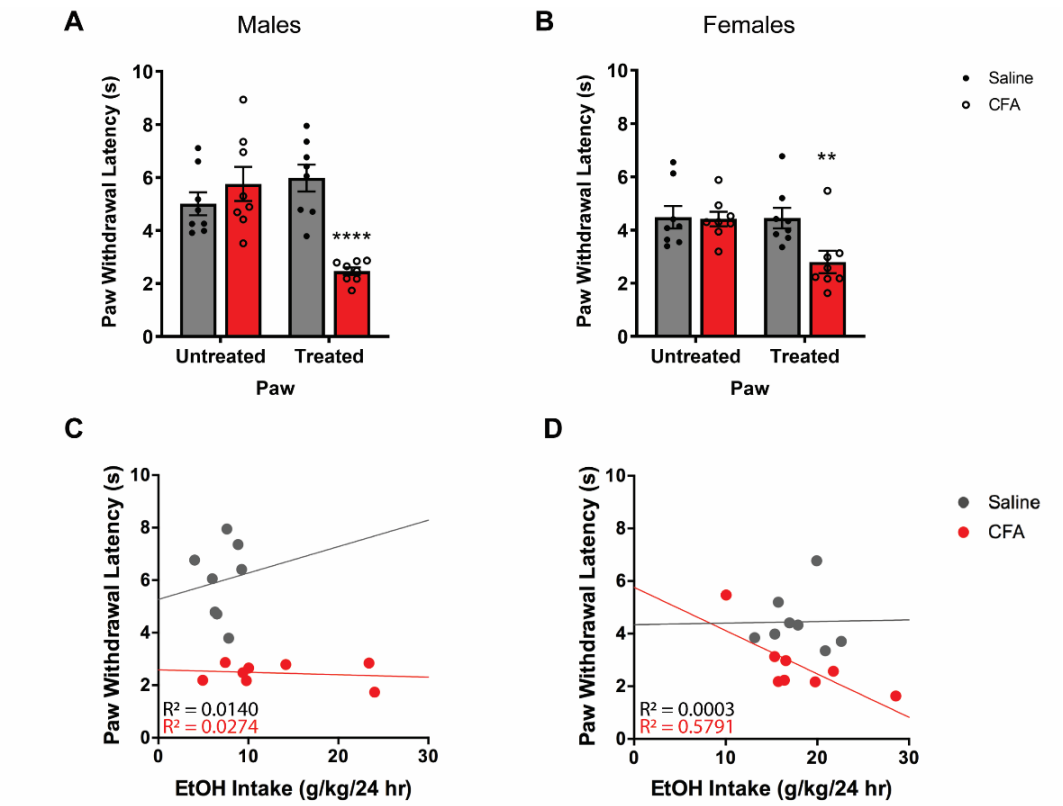

Figure 26. Pain Sensitivity of Saline- and CFA-Treated C57BL6/J Mice During Acute Withdrawal.

(A-B) Thermal nociceptive sensitivity of saline- and CFA-treated C57BL/6J mice was tested 24 hrs after last alcohol exposure. Using the Hargreaves test, paw withdrawal latency (s) was measured in response to a thermal nociceptive stimulus for substance-treated and untreated paws of (A) male (Two-way mixed-model ANOVA with Sidak's post hoc: Paw x Inflammatory Pain interaction [$F(1, 14) = 27.46$, $p = 0.0001$], main effect of Paw [$F(1, 14) = 8.155$, $p = 0.0127$] and Inflammatory Pain [$F(1, 14) = 7.010$, $p = 0.0191$]) and (B) female (Two-way mixed-model ANOVA with Sidak's post hoc: Paw x Inflammatory Pain interaction [$F(1, 14) = 10.15$, $p = 0.0066$], main effect of Paw [$F(1, 14) = 11.00$, $p = 0.0051$], no main effect of Inflammatory Pain [$F(1, 14) = 3.276$, $p = 0.0918$]) mice.

