BIOLOGICAL SEX AND OVARIAN HORMONES MATTER IN ADDICTION

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CREDITS

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Abstract

Females have been underrepresented in clinical and preclinical research of addictive drugs and substance use disorders (SUD), which has led to huge gaps in our knowledge regarding the disease process in females and sex-informed treatment strategies. The goal of this dissertation was to investigate sex differences in development, neurobiological basis, and treatment of SUDs with a focus on cocaine and opioids. In Chapter I, I discuss the evidence for and against one of the most widely cited gender/sex differences in SUD research, the telescoping effect. Chapter II and III show original research that firmly establishes the biological basis of the telescoping effect using a rat model of cocaine use disorder (CUD). Chapter IV contributes additional evidence for the possibility that the neuroadaptations underlying addiction maybe sex-dependent. Chapter V evaluates the efficacy of the patented nutritional supplement SMAASH-C at reducing cue-induced relapse vulnerability and cocaine-induced toxicity in males and females and finds the beneficial effects to be sex-specific highlighting the importance of considering biological sex when evaluating candidate treatments for CUD. In Chapters VI to VIII, I pivot to sex differences in opioid use disorder (OUD) using a rat model with fentanyl. More specifically in Chapter VI, I show original research that evaluates sex differences across a broad range of fentanyl doses, including low (0.25 and 0.75 μg/kg/infusion) and high (1.5 and 3.0 μg/kg/infusion) doses expected to minimized and maximize the expression of addiction-like features (e.g., vulnerability to relapse and physical dependence), respectively. Chapter VII demonstrates that the role of estradiol in vulnerability to opioid addiction in females is similar to that of psychostimulants and alcohol. Chapter VIII evaluates sex differences in time-course for the incubation of fentanyl-craving and the efficacy of R-ketamine as an anti-craving treatment for OUD. Notably, we show that the beneficial effects of R-ketamine are also sex-specific thereby highlighting the importance of considering biological sex when evaluating candidate treatments for OUD as well. Finally, Chapter IX highlights some of the most exciting findings presented in this dissertation and provides preliminary data on the directions we are taking to follow up on these exciting advances in addiction research. Together, I hope this dissertation will encourage clinical and preclinical researchers in the field to consider biological sex as an important risk factor in addiction as I strongly believe a better understanding of the disease process in females is necessary for continuing to move the science forward and producing a more representative and translationally relevant body of knowledge on addiction.

Introduction

Females have been underrepresented in clinical and preclinical research on the use of addictive drugs and substance use disorders (SUD) (Gunn et al., 2022; Montemitro et al., 2022; Allegra et al., 2022), which has led to huge gaps in our knowledge regarding the disease process in females and sex-informed treatment strategies. Although males have historically had higher rates of substance use and SUD than females, the epidemiology is rapidly changing, with an increasing proportion of those initiating drug use and those with SUD being female (U.S. Department of Health and Human Services, 2020). Additionally, there is increasing evidence of sex differences in every stage of the addiction process (see Towers et al., 2023a; Becker et al., 2022 for reviews). As a woman in the field, the aim of this thesis titled "Biological Sex and Ovarian Hormones Matter in Addiction Research" is to highlight the importance of investigating sex differences in development, neurobiological basis, and treatment of SUDs. Eight chapters are included, each featuring important sex differences in addiction research with a focus on cocaine and opioids.

In Chapter I, we provide an overview of the evidence for and against one of the most widely cited gender/sex differences in SUD research, the telescoping effect (Towers et al., 2023b). This phenomenon describes the faster progression from initial drug use to meeting the criteria for a SUD or seeking treatment in women compared to men. We draw upon data from clinical and preclinical studies using multiple drug classes including opioids, psychostimulants, alcohol, and cannabis. We also discussed the contribution of biological factors, underlying neurobiological mechanisms, and potential targets to prevent the development of SUD in women.

In Chapter II, we used a rat model of cocaine use disorder (CUD) to determine whether two key features of addiction in humans, an enhanced motivation for cocaine (as assessed under a progressive-ratio reinforcement schedule) and compulsive use (as assessed using a histamine punishment procedure), emerge sooner during withdrawal from extended-access cocaine self-administration in females versus males (Towers et al., 2021a). We found motivation for cocaine increased from baseline earlier during withdrawal in females than males (at 7 versus 14 days) and although histamine decreased progressive-ratio responding for cocaine in both sexes, effects were greatest in males in the 7-day withdrawal group. Males reached the female-level of resistance to histamine-punishment by 14 days of withdrawal. These findings indicate that

female rats developed addition-like features sooner during withdrawal than male rats, which provides support for the telescoping effect observed in humans being biologically-based.

In Chapter III, we provide additional evidence for the biological basis of the telescoping effect with cocaine (Towers et al., 2023a). This study focused on another key feature of addiction in humans, cocaine-craving/vulnerability to relapse, as assessed using an extinction/ cue-induced reinstatement procedure. We found that cocaine-craving (total extinction and cue-induced reinstatement responding) progressively increased from early to later withdrawal time-points in males; whereas, in females, cocaine-craving was already elevated during early withdrawal (after 2 days) and did not further increase at later withdrawal time-points (after 14 days). We also showed that gene expression changes differed markedly between the sexes such that males showed the expected relapse- and withdrawal-associated changes in *Bdnf-IV*, *Grin2a*, *Grin2b*, and *Grin1* expression, but females only showed a modest increase *Grin1* expression at the intermediate withdrawal timepoint. These findings indicate the time-course for the incubation cocaine-craving also appears to be accelerated in females and the molecular mechanisms also likely differ in females versus males.

