EXPLORING SEX DIFFERENCES IN CONDITIONED PLACE AVERSION

by

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Abstract

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Substance use disorder is a chronic condition characterized by cycles of intoxication, withdrawal, and relapse. Negative affective and somatic symptoms commonly accompany cessation of substance use. These symptoms can be incredibly aversive, to the extent that they can be classically conditioned to be associated with cues or environments. Women show greater cue-reactivity to drug-associated stimuli and progress from casual drug use to abuse at a faster rate compared to men. Human and animal literatures strongly suggests that these elevated vulnerabilities in females are associated with changes in levels of circulating ovarian hormones. Studies investigating sex differences and hormonal influences during opioid withdrawal (OW) and their motivational influences are severely lacking, and the few that address sex as a variable report disparate findings. A sizable portion of the existing knowledge on OW has been obtained from research that is limited to men and male subjects and extrapolated to women, including scales assessing withdrawal. Thus, the objective of the present study was to investigate sex differences and hormonal influences in the motivational consequences of opioid withdrawal using a conditioned place aversion paradigm. Specifically, our aim was to ascertain (1) the extent to which gonadally intact, adult male and female rats displayed aversion to environments associated with an acute naloxone precipitated withdrawal after morphine pretreatment during a two- and four-day conditioning cycle, (2) the degree to which elevations in (or elimination of) circulating levels of estradiol influence conditioned place aversion, and (3) the differences in magnitude of conditioned place aversion after varying the dose of morphine and conditioning

doses of naloxone in intact females. Overall, our findings suggest the following: (1) Both male and female rats developed significant naloxone-precipitated conditioned place aversion of similar magnitude after two and four days of conditioning. However, this test failed to yield significant sex differences. (2) Estradiol treatment did not appear to affect naloxone-precipitated conditioned place aversion in ovariectomized female rats, as no significant differences were found between estradiol and vehicle (peanut oil)-treated groups. (3) Female rats demonstrated significant conditioned place aversion across various doses of morphine and naloxone.

Keywords: Opioid withdrawal, Sex differences, Conditioned Place Aversion (CPA)

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CHAPTER 1

INTRODUCTION

1.1 Opioid Use and Opioid Use Disorder

In the year 2020, drug overdose deaths rose by nearly 30% in the United States, the highest recorded number to date (Centers for Disease Control and Prevention, 2021). According to provisional data from the National Center for Health Statistics, opioid drug use was a significant driving force behind the rise in overdose deaths. From 1999 to 2020, deaths due to opioid overdose increased from 50,963 to 69,710. More specifically, deaths involving prescription opioids have more than quadrupled (Centers for Disease Control, 2021).

Prescription opioids are considered safe when used for short-term pain relief, however chronic use of common prescription opioids like morphine have been known to lead to dependence and addiction. Drug addiction, otherwise known as substance use disorder, is a chronic illness defined by cycles of intoxication, withdrawal, and relapse. Per the DSM-5, opioid use disorder (OUD) is considered a spectrum disorder the severity of which is dependent on the number and intensity of symptoms present (American Psychiatric Association, 2013). Unlike other substances of abuse, OUD may not necessarily begin with recreational use, the combination of self-medication for chronic pain and negative reinforcement in the form of somatic and affective withdrawal symptoms have been shown to influence the progression of opioid use disorder (Koob, 2020).

Historically, men have been more likely to report use and abuse illicit substances overall (Center for Behavioral Health Statistics and Quality, 2017), but more recent data reveals that

women develop substance use disorders at similar rates to men. Although, overdose deaths due to opioid use are higher in men than women (National Institute on Drug Abuse, 2020), reports from the Centers for Disease Control have shown a 583% increase in the rate of overdoses for women between 1999 and 2016 (Mazure & Fiellin, 2018). Interestingly, women seeking treatment for substance use have reported a more rapid escalation in drug consumption and a shorter latency from "first use" to addiction compared to men seeking treatment (Becker & Chartoff, 2019). This phenomenon of "telescoping" has been characterized as a rapid escalation from casual drug use to substance abuse (Becker & Chartoff, 2019).

1.2 Sex differences in Opioid Use Disorder

Each individual cell possesses a biological sex, a trait which encompasses various dimensions. Biological sex is comprised of several components including genetic, epigenetic, cellular, hormonal, and morphological. According to our current understanding of sexual differentiation, two primary factors contribute the process: sex chromosomes and steroid hormones (Sanchis-Segura & Becker, 2016). Due to this dynamism, it is important to assess sex differences across four measures, as described by Becker and Koob: 1) qualitative differences (i.e., phenotypic differences), 2) quantitative differences (different averages in a particular trait), 3) population differences and., 4) convergent differences (wherein the trait or expression of trait is the same, but the underlying biological mechanism differs) (Becker & Koob, 2016). A growing number of studies demonstrate the influence of sex in the risk for abuse of nonmedical prescription opioid medications (Back et al., 2010; Fillingim et al., 2009; Green et al., 2009). While men typically use and abuse illicit opiates like heroin,

women tend to use and abuse prescription opioids, therefore, use among women is most commonly initiated with prescription opioids (National Institute on Drug Abuse [NIDA], 2020; Bawor et al., 2015). In 2018, women in the US had 1.5 times the odds of filling an opioid prescription than men, this trend of higher prescription rates was consistent across all age groups (Green et al., 2009). This may be a result of increased prevalence of pain conditions and chronic illnesses among women, however, the extent to which these conditions may contribute to prescription opioid misuse among women is unclear (National Institute on Drug Abuse [NIDA], 2020; Terplan, 2017).

The cycle of addiction has been characterized by three distinct stages: initiation/escalation, withdrawal, and preoccupation/craving (Back et al., 2010) . Clinical studies have highlighted sex differences in these stages during drug consumption. Women report more intense craving for opioids after exposure to drug paired cues than men (Yu et al., 2007). Women and girls report initiation of drug use as a coping mechanism (negative reinforcement), while men report initiation of drug use to bond or engage socially (Vigna-Taglianti et al., 2016). Women transition from casual opioid use to dependence at an accelerated rate compared to men (Back et al., 2011). The rapid escalation from use to misuse of opioids (telescoping) among women is accompanied by more severe affective and physical withdrawal symptoms compared to men (Terplan, 2017). These sex differences highlight the need for considering sex as a contributing factor in the variation in patterns of opioid consumption and the development of opioid use disorder.

Similar sex differences in opioid use are also evident in preclinical studies. Compared to male rodents, oral and IV-self administration of opioids are acquired at lower doses, under more varied contexts, and more quickly in female rodents (Alexander et al., 1978; Cicero et

al., 2003; Lynch & Carroll, 1999). Female rodents consume greater amounts of opioids and pay a higher behavioral "price" (achieve higher breakpoints on a progressive ratio schedule of reinforcement) than male rodents (Cicero et al., 2003). Moreover, conditioned place preference (CPP) studies suggest that females demonstrate greater preferences for environments associated with lower doses of morphine compared to males (Karami & Zarrindast, 2008). Studies have also demonstrated that females exhibit conditioned place preference (CPP) at lower cocaine doses than males (Russo et al., 2003; Zakharova et. al., 2009). Overall, evidence from the rodent literature lends support to the clinical findings which suggests that females are more severely affected than men by the chronic use of opioid drugs.

1.3 Opioid withdrawal

Opioid use cessation can induce a withdrawal syndrome which is marked by negative somatic and emotional symptoms (e.g., hypersensitivity to pain, nausea, vomiting, dysphoria, anxiety, depression, irritability, craving) (Fishman, 2008; McHugh et al., 2016). These symptoms are so aversive that opioid withdrawal often serves as a powerful motivator for continued drug use and/or relapse. Interestingly, some aspects of withdrawal can be classically conditioned. Classical conditioning (or Pavlovian conditioning) is a paradigm in which a previously neutral stimulus can be conditioned to evoke a response after it is repeatedly paired with a stimulus that already elicits a response that is automatic or reflexive. Classical conditioning is comprised of three fundamental elements: 1) Unconditioned stimulus (US), a stimulus that naturally/automatically triggers a response, 2) Unconditioned response (UR), a naturally occurring response to the unconditioned stimulus, 3) Conditioned stimulus (CS), a neutral stimulus that is repeatedly paired with the unconditioned stimulus to

evoke a response. Classical conditioning can be used to evoke both positive and negative effects, depending on the pairing of the stimuli. For example, anecdotal accounts from people suffering with opioid use disorder revealed conditioned responses including lacrimation and feeling nauseated when exposed to the smell of peppermint (odor used as the neutral stimuli) (McLellan et al., 1986; O'Brien et al., 1976). These responses have also been classically conditioned in opioid-dependent animals (Goldberg & Schuster, 1970; Irwin & Seevers, 1956; O'Brien et al., 1976; Wikler & Pescor, 1967) and can even be achieved in non-opioid dependent animals (acute withdrawal).