(C-D) Pain sensitivity correlations with average daily alcohol intake in (C) males and (D) females.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

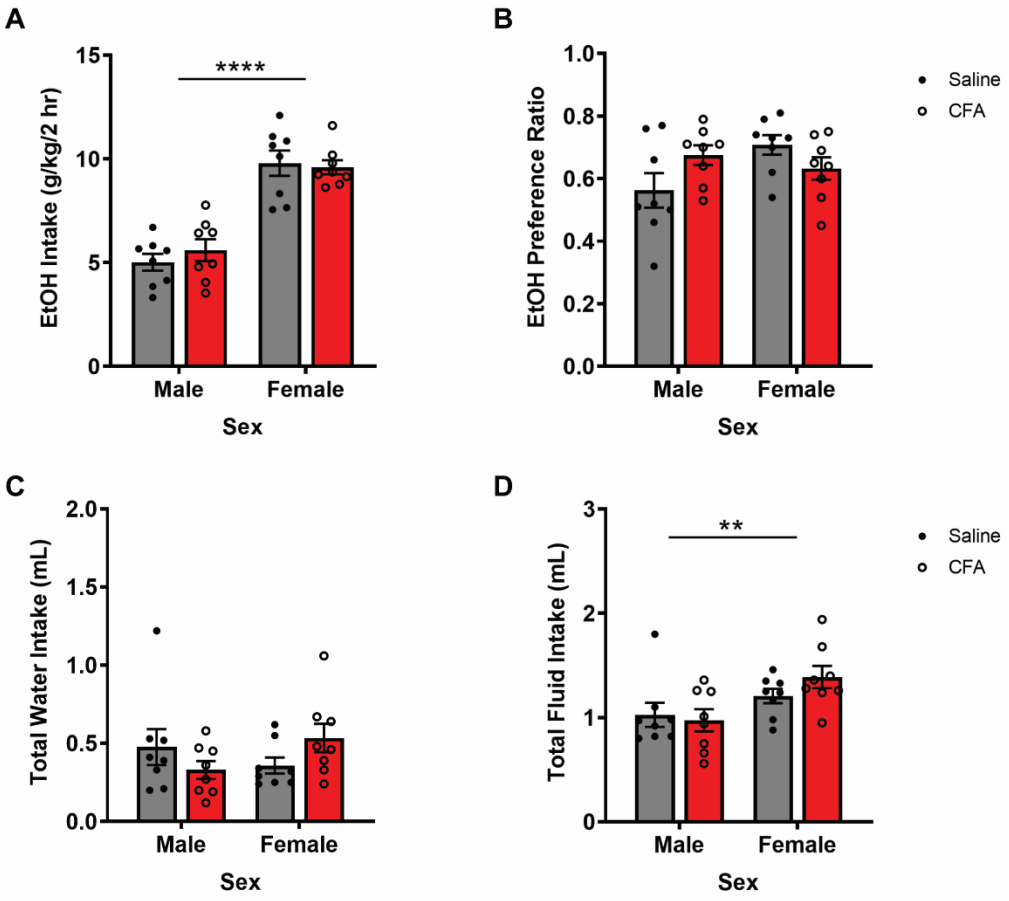

Figure 27. Average Alcohol and Water consumption of Saline- or CFA-Treated C57BL/6J Mice over a 2-hr Terminal Drinking Session.

(A) Average alcohol intake (g/kg/2 hr) in saline- or CFA-treated male (n = 8) and female (n = 8) C57BL/6J mice during a final 2-hr drinking session (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 0.6611$, $p = 0.4230$], main effect of Sex [$F(1, 28) = 83.19$, $p < 0.0001$], no main effect of Inflammatory Pain [$F(1, 28) = 0.1574$, $p = 0.6945$]).

(B) Average preference ratio in saline- or CFA-treated males (n = 8) and females (n = 8) during a final 2-hr drinking session (Two-way mixed-model ANOVA with Sidak's post hoc: Sex x Inflammatory Pain interaction [$F(1, 28) = 5.599$, $p = 0.0251$], no main effect of Sex [$F(1, 28) = 1.673$, $p = 0.2064$] and Inflammatory Pain [$F(1, 28) = 0.2239$, $p = 0.6397$]).

(C) Average water intake (g/kg/2 hr) in saline- or CFA-treated males (n = 8) and females (n = 8) during a final 2-hr drinking session (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 3.800$, $p = 0.0613$] or main effect of Sex [$F(1, 28) = 0.2640$, $p = 0.6114$] and Inflammatory Pain [$F(1, 28) = 0.03288$, $p = 0.8574$]).

