SEX DIFFERENCES IN ALCOHOL- AND OXYCODONE-SEEKING BEHAVIORS

by

HANNAH DREW FULENWIDER

(Under the Direction of Jesse Schank)

ABSTRACT

Alcohol and opioid use disorders (AUD, OUD) are prevalent public health issues in the United States, with millions of men and women currently diagnosed. Unfortunately, most preclinical studies of addictive-like behaviors have been conducted using only male subjects. Given the extensive epidemiological data suggesting a sex difference in the progression and manifestation of AUD and OUD, an increased number of studies assessing addictive-like behaviors in female subjects is greatly needed. Therefore, the goals of our current studies were to characterize sex differences in compulsive-like ethanol consumption using the aversion-resistant ethanol intake model, identify the neuroanatomical loci differentially activated in males and females during this behavior, and to establish a model of oral oxycodone self-administration while characterizing sex differences in oxycodone-seeking. We found that female mice exhibit aversion-resistant ethanol intake to a higher degree than males and that this effect does not differ throughout the progression of the estrous cycle. We also observed increased neuronal activation within the ventral tegmental area (VTA) and posterior insular cortex (PIC) of males, but not females, during consumption of quinine-adulterated ethanol, suggesting the potential involvement of these regions in sex differences in quinine-

ethanol intake. We also demonstrated that females self-administer significantly more oral oxycodone than males and that this behavior is unaffected by estrous cycle phase. Additionally, both males and females reinstate strongly to oxycodone-seeking following stress exposure, and this behavior is significantly attenuated following systemic treatment with a neurokinin-1 receptor antagonist. Collectively, these findings identified significant sex differences in compulsive-like ethanol intake and in oral oxycodone self-administration. Interestingly, these behaviors are unaffected by fluctuating levels of systemic sex hormones, suggesting that the presence of threshold levels of circulating estradiol or progesterone and/or developmental effects contribute to these sex differences.

INDEX WORDS: Ethanol, Opioids, Compulsive, Aversion-Resistance, Reinforcement, Reinstatement, Relapse, Neurokinin-1 Receptor, Salience, Reward, Aversion, Decision-Making, Motivational Conflict, Lateral Septum, Ventral Tegmental Area, Posterior Insular Cortex, c-Fos

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CHAPTER 1: INTRODUCTION

1.1 ALCOHOL AND OPIOID USE DISORDERS

Epidemiological Data

Alcohol use disorder (AUD) is highly prevalent in the United States, with approximately 14 million adults currently diagnosed [1]. Opioid use disorder (OUD) is also a severe public health problem, with over 2 million adults diagnosed in the US [2]. Opioids include licit and illicit formulations and consist of three general classes: natural, semi-synthetic, and synthetic. Natural opioids, such as morphine and heroin, are derivatives of opium extracted from the poppy plant, whereas semi-synthetic opioids, such as hydrocodone and oxycodone, are derivatives of opioid alkaloids, and synthetic opioids such as fentanyl are 100% synthetic in origin [3]. As outlined above, while both AUD and OUD are prominent substance use disorders, they can also be comorbidly expressed. For example, approximately 58% of those diagnosed with OUD also meet criteria for AUD, while approximately 2% of those diagnosed with AUD also meet OUD diagnostic criteria [4].

AUD has been shown to significantly increase the risk of heart and liver disease, impaired immune system functioning, pancreatitis, as well as several types of cancer [5]. In contrast, those with OUD are significantly more at risk for developing conditions such as palatal/nasal necrosis, hepatitis and human immunodeficiency virus (HIV), as well as other systemic infections due to the intranasal and intravenous (i.v.) routes of administration (ROAs) often utilized by abusers [6]. In addition to these complications,

approximately 88,000 alcohol-related deaths and 48,000 opioid overdose-induced deaths occur annually [2, 7], and the economic burden of AUD is approximately $249 billion [8, 9] and over $78 billion for OUD [10]. Collectively, these data demonstrate a need for increased preclinical work on opioid- and alcohol-seeking to develop an improved understanding of these disorders.

The presentation of AUD and OUD is complex and can vary significantly between persons diagnosed. With the terms “abuse” and “dependence” no longer in use by the American Psychiatric Association, AUD and OUD are now considered spectrum disorders. In other words, disorder severity is determined based on the number of symptoms presented for a given patient, so that an increased number of symptoms indicates increased severity [11, 12]. The DSM-5 includes 11 diagnostic criteria for each of these disorders, ranging from the inability to control intake to the development of physical tolerance (See Tables 1.1 and 1.2 for lists of all criteria). While specific symptoms vary among those with AUD/OUD, consistent features of these disorders include their identification as chronic, relapsing conditions in which an individual exhibits compulsive and excessive drug intake, even in the face of negative consequences [13, 14].

AUD and OUD Development

A major theory posits that the development of addiction is a dynamic and cyclical process that can be divided into three main phases: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation [13-18]. The binge and intoxication phase is driven largely by positive reinforcement, such that an individual is motivated to consume a drug because of its rewarding properties [13-17]. The withdrawal

and negative affect stage is attributable to negative reinforcement, or the desire to consume a drug in order to alleviate a pre-existing or withdrawal-induced aversive state that follows the cessation of drug use after the establishment of dependence [13-17]. Lastly, the preoccupation and anticipation stage encompasses craving and is driven by conditioned reinforcement, for example exposure to drug-associated stimuli [13-17]. As a person progresses through these stages, the reward circuitry is desensitized, while anti-reward and stress circuits are sensitized, resulting in a dependent phenotype [13-17]. Specific regions and circuits involved in these processes are discussed in more detail in section 1.6.

1.2 PHARMACOLOGY OF ALCOHOL

Acute Alcohol Exposure

Alcohol modulates the activity of various neurotransmitter systems within the central nervous system (CNS), including gamma-aminobutyric acid (GABA), glutamate, dopamine (DA), and serotonin (5-HT) [19, 20]. GABA and glutamate are the primary inhibitory and excitatory neurotransmitters within the CNS, respectively. Alcohol's activation of ionotropic $GABA_A$ receptors leads to an influx of chloride ions, hyperpolarizing the cell and resulting in neuronal inhibition [21]. Conversely, alcohol inhibits the activity of the NMDA receptor, preventing depolarization and neuronal excitation [3, 20]. This potentiation of inhibitory signaling and attenuation of excitatory signaling results in many symptoms associated with alcohol intoxication, including sedation, anxiolysis, and ataxia [22-24].

In addition to modulating global neuronal excitation and inhibition, alcohol targets the dopaminergic and opioid systems. Alcohol increases mesolimbic DA release through several mechanisms, all of which are believed to be indirect [3]. This circuit is comprised of DA projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), and virtually all drugs of abuse cause increased DA release within this circuit to exert their reinforcing and euphoric effects [25]. Specifically, alcohol binds to inhibitory $GABA_A$ and mu opioid receptors (MORs) present on GABAergic interneurons within the VTA to disinhibit DA release to the NAc [3, 25].

Alcohol also modulates the activity of the endogenous opioid system. For example, acute alcohol administration stimulates beta-endorphin release within the NAc and VTA [26], while causing increases in both beta-endorphin and dynorphin release in the CeA [27]. Beta-endorphin release is thought to contribute to alcohol's reinforcing properties while dynorphin release is likely involved in the processing of alcohol's aversive effects [27].

Lastly, alcohol has been demonstrated to affect signaling within the 5-HT system. Acute alcohol administration causes increased 5-HT release, specifically within the mesolimbic dopamine circuit [28]. Alcohol has also been shown to interact with certain 5-HT receptors directly to modulate dopaminergic signaling. For example, alcohol binds the excitatory ionotropic $5\text{-}HT_3$ receptor on dopaminergic cells within the VTA to potentiate mesolimbic dopamine release, which is likely an additional mechanism contributing to alcohol reinforcement [29, 30].

Current Pharmacotherapies for AUD

There are currently three medications approved by the Food and Drug Administration (FDA) for medication-assisted treatment of AUD: naltrexone, acamprosate, and disulfiram [31]. Naltrexone (ReVia, Vivitrol) is a mu opioid receptor (MOR) that can reduce excessive alcohol intake, craving, and risk of relapse [31-33]. However, this treatment has only been shown to be effective in a subset of patients with certain polymorphisms at the OPRM1 gene, which codes for the MOR [34, 35]. For example, in patients of Asian descent, naltrexone treatment reduces risk of relapse for those with one to two copies of the Asp40 allele significantly more than in those homozygous for the Asn40 allele of OPRM1 [36]. However, a separate study with subjects of European descent has demonstrated that naltrexone caused increases in alcohol craving and risk of relapse in subjects homozygous for the Asp40 allele [37], demonstrating that OPRM1 polymorphisms have differential responses to naltrexone treatment depending on patient population [35].

Acamprosate (Campral), or calcium-bis (*N*-acetylhomotaurinate), has been shown to decrease risk of relapse, though the mechanism responsible for this effect remains unknown [38]. For example, decades of work have suggested that acamprosate decreases risk of relapse through *N*-acetylhomotaurinate's modulation of glutamatergic signaling [39, 40]. However, a more recent study has demonstrated that the calcium, rather than the *N*-acetylhomotaurinate, component of this drug is what inhibits relapse-like behavior [41]. Additonally, this medication has only proved effective in a subset of patients, with certain patients even reporting increased craving and risk of relapse following acamprosate treatment [42-44].

In contrast to each of the previously discussed medications, disulfiram (Antabuse) acts outside of the central nervous system to affect alcohol metabolism [19]. Following consumption, alcohol is metabolized by alcohol dehydrogenase to produce acetaldehyde, which is then converted to acetic acid by acetaldehyde dehydrogenase [19]. Disulfiram inhibits acetaldehyde dehydrogenase activity, so that if alcohol is consumed while on this medication, acetaldehyde accumulates in the blood, causing aversive symptoms such as vomiting and tachycardia [19, 31, 33]. These effects are intended to serve as a deterrent to alcohol consumption; unfortunately, though, patient compliance is a primary obstacle to this medication's efficacy [19, 31, 39].

In addition to the issues outlined above, these therapies are grossly underutilized, in that only 3% of treatment-seeking AUD patients have been prescribed with one of these medications [45]. Therefore, the identification of additional therapeutic targets and the deployment of newly established medications remains essential in improving AUD treatment efficacy.

1.3 PHARMACOLOGY OF OPIOIDS: OXYCODONE

Endogenous Opioid System

There are three principle classes of endogenous opioid receptors: mu, delta, and kappa (MOR, DOR, and KOR, respectively). Each type is widely expressed throughout the peripheral and central nervous systems, with high density in regions involved in nociception and reward processing. Their interactions with the endogenous opioid peptides beta-endorphin, enkephalin, and dynorphin modulate many physiological and behavioral processes [46, 47]. MOR, DOR, and KOR are all coupled to inhibitory G

proteins so that their activation results in neuronal inhibition and/or prevention of neurotransmitter release [46, 48-50]. While agonism of each opioid receptor subtype results in analgesia, their effects on affect and reward processing differ significantly.

MORs preferentially bind beta-endorphin and are highly expressed in the VTA and NAc. Their activation leads to disinhibited mesolimbic DA release and the subjective experience of reward [47, 51, 52]. DORs preferentially bind enkephalin and are highly expressed within the prefrontal cortex, NAc, amygdala, and hippocampus. Activation of the DOR system has strong anxiolytic and antidepressant effects [47, 51]. In contrast to MORs and DORs, the KOR system is associated with anti-reward, dysphoria, and stress [53]. KORs preferentially bind dynorphin and are densely expressed in the bed nucleus of the stria terminalis (BNST), amygdala, hypothalamus, NAc and VTA [52]. KOR activation has been shown to attenuate mesolimbic DA release [53] and facilitate stress-induced drug-seeking [54-56].

In addition to the mu, delta, and opioid receptors, the nociceptin opioid peptide (NOP) receptor system is also considered to be a part the opioid receptor family. This receptor is also G_i-coupled, but in contrast to the MOR, DOR, and KOR, its activation generally promotes nociception. Interestingly, activation of the NOP receptor has also been shown to be anxiolytic [47, 57]. The NOP receptor has been demonstrated to have low affinity for endogenous and exogenous opioids [57], and its activation has been shown to decrease reward sensitivity and withdrawal severity for several classes of drug [46, 57].

Opioid Receptor Agonists: Oxycodone

Opioid agonists are a class of drug that mimic the activity of endogenous opioid peptides by targeting MORs, DORs, and KORs and include various licit and illicit formulations, such as heroin, fentanyl, morphine, and oxycodone [47]. Oxycodone is one of the most commonly prescribed and abused opioids in the United States [58, 59]. Oxycodone is a semi-synthetic opioid derived from thebaine, first synthesized in 1916 in Germany and made available in the United States in 1939 [60, 61] Oxycodone was then approved by the FDA in 1950 under the brand name Percodan, an immediate-release formulation in which oxycodone is combined with aspirin [62].

Oxycodone was then made available in several different formulations, including immediate and controlled release (OxyIR and OxyContin, respectively), as well as in combination with other nonsteroidal anti-inflammatory drugs such as acetaminophen (Percocet) [63, 64]. As the availability of oxycodone increased, drug use and diversion also increased, in that by 2011, oxycodone consumption in the United States was approximately 500% higher than in 1999 [65]. This trend began shortly after the introduction of a controlled-release formulation of oxycodone, OxyContin, developed by Purdue Pharma in 1995 [65]. While OxyContin was often useful if taken as prescribed, the drug still had relatively high abuse potential, and many non-medical users began to take advantage of OxyContin's higher drug load by crushing the tablets to be chewed, swallowed, snorted, or injected [58, 66]. These methods of misuse led to faster onset of action as well as increased intensity of oxycodone's reinforcing effects [58].

Oxycodone is a potent MOR agonist, with a weak affinity for DOR and KOR [48]. As each of these receptors are G_i-coupled, their activation results in neuronal

inhibition and decreased neurotransmitter release [46]. Oxycodone exerts its analgesic effects primarily through agonism of MORs within the ascending and descending pathways. More specifically, activation of MORs on substance P (SP)-expressing neurons in the dorsal horn of the spinal cord decreases afferent nociceptive input, while activation of MORs on GABAergic interneurons within the periaqueductal gray (PAG) disinhibits serotonergic and noradrenergic release to the dorsal horn, resulting in analgesia [67, 68].

Oxycodone's actions at the MOR are also responsible for the off-target reinforcing effects and high abuse potential of this drug [69]. Specifically, acute activation of MORs within the VTA results in disinhibited DA release to induce euphoria and a subjective "high" [46]. At greater concentrations, oxycodone also activates MORs in various brainstem nuclei, such as the pre-Botzinger complex, parabrachial nucleus, and dorsal rostral pons, resulting in opioid overdose-induced respiratory depression [70].

Current Pharmacotherapies for OUD

While ongoing research continues to identify potential targets for therapeutics, three medications are currently approved by the FDA for OUD treatment: methadone, buprenorphine, and naltrexone [31]. Methadone and buprenorphine are essentially replacement therapies, in that they target the same set of receptors as opioids to alleviate withdrawal symptoms without inducing euphoria and impairment. When taken as prescribed, these medications have proven to prevent relapse. However, there is still a significant risk for misuse, abuse, and overdose, and patients have not been demonstrated to respond well to tapering of these medications [31, 71]. In comparison, naltrexone has no potential for misuse or abuse, as it blocks the reinforcing effects of opioids [31]. However, one of the principle issues in OUD therapy is that these pharmacotherapies are

still underutilized, in that only 29% of OUD patients have been prescribed with one of these medications as part of treatment [45]. As with AUD, continued research to identify additional therapeutic targets and more effective deployment is essential in order to improve OUD treatment outcome.

1.4 SEX DIFFERENCES IN ALCOHOL AND OPIOID USE DISORDERS: PREVALENCE, ETIOLOGY, AND PRESENTATION

Epidemiological Data

The ratio of men to women diagnosed with an AUD is approximately 1.8:1, demonstrating a pronounced gender gap in the prevalence of this disorder [1]. However, epidemiological data from 1980s show that this ratio was approximately 5:1 at this time [72-74]. This narrowing of the gender gap is due to an increase in AUD prevalence among women rather than a decrease among men [74]. Similar patterns are observed with respect to OUD, in that epidemiological data reveal a higher incidence of opioid-induced deaths in men than women. However, the rate of increase for overdose-induced deaths is significantly higher in women. Specifically, a seven-fold increase in prescription opioid overdose-induced deaths between 1999 and 2016 has been observed among women, compared to a four-fold increase among men [75].

Women with AUD or OUD have also been shown to exhibit significantly more health problems compared to men. For example, women with AUD are more likely than men to experience cardiomyopathy, cerebrovascular disease, hypertension, acute liver failure, hepatitis, and cirrhosis [76-78]. Women with OUD also exhibit significantly more

medical problems when compared to men with this disorder, evidenced by higher medical addiction severity index (ASI) scores [79, 80].

Epidemiological data suggest that the etiology and manifestation of AUD and OUD vary between men and women as well. For example, while women with these disorders typically initiate drug use at a later age than men, they tend to transition from a state of recreational use to dependence more quickly [81, 82]. Additional data demonstrate that the number of years between initial alcohol or opioid use and treatment entry is significantly lower for women when compared to men [83, 84]. Overall, this phenomenon, referred to as the telescoping effect, suggests that women may develop AUDs and OUDs at a more rapid rate than men [83-85]. Women receiving treatment for OUD also report higher subjective opioid craving, a major contributor to risk of relapse [79, 86, 87]. Lastly, the proportion of women with comorbid psychiatric disorders, namely depression, anxiety, and post-traumatic stress disorder, is also significantly higher compared to men with AUD or OUD [77, 82, 88-90]. These factors must be taken into account in developing more effective pharmacotherapeutics and overall treatment strategies for these populations.

Collectively, these data provide substantial evidence for sex differences in the etiology and presentation of addiction, highlighting the importance of preclinical studies assessing these behaviors in both male and female subjects in order to develop more effective therapeutics for each sex.

Preclinical Data

Several animal models of voluntary ethanol consumption and operant ethanol self-administration have shown that females consume significantly more ethanol than males

[91-94]. Females have also been shown to consume significantly more quinine-adulterated ethanol than males with no prior ethanol exposure [92].

The roles of estradiol and progesterone on sex differences in reward and aversion sensitivity have been most extensively studied for psychostimulants and generally suggest that estradiol potentiates while progesterone attenuates drug-seeking and reward sensitivity [95-105]. Recent work has also identified a role of these hormones in certain alcohol-seeking behaviors. In various models of ethanol self-administration and voluntary intake, estrous cycle phase has been repeatedly demonstrated to have no effect on ethanol intake in naturally cycling animals [91, 106-108]. However, ovariectomy has been shown to significantly attenuate binge-like ethanol intake in females [91]. Females also exhibit increased sensitivity to ethanol reward compared to males, an effect that is attenuated after ovariectomy [109]. Additional work has shown that ethanol-induced increases in cortical dopamine levels is significantly attenuated following ovariectomy [110], and that estradiol potentiates VTA DA release [111], providing further evidence of the involvement of estradiol in ethanol reward processing. Interestingly, neonatal estrogenization of female pups, which results in masculinization of the brain, has been shown to decrease ethanol intake in females, causing them to be more phenotypically similar to males [112]. Collectively, these data suggest that sex differences in ethanol-seeking and -taking are likely due to a combination of organizational and activational effects of this hormone.

Similar findings have been reported for opioids, in that females self-administer significantly more oxycodone, heroin, and morphine when compared to males, regardless of ROA employed [113-116]. Females have also been shown acquire heroin self-

administration more quickly than males [117, 118], where acquisition is measured by the number of self-administration sessions required for an animal to reach a specific criterion level of intake [119]. Females have also exhibited increased sensitivity to opioid reward [120, 121], and decreased sensitivity to opioid-induced aversive effects such as sedation [122]. However, our group and others have demonstrated that estrous cycle phase has no effect on oxycodone-seeking [113, 114]. Interestingly, though, ovariectomized rats have been demonstrated to acquire heroin self-administration at rates more similar to those observed in males, an effect that is reversed with chronic, systemic estradiol treatment [123]. These findings suggest that sex differences in opioid-seeking are likely mediated by a combination of organizational and activational hormonal effects.

Lastly, data on sex differences in alcohol and opioid withdrawal severity remain equivocal [124]. For example, epidemiological data have suggested that women with AUD or OUD may experience more severe [79, 125], less severe [77, 126], or equally severe withdrawal symptoms compared to men [79]. Several preclinical studies have revealed that withdrawal symptoms for both alcohol and opioids, such as wet-dog shakes, weight loss, seizure, increased corticosterone levels, and anxiety-like behaviors, are markedly more severe in male subjects [127-134]. However, additional studies have observed no sex differences in alcohol and opioid withdrawal severity [135-137]. Taken together, these data demonstrate that the effects of sex on motivational and physical withdrawal severity is rather complex and warrants further study.

1.5 BRAIN REGIONS INVOLVED IN ADDICTIVE BEHAVIORS

A central challenge in investigating both AUD and OUD is characterizing the transition from controlled to uncontrolled use, an undoubtedly complex process, recruiting multiple circuits involved in executive functioning, reward, and stress systems [13].

During initial drug use, an individual is motivated by the positive and rewarding effects of that drug, mediated by increased dopaminergic release from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), or ventral striatum, contributing to reinforcement-driven responding [14]. In fact, virtually all drugs of abuse enhance activity within this circuit to induce reinforcement [25]. When dopaminergic input to the NAc is combined with glutamatergic input from the hippocampus and medial prefrontal cortex (mPFC), an individual begins to form associations between drug-related stimuli and reward to influence goal-directed drug-seeking [138]. Following chronic use, this mesolimbic dopamine projection is biased toward the dorsal striatum, resulting in a shift from goal-directed to habitual drug-seeking [138-141]. Habit-driven responding is thought to contribute to compulsive-like consumption, or the consumption of drug despite aversive consequences [142]. Glutamatergic projections from the mPFC and AIC to the NAcc have been shown to promote aversion-resistant ethanol intake in male rats when the aversive stimulus is footshock or quinine, with no effect on punishment-free ethanol intake [143].

In addition to a shift toward habit-driven behaviors, chronic drug use is associated with a compensatory decrease in dopaminergic activity within the reward circuitry and increase in activity within the bed nucleus of the stria terminalis (BNST) and central

amygdala (CeA) [13]. The lateral habenula (LHb) and rostromedial tegmental nucleus (RMTg) are regions strongly activated following the omission of an anticipated reward, during the presentation of a noxious stimulus, and during the expression of certain withdrawal symptoms [144-147]. Each of these regions sends dense GABAergic projections to the VTA to inhibit mesolimbic DA release, inducing dysphoria and aversion [148].

An additional theory centers largely on dysregulation within subregions of the prefrontal cortex, maintaining that addiction is a product of impaired response inhibition and salience attribution [149-151]. Impaired response inhibition is the inability to inhibit maladaptive behaviors, contributing to a loss of control over drug-seeking and -taking. [149-151]. Under normal or healthy conditions, response inhibition is executed through the activation of regions such as the anterior cingulate cortex (ACC) during the detection of conflict. ACC activation then results in the recruitment of regions such as the dorsolateral prefrontal cortex (DLPFC) to regulate attention bias to the appropriate stimuli. In individuals with substance use disorders, the ACC is thought to be hypoactive, impairing conflict detection and subsequent inhibitory control of drug-seeking [149-151].

Impaired response inhibition can also be attributed to an imbalance in the activity of two subregions within the mPFC: the infralimbic and prelimbic cortices (ILC, PLC). These regions are involved in regulating behavioral flexibility, response inhibition, and decision-making under conflict [152-155]. It has been demonstrated that ILC activation typically inhibits, while PLC activation promotes drug-seeking [152, 156]. Therefore, a hyperactive PLC and/or hypoactive ILC may also contribute to impaired inhibition of drug-seeking behaviors [152].

Following chronic drug use, an individual's exaggerated sensitivity to and attentional bias for the drug of abuse and all drug-related stimuli can occur [149-151]. Salience attribution toward other nondrug-related stimuli is simultaneously decreased, potentiating the focus on obtaining and consuming the drug of abuse [149-151]. Regions proposed to be involved in these processes include many of those involved in the preoccupation/anticipation stage of addiction [13, 14], namely the AIC, ACC, orbitofrontal, and medial prefrontal cortices (OFC, mPFC) [149, 150]. High attentional bias to drug-related stimuli, and associated high activity within these prefrontal cortical regions, has been shown to positively correlate with compulsive drug-seeking in individuals with substance use disorders [157].

1.6 RODENT MODELS OF DRUG-SEEKING

AUD and OUD are disorders that cannot be recapitulated in preclinical models using a single paradigm. Rather, assays must be used to investigate specific behaviors associated with these disorders.

Drug Reward and Reinforcement

The operant self-administration paradigm is arguably one of the most translationally relevant animal models of drug-seeking. This assay involves training an animal to associate an operant response, such as a lever press, with the delivery of a reinforcer. Depending on the apparatus and experimental setup, the reinforcer can be delivered via intravenous or oral ROAs. This assay also allows for the investigation of various drug taking behaviors such as acquisition of the operant response, maintenance of drug self-administration, motivation, extinction, and relapse-like behavior, where acquisition is the

number of sessions required for an animal to reach a specific criterion level of intake, and maintenance the establishment of stable, high levels of drug intake [119, 152].

Specifically, acquisition and maintenance can each be assessed using a fixed-ratio (FR) schedule of reinforcement, in which a set number of responses must be completed in order to obtain a reinforcer, while motivation to consume a drug can be assessed using a progressive ratio (PR) schedule of reinforcement. During PR sessions, the number of responses required for reinforcer delivery increases within the session. The highest number of responses an animal completes for the delivery of a single reinforcer is the breakpoint, and a higher breakpoint is interpreted as increased motivation [14, 119, 158, 159].

Of note, during training, reinforcer delivery is often paired with a discrete cue, such as a light or tone, to strengthen the association between the operant response and drug availability. The pairing of drug delivery with a discrete cue also allows for subsequent investigation of cue-primed reinstatement, an animal model of relapse (described in more detail below). Similarly, this stage of self-administration can occur in a specific context with salient environmental cues, to allow for subsequent context-primed reinstatement studies [119, 152]. Reinstatement is an established animal model of relapse, in which animals are re-exposed to the drug of abuse or exposed to a stressor or drug-related cue/context following self-administration training and extinction to assess reinstatement of drug-seeking [152, 159-163]. Specifically, after animals have formed the association between an operant response and reinforcer delivery, this reinforcer is removed. Eventually, animals will learn that the operant response no longer has a consequence and will decrease their responding accordingly, a process known as extinction. If cue-primed

reinstatement is being investigated, the discrete cue that was previously paired with drug delivery is also removed during extinction sessions. If context-primed reinstatement is being investigated, extinction sessions occur in a self-administration chamber with different environmental cues from those associated with the chamber in which drug was administered. In contrast, extinction sessions for drug- and stress-primed reinstatement occur in identical environments and with identical cue presentation as those in which drug administration previously occurred [152]. Following extinction, animals are then exposed to a specific stimulus: cue, context, stress, or a small dose of the previously administered drug, all of which are the primary triggers for relapse in the human population [160, 164, 165]. Each of these stimuli has been shown to reliably induce reinstatement of drug-seeking [152, 160], demonstrating the efficacy of the reinstatement model for investigating relapse-like behavior.

In contrast to self-administration, two-bottle choice is a model of voluntary drug intake that does not require completion of an operant behavior. Essentially, an animal is provided access to one bottle containing water and one bottle containing drug solution. Of note, this assay is most commonly used to study alcohol consumption [166]; however, this model has been used to assess voluntary consumption of the opioids morphine, oxycodone, and fentanyl [167-169]. The primary measures obtained from this assay are drug preference and intake expressed as mg or g/kg of body weight. Therefore, two-bottle choice can be used to assess maintenance and escalation of drug intake. Schedules of exposure can also be manipulated by the experimenter to affect consumption. For example, in comparison to continuous access schedules, schedules in which alcohol availability is restricted tend to induce escalated, binge-like consumption [158, 159].

Place conditioning procedures can be used to assess an animal's sensitivity to the rewarding or aversive properties of a drug. For these assays, animals are treated with vehicle or drug before being confined to one side of a two-chambered apparatus. Each side contains saliently different contextual cues so that the animal can distinguish between the drug- and vehicle-paired environments. Following several drug and vehicle conditioning sessions, animals are then allowed to roam the entirety of the two-chambered apparatus. If an animal found the drug rewarding, more time will be spent in the drug-paired compartment. In contrast, if an animal found the drug aversive, more time will be spend in the vehicle-paired compartment, serving as a reliable measure drug-induced reward or aversion [119, 159].

Aversion-Resistance

Aversion-resistant intake is the continued consumption of a drug despite negative consequences [142]. This model serves as a model of compulsive-like consumption, and therefore has high translational value. For this assay, animals undergo ethanol self-administration or two-bottle choice before the ethanol solution is presented with an aversive stimulus, such as the bitter tastant quinine, most often used in two-bottle choice models, or a mild footshock, used in self-administration models [142]. If animals continue to consume drug despite adulteration with quinine or pairing with footshock, this behavior is considered inflexible and compulsive-like [142]. This model is most commonly used with ethanol self-administration or two-bottle choice, [106, 143, 170-173]. However, future studies using the aversion-resistant model to assess mechanisms involved in compulsive-like opioid intake would prove highly relevant.

1.7 SUMMARY

Both AUD and OUD are prevalent public health issues within the United States, with millions of men and women currently diagnosed [1, 2]. While decades of research have provided great insight into the etiology of addiction and substance use disorders, further studies are still needed to identify more effective therapeutics targeted for specific patient populations. As the majority of preclinical addiction work has been conducted using only male subjects, literature exploring mechanisms mediating the development and expression of various addictive behaviors in females is lacking. Given the extensive epidemiological data suggesting a sex difference in the progression and manifestation of AUD and OUD [79, 82, 83, 88, 174, 175], an increased number of preclinical studies assessing addictive-like behaviors in female subjects is greatly needed. Therefore, the goals of our current studies were to characterize sex differences in compulsive-like ethanol consumption using the aversion-resistant ethanol intake model, identify the neuroanatomical loci differentially activated in males and females during this behavior, and to establish a model of oral oxycodone self-administration while characterizing sex differences in oxycodone-seeking.

Table 1.1 AUD diagnostic criteria. The presence of 2-3 symptoms indicates mild AUD diagnosis, whereas presence of 4-5 indicates a moderate AUD. Presence of 6 or more in a results in diagnosis of severe AUD. Adapted from the APA DSM-5 (2016).

In the past year have you:
Had times when you ended up drinking more, or longer, than you intended?
More than once wanted to cut down or stop drinking, or tried to, but couldn't?
Spent a lot of time drinking? Or being sick or getting over other aftereffects?
Wanted a drink so badly you couldn't think of anything else?
Found that drinking – or being sick from drinking – often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
Continued to drink even though it was causing trouble with your family or friends?
Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?
More than once gotten into situations while or after drinking that increased your chance of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?
Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?
Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?

Table 1.2 OUD diagnostic criteria. The presence of 2-3 symptoms indicates mild OUD diagnosis, whereas presence of 4-5 indicates a moderate OUD. Presence of 6 or more in a results in diagnosis of severe OUD. Adapted from the APA DSM-5 (2016).

In the past year have you:
Taken larger amounts or taking drugs over a longer period than intended?
Experienced persistent desire or unsuccessful efforts to cut down on opioid use?
Spent a great deal of time obtaining or using the opioid? Or recovering from its effects?
Experienced craving or a strong desire or urge to use opioids?
Had problems fulfilling obligations at work, school or home?
Continued opioid use despite having recurring social or interpersonal problems?
Given up or reduced activities because of opioid use?
Used opioids in physically hazardous situations?
Continued opioid use despite ongoing physical or psychological problems likely to have been caused or worsened by opioids?
Experienced tolerance (i.e. needed increased amounts or diminished effect with continued use of the same amount)?
Experienced withdrawal (opioid withdrawal syndrome) or taking opioids (or a closely related substance) to relieve or avoid withdrawal symptoms?