Studies examining sex differences in opiate withdrawal (OW) are scarce, and the limited number of studies that have explored sex as a variable have produced conflicting findings. While some studies report that male rodents experience more severe OW than females (Cicero et al., 2002; Towers et al., 2019), others have found that females are more sensitive than males (Ali et al., 1995; Papaleo & Contarino, 2006). Moreover, some studies have reported no sex differences, or even reported that females affected by morphine withdrawal after acute morphine (MOR) administration or naloxone-precipitated withdrawal experienced more severe symptoms (Ali et al., 1995; Becker & Chartoff, 2019; Bodnar & Kest, 2010; Cicero et al., 2002; Craft et al., 1999; el-Kadi & Sharif, 1994; Papaleo & Contarino, 2006). These discrepancies may be due, in part, to differences in methodology such as varied drug dose, number/route of drug administration, withdrawal signs being measured, and failure to account for estrous cycle phase in female subjects (Becker & Chartoff, 2019; Bobzean et al., 2014). Additionally, much of the literature as it relates to opiate withdrawal has been gleaned from studies exclusively in males and then applied to females, as most withdrawal scales have been developed using men and male subjects (Arfken et al., 2001; Puigdollers et al.,

2004). The paucity of research on opiate withdrawal in females is concerning, particularly considering previous research that suggests that factors such as sex and ovarian hormone activity influence pain sensitivity (Berkley, 1997; Fillingim et al., 2009; Green et al., 2009; Gureje et al., 1998; Mogil, 2012; Stoffel et al., 2003), pain inhibition (Craft, 2003; Negus et al., 2002; Stoffel et al., 2003), nociception processing/opioid analgesia (Amandusson & Blomqvist, 2013; Mogil, 2012), and sensitivity to stress (Hodes, 2018). These variables are known to promote opioid use and relapse, further highlighting the need for research to better identify and understand sex differences.

1.4 Sex hormones, Motivation, and the Mesolimbic Reward Pathway

Motivation is described as an internal state that drives an animal to conduct various behaviors associated with reward or avoidance of aversive stimuli. Sex differences in neural systems involved in motivational behaviors are thought to be a result of what is considered reproductive success in males as opposed to females. The primary evolutionary motivation for reproductive success in male mammals is to gain access to females and inseminate as many females as possible to increase their chances of reproductive success. This drive, in males, is activated by testosterone and its metabolites. Conversely, primary reproductive motivation and success in female mammals is the production and care for offspring, behaviors primarily managed by circulating levels of estradiol and progesterone. Perhaps because of differences in selection pressures in male and female reproduction strategies, there exist differences in neural systems that mediate motivational behaviors (Becker et al., 2007). Sex differences in motivation have been demonstrated along two dimensions: estrous cycle and neuroanatomy. While gonadal hormones remain constant in males, females experience cyclical fluctuations defined by periods of high and low levels of progesterone and estradiol. The human female menstrual cycle lasts

approximately 28 days and is divided into the follicular phase and the luteal phase (*Figure. 1)*. During the early follicular phase, menses occurs and is characterized by low levels of progesterone and estrogen. Estrogen gradually increases and peaks just prior to ovulation, while progesterone remains low until after ovulation. During the luteal phase, progesterone rises and peaks 3 - 8 days post-ovulation. In female rodents, the estrous cycle lasts 4-5 days on average and is comprised of four stages (proestrus, estrus, metestrus, and diestrus; *Figure. 1*). Each of the four stages is defined by periods of relatively elevated levels of estradiol (proestrus/estrous) and relatively low levels (metestrus/diestrus). Circulating gonadal hormones modulate motivation in females, increasing their vulnerability to substance abuse. Sexual dimorphisms in motivation may be a result of differences in reward-related learning.

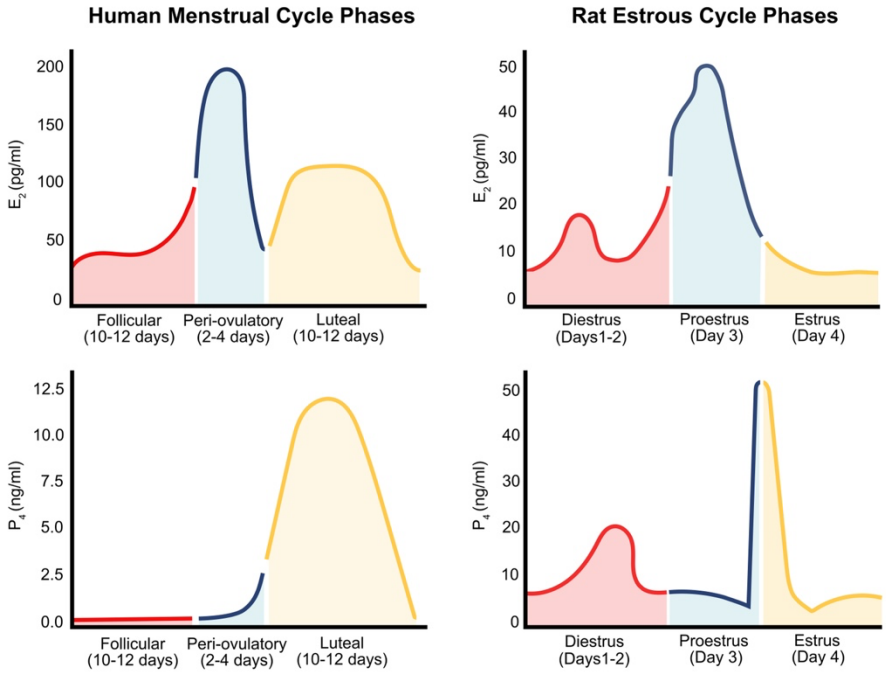

Figure 1. **Contrasts the human menstrual cycle (left) and rat estrous cycle (right).** The human menstrual cycle lasts approximately 28 days and is regulated by circulating hormones estrogen (E2) (top) and progesterone (P4) (bottom). The menstrual cycle can be subdivided into four phases: the menstrual phase, the follicular phase, ovulation, and the luteal phase. During the peri-ovulatory phase, levels of E2

peak. This peak is followed by a steep drop and plateau mid-luteal phase. P4 levels increase gradually towards the end ovulation and peak in the middle of the luteal phase. The rat estrous cycle lasts 4-5 days and can also be divided into four phases: proestrus, estrous, metestrus, and diestrus. During proestrus, E2 levels rise and peak while P4 levels peak during mid-proestrus. (Reprinted without permission Kokane & Perrotti 2020).

The mesolimbic reward pathway is crucial in mediating reinforcing and pleasurable behaviors such as eating, drinking, and mating (Becker, 2016; Gardner, 2011; Kokane & Perrotti, 2020). Primarily based on data collected from men and male animals, ascending dopamine (DA) neurons in the ventral tegmental area (VTA) and substantia nigra project and transmit signals to various forebrain structures including the prefrontal cortex, striatum, hippocampus, nucleus accumbens (NAcc), and amygdala. These regions are responsible for modulating and reinforcing behaviors that are critical for an organism's survival. However, persistent drug use leads to enhanced activation of these structures as well as long-term structural and functional modification, such changes perpetuate the cycle of drug use and abuse (Nestler, 2016; Zorrilla & Koob, 2019). Projections of dopamine neurons arising from the ventral tegmental area (VTA) are involved in regulating motivational processes related to aversive or rewarding stimuli. The primary target of dopaminergic projections of the VTA is the NAcc, a region involved in mediating the effects of rewarding stimuli, be it naturally occurring rewards in the environment, or drugs of abuse. The NAcc is comprised of the core and the shell which differ in their afferent input and efferent projections, each region regulates specific functions. The amygdala is critical in processing emotional memory and is therefore motivationally significant in both aversive and appetitive contexts. The amygdala is comprised of numerous nuclei: the central, medial, and basolateral amygdala (CeA, MeA, and BLA respectively). Notably, the CeA has been shown to modulate behaviors associated with motivational and affective signs of opiate withdrawal (Stinus et. al., 1990).

Cessation of opioid use is accompanied by both somatic and affective withdrawal symptoms. The aversive nature of opioid use withdrawal symptoms and the avoidance of experiencing them have been implicated in persistent drug use as a means of alleviation (Koob, 2009). Repercussions of opioid withdrawal extend beyond acute unconditioned effects, rodents have demonstrated conditioned aversive responses to previously neutral environmental stimuli through use of a classical conditioning paradigm, conditioned place aversion (CPA). CPA is a sensitive measure of aversive properties of a particular stimulus wherein a neutral environment, like a tactilely distinct chamber, is paired with noxious effects of an unconditioned stimulus, like naloxone-precipitated opiate withdrawal.