In Chapter IV, we provide additional evidence for the possibility that the neuroadaptations underlying addiction maybe sex-dependent (Towers et al., 2023d). More specifically, a hallmark neuroadaptation of SUD is impaired dopamine 2 (D2) receptor signaling in the ventral striatum (e.g., nucleus accumbens, NAc). This is one of, if not, the most replicated finding in human imagining studies. These human studies have used multiple addictive drugs (i.e., alcohol, cocaine, opioids, methamphetamine, tobacco/nicotine) and repeatedly shown decreased striatal D2 receptor binding in individuals with a SUD relative to healthy controls. However, the vast majority of the human data is from males, and findings in smokers suggest this molecular shift may not translate to females. In this study, we used our rat model of CUD and defined the development of an addiction-like phenotype as the expression of enhanced motivation for cocaine in the extended-access group relative to the short-access, control group. We used this model to determine whether there were sex differences in the role of D2 receptors in motivating cocaine use prior to and following the development of an addiction-like phenotype (relative to short-access, controls). We found that in males, NAc infused eticlopride (D2 receptor antagonist) was more effective at decreasing motivation for cocaine prior to than following the development of an addiction-like phenotype, particularly at low eticlopride doses. In contrast, in

females, there were no differences in the effectiveness of eticlopride prior to and following the development of an addiction-like phenotype. These findings align with the data in smokers and indicate that males, but not females, become less sensitive to NAc-D2 receptor antagonism with the development of an addiction-like phenotype.

In Chapter V, we present a study that used our rat model of CUD to determine the efficacy of the patented nutritional supplement SMAASH-C at reducing cue-induced relapse vulnerability and cocaine-induced toxicity in males and females (Towers et al., under review). We found in females, both the 2-week and the 6-week SMAASH-C treatment regimens over abstinence reduced cocaine-seeking (extinction or cue-induced reinstatement), and these effects were most apparent during estrus, when drug-seeking was heightened. Despite the lack of efficacy of SMAASH-C to reduce cocaine-seeking in males, two-weeks of SMAASH-C treatment improved a maker of cocaine-induced liver toxicity (AST) and six-weeks of SMAASH-C treatment improved a marker of cocaine-induced pancreatic toxicity (amylase). Thus, the beneficial effects of oral SMAASH-C treatment over abstinence following chronic cocaine self-administration appears to be sex-specific. This study highlights the importance of considering biological sex when evaluating candidate treatments for CUD.

In Chapter VI, we pivoted to focusing on sex differences in opioid use disorder (OUD) using a rat model with fentanyl. We present a study that evaluated sex differences across a broad range of fentanyl doses, including low (0.25 and 0.75 μg/kg/infusion) and high (1.5 and 3.0 μg/kg/infusion) doses expected to minimized and maximize the expression of addiction-like features (e.g., vulnerability to relapse and physical dependence), respectively (Towers et al., 2022). Surprisingly, we found despite markedly higher intake in the high- versus low-dose groups, each group responded similarly during relapse testing (extinction and cue-induced reinstatement). However, number of infusions, or frequency of use, during extended-access was predictive of later vulnerability to relapse; whereas, total intake impacted physical dependence given that weight loss only occurred following the discontinuation of fentanyl self-administration at the three highest doses. Additionally, similar to previous findings with other addictive drugs, females were more vulnerable to expressing addiction-like features than males. More specifically, females self-administered more fentanyl each day and within each binge (active trial) than males. Females had longer lasting weight loss during withdrawal than males, indicating a prolonged physical dependence. Relapse vulnerability was also higher in females

than males and highest in females tested during estrus. Together, these findings indicate that sex is an important risk factor for patterns and levels of fentanyl intake, relapse, and physical dependence.

In Chapter VII, we used the same rat model of OUD to determine the impact of estradiol on vulnerability to opioid addiction in females (Towers et al., 2023c). A major theory of sex differences in SUDs is that these differences are due to ovarian hormones with estradiol enhancing vulnerability in females. However, the vast majority of this evidence is for psychostimulants and alcohol and the evidence with opioids is sparse. Here, we found ovariectomized (OVX) females with estradiol replacement (OVX+E) self-administered markedly higher levels of fentanyl under extended, intermittent-access conditions compared to ovariectomized females with vehicle replacement (OVX+V). OVX+E females also showed a longer time-course of physical dependence, a greater increase in motivation for fentanyl, and an enhanced sensitivity to the reinstating effects of fentanyl-associated cues compared to OVX+V females. Severe health complications were also observed in OVX+E, but not OVX+V females, during withdrawal. Together, these results indicate that, as with findings with psychostimulants and alcohol, estradiol enhances vulnerability in females to developing opioid addiction-like features and serious opioid-related health complications in females.

In Chapter VIII, we evaluate sex differences in the time-course for the development of an enhanced vulnerability to relapse as measured by levels of drug-seeking in response to fentanyl associated cues during early (withdrawal day 0 and 1) versus protracted withdrawal (withdrawal day 14) when levels are expected to be low versus high, respectively. As a second goal of this study, we determined the efficacy of R-ketamine, a novel potential treatment to substance use disorder, at reducing vulnerability to relapse. To our surprise, we found that in both sexes, vulnerability to fentanyl-associated cues was similarly high during both early and protracted withdrawal. Our relapse phenotype was robust and was not only similarly high across withdrawal time-points, but also between males and females, estrous cycle phases (in females), and following single or repeated cycles of fentanyl self-administration, withdrawal, and relapse testing. Despite these high levels of vulnerability to cue-induce relapse, R-ketamine persistently attenuated vulnerability to fentanyl-associated cues over protracted withdrawal in males, but not females. Thus, the beneficial effects of R-ketamine treatment appear to be sex--specific.

Lastly, in Chapter IX, I highlight some of the most exciting findings from this dissertation and provide preliminary data on the directions we are taking in our future studies. Specifically, one of the major theories that we explore is that the accumulation of synaptic, Ca^{2+}-permeable AMPA- type glutamate receptors (CP-AMPAR) in the nucleus accumbens core (NAc) mediates the development of enhanced motivation for cocaine and occurs sooner during withdrawal in females thereby underlying the telescoping effect. This idea is supported by preliminary data showing that similar to our previous studies, motivation for cocaine is increased from baseline (or prior to extended-access cocaine self-administration) in vehicle-treated females, but not males, following extended-access cocaine self-administration and a 7-day withdrawal period. Additionally, as expected, this motivational shift is blocked in females by inhibiting the accumulation of CP-AMPAR NAc and induced in males by facilitating the accumulation of CP-AMPA. We intend to finish this study and also to use RNA- and ChIP-sequencing to explore mechanisms underlying the telescoping effect more broadly. I also address our ongoing efforts to expand the preclinical research on the telescoping effect to included opioids and present preliminary data indicating females also develop an opioid addiction-like phenotype and opioid-related health consequences more readily than males, similar to humans and previous preclinical studies with cocaine.