CHAPTER 2: SEX DIFFERENCES IN AVERSION-RESISTANT ETHANOL INTAKE IN MICE

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2.1 ABSTRACT

Compulsive ethanol intake, characterized by persistent consumption despite negative consequences, is an addictive behavior identified by the DSM-5 as a central criterion in diagnosing alcohol use disorders (AUD). Epidemiological data suggest that females transition from recreational alcohol use to AUD more rapidly than males. Because of this potential sex difference in the etiology of AUD, it is critical to assess addictive behaviors such as compulsive intake in both males and females in preclinical studies. We used the model of aversion-resistant ethanol consumption to assess compulsive-like ethanol intake. In these experiments, C57BL6/J mice were first provided with continuous access two-bottle choice between water and ethanol to establish baseline intake. Ethanol solution was then adulterated with increasing concentrations of the bitter tastant quinine hydrochloride. Animals that consume ethanol solution despite its pairing with this negative stimulus are thought to be exhibiting compulsive-like behavior. We found that higher concentrations of quinine were required to suppress ethanol consumption in female mice relative to males. We found no effect of estrous cycle phase on baseline ethanol intake or on quinine-adulterated ethanol intake in females. Collectively, these data suggest that females exhibit a higher degree of aversion-resistance than male mice. Because we observed no effect of estrous cycle phase, it is likely that the presence of threshold levels of estradiol or progesterone, as opposed to their natural fluctuation across the estrous cycle, mediates increased aversion-resistance in females. Alternatively, or in

combination, developmental effects of sex hormones could contribute to aversion-resistant ethanol intake.

2.2 INTRODUCTION

According to the National Institute on Alcohol Abuse and Alcoholism, approximately 9.8 million men and 5.3 million women in the United States suffer from an alcohol use disorder (AUD). While these current statistics reveal that approximately twice as many males are diagnosed with AUDs compared to females, epidemiological data from the early 1980s show that the ratio was 5:1 at that time [72-74]. This closing gender gap is due to an increasing prevalence in the number of women diagnosed with AUDs rather than a decrease in the number of men diagnosed [74]. Additionally, epidemiological evidence suggests that females may transition from recreational alcohol use to a state of dependence more rapidly than males [74, 84], despite the fact that most women begin drinking at a later age than men [83]. This phenomenon, referred to as the telescoping effect [83], suggests differences in the etiology of AUDs between males and females, and demonstrates the importance of including both male and female subjects in preclinical investigations of this disorder.

Many preclinical rodent studies have examined differences between males and females in alcohol intake. While some studies of sex differences have found that female rodents consume significantly more ethanol [93, 176-182], other studies have found that males consume more [183], or have observed no differences between males and females during voluntary ethanol intake [184, 185]. These inconsistencies are perhaps attributable to differences in strain and age of rodents, or the specific drinking paradigm used. Notably, much of this work was performed using rats or strains of mice other than those used in our study.

In our experiments, we used male and female C57BL6/J mice, and several studies have demonstrated that female C57BL6/J mice consume significantly more ethanol compared to males, a phenomenon most strongly observed during drinking in the dark (DID) models, which induce binge-like levels of ethanol consumption [91, 92]. A similar effect has been observed in mice exposed to an intermittent access schedule of ethanol two-bottle choice, in that females escalate their intake to a greater degree than males [186]. It is often hypothesized that sex differences in these behaviors may be due to increased sensitivity to ethanol's rewarding properties and decreased sensitivity to its aversive properties in females, which has been observed in some studies [109, 185].

A major DSM-5 criterion in the diagnosis an AUD is compulsive intake, or the consumption of alcohol despite negative consequences such as trouble with family, friends, or work [187]. Aversion-resistant ethanol intake is a well-established animal model of this addictive behavior [142]. In this model, animals are first trained to voluntarily consume ethanol using assays such as two-bottle choice to establish a baseline level of intake [142]. Once a stable baseline is established, the ethanol solution is then paired with an aversive stimulus, such as the bitter tastant quinine hydrochloride [142, 143, 171, 173, 188-192] or mild footshock [143]. If an animal continues to consume ethanol despite its presentation with the aversive stimulus, this animal is considered aversion-resistant and to be exhibiting compulsive-like ethanol consumption [142]. While a number of studies [143, 191, 193, 194] have provided excellent data characterizing the behavioral and neuronal mechanisms associated with the aversion-resistant phenotype, these studies have used only male subjects. Because of the sex differences in the etiology

of AUDs described above, it is critical that more studies on this addiction-like behavior be conducted using female subjects.

Currently, only one study has assessed the aversion-resistant phenotype in female mice [92]. These data demonstrated that males and females exhibited the same degree of aversion-resistant ethanol intake, measured by consumption of quinine-adulterated ethanol as a percentage of baseline ethanol intake [92]. Of note, this lack of sex difference in quinine-adulterated ethanol intake only emerged following 15 days of binge-like ethanol consumption [92]. When quinine-adulterated ethanol was introduced initially, females consumed significantly more of this solution than males [92]. These data suggest that sex differences in consumption of quinine-adulterated ethanol may be observed under specific experimental conditions and are affected by the schedule and length of ethanol exposure. Therefore, the aims of the current studies are to characterize aversion-resistant ethanol intake in female mice in comparison to males, following continuous access two-bottle choice, and to assess the role of the estrous cycle in this behavior.

2.3 MATERIALS AND METHODS

Animals

Adult male and female C57BL6/J mice aged 10–12 weeks at the start of experimentation were used for all studies. Animals were singly housed on a reverse 12:12 light/dark cycle. All procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with NIH guidelines.

Ethanol two-bottle choice

Mice were presented with two bottles in the home cage, one containing water and one containing increasing concentrations of ethanol on a continuous access schedule. Bottles were weighed every 24 hours to calculate g/kg intake, and bottle position was switched daily to prevent the development of a side preference. After 2–3 days of water only availability in both bottles, ethanol exposure began with 3% (v/v), and each ethanol concentration was presented for 4 days before being increased in increments of 3%.

Aversion-resistant intake

For this experiment, mice (n = 5–6/sex) were exposed to ethanol concentrations ranging from 3–24% (v/v) on the continuous access schedule, with each concentration available for 4 days before being increased by 3%. Because g/kg intake in males and females stabilized after the 15% concentration (see Fig. 2.1A), animals were reintroduced to the 15% ethanol solution to establish baseline intake. Animals were then exposed to 15% ethanol adulterated with increasing concentrations of the bitter tastant quinine hydrochloride (0.03, 0.1, and 0.3 mM). Previous work has demonstrated that rats and mice are sensitive to similar quinine concentrations. More specifically, rats have been shown to exhibit sensitivity to quinine concentrations as low as 0.01 g/l [195], therefore we selected the 0.03 mM for our first concentration to present in our quinine-adulterated ethanol experiments. Each quinine concentration was presented twice, with 4 days of quinine-free ethanol two-bottle choice between test days.

Quinine sensitivity

A separate cohort of mice (n = 5–6/sex) were individually housed and presented with two bottles in the home cage. Both bottles contained water and were weighed every

24 hours. As in ethanol two-bottle choice studies, bottle position was switched daily. Water intake was calculated as ml/kg consumed. Once a stable baseline was established, one water bottle was adulterated with increasing concentrations of quinine hydrochloride (0.001, 0.003, 0.01 and 0.03 mM). Each concentration was presented twice, and 4 days of water washout occurred between test days.

Estrous cycle monitoring

The estrous cycle in mice consists of four phases, each lasting approximately 24 hours [196]. Proestrus is characterized by peak estradiol levels and relatively high progesterone levels. Proestrus is followed by the estrus phase, in which estradiol and progesterone levels begin to decrease. This is followed by metestrus, in which levels of estradiol and progesterone are extremely low. Metestrus is in turn followed by the diestrus phase, which is characterized by peak progesterone levels and low estradiol levels. Progesterone levels begin to decrease at the end of diestrus, triggering reentry to the proestrus phase [196]. A separate cohort of adult females (n = 10) underwent continuous access (3–15%, v/v) two-bottle choice until intake stabilized at the 15% concentration. Once stable, estrous cycle was monitored daily by vaginal lavage as previously described [196]. Briefly, 20 µl of 0.9% sterile saline was pipetted slowly into the vaginal opening and aspirated and expelled several times to collect the sample. Samples were immediately pipetted onto a clean microscope slide. Slides were air dried and stained with Toluidine Blue O (Millipore Sigma) and imaged at 20x using a Zeiss light microscope. Phase of estrous cycle was determined based on qualitative analysis of predominant cell type present in the sample. For example, proestrus was identified based on the presence of predominantly nucleated epithelial cells. Similarly, estrus was

identified by the presence of primarily cornified epithelial cells, metestrus by the presence of a mixture of cornified epithelial cells and leukocytes, and diestrus by the presence of primarily leukocytes [197].

Statistics

Statistical analyses were performed using Statistica software, and figures were created using GraphPad Prism. For analysis of baseline ethanol intake, two-way ANOVA was conducted using the between subjects factor of sex and the within subjects factor of ethanol concentration. The dependent variable was g/kg ethanol consumption. For analysis of aversion-resistant intake, two-way ANOVA was conducted using the between subjects factor of sex and the within subjects factor of quinine concentration. The dependent variable was percent change from baseline or g/kg ethanol consumption. For analysis of quinine sensitivity, two-way ANOVA was conducted using the between subjects factor of sex and the within subjects factor of quinine concentration. The dependent variable was percent change in water intake. For analysis of the effect of estrous cycle, repeated measures one-way ANOVA was conducted using the main factor of estrous cycle phase. The dependent variable was g/kg ethanol intake or percent change in ethanol intake. All post-hoc analyses were conducted using Newman–Keuls test. Bartlett's test was conducted for all data sets to test normality and homogeneity of the distributions. This analysis did not detect any abnormal distributions. Thus, standard parametric ANOVAs were used for analysis.

2.4 RESULTS

Male and female mice do not differ in baseline ethanol intake

Ethanol intake was monitored in males and females (n = 5–6/sex) every 24 hours for 6 weeks for concentrations ranging from 3% to 24% ethanol (v/v). Two-way ANOVA revealed no effect of sex on g/kg ethanol intake (Fig. 2.1A; $F_{1,10} = 0.12$, $P = 0.74$). There was, however, a main effect of ethanol concentration ($F_{7,70} = 31.4$, $P < 0.0001$), as intake tended to increase with increasing ethanol concentrations. Specifically, for all concentrations greater than 6%, intake was significantly increased relative to intake at the initial 3% concentration ($P < 0.0001$). Post-hoc analysis also revealed that g/kg intake stabilized after the 15% ethanol concentration, evidenced by the fact that g/kg intake of 18%, 21% and 24% solutions was not significantly different compared to the 15% concentration ($P > 0.99$, P = 0.88, $P > 0.99$, respectively). There was no significant interaction between sex and ethanol concentration ($F_{7,70} = 0.70$, $P = 0.67$). Lastly, intake was measured after mice were reintroduced to the 15% ethanol concentration. After intake stabilized, the average of the last 3 days was used to calculate baseline intake. Unpaired t-test revealed no effect of sex on g/kg ethanol intake ($t_9 = 0.42$, $P = 0.68$; Fig. 2.1B) after intake had stabilized at this concentration. These data indicate that males and females did not differ in ethanol intake at baseline under the conditions used in our experiments.

Female mice consume more quinine-adulterated ethanol than males

After baseline intake at the 15% ethanol concentration was established, all animals (n = 5–6/sex) were presented with increasing concentrations of quinine (0.03, 0.1 and 0.3mM). Data were represented as a percent change from the group's average intake

under baseline conditions. Two-way ANOVA revealed a main effect of sex ($F_{1,9} = 27.0$, $P < 0.001$), a main effect of quinine concentration ($F_{3,27} = 7.4$, $P < 0.001$), and a trend-level interaction between these factors ($F_{2,18} =$, $P = 0.06$; Fig. 2.2A). Post-hoc analysis revealed no difference between males and females at baseline ($P > 0.99$) or at the highest (0.3 mM) quinine concentration ($P = 0.45$). However, at the 0.03 and 0.1mM quinine concentrations, males and females were significantly different, with males showing a greater level of suppression ($P < 0.01$ and $P < 0.05$, respectively). Post-hoc analysis also revealed that males' percent change in intake was significantly decreased at all quinine concentrations when compared to baseline ($P < 0.05$ for all comparisons). However, percent change in intake compared to baseline in female mice was not significantly different at the 0.03 or 0.1mM quinine concentrations ($P = 0.88$ and $P = 0.99$, respectively), but did significantly decrease at the 0.3 mM quinine concentration ($P < 0.05$).

A number of these effects were also detected when data were expressed as g/kg intake. Specifically, two-way ANOVA revealed a main effect of sex ($F_{1,9} = 34.1$, $P < 0.001$) and a main effect of quinine concentration ($F_{3,27} = 6.0$, $P < 0.01$; Fig. 2.2B), but no significant interaction was observed ($F_{3,27} = 1.6$, $P = 0.22$). Overall, female mice consumed more ethanol than male mice and quinine induced a concentration dependent suppression of alcohol intake. Pairwise group comparisons using Newman–Keuls tests indicated that alcohol consumption in male mice decreased significantly when adulterated with 0.3 mM quinine compared to baseline intake ($P = 0.02$). This concentration of quinine induced a trend level suppression of alcohol intake in female mice ($P = 0.07$). Males and females significantly differed in consumption when alcohol was adulterated

with 0.03 mM quinine ($P = 0.01$) and this effect was trend level at 0.1 mM quinine ($P = 0.09$). Taken together, these data suggest that females exhibit a higher degree of quinine-adulterated ethanol intake when compared to males.

Males and females do not differ in sensitivity to quinine

After baseline water intake was established, all animals were presented with increasing concentrations of quinine hydrochloride in the absence of ethanol (0.001, 0.003, 0.01, and 0.03 mM, Fig. 2.3). Two-way ANOVA revealed a main effect of quinine concentration ($F_{4,36} = 4.1$, $P < 0.01$), but no effect of sex ($F_{1,9} = 0.002$, $P = 0.97$), nor a significant interaction between these factors ($F_{4,36} = 0.59$, $P = 0.67$). Post-hoc analysis demonstrated that intake at the 0.03 mM quinine concentration was significantly lower when compared to baseline ($P < 0.05$). These data indicate that males and females do not differ in their sensitivity to the bitter tastant quinine.

Estrous cycle phase does not affect baseline ethanol consumption or quinine-adulterated intake

After stable baseline consumption was reached at 15% ethanol concentration, ethanol intake was measured throughout two complete estrous cycles (see Fig. 2.4 for representative examples of vaginal cytology for each phase). One-way ANOVA revealed no significant effect of estrous cycle ($F_{3,15} = 0.11$, $P = 0.95$) on g/kg ethanol intake (Fig. 2.5A). Quinine-adulterated ethanol (15% v/v ethanol; 0.1 mM concentration quinine) was then presented twice on each phase of the estrous cycle. Data were expressed as percent change from the baseline intake at that specific phase of estrous (Fig. 2.5B). For example, quinine-adulterated ethanol intake during the proestrus phase was expressed as a percent change from quinine-free ethanol intake during the proestrus phase. One-way ANOVA

revealed no effect of estrous cycle phase on the percent change in ethanol intake ($F_{3, 15}$ = 0.26, P = 0.85). These data indicate that estrous cycle phase does not affect quinine-adulterated ethanol intake. This cohort did exhibit a mild suppression of ethanol intake (approximately 20%) when quinine was introduced to the ethanol. Using g/kg consumption as a measure, two-way ANOVA revealed a main effect of quinine on alcohol intake ($F_{1,9}$ = 9.7, P = 0.01). However, there was no main effect of estrous cycle ($F_{3,27}$ = 2.5, P = 0.08), nor an interaction between these two factors ($F_{3,27}$ = 0.5, P = 0.68). Notably, this decrease was of a lesser magnitude than the decrease observed in male mice at this concentration of quinine in the initial experiment (approximately 60% decrease), and when males and females were run concurrently in the same cohort of the initial experiment, a strong sex difference was observed.

2.5 DISCUSSION

The primary finding from our experiments is that female C57BL6/J mice consume higher levels of quinine-adulterated ethanol solution when compared to males. Ethanol consumption despite its pairing with a negative stimulus is an established animal model of compulsive-like intake [142], which is a major criterion in AUD diagnosis [187]. Therefore, these data provide preclinical evidence suggesting potential sex differences in the propensity to exhibit compulsive ethanol consumption.

Interestingly, males and females in our experiments did not differ in baseline intake across a range of ethanol concentrations. While it is often found that females consume more ethanol than males, sex differences in consumption vary depending on the ethanol concentration presented, the length and schedule of exposure used, and general

cohort variability. For example, female C57BL6/J mice have been shown to consume significantly more ethanol than males in the drinking in the dark (DID) model, an assay that induces binge-like levels of intake [91, 92]. However, in other labs using the same DID assay, whether or not females consumed more ethanol was inconsistent between cohorts, suggesting that inter-cohort variability may play a significant role in whether or not sex differences are observed [198]. It has also been found that female mice consume significantly more ethanol during schedules of limited, restricted, or continuous access two-bottle choice over periods of time ranging from 2 to 4 weeks [199-201]. In contrast, a study assessing ethanol intake during 2 weeks of restricted access found no significant sex difference in C57BL6/J mice [202]. Yoneyama and colleagues also found that whether or not a sex difference in ethanol intake during continuous access was observed was dependent upon the concentration of ethanol presented [203]. Collectively, these data demonstrate that the findings on sex differences in baseline ethanol intake are highly variable.

Our finding that males and females do not differ in baseline ethanol consumption, but do significantly differ in quinine-adulterated ethanol intake is intriguing to consider in the context of the findings by Seif et al. (2013), which identify unique neural circuitries activated during baseline consumption and aversion-resistant intake. While this specific study assessed aversion-resistant intake in males only, identification of a unique circuit for the expression of aversion-resistant intake raises the possibility that while males and females may not differ in the neurocircuitry mediating baseline consumption, sex differences may emerge in the neurocircuitry mediating quinine-adulterated ethanol intake. For example, glutamatergic projections from the medial prefrontal cortex and the

insula to the nucleus accumbens core promote aversion-resistant ethanol consumption while having no effect on punishment-free consumption [143]. Brain regions involved in the aversion circuitry may also contribute to this behavior, such as the rostromedial tegmental nucleus [204] and the lateral habenula [205]. These regions are strongly activated in response to aversive stimuli, including the aversive properties of ethanol, but their role in aversion-resistant ethanol intake in females is currently unknown. Our future studies will examine neuronal activation in the specific neurocircuitry that mediates aversion-resistant intake, as there may be a difference in the magnitude of effect between sexes. Additionally, it would be very interesting to know if these sex differences generalize to aversion-resistant intake that is induced by other experimental methods, such as footshock suppressed operant self-administration of ethanol, for example.

Another novel finding from this study is apparent aversion-resistant intake in females after only 6 weeks of continuous ethanol two-bottle choice. Previous work has demonstrated that to induce the aversion-resistant phenotype in males, rodents must be exposed to models such as continuous access two-bottle choice for approximately 8 months [206], intermittent access two-bottle choice for 3–4 months [195], restricted access two-bottle choice for 2 or 8 weeks [172], a 24-hour forced alcohol intake session [170], chronic intermittent ethanol vapor treatment [191], or drinking in the dark [92]. In our model of 6 weeks of continuous access two-bottle choice, males were sensitive to quinine adulteration and decreased their ethanol intake under these conditions. Based on previous literature, other methods of ethanol exposure such as those outlined above would be necessary to induce the aversion-resistant phenotype in male C57BL6/J mice. Taken together with the existing literature, our model demonstrates that females may

express inherent differences in aversion-resistant alcohol intake when compared to male rodents. One outstanding question is if males and females respond differently in aversion-resistant intake following the induction of dependence, and future experiments will examine these factors.

After observing a sex difference in quinine-adulterated ethanol intake, we conducted a control experiment in a separate cohort of animals to ensure that this difference was not due to a difference in sensitivity to the bitter tastant quinine. These results demonstrated that both males and females significantly decrease their water intake when water was adulterated with 0.03 mM quinine (see Fig. 3). Because this is the same quinine concentration at which males exhibited a significant decrease in ethanol intake, while females did not, no further concentrations of quinine were tested. These data indicate that the sex differences in quinine-adulterated ethanol consumption are not due to a difference in taste sensitivity to quinine. Slight fluctuations in intake at lower concentrations of quinine are likely due to random variation in intake across days of the experiment. Importantly, there was no significant difference between male and female intake at any concentration used in this experiment. Of note, a previous study has found that male mice do not decrease intake when presented with 0.1 mM quinine-adulterated water, in contrast to our finding that males and females are sensitive to an even lower concentration of quinine (0.03 mM) [172]. However, a separate study has found that C57BL6/J males and females demonstrate sensitivity to both 0.03 mM and 0.1 mM quinine-adulterated water solutions [207], which is more in line with our observations.

We also assessed the role of circulating estradiol and progesterone in this behavior by monitoring estrous cycle. After stabilizing on the 15% ethanol concentration,

vaginal lavages were conducted prior to the start of the dark cycle to determine estrous cycle phase. We found that estrous cycle did not affect baseline intake, consistent with several previous studies investigating the role of estrous cycle phase on ethanol consumption [91, 107, 108, 182, 208]. We also found that estrous cycle phase had no effect on quinine-adulterated ethanol consumption, in that females across all phases consumed similar amounts of quinine-adulterated ethanol solution. While this cohort did exhibit a slight suppression (approximately 20%) when compared to their quinine-free baseline, this decrease was considerably less than the decrease observed in males in the initial experiment (approximately 60%). More importantly, the amount of ethanol/quinine intake did not significantly differ across the estrous cycle. These findings suggest that the fluctuation of estradiol and progesterone during estrous cycle phases does not significantly affect consumption of quinine-adulterated ethanol.

While we found no effect of estrous cycle on this addictive behavior, it is still possible that circulating estradiol and/or progesterone contribute to sex differences in aversion-resistant ethanol intake. For example, previous work by Satta and colleagues has demonstrated that while estrous cycle phase had no effect on binge-like alcohol intake, ovariectomy significantly attenuated this behavior [91]. Such findings suggest that a minimum threshold level of circulating estradiol and/or progesterone is required to induce sex differences in certain addictive behaviors, rather than naturally fluctuating levels resulting from progression through the estrous cycle. Alternatively, or additionally, developmental effects of sex hormones may contribute to the expression of aversion-resistant drinking.

These studies identify a significant sex difference in quinine-adulterated ethanol intake. Specifically, females consume higher levels of quinine-adulterated ethanol solution when compared to males. However, we observed no effect of estrous cycle phase on this behavior. Thus, the presence of threshold levels of circulating estradiol or progesterone and/or developmental sex differences play a significant role in compulsive ethanol intake. Future experiments will specifically examine these mechanisms.

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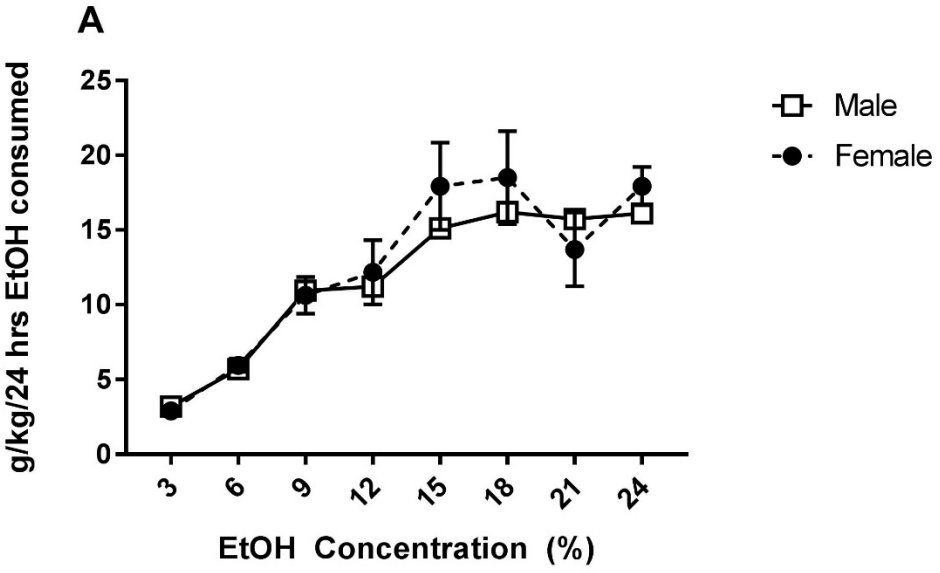

A
g/kg/24 hrs EtOH consumed
25
20
15
10
5
0
3
6
9
12
15
18
21
24
EtOH Concentration (%)
Male
Female

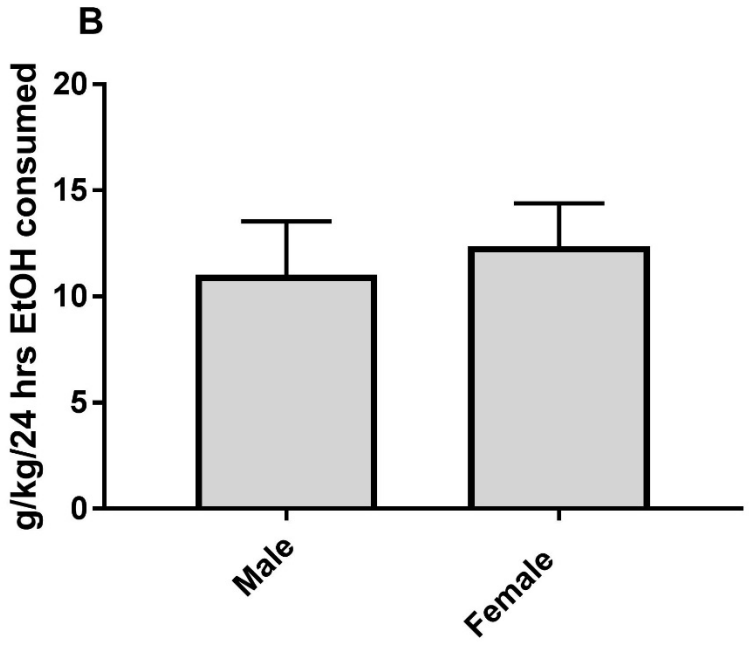

B
g/kg/24 hrs EtOH consumed
20
15
10
5
0
Male
Female

Figure 2.1 Effect of sex on baseline ethanol intake. (A) Baseline ethanol consumption for ethanol concentrations ranging 3-24% (v/v). Ethanol intake increased with increasing concentration of alcohol and plateaued at 15%. There was no effect of sex on ethanol intake. (B) Average of the last two days of ethanol intake at the 15% concentration. There was no difference between males and females ($P = 0.68$). n=5-6/sex.

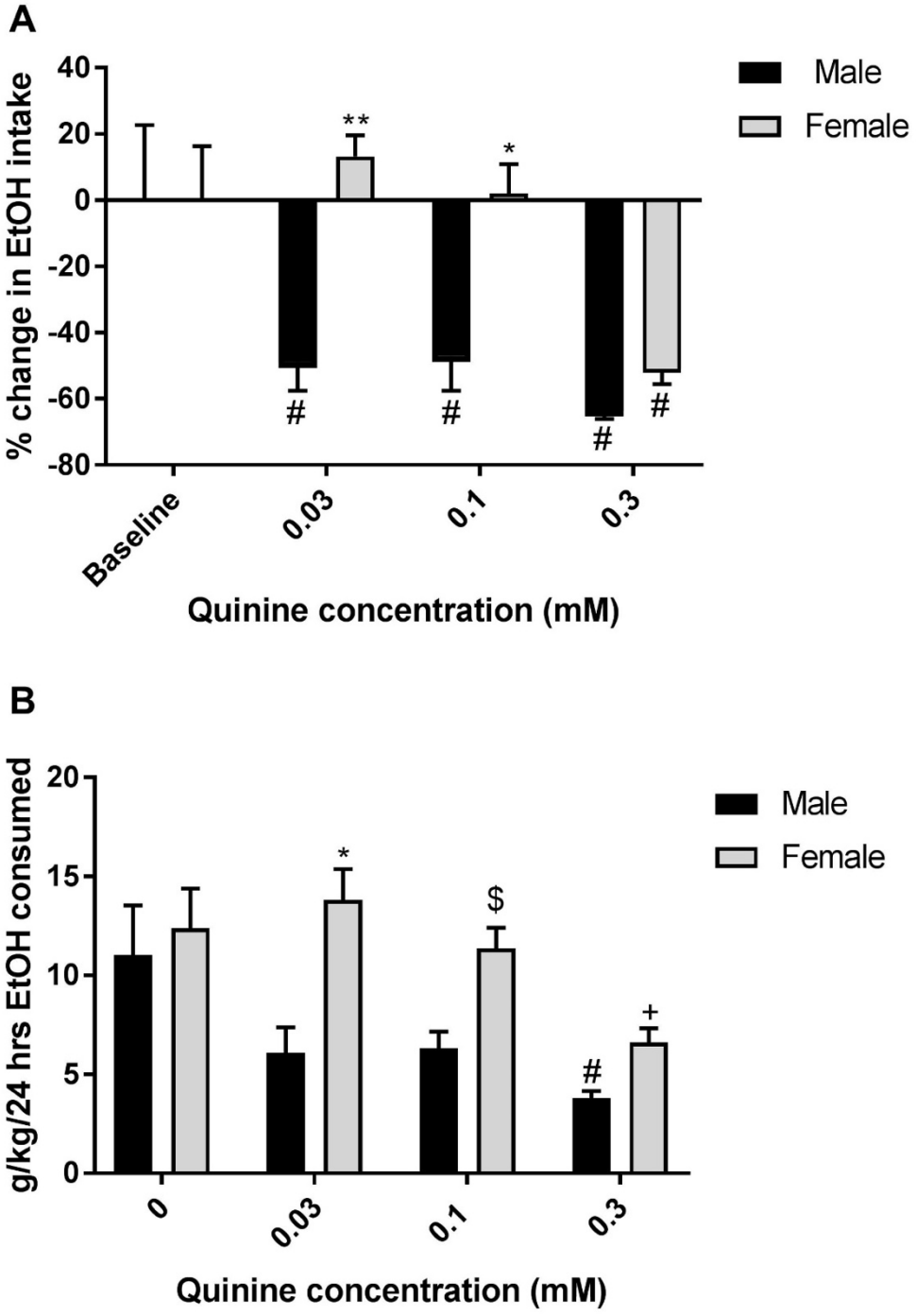

A
% change in EtOH intake
40
20
0
-20
-40
-60
-80
**
*
#
#
#
#
Male
Female
Baseline
0.03
0.1
0.3
Quinine concentration (mM)
B
g/kg/24 hrs EtOH consumed
20
15
10
5
0
*
$
#
+
Male
Female
0
0.03
0.1
0.3
Quinine concentration (mM)

Figure 2.2 Effect of sex on quinine-adulterated ethanol intake. (A) Intake expressed as percent change from baseline (last 3 days of 15% ethanol consumption prior to quinine exposure). Intake of males and females was significantly different at the 0.03 and 0.1mM quinine concentrations ($P < 0.01$, $P < 0.05$). However, males and females did not differ at the highest (0.3 mM) quinine concentration ($P = 0.45$). Quinine suppressed ethanol intake in males at all concentrations examined, but did not affect intake in female mice until the highest concentration of quinine was presented. (B) Intake expressed as g/kg. Females consumed more alcohol than males overall and quinine induced a concentration dependent suppression of alcohol intake. Post-hoc analysis revealed that intake at the 0.3mM quinine concentration was significantly less than intake at baseline for males ($P < 0.05$) and reached trend level for females ($P < 0.1$). Females consumed significantly more alcohol compared to males when the solution was adulterated with 0.03mM quinine ($P < 0.05$) and this effect was trend level for 0.1mM quinine solution ($P < 0.1$). *$P < 0.05$, compared to males. **$P < 0.01$, compared to males. \$$P < 0.10$ compared to males. #$P < 0.05$, compared to baseline. +$P < 0.10$, compared to baseline. n = 5–6/sex.

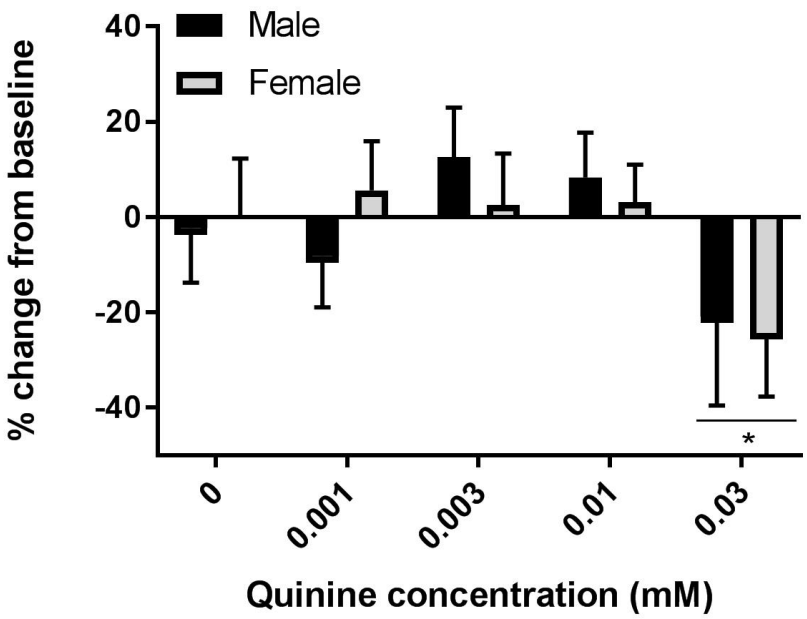

40
20
0
-20
-40
Male
Female
% change from baseline
*
0
0.001
0.003
0.01
0.03
Quinine concentration (mM)

Figure 2.3 Effect of sex on quinine sensitivity. Data expressed as percent change from baseline water intake. Males and females did not differ in their sensitivity to the bitter tastant quinine. Intake at the 0.03mM quinine concentration decreased significantly when compared to baseline intake ($P < 0.05$). There was no effect of sex on consumption of quinine solution. *$P < 0.05$, compared to baseline. n = 5–6/group.