Research Objectives

Preclinical studies assessing the impact of ovarian hormones on the aversive effects following withdrawal from opioids are limited and findings are disparate. The goal of this work was to identify and characterize sex and estrous cycle differences in acute conditioned morphine withdrawal. Furthermore, we sought to uncover the possibility of convergent mechanisms through ovariectomy and exogenous hormone treatment.

CHAPTER 2 METHODS

2.1. Subjects

115 Adult, intact, male and female, experimentally naïve Long Evans rats were group housed (3 per cage) with same-sex cage mates, see *Figure 2.* for subject flow. Animals were housed in a humidity and temperature-controlled environment under a 12-hour reversed light/dark cycle wherein lights were on at 7p.m. and off at 7 a.m. Food and water were available ad libitum throughout the study. Cage changes were carried out every Monday and Thursday by

the researcher. All experimental procedures and animal maintenance were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Texas at Arlington IACUC.

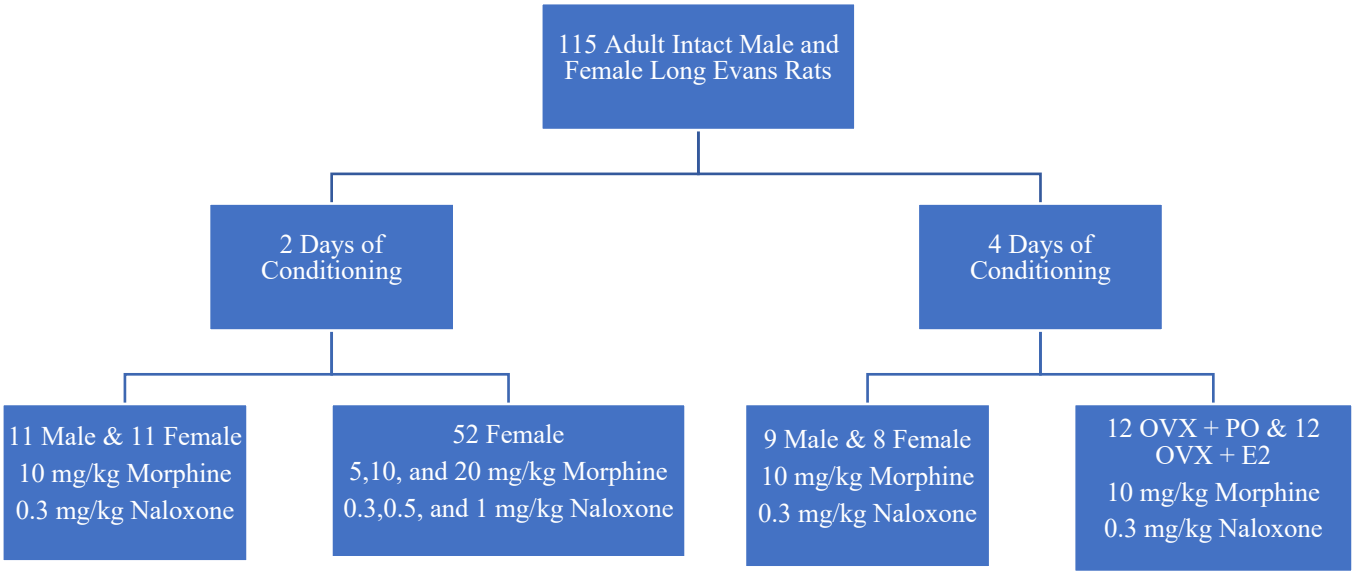

Figure 2. Illustrates subject flow. 115 Adult intact Long Evans rats were subdivided into two and four – day conditioning cycles. Additional groups were also used for a dose response procedure and a subset of females were also ovariectomized and treated with exogenous estradiol (E2) or Peanut oil (PO).

2.2. Vaginal lavage testing

To determine and predict estrous cycle phases (estrus, metestrus, diestrus, proestrus), all females underwent 10 days (at least two cycles) of consecutive vaginal lavage testing wherein vaginal secretions were collected via a micropipette. To reduce variability, lavage samples were collected at approximately the same time every day. Estrous cycle phases were determined by examining morphology and composition of cells collected from the vagina. The changes in cell morphology (nucleated epithelial cells, cornified cells, and leukocytes) and composition across the cycle are associated with fluctuations of estradiol and progesterone, therefore this technique

can be used as a noninvasive method of tracking fluctuating levels of hormones (*Figure* 3). The vaginal orifice was flushed with 30 µL of 0.9 % saline using a micropipette and vaginal secretions were collected on a microscope slide. Great care was taken to avoid stimulating the cervix which may induce pseudopregnancy and a persistent diestrus phase. Unstained secretions were then observed under a light microscope and stages of the estrous cycle were determined. Vaginal lavage images were observed at 10X and 20X magnification. Literature suggests that vaginal lavage testing may impact behavioral response (Walker et al., 2002), hence lavage testing took place prior to the CPA procedure and at times not associated with behavioral testing or transport procedures. To control for handling effects associated with the vaginal lavage procedure, male rats underwent a procedural control wherein they experienced similar handling.

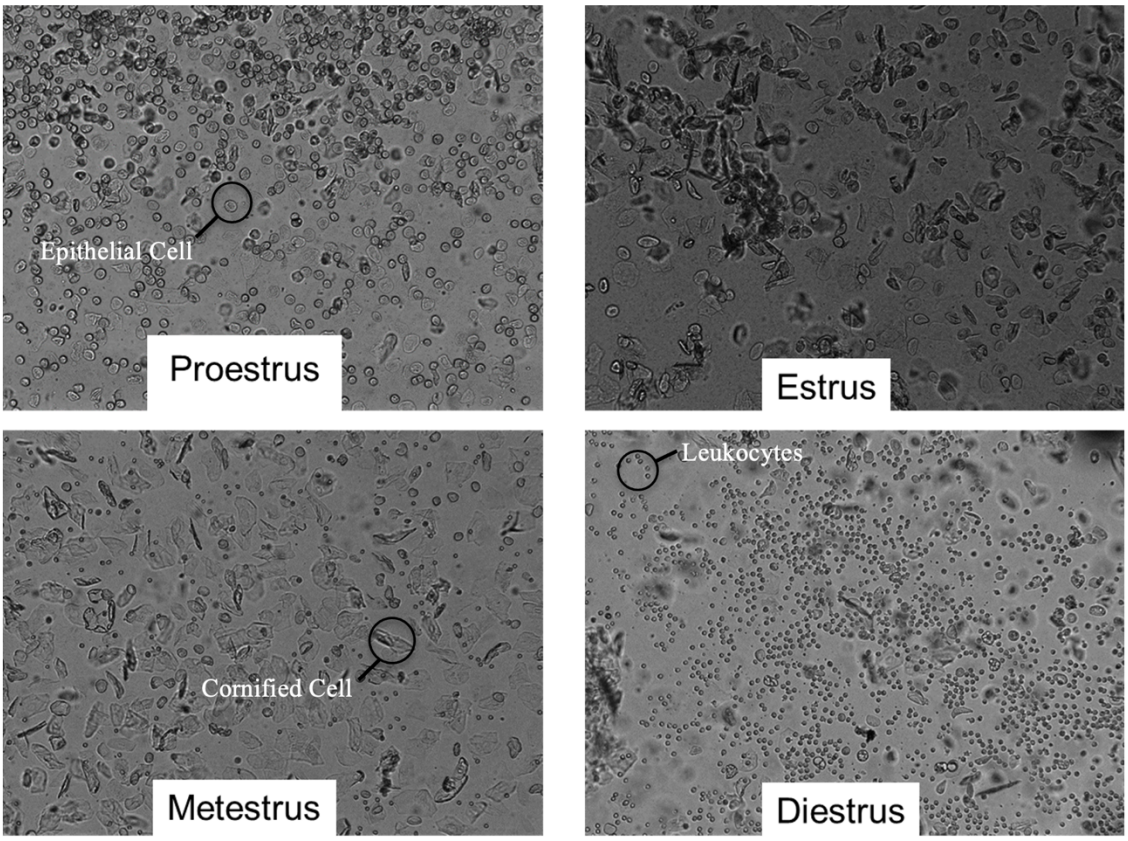

Figure 3. **Illustrates examples of the variance in cell morphology and composition across the estrous cycle in naturally cycling females.** Estrous cycle phase was determined by examining morphology and composition of cells collected from the vagina. During proestrus the vaginal lavage sample contains a

high concentration of nucleated epithelial cells, as shown in the top left panel. During estrus lavage samples contain anucleated cornified epithelial cells as shown in the top right panel. Metestrus is characterized by a mix of nucleated epithelial cells, leukocytes, and cornified epithelial cells as shown in the bottom left panel. Diestrus lavage samples largely contain leukocytes as shown in the bottom left panel. Changes in cell morphology and composition over the estrous cycle are associated with estradiol fluctuations.