Together, I hope the body of work included in these chapters shows the importance of considering biological sex in development, neurobiological basis, and treatment of SUD. I also hope these studies will encourage clinical and preclinical researchers in the field to consider biological sex as an important risk factor in addiction research. I strongly believe a stronger understanding of the disease process in females is necessary to continue to move the science forward and to produce a more representative and translationally relevant body of knowledge on addiction. I am hopeful that this knowledge will help lead to the development of equitable treatment strategies for women and men.

Chapter I

Sex/Gender Differences in The Time-Course for the Development of Substance Use Disorder: A Focus on the Telescoping Effect

1 Introduction

Despite higher rates of drug use and substance use disorder (SUD) in men, women are more vulnerable than men on many aspects of the disease. One striking example is "the telescoping effect" which reflects an accelerated course in women versus men for the transition from initiation of substance use to meeting criteria for SUD and/or seeking treatment for SUD. This phenomenon was originally described for alcohol more than 30 years ago (Ashley et al. 1977; Hesselbrock et al. 1985; Piazza et al. 1989) and the observation has been replicated in multiple subsequent studies with alcohol (Diehl et al. 2007; Johnson et al. 2005; Randall et al. 1999; Lewis and Nixon, 2014; Hesselbrock et al. 1985; Piazza et al. 1989; Mann et al. 1992, 2005; McCance-Katz et al. 1999; Hernandez-Avilaet al. 2004) as well as with other drug classes, including stimulants (e.g., cocaine, nicotine/tobacco, methamphetamine; McCance-Katz et al. 1999; Griffin et al. 1989; White et al. 1996; Brecht et al. 2004; Sofuoglu et al. 1999; Thorner et al. 2007; O'Brien and Anthony, 2005; Haas and Peters, 2000), opioids (Hernandez-Alvila et al. 2004; Lewis et al. 2014; Peltier et al. 2021; Anglin et al. 1987; Adelson et al. 2018; Hser et al. 1987a,b; Back et al. 2011b; DiFranza et al. 2002), and cannabis (Hernandez-Avila et al. 2004; Lewis et al. 2014; Khan et al. 2013; Ehlers et al. 2010; Haas and Peters, 2000). It has also been reported for non-pharmacological addictions, such as gambling (Tavares et al. 2003; Ladd and Petry, 2002; Ibanez et al. 2003; Grant et al. 2012).

The telescoping effect in women has been widely noted in studies of SUD yet there are some inconsistent reports that show either no difference between men and women in the time-course for the development of SUD (Alvanzo et al. 2011; Stoltman et al. 2015; DiFranza et al. 2007) or the reverse, a faster course in men than women (Keyes et al. 2010; Slutske et al. 2015). Changes in sociocultural factors, such as a progressive destigmatization of drug use in women over time, have been proposed to account for differences observed between women and men in the original telescoping studies versus more recent ones (Nicolaides, 1996). Recent studies, using population-based surveys, may be further confusing the literature since sex/gender differences in the time-course for the development of SUD are confounded by differences in the likelihood of developing SUD and seeking treatment for SUD, both of which are greater in men than women (Wagner and Anthony, 2007; Greenfield, 2007). Some notable exceptions are for psychotherapeutics (i.e., non-medical use of pain relievers, sedatives, stimulants, and tranquilizers) and tobacco; in these cases, women are more likely than men to develop a SUD

(Cotto et al. 2010; Lopez-Quintero et al. 2011). The telescoping effect has also been replicated in several studies conducted during the past ten years (Lewis and Nixon, 2014; Lewis et al. 2014; Grant et al. 2012; Peltier et al. 2021; Khan et al. 2013; Sylvestre et al. 2018), with robust effects within treatment seeking populations (e.g. Randall et al. 1999; Hernandez-Avila et al. 2004; Ibanez et al. 2003). The validity of the telescoping effect is also strongly supported by results from preclinical studies which show that, like the human situation, female animals develop addiction-like features more readily than male animals (Kerstetter et al. 2012; Perry et al. 2013b, 2015; Kawa and Robinson, 2019; Lynch and Taylor, 2004; Towers et al. 2021).

Thus, the purpose of this review is to evaluate evidence for and against the telescoping effect in women and determine the conditions/populations for which the telescoping effect is most relevant. We also discuss preclinical findings of sex differences in order to establish a biological basis for the telescoping effect. This evidence is divided into findings from *animal models of substance use* (see **Table 1** for a glossary of terms), which generally use short-access drug self-administration (1-2 hr/day) and focus on differences in the acquisition of drug self-administration or maintenance levels of intake or motivation for the drug, versus *animal models of SUD*, which typically use extended-access drug self-administration (≥ 6 hr/day) and focus on differences in the development and/or expression of addiction-like features like those observed in humans with a SUD (e.g., escalation of drug use, compulsive drug use despite punishment, an enhanced motivation for the drug, enhanced drug-craving/vulnerability to relapse). Mechanisms underlying the telescoping effect are also explored, including the potential for ovarian hormones to drive an enhanced vulnerability in women and female laboratory animals during both initial substance use and with the development of SUD. We also discuss neurobiological mechanisms of substance use and SUD in women and men and male and female laboratory animals focusing on the mesolimbic dopamine reward pathway and corticomesolimbic glutamatergic pathways considering the critical roles these pathways play in the rewarding/reinforcing effects of addictive drugs and SUD. The potential role of sex chromosomes and other signaling pathways, including the potential for stress and the HPA-axis to enhance vulnerability in females, are also briefly discussed. We conclude with implications for sex-specific interventions for SUD and future research directions.

Table 1. Glossary of the key terms used in this review.