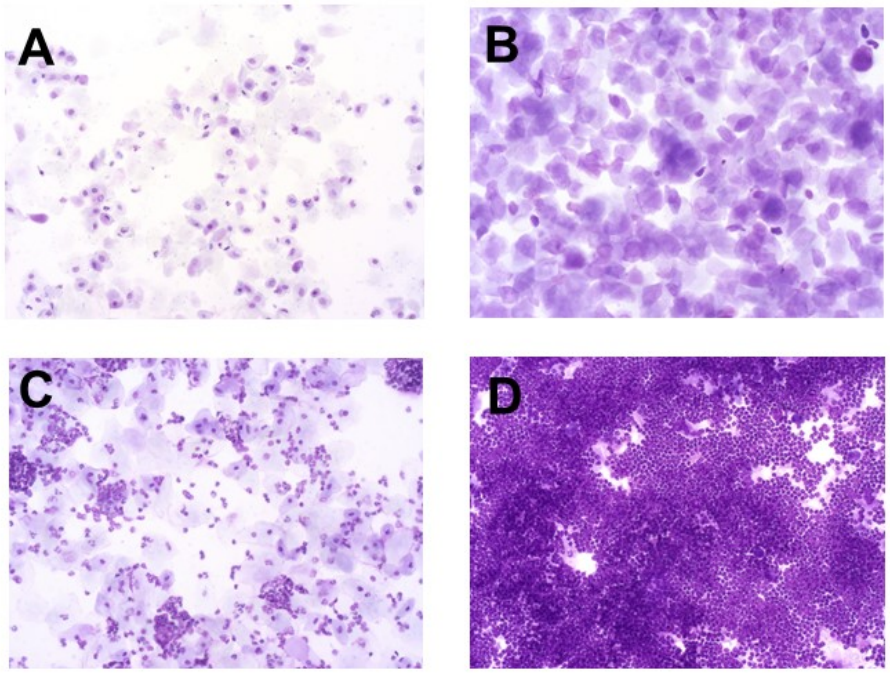

A
B
C
D

Figure 2.4 Representative images of each phase of the estrous cycle. (A) Proestrus: characterized by the presence of predominantly nucleated epithelial cells. (B) Estrus: characterized by the presence of predominantly cornified epithelial cells. (C) Metestrus: characterized by a mixture of cornified epithelial cells and leukocytes. (D) Diestrus: characterized by the presence of predominantly leukocytes. Images taken at 20x.

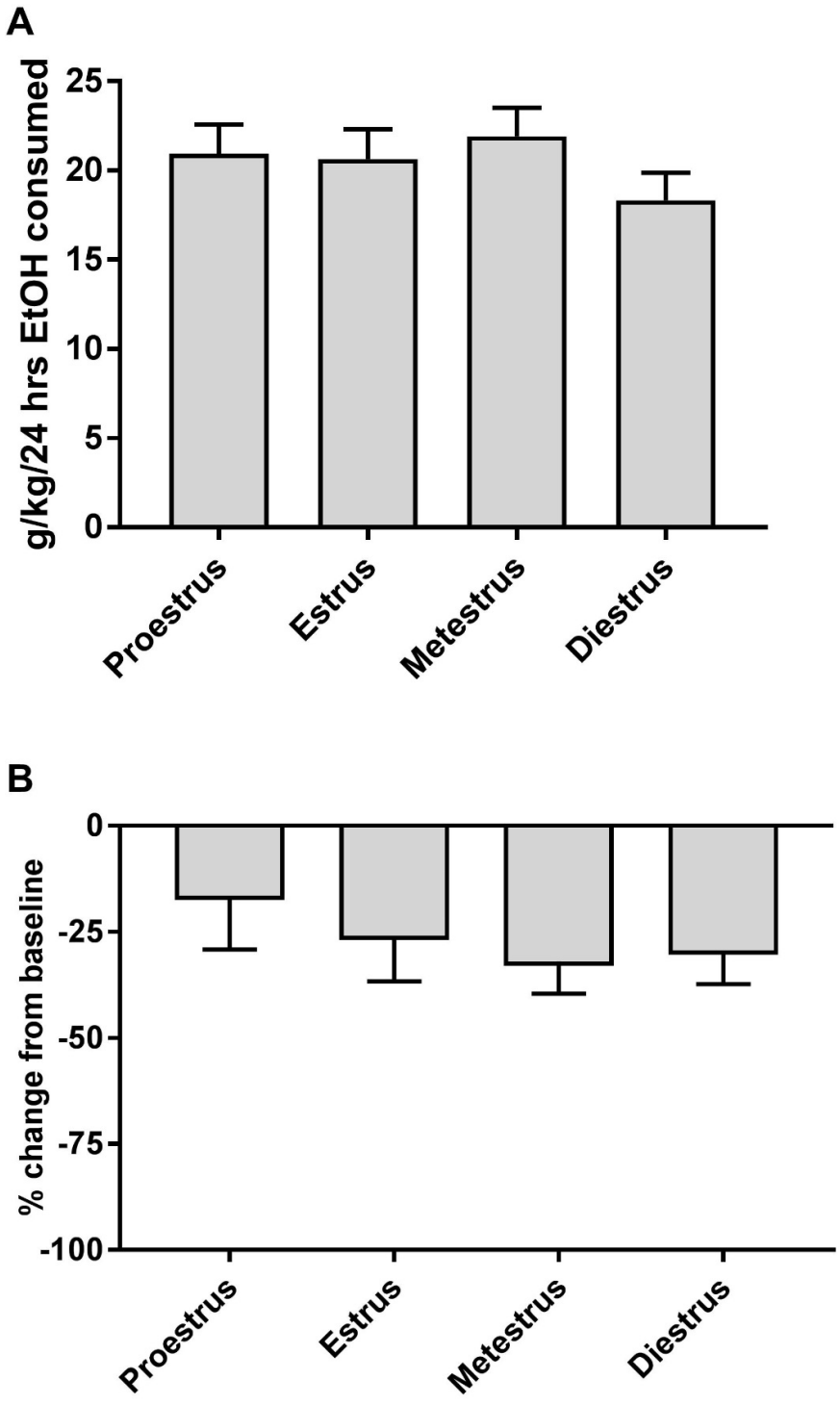

A
g/kg/24 hrs EtOH consumed
25
20
15
10
5
0
Proestrus
Estrus
Metestrus
Diestrus
B
% change from baseline
0
-25
-50
-75
-100
Proestrus
Estrus
Metestrus
Diestrus

Figure 2.5 Effect of estrous cycle phase on baseline ethanol intake and quinine-adulterated ethanol intake. (A) Baseline intake. Data expressed as g/kg ethanol intake across the estrous cycle. Estrous cycle phase had no effect on baseline ethanol intake. (B) Quinine-adulterated intake. Data expressed as percent change from baseline intake during each phase. Estrous cycle phase had no effect on quinine-adulterated ethanol intake. n = 10.

CHAPTER 3: SEX DIFFERENCES NEURONAL ACTIVATION FOLLOWING QUININE-ADULTERATED ETHANOL INTAKE

3.1 INTRODUCTION

Alcohol use disorder (AUD) is a prevalent public health problem in the United States, with approximately 9 million men and 5 million women currently diagnosed [1]. While these statistics demonstrate that a men to women ratio of AUD prevalence of 1.8:1, identifying a significant gender gap, in the 1980s, this ratio used to be 5:1 [72-74]. Of note, this narrowing gender gap is due to an increase in women diagnosed with AUD diagnosis, not a decrease in the number of men diagnosed [74]. In addition to an increased number of women developing AUD, certain epidemiological evidence suggest that the etiology of AUDs differs between men and women. For example, women undergoing treatment for AUD report higher levels of alcohol craving in comparison to their male counterparts, a symptom that is generally predictive of the risk of relapse [88]. Additionally, the period of time between initial alcohol use and treatment entry is significantly shorter for females than males with AUD, suggesting that women may develop dependence more quickly [74, 81, 83]. Collectively, these epidemiological data demonstrate a need for increased preclinical studies using both male and female subjects to identify and characterize any potential sex differences that could significantly influence treatment strategy.

AUD is a chronic, relapsing disorder, characterized by symptoms such as the inability to control intake, and continued intake despite negative consequences [187]. Alcohol consumption despite negative consequences such as issues with work, family, or health, is considered compulsive intake and is a central component of AUD [13, 209].

This specific behavior can be modeled preclinically using assays in which animals are presented with ethanol that is paired with an aversive stimulus, such as the bitter tastant quinine hydrochloride or mild footshock [142]. Animals that continue to consume ethanol despite its pairing with a negative stimulus are considered to be exhibiting aversion-resistant intake (also referred to as inflexible or compulsive-like intake) [142].

Aversion-resistant intake has been utilized to identify several promising targets that contribute to this addictive-like behavior in male subjects. For example, Seif et al have identified a faciliatory role of insular and medial prefrontal cortex (mPFC) projections to the nucleus accumbens core (NAcc) in aversion-resistant intake in male rats [143]. Interestingly, these findings applied to aversion-resistance when the negative stimulus was either footshock or quinine, illustrating the importance of these circuits in aversion-resistance regardless of the sensory modality of the negative stimulus used [143]. Additionally, manipulations of these circuits had no effect on punishment-free ethanol intake, demonstrating that aversion-resistant intake recruits a unique circuitry from that involved in punishment-free ethanol consumption [143].

While several potential mechanisms and regions involved in aversion-resistant ethanol intake have been characterized using male subjects, the neurocircuitry mediating this behavior females is not yet understood. Therefore, in this study, we aimed to identify potential sex differences in gross neuronal activation using c-Fos mapping. c-Fos is an inducible transcription factor (ITF) expressed in relatively low levels throughout the brain under basal conditions. Following neuronal activation in response to an external stimulus, *c-fos* mRNA will be transcribed within 30 minutes and c-Fos protein translated within 90-120 minutes [210, 211], allowing for visualization of neuronal activation using

in situ hybridization or immunohistochemistry (IHC). Brain regions examined were selected based on their established roles in reward and aversion processing, decision-making, and salience attribution, in which salience is the denoted importance or relevance of a given stimulus [212, 213]. Specifically, the anterior and posterior insular cortices (AIC, PIC), prelimbic and infralimbic cortices (PLC, ILC), and anterior cingulate cortex (ACC) have been shown to be dysregulated following chronic drug use and to have roles in craving, salience attribution, attention bias, and behavioral inhibition [138, 214-225]. The nucleus accumbens core and shell (NAcc, NAcs) and the ventral tegmental area (VTA) also have established roles in salience attribution, as well as in reward and aversion processing [25, 225-228]. The dorsal and ventral lateral septum (dLS, vLS) and the dorsal and ventral bed nucleus of the stria terminalis (dBNST, vBSNT) are regions that have been shown to modulate alcohol intake and stress-related behaviors [191, 229-235]. The anterior and posterior portions of the paraventricular nucleus of the thalamus (aPVT, pPVT) have been shown to be involved in decision-making under motivational conflict as well as in certain drug-seeking behaviors [236-240]. Lastly, the lateral habenula (LHb) and rostromedial tegmental nucleus (RMTg) have established roles in aversion and anti-reward processing [148, 204, 226, 228, 241, 242]. The identification of sex differences in neuronal activation during quinine-adulterated ethanol consumption will allow for future studies investigating the specific mechanisms responsible for aversion-resistant ethanol intake in females.

3.2 MATERIALS AND METHODS

Animals

Adult male and female C57BL6/J mice aged 10 weeks at the start of experimentation were used in these studies. Animals were singly housed on a reverse 12:12 light/dark cycle. All procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with NIH guidelines.

Ethanol two-bottle choice

Males and females (n=8/sex) were presented with two bottles in the home cage, one containing water and one containing increasing concentrations of ethanol on a continuous access schedule. Bottles were weighed every 24 hours (at the start of the dark cycle) to calculate g/kg intake, and bottle position was switched daily to prevent the development of a side preference. After 2–3 days of water only availability in both bottles, ethanol exposure began with 3% (v/v), and each ethanol concentration was presented for 4 days before being increased in increments of 3%.

Aversion-resistant ethanol intake

Following baseline ethanol consumption for concentrations ranging from 3-24% (v/v), males and females were reintroduced to the 15% ethanol solution for two weeks to establish a stable baseline. Animals were then exposed to 15% ethanol adulterated with increasing concentrations of the bitter tastant quinine hydrochloride (0.03, 0.1, and 0.3 mM). Previous work in our lab has demonstrated significant sex differences in aversion-resistant ethanol intake when quinine was at 0.03 and 0.1mM concentrations. Specifically, we have observed that while males significantly suppress their intake of

ethanol solution when it is adulterated with these quinine concentrations, females do not [106]. When the 0.3mM concentration is introduced, both males and females significantly suppress their ethanol consumption [106]. Therefore, 0.03, 0.1, and 0.3mM quinine concentrations (adulterated in 15% ethanol) were used for the following experiment. Each quinine concentration was presented twice, with 2 days of quinine-free ethanol two-bottle choice occurring between test days. Because we did not observe a significant sex difference in the percent change in ethanol intake with any of the previously mentioned quinine concentrations, an intermediate quinine concentration of 0.2mM was also tested.

Quinine-adulterated ethanol intake before perfusion

Beginning at the last two weeks of quinine-free baseline ethanol intake an additional measure was taken to assess intake in the first 2.5 hours of the dark cycle. Because a significant sex difference was observed in aversion-resistant ethanol intake at the 0.2mM quinine concentration (see Results), this concentration was used during the final 2.5-hour test period before tissue collection. Males and females (n=8/sex) were randomly assigned to undergo quinine-free or quinine-adulterated ethanol two-bottle choice (n=4/group) for the 2.5 hours prior to euthanasia.

Immunohistochemistry

Mice were injected with an overdose of ketamine/xylazine and intracardially perfused with 4% paraformaldehyde (PFA). Brains were extracted and post-fixed overnight at 4°C in 4% PFA before being transferred to 30% (w/v) sucrose solution. Tissue was then frozen on crushed dry ice and stored at -80°C until processing. 30µm sections were collected using a cryostat and stored in cryopreservant at -20°C until IHC was performed. Free-floating 30-µm tissue sections were washed 3 times in 1xPBS with

0.3% Triton (PBS-Tx), incubated with 0.3% hydrogen peroxide, and blocked using 2.5% normal horse serum at room temperature (ImmPress Horse Anti-Rabbit IgG Polymer Kit, Peroxidase, Vector Laboratories). Sections were then incubated with rabbit anti-cFos (Cell Signaling Technology #2250) diluted 1:5000 in PBX-Tx (0.3%) for 72 hours at 4°C on a shaker. Sections were washed 3 times with PBS-Tx (0.3%) and incubated with undiluted Rabbit ImmPress Horse HRP reagent at room temperature (ImmPress Horse Anti-Rabbit IgG Polymer Kit, Peroxidase, Vector Laboratories). Sections were washed once in PBS-Tx (0.3%), twice in 1xPBS (no Triton), and developed using diaminobenzidine (DAB) substrate (Vector Laboratories) for 3.5 minutes (aPVT, pPVT, and LHb) or 10 minutes (all other regions). Tissue was mounted onto gelatin-coated slides and allowed to dry at room temperature overnight. Slides were then placed through an ethanol dehydration series, cleared with xylene, and coverslipped (index #1.0) using Cytoseal (Fisher Scientific).

All regions examined were imaged at 40x using a Zeiss light microscope and quantified using ImageJ by an experimenter blind to treatment. Data are expressed as number of c-Fos-positive cells per field or as percent change relative to quinine-free ethanol groups, as indicated in Results.

Statistics

Statistical analyses were performed using Statistica software, and figures were created using GraphPad Prism. For analysis of baseline ethanol intake, unpaired student's t-test was conducted to assess sex differences in the average g/kg ethanol consumption for the 15% concentration. For analysis of aversion-resistant intake (0.03, 0.1, and 0.3mM quinine concentrations), mixed model two-way ANOVA was conducted using the

between subjects factor of sex and the within subjects factor of quinine concentration. The dependent variable was percent change from baseline ethanol consumption. For analysis of aversion-resistant ethanol intake at the 0.2mM quinine concentration, unpaired student's t-test was used. In contrast to data collected over a 24-hour period, data from the first 2.5-hours of intake revealed no sex difference in quinine-adulterated ethanol consumption. Therefore, we chose to analyze the effect of timepoint, i.e. 2.5-hour versus 24-hour sessions, on sex differences in quinine-adulterated intake. For analysis of the effect of timepoint, mixed model two-way ANOVA was conducted using the main factors of sex and timepoint. For analyses of neuronal activation, two-way ANOVA was conducted using the main factors of sex and quinine. The dependent variable was number of c-Fos-positive cells per field. For analysis of percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test was used. When appropriate, post-hoc analyses were conducted using Newman–Keuls test. Grubb's outlier test was conducted for each group and for all regions examined. Any data point identified as a statistically significant outlier was removed from that specific data set. The following data points were removed due to their identification as statistically significant outliers: one male-ethanol for PIC, one male-ethanol for ACC, one male-ethanol for vLS, one male-ethanol for LHb, one male-ethanol for VTA, and one female-quinine-ethanol for VTA.

3.3 RESULTS

Females consume significantly more quinine-free ethanol than males

Ethanol intake was monitored for 6 weeks during continuous access ethanol two-bottle choice. Intake in g/kg stabilized for males and females stabilized at the 15% concentration, and baseline intake was calculated as the average of the last 3 days of consumption. At baseline, females consumed significantly more ethanol in g/kg than males ($t_{14} = 2.96$, $P = 0.01$, Figure 3.1).

Females consume significantly more quinine-adulterated ethanol than males over a 24-hour period

Upon presentation of 0.03, 0.1, and 0.3mM quinine-adulterated ethanol solutions, no sex difference in aversion-resistant ethanol intake was observed. Specifically, both males and females exhibited aversion-resistance at the 0.03 and 0.1mM quinine concentrations, and both males and females significantly suppressed their ethanol intake at the 0.3mM quinine concentration (Figure 3.2A). Mixed model two-way ANOVA revealed a main effect of quinine concentration ($F_{2,28} = 11.27$, $P < 0.001$), no effect of sex ($F_{1,14} = 0.47$, $P = 0.51$), and no interaction between these factors ($F_{2,28} = 0.54$, $P = 0.59$). When an intermediate quinine concentration of 0.2mM was presented, males suppressed their intake to a higher degree than females ($t_{14} = 2.17$, $P = 0.048$, Figure 3.2B).

Males and females do not differ in quinine-free or quinine-adulterated ethanol intake during the first 2.5 hours of the dark cycle

While females exhibited aversion-resistant ethanol intake to a higher degree than males for the 24-hour measure (Figure 3.2B), no sex difference was observed at the 2.5-hour measure (Figure 3.3). Mixed model two-way ANOVA revealed a main effect of sex

($F_{1,14} = 4.86$, $P = 0.04$), a main effect of timepoint ($F_{1,14} = 5.27$, $P = 0.04$), and no significant interaction between these factors ($F_{1,14} = 1.69$, $P = 0.21$). Post-hoc analysis using Bonferroni correction revealed that there was no difference in aversion-resistance at the 2.5-hour measure ($P > 0.9999$), but that males suppressed intake to a higher degree than females at the 24-hour measure ($P = 0.04$).

Quinine-free and quinine-adulterated ethanol intake prior to perfusion

Males and females were randomly assigned to quinine-free or quinine-adulterated ethanol groups (n=4/group) for 2.5 hours of two-bottle choice before being perfused. At this timepoint, no sex differences in quinine-free or quinine-adulterated ethanol intake were observed, consistent with the data above. Two-way ANOVA revealed no main effect of sex ($F_{1,12} = 0.22$, $P = 0.65$), no effect of quinine ($F_{1,12} = 0.56$, $P = 0.47$), and no interaction between these factors ($F_{1,12} = 0.06$, $P = 0.80$, Figure 3.4).

Fos-mapping results

Following 2.5 hours of quinine-free or quinine-adulterated ethanol two-bottle choice, mice were perfused and brains collected for c-Fos IHC to visualize neuronal activation across various brain regions. Regions imaged and analyzed include: AIC, PIC, PLC, ILC, ACC, NAcc, NAcs, dLS, vLS, dBNST, vBNST, aPVT, pPVT, LHb, VTA, and RMTg. These regions were assessed based on their involvement in decision-making, specifically decision-making under conflict, salience, reward/motivation, and aversion [143, 148, 154, 212, 214, 219, 226, 228, 230, 243].

Cortical Regions

Within the AIC, two-way ANOVA revealed no effect of sex ($F_{1,9} = 0.05$, $P = 0.82$), no effect of quinine ($F_{1,9} = 1.83$, $P = 0.21$), and no interaction between these

factors ($F_{1,9} = 0.05$, $P = 0.83$) for the AIC (Figure 3.5). We also expressed c-Fos data for the quinine-adulterated ethanol groups as a percent change relative to that observed in mice consuming quinine-free ethanol. Unpaired student's t-test of these data revealed no sex difference in neuronal activation ($t_{1,6} = 0.31$, $P = 0.76$). Overall, neuronal activation within the AIC did not differ between males and females in response to ethanol or quinine-adulteration.

Two-way ANOVA of revealed no effect of sex ($F_{1,9} = 2.62$, $P = 0.14$), a main effect of quinine ($F_{1,9} = 6.61$, $P = 0.03$), and a significant interaction between these factors ($F_{1,9} = 16.66$, $P < 0.01$) for neuronal activation within the PIC. Post-hoc analyses using Newman-Keuls revealed that males consuming quinine-adulterated ethanol exhibited significantly higher levels of neuronal activation when compared to males consuming quinine-free ethanol ($P < 0.05$) and when compared to females consuming quinine-adulterated ethanol ($P < 0.01$). When c-Fos data for quinine-ethanol groups were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test also revealed a significant sex difference ($t_{1,5} = 5.67$, $P < 0.01$). These data demonstrate that males exhibit significant increases in neuronal activation following quinine-adulterated ethanol intake, whereas females do not.

Two-way ANOVA for the PLC revealed no effect of sex ($F_{1,9} = 0.28$, $P = 0.61$), no effect of quinine ($F_{1,9} < 0.001$, $P > 0.99$), and no interaction between these factors ($F_{1,9} = 0.17$, $P = 0.69$). When data for quinine-ethanol groups were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed no difference between males and females ($t_{1,5} = 1.07$, $P = 0.33$). These data demonstrate that

PLC activation does not vary between males and females nor in response to quinine-adulteration.

Within the ILC, two-way ANOVA revealed no effect of sex ($F_{1.8} = 0.05$, $P = 0.83$), no effect of quinine ($F_{1,8} = 1.90$, $P = 0.21$), and no interaction between these factors ($F_{1,8} = 0.003$, $P = 0.95$). Data for the quinine-ethanol groups were also expressed as a percent change relative to quinine-free ethanol groups. Unpaired student's t-test revealed no difference between males and females ($t_{1,5} = 0.27$, $P = 0.80$). These data demonstrate that ILC activation does not vary between males and females or as a result of quinine-adulteration.

While no statistically significant effects of sex ($F_{1,10} = 1.66$, $P = 0.23$) or quinine ($F_{1,10} = 1.22$, $P = 0.30$), nor an interaction between these effects ($F_{1,10} = 2.80$, $P = 0.13$) were observed within the ACC, there appeared to be a sex difference in neuronal activation in the quinine-free ethanol groups that did not extend to the quinine-adulterated ethanol groups (Figure 3.9A). When we then expressed c-Fos data for the quinine-adulterated ethanol groups as a percent change relative to that observed in mice consuming quinine-free ethanol, unpaired student's t-test revealed a significant sex difference in the percent change in neuronal activation relative to quinine-free ethanol groups ($P = 0.02$). These data suggest that males exhibit a greater increase in neuronal activation within the ACC during quinine-adulterated ethanol intake than females, when corrected for baseline differences in activation (Figure 3.9B).

Subcortical Regions

Within the NAcc, two-way ANOVA revealed no effect of sex ($F_{1,10} = 0.11$, $P = 0.75$) or quinine ($F_{1,10} = 0.09$, $P = 0.77$) on neuronal activation (Figure 3.10A).

Additionally, no interaction between these two factors was observed ($F_{1,10} = 2.61$, $P = 0.14$). When data were expressed as a percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test revealed a trend-level difference between males and females consuming quinine-adulterated ethanol ($P = 0.07$, Figure 3.9B).

For the NAcs, two-way ANOVA revealed no effect of sex ($F_{1,10} = 0.91$, $P = 0.36$) or quinine ($F_{1,10} = 0.49$, $P = 0.50$, Figure 3.10A). However, a trend-level interaction was observed ($F_{1,10} = 4.70$, $P = 0.056$). When data were expressed as a percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test revealed a significant difference between males and females ($P = 0.02$, Figure 3.10B). As observed in the ACC, males demonstrate a greater increase in neuronal activation within the NAcs during quinine-adulterated ethanol intake than females when corrected for differences in baseline activation.

Two-way ANOVA of neuronal activation within the dorsal portion of the lateral septum (dLS) revealed no effect of sex ($F_{1,11} = 2.19$, $P = 0.17$), a trend-level effect of quinine ($F_{1,11} = 4.47$, $P = 0.052$), and no interaction between these two factors ($F_{1,11} = 0.37$, $P = 0.56$, Figure 3.11A). When data were expressed as a percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test revealed a significant difference between males and females ($P = 0.03$, Figure 3.11B). These data suggest that males exhibit a greater increase in neuronal activation within the dLS during quinine-adulterated ethanol consumption compared to females when corrected for differences in baseline activation.

For the ventral portion of the lateral septum (vLS), two-way ANOVA revealed a main effect of sex ($F_{1,10} = 7.13$, $P = 0.02$), no effect of quinine ($F_{1,10} = 0.86$, $P = 0.38$), and no interaction between these factors ($F_{1,10} = 0.06$, $P = 0.80$, Figure 3.12A). When data were expressed as a percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test revealed no significant difference between males and females ($P = 0.11$, Figure 3.12B). These findings demonstrate that in the vLS, females exhibit significantly higher neuronal activation than males, regardless of whether mice were consuming quinine-free or quinine-adulterated ethanol.

While average neuronal activation was relatively low for all groups, analysis of the dorsal portion of the BNST (dBNST) using two-way ANOVA revealed a significant effect of sex ($F_{1,9} = 5.37$, $P = 0.046$) and of quinine ($F_{1,9} = 8.06$, $P = 0.02$), but no interaction between these factors ($F_{1,9} = 0.03$, $P = 0.87$, Figure 3.14A). When data were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed a significant sex difference ($t_{1,5} = 2.67$, $P = 0.04$). These data demonstrate that males exhibit higher neuronal activation within the dBNST during quinine-free and quinine-adulterated ethanol intake, and that quinine-ethanol induced increased activation in the dBNST in males and in females.

Within the vBNST, two-way ANOVA revealed no effect of sex ($F_{1,10} = 0.51$, $P = 0.49$), no effect of quinine ($F_{1,10} = 0.58$), and no interaction between these factors ($F_{1,10} = 1.63$, $P = 0.23$, Figure 3.15A). When data were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed no sex difference ($t_{1,6} = 1.62$, $P = 0.16$).

Diencephalon

Both males and females consuming quinine-free and quinine-adulterated ethanol displayed strong neuronal activation within the aPVT (Figure 3.16A). Two-way ANOVA revealed no effect of sex ($F_{1,11} = 0.92$, $P = 0.36$), no effect of quinine ($F_{1,11} = 1.67$, $P = 0.22$), and no interaction between these factors ($F_{1,11} = 2.49$, $P = 0.14$). When data were expressed as a percent change in neuronal activation relative to quinine-free ethanol groups, unpaired student's t-test revealed a trend-level difference between males and females ($P = 0.06$, Figure 3.16B). These data suggest that males may exhibit a greater increase in neuronal activation within the aPVT during quinine-adulterated ethanol intake than females when corrected for baseline differences in activation.

Two-way ANOVA of the pPVT revealed no effect of sex ($F_{1,12} = 0.002$, $P = 0.96$), a trend-level effect of quinine ($F_{1,12} = 3.40$, $P = 0.09$), and no interaction between these factors ($F_{1,12} = 0.27$, $P = 0.61$, Figure 3.17). When data were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed no sex difference ($t_{1,6} = 0.98$, $P = 0.37$). These data suggest that quinine-adulterated ethanol consumption lead to a modest increase in neuronal activation within the pPVT when compared to quinine-free ethanol intake, regardless of sex.

Neuronal activation within the LHb was relatively low among all groups, with the number of c-Fos-positive cells per field ranging from 0.5 ± 0 to 5.5 ± 3.2, and two-way ANOVA revealed no effect of sex ($F_{1,11} = 0.40$, $P = 0.54$), no effect of quinine ($F_{1,11} = 0.15$, $P = 0.71$), and no interaction between these factors ($F_{1,11} = 2.29$, $P = 0.16$). When data were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed no sex difference ($t_{1,6} = 1.87$, $P = 0.11$). Collectively, that

neuronal activation within the LHb is low among both males and females consuming quinine-free or quinine-adulterated ethanol.

Midbrain

Two-way ANOVA of the VTA revealed no effect of sex ($F_{1,9} = 2.42$, $P = 0.15$), a trend-level effect of quinine ($F_{1,9} = 3.47$, $P = 0.096$), and a significant interaction between these factors ($F_{1,9} = 10.54$, $P = 0.01$, Figure 3.19). Post-hoc analysis using Newman-Keuls revealed no significant difference in neuronal activation between males and females consuming quinine-free ethanol ($P = 0.48$) or between females consuming quinine-free and quinine-adulterated ethanol ($P = 0.35$). However, significant differences between males and females consuming quinine-adulterated ethanol ($P = 0.02$) and between males consuming quinine-free and quinine-adulterated ethanol ($P = 0.02$) were observed. When data were analyzed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed a significant difference between males and females ($t_{1,4} = 3.22$, $P = 0.03$). These data demonstrate that males consuming quinine-adulterated ethanol exhibit a significant increase in neuronal activation within the VTA, whereas females do not.

Neuronal activation was quite low for all groups within the RMTg, with the number of Fos-positive cells per field ranging from and 0 ± 0 to 2.6 ± 1.3. Two-way ANOVA revealed no effect of sex ($F_{1,10} = 1.79$, $P = 0.21$), no effect of quinine ($F_{1,10} = 0.0004$, $P = 0.99$), and no interaction between these factors ($F_{1,10} = 1.08$, $P = 0.32$). When data were expressed as a percent change relative to quinine-free ethanol groups, unpaired student's t-test revealed no sex difference ($t_{1,5} = 1.73$, $P = 0.14$). These findings demonstrate that males and females consuming quinine-free and quinine-adulterated

ethanol did not exhibit any significant differences in neuronal activation within the RMTg.

3.4 DISCUSSION

In the experiments described above, we found that when exposed to continuous access ethanol two-bottle choice, females consumed significantly more ethanol than males (Figure 3.1). While this finding is different from that observed in our previous study of sex differences in aversion-resistant ethanol intake [106], females have been shown to consume significantly more g/kg ethanol than males in several other studies [91, 92, 199-201]. Several studies have also suggested females may be more sensitive to ethanol's rewarding effects and less sensitive to its aversive properties [109, 185], potentially contributing to the increased ethanol intake often observed in females. However, it must be noted that females do not always consume significantly more ethanol than males [106, 183-185]. Preclinical studies of sex differences in ethanol intake have been conducted using rodents of differing ages and strains and have exposed subjects to varying ethanol concentrations and schedules of access. These factors likely contribute to differences in the literature [198, 199, 202, 203]. Of note, the only differences between the cohorts in Chapter 2 and the cohort in Chapter 3 for our experiments is that mice aged 10-12 weeks at the start of experimentation were used in Chapter 2 experiments, whereas all mice were 10 weeks at the start of experimentation for the experiment in Chapter 3. This slight age difference may contribute, at least in part, to the variability in baseline ethanol consumption between these cohorts.