2.3. Ovariectomy and hormone treatment

To assess the impact of circulating ovarian hormones on conditioned place aversion and the possibility of underlying convergent mechanisms, ovariectomies were performed on a subgroup of females. Female rodents were anesthetized with an isoflurane-oxygen mixture and ovariectomized (OVX). The OVX procedure first involved shaving and carefully sterilizing both sides of the lower abdomen with iodine. An incision was made on the lower abdomen, slightly below the ribs. An additional incision was made through the muscle, the ovary was clamped with a hemostat, excised through the incision and the fallopian tube was cauterized with sterile surgical scissors. Following removal of the ovary, the muscle layer was sutured shut and wound clips were applied to the skin incision. The procedure was repeated on the contralateral side. Approximately 4-5 days following the OVX procedure, female animals were assigned to one of two groups: vehicle (0.1 ml peanut oil; PO), or 5µg 17β-Estradiol 3 benzoate dissolved in 0.1 ml peanut oil. Hormone and vehicle treatment was administered once subcutaneously (s.c.) approximately thirty minutes prior to each naloxone conditioning session. Male rats received identical treatment to female rats with the exception of estradiol injections and surgeries.

2.4. Drugs and administration

Morphine sulfate (Spectrum Chemical Manufacturing Corp.) was dissolved in 0.9% sterile saline. Subcutaneous injections (s.c.) of morphine were administered at a volume of 1 ml/kg at a dose of 10 mg/kg. Similarly, saline (0.9% wt/vol) was injected at a volume of 1 ml/kg.

Naloxone Hydrochloride (Sigma-Aldrich) was also dissolved in 0.9% sterile saline and injected subcutaneously at a volume of 1 ml/kg and a dose of 0.3 mg/kg.

2.4a. Dose response

A dose response as it relates to conditioned place aversion has not been conducted in female rodents, therefore a dose response was conducted for 5, 10, and 20 mg/kg of Morphine and 0.3, 0.5, and 1 mg/kg of Naloxone. Morphine sulfate and Naloxone Hydrochloride were administered (s.c.). A breakdown of the conditions of each group can be found below, in *Table 1*.

Table 1.

Breakdown of conditions and groups.

Group	Dose	Days of Conditioning
Female: Intact Female: OVX + Acute Peanut Oil Vehicle Female: OVX + Acute Estradiol Benzoate Male: Intact	10 mg/kg Morphine Sulfate, 0.3 mg/ kg Naloxone Hydrochloride	4 days
Female: Intact Male: Intact	5, 10, and 20 mg/kg Morphine and 0.3, 0.5, and 1 mg/kg Naloxone Hydrochloride. 10 mg/kg of Morphine and 0.3 mg/kg Naloxone Hydrochloride	2 days

2.5. Conditioned Place Avoidance Procedure (CPA)

The conditioned place avoidance (CPA) procedure was performed using a standard three-chambered testing apparatus, comprised of two large chambers and one small shuttle chamber (Med Associates, Georgia, VT). The two large chambers are contextually distinct, one with black walls and steel rod flooring and the other with white walls and wire mesh flooring. The two large chambers are separated by a smaller gray chamber and each chamber is separated by guillotine doors that can be lifted to allow free access to the entire apparatus. A 60 W light is mounted at the top of each chamber, and all three chambers are equipped with infrared photobeam detectors

that automatically collect data during the behavioral test. Animals were further subdivided into two groups. One group underwent two days of conditioning and the other underwent four (*Figure 4*). The procedure took place over a period of four to six days and consisted of three phases: 1) preconditioning, 2) conditioning, and 3) posttest [*Table 2*]. During preconditioning, rats were allowed to freely explore the entire apparatus for 15 minutes. The pretest phase was to determine whether an animal had any preexisting preference or aversion towards any of the chambers prior to conditioning. All rats were then randomly assigned to naloxone/saline chambers for the subsequent conditioning sessions. During the conditioning phase, rats were pretreated with subcutaneous injections of morphine (MOR) or vehicle (0.9% saline; SAL) in their home cages. Four hours following home cage injections, rats received a s.c. injection of naloxone or SAL and confined to one of the two large chambers of the apparatus for approximately 22 minutes. During the posttest phase rats freely explored the apparatus for 15 minutes in a drug free state. Each phase was conducted on consecutive days. Dose responses for both morphine and naloxone were conducted to identify any dose dependent effects between sex and estrous cycle stages.

Table 2.

Summary of experimental protocol for conditioned place aversion.

Summary of Experimental protocol for naloxone-induced conditioned place aversion						
Experimental Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
CPA Phase:	Pre-test	Conditioning	Conditioning	Conditioning	Conditioning	Post Test
Pretreatment:	-	Saline	Morphine	Saline	Morphine	-
Treatment:	-	Vehicle	Naloxone	Vehicle	Naloxone	-

Estradiol or Peanut Oil Injection → 30 Min → **Naloxone or Saline Injection** → **Conditioning or Test**

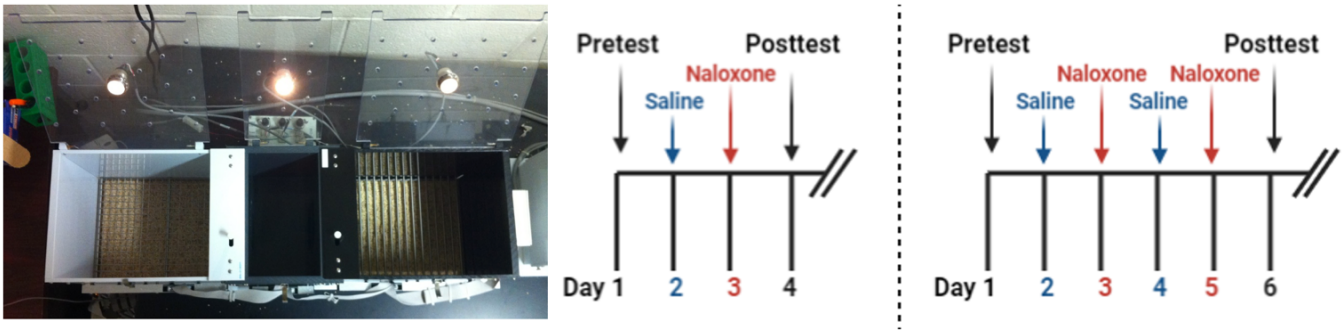

***Figure 4*. CPA Apparatus (Left). Timeline of the CPA paradigm (Right).** Pretest (Day 1): rats freely explore the apparatus in a drug free state. Saline-Naloxone conditioning took place on days 2-3 or 2-5 (2 or 4 days of conditioning). The Posttest was conducted 24 hours after the last conditioning session and was identical to the Pretest.

Drug Treatments: Morphine sulfate (10mg/kg), Naloxone (0.3mg/kg) and Saline were administered subcutaneously in a volume of 0.1ml per 100g body weight. The doses of Morphine and Naloxone were selected based on published findings in male rats (Azar et al., 2003).

2.6 Tissue fixation and sectioning

Immediately after the completion of the posttest phase, animals were sacrificed via exsanguination. Prior to the exsanguination procedure, all animals were deeply anesthetized with sodium pentobarbital (60mg/kg) they were then intracardially exsanguinated with ice cold 0.01 M PBS (Phosphate Buffered Saline) and perfused with 4% paraformaldehyde in 0.01 PBS. Brain tissue were extracted and stored at 4 °C in 4% paraformaldehyde overnight. Brains were transferred to 20% glycerol for at least 24 hours prior to sectioning.

2.7 Data Analyses

Statistical analyses were all performed using GraphPad Prism (version 9) with a significance level of $p < 0.05$. CPA scores (conditioned place aversion scores) were calculated by subtracting time spent in each compartment during the pretest from time spent in each

compartment during the posttest (posttest-pretest). Negative scores were indicative of avoidance of a particular compartment. Animals that displayed significant aversion or preference for one compartment (over 45% of time spent) during the pretest were excluded to control for any preexisting place aversion or preference.