(D) Average total fluid intake (mL) in saline- or CFA-treated males (n = 8) and females (n = 8) during a final 2-hr drinking session (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 1.341$, $p = 0.2566$], main effect of Sex [$F(1, 28) = 8.726$, $p = 0.0063$], no main effect of Inflammatory Pain [$F(1, 28) = 0.4069$, $p = 0.5288$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

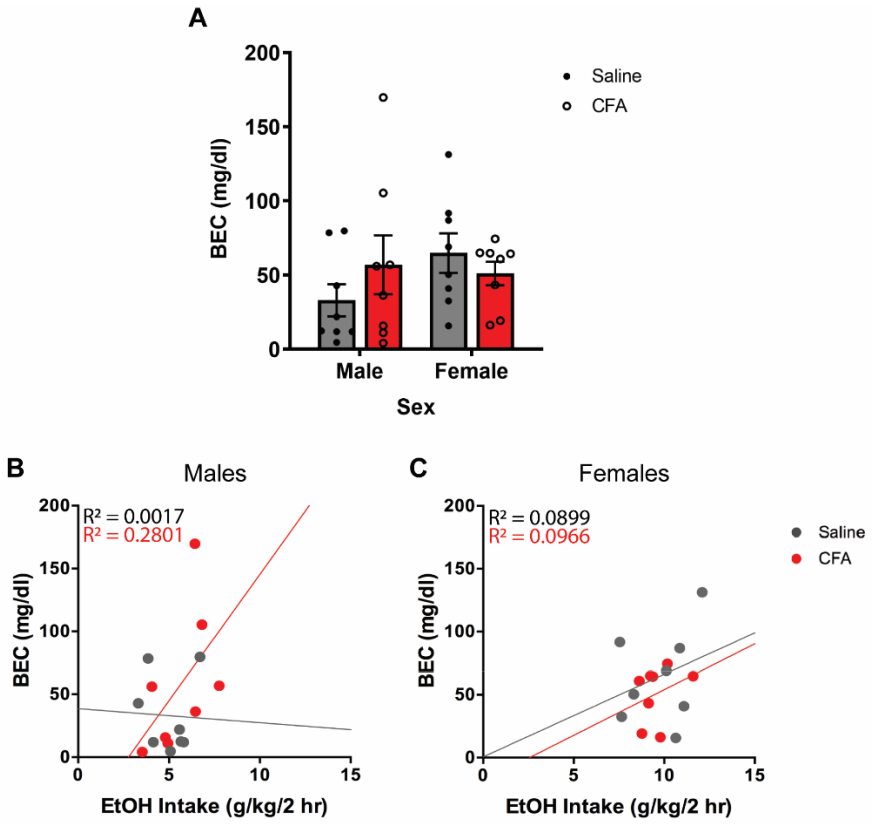

Figure 28. Blood Ethanol Content of Saline- or CFA-Treated C57BL/6J Mice following a 2-hr Terminal Drinking Session.

(A) Blood ethanol content (mg/dl) of saline- and CFA-treated C57BL/6J mice was measured 2-hrs into the final alcohol exposure day in male and female mice (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 1.890$, $p = 0.1801$] or main effect of Sex [$F(1, 28) = 0.8967$, $p = 0.3518$] and Inflammatory Pain [$F(1, 28) = 0.1365$, $p = 0.7146$]).

(B-C) BEC was correlated with average daily alcohol intake in (B) males and (C) females.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