Term	Definition
Addition-like feature	The expression of a behavior in an animal that resembles a criterion, or symptom, of SUD in humans as defined by the DSM-5 (American Psychiatric Association, 2013). Some of the more commonly modeled features include escalation of drug intake over time, binge/abstinent patterns of drug intake, physical dependence, an enhanced motivation to obtain the drug, compulsive drug use despite adverse consequences, preference for the drug over a non-drug rewards, and enhanced drug-craving/vulnerability to relapse (Lynch 2018).
Addition-like phenotype	The expression in an animal of one or more characteristics (or addiction-like features) that resemble features of SUD in humans as defined by the DSM-5. For example, the development of an enhanced motivation for the drug has been used to define the development of an addiction-like phenotype since, as in humans, once this feature emerges, it appears to represent a relatively permanent shift to a higher motivational state (Lynch 2018).
Acquisition procedure	A procedure that uses a set of performance criteria to define the time-point when an animal has learned a new behavior, such as lever pressing to obtain infusions of a drug. Acquisition procedures can be a strong tool for investigating individual differences in sensitivity to the reinforcing effects of a drug. These effects are ideally studied under low dose conditions and the question asked is "which animals can detect the reinforcing effects of this low dose of the drug". A faster speed of acquisition and/or greater percent group acquisition is then used to define an enhanced vulnerability to substance use (Lynch et al. 2010).
Animal model of substance use	A model used to assess initial vulnerability to use addictive drugs. Short-access drug self-administration procedures (1-2 hr/day access) are commonly used and focus on rates and/or percent group acquisition of drug self-administration, maintenance levels of drug use, or motivation to obtain the drug, as assessed using a progressive-ratio schedule or a within-session threshold procedure, following acquisition.
Animal model of substance use disorder	A model that has been validated to induce an addiction-like phenotype in animals like that observed in humans with SUD. Extended-access drug self-administration procedures ($\geq$ 6 hr/day access) are the gold-standard for inducing addiction-like features in animals (Lynch 2018).
Binge/abstinent pattern	A binge-abstinent of pattern of drug self-administration is characterized by cycles of heavy/prolonged periods of drug use (binge intake) separated by periods of self-imposed abstinence.
Choice procedure	A procedure used to determine percent choice, or preference, for one reinforcer over another (or for different magnitudes of a reinforcer). Choice procedures can be a powerful approach for determining individual differences in vulnerability to developing a preference for the drug over other non-drug rewards, such as a highly palatable food reward, and for determining potential interventions that reverse a drug preference back to a non-drug one.
Compulsive drug use	A core feature of addiction in humans that is modelled in animals using punishment or choice procedures. The development of this addiction-like feature has been defined as continued drug use despite adverse consequences (e.g., coincident shock) or an exclusive choice (>90%) of the drug over an alternative

	non-drug reward (Lynch 2018). This addiction-like feature emerges following abstinence l (7 days or more) from extended-access self-administration and the magnitude of its expression increases with longer periods of abstinence (Towers et al. 2021a).
Enhanced motivation to obtain the drug	A core feature of addiction in humans that is modelled in animals using either a progressive ratio schedule or the threshold procedure. This feature has been defined as ≥15% increase in motivation for the drug relative to short-access controls or baseline prior to extended-access self-administration and abstinence (Lynch 2018). This addiction-like feature emerges following abstinence (7 days or more) from extended-access self-administration and the magnitude of its expression increases with longer periods of abstinence (Towers et al. 2021a).
Enhanced drug-craving/vulnerability to relapse	A core feature of addiction in humans that is modelled in animals using an extinction/reinstatement procedure or a cue-induced drug-seeking procedure. This addiction-like feature is typically assessed following extended-access self-administration and a period of protracted abstinence (>14 days) since these conditions induce high levels of drug-seeking relative to short-access controls and earlier abstinence time-points. The expression of this addiction-like feature progressively increases, or incubates, over abstinence (Lynch 2018).
Escalation of drug intake	Escalation of drug intake occurs in animals given extended-access, but not short-access, to the drug and is characterized by a gradual increase in drug intake over time. It is ideally studied following acquisition of drug self-administration, to ensure that increases in intake are reflective of escalation rather than acquisition, and is thought to resemble the loss of control over drug intake feature observed in humans with SUD (Koob 2021).
Fixed-ratio schedule	A schedule of reinforcement in which a set number of responses (e.g., 1, 2, or 10) produce a reinforcer delivery, such as a drug infusion.
Gender	The characterization of women or men that is socially constructed and varies over time and between cultures (Committee on Understanding the Biology of Sex and Gender Differences 2001).
Incubation effect	The incubation effect refers to a progressive increase in drug-seeking from early to later periods of abstinence following extended-access self-administration. A similar phenomenon has also been reported in humans with SUD (Li et al. 2015) and is thought to reflect the development of an enhanced vulnerability to relapse. A similar incubation effect has also been observed for the development of other addiction-like features, including compulsive drug use and an enhanced motivation to obtain the drug (Towers et al. 2021a; Gancarz-Kausch et al. 2014).
Intermittent-access procedure	A drug self-administration procedure wherein access to the drug is intermittently available, such as in 5-min trials with unrestricted, fixed-ratio 1 access, or in discrete trials, With the most commonly used procedures, animals either have unrestricted, fixed-ratio 1 access to the drug infusions in 5 min trials that initiate every 30 minutes for 6 or more hours/day or to single infusions of the drug in discrete trials that initiate every 15 min 12-24-hr/day (Fitch and Roberts 1993; Zimmer et al. 2012). Intermittent-access self-administration results in a binge-abstinent pattern of drug intake and spiking brain drug levels (Zimmer et al. 2012).