CHAPTER 3 RESULTS

3.1 Conditioned Place Aversion in Intact Males and Females

The overarching goal of this study was to explore sex and hormonally mediated differences in acute conditioned morphine withdrawal using a conditioned place aversion paradigm (CPA). Data below illustrates aversion scores in intact male and female rats after undergoing the conditioned place aversion procedure for a two-day conditioning cycle or a four-day conditioning cycle. During the two-day conditioning procedure shown in *Figure 5*. a paired t-test revealed significant aversion for females, $t(10) = 3.74$, $p = 0.004$, d = 1.77, meaning they spent less time in the chamber associated with the naloxone injection compared to the chamber associated with the saline injection. Similarly, females also displayed significant aversion during the four-day conditioning cycle, $t(7) = 4.79$, $p = 0.002$., d = 2.94. Males displayed significant aversion scores during the two-day conditioning cycle as revealed by a paired t-test, $t(9) = 2.50$, $p = 0.034$, d = 1.36 and the four-day conditioning cycle, $t(8) = 3.75$, $p = 0.006$, d = 1.18 . When comparing aversion scores between males and females, no significant sex difference was found in aversion scores. During the two-day cycle, females on average, demonstrated elevated levels of aversion (M = - 80.37, SEM = 20.37) compared to males (M = - 65.47, SEM = 32.26), however there was no significant effect of sex as revealed by an independent samples t-test, $t(19) = 0.40$, $p = 0.69$ (ns), d = - 0.174. During the four-day conditioning procedure, also shown in *Figure 5*., an independent samples t-test revealed no significant sex difference in time spent in

the naloxone paired chamber. Consistent with the two-day conditioning procedure, males and females demonstrated similar aversion scores. Female animals, again, displayed slightly elevated scores on average (M = -145.70, SEM = 27.29) compared to males (M = -135.90, SEM = 26.68), however no significant sex differences were found, $t(15) = 0.26$, $p = 0.80$ (ns), d = - 0.124.

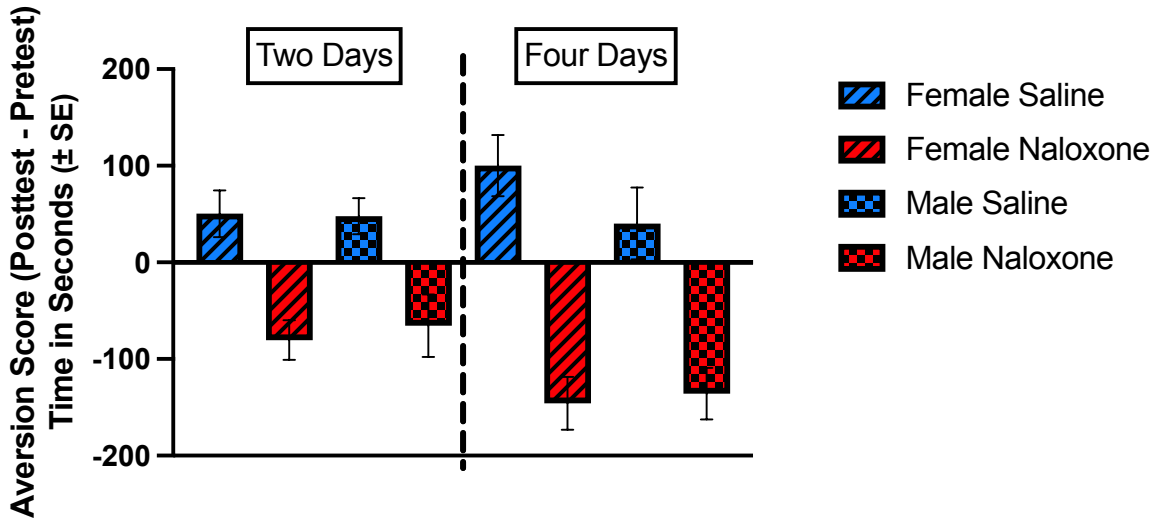

Figure 5. Data are expressed as CPA scores (posttest - pretest) across two days of conditioning (left) and four days of conditioning (right). Intact females are presented as striped and intact males are presented as checkered. Both male and female animals spent significantly less time in the naloxone paired chamber, however no significant differences were found between sexes.

3.2 Conditioned Place Aversion and the Estrous Cycle

While these data did not demonstrate any robust sex differences in acute precipitated morphine withdrawal-induced CPA, data within females did reveal some trends that may implicate the influence of hormonal cycles as shown in *Figure 6*. This study compared the effects of the estrous cycle by assessing the differences between females in proestrus/estrus (High E2) and diestrus (Low E2). Females in the high E2 group displayed significant aversion scores (spending less time in the naloxone paired compartment than the saline paired compartment) as revealed by a paired t-test, $t(6) = 2.81$, $p = 0.031$, d = - 1.061, while females in

the low E2 group did not, $t(2) = 1.40$, $p = 0.297$, d = - 0.81. While there was no significant difference in time spent in the naloxone paired compartment between high and low E2 groups, $t(8) = 1.04$, $p = 0.33$ (ns), d = -1.278, females in the high E2 group spent less time on average in the naloxone paired chamber (M = -77.19, SEM = 14.75) than females in the low E2 group (M = 40.59. SEM = 44.10). Findings from the investigation in intact females indicated that the predominance of estrogen and/or progesterone during particular phases of the estrous cycle may affect the expression of morphine withdrawal-induced CPA and therefore the severity of opioid withdrawal.

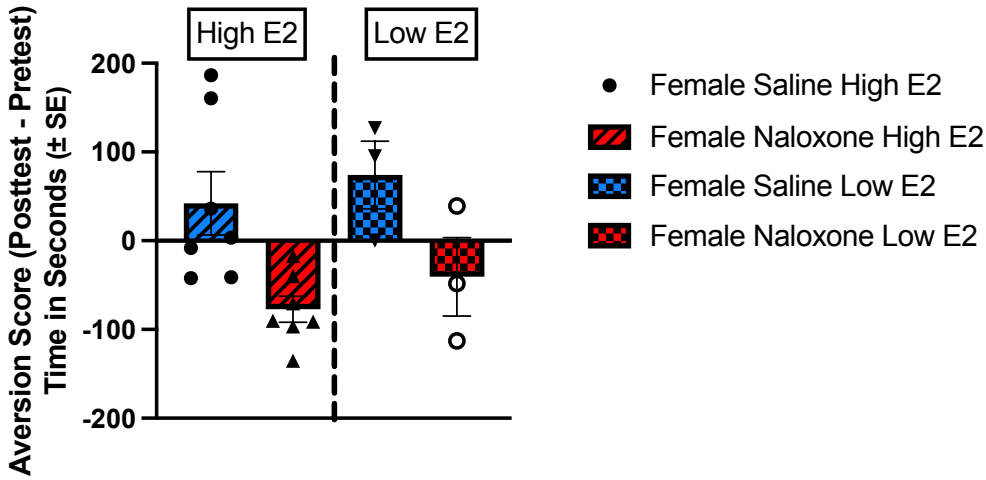

Figure 6. Data are expressed as CPA scores (posttest - pretest) across two days of conditioning in intact females during high levels of circulating estradiol (E2) (left) and low levels of circulating E2 (right). Intact females displaying high levels of E2 are presented as striped and intact females displaying low levels of E2 are presented as checkered. Both groups spent significantly less time in the naloxone paired chamber, however no significant differences were found between groups.

3.3 Conditioned Place Aversion in Ovariectomized Females

It is difficult to draw these conclusions due to small sample size and the inability to "assign" naturally cycling rats to estrous cycle phases. Accordingly, an additional group of females were ovariectomized and levels of estradiol were artificially manipulated to directly

examine effects of estradiol on morphine-withdrawal naloxone induced CPA. Females across all groups displayed significant CPA, $t(7) = 4.79$, $p = 0.002$, d = 2.94 (intact females; four-day conditioning cycle), $t(11) = 5.62$, $p = 0.0002$ (OVX + PO), d = -1.382, $t(11) = 8.33$, $p < 0.0001$ (OVX + EB), d = -2.418. A one-way Analysis of Variance (ANOVA) was performed to compare intact, OVX + PO, and OVX + EB groups and determine the effect of acute estradiol administration on conditioned place aversion. As shown in *Figure 7.,* no significant differences were found between intact females (four-day conditioning procedure), OVX females pretreated with estradiol, and OVX females given peanut oil, $F(2, 29) = 0.32$, $p = 0.73$ (ns)., $\eta_p^2 = 0.021$. Females pretreated with estradiol prior to conditioning displayed slightly elevated CPA scores ($M = -166.30$, $SEM = 16.20$), compared to females given PO ($M = -148.50$, $SEM = 19.06$).