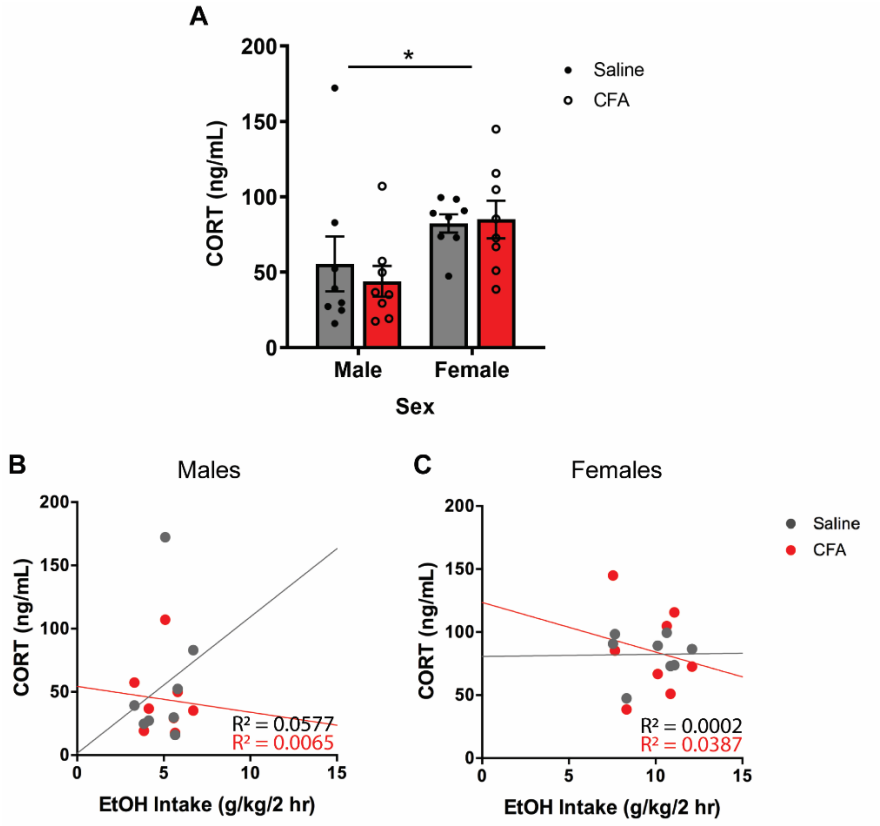

Figure 29. Plasma Corticosterone of Saline- or CFA-Treated C57BL/6J Mice following a 2-hr Terminal Drinking Session.

(A) Plasma corticosterone (CORT [ng/mL]) of saline- and CFA-treated C57BL/6J mice was measured 2-hrs into the final alcohol exposure day in male and female mice (Two-way mixed-model ANOVA with Sidak's post hoc: no Sex x Inflammatory Pain interaction [$F(1, 28) = 0.3202$, $p = 0.5760$], main effect of Sex [$F(1, 28) = 7.298$, $p = 0.0116$], no main effect of Inflammatory Pain [$F(1, 28) = 0.1243$, $p = 0.7270$]).

(B-C) CORT levels were correlated with average daily alcohol intake in (B) males and (C) females.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

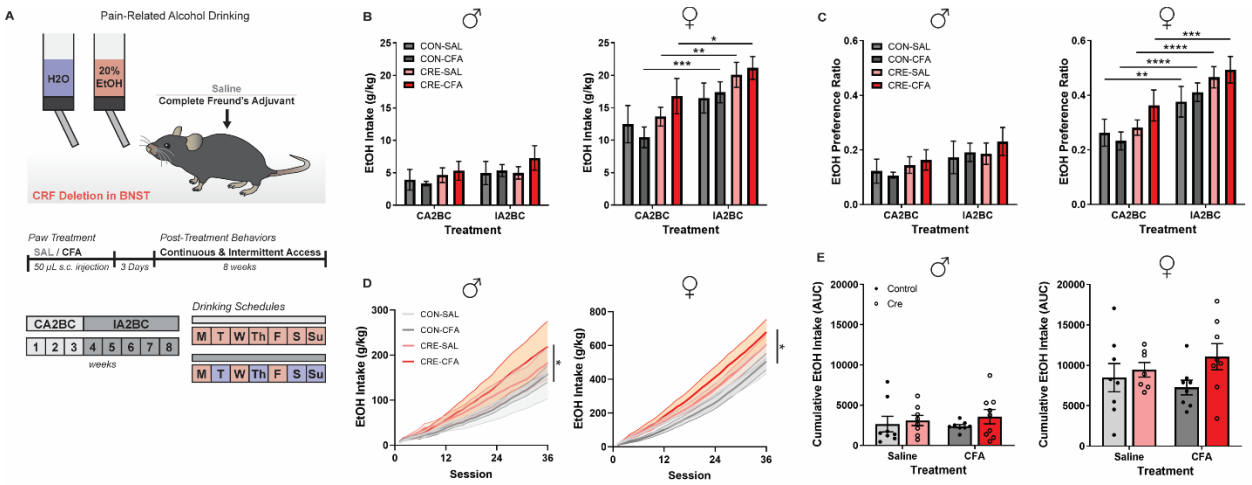

Figure 30. CRF Deletion in BNST Alters Pain-Alcohol Interactions

(A) Schematic of pain-related alcohol drinking with experimental timeline.