Long-access procedure	A drug self-administration procedure that allows continuous, fixed-ratio 1 access to the drug for ≥6 hr/day. This results in high levels of drug intake and an escalating pattern of drug use (Ahmed and Koob 1998).
Physical dependence	A core feature of addiction in humans that is assessed in animal models following chronic drug self-administration and defined by withdrawal-induced weight loss and somatic signs of withdrawal (e.g., abdominal constriction, salivation, ptosis, paw tremors; Lynch et al. 2010).
Preference for the drug over a non-drug reward	A core feature of addiction in humans that is modelled in animals using a choice procedure. The development of this addiction-like feature is defined as an exclusive choice (>90%) for the drug versus a non-drug reward (Lynch 2018).
Progressive-ratio schedule	A schedule of reinforcement that requires the animal to emit an increasing amount of work (or lever pressing) to obtain each subsequent delivery of the drug within a session. The breakpoint, or the point that the animal stops responding, is used as a measure of motivation to obtain the drug.
Punishment procedure	Punishment procedures decrease the probability of responding for the reinforcer. For example, when an aversive stimulus, such as electric shock, is paired with the delivery of the drug, drug-taking decreases. Punishment procedures have also been used to demonstrate compulsive use, a core feature of addiction in humans, wherein animals show a reduced sensitivity to punishment and continue to self-administer high levels of the drug.
Reinstatement procedure	A model of relapse/drug-craving whereby the animal is tested on responding on a lever that was formerly associated with the drug under non-reinforced conditions (extinction), and once responding has reached a certain level of non-responsiveness, the reinstatement of drug-seeking (responding on this same lever) is examined in response to presentations of drug-associated cues, a small "priming" dose of drug, or stress.
Sex	The characterization of an individual as female or male according to their reproductive organs and functions derived from their chromosomal complement (generally XX for female and XY for male; Committee on Understanding the Biology of Sex and Gender Differences, 2001).
Short-access procedure	A drug self-administration procedure wherein animals have access to the drug for 1-2 hr/day. Such access results in relatively stable and low levels of drug intake from day-to-day.
Telescoping effect	A phenomenon that describes a faster progression in females compared to males from initial drug use to meeting the criteria and/or seeking treatment for a SUD (Piazza et al. 1989).
Threshold procedure	A procedure used to examine motivation to obtain a reinforcer. For example, the demand for a drug is measured by varying the price (response requirement) and the value (dose) of the drug within a session (Zimmer et al. 2012).

Human studies were selected based on Pub Med and Google Scholar searches using the key words telescoping, time-course, trajectory, alcohol, cocaine, methamphetamine, opioids, fentanyl, heroin, morphine, oxycodone, cannabis, smoking, nicotine, tobacco, illicit drug use,

initiation of use, regular use, problem use, addiction, and SUD. Preclinical studies were identified using these terms as well as acquisition, reinforcing effects, self-administration, addiction phenotype, relapse, enhanced motivation, compulsive use, escalation, binge intake, and extended-access self-administration. Human and animal studies of biological factors and neurobiological mechanisms focused on these terms as well as ovarian hormones, estrous cycle, menstrual cycle, luteal, follicular, estradiol, progesterone, dopamine, glutamate, excitability, nucleus accumbens (NAc), ventral tegmental area (VTA), medial prefrontal cortex (mPFC). Throughout this review, the term *sex* refers to biological differences between women and men and male and female laboratory animals related to sex hormones, chromosomes, gene expression, anatomy, or physiology (Committee on Understanding the Biology of Sex and Gender Differences, 2001). The term *gender* refers to socially determined differences between women and men roles that vary over time and between cultures (Committee on Understanding the Biology of Sex and Gender Differences, 2001).

2 Sex Differences in The Progression to Addiction

2.1 Evidence for and Against a Telescoping Effect in Women

The original reports of a telescoping effect were based on self-reports and structured interviews from men and women with an alcohol use disorder (AUD; i.e., abuse or dependence based on DSM-III/IV) detailing the time-line of onset of major alcohol-related life events. These events include first drink, first intoxication, continuous consumption, onset of dependence, and first inpatient treatment which have been shown to occur in a chronological sequence with a high level of predictability in both women and men (Schuckit et al. 1995). Using this framework, these studies consistently show that women progress more rapidly from regular alcohol use to developing problematic alcohol use or an AUD (**Table 2 a-b;** Diehl et al. 2007; Johnson et al. 2005; Randall et al. 1999; Hesselbrock et al. 1985). Women also have a shorter course from the onset of problematic use/AUD to seeking treatment for the disorder than men (Lewis and Nixon, 2014; Piazza et al. 1989; Randall et al. 1999; Diehl et al. 2007; Hernandez-Avila et al. 2004; Man et al. 1992, 2005; Ashley et al. 1977; McCance-Katz et al. 1999). This faster progression to treatment seeking may be attributable to an earlier onset of severe SUD (5 or more DSM-V symptoms) considering that at treatment entry, women have more severe clinical profiles than men (e.g., more medical, psychological, behavioral, and social problems; Greenfield et al. 2010).

This conclusion is further supported by studies showing that women have an accelerated course and/or an enhanced sensitivity to alcohol-related health consequences as compared to men. Some of the differences in health decline include a faster course in women than men for the development of alcohol-associated cirrhosis (Loft et al. 1987) and brain atrophy (Hommer et al. 1996; Hommer et al. 2001; Mann et al. 1992; Mann et al. 2005), as well as greater alcohol-associated effects on cardiac and skeletal muscle in women than men (Fernandez-Sola et al. 1997; Urbano-Marquez et al. 1995).

Similar methods have been used to establish sex/gender differences in transitions from initial use to regular use, problematic use, and SUD and/or treatment for SUD with other addictive drugs, including opioids, psychostimulants, cannabis, and tobacco (**Table 2 a-b**). These studies show that compared to men, women have a shorter duration of opioid (Aldelson et al. 2018; Hernandez-Avila et al. 2004; Hser 1987a,b; Peltier et al. 2021), psychostimulants (cocaine and methamphetamine; Brecht et al. 2004; Griffin et al. 1989; Haas and Peters, 2000; McCance-Katz et al. 1999; O'Brien and Anthony, 2005; Sofuoglu et al. 1999; White et al. 1996), and cannabis use (Hernandez-Avila et al. 2004) prior to entering treatment and a faster progression from initial use of opioids (Back et al. 2011b; Anglin et al. 1987; Lewis et al. 2014), cocaine (White et al. 1996; Sofuoglu et al. 1999; O'Brien and Anthony, 2005), tobacco (DiFranza et al. 2002; Sylvestre et al. 2018; Scragg et al. 2008; Thorner et al. 2007), and cannabis (Ehlers et al. 2010; Khan et al. 2013; Lewis et al. 2014) to regular or problem use. The same pattern has also been reported for gambling wherein women show a faster progression from the initiation of gambling to developing a problem with gambling or to meeting criteria for pathological gambling compared to men (Ladd and Petry, 2002; Ibáñez et al. 2003; Tavares et al. 2003; Grant et al. 2012). As with findings with alcohol, women with SUD have more severe clinical profiles than men with SUD at treatment entry (Arfken et al. 2001; Fernandez-Montalvo et al. 2014), and show an accelerated course and/or enhanced vulnerability to drug-related medical consequences including a greater risk of infectious diseases with opioid use (i.e, hepatitis C; Iversen et al. 2010 and AIDS; Des Jarlaise et al. 2012), an earlier age for onset of psychotic disorders with cannabis use (Large et al. 2011), overall greater risk for cocaine-induced death (de la Fuente et al. 2014), shorter time interval between onset of cocaine use and its fatal outcome (Origer et al. 2014; see Agabio et al. 2016 for review), and increased susceptibility to smoking-associated lung cancer (Hansen et al. 2018; Kiyohara et al. 2010).