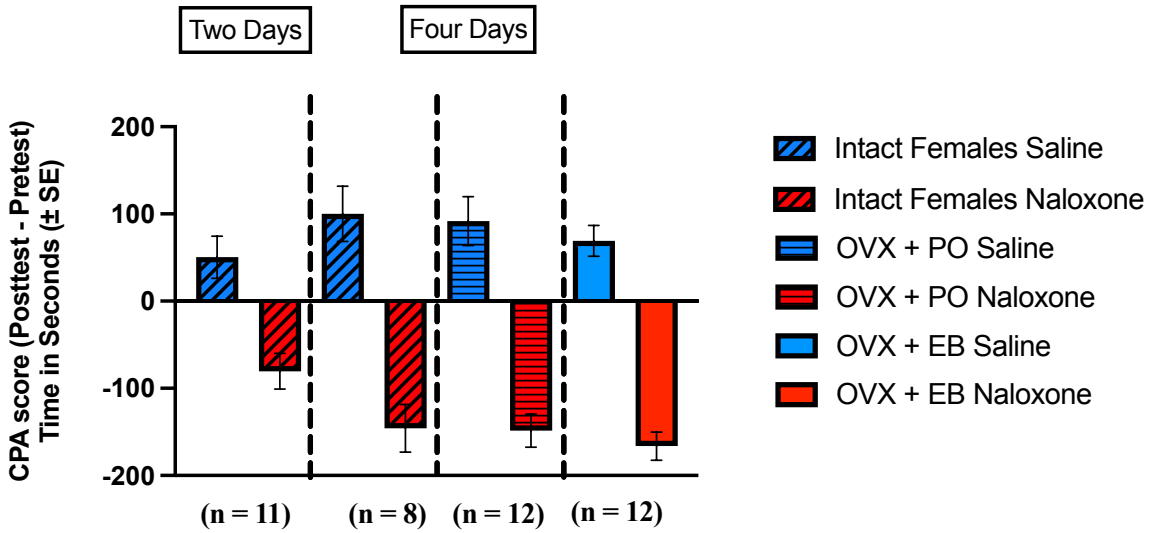

Figure 7. Data are expressed as CPA scores (posttest - pretest) across two days of conditioning (left) and four days of conditioning (right). All female animals spent significantly less time in the naloxone paired chamber than the saline paired chamber. No significant differences were found between intact, ovariectomized, and ovariectomized + hormone treated animals.

3.4 Conditioned Place Aversion Dose Response in Intact Females

To extend, compare, and determine an ideal dose for conducting the CPA procedure in females, a dose response was conducted. Per a two-way analysis of variance (ANOVA), the

effect of morphine dose and naloxone dose did not have a statistically significant interaction effect on CPA scores as shown in *Figure 8*., $F(4,43) = 1.45$, $p = 0.24$, $\eta_p^2 = 0.127$. Naloxone dose also did not induce a significant difference in CPA scores, $F(2,43) = 0.54$, $p = 0.59$, $\eta_p^2 = 0.025$. Finally, Morphine dose also did not induce a significant difference in CPA scores, $F(2,43) = 0.74$, $p = 0.48$, $\eta_p^2 = 0.033$.

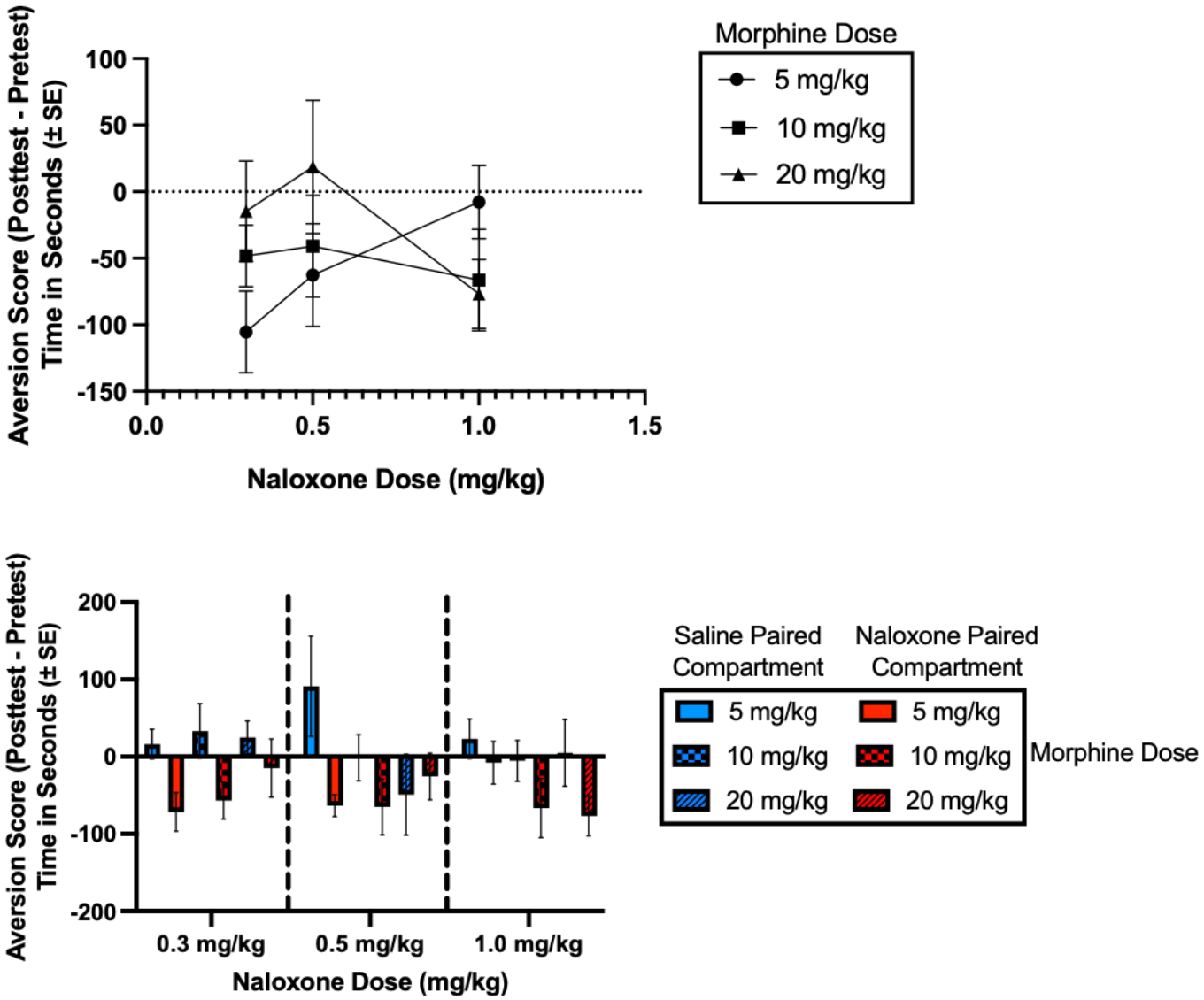

Figure 8. Data are expressed in CPA scores (posttest - pretest) across two days of conditioning. The top panel strictly displays time spent in the naloxone paired chamber across various naloxone and morphine doses. The bottom panel displays time spent in the naloxone paired chamber and saline paired chamber across various doses. No significant differences were found based on naloxone or morphine dose.

CHAPTER 4: DISCUSSION

4.1 Summary of Findings

The objective of the present study was to determine the extent, if any, to which sex and circulating gonadal hormones influence the expression of acute precipitated morphine withdrawal induced conditioned place aversion in a preclinical rodent model. At present there are no studies exploring sex differences in conditioned place aversion using this particular paradigm, therefore our study aimed to extend and compare present findings observed in males. It was hypothesized that animals undergoing the four-day conditioning cycle would demonstrate increased aversion (spend less time in the compartment associated with acute morphine withdrawal following a naloxone injection) compared to animals undergoing the two-day conditioning cycle. A significant difference was found between the two and four-day conditioning cycle, in that the four-day cycle elicited increased levels of aversion compared to the two-day cycle. It was also hypothesized that compared to males, females would demonstrate elevated levels of aversion. Unexpectedly, our study found that males and females express similar conditioned place aversion scores in both the two- and four-day conditioning procedures. We also hypothesized that CPA scores would vary across the estrous cycle. In intact females with high levels of circulating estradiol, significant CPA was demonstrated while those with low levels of circulating estradiol did not. However, due to small sample size and the inability to "assign" naturally cycling females to estrous cycle phases, it was difficult to draw any firm conclusions. Therefore, we sought to isolate the effects of estradiol by manipulating estradiol levels in ovariectomized rats. It was hypothesized that ovariectomized (OVX) females treated with estradiol 30 minutes prior to naloxone conditioning would demonstrate increased CPA scores compared to OVX females treated with the peanut oil vehicle. Females treated with

estradiol and females treated with peanut oil both developed significant levels of CPA, however there were no significant differences between the groups. Overall, the results of this study did not reveal a significant effect of elevations of estradiol levels alone on conditioned place aversion in non-opioid dependent animals.

4.2 Interpretation of Findings

Substance use disorder is often characterized by the compulsion to seek a drug to maintain the desired pleasurable effects. However, drug users also experience negative affective and physical symptoms when the substance is not present, oftentimes leading to continued use to alleviate these symptoms (Wikler A., 1965). In the context of drug addiction, this avoidance of negative affective and physical symptoms during periods of abstinence is alone a motivation for further drug use by way of negative reinforcement. While men have historically reported more instances of opioid use and abuse, women escalate from casual consumption to abuse at an increased rate compared to men. Women also report experiencing more aversive affective symptoms during opiate withdrawal and as a result, an increased likelihood of relapse (Hernandez-Avila et al., 2004). The present study aimed to investigate and explore the difference, if any, in acute precipitated morphine withdrawal between sexes and hormonal states, by using the conditioned place aversion (CPA) paradigm. Overall, the results did not suggest that sex or estradiol levels have any significant effect on conditioned place aversion. Although not statistically significant, there exist intriguing trends that warrant further investigation.