When 15% ethanol solution was adulterated with 0.03, 0.1, and 0.3mM quinine hydrochloride, no sex differences in aversion-resistance were observed (Figure 3.2A). However, upon the presentation of ethanol solution adulterated with an intermediate quinine concentration (0.2mM), males decreased their intake significantly more than females (Figure 3.2B). While these findings contrast with those from our previous study (see Chapter 2), demonstrating inter-cohort variability for the quinine concentration at which sex differences in aversion-resistance is observed, the observation that females exhibit aversion-resistance to a higher degree than males remains consistent [106]. Interestingly, this sex difference was only observed over the course of a 24-hour two-bottle choice session, in that males and females do not display different levels of quinine-adulterated alcohol intake within first 2.5 hours of the dark cycle (Figure 3.3). These data suggest that when quinine-adulterated ethanol is presented, males decrease their intake over the course of 24 hours, whereas females do not.

Because we observed a sex difference in aversion-resistance at the 0.2mM quinine concentration, this solution was used in the test session prior to perfusion and tissue collection. Mice were randomly assigned to quinine-free or quinine-adulterated ethanol groups before undergoing two-bottle choice for 2.5 hours before perfusion. As observed in prior test sessions (see Figure 3.3), no sex differences in quinine-free or quinine-adulterated ethanol intake were observed (Figure 3.4). Of note, this lack of sex difference in 2.5-hour intake may strengthen our findings from subsequent c-Fos IHC, in that despite similar levels of exposure to quinine-ethanol solution, there are sex differences in regional activation following this exposure.

We then conducted c-Fos mapping to identify any potential sex differences in neuronal activation during consumption of quinine-free or quinine-adulterated ethanol. The only region in which females exhibited significantly higher neuronal activation than males in both groups was the vLS (Figure 3.9C). This region has reciprocal connections with various regions associated with addictive-like behaviors including, but not limited to, the NAc, VTA, and amygdala [244]. The vLS contains primarily GABAergic neurons and expresses high levels of receptors for oxytocin, vasopressin, corticotropin releasing factor (CRF), androgen, estrogen, and serotonin [230, 244-247]. Of note, CRF_2 and $5HT_{1A}$ receptors are the receptor subtypes for CRF and serotonin, respectively, that are present in this region [244]. Interestingly, the serotonin system has been shown to differ in males and females under drug-free conditions [248-250], and certain ethanol-induced alterations in this system also seem to be sexually dimorphic [251]. For example, following ethanol exposure during the early postnatal period, females exhibit a significant increase, and males a significant decrease, in serotonin levels within the septum [252]. As serotonin has an established role in various ethanol-seeking and -taking behaviors [29], altered activity within the vLS serotonin system may contribute to the observed increase in quinine-free and quinine-adulterated ethanol intake among females in this cohort.

We also observed a significant sex difference in neuronal activation within the dBNST in quinine-free and quinine-adulterated ethanol groups (Figure 3.14), in that males exhibited increased neuronal activation relative to females. The BNST is regarded as a sexually dimorphic region [253]and has been shown to respond strongly to aversive stimuli, including the aversive properties of alcohol [254, 255]. Previous work has

demonstrated that males may be more sensitive to alcohol's aversive properties [185, 256], and it is possible that the dBNST contributes to this increased sensitivity.

Within the dLS and dBNST, we observed a main effect quinine on neuronal activation (Figures 3.12A and 3.14A), in that quinine-adulterated ethanol groups exhibited higher activation than quinine-free ethanol groups. The dLS is a heterogenous region that expresses various neuropeptides, including somatostatin, substance P, enkephalin, neurotensin, and CRF [246, 247, 257], and activation of this region has been associated with the expression certain anxiety-like and defensive behaviors [258]. It is possible that the dLS is also activated following exposure to aversive stimuli, such as quinine, as a mechanism to promote avoidance. In addition to several neuropeptides such as SP, CRF, and neuropeptide Y (NPY), the dBNST receives noradrenergic projections from the ventral noradrenergic bundle and dopaminergic projections from the VTA, contributing to motivational salience [259, 260]. As described above, the BNST has also been shown to be contribute to aversion processing [254, 261]. Therefore, the increased activation of the dBNST observed in quinine-ethanol groups is likely attributable to the detection of the aversive, bitter tastant.

We also observed a trend-level effect of quinine on neuronal activation within the pPVT (Figure 3.17A), a region shown to be involved in decision-making during motivational conflict [239]. It must also be noted that the pPVT receives dense glutamatergic projections from the posterior insular cortex (PIC), a circuit with established roles in processing aversive taste [262]. Therefore, the increased pPVT activation in both males and females within the quinine-ethanol group suggests similar perception of quinine as a bitter tastant.

In several regions, we observed sex-specific increases in neuronal activation during quinine-adulterated ethanol consumption. For example, we identified a significant change in neuronal activation of the ACC in males consuming quinine-adulterated ethanol compared to males consuming quinine-free ethanol, whereas females in the quinine-adulterated ethanol group did not differ from females in the quinine-free group (Figure 3.5). This region is associated with value-based decision-making and salience attribution [213]. We observed similar effects in the NAc (Figure 3.7), which in addition to reward processing, is also involved in motivational salience [212]. Thus, it must be considered that sex differences in neuronal activations of these regions may be due to differences in salience attribution rather than reward sensitivity. Lastly, preclinical studies have demonstrated that stressful and aversive stimuli induce dopaminergic release into the NAcs [263, 264], which may also mediate the observed increase in NAcs activity in males during quinine-adulterated ethanol intake.

When data for neuronal activation within the aPVT were expressed as a percent change relative to quinine-free ethanol, we observed a trend-level difference between males and females in the quinine-adulterated ethanol group (Figure 3.16). Given the aPVT's role in decision-making under motivational conflict [239], the trend-level increase in neuronal activation observed in this region could contribute to males' decreased quinine-adulterated ethanol consumption.

Perhaps one of the most unexpected findings of this study was increased neuronal activation within the VTA of male mice consuming quinine-adulterated ethanol (Figure 3.17). Because neuronal activation within the VTA is typically associated with reward processing [25, 225], it was initially surprising that we observed increased activity in this

region for males, which exhibit aversion-resistance to a lesser degree than females (Figure 3.2B). However, it must be considered that the dopaminergic VTA neurons do not exclusively respond to stimuli with a positive valence [265]. For example, a subpopulation of VTA dopaminergic neurons are activated during the presentation of noxious or aversive stimuli [266, 267]. In addition to the potential contribution of dopaminergic neurons in the observed increase in VTA neuronal activation, GABAergic or glutamatergic neuronal activation must be considered as well. While the VTA consists primarily of dopaminergic cells (~65%), approximately 33% of VTA neurons are GABAergic, and the remaining 2% glutamatergic [268, 269]. It has been shown that exposure to an aversive stimulus such as footshock activates GABAergic interneurons within the VTA , inhibiting the activity of dopaminergic neurons in this region [270]. Lastly, the glutamatergic population in VTA has been shown to contribute to salience attribution, in that they are activated following exposure to rewarding and aversive stimuli [271]. Future directions will include triple-labeling of tyrosine hydroxylase (TH), glutamate decarboxylase (GAD), and c-Fos to identify whether dopaminergic, glutamatergic, or GABAergic VTA neurons are more strongly activated in males during quinine-adulterated ethanol intake.

We observed similar effects within the PIC, a region that has been shown to be hypoactive and/or reduced in size in humans with substance use disorders [218, 222, 272]. As this region has established roles in salience attribution, attention bias, and inhibitory control [223], PIC hypoactivity could contribute to decreased behavioral inhibition, and increased craving and drug-seeking. Interestingly, decreased PIC activation in women is associated with an increase in expectancy of alcohol's positive

effects, which is predictive of problem drinking [243]. It has also been demonstrated that a glutamatergic PIC-NAcc circuit serves to inhibit consummatory behaviors [273]. Subsequent experiments using c-Fos IHC in combination with retrograde and anterograde tract tracing will be conducted to elucidate the role of this circuit in quinine-adulterated ethanol consumption.

It is necessary to acknowledge an additional role of the insula, including the PIC, in taste processing, specifically that of aversive tastants [214, 273]. For example, it has been shown that intra-oral infusion of quinine leads to activation in the AIC and PIC [273], which suggests that while our behavioral data demonstrate no sex difference in sensitivity to the bitter tastant quinine, quinine-adulterated ethanol solution may be processed as more salient aversive stimulus in males than in females. Further experiments examining sex differences in PIC activation during quinine-water versus quinine-ethanol consumption will assess whether this effect is observed during ethanol-free conditions.

In summary, we found that activity was increased within the dLS, dBNST, and pPVT of animals exposed to quinine-adulterated ethanol, regardless of sex. We also found that activity of the vLS and dBNST were significantly different between males and females, regardless of whether animals were consuming quinine-free or quinine-adulterated ethanol. Lastly, we identified two regions in which quinine-ethanol-induced increases in activity were sex dependent, in that only males exhibited increased activity in the PIC and VTA during quinine-adulterated ethanol intake.

Overall, we demonstrated that females require higher quinine concentrations to suppress their ethanol intake, and that this compulsive-like behavior may be due to increased activity within the vLS. Additionally, greater sensitivity to quinine-adulteration

in males may be due to increased neuronal activation in the PIC and/or VTA. Retrograde and anterograde tract tracing in conjunction with c-Fos IHC will be utilized to identify neurons activated during quinine-adulterated ethanol intake in males that also receive projections from and/or project to the VTA and PIC. These circuits can then be pharmacologically or chemogenetically manipulated in females in efforts to decrease quinine-adulterated intake. These studies will serve to better characterize the mechanisms that contribute to compulsive-like ethanol consumption in females.

3.5 ACKNOWLEDGEMENTS

I would like to thank Kimberly Whiting for her assistance in quantifying all c-Fos IHC images for this study. I would also like to thank Kimberly Freeman and Rachael Hart-Earls for their assistance in developing and optimizing IHC protocols.

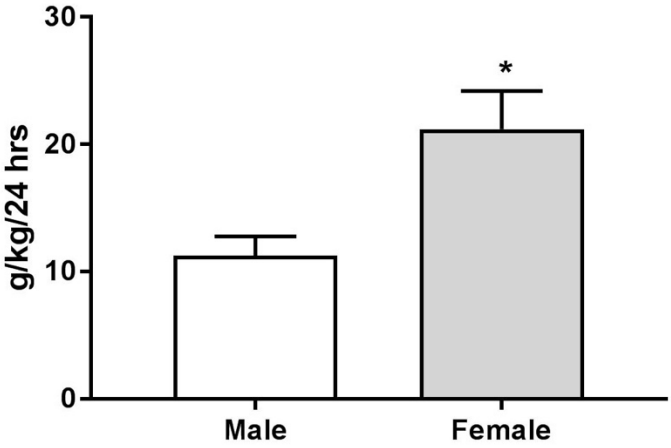
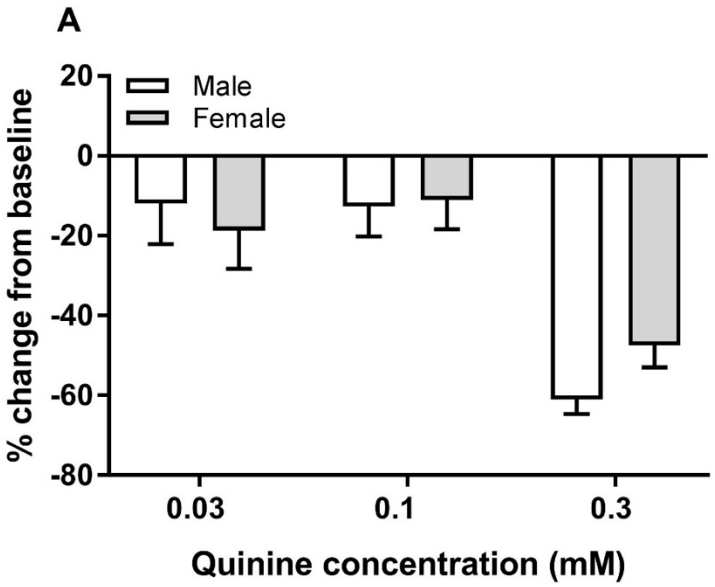

30
20
10
0
g/kg/24 hrs
*
Male
Female

Figure 3.1 Effect of sex on baseline ethanol intake. Average of the last 3 days of ethanol intake at the 15% concentration. Females consumed significantly more g/kg ethanol than males. *P = 0.01, n=8/sex.


A
Male
Female
20
0
-20
-40
-60
-80
% change from baseline
0.03
0.1
0.3
Quinine concentration (mM)

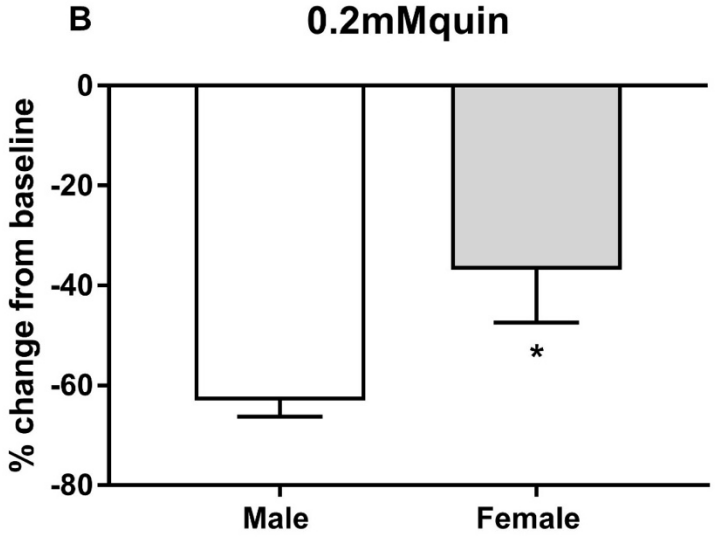

B
0.2mMquin
0
-20
-40
-60
-80
% change from baseline
*
Male
Female

Figure 3.2 Effect of sex on quinine-adulterated ethanol intake. (A) Mice were exposed to increasing concentrations of quinine hydrochloride adulterated in 15% ethanol. Data represented as percent change from baseline ethanol intake. No sex difference was observed in the percent change in ethanol intake at the 0.03, 0.1, or 0.3mM quinine concentrations. (B) Mice were exposed to an intermediate quinine concentration of 0.2mM adulterated in 15% ethanol. Data expressed as percent change from baseline ethanol intake. Unpaired student's t-test revealed a significant sex difference in the percent change in ethanol intake at the 0.2mM quinine concentration. **P* = 0.048, n=8/sex.

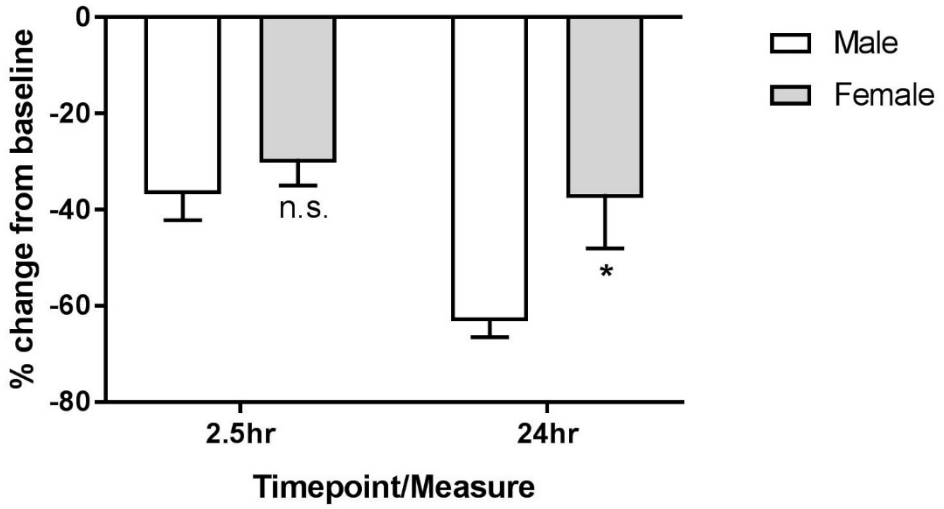

0
-20
-40
-60
-80
% change from baseline
n.s.
*
2.5hr
24hr
Timepoint/Measure
Male
Female

Figure 3.3 Effect of timepoint on sex difference in quinine-adulterated ethanol intake. Measures of ethanol intake were taken 2.5 hours following the introduction of solutions (at the start of the dark cycle) and every 24 hours. Mice were presented with 0.2mM quinine adulterated in 15% ethanol. Data represented as percent change in ethanol intake relative to baseline at the 2.5-hour and 24-hour timepoints. Sex difference in aversion-resistant ethanol intake was only observed at the 24-hour measure/timepoint. *P = 0.04, compared to males. n=8/sex.

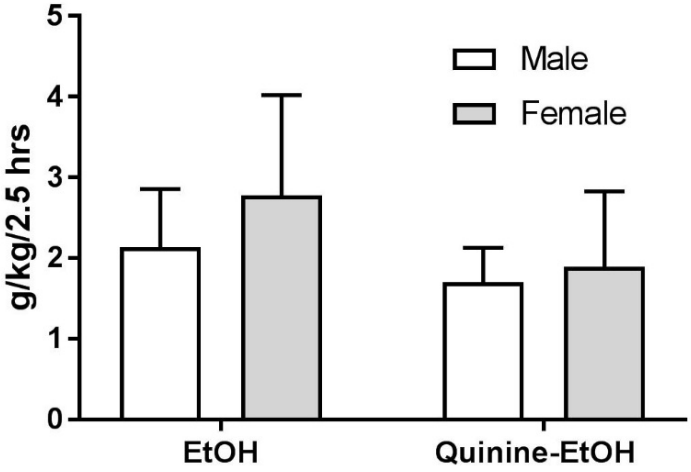

5
4
3
2
1
0
g/kg/2.5 hrs
Male
Female
EtOH
Quinine-EtOH

Figure 3.4 Quinine-free and quinine-adulterated ethanol intake before perfusion. Males and females were randomly assigned to undergo quinine-free 15% ethanol two-bottle choice or 0.2mM quinine-adulterated 15% ethanol two-bottle choice for 2.5 hours before being perfused. No sex difference or effect of quinine was observed during the 2.5-hour period before perfusion. Data expressed as mean ± SEM. n=4/group.

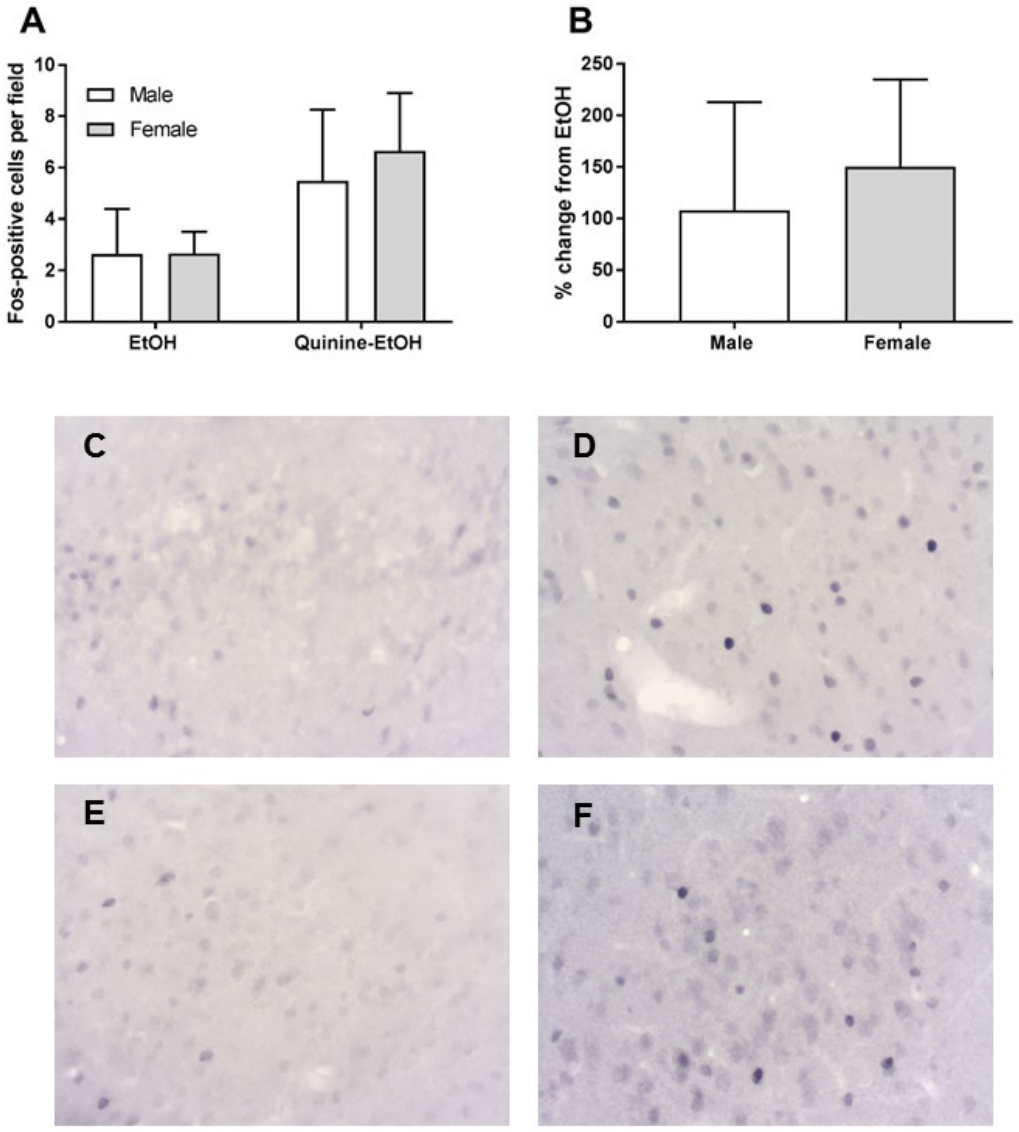

A
Fos-positive cells per field
10
8
6
4
2
0
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
250
200
150
100
50
0
Male
Female
C
D
E
F

Figure 3.5 Neuronal activation in the AIC. (A) Data expressed as mean ± SEM. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. n=2-4/group.

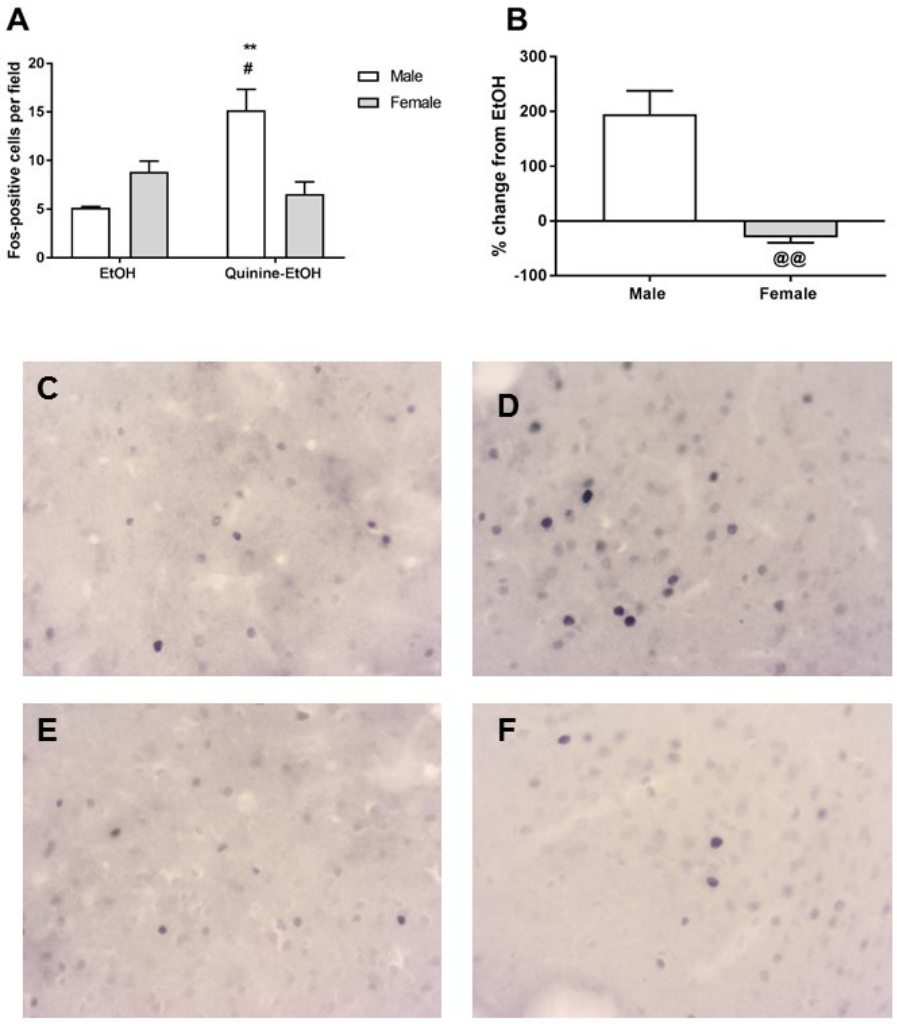

A
Fos-positive cells per field
20
15
10
5
0
**
#
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
300
200
100
0
-100
@@
Male
Female
C
D
E
F

Figure 3.6 Neuronal activation in the PIC. (A) Average number of Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. #$P < 0.05$, compared to EtOH males. **$P < 0.01$, compared to quinine-EtOH females. @@$P < 0.01$, compared to males. n= 2-4/group.

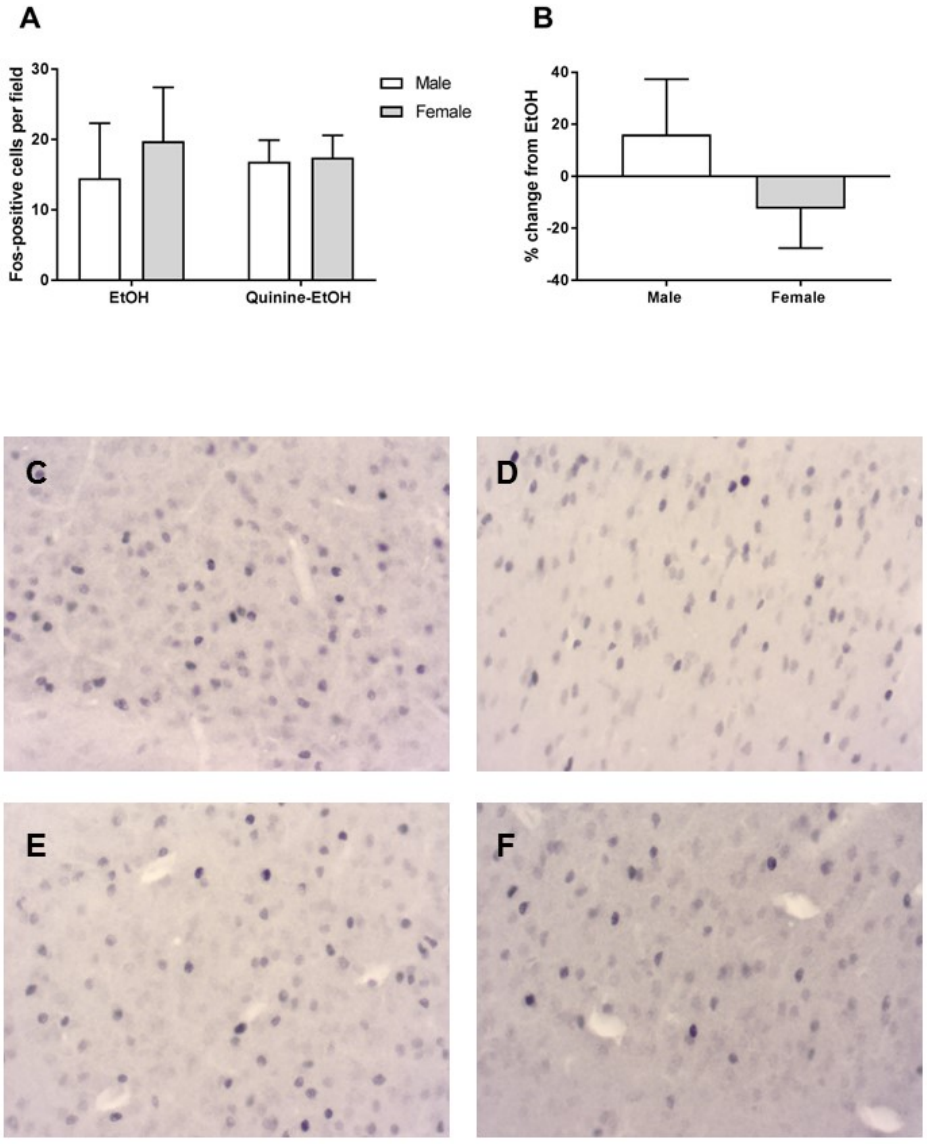

A
Fos-positive cells per field
30
20
10
0
EtOH
Quinine-EtOH
Male
Female
B
% change from EtOH
40
20
0
-20
-40
Male
Female
C
D
E
F

Figure 3.7 Neuronal activation in the PLC. (A) Average number of Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n= 3-4/group.

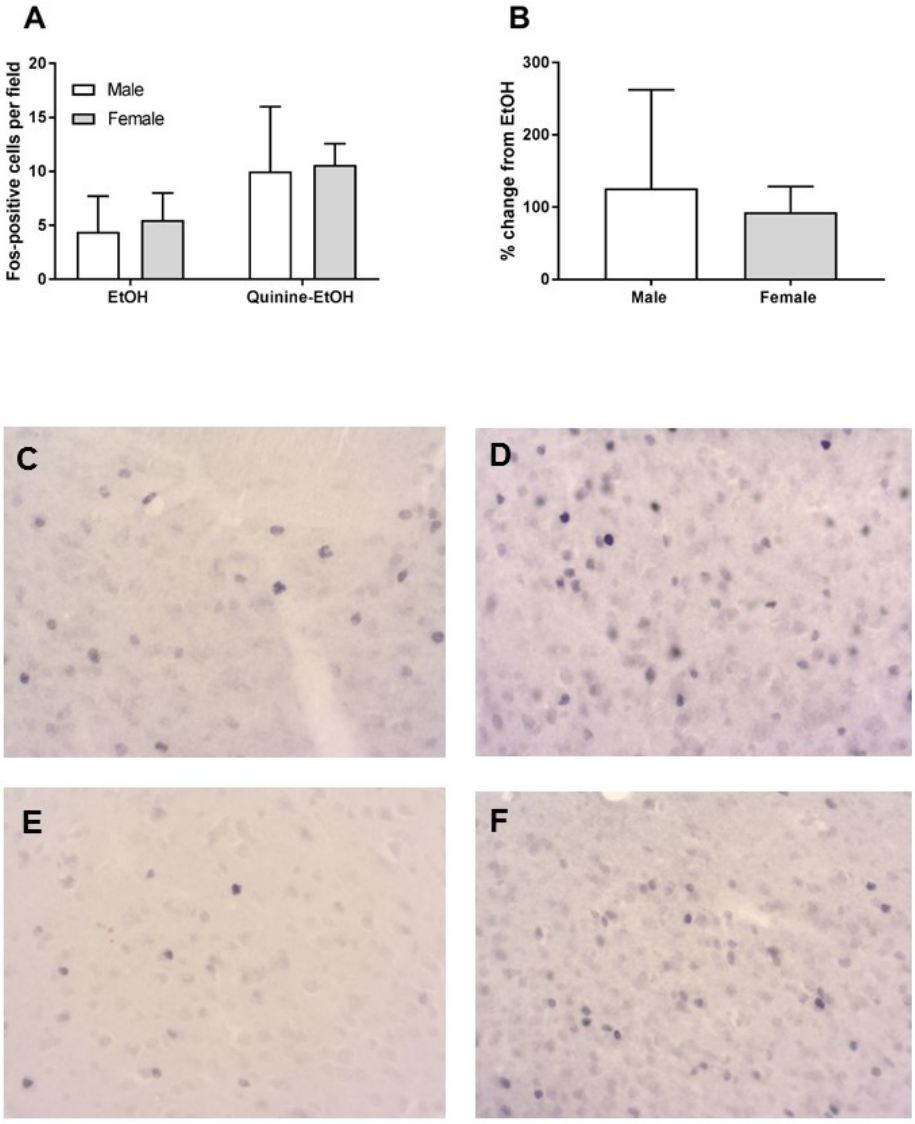

A
Fos-positive cells per field
20
15
10
5
0
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
300
200
100
0
Male
Female
C
D
E
F

Figure 3.8 Neuronal activation in the ILC. (A) Average number of Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n= 2-4 /group.