(B) Comparisons of average daily ethanol intake for (Left) male (n = 8-9; Two-way mixed-model ANOVA: no Drinking Protocol x Virus/Inflammatory Pain interaction [F(3, 29) = 0.6600, p =0.5833], main effect of Drinking Protocol [F(1, 29) = 7.266, p = 0.0116], no main effect of Virus/Inflammatory Pain [F(3, 29) = 0.5242, p = 0.6691]) and (Right) female (n = 7-8; Two-way mixed-model ANOVA: no Drinking Protocol x Virus/Inflammatory Pain interaction [F(3, 27) = 0.8899, p =0.4589], main effect of Drinking Protocol [F(1, 27) = 48.36, p < 0.0001], no main effect of Virus/Inflammatory Pain [F(3, 27) = 1.418, p = 0.2593]) subjects during CA2BC and IA2BC.

(C) Average ethanol preference for (Left) male (n = 8-9; Two-way mixed-model ANOVA: no Drinking Protocol x Virus/Inflammatory Pain interaction [F(3, 29) = 0.3407, p =0.7961], main effect of Drinking Protocol [F(1, 29) = 14.34, p = 0.0007], no main effect of Virus/Inflammatory Pain [F(3, 29) = 0.3888, p = 0.7619]) and (Right) female (n = 7-8; Two-way mixed-model ANOVA: no Drinking Protocol x Virus/Inflammatory Pain interaction [F(3, 27) = 1.333, p =0.2842], main effect of Drinking Protocol [F(1, 27) = 101.5, p < 0.0001], no main effect of Virus/Inflammatory Pain [F(3, 27) = 1.518, p = 0.2324]) subjects during CA2BC and IA2BC.

(D) Cumulative distribution functions of ethanol intake for (Left) male and (Right) female subjects (CON-SAL (M): 2643 [1873-3413], CON-CFA (M): 2386 [2179-2593], CRE-SAL (M): 3090 [2609-3572], CRE-CFA (M): 3565 [2794-4335]; CON-SAL (F): 8476 [7167-9784], CON-CFA (F): 7257 [6547-7967], CRE-SAL (F): 9449 [8762-10136], CRE-CFA (F): 11065 [9866-12264]).

(E) Averaged AUC of cumulative ethanol intake for (Left) male (n = 8-9; Two-way ANOVA: no Inflammatory Pain x Virus interaction [F(1, 29) = 0.2343, p =0.6320] or main effect of Inflammatory Pain [F(1, 29) = 0.02055, p = 0.8870] and Virus [F(1, 29) = 1.164, p = 0.2895]) and (Right) female (n = 7-8; Two-way ANOVA: no Pain x Virus interaction

[$F(1, 27) = 1.055$, $p = 0.3134$] or main effect of Sex [$F(1, 27) = 0.02071$, $p = 0.8866$] and Virus [$F(1, 27) = 3.003$, $p = 0.0945$]) Floxed-CRF mice, with corresponding cumulative distribution functions displayed in (D).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