Our first experiment assessed the expression of conditioned place aversion in acute naloxone precipitated withdrawal between intact male and female Long-Evans rats. Consistent with a recently published report using a Y-maze test, both the two-day conditioning cycle and the four-day conditioning cycle yielded no significant differences between intact male and female

rats (Fournier et al., 2023). Because this apparatus is comprised of three chambers, one saline-paired chamber, one naloxone-paired chamber, and one neutral chamber it was possible to assess avoidance of the naloxone-paired chamber and preference for the saline-paired chamber. While there were no significant differences in the preference for the saline-paired chamber, females on average spent more time in the saline paired compartment compared to males. Altogether, these data suggest that intact male and female Long-Evans rats are equally sensitive to the aversive effects of acute precipitated naloxone induced withdrawal and furthermore appear to express similar cue associated memory as it relates to acute opiate withdrawal.

Intact females were also subdivided based on the stage of estrous cycle to determine whether high or low levels of circulating estradiol may influence the expression of conditioned place aversion. Females in the high estradiol group expressed significant aversion, meaning they spent significantly more time in the saline-paired compartment compared to females in the low estradiol group. These data initially implied that estradiol may affect the expression of naloxone precipitated acute morphine withdrawal-induced CPA. However, upon further investigation using ovariectomized females, it appears that estradiol alone may not influence expression of CPA.

4.3 Limitations and Future Directions

Initial findings reveal no significant sex differences in naloxone induced conditioned place aversion after an acute morphine injection however, future studies are needed to expand and verify these preliminary findings. Future studies aim to determine whether sex differences can be found after chronic morphine exposure and immunohistochemical analyses are currently underway to determine the influence, if any, of estradiol on naloxone-induced CPA and resulting c-Fos signaling in the mesolimbic reward pathway.

The current experiment is limited to acute morphine administration. While anxiogenic behavior was exhibited after acute morphine pretreatment and naloxone administration, addiction is a chronic escalating process that extends beyond an acute exposure to a given drug of abuse. The neuroadaptive process that takes place between initial substance exposure, abuse, and dependence involves, but is not limited to, elevation in levels of adenylate cyclase activity in the locus coeruleus and nucleus accumbens, areas associated with opiate withdrawal, the motivational effects of withdrawal and reduction in the number of neurofilament protein in the ventral tegmental area (VTA), the cell bodies of which project to the nucleus accumbens (Nestler, 1996). Neuroadaptations that accompany chronic opioid use and dependence are therefore not accounted for in this study, consequently it is difficult to draw definitive conclusions about sex differences in aversive motivational behavior without considering the motivational consequences of long-term use or dependence.

In summary, similar levels of place aversion were expressed in intact male and intact female Long-Evans rats in a three-chamber conditioned place aversion paradigm. No differences in CPA expression were found on the level of sex (in intact animals) or presence of estradiol (in ovariectomized animals). While the present study did not find such differences, it did find persistent place aversion induced by negative reinforcement as a result of acute precipitated opiate withdrawal which is a critical component and motivation for relapse. Finally, this study does not discount the need for better understanding of the possibility of underlying mechanisms that may contribute sex dependent differences (i.e. convergent sex differences) that influence each stage of addiction.

References

Alexander, B. K., Coambs, R. B., & Hadaway, P. F. (1978). The effect of housing and gender on morphine self-administration in rats. *Psychopharmacology*, *58*(2), 175–179. https://doi.org/10.1007/BF00426903

Ali, B. H., Sharif, S. I., & Elkadi, A. (1995). Sex differences and the effect of gonadectomy on morphine-induced antinociception and dependence in rats and mice. *Clinical and Experimental Pharmacology & Physiology*, *22*(5), 342–344. https://doi.org/10.1111/j.1440-1681.1995.tb02012.x

Amandusson, Å., & Blomqvist, A. (2013). Estrogenic influences in pain processing. *Frontiers in Neuroendocrinology*, *34*(4), 329–349. https://doi.org/10.1016/j.yfrne.2013.06.001

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders*. American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596

Arfken, C. L., Klein, C., di Menza, S., & Schuster, C. R. (2001). Gender differences in problem severity at assessment and treatment retention. *Journal of Substance Abuse Treatment*, *20*(1), 53–57. https://doi.org/10.1016/s0740-5472(00)00155-0

Azar, M. R., Jones, B. C., & Schulteis, G. (2003). Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. *Psychopharmacology*, *170*(1), 42–50. https://doi.org/10.1007/s00213-003-1514-y

Back, S. E., Payne, R. L., Simpson, A. N., & Brady, K. T. (2010). Gender and prescription opioids: findings from the National Survey on Drug Use and Health. *Addictive Behaviors*, *35*(11), 1001–1007. https://doi.org/10.1016/j.addbeh.2010.06.018

Back, S. E., Payne, R. L., Wahlquist, A. H., Carter, R. E., Stroud, Z., Haynes, L., Hillhouse, M., Brady, K. T., & Ling, W. (2011). Comparative Profiles of Men and Women with Opioid Dependence: Results from a National Multisite Effectiveness Trial. *The American Journal of Drug and Alcohol Abuse*, *37*(5), 313–323. https://doi.org/10.3109/00952990.2011.596982

Becker, J. B. (2016). Sex differences in addiction. *Dialogues in Clinical Neuroscience*, *18*(4), 395–402. https://doi.org/10.31887/DCNS.2016.18.4/jbecker

Becker, J. B., Berkley, K. J., Geary, N., Hampson, E., Herman, J. P., & Young, E. (2007). *Sex Differences in the Brain*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780195311587.001.0001

Becker, J. B., & Chartoff, E. (2019). Sex differences in neural mechanisms mediating reward and addiction. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *44*(1), 166–183. https://doi.org/10.1038/s41386-018-0125-6

Becker, J. B., & Koob, G. F. (2016). Sex differences in animal models: Focus on addiction. In *Pharmacological Reviews* (Vol. 68, Issue 2, pp. 242–263). American Society for Pharmacology and Experimental Therapy. https://doi.org/10.1124/pr.115.011163

Berkley, K. J. (1997). Sex differences in pain. *The Behavioral and Brain Sciences*, *20*(3), 371–380; discussion 435-513. https://doi.org/10.1017/s0140525x97221485

Bobzean, S. A. M., DeNobrega, A. K., & Perrotti, L. I. (2014). Sex differences in the neurobiology of drug addiction. *Experimental Neurology*, *259*, 64–74. https://doi.org/10.1016/j.expneurol.2014.01.022

Bodnar, R. J., & Kest, B. (2010). Sex differences in opioid analgesia, hyperalgesia, tolerance and withdrawal: central mechanisms of action and roles of gonadal hormones. *Hormones and Behavior*, *58*(1), 72–81. https://doi.org/10.1016/j.yhbeh.2009.09.012

Cicero, T. J., Aylward, S. C., & Meyer, E. R. (2003). Gender differences in the intravenous self-administration of mu opiate agonists. *Pharmacology Biochemistry and Behavior*, *74*(3), 541–549. https://doi.org/10.1016/S0091-3057(02)01039-0

Cicero, T. J., Nock, B., & Meyer, E. R. (2002). Gender-linked differences in the expression of physical dependence in the rat. *Pharmacology, Biochemistry, and Behavior*, *72*(3), 691–697. https://doi.org/10.1016/s0091-3057(02)00740-2

Craft, R. M. (2003). Sex differences in opioid analgesia: "from mouse to man". *The Clinical Journal of Pain*, *19*(3), 175–186. https://doi.org/10.1097/00002508-200305000-00005

Craft, R. M., Stratmann, J. A., Bartok, R. E., Walpole, T. I., & King, S. J. (1999). Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology*, *143*(1), 1–7. https://doi.org/10.1007/s002130050911

el-Kadi, A. O., & Sharif, S. I. (1994). The influence of various experimental conditions on the expression of naloxone-induced withdrawal symptoms in mice. *General Pharmacology*, *25*(7), 1505–1510. https://doi.org/10.1016/0306-3623(94)90181-3

Fillingim, R. B., King, C. D., Ribeiro-Dasilva, M. C., Rahim-Williams, B., & Riley, J. L. (2009). Sex, gender, and pain: a review of recent clinical and experimental findings. *The Journal of Pain*, *10*(5), 447–485. https://doi.org/10.1016/j.jpain.2008.12.001

Fishman, M. (2008). Precipitated withdrawal during maintenance opioid blockade with extended release naltrexone. *Addiction (Abingdon, England)*, *103*(8), 1399–1401. https://doi.org/10.1111/j.1360-0443.2008.02252.x