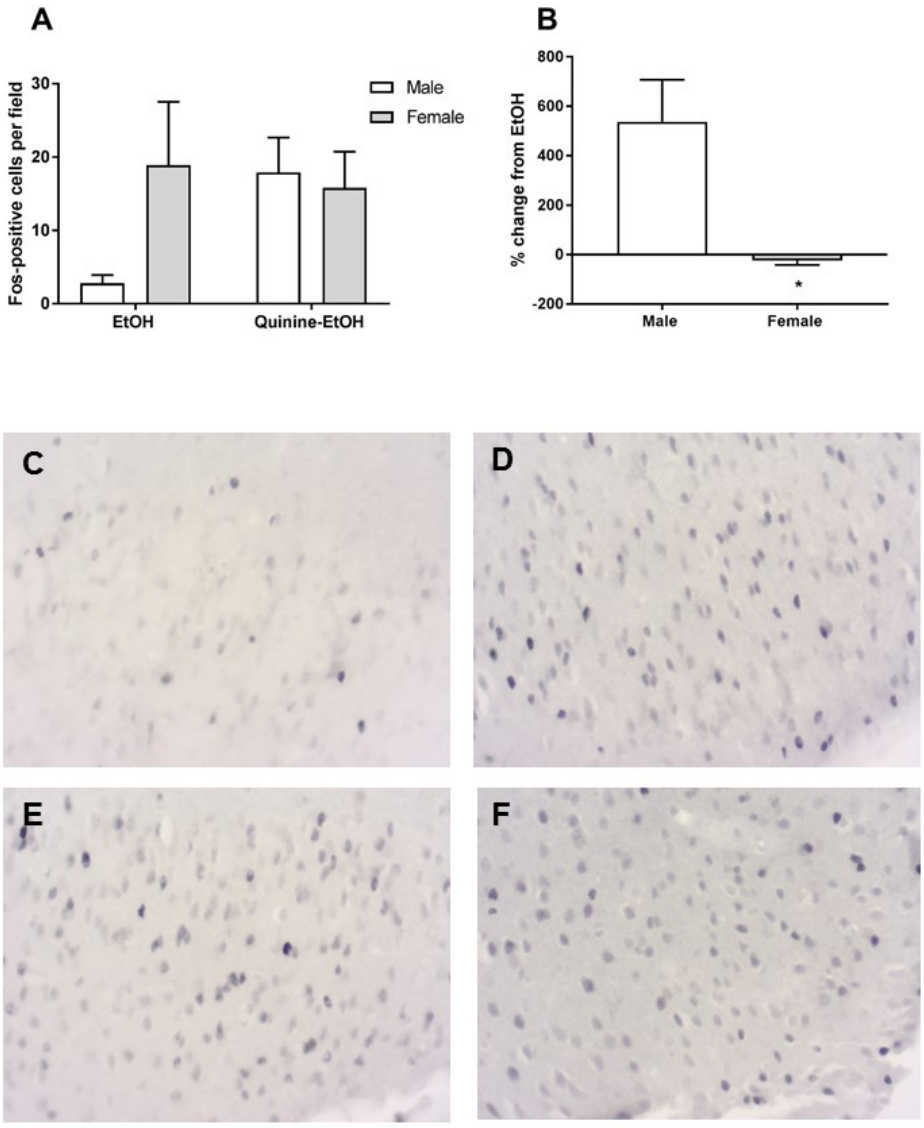

A
Fos-positive cells per field
30
20
10
0
EtOH
Quinine-EtOH
Male
Female
B
% change from EtOH
800
600
400
200
0
-200
*
Male
Female
C
D
E
F

Figure 3.9 Neuronal activation in the ACC. Males exhibit an increase in ACC neuronal activation during quinine-adulterated ethanol intake, whereas females do not. (A) Data expressed as mean ± SEM. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. $*P < 0.05$. n=3-4/group.

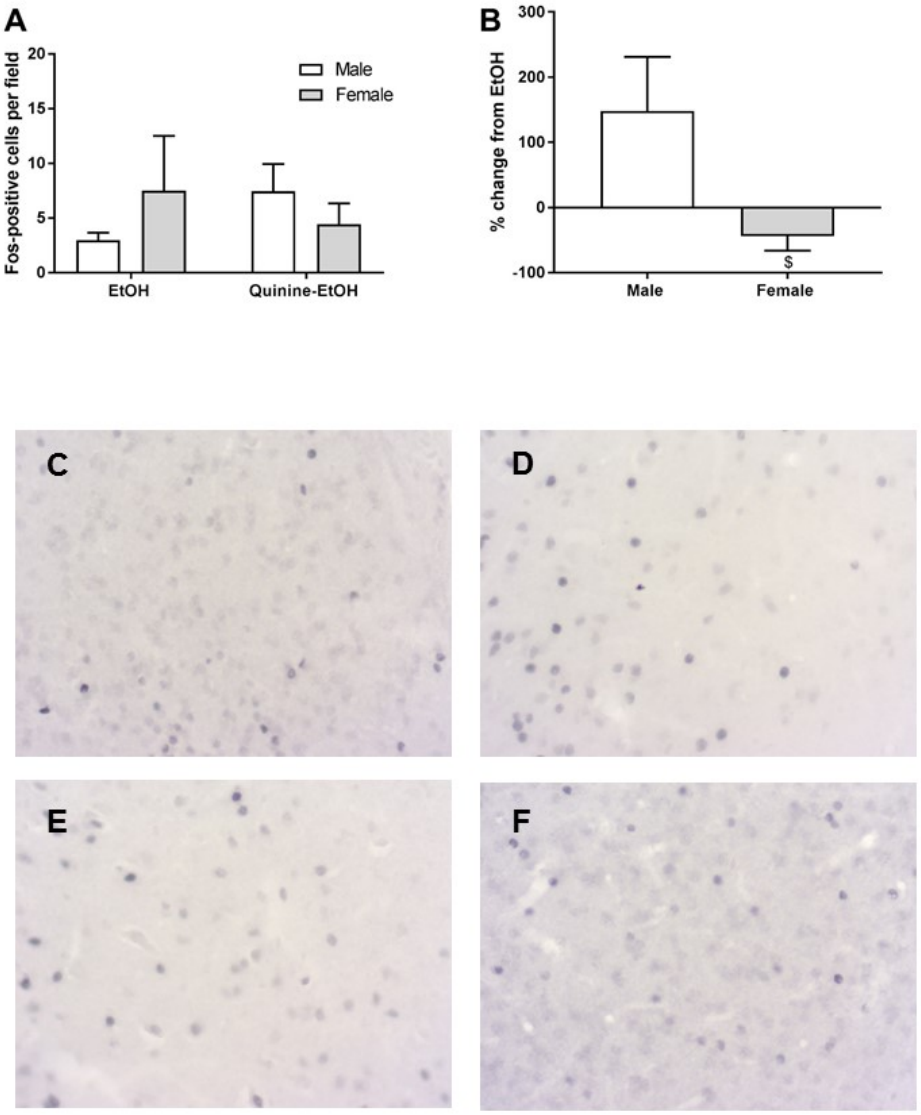

A
Fos-positive cells per field
20
15
10
5
0
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
300
200
100
0
-100
$
Male
Female
C
D
E
F

Figure 3.10 Neuronal activation in the NAcc. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. $*P* = 0.07. n=3-4/group.

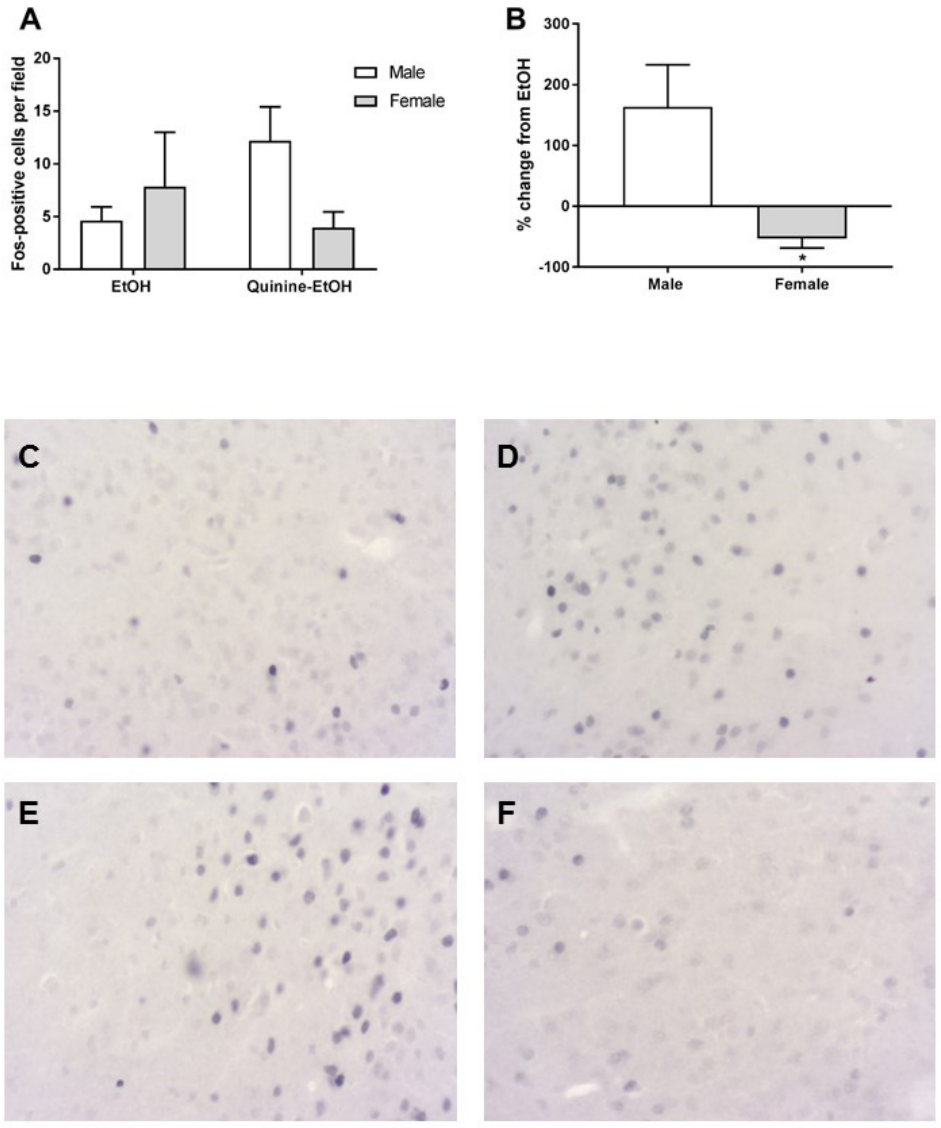

A
Fos-positive cells per field
20
15
10
5
0
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
300
200
100
0
-100
*
Male
Female
C
D
E
F

Figure 3.11 Neuronal activation in the NAcs. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n=3-4/group.

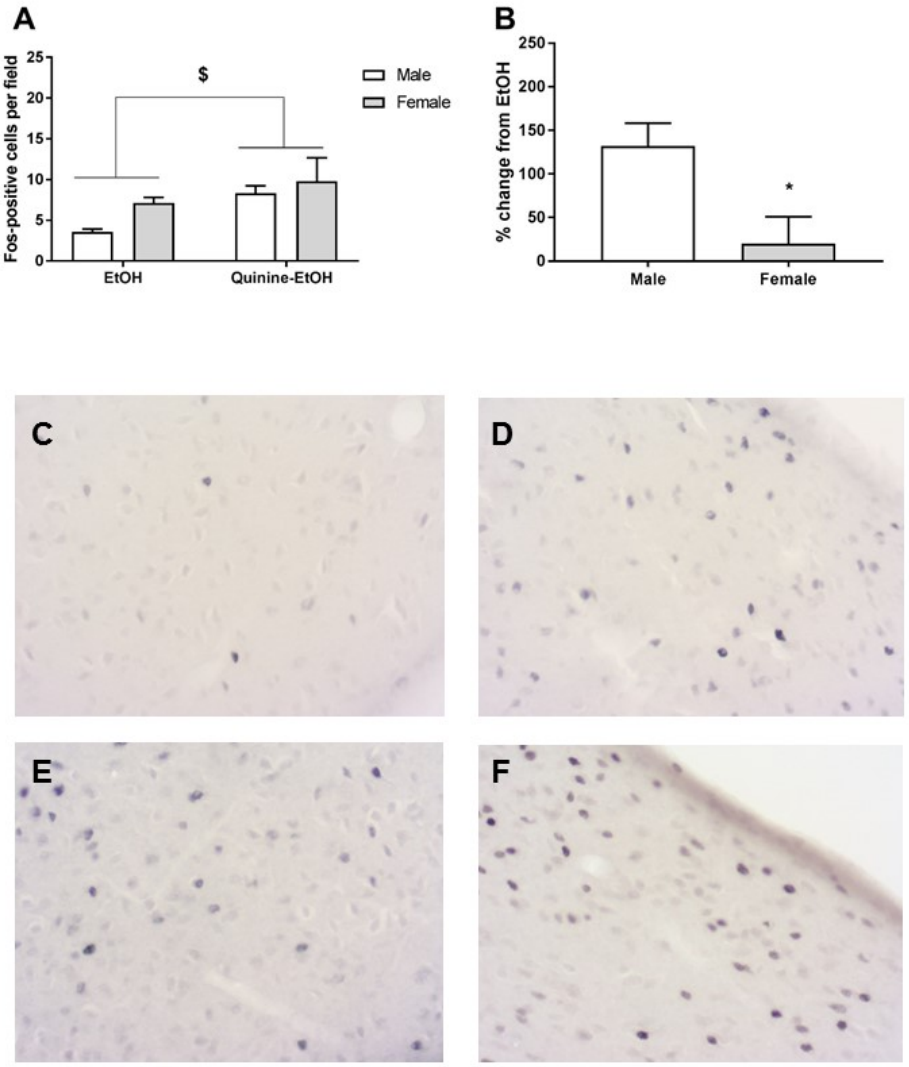

A
Fos-positive cells per field
25
20
15
10
5
0
$
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
250
200
150
100
50
0
*
Male
Female
C
D
E
F

Figure 3.12 Neuronal activation in the dLS. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. $\$P = 0.052$, compared to quinine-free EtOH groups. $*P < 0.05$, compared to males. n=3-4/group.

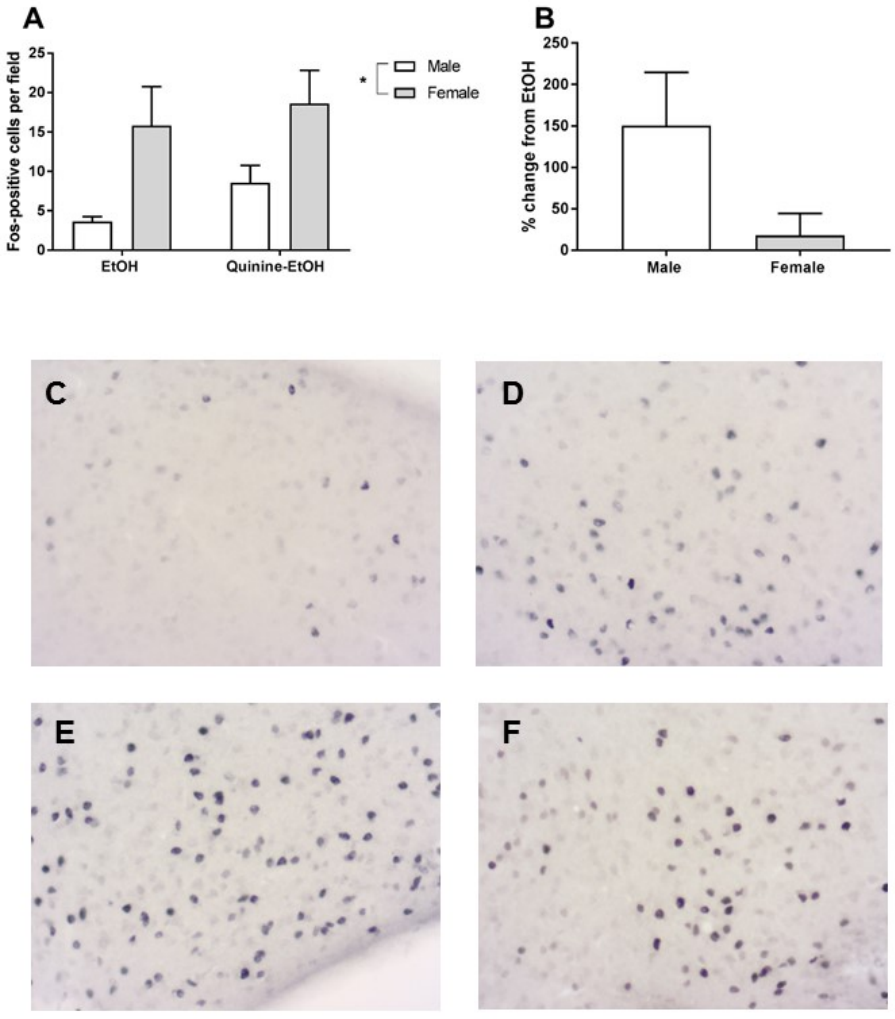

A
Fos-positive cells per field
25
20
15
10
5
0
EtOH
Quinine-EtOH
*
Male
Female
B
% change from EtOH
250
200
150
100
50
0
Male
Female
C
D
E
F

Figure 3.13 Neuronal activation in the vLS. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. *****$P < 0.05$, compared to males. n=2-4/group.

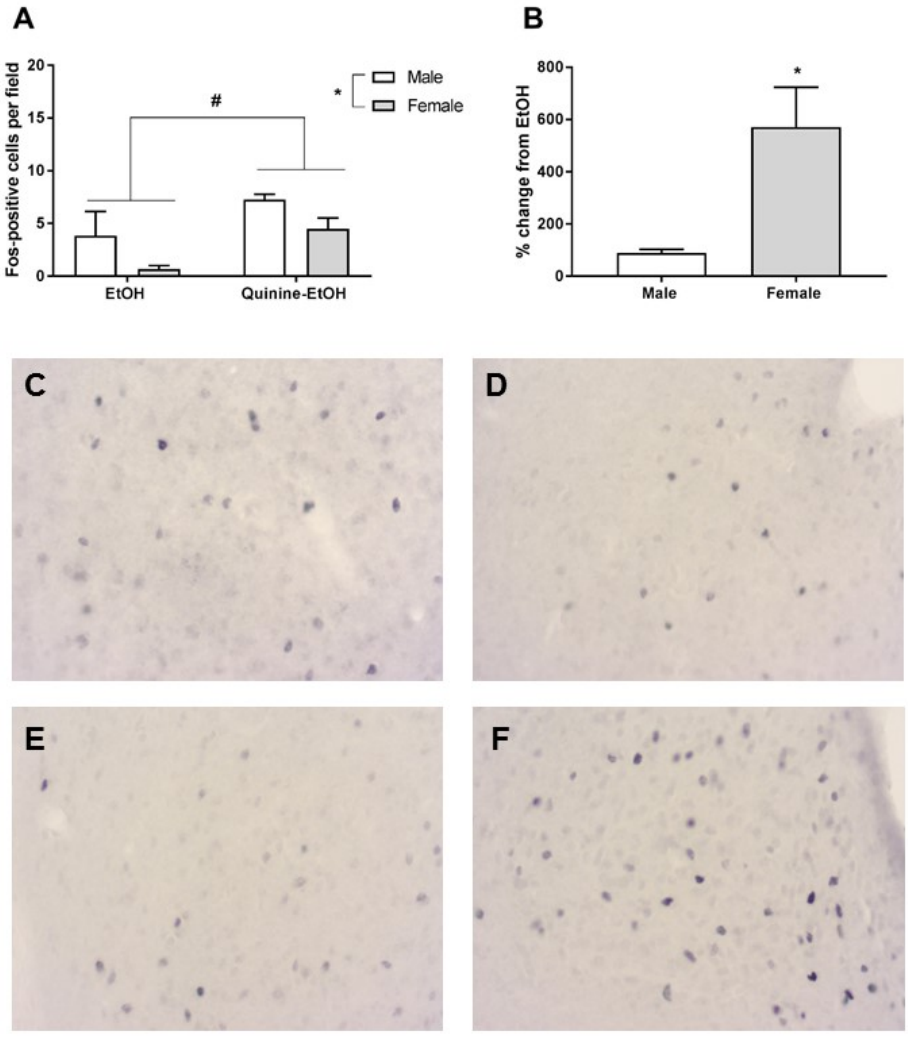

A
Fos-positive cells per field
20
15
10
5
0
#
*
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
800
600
400
200
0
*
Male
Female
C
D
E
F

Figure 3.14 Neuronal activation in the dBNST. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. *****$P < 0.05$, compared to males. #$P < 0.05$, compared to quinine-free EtOH groups. n=3-4/group.

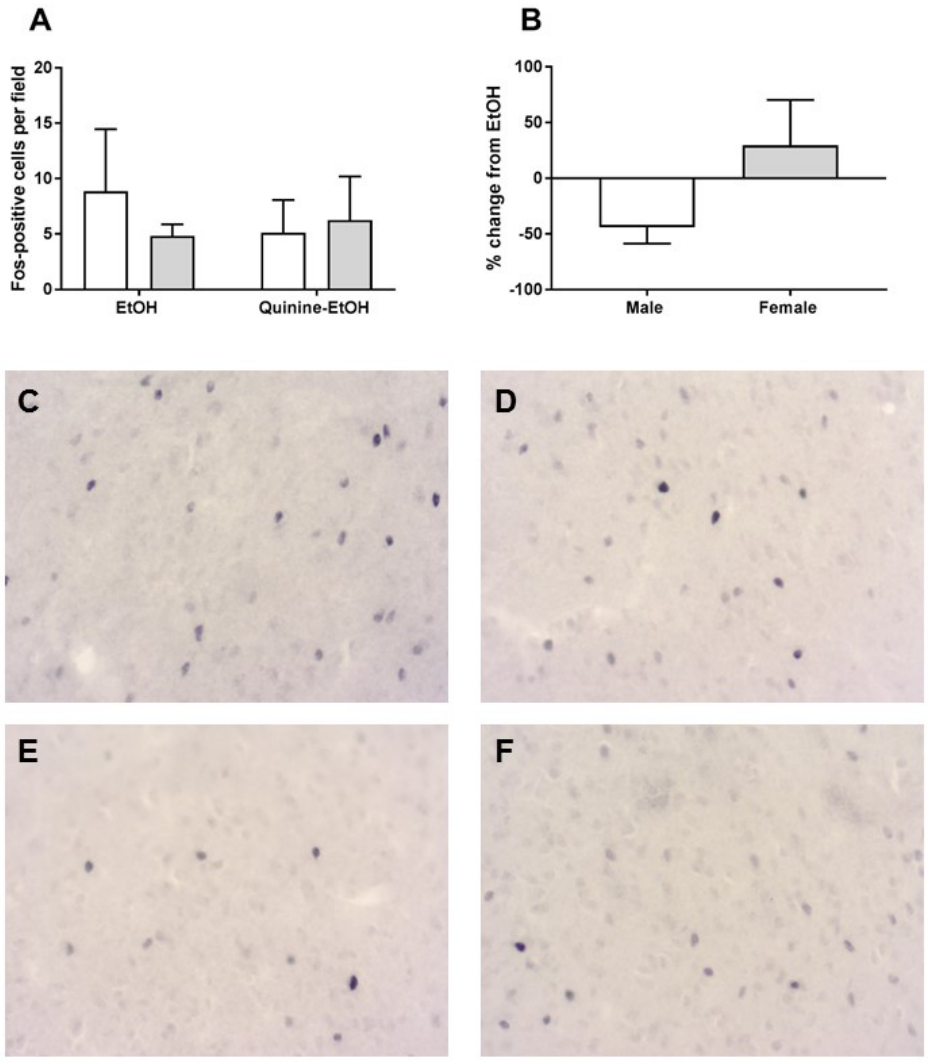

A
Fos-positive cells per field
20
15
10
5
0
EtOH
Quinine-EtOH
B
% change from EtOH
100
50
0
-50
-100
Male
Female
C
D
E
F

Figure 3.15 Neuronal activation in the vBNST. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n=3-4/group.

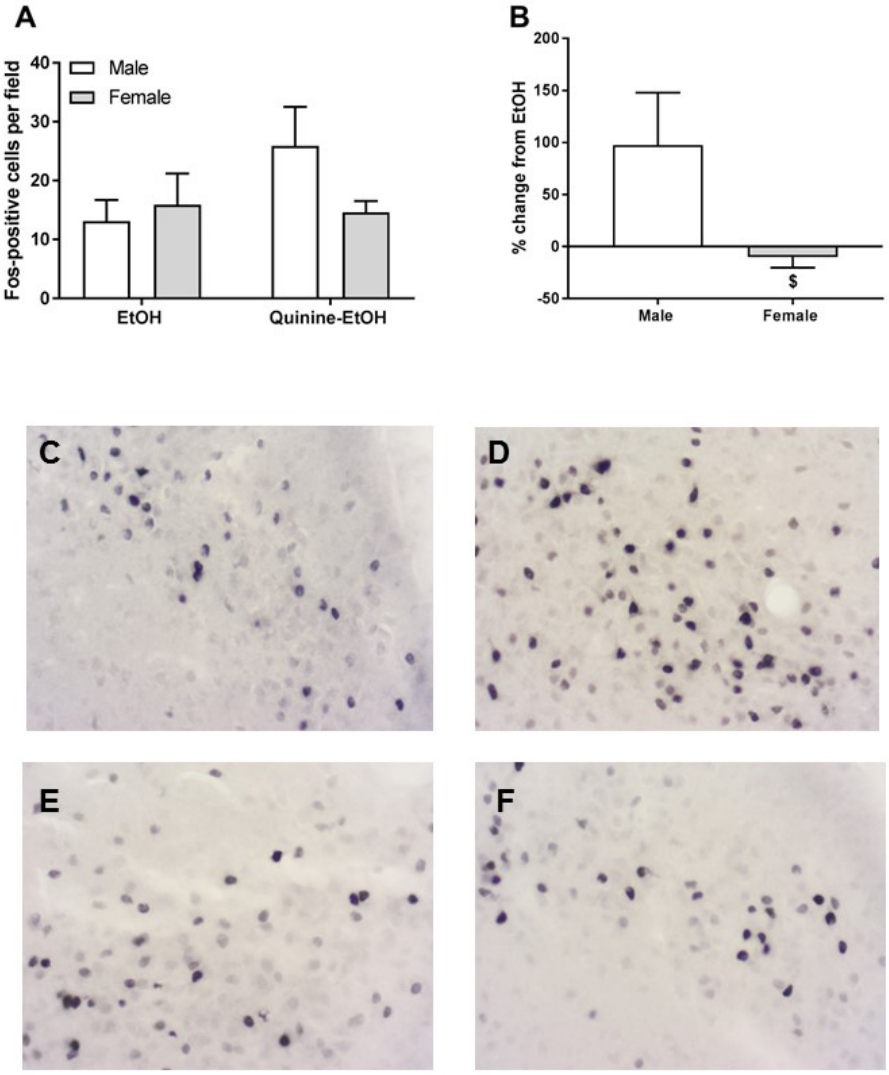

A
Male
Female
Fos-positive cells per field
40
30
20
10
0
EtOH
Quinine-EtOH
B
% change from EtOH
200
150
100
50
0
-50
$
Male
Female
C
D
E
F

Figure 3.16 Neuronal activation in the aPVT. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. **$***P* = 0.06. n=3-4/group.

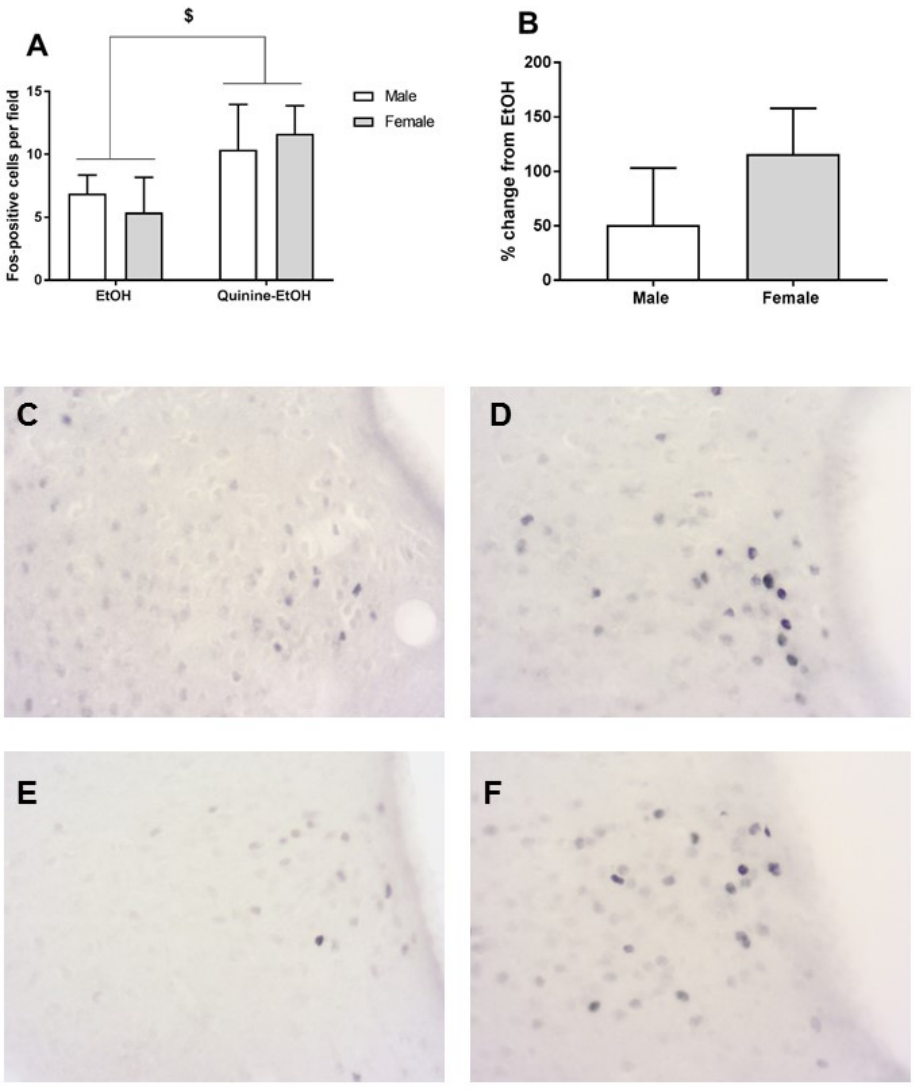

A
$
Fos-positive cells per field
15
10
5
0
EtOH
Quinine-EtOH
Male
Female
B
% change from EtOH
200
150
100
50
0
Male
Female
C
D
E
F

Figure 3.17 Neuronal activation in pPVT. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. **$***P* = 0.09, compared to quinine-free EtOH groups. n=4/group.