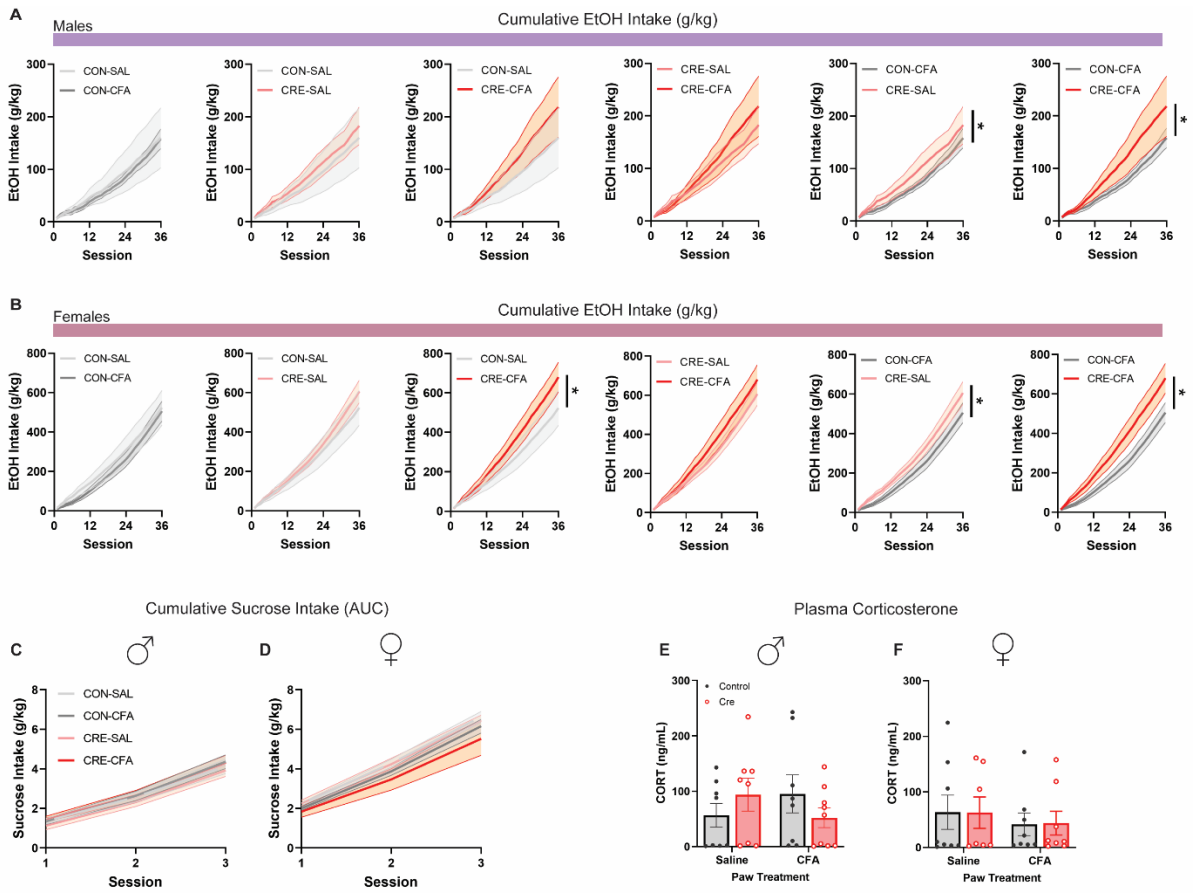

Figure 31. CRF Deletion in BNST Alters Pain-Related Alcohol Drinking Behaviors but not Sucrose Drinking Behaviors or Plasma Corticosterone Levels

(A) Cumulative distribution of ethanol intake for male subjects with individual group comparisons (CON-SAL (M): 2643 [1873-3413], CON-CFA (M): 2386 [2179-2593], CRE-SAL (M): 3090 [2609-3572], CRE-CFA (M): 3565 [2794-4335]).

(B) Cumulative distribution of ethanol intake for female subjects with individual group comparisons (CON-SAL (F): 8476 [7167-9784], CON-CFA (F): 7257 [6547-7967], CRE-SAL (F): 9449 [8762-10136], CRE-CFA (F): 11065 [9866-12264]).

(C-D) Cumulative distribution of sucrose intake for (C) male (CON-SAL (M): 5.312 [3.839-6.785], CON-CFA (M): 5.495 [4.089-6.901], CRE-SAL (M): 4.849 [3.491-6.206], CRE-CFA (M): 5.542 [3.936-7.149]) and (D) female subjects (CON-SAL (F): 8.707 [7.552-9.862], CON-CFA (F): 7.956 [6.691-9.220], CRE-SAL (F): 8.599 [7.248-9.950], CRE-CFA (F): 7.156 [3.912-10.40]).