Fournier, M. L., Faugere, A., Barba-Vila, O., & Le Moine, C. (2023). Male and female rats show opiate withdrawal-induced place aversion and extinction in a Y-maze paradigm. *Behavioural Brain Research*, *437*, 114122. https://doi.org/10.1016/J.BBR.2022.114122

Gardner, E. L. (2011). *Addiction and Brain Reward and Antireward Pathways* (pp. 22–60). https://doi.org/10.1159/000324065

Goldberg, S. R., & Schuster, C. R. (1970). Conditioned nalorphine-induced abstinence changes: persistence in post morphine-dependent monkeys. *Journal of the Experimental Analysis of Behavior*, *14*(1), 33–46. https://doi.org/10.1901/jeab.1970.14-33

Green, T. C., Grimes Serrano, J. M., Licari, A., Budman, S. H., & Butler, S. F. (2009). Women who abuse prescription opioids: findings from the Addiction Severity Index-Multimedia Version Connect prescription opioid database. *Drug and Alcohol Dependence*, *103*(1–2), 65–73. https://doi.org/10.1016/j.drugalcdep.2009.03.014

Gureje, O., Von Korff, M., Simon, G. E., & Gater, R. (1998). Persistent pain and well-being: a World Health Organization Study in Primary Care. *JAMA*, *280*(2), 147–151. https://doi.org/10.1001/jama.280.2.147

Hernandez-Avila, C. A., Rounsaville, B. J., & Kranzler, H. R. (2004). Opioid-, cannabis- and alcohol-dependent women show more rapid progression to substance abuse treatment. *Drug and Alcohol Dependence*, *74*(3), 265–272. https://doi.org/10.1016/J.DRUGALCDEP.2004.02.001

Hodes, G. E. (2018). A primer on sex differences in the behavioral response to stress. *Current Opinion in Behavioral Sciences*, *23*, 75–83. https://doi.org/10.1016/j.cobeha.2018.03.012

Irwin, S., & Seevers, M. H. (1956). Altered response to drugs in the post addict (Macaca mulatta). *J Pharmacol Exp Ther*, *116*, 31–32.

Karami, M., & Zarrindast, M. R. (2008). Morphine sex-dependently induced place conditioning in adult Wistar rats. *European Journal of Pharmacology*, *582*(1–3), 78–87. https://doi.org/10.1016/j.ejphar.2007.12.010

Kokane, S. S., & Perrotti, L. I. (2020). Sex Differences and the Role of Estradiol in Mesolimbic Reward Circuits and Vulnerability to Cocaine and Opiate Addiction. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/fnbeh.2020.00074

Koob, G. F. (2009). Neurobiological substrates for the dark side of compulsivity in addiction. *Neuropharmacology*, *56 Suppl 1*(Suppl 1), 18–31. https://doi.org/10.1016/j.neuropharm.2008.07.043

Koob, G. F. (2020). Neurobiology of Opioid Addiction: Opponent Process, Hyperkatifeia, and Negative Reinforcement. *Biological Psychiatry*, *87*(1), 44–53. https://doi.org/10.1016/j.biopsych.2019.05.023

Lynch, W. J., & Carroll, M. E. (1999). Sex differences in the acquisition of intravenously self-administered cocaine and heroin in rats. *Psychopharmacology*, *144*(1), 77–82. https://doi.org/10.1007/s002130050979

Mazure, C. M., & Fiellin, D. A. (2018). Women and opioids: something different is happening here. *Lancet (London, England)*, *392*(10141), 9–11. https://doi.org/10.1016/S0140-6736(18)31203-0

McHugh, R. K., Weiss, R. D., Cornelius, M., Martel, M. O., Jamison, R. N., & Edwards, R. R. (2016). Distress Intolerance and Prescription Opioid Misuse Among Patients With Chronic Pain. *The Journal of Pain*, *17*(7), 806–814. https://doi.org/10.1016/j.jpain.2016.03.004

McLellan, A. T., Childress, A. R., Ehrman, R., O'Brien, C. P., & Pashko, S. (1986). Extinguishing conditioned responses during opiate dependence treatment turning laboratory findings into clinical procedures. *Journal of Substance Abuse Treatment*, *3*(1), 33–40. https://doi.org/10.1016/0740-5472(86)90006-1

Mogil, J. S. (2012). Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nature Reviews. Neuroscience*, *13*(12), 859–866. https://doi.org/10.1038/nrn3360

Negus, S. S., Zuzga, D. S., & Mello, N. K. (2002). Sex differences in opioid antinociception in rhesus monkeys: antagonism of fentanyl and U50,488 by quadazocine. *The Journal of Pain*, *3*(3), 218–226. https://doi.org/10.1054/jpai.2002.124734

Nestler, E. J. (1996). Under siege: The brain on opiates. In *Neuron* (Vol. 16, Issue 5). https://doi.org/10.1016/S0896-6273(00)80110-5

Nestler, E. J. (2016). Reflections on: "A general role for adaptations in G-Proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function". *Brain Research*, *1645*, 71–74. https://doi.org/10.1016/j.brainres.2015.12.039

O'Brien, C. P., Testa, T., O'Brien, T. J., & Greenstein, R. (1976). Conditioning in human opiate addicts. *The Pavlovian Journal of Biological Science*, *11*(4), 195–202. https://doi.org/10.1007/BF03000314

Papaleo, F., & Contarino, A. (2006). Gender- and morphine dose-linked expression of spontaneous somatic opiate withdrawal in mice. *Behavioural Brain Research*, *170*(1), 110–118. https://doi.org/10.1016/j.bbr.2006.02.009

Puigdollers, E., Domingo-Salvany, A., Brugal, M. T., Torrens, M., Alvarós, J., Castillo, C., Magrí, N., Martín, S., & Vázquez, J. M. (2004). Characteristics of heroin addicts entering methadone maintenance treatment: quality of life and gender. *Substance Use & Misuse*, *39*(9), 1353–1368. https://doi.org/10.1081/ja-120039392

Sanchis-Segura, C., & Becker, J. B. (2016). Why we should consider sex (and study sex differences) in addiction research. *Addiction Biology*, *21*(5), 995–1006. https://doi.org/10.1111/adb.12382

Stoffel, E. C., Ulibarri, C. M., & Craft, R. M. (2003). Gonadal steroid hormone modulation of nociception, morphine antinociception and reproductive indices in male and female rats. *Pain*, *103*(3), 285–302. https://doi.org/10.1016/s0304-3959(02)00457-8

Terplan, M. (2017). Women and the opioid crisis: historical context and public health solutions. *Fertility and Sterility*, *108*(2), 195–199. https://doi.org/10.1016/j.fertnstert.2017.06.007

Towers, E. B., Tunstall, B. J., McCracken, M. L., Vendruscolo, L. F., & Koob, G. F. (2019). Male and female mice develop escalation of heroin intake and dependence following extended access. *Neuropharmacology*, *151*, 189–194. https://doi.org/10.1016/j.neuropharm.2019.03.019

Vigna-Taglianti, F. D., Burroni, P., Mathis, F., Versino, E., Beccaria, F., Rotelli, M., Garneri, M., Picciolini, A., & Bargagli, A. M. (2016). Gender Differences in Heroin Addiction and Treatment: Results from the VEdeTTE Cohort. *Substance Use & Misuse*, *51*(3), 295–309. https://doi.org/10.3109/10826084.2015.1108339

Walker, Q. D., Nelson, C. J., Smith, D., & Kuhn, C. M. (2002). Vaginal lavage attenuates cocaine-stimulated activity and establishes place preference in rats. *Pharmacology Biochemistry and Behavior*, *73*(4), 743–752. https://doi.org/10.1016/S0091-3057(02)00883-3

Wikler A. (1965). Conditioning factors in opiate addictions and relapse. In D. Wilner & G. Kassebaum (Eds.), *Narcotics* (pp. 85–100). McGraw-Hill.

Wikler, A., & Pescor, F. T. (1967). Classical conditioning of a morphine abstinence phenomenon, reinforcement of opioid-drinking behavior and "relapse" in morphine-addicted rats. *Psychopharmacologia*, *10*(3), 255–284. https://doi.org/10.1007/BF00401386

Yu, J., Zhang, S., Epstein, D. H., Fang, Y., Shi, J., Qin, H., Yao, S., Le Foll, B., & Lu, L. (2007). Gender and stimulus difference in cue-induced responses in abstinent heroin users. *Pharmacology Biochemistry and Behavior*, *86*(3), 485–492. https://doi.org/10.1016/j.pbb.2007.01.008

Zorrilla, E. P., & Koob, G. F. (2019). Impulsivity Derived From the Dark Side: Neurocircuits That Contribute to Negative Urgency. *Frontiers in Behavioral Neuroscience*, *13*. https://doi.org/10.3389/fnbeh.2019.00136