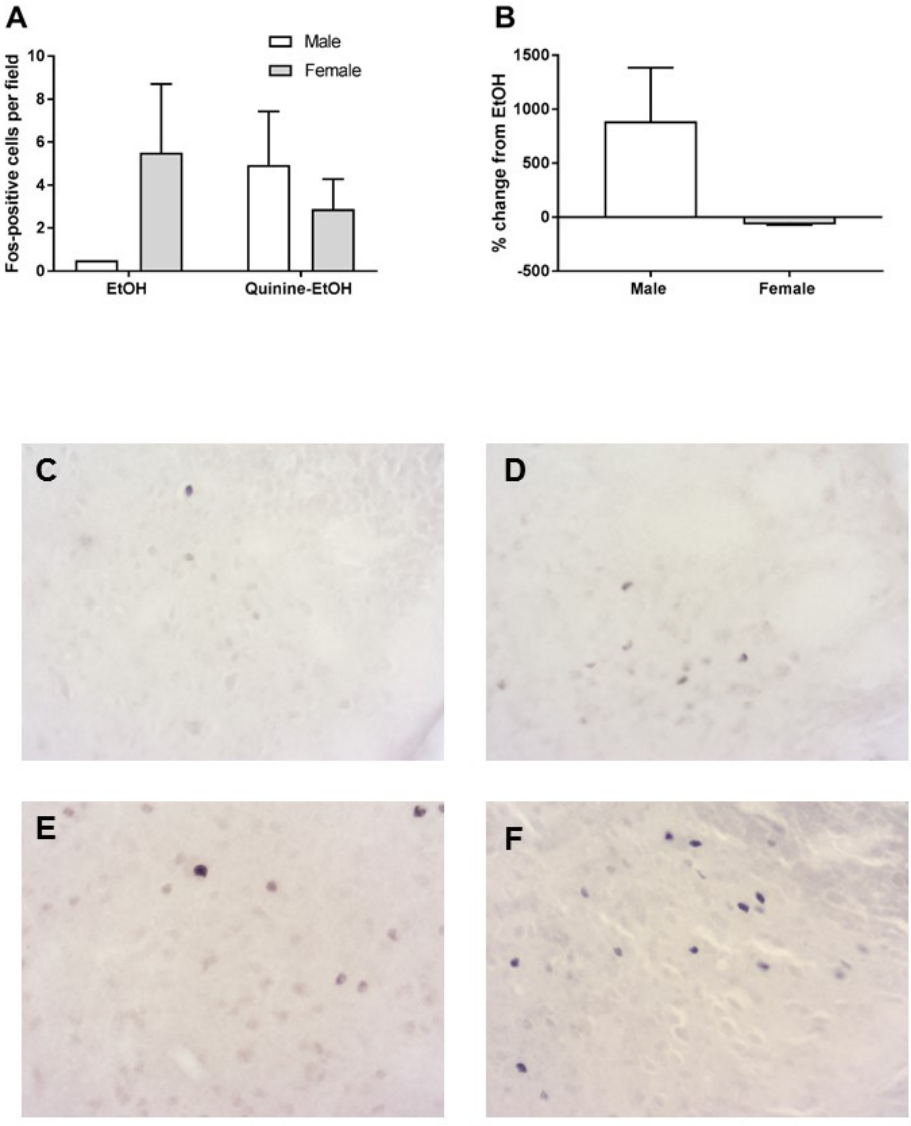

A
Male
Female
Fos-positive cells per field
10
8
6
4
2
0
EtOH
Quinine-EtOH
B
% change from EtOH
1500
1000
500
0
-500
Male
Female
C
D
E
F

Figure 3.18 Neuronal activation in the LHb. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n=2-4/group.

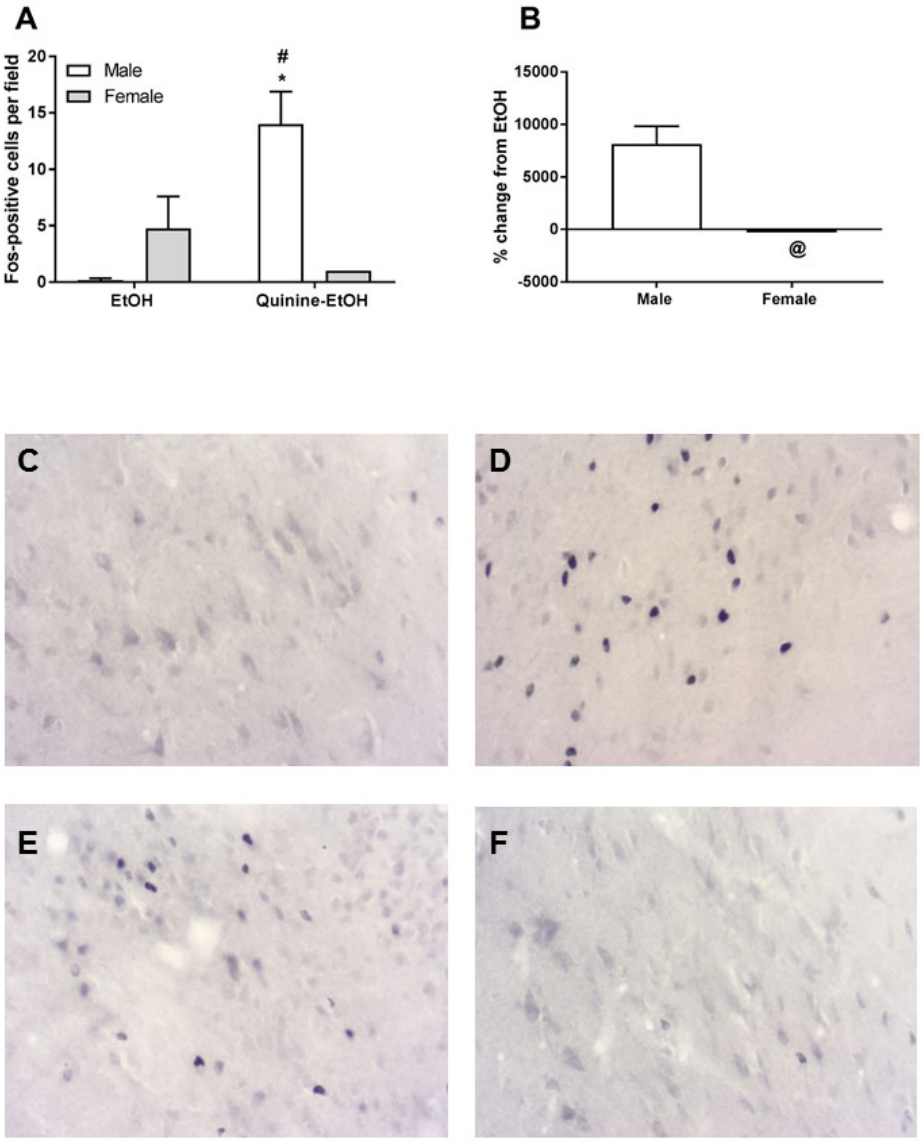

A
Fos-positive cells per field
20
15
10
5
0
Male
Female
#
*
EtOH
Quinine-EtOH
B
% change from EtOH
15000
10000
5000
0
-5000
@
Male
Female
C
D
E
F

Figure 3.19 Neuronal activation in the VTA. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. **P* < 0.05, compared to EtOH males. #*P* < 0.05, compared to quinine-EtOH females. @*P* < 0.05, compared to males. Images taken at 40x. n=2-4/group.

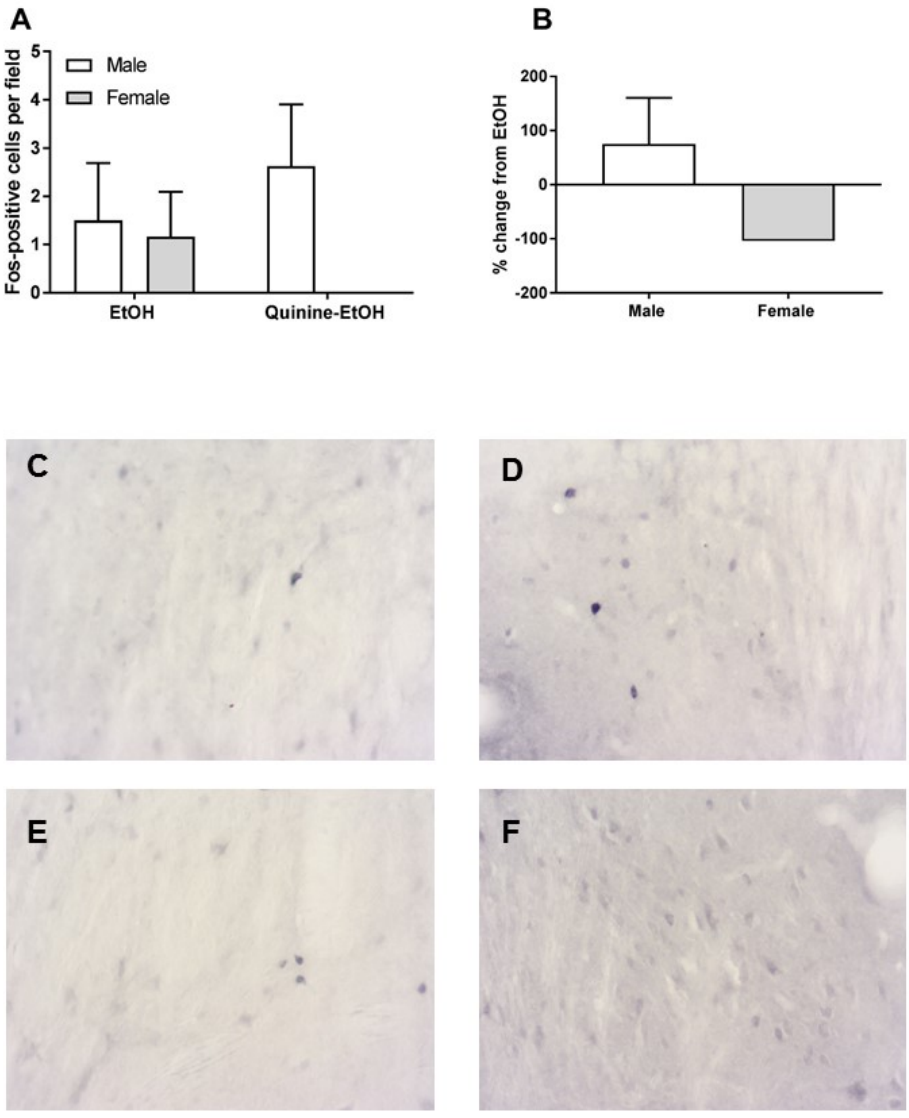

A
Fos-positive cells per field
Male
Female
EtOH
Quinine-EtOH
B
% change from EtOH
Male
Female
C
D
E
F

Figure 3.20 Neuronal activation in the RMTg. (A) Average number Fos+ cells/field. (B) Data expressed as percent change relative to quinine-free ethanol groups. All data expressed as mean ± SEM. Representative images of c-Fos IHC for male-EtOH (C), male-quinine-EtOH (D), female-EtOH (E), and female-quinine-EtOH (F) groups. Images taken at 40x. n=2-4/group.

Table 3.1 Summary of Fos-mapping data. A-P (mm) represents the approximate anterior-posterior coordinates relative to Bregma for regions imaged. EtOH represents groups consuming quinine-free EtOH. QEtOH represents groups consuming quinine-adulterated EtOH. Data represented as mean ± SEM. *****$P < 0.05$, compared to males. ^$P = 0.052$, compared to EtOH groups. **$**$P = 0.09$, compared to EtOH groups. ✢$P < 0.05$, compared to EtOH groups. #$P < 0.05$, compared to EtOH males. ¤P < 0.05, compared to quinine-EtOH females. n=2-4/group.

Region	A-P (mm)	Males-EtOH	Males-QEtOH	Females-EtOH	Females-QEtOH
AIC	+1.2	2.64 ± 1.76	5.49 ± 2.77	2.66 ± 0.84	6.65 ± 2.25
PIC ✢	-0.1	5.13 ± 0.13	15.17 ± 2.17 #¤	8.84 ± 1.11	6.56 ± 1.25
PLC	+1.4	14.5 ± 7.82	16.83 ± 3.09	19.75 ± 7.67	17.44 ± 3.14
ILC	+1.4	4.42 ± 3.31	10.0 ± 6.01	5.5 ± 2.5	10.63 ± 1.95
ACC	+0.4	2.81 ± 1.13	17.9 ± 4.77	18.92 ± 8.61	15.82 ± 4.91
NAcc	+1.2	2.96 ± 0.71	7.44 ± 2.50	7.5 ± 5.0	4.44 ± 1.90
NAcs	+1.2	4.63 ± 1.30	12.21 ± 3.20	7.83 ± 5.17	3.96 ± 1.51
dLS ^	+0.4	3.58 ± 0.36	8.31 ± 0.93	7.13 ± 0.69	9.79 ± 2.89
vLS *	+0.4	3.63 ± 0.63	8.56 ± 2.20	15.83 ± 4.92	18.65 ± 4.17
dBNST *✢	+0.1	3.83 ± 2.32	7.25 ± 0.52	0.67 ± 0.33	4.5 ± 1.02
vBNST	+0.1	8.83 ± 3.25	5.13 ± 1.48	4.83 ± 0.60	6.25 ± 1.97
aPVT	-0.2	13.13 ± 3.6	25.92 ± 6.63	15.88 ± 5.34	14.6 ± 1.95
pPVT $	-0.9	6.88 ± 1.48	10.38 ± 3.60	5.38 ± 2.79	11.63 ± 2.25
LHb	-1.2	0.5 ± 0	4.94 ± 2.49	5.5 ± 3.20	2.88 ± 1.41
VTA $	-3.2	0.17 ± 0.17	14 ± 2.89 #¤	4.75 ± 2.85	1.0 ± 0
RMTg	-3.8	1.5 ± 1.19	2.63 ± 1.28	1.17 ± 0.93	0 ± 0

CHAPTER 4: SEX DIFFERENCES IN ORAL OXYCODONE SELF-ADMINISTRATION AND STRESS-PRIMED REINSTATEMENT IN RATS

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4.1 ABSTRACT

The opioid epidemic has become a severe public health problem, with approximately 130 opioid-induced deaths occurring each day in the United States. Prescription opioids are responsible for approximately 40% of these deaths. Oxycodone is one of the most commonly abused prescription opioids, but despite its prevalent misuse, the number of preclinical studies investigating oxycodone-seeking behaviors are relatively limited. Furthermore, preclinical oxycodone studies that include female subjects are even more scarce, and it is critical that future work includes both sexes. Additionally, the oral route of administration is one of the most common routes for recreational users, especially in the early stages of drug experimentation. However, currently only two studies have been published investigating operant oral oxycodone self-administration in rodents. Therefore, the primary goal of the present study was to establish an oral oxycodone operant self-administration model in adult male and female rats, as well as to examine a potential mechanism of stress-primed reinstatement. We found that females consumed significantly more oral oxycodone than males in operant self-administration sessions. We also found that active oxycodone self-administration was reduced by mu opioid receptor antagonism and by substitution of water for oxycodone solution. Lastly, we induced stress-primed reinstatement and found that this behavior was significantly attenuated by antagonism of the neurokinin-1 receptor, consistent with our prior work examining stress-induced reinstatement of alcohol- and cocaine-seeking.

4.2 INTRODUCTION

Prescription opioid abuse is a severe public health issue in the United States, with approximately 17,000 prescription opioid-related deaths annually [274], and an estimated economic burden of $78.5 billion [275]. The current overdose rate is five times higher than in 1999 [274], highlighting the acceleration of the opioid addiction crisis over the last two decades. In addition to prescription opioid abuse being an issue in and of itself, epidemiological data suggest that it also increases the risk of later heroin abuse by approximately 40-fold [276-278]. As of 2013, approximately 75% of people seeking treatment for heroin addiction first began with nonmedical use of prescription opioids [279].

Oxycodone is one of the most frequently prescribed opioid analgesics, with approximately 59 million oxycodone containing prescriptions filled in 2013. This drug is available in several formulations, including under the trade name Roxicodone, as a high dose, slow release pill (OxyContin), and in combination with NSAIDs (Percocet) [280], each of which act as a potent mu opioid receptor (MOR) agonist [67]. While MOR agonism is responsible for the strong analgesic effects of oxycodone, this property is also responsible for its off-target reinforcing effects, giving this drug high abuse potential [69]. Because of these properties, and as the availability of oxycodone has continued to increase over time, misuse, dependence, and incidents of overdose have increased in parallel, culminating in our current epidemic [69, 281, 282]. [283]

Despite the prevalence of this issue, preclinical studies investigating oxycodone are underrepresented in the current addiction literature. A PubMed search for “rat oxycodone self-administration” yields only 27 results, compared with 583

results obtained for “rat morphine self-administration.” The vast majority of these oxycodone studies assessed intravenous (i.v.) self-administration (see, for example [113, 284-288]). While these studies and others have provided significant findings regarding i.v. intake, epidemiological data suggest that one of the preferred routes of administration (ROA) for prescription opioid abuse is oral, in that 72% of chronic abusers and 97% of recreational abusers report a preference for this method of use [58]. While many opioid abusers also report a high preference for snorting prescription opioids, the pharmacokinetic profile of which is more similar to an i.v. ROA [289], it should be noted that the majority of this population initiated their abuse using the oral ROA [290]. However, only two operant self-administration studies to date have investigated oxycodone-seeking using the oral ROA in rodents [167, 291]. Therefore, an increase in preclinical studies assessing oral oxycodone administration is crucial.

In addition to the need for more studies assessing oral oxycodone intake, inclusion of female subjects is essential to elucidate the role of sex in the progression of oxycodone use disorders. Currently, only two rodent oxycodone self-administration studies using both male and female subjects have been published [113, 167]. While a marked increase in prescription opioid-induced overdoses has been observed since 1999, this effect is even more pronounced in females, in that a seven-fold increase has been observed in females compared with a four-fold increase in males [292]. Some data also suggest that women transition from a state of regular opioid abuse to dependence more quickly than men, often referred to as the telescoping effect [84]. Lastly, women with opioid use disorder report significantly higher subjective craving for opioids than men [79], a factor that is known to contribute significantly to incidence of relapse [86].

Several therapeutic targets such as the MOR and dopamine D3 receptor have been identified for their potential to prevent certain oxycodone-seeking behaviors [285, 288, 293]. However, these studies have been conducted using solely male subjects and the i.v. ROA. The neurokinin 1 receptor (NK1R) is associated with stress, anxiety, and drug-seeking behaviors [294], and represents a valuable target for influencing opiate-related behaviors. Specifically, work from our group has demonstrated that NK1R antagonism significantly attenuates stress-primed reinstatement for various classes of drugs [295-297]. Additionally, NK1R antagonism has been shown to attenuate the reinforcing properties of opioids [298-301], making the NK1R system an especially promising target for development of therapeutics for those suffering from opioid use disorders. Therefore, a secondary objective of our study was to examine the effect of NK1R antagonism on stress-primed reinstatement of oxycodone seeking.

4.3 MATERIALS AND METHODS

Animals

Adult male and female Wistar rats (Charles River, Wilmington, MA) aged 10 weeks at the start of experimentation were used to conduct an oxycodone concentration response curve, baseline self-administration experiment, and estrous cycle monitoring. Adult male and female Long Evans rats (Charles River, Wilmington, MA) aged 10 weeks at the start of experimentation were used to assess baseline self-administration, estrous cycle monitoring, naloxone treatment, extinction, and stress-primed reinstatement experiments. After 1 week of acclimation to the animal facility, animals were handled daily for 3 days before beginning self-administration training. Animals were pair housed

on a reverse 12:12 light/dark cycle, and all experiments were conducted during the dark phase. Food and water were provided ad libitum, except where stated. All procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

Oral self-administration

Med Associates (Fairfax, VT) self-administration chambers were used for all experiments. Upon being placed in the chambers, levers were extended indicating the start of the 1-hour self-administration session. Subjects had access to an inactive lever and an active lever, in which inactive lever presses were recorded but had no consequence, and active lever presses resulted in the delivery of a 100-µl reinforcer into a trough. Following each session, a paper towel was placed in the trough to check for excess, non-consumed liquid to ensure that each subject was consuming all of the delivered liquid reinforcer. If a subject did not consume all of the delivered reinforcers, this animal was dropped from the study.

Concentration response curve

Subjects were water deprived for 22 hours/day for the first three self-administration sessions to encourage lever pressing. Rats were trained on a fixed-ratio 1 (FR1) schedule with 0.2 % (w/v) saccharin as the reinforcer. Following the first three days of water deprivation, animals self-administered saccharin for two additional sessions. Oxycodone (NIDA Drug Supply Program, Research Triangle Park, NC) solution was then introduced as the reinforcer at a concentration of 0.03 mg/ml (dissolved in tap water). Upon the introduction of oxycodone, sessions were conducted

using an FR1 schedule, with the addition of a cue light and 20-second timeout period following an active lever press. Rats self-administered the 0.03 mg/ml oxycodone concentration for five consecutive sessions before being exposed to increasing concentrations of oxycodone (0.1, 0.3, and 1.0 mg/ml). Each concentration was presented as the reinforcer for five consecutive sessions of FR1 self-administration. Following the 1.0 mg/ml concentration, the 0.1 mg/ml concentration was re-introduced to investigate whether lever presses would increase in efforts to titrate the dose of oxycodone consumed. For subsequent experiments, only 0.3 mg/ml oxycodone (no saccharin) was used in initial self-administration training, and the timeout period following active lever press was reduced from 20 to 5 seconds (see below).

Baseline self-administration.

As in the previously described experiment, subjects were water deprived for 22 hours/day for the first three self-administration sessions. Subjects self-administered oxycodone solution in 1-hour sessions on an FR1 schedule so that for every active lever press, 100 µl of 0.3 mg/ml oxycodone solution (dissolved in tap water) was delivered. The 0.3 mg/ml concentration was presented for 3-5 additional days following the end of water deprivation before being reduced to 0.1 mg/ml. Oxycodone concentrations and schedule used were determined based on data collected from preliminary experiments presented in Figures 4.1 and 4.2. When the oxycodone concentration was reduced to 0.1 mg/ml, animals continued self-administering on an FR1 schedule, and a 5-second timeout with cue light illumination was introduced. Sessions were 1 hour in duration and were performed once daily for 5 days/week.

Estrous cycle monitoring

The estrous cycle in rodents consists of four phases, each phase lasting approximately 24 hours [196]. Proestrus is characterized by peak estradiol and relatively high progesterone levels. This phase is followed by estrus, the phase in which estradiol levels begin to decrease and progesterone levels are relatively low. Estrus is then followed by the metestrus phase, which is characterized by low levels of both hormones. Metestrus is followed by diestrus, in which estradiol levels are low, and progesterone levels peak [196]. After self-administration rates stabilized (less than 20% variation in active lever presses over 3 consecutive days, following a minimum of 15 sessions), estrous cycle was monitored daily via vaginal lavage for four consecutive days. Immediately following self-administration sessions, animals were lightly restrained, and 100 μl 0.9% sterile saline was pipetted into the vaginal opening. The solution was aspirated and expelled several times, and the sample was pipetted directly onto a clean microscope slide. Slides were allowed to dry at room temperature and were stained with Toluidine Blue O for visualization using a Zeiss light microscope at 20x. For representative images of vaginal cytology for each estrous cycle phase, see Figure 4.3. Estrous cycle was also monitored in drug-naïve animals using the same methodology (see Figure 4.7).

Progressive ratio

After responding stabilized (as defined above) on the 0.1 mg/ml oxycodone concentration, a progressive ratio (PR) session was conducted. Under this schedule, the number of active lever presses required to result in the delivery of a reinforcer increases over the course of the session using the following schedule: 1, 2, 3, 4, 6, 8, 10, 12, 16,

and continuing to increase by 4. The session terminated if 30 minutes passed without the completion of the response requirement. We have used this specific PR schedule in studies examining oral alcohol self-administration [302]. The largest response requirement that the subject completed for one reinforcer is referred to as the breakpoint.

Naloxone pretreatment

Animals (n=4-6/sex) self-administered 0.1 mg/ml oxycodone before being treated with 0, 1, 3, or 10 mg/kg naloxone (Sigma-Aldrich; 1 ml/kg injection volume, 0.9% saline vehicle, i.p.) [303] 15 minutes before beginning the self-administration session. Of note, animals had undergone a total of 26 self-administration sessions before their first challenge with naloxone (eight sessions with the 0.3 mg/ml concentration and 18 sessions with the 0.1 mg/ml concentration). Doses and time of injection were selected based on previous literature [304-307]. Doses of naloxone were administered in a counterbalanced design, with 2 days of self-administration occurring between each treatment day.

Because we observed no effect of single dose of naloxone on oxycodone self-administration, we conducted a follow-up experiment in which repeated 10 mg/kg naloxone treatments were administered. This second set of naloxone treatments occurred 7 sessions after final naloxone pretreatment described above. Subjects were injected with vehicle or 10 mg/kg naloxone 15 minutes before self-administration sessions for 3 consecutive days. Subjects underwent 3 days of normal self-administration before receiving the opposite pretreatment in a counterbalanced design.

Replacement of oxycodone solution with water

Following naloxone treatments, animals were allowed to self-administer for at least 3 sessions, or until active lever presses were stable for males and females (<20% variability over last 3 sessions) on the 0.1 mg/ml oxycodone concentration. Once stable, oxycodone solution was replaced with water as the reinforcer (n=4-6/sex). This was to investigate whether subjects were able to distinguish between water and 0.1 mg/ml oxycodone by decreasing the active lever presses per session when water replaced oxycodone as the reinforcer. Subjects were said to have met extinction criterion when the number of active lever presses for water was at least 50% lower than the active presses for baseline oxycodone. Data from animals that did not meet extinction criterion were excluded.

Extinction and Reinstatement

Animals (n=10/sex) were allowed to self-administer 0.1 mg.ml oxycodone for 23 days before beginning extinction. Extinction sessions were run three times per day. Between each 1-hour extinction session, rats were placed in the home cage with food and water access for 10 minutes. During extinction sessions, oxycodone solution was replaced with water. This specific approach (water delivery as opposed to no lever press consequence) helped to confirm that rats were actively pressing for oxycodone containing solutions during previous baseline self-administration. The cue light continued to be activated following presses on the previously active lever. Animals underwent three extinction sessions per day until 75% of subjects met extinction criterion (active lever presses less than half of baseline), which took a total of 10 days, or 30 sessions. The animals that had not extinguished by this time point underwent three additional days of

three extinction sessions per day, and if criterion was not met by this point, self-administration data were excluded for these subjects. The data from two female and one male subject were excluded based on this criterion. In efforts to establish most conservative comparison between extinction and reinstatement responding, extinction response rate was quantified as the number of active lever presses exhibited during the first of the 3 extinction sessions that occurred on the last day before reinstatement testing.

For the first stress-primed reinstatement session, rats were placed in self-administration chambers, with levers withdrawn, and exposed to 15 minutes of unpredictable footshocks (0.8 mA intensity, 0.5-second duration, 45-second average intershock interval). Immediately following the end of footshock, levers were extended, and animals underwent a 1-hour reinstatement session, in which only water was delivered. Because males and females appeared to differ slightly in their degree of reinstatement following 0.8 mA footshock, we conducted two additional reinstatement sessions at different shock intensities (first, 0.4 mA, and then, 1.0 mA) to investigate whether shock intensity would affect reinstatement responding differently in males and females. Each stress-primed reinstatement session was separated by one day (three sessions) of extinction. The reinstatement data from one male subject were excluded due identification as a statistically significant outlier using Grubb's test. After the reinstatement sessions detailed above, animals were given an additional day (three sessions) of extinction. Next, subjects received i.p. injections of vehicle (2-hydroxypropyl-β-cyclodextrin; Sigma-Aldrich, 45% w/v) or the NK1R antagonist L822429 (15 mg/kg, 1 ml/kg injection volume; synthesized by K. Cheng and K. Rice at NIDA/NIAAA) 45 minutes prior to footshock exposure (0.8 mA, as above). Injection

time and dose were based on previous studies [295]. Following the 15-minute footshock session, levers were extended, and animals underwent a 1-hour reinstatement session as described above. As with the previous stress-primed reinstatement experiments, each test day was separated by 1 day (three sessions) of extinction. L822429 and vehicle pretreatments were administered using a counterbalanced design.

Serum oxycodone concentration analysis

Oxycodone serum concentration was analyzed as previously described [113, 308-311]. Rats were sacrificed via live decapitation and trunk blood was collected 15 minutes following intragastric gavage of 0.87 mg/kg (males) or 1.49 mg/kg oxycodone (females) at 1 ml/kg volume, dissolved in tap water. Doses administered were determined based on the average mg/kg oxycodone consumed for each sex during baseline oxycodone self-administration. Samples were collected in 1.5 ml tubes and centrifuged at room temperature at 7500 rpm. Serum was then stored at -20° until analysis by gas chromatography-mass spectrometry.

Statistics

GraphPad Prism software was used for graphing all results and Statistica software was used for data analyses. Data were analyzed using mixed model two-way ANOVA, repeated measures one-way ANOVA, or unpaired student's t-test as indicated in the Results section. When appropriate, post-hoc analyses were conducted using Bonferroni correction (Figures 4.1 and 4.9) or Fisher's LSD test (Figure 4.2). Planned Comparisons analyses were conducted for data represented in Figures 4.5A, 4.5E, and 4.5G.

4.4 RESULTS

Self-administration concentration response-curve: Wistar

Subjects were exposed to increasing concentrations of oxycodone solution and allowed to self-administer each solution on an FR1 schedule for 5 consecutive days. Data points represent the average of the last 3 days of self-administration for each oxycodone concentration (Figure 4.1). Repeated measures two-way ANOVA for active lever presses revealed a main effect of oxycodone concentration ($F_{5,40} = 9.69$, $P < 0.0001$), no effect of sex ($F_{1,8} = 2.74$, $P = 0.14$), and no interaction between these factors ($F_{5,40} = 1.09$, $P = 0.38$). Post-hoc analysis across the factor of oxycodone concentration using Bonferroni correction revealed that active lever presses for the 0.03 and 0.1 mg/ml oxycodone concentrations were significantly different than active presses for water ($P < 0.001$, $P = 0.024$, respectively). For inactive lever presses, repeated measures two-way ANOVA revealed a main effect of oxycodone concentration ($F_{5,40} = 3.87$, $P < 0.01$), no effect of sex ($F_{1,8} = 1.18$, $P = 0.31$), and no interaction between these factors ($F_{5,40} = 1.10$, $P = 0.37$). Post-hoc analysis across the factor of oxycodone concentration using Bonferroni correction revealed that inactive lever presses for the 0.03 mg/ml oxycodone concentration were significantly different than inactive presses for water ($P < 0.01$). However, no differences were observed for inactive presses for all other oxycodone concentrations when compared to water ($P > 0.9999$ for all concentrations).

Analysis of reinforcers earned using repeated measures two-way ANOVA revealed a main effect of oxycodone concentration ($F_{5,40} = 7.42$, $P < 0.0001$), no effect of sex ($F_{1,8} = 2.91$, $P = 0.13$), and no interaction between these factors ($F_{5,40} = 1.93$, $P = 0.11$). Post-hoc comparisons across the factor of oxycodone concentration using

Bonferroni correction revealed no significant differences in reinforcers earned for the 0.03, 0.1, 0.3, 1, or reintroduced 0.1 mg/ml oxycodone concentrations when compared to water ($P = 0.20$, $P = 0.10$, $P > 0.9999$, $P > 0.9999$, $P = 0.29$, respectively).

Lastly, analysis of oxycodone intake (measured in mg/kg) revealed that females consumed significantly more oxycodone than males. Repeated measures two-way ANOVA revealed a main effect of oxycodone concentration ($F_{4,32} = 40.26$, $P < 0.0001$), a main effect of sex ($F_{1,8} = 5.74$, $P = 0.044$), and a significant interaction between these factors ($F_{4,32} = 3.76$, $P = 0.013$). Intake was influenced by concentration of the solution available, which higher levels of intake at higher solution concentration. Also, females consumed significantly more oxycodone at the 1 mg/ml concentration when compared to males ($P = 0.011$).

To assess responding for 0.1 mg/ml oxycodone versus water, the average active lever presses for the last 3 days of re-introduced 0.1 mg/ml oxycodone were compared to average of last 3 days of water. Repeated measures two-way ANOVA revealed a main effect of oxycodone ($F_{1,8} = 5.33$, $P < 0.049$), no effect of sex ($F_{1,8} = 3.21$, $P = 0.11$), and no interaction between these factors ($F_{1,8} = 1.68$, $P = 0.23$), providing evidence that 0.1 mg/ml oxycodone was distinguishable from water. Average inactive lever presses for the last 3 days of re-introduced 0.1 mg/ml oxycodone were compared to average of last 3 days of water. Repeated measures two-way ANOVA revealed no effect of oxycodone ($F_{1,8} = 0.18$, $P = 0.68$), no effect of sex ($F_{1,8} = 0.84$, $P = 0.39$), and no interaction between these factors ($F_{1,8} = 1.15$, $P = 0.32$).

Baseline self-administration: Wistar

Baseline self-administration data (n=5-6/sex) in the Wistar strain revealed that females consumed significantly more oxycodone than males (Figure 4.2). Repeated measures two-way ANOVA of active lever presses (Figure 4.2A) revealed main effect of sex ($F_{1,9} = 6.16$, $P = 0.03$), a main effect of time ($F_{14,126} = 2.72$, $P < 0.01$), but no interaction between these factors ($F_{14,126} = 0.97$, $P = 0.49$). Post-hoc analysis using Fisher's LSD revealed significant sex differences in active lever presses on sessions 1, 3, and 5-14 ($P < 0.05$ for all comparisons). Unpaired student's t-test comparing the average active presses over the last three days of self-administration revealed a significant sex difference (Figure 4.2B, $t_{1,9} = 2.49$, $P = 0.03$).