(E-F) Average plasma corticosterone levels for (E) male (n = 8-9; Two-way ANOVA: no Inflammatory Pain x Virus interaction [F(1, 29) = 2.362, p = 0.1351] or main effect of Inflammatory Pain [F(1, 29) = 0.003521, p = 0.9531] and Virus [F(1, 29) = 0.01273, p = 0.9109]) and (F) female subjects (n = 7-8; Two-way ANOVA: no Inflammatory Pain x

Virus interaction [$F(1, 27) = 0.003371$, $p = 0.9541$] or main effect of Inflammatory Pain [$F(1, 27) = 0.6430$, $p = 0.4296$] and Virus [$F(1, 27) = 0.0007458$, $p = 0.9784$]).

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

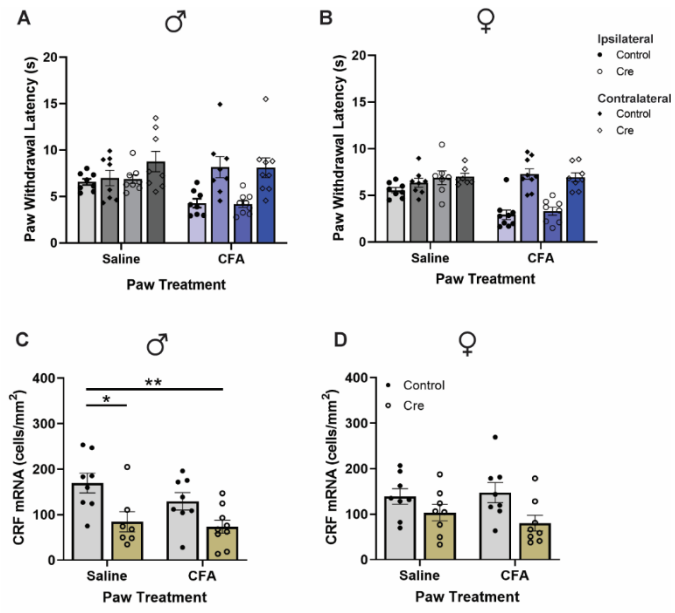

Figure 32. Effects of Persistent Inflammatory Pain on CRF Deletion in BNST

(A-B) Thermal nociceptive sensitivity of **(A)** male (n = 8-9) (Two-way ANOVA with Tukey's post hoc: no Inflammatory Pain x Virus/Paw interaction [$F(3, 58) = 2.428$, $p = 0.0745$] or main effect of Inflammatory Pain [$F(1, 58) = 4.000$, $p = 0.0502$]; main effect of Virus/Paw [$F(3, 58) = 7.240$, $p = 0.0003$]) and **(B)** female (n = 7-9) (Two-way ANOVA with Tukey's post hoc: Inflammatory Pain x Virus/Paw interaction [$F(3, 56) = 9.186$, $p < 0.0001$], main effect of Inflammatory Pain [$F(1, 58) = 14.57$, $p = 0.0003$] and Virus/Paw [$F(3, 58) = 14.82$, $p < 0.0001$]) Floxed-CRF mice. **(C-D)** Quantification of CRF mRNA expression in BNST of **(C)** male (n = 8-9) (Two-way ANOVA with Tukey's post hoc: no Inflammatory Pain x Virus interaction [$F(1, 29) = 1.882$, $p =0.1807$] or main effect of Inflammatory Pain [$F(1, 29) = 1.038$, $p = 0.3167$]; main effect of Virus [$F(1, 29) = 22.09$, $p < 0.0001$]) and **(D)** female (n = 7-8) (Two-way ANOVA with Tukey's post hoc: no Inflammatory Pain x Virus interaction [$F(1, 27) = 1.161$, $p =0.2907$] or main effect of Inflammatory Pain [$F(1, 27) = 0.4011$, $p = 0.5319$]; main effect of Virus [$F(1, 27) = 6.014$, $p = 0.0209$]) subjects by cells/mm^2.