Table 2a. Summary of human studies on the telescoping effect within treatment-seeking individuals.

Source	Drug	Subjects	Telescoping Findings: Time (in years unless stated otherwise) Between Events
Diehl et al. (2007)	Alcohol	106W/106M	**W<M:** regular use to dependence (10.0 vs 11.6) **W<M:** dependence to treatment (4.5 vs 7.9)
Johnson et al. (2005)	Alcohol	785W/1252M	**W<M:** regular use to problematic use in the older (30+; 7.6 vs 10.9), but not younger age group (<29; 4.9 vs 5.2)
Randall et al. (1999)	Alcohol	419W/1307M	**W<M:** regular use to problematic use (0.9 vs 2.3) **W<M:** loss of control over use to severe alcohol-related problems (5.5 vs 7.8) **W<M:** regular use to seeking treatment (11.6 versus 15.8)
Lewis and Nixon (2014)	Alcohol	257W/274M	**W<M:** milestones (first use, first intoxication, regular use, problematic use) to treatment (18.1 vs 23.0, 15.5 vs 20.7, 13.0 vs 18.2, 10.3 vs 14.5) **W=M:** milestones (first use, first intoxication, regular use) to problematic use/dependence (8.9 vs 9.7, 6.3 vs 7.4, 3.2 vs 4.5)
Ashley et al. (1977)	Alcohol	135W/736M	**W<M:** problematic use to treatment (14.1 vs 20.2)
Hesselbrock et al. (1985)	Alcohol	90W/231M	**W<M:** initial use to problematic use/dependence (7.4 vs 15.0)
Piazza et al. (1989)	Alcohol	33W/105M	**W<M:** problematic use to treatment (10.4 vs 14.7) **W=M:** initial use to first intoxication (2.9 vs 1.7) **W=M:** first intoxication to problematic use (14.0 vs 14.7)
Mann et al. (1992)	Alcohol	14W/51M	**W<M:** initial use to treatment (3.8 vs 9.2)
Mann et al. (2005)	Alcohol	42W/34M	**W<M:** problematic use/dependence to treatment (5.6 vs 10.4)
McCance-Katz et al. (1999)	Alcohol, Cocaine	92W/206M	**W<M:** initial alcohol use to treatment (8.8 vs 11.4) **W<M:** initial cocaine use to treatment (5.2 vs 5.8)
Hernandez-Avila et al. (2004)	Alcohol, Cannabis, Opioids	156W/115M	**W<M:** regular alcohol use to treatment (14.5 vs 19.0) **W<M:** regular cannabis use to treatment (13.0 vs 18.0) **W<M:** regular opioid use to treatment (8.0 vs 12.0)
Griffin et al. (1989)	Cocaine	34W/95M	**W<M:** initial use to treatment (9.0 vs 10.2)
White et al. (1996)	Cocaine	27W/60M	**W<M:** initial use to problematic use (1.6 vs 3.3) **W<M:** initial use to treatment (5.1 vs 10.4)
Haas and Peters (2000)*	Alcohol, Cocaine, Cannabis	42W/118M	**W< M:** initial cocaine use to problematic use (4.3 vs 9.8) **W=M:** initial alcohol or cannabis use to problematic use (2.2 vs 1.9)
Lewis et al. (2014)	Cocaine, Cannabis,	288W/255M	**W=M:** regular cocaine use to problematic use (1.1 vs 1.8)

	Opioids		**W<M:** regular opioid use to problematic use (0.5 vs 2.7) **W<M:** regular cannabis use to problematic use (0.7 vs 2.0[#])
Tavares et al. (2003)	Gambling	70W/70M	**W<M:** milestones (social, intense, and problematic gambling) to treatment (5.0 vs 7.9, 0.8 vs 4.3, 1.9 vs 6.7)
Ladd and Petry (2002)	Gambling	45W/70M	**W<M:** problematic gambling to treatment (4.4 vs 14.6)
Ibanez et al. (2003)	Gambling	22W/47M	**W<M:** initial gambling to problematic gambling (4.2 vs 11.0)
Grant et al. (2012)	Gambling	34W/37M	**W<M:** initial gambling to problematic gambling (8.3 vs 12.0)
Brecht et al. (2004)	METH	154W/196M	**W<M:** initial use to regular use (1.6 and 2.6 years[#])
Peltier et al. (2021)	Opioids	2794W/45614M	**W<M:** age diagnosed with OUD (44.9 vs 51.0)
Anglin et al. (1987)	Opioids	264W/282M	**W<M:** months from initial use to daily use (14 vs 21)
Adelson et al. (2018)	Opioids	494W/762M	**W<M:** initial heroin use to treatment (12.9 vs 14.8)
Hser et al. (1987)	Opioids	264W/282M	**W<M:** months from daily use to treatment (82.5 vs 98.0)

*this treatment population underwent forced treatment due to a drug court program. [#]trend for significant difference (P<0.1). M=men. W=women. n.s.= non-significant, METH, methamphetamine