Repeated measures two-way ANOVA of reinforcers earned (Figure 4.2C) revealed a trend-level effect of sex ($F_{1,9} = 4.97$, $P = 0.05$), a main effect of time ($F_{14,126} = 3.19$, $P < 0.01$), and a trend-level interaction ($F_{14,126} = 1.73$, $P = 0.06$). Post-hoc analysis using Fisher's LSD revealed significant sex differences in reinforcers earned on sessions 8-13 ($P < 0.05$ for all comparisons). Unpaired student's t-test comparing the average reinforcers earned over the last three days of self-administration also revealed a trend-level sex difference (Figure 4.2D, $t_{1,9} = 2.20$, $P = 0.06$).

Repeated measures two-way ANOVA of mg/kg intake (Figure 4.2E) revealed a main effect of sex ($F_{1,9} = 13.27$, $P < 0.01$), a main effect of time ($F_{14,126} = 2.45$, $P < 0.01$), and a trend-level interaction ($F_{14,126} = 1.67$, $P = 0.07$). Post-hoc analysis using Fisher's LSD revealed significant sex differences in oxycodone intake for sessions 1-15 ($P < 0.05$ for all comparisons). Unpaired student's t-test comparing the average mg/kg intake over

the last three days of self-administration also revealed a significant sex difference (Figure 4.2F, $t_{1,9} = 3.62$, $P < 0.01$).

Estrous cycle monitoring: Wistar

Estrous cycle was monitored every 24 hours via vaginal lavage immediately following oxycodone self-administration sessions for 4 consecutive days (n=9). For representative images of vaginal cytology samples for each estrous cycle phase, see Figure 4.3. Estrous cycle phase did not affect oxycodone self-administration in the Wistar strain (Figure 4.4A), in that repeated measures one-way ANOVA revealed no effect of estrous cycle phase number of active lever presses ($F_{3,32} = 0.02$, $P = 0.99$). Additionally, no effect of oxycodone self-administration was observed on estrous cycle phase (Figure 4.4B), evidenced by normal progression through the estrous cycle. Specifically, the average percent time spent in each estrous cycle phase was 25 ± 4.17 for proestrus, 22.22 ± 2.78 for estrus, 27.78 ± 2.78 for metestrus, and 25 ± 0 for diestrus. (Data reported as mean ± SEM).

Baseline self-administration: Long Evans

Similarly to the Wistar strain, baseline self-administration data (n=4-6/sex) in Long Evans rats revealed a significant sex difference, in that females consumed significantly more oxycodone than males, as measured by active lever presses, reinforcers earned, and mg/kg intake. Repeated measures two-way ANOVA of active presses (Figure 4.5A) revealed a main effect of time ($F_{23,184} = 2.06$, $P < 0.01$), and a significant interaction between these two factors ($F_{23,184} = 1.98$, $P < 0.01$), but no effect of sex ($F_{1,8} = 4.53$, $P = 0.07$). Planned comparisons analysis demonstrated that females pressed significantly more than males on sessions 19 ($P = 0.01$), 21 ($P = 0.03$), and 23 (P

= 0.01). Unpaired t-test comparing the average active presses over last three days of self-administration revealed a significant sex difference (Figure 4.5B, $t_8 = 3.63$, $P < 0.01$).

While repeated measures 2-way ANOVA of inactive lever presses (Figure 4.5C) revealed a main effect of time ($F_{23,184} = 3.16$, $P < 0.0001$), males and females did not differ in the number of inactive lever presses inactive lever presses, indicated by no significant effect of sex ($F_{1,8} = 0.03$, $P = 0.86$), and no interaction ($F_{23,184} = 0.87$, $P = 0.64$). Similarly, unpaired t-test comparing the average inactive presses earned over the last three days of self-administration revealed no significant difference between males and females (Figure 4.5D; $t_8 = 1.17$, $P = 0.28$).

Additionally, to ensure that active lever presses were consistently higher than inactive presses for both sexes, a repeated measures three-way ANOVA was conducted with the factors of sex, time, inactive/active lever. This analysis revealed a main effect of lever press, in that active lever presses were significantly higher than inactive lever presses ($F_{1,16} = 49.20$, $P < 0.0001$) over the course of oxycodone self-administration.

Repeated measures two-way ANOVA of reinforcers (Figure 4.5E) revealed a main effect of sex ($F_{1,8} = 6.01$, $P = 0.040$), a main effect of time ($F_{23,184} = 4.64$, $P < 0.0001$), but no interaction between these two factors ($F_{23,184} = 1.45$, $P = 0.09$). Planned comparisons analysis demonstrated that females received significantly more reinforcers than males on sessions 3 ($P = 0.03$), 17-20 ($P = 0.02$, $P = 0.04$, $P = 0.047$, $P = 0.03$, respectively), and 23 ($P < 0.01$). Unpaired t-test comparing the average reinforcers earned over the last three days of baseline oral self-administration (Figure 4.5F) also revealed a significant sex difference ($t_8 = 3.63$, $P < 0.01$).

Repeated measures two-way ANOVA of mg/kg intake (Figure 4.5G) revealed a main effect of sex ($F_{1,8} = 14.28$, $P < 0.01$), a main effect of time ($F_{23,184} = 2.61$, $P < 0.001$), but no interaction between these two factors ($F_{23,184} = 1.13$, $P = 0.32$). Planned comparisons analysis demonstrated that females consumed significantly more oxycodone than males on sessions 3-5 ($P = 0.01$, $P = 0.03$, $P = 0.02$) and sessions 9-24 ($P = 0.03$ for sessions 9, 10, 12, 13, and 22; $P = 0.02$ for sessions 15, 16, 18, 21, and 24; $P < 0.01$ for sessions 11, 17, 19, 20, and 23). Unpaired t-test revealed a significant sex difference in mg/kg intake (Figure 4.5H, $t_8 = 4.69$, $P < 0.01$).

Progressive ratio: Long Evans

After responding stabilized on the FR1 self-administration schedule, subjects underwent a single PR session, a measure of motivation for drug delivery. While the average breakpoint of females was higher than that of male subjects (mean ± SEM: females: 17.3 ± 2.7, males: 11.5 ± 2.1), unpaired t-test failed to detect a significant difference (Figure 4.5I, $t_8 = 1.57$, $P = 0.15$). Similarly, the average number of active lever presses during PR was higher in females (mean ± SEM: females: 87.5 ± 17.4, males: 51 ± 10.9), but unpaired t-test did not reveal a significant difference between sexes (Figure 4.5J, $t_8 = 1.56$, $P = 0.16$). Lastly, inactive lever presses did not differ between males and females for this session (Figure 4.5K, $t_8 = 0.67$, $P = 0.52$).

Estrous cycle monitoring: Long Evans

Estrous cycle was monitored every 24 hours via vaginal lavage immediately following oxycodone self-administration sessions for 4 consecutive days. Results of estrous cycle monitoring of Long Evans rats (n=6) pointed to a dysregulation of the estrous cycle, in that the order in which phases were observed and the percentage of time

spent in each phase seemed to be disrupted (Figure 4.6A-B). More specifically, the average percent time spent in each phase was 50.0 ± 12.9 SEM for proestrus, 29.2 ± 15.0 SEM for estrus, 20.8 ± 4.2 SEM for metestrus, and 0 for diestrus (Figure 4.6A). When estrous cycle was monitored for 4 consecutive days in age-matched, drug-naïve controls (n=6), we also found evidence of estrous cycle dysregulation, in that 4/6 rats were not observed in diestrus, and the percent time spent in each phase was irregular (Figure 4.7).

While our results assessing the effect of oxycodone self-administration on the estrous cycle remain inconclusive, we found strong evidence that estrous cycle phase did not affect oxycodone self-administration (Figure 4.6C-F). One-way ANOVA revealed no effect of estrous cycle phase on active lever presses (Figure 4.6C, $F_{2,10} = 0.09$, $P = 0.92$), inactive lever presses (Figure 4.5D, $F_{2,10} = 2.25$, $P = 0.16$), reinforcers earned (Figure 4.6E, $F_{2,10} = 0.03$, $P = 0.97$), or intake in mg/kg (Figure 4.6F, $F_{2,12} = 0.04$, $P = 0.96$).

Overall, these data demonstrate that oral oxycodone self-administration is not affected by estrous cycle phase. However, the effect of oral oxycodone self-administration in Long Evans rats remains unclear, in that estrous cycle phase also appeared to be somewhat abnormal in drug-naïve controls.

Naloxone treatment: Long Evans

Pretreatments with single administration of naloxone (0, 1, 3, 10 mg/kg) had no effect on oral oxycodone self-administration response rates (Figure 4.8A). Repeated measures two-way ANOVA revealed a main effect of sex ($F_{1,8} = 10.55$, $P = 0.012$), but no effect of naloxone treatment ($F_{3,24} = 0.45$, $P = 0.72$), nor an interaction between these two factors ($F_{3,24} = 0.33$, $P = 0.81$). Repeated measures two-way ANOVA also revealed that naloxone treatment had no effect on inactive lever presses (Figure 4.8B, $F_{3,24} = 1.68$,

$P = 0.20$). Additionally, there was no effect of sex on inactive lever presses ($F_{1,8} = 0.46$, $P = 0.52$) and no interaction between sex and naloxone treatment ($F_{3,24} = 0.50$, $P = 0.69$).

We hypothesized that the absence of a naloxone effect on active lever pressing may be the result of a delay in experiencing oxycodone's reinforcing properties following its oral self-administration. Thus, it may take multiple treatment days for the animal to learn that the reinforcing value of the oxycodone solution has been reduced because the animal may not fully sense this reduction in value until after the completion of the session. Therefore, we administered naloxone over 3 consecutive days, which effectively attenuated oxycodone self-administration rates in males and females (Figure 4.8C). Repeated measures two-way ANOVA revealed a main effect of sex ($F_{1,8} = 14.15$, $P < 0.01$), a main effect of naloxone treatment ($F_{1,8} = 14.98$, $P < 0.01$), but no interaction between these two factors ($F_{1,8} = 1.47$, $P = 0.26$). This result was specific for active lever presses, in that repeated naloxone treatment had no effect on inactive lever presses (Figure 4.8D, $F_{1,8} = 0.61$, $P = 0.46$). Consistent with previous data, there was no effect of sex on inactive lever presses ($F_{1,8} = 0.003$, $P = 0.96$) and no interaction between sex and naloxone treatment ($F_{1,8} = 1.75$, $P = 0.22$).

Replacement of oxycodone solution with water: Long Evans

Following naloxone treatments, animals underwent 3 days of oxycodone self-administration at the 0.1 mg/ml concentration. Once responding was stable (<20% variability over last 3 sessions) for males and females, oxycodone solution was replaced with water. For males and females, the number of active lever presses per session significantly decreased when oxycodone was replaced with water (Figure 4.9A). Data is expressed as the average number of active lever presses over the last 3 days of oxycodone

oral self-administration. Repeated measures two-way ANOVA revealed main effect of sex ($F_{1,8} = 12.96$, $P < 0.01$), a main effect of water replacement ($F_{1,8} = 67.71$, $P < 0.0001$), and a significant interaction between these factors ($F_{1,8} = 6.7$, $P = 0.03$). Post-hoc analysis using Bonferroni correction revealed that both males and females pressed significantly more for oxycodone than water ($P = 0.04$, $P < 0.001$, respectively). Additionally, while females pressed significantly more than males for oxycodone ($P < 0.01$), there was no sex difference in active lever presses for water ($P = 0.49$). The majority of these effects were specific to active lever presses. Specifically, repeated measures two-way ANOVA of average inactive lever presses revealed no effect of sex ($F_{1,8} = 2.29$, $P = 0.17$), a main effect of water replacement ($F_{1,8} = 10.27$, $P = 0.013$), and no interaction between these factors ($F_{1,8} = 0.01$, $P = 0.93$). While a main effect of water replacement was observed for inactive lever presses, post-hoc analysis across this factor revealed a significant difference between active presses at the 0.1 mg/ml oxycodone concentration and water ($P = 0.01$).

Extinction and Reinstatement: Long Evans

A separate cohort of male and female Long Evans rats (n=10/sex) was trained to orally self-administer 0.1 mg/ml oxycodone for at least two weeks or until stable (28 total sessions). Consistently with previous cohorts, female rats self-administered significantly more oxycodone when compared to males (Figure 4.10A, unpaired t-test of the average mg/kg consumed over the last 3 sessions of self-administration revealed a significant sex difference: $t_{18} = 65.2$, $P < 0.0001$). When water was substituted for oxycodone solution, lever pressing extinguished over time (Figure 4.10B). Repeated measures two-way ANOVA of the average lever presses at baseline compared to extinction revealed a main

effect of extinction ($F_{1,18} = 65.2$, $P < 0.0001$), indicating that lever responding decreased when water was substituted for oxycodone during extinction. However, there was no effect of sex ($F_{1,18} = 0.05$, $P = 0.82$), nor an interaction between these two factors (Figure 5A, $F_{1,18} = 0.13$, $P = 0.73$). This effect was specific for active lever presses, in that repeated measures two-way ANOVA of average inactive lever presses at baseline compared to extinction revealed no effect of extinction ($F_{1,18} = 2.68$, $P = 0.12$), no effect of sex ($F_{1,18} = 0.05$, $P = 0.82$), and no interaction between these factors ($F_{1,18} = 1.40$, $P = 0.25$, Figure 4.10C). These data support the hypothesis that oral oxycodone functions as an effective reinforcer and that its self-administration is extinguished when substituted with water.

After extinction, subjects underwent 15-minute footshock exposure (0.4, 0.8, or 1.0 mA) before the 1-hour reinstatement session (Figure 4.10D). Repeated measures two-way ANOVA of active lever presses revealed a main effect of shock ($F_{3,51} = 27.36$, $P < 0.0001$), no effect of sex ($F_{1,17} = 0.38$, $P = 0.55$), nor an interaction between these two factors ($F_{3,51} = 1.54$, $P = 0.22$). Post-hoc comparisons using Bonferroni correction across the factor of shock intensity indicated a significant increase in responding relative to extinction at all shock intensities used ($P < 0.0001$ for all comparisons). This effect was specific to active lever presses, in that repeated measures two-way ANOVA of inactive lever presses (Figure 4.10E) revealed no effect of shock ($F_{3,51} = 2.56$, $P = 0.07$), no effect of sex ($F_{1,17} = 0.30$, $P = 0.59$), and no interaction between these factors ($F_{3,51} = 0.09$, $P = 0.97$).

Collectively, these data indicate that males and females reinstate strongly following footshock stress exposure, and that this behavior is not sensitive to variations in

shock intensity within the range used. While both males and females reinstated following each shock intensity, males exhibited peak responding at the 0.8 mA intensity, whereas females exhibited peak responding at the 1.0 mA. Additionally, responding at the 0.8 mA appeared to be slightly greater in males than females. These data suggest that males may be more sensitive to some aspects of footshock-induced reinstatement, but further experiments with larger group sizes will be necessary to detect these subtle differences.

Systemic NK1R antagonism: Long Evans

Subjects were administered vehicle or 15 mg/kg L822429 (i.p.) 45 minutes before a 15-minute footshock session (0.8 mA) and 1-hour reinstatement session (Figure 5E). Dose and time of injection were based on previous experiments demonstrating efficacy for attenuation of stress-induced reinstatement of drug/alcohol seeking, and minimization of non-specific effects in the Long Evans strain [295]. Repeated measures two-way ANOVA of active lever presses revealed a main effect of NK1R antagonism ($F_{1,17} = 10.02$, $P < 0.001$), but no effect of sex ($F_{1,17} = 2.29$, $P = 0.14$), and no interaction between these two factors ($F_{1,17} = 2.74$, $P = 0.12$). This indicates that antagonist pretreatment reduced reinstatement responding in both male and female rats. Repeated measures two-way ANOVA of inactive lever presses revealed a main effect of sex ($F_{1,17} = 6.21$, $P = 0.015$), no effect of NK1R antagonism ($F_{1,17} = 0.58$, $P = 0.45$), and no interaction between these factors ($F_{1,17} = 0.28$, $P = 0.60$).

These data demonstrate that systemic NK1R antagonism significantly attenuates stress-primed reinstatement in Long-Evans rats. Consistent with the results shown in Figure 4.10D, reinstatement responding in males appeared to be slightly

higher than female rats under vehicle pretreatment conditions, but the effect of sex did not reach statistical significance.

Oxycodone serum concentration: Long Evans

After completing stress-primed reinstatement sessions, subjects (n=11-12/sex) were intragastrically gavaged with 0.87 mg/kg (males) or 1.49 mg/kg oxycodone (females). Doses administered for oral gavage were determined based on the average mg/kg oxycodone consumed for each sex during baseline self-administration. The average serum concentrations were 2.36 ± 0.4 ng/ml and 15.42 ± 3.9 ng/ml for males and females respectively (with data represented as mean ± SEM). These concentrations are similar to those obtained by humans following oral consumption of oxycodone.

4.5 DISCUSSION

In addition to establishing a protocol for operant oral oxycodone self-administration in rats, these experiments revealed a significant sex difference in oxycodone intake. Females consumed significantly more than males, a phenomenon that has been observed under certain conditions in several preclinical studies of drugs of abuse [94, 116, 117, 312]. Mavrikaki and colleagues specifically demonstrated that females self-administer significantly more i.v. oxycodone than males [113]. These data, in conjunction with our current findings, provide strong evidence of a sex difference in oxycodone self-administration. Evidence that rats in our study self-administered oral oxycodone solution for its reinforcing properties includes the inverted U-shaped concentration-response curve generated across increasing concentrations of oxycodone solution (Figure 4.1), extinguished lever pressing when water was substituted for

oxycodone in multiple cohorts (Figures 4.1E, 4.10B), and disruption of lever pressing following MOR antagonist treatment (Figure 4.7C). Additionally, we were able to induce reinstatement of extinguished oxycodone seeking by exposing subjects to footshock stress, and this response was attenuated by NK1R antagonism (Figure 4.10D and 4.10F).

Some studies have shown that fluctuations in systemic hormone levels during the estrous cycle affects various drug-seeking behaviors. Generally, higher systemic estradiol levels tend to facilitate, and progesterone to inhibit, drug-related behaviors [95, 96, 313-315]. In contrast, a study assessing the role of estrous cycle phase in i.v. oxycodone self-administration found no effect of estrous cycle on drug-seeking [113]. Consistent with these data, we found no effect of estrous cycle phase on oral oxycodone self-administration. Though estrous cycle did not affect drug-seeking behavior (Figure 4.6C), our data suggest that chronic oral oxycodone self-administration may lead to dysregulation of the estrous cycle (Figure 4.6A,B) However, because the cycle also appeared to be irregular in drug-naïve controls (Figure 4.7), the role of oxycodone self-administration on estrous cycle regularity in Long Evans rats remains ambiguous. Interestingly, we did not observe estrous cycle dysregulation in a different strain of rats (Wistar) exposed to chronic oral oxycodone self-administration (Figure 4.4). Of note, a paper by Goldman et al [316] revealed that in addition to the presence of predominantly leukocytes, the diestrus phase can also be characterized by the presence of a combination of leukocytes and epithelial cells in the Long Evans strain. Thus, determination of estrous cycle phase in Long Evans rats may be particularly challenging.

As oxycodone is a potent MOR agonist [317], we sought to determine whether administration of an opioid receptor antagonist could disrupt oral self-administration of

this drug. We found that single pretreatments of naloxone had no effect on oxycodone self-administration. While initially a surprising finding, it is important to consider the role of oxycodone ROA in the response to systemic naloxone treatment. In other words, because subjects were self-administering oxycodone orally, we speculate that they experienced a longer delay between operant response and sensation of drug effects than would occur during i.v. self-administration. Thus, it is possible that the animals were unable to learn the effect of naloxone pretreatment on oxycodone reinforcement until later in the session, or even after the session had concluded. Therefore, we conducted repeated naloxone pretreatments for 3 consecutive days, and found that oxycodone-seeking was significantly attenuated. Thus, naloxone was capable of disrupting operant responding for oxycodone, but this response required multiple exposures. The fact that lever pressing was decreased, as opposed to increased, is consistent with our dose-response data suggesting that 0.1 mg/ml oxycodone is near the peak, or on the ascending limb, of the dose-response function. We do not think that this attenuation in oxycodone self-administration is due to off target or general sedative effects because 10 mg/kg naloxone had no effect on responding when given acutely. Additionally, no somatic withdrawal symptoms were observed following any of the acute naloxone treatments or the repeated naloxone treatments, suggesting that animals were not physically dependent on oxycodone during these treatments. However, it is important to note that the first day of single-dose naloxone treatment occurred following 26 self-administration sessions, and the first day of the repeated naloxone treatments occurred 14 sessions after that, which may have also contributed to the increased efficacy of naloxone treatment.

The NK1R is known to have a role in stress and anxiety responses, as well as in various drug-seeking behaviors [294]. This system is particularly relevant in studying opioid abuse, in that antagonism of this receptor has been repeatedly shown to alter opioid-seeking behaviors. The majority of studies have found that NK1R antagonism attenuates the reinforcing properties of opiate drugs [298-301]. In contrast, Walsh et al have demonstrated that NK1R antagonism with aprepitant potentiates oxycodone's reinforcing effects when consumed orally or intranasally in human recreational opioid users [318]. This unexpected result could be the result of complex factors including the oxycodone experience of experimental subjects, where the dose of oxycodone falls on the dose-response function, and the potency of NK1R antagonism of the specific dose and compound used. However, the NK1R system has been consistently shown to mediate stress-primed reinstatement for both cocaine and alcohol [295-297]. To our knowledge, we are the first to assess the role of the NK1R in reinstatement of drug seeking for any opiate drug. We found that systemic delivery of a NK1R antagonist significantly attenuated reinstatement of oxycodone-seeking following stress for both male and female subjects. This finding provides further support for targeting the NK1R system in the development of therapeutics for those suffering from substance use disorders and suggests that its ability to suppress stress-elicited drug seeking extends to all classes of drugs examined thus far. In both the initial shock titration experiment, and in the antagonist experiment, it appeared that males exhibited greater lever-pressing behavior following exposure to 0.8 mA shock intensity. While this was not a statistically significant effect, it suggests that males may be slightly more sensitive to stress-induced reinstatement of oxycodone seeking than females. It should also be noted that estrous

cycle phase was not monitored during the reinstatement test days, and reinstatement behavior could be more intense on specific days of the estrous cycle. Future studies will assess the role of ovarian hormones in reinstatement to oxycodone-seeking and will follow up on this subtle effect of sex on stress-induced oxycodone seeking.

Lastly, we found that following intragastric gavage of the same amount of oxycodone that was consumed during baseline self-administration produced blood levels that were comparable to those reached following oral oxycodone intake in humans [67], suggesting that rats voluntarily self-administer pharmacologically relevant doses.

Collectively, these data demonstrate that operant self-administration can be used to study oral oxycodone intake in rats. Based on typical drug-taking behaviors in humans, this preclinical model has high translational value and can be used as a platform for early development of pharmacotherapy for prescription opiate dependence as well as studying the transition from oral to intravenous routes of opioid administration that frequently occurs in human opioid abusers. Additionally, we observed a significant sex difference in oxycodone consumption that will be important to consider in the interpretation of future studies. Mechanistically, we identified a role of the NK1R system in stress-primed reinstatement, consistent with the effect of this receptor on stress-induced seeking of other drug classes, as well as its role in reward/reinforcement for other opiate drugs.

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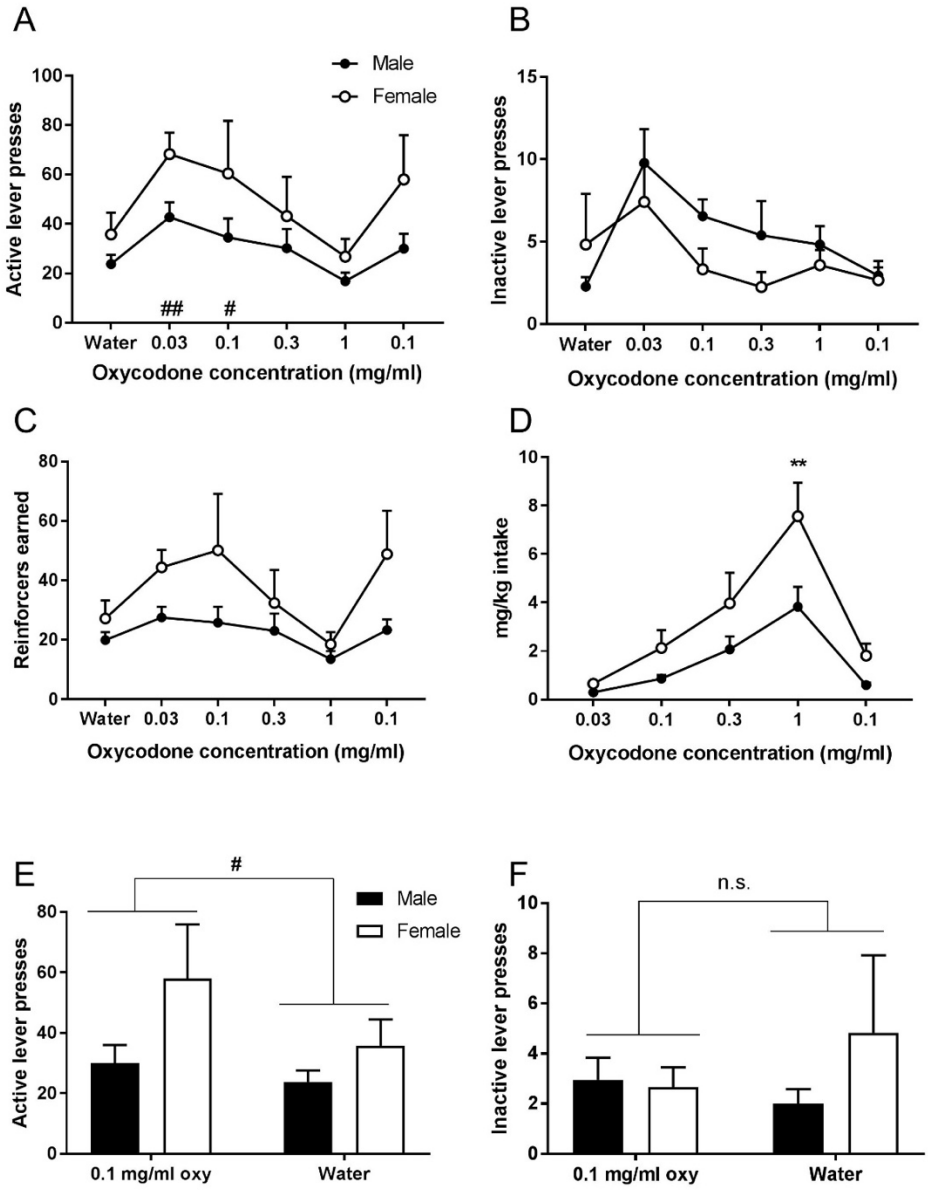
A
Male
Female
Active lever presses
100
80
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40
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0
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Water 0.03 0.1 0.3 1 0.1
Oxycodone concentration (mg/ml)
B
Inactive lever presses
15
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5
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Water 0.03 0.1 0.3 1 0.1
Oxycodone concentration (mg/ml)
C
Reinforcers earned
80
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Water 0.03 0.1 0.3 1 0.1
Oxycodone concentration (mg/ml)
D
mg/kg intake
10
8
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0.03 0.1 0.3 1 0.1
Oxycodone concentration (mg/ml)
E
#
Male
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Active lever presses
80
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0.1 mg/ml oxy
Water

Figure 4.1 Oral oxycodone self-administration: concentration-response curve. Data for each concentration represent the average of the last 3 days of self-administration. (A) Average active lever presses. (B) Average inactive lever presses. (C) Average reinforcers earned. (D) Average intake in mg/kg. (E) Average active lever presses for the last 3 days of re-introduced 0.1 mg/ml oxycodone compared to average of last 3 days of water. (F) Average inactive lever presses for the last 3 days of re-introduced 0.1 mg/ml oxycodone compared to average of last 3 days of water. Data expressed as mean values ± SEM. ##$p<0.01$, #$p<0.05$, compared to water. **$p<0.01$, compared to males. n=4-6/sex.

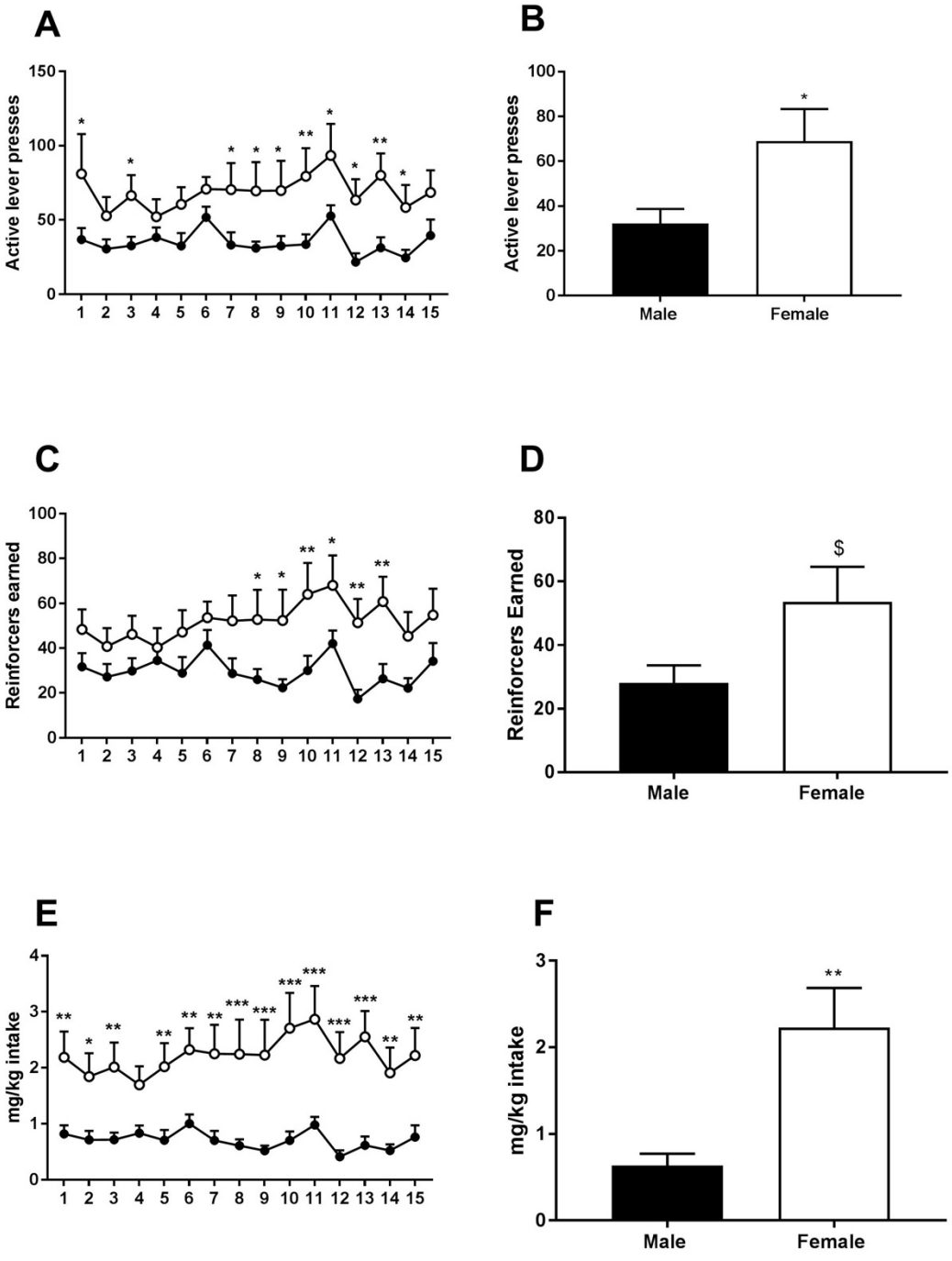

A
Active lever presses
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Active lever presses
100
80
60
40
20
0
Male
Female
C
Reinforcers earned
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
Reinforcers Earned
80
60
40
20
0
$
Male
Female
E
mg/kg intake
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
F
mg/kg intake
3
2
1
0
Male
Female

Figure 4.2 Baseline oral oxycodone self-administration in Wistar rats. (A) Total active lever presses. (B) Average active lever presses over the last 3 days of baseline self-administration. (C) Total reinforcers earned. (D) Average reinforcers earned over the last 3 days of baseline self-administration. (E) Total mg/kg intake. (F) Average mg/kg intake over the last 3 days of baseline self-administration. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, \$$P = 0.06$, compared to males. n=5-6/sex.