Data are shown as mean ±SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

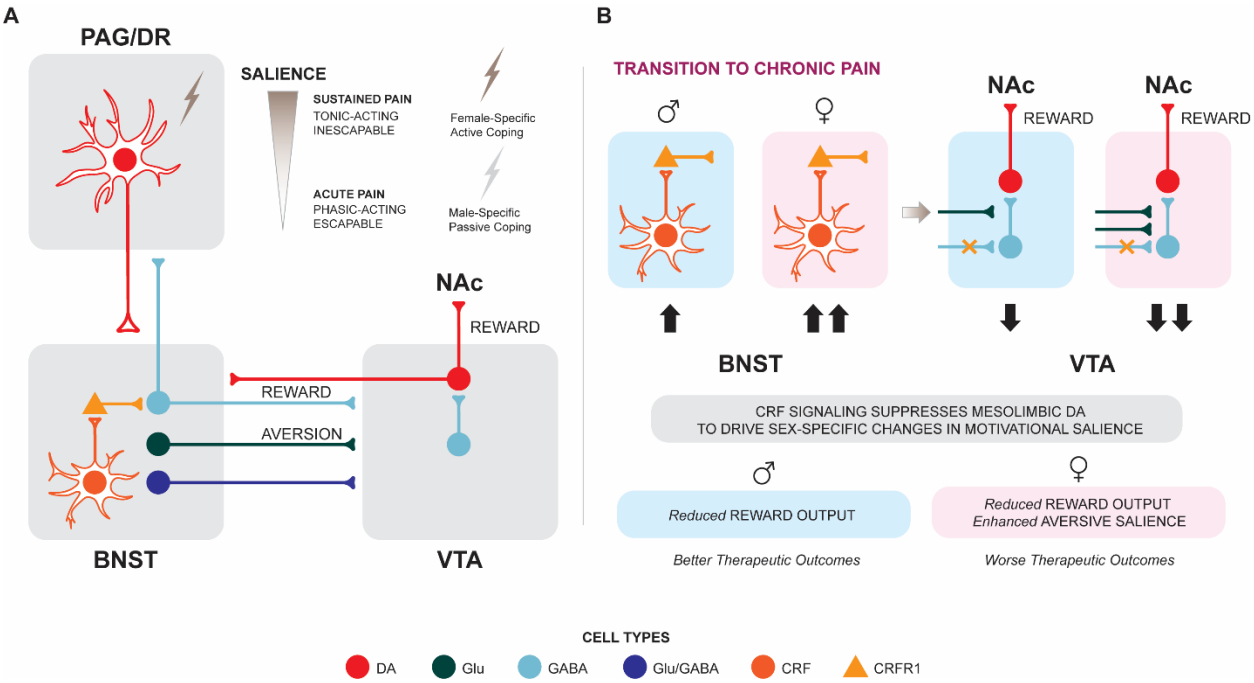

Figure 33. Proposed Model of PAG/DR^{DA+} and BNST^{CRF+} Circuitry for Sex-Specific Pain and Drug Use

(A) Functional connectivity map by cell type featuring PAG/DR^{DA+} and BNST^{CRF+} neurons and supporting circuits in the VTA and NAc. We hypothesize that PAG/DR^{DA+} neurons are recruited by contextual salience to express pain responses through the BNST-VTA-NAc circuit in a sex-specific manner. This DA signaling system may specifically recruit BNST^{CRF+} neurons via D1-like and D2-like receptors to represent the salient features of rewarding and aversive stimuli in relation to the environment. These DA and CRF circuit mechanisms are likely to be important for adaptive behaviors and associative learning, acting as a link between the valence of a cue and the environment to inform functional outcomes.

(B) Enhanced CRF signaling in the BNST is posited to support the transition from acute pain to chronic pain. Following the induction of a chronic pain state, local CRF and CRF_{R1} production are augmented in the BNST to suppress the mesolimbic DA system. This mechanism could explain observed sex differences in the processing of reward and aversion among chronic pain patients. Female-specific increases in BNST representations of aversive information are specifically thought to correspond to negative experiences with analgesic drug use and worse therapeutic outcomes in women.

Abbreviations: BNST: bed nucleus of the stria terminalis, DR: dorsal raphe, NAc: nucleus accumbens, PAG: periaqueductal gray; VTA: ventral tegmental area; DA: dopamine, Glu: glutamate, GABA: γ-aminobutyric acid, CRF: corticotropin releasing factor, CRFR1: corticotropin releasing factor receptor 1.

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