However, not all studies have observed a telescoping effect in women (Alvanzo et al. 2011; DiFranza et al. 2007) and findings from non-treatment seeking populations, particularly population-based studies, have been mixed (**Table 2 b**; e.g., Ehlers et al. 2010; Keyes et al. 2010; Khan et al. 2013; Slutske et al. 2015; Stoltman et al. 2015; Back et al. 2011b). Probably the most controversial findings are from the Keyes et al. (2010) study which was a large-scale study of alcohol use trajectories based on population-level data from two US national surveys (conducted in 1991-1992 and 2001-2002) of five birth cohorts (1934-1943, 1944-1953, 1954-1963, 1964-1973, and 1974-1983). They analyzed survival probabilities over time for the transition from initial alcohol use to developing an AUD and from the onset of an AUD to seeking treatment for the disorder. In contrast to predicted effects, men transitioned faster than women from initial alcohol use to AUD and from developing AUD to seeking treatment for the disorder. However, another interpretation is that the data reflect a greater risk in men for developing an AUD and a lower likelihood in women of seeking treatment for AUD. Indeed, the analysis of alcohol use trajectories in the individuals that actually developed an AUD are consistent with previous reports of a telescoping effect. In addition, the mean number of years between initial alcohol use

and the development of AUD was shorter in women than men (i.e., alcohol dependence as defined by the DSM-IV; 3.7 years versus 4.2 years). Similarly, when the analysis was limited to individuals that sought treatment for an AUD, women had fewer years between the onset of an AUD to seeking treatment for the disorder (6.1 versus 7 years). These differences were modest, however, particularly for the time-course for developing an AUD, and the effect was limited to one of the five birth cohorts (cohort 2). The effect for treatment was statistically significant when data were collapsed across all the cohorts, but analysis within each cohort only yielded significance for cohort 5 (19.4 versus 23.5).

Table 2b. Summary of human studies on the telescoping effect within nontreatment-seeking individuals.

Source	**Drug**	**Subjects**	**Telescoping Findings: Time (in years unless stated otherwise) between events**
Alvanzo et al. (2011)	Alcohol	11862W/9244M	**W=M:** initial use to dependence (4.9 vs 5.4)
Keyes et al. (2010)	Alcohol	30125W/23113M	**W=M:** initial use to dependence in overall sample (5.6 and 5.8) **W<M:** initial use to dependence in cohort 2 only (3.7 vs 4.2) **W<M:** dependence to treatment in overall sample (6.1 vs 7.0) and in one of 5 cohorts (cohort 5, 19.4 vs 23.5)
Huggett et al. (2018)	Alcohol, Tobacco	1477W/1297M	**W≤M:** initial alcohol use to dependence (3.3 vs 3.8[#]) **W=M:** initial tobacco use to dependence (4.5 vs 4.5)
Khan et al. (2013)	Cannabis	1217W/2080M	**W<M:** initial use to dependence (2.2 vs 2.6)
Ehlers et al. (2010)	Cannabis	177W/172M	**W<M:** initial use to dependence (44.7 vs 49.3)
Sofuoglu et al. (1999)	Cocaine	21W/23M Study 1 12W/11M Study 2	**W<M:** initial use to dependence in two human laboratory studies (Study 1, 9.2 vs 11.3; Study 2, 7.4 vs 13.0)
O'Brien and Anthony (2005)	Cocaine	59488W/54753M	**W<M:** initial use to dependence (defined by risk within 24 months of first use, W 3-4 times more likely than M)
Slutske et al. (2015)	Gambling	2662W/2001M	**W>M:** initial gambling to weekly/problematic gambling, disordered gambling symptoms, and diagnosis of disordered gambling (8.6 vs 8.1, 10.9 vs 8.3, 12.9 vs 10.9)
DiFranza et al. (2002)	Tobacco	679W/M[+]	**W<M:** days from monthly smoking to dependence symptoms (21 days vs 183 days)
Scragg et al. (2008)	Tobacco	14925W/10070M[+]	**W<M:** initial use to dependence (W had less use than M prior to symptoms onset)

DiFranza et al. (2007)	Tobacco	647W/599M[+]	**W=M:** days from initial use to nicotine/tobacco dependence, symptoms, and autonomy loss (no sex effect, data not stated)
Sylvestre et al. (2018)	Tobacco	471W/368M[+]	**W<M:** initial use to dependence symptoms (21 days vs 183 days)
Thorner et al. (2007)	Tobacco	378W/261M[+]	**W<M:** initial use to daily use (0.9 vs 1.3)
Stoltman et al. (2015)	Opioids	165W/389M	**W=M:** initial heroin use to problematic use (2.1 vs 2.5)
Back et al. (2011b)	Opioids	12W/12M	**W<M:** initial use to regular use (5.0 vs 8.1)

[+]Conducted in children/adolescents. [#]trend for significant difference (P<0.1). M=men. W=women. n.s., non-significant.

These data, together with mixed reports of a telescoping effect in non-treatment seeking populations (Stoltman et al. 2015; Back et al. 2011b), indicate that the telescoping effect may be most relevant within treatment-seeking populations, which presumably include only individuals that develop a severe SUD requiring treatment. This idea is also consistent with findings from a population-level study showing that adolescent and young adult females are less likely than their male counterparts to have a mild to moderate illicit drug use disorder (other than cannabis), but equally likely, if not more likely, to have a severe illicit drug use disorder (i.e., classified as dependence according to DSM-IV; Cotto et al. 2010). It is also supported by population-level data (Wave I and II of the National Epidemiologic Survey on Alcohol and Related Conditions) showing that women with a history childhood maltreatment, which is known to be associated with greater addiction severity (see Puetz and McCrory, 2015 for review), had a faster progression from the onset of drinking to developing an AUD than women without childhood maltreatment and men with and without this history (Schuckher et al. 2018). Importantly, this vulnerable in-treatment population is the population that needs to be studied for insights into prevention and treatment.