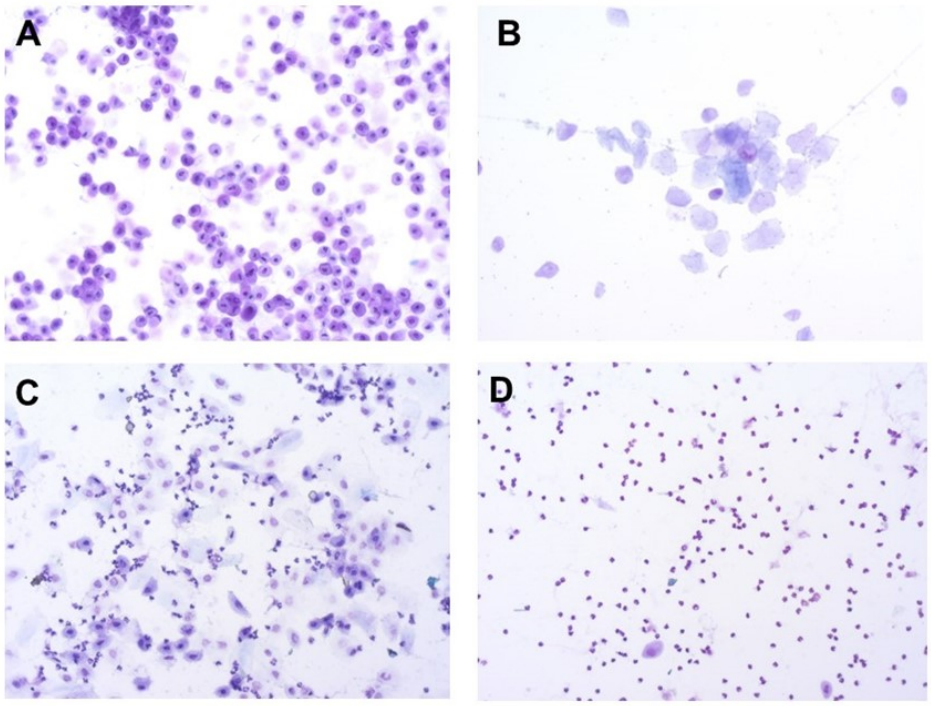

A
B
C
D

Figure 4.3 Representative samples of vaginal cytology for each estrous cycle phase. (A) Proestrus, characterized by the presence of primarily nucleated epithelial cells. (B) Estrus, characterized by the presence of primarily cornified epithelial cells. (C) Metestrus, characterized by the presence of a combination of cornified epithelial cells and leukocytes. (D) Diestrus, characterized by the presence of primarily leukocytes. Images taken at 20x.

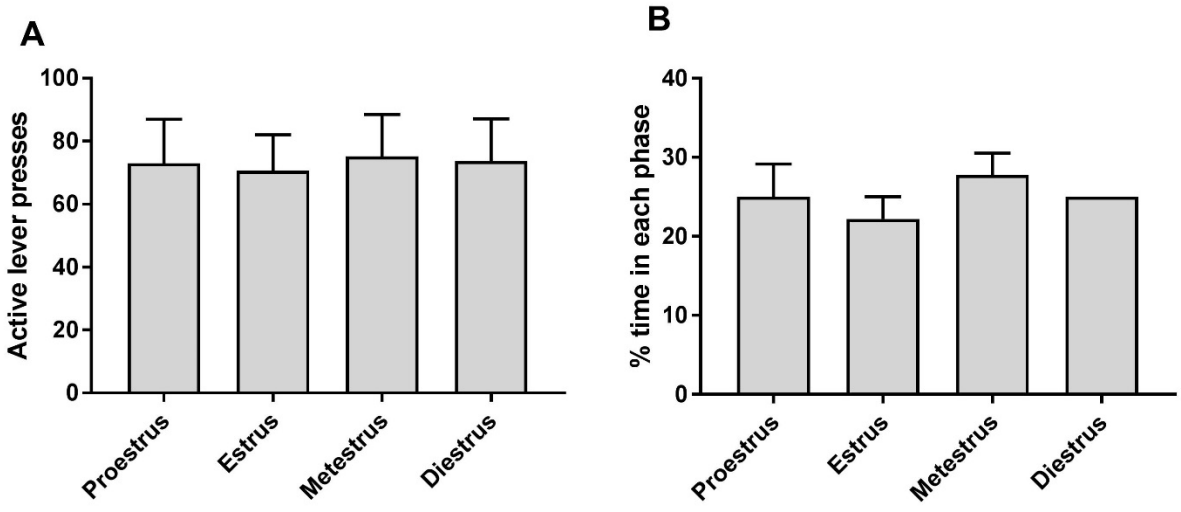

A
Active lever presses
100
80
60
40
20
0
Proestrus
Estrus
Metestrus
Diestrus
B
% time in each phase
40
30
20
10
0
Proestrus
Estrus
Metestrus
Diestrus

Figure 4.4 Estrous cycle monitoring in Wistar rats undergoing oxycodone self-administration. (A) Active lever presses for each self-administration during each estrous cycle phase. (B) Percent time spent in each estrous cycle phase. n=9.

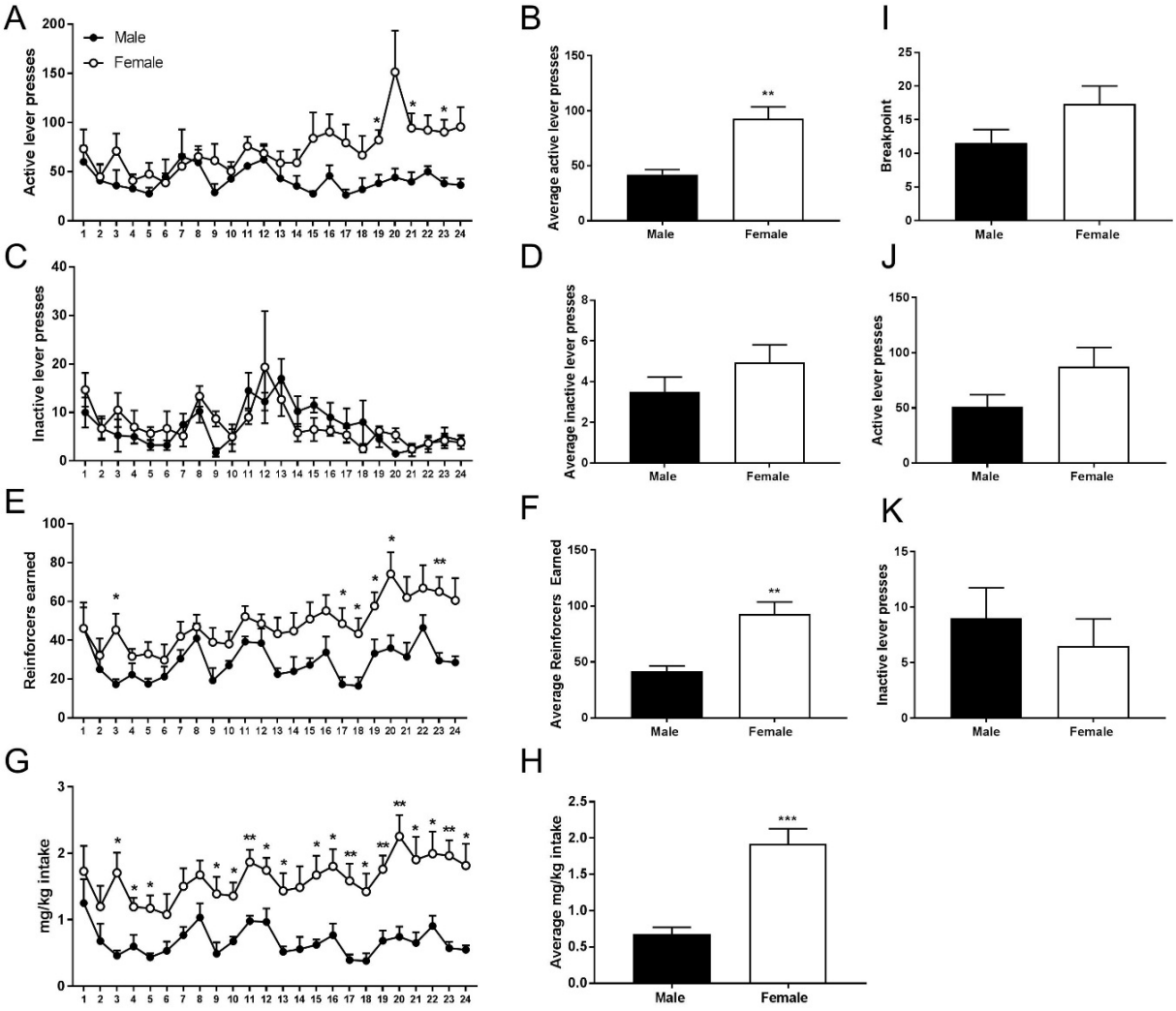
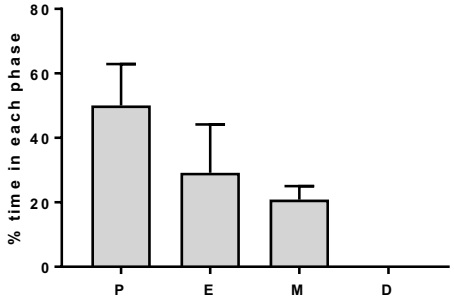

A
Male
Female
Active lever presses
B
Average active lever presses
Male
Female
I
Breakpoint
C
Inactive lever presses
D
Average inactive lever presses
J
Active lever presses
E
Reinforcers earned
F
Average Reinforcers Earned
K
Inactive lever presses
G
mg/kg intake
H
Average mg/kg intake

Figure 4.5 Baseline oral oxycodone self-administration and progressive ratio in Long Evans rats (A) Total active lever presses. (B) Average active lever presses over last three days of baseline self-administration. (C) Total inactive lever presses. (D) Average inactive lever presses over last three days of baseline self-administration. (E) Total reinforcers earned. (F) Average reinforcers earned over the last three days of baseline self-administration. (G) Total mg/kg consumed (H) Average mg/kg intake over the last three days of baseline self-administration. (I) Breakpoints obtained in progressive ratio (PR) session. (J) Total active lever presses during PR session. (K) Total inactive lever presses during PR session. Data expressed as mean values ± SEM. **$p<0.01$, *$p<0.05$, compared to males. n=4-6/sex.

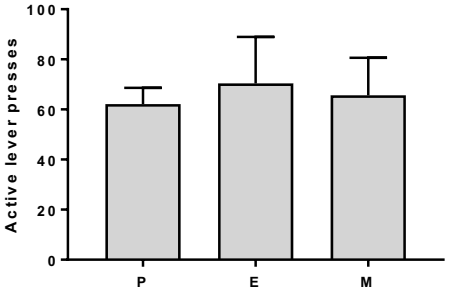
A



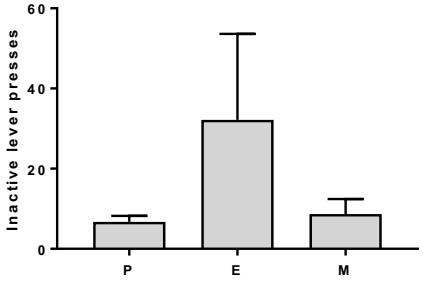
B

Subject ID	Day 1	Day 2	Day 3	Day 4
307	P	M	P	P
308	M	P	P	P
309	P	E	E	E
310	P	M	P	P
311	E	E	E	M
312	P	E	M	P

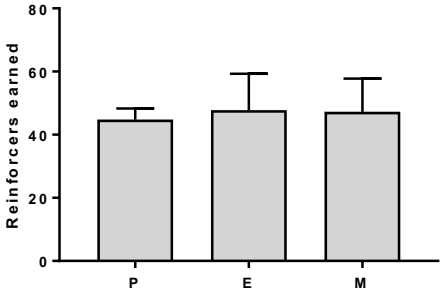
C



D



E



F

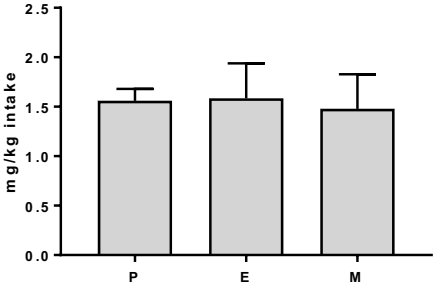
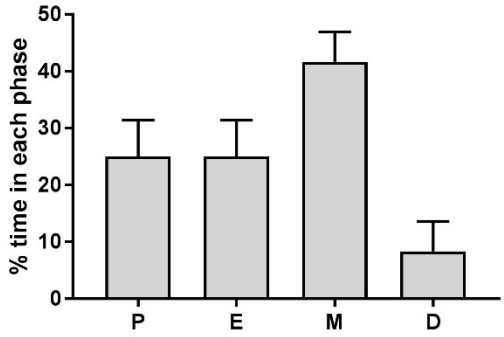


Figure 4.6 Estrous cycle monitoring. (A) Percent time spent in each estrous cycle phase. (B) Estrous cycle phases for individual subjects over four days of estrous cycle monitoring. (C) Active lever presses for each estrous phase. (D) Inactive lever presses for each estrous phase. (E) Reinforcers earned for each estrous phase. (F) Intake in mg/kg for each estrous phase. Data expressed as mean values ± SEM. n=6. P: proestrus, E: estrus, M: metestrus, D: diestrus. n=6.

A



B

Rat ID	Day 1	Day 2	Day 3	Day 4
501	M	P	E	M
502	M	P	E	M
503	P	M	M	P
504	D	P	E	M
505	M	D	E	M
506	E	M	P	E

Figure 4.7 Estrous cycle monitoring in drug-naïve Long Evans rats. (A) Percent time in each phase. On average, the percent time spent in each phase was 25 ± 15.8 SEM for proestrus, 25 ± 15.8 SEM for estrus, 41.7 ± 12.9 SEM for metestrus, and 8.3 ± 12.9 SEM for diestrus. (B) Estrous cycle phases for individual subjects over four consecutive days. P: proestrus, E: estrus, M: metestrus, D: diestrus. n=6

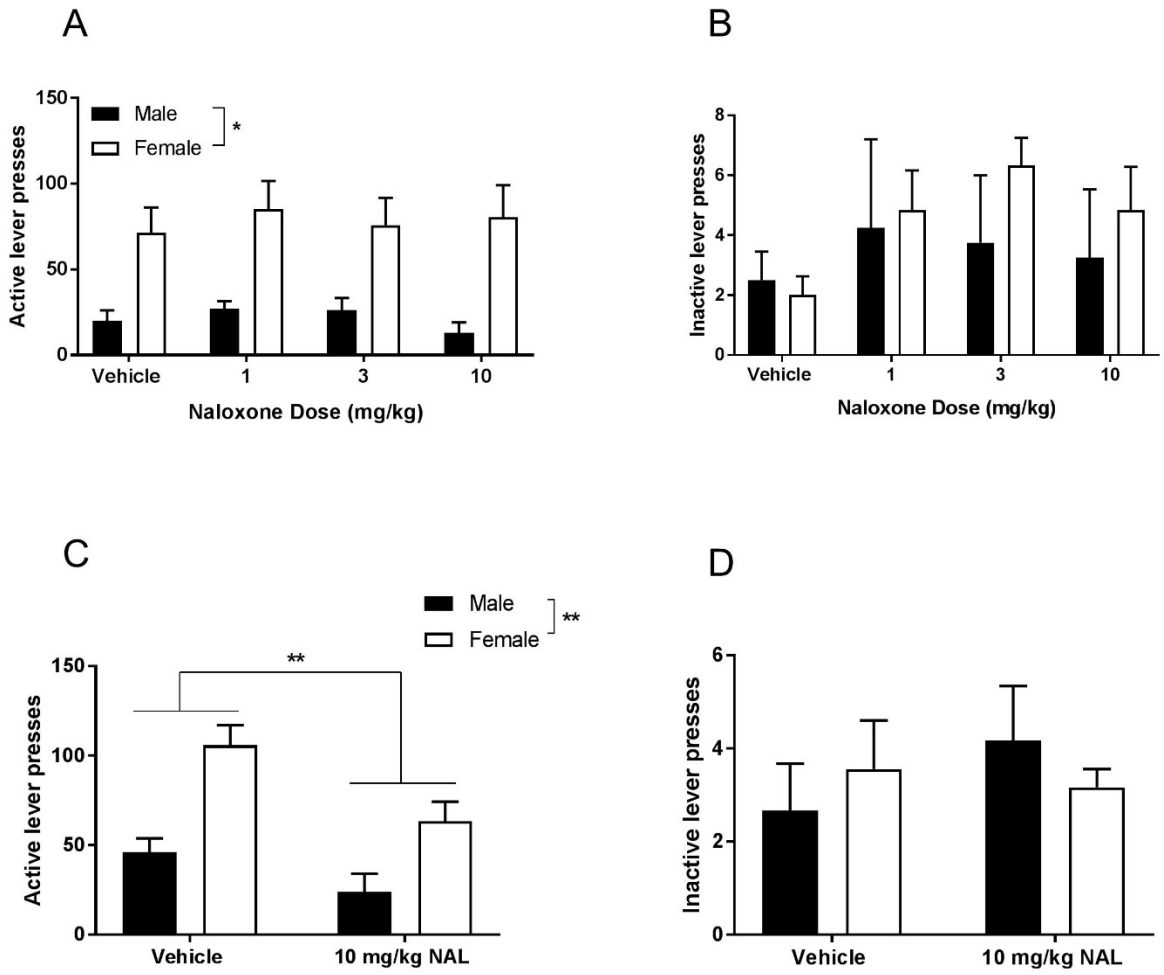

A
Male
Female
*
Active lever presses
150
100
50
0
Vehicle
1
3
10
Naloxone Dose (mg/kg)
B
Inactive lever presses
8
6
4
2
0
Vehicle
1
3
10
Naloxone Dose (mg/kg)
C
Male
Female
**
**
Active lever presses
150
100
50
0
Vehicle
10 mg/kg NAL
D
Inactive lever presses
6
4
2
0
Vehicle
10 mg/kg NAL

Figure 4.8 Effect of naloxone pretreatment on oral oxycodone self-administration. (A) Active lever presses following single naloxone pretreatment at 1, 3, and 10mg/kg doses. (B) Inactive lever presses following single naloxone pretreatment at 1, 3, and 10 mg/kg doses. (C) Average active lever presses after three consecutive days of 10 mg/kg naloxone pretreatment. (D) Average inactive lever presses after three consecutive days of 10 mg/kg naloxone pretreatment. Data expressed as mean values ± SEM. $^{**}P < 0.01$, compared with vehicle. n = 4-6/sex

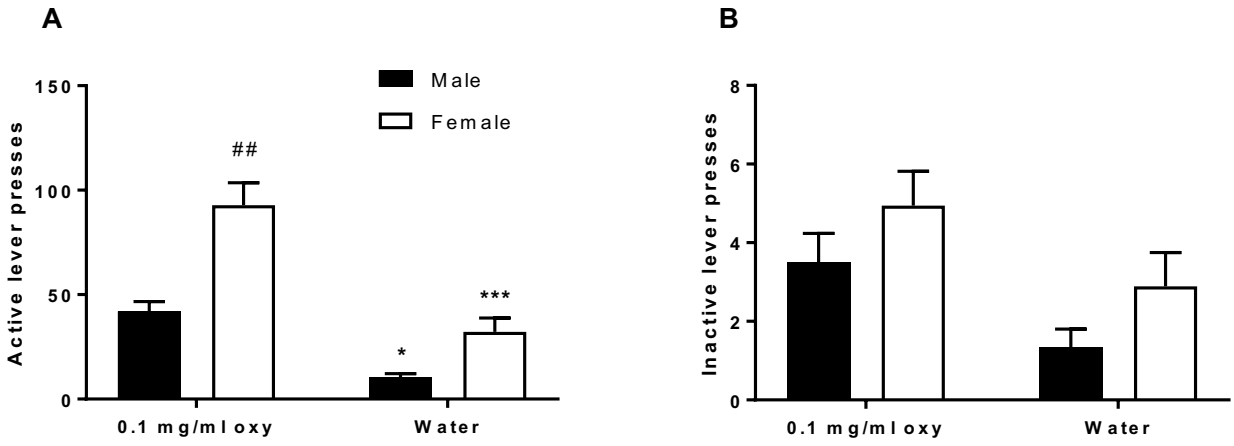

A
Male
Female
Active lever presses
150
100
50
0
##
*
***
0.1 mg/ml oxy
Water
B
Inactive lever presses
8
6
4
2
0
0.1 mg/ml oxy
Water

Figure 4.9 Extinction of oxycodone-seeking. When 0.1 mg/ml oxycodone solution was replaced with water, the number of active lever presses per session significantly decreased. (A) Average active lever presses over the last 3 days of self-administration. (B) Average inactive lever presses over the last 3 days of self-administration. Data represented as mean values ± SEM. ##$P < 0.01$, compared to males. *$P < 0.05$ and ***$P < 0.005$, compared to oxycodone. n=4-6/sex.

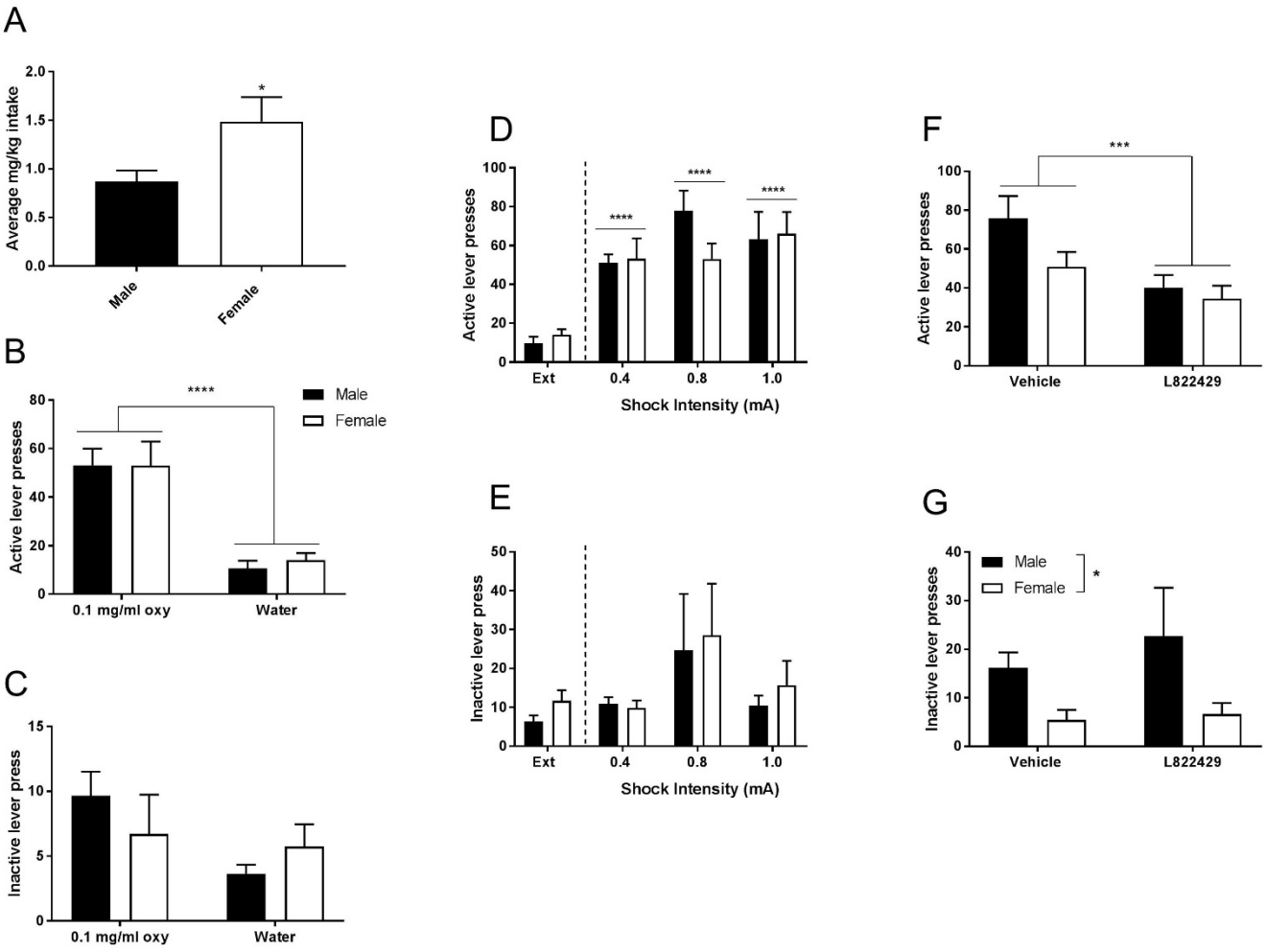

A
Average mg/kg intake
2.0
1.5
1.0
0.5
0.0
*
Male
Female
B
Active lever presses
80
60
40
20
0
****
Male
Female
0.1 mg/ml oxy
Water
C
Inactive lever press
15
10
5
0
0.1 mg/ml oxy
Water
D
Active lever presses
100
80
60
40
20
0
****
****
****
Ext
0.4
0.8
1.0
Shock Intensity (mA)
E
Inactive lever press
50
40
30
20
10
0
Ext
0.4
0.8
1.0
Shock Intensity (mA)
F
Active lever presses
100
80
60
40
20
0
***
Vehicle
L822429
G
Inactive lever presses
40
30
20
10
0
Male
Female
*
Vehicle
L822429

Figure 4.10 Extinction and stress-primed reinstatement. (A) Average mg/kg intake over last three oxycodone self-administration sessions. (B) Average active lever presses over last three oxycodone self-administration sessions compared with the average active lever presses over the last three extinction (water only) sessions. (C) Average inactive lever presses over the last three oxycodone self-administration sessions compared with average active lever presses over the last three extinction (water only) sessions. (D) Effect of shock intensity on stress-primed reinstatement, active lever presses. (E) Effect of shock intensity on stress-primed reinstatement, inactive lever presses. (F) NK1R antagonism pretreatment and stress-primed reinstatement, active lever presses. (G) NK1R antagonism pretreatment and stress-primed reinstatement, inactive lever presses. Data expressed as mean values ± SEM. *P < .05, compared with extinction; ***P < .001, compared with vehicle; ****P < .0001, compared with extinction. n = 9-10/sex

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Sex Differences in Quinine-Adulterated Ethanol Intake

We have demonstrated that while sex differences in baseline, or punishment-free, ethanol intake vary between cohorts, females consistently require higher quinine concentrations to reduce ethanol consumption when compared to males. While we did not observe an effect of estrous cycle phase on quinine-adulterated ethanol intake, it is possible that circulating sex hormones still contribute to this behavior in females. For example, Satta and colleagues have shown that females consume significantly more ethanol than males in a model of binge-like consumption and that estrous cycle phase did not affect this behavior [91]. However, following ovariectomy, binge-like consumption significantly decreased [91], suggesting that a threshold level of estradiol and/or progesterone may still be required for the expression of certain alcohol-related behaviors. Subsequent experiments using ovariectomy combined with hormone replacement will identify the role of estradiol and progesterone in quinine-adulterated ethanol intake.

However, it is also possible that sex differences in quinine-adulterated ethanol intake are developmental in origin. Future experiments will utilize perinatal estradiol or testosterone treatment, resulting in brain masculinization [319], to determine the organizational effect of sex hormones on later quinine-adulterated ethanol intake. Collectively, these experiments will allow for the characterization of the organizational and activational effects of sex hormones in quinine-adulterated ethanol intake.

Sex Differences in Neuronal Activation during Quinine-Adulterated Ethanol Intake

Interestingly, while a sex difference in quinine-adulterated ethanol intake was observed over the course of 24 hours, no difference was observed during the first 2.5 hours of intake. These data, while initially surprising, may serve to strengthen the findings of our c-Fos-mapping experiment, in that males and females exhibit distinct differences in regional activation despite similar levels of quinine-ethanol exposure over this restricted time frame. Specifically, we observed a sex-specific increase in neuronal activation within the PIC and VTA during quinine-adulterated ethanol intake, in that males exhibited increased activity in these regions compared to males consuming quinine-free ethanol, as well as compared to females consuming quinine-adulterated ethanol.

The VTA is involved in various drug-seeking processes, such as salience attribution, reward, and aversion processing, and consists primarily of dopaminergic neurons [225, 266, 269]. However, this region also contains GABAergic and glutamatergic neuronal populations [269], warranting additional IHC studies to identify the type of neuron more strongly activated in males during quinine-adulterated ethanol intake. Additionally, retrograde and anterograde tract tracing in conjunction with c-Fos IHC will be utilized to identify neurons activated during quinine-adulterated ethanol intake in males that also receive projections from and/or project to the VTA. These circuits can then be pharmacologically or chemogenetically manipulated in females in efforts to decrease quinine-adulterated intake.

The PIC is a region that is associated with behavioral inhibition, salience attribution, and aversion processing [214, 223, 273]. Activation of a glutamatergic PIC-

NAcc circuit has also been shown to inhibit consummatory behaviors [273], therefore the increased activity within the PIC observed in males consuming quinine-ethanol solution may be responsible for the decrease in intake that is observed in males over a 24-hour session. Subsequent experiments will combine c-Fos IHC with anterograde and retrograde tract tracing to examine PIC afferents and/or efferents that are activated during quinine-adulterated ethanol intake.

A major caveat of our c-Fos-mapping experiment was the lack of water-only and quinine-adulterated water groups. To compare neuronal activation between ethanol, quinine-ethanol, water, and quinine-water groups, it would be necessary for all groups undergo 6 weeks of continuous access ethanol two-bottle choice prior to testing/tissue collection. It must then be considered that animals in both water-only and quinine-water groups may experience motivational withdrawal following ethanol removal, which would likely lead to increased activation within regions associated with withdrawal and anti-reward processing. Therefore, future directions will include the completion of c-Fos mapping in males and females exposed to water, quinine-adulterated water, ethanol, or quinine-adulterated ethanol for two weeks prior to perfusion/tissue collection to conduct between-group comparisons, rather than repeated measure, within-group comparisons in attempts to circumvent this issue. These data will allow for the distinction between regions activated during quinine-adulterated ethanol intake and those activated in response to the detection of the bitter tastant quinine.

Sex Differences in Oral Oxycodone Self-Administration and Stress-Primed Reinstatement

In our development of a model of oral oxycodone self-administration in rats, we found that females consistently consumed significantly more drug than males.

Interestingly, females did not exhibit significantly higher breakpoints during PR sessions, suggesting that this increased intake is not due to increased motivation among females. Previous work have suggested that in comparison to males, females may be more sensitive to opioid reward, while also being less sensitive to its aversive, sedative properties [120-122]. Future studies will assess sex differences in sensitivity to oxycodone's rewarding and aversive effects through dose-response CPP and CTA experiments. Additional experiments will aim to identify the mechanisms mediating sex differences oxycodone-seeking by comparing MOR, DOR, KOR, and NK1R expression and sensitivity between males and females.

Future directions will also include modifications of our oral oxycodone self-administration model to allow for co-administration of ethanol and oxycodone solutions. Opioid and ethanol co-abuse is a prevalent problem [4], and this model will allow for the investigation of mechanisms involved in opioid- and ethanol-seeking. Prescription opioid misuse also increases the later risk of heroin use by approximately 40-fold [277, 278]. Therefore, additional experiments will investigate the effect of prior oral oxycodone self-administration on subsequent i.v. heroin self-administration.

Lastly, we observed that systemic NK1R antagonism significantly attenuated stress-primed reinstatement in males and females. To identify the brain regions involved in this response, future experiments will involve double labeling IHC of the NK1R and c-Fos in brain regions involved in stress and drug-seeking behaviors. Following the identification of candidate regions, a NK1R antagonist will be administered directly to the brain region of interest to determine whether this is sufficient to attenuate stress-primed reinstatement. We will also infuse an adeno-associated viral vector

overexpressing the NK1R into the same region of interest to see if this potentiates stress-primed oxycodone-seeking behaviors, allowing for an increased understanding of the NK1R in this behavior and contributing to the potential for this receptor as a therapeutic target in OUD.

Summary

Overall, we have identified significant sex differences in compulsive-like ethanol intake and in oral oxycodone self-administration, in that females consume significantly more quinine-adulterated ethanol and self-administer significantly more oral oxycodone than males. However, we found that neither of these behaviors are affected by fluctuating levels of sex hormones in naturally cycling females, suggesting that a threshold level of systemic estradiol/progesterone, an organizational effect of these hormones, or a combination of activational and organizational effects contribute to sex differences in drug-seeking behaviors.

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