While the mechanisms underlying the telescoping effect are not yet known, it is likely that both sociocultural-gender differences and biological-sex differences contribute. For example, gender differences in the use of the health care system have been suggested as a potential explanation for the telescoping effect since women seek care sooner after initiating substance use or developing a SUD disorder than men. Social stigma against substance use and SUD in women may also cause women to seek treatment earlier after initiating substance use and/or developing a

SUD than men. This does not appear to be the case, however, since in contrast to the gender difference for seeking medical care overall, women are not more likely than men to seek treatment for a SUD (Greenfield et al. 2007; Center for Behavioral Health Statistics and Quality, 2015). Women are also more likely than men to be primary caregivers, and fear of losing custody of children is commonly reported as a barrier to seeking care for SUD (Pool et al. 2001; Mackay et al. 2020). Greater socio-relational impairment in women than men has also been reported to serve as a barrier to seeking treatment for a SUD in women. These gender differences may explain the disparity in SUD treatment between men and women and further support the conclusion that women who enter SUD treatment represent a vulnerable population that develops a severe SUD. This explanation also fits the data indicating that at the start of treatment for SUD, women have more severe SUDs and have more psychiatric and medical comorbidities than men. Biological factors also likely contribute to this vulnerability in women and the telescoping effect considering that similar behavior has been reported in female versus male laboratory animals (as detailed below).

2.2 Sex Differences in Animal Models of Initial Vulnerability to Substance Use

Preclinical studies of sex differences in addiction have focused predominately on vulnerability during early phases of the addiction process, such as acquisition of drug self-administration under short access conditions. These differences are ideally studied under low drug doses which maximize individual differences; low doses are also less likely than high doses to induce negative side-effects that may counter the reinforcing effects of the drug or impact the animal's ability to respond (Lynch et al. 2010). Results from studies comparing male and female rats have consistently revealed faster rates of acquisition and greater percent group acquisition in females than males under low dose conditions (e.g., Carroll et al. 2002; Lynch, 2008; Roth and Carroll, 2004). While most of this work has focused on cocaine, similar findings have been reported for other classes of drugs including opioids, alcohol, and cannabis, and for other psychostimulants such as nicotine and methamphetamine (for reviews see Becker and Hu, 2008; Lynch, 2006; Carroll et al. 2004). Females also typically self-administer more drug under short-access conditions (fixed-ratio 1, 1-2-hr/day) than males (e.g., Smith et al. 2021; Roberts et al. 1989), but this measure is less sensitive to individual differences and sex differences are not always observed (e.g., Towers et al. 2019; Roth and Carroll, 2004). The direction of effects can also be

difficult to interpret from maintenance levels of intake since lower intake may reflect less sensitivity to the reinforcing effects of the drug (e.g., the dose may function as a reinforcer in only a subset of the animals) or greater sensitivity (e.g., less drug is needed to maintain a preferred level of effect). Motivation to obtain the drug, as assessed under progressive-ratio schedules or the threshold procedure, is sensitive to individual differences and is a linear measure of reinforcing effects (i.e., larger doses maintain higher levels of responding). Numerous studies have shown that females are more motivated to obtain infusions of addictive drugs, and this effect has been observed at both low and high drug doses and for multiple addictive drugs (e.g., Mello et al. 2007; Roth and Carroll, 2004; for review see Lynch 2006; 2018). These findings indicate that females have an enhanced sensitivity to reinforcing effects of addictive drugs (**Figure 1**).

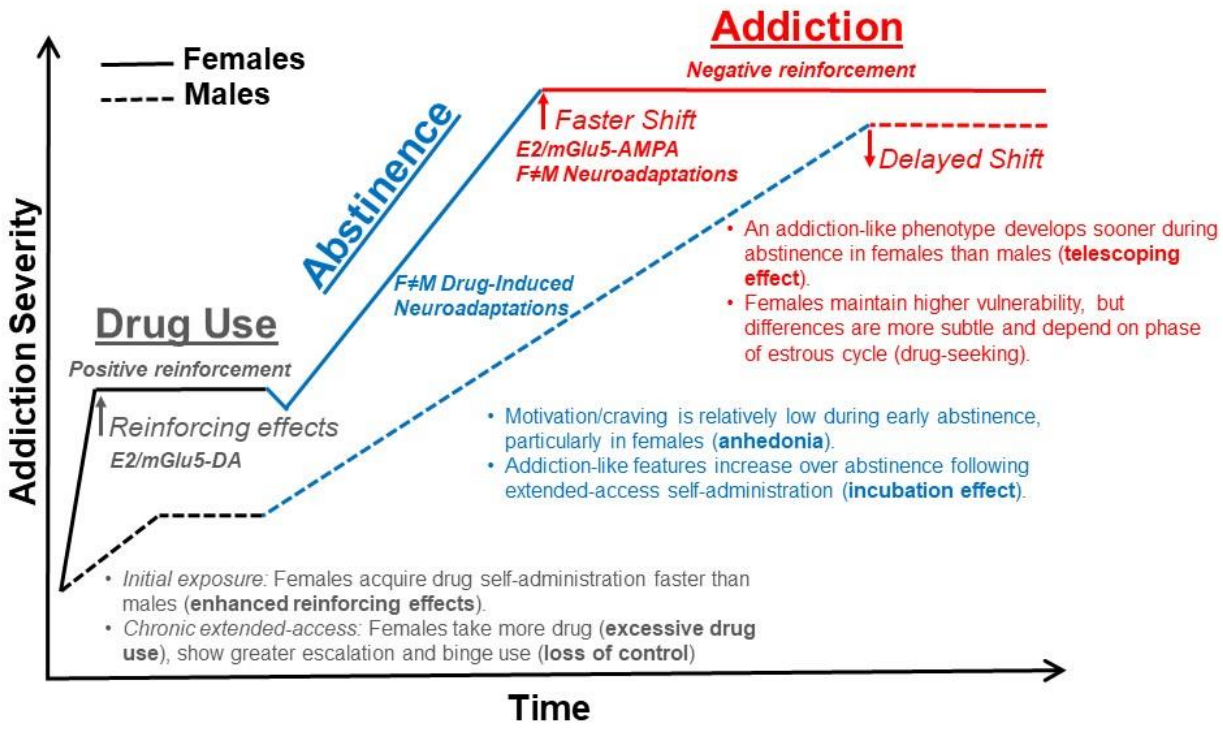

Figure 1. Biological basis for the faster course from drug use to addiction/SUD in females. Females are more sensitive to the positive reinforcing effects of drugs and acquire drug self-administration faster than males. This is mediated through interactions of estradiol and mGlu5, both of which increase drug-evoked dopamine signaling in the mesolimbic reward pathway of females. Craving and motivation to use addictive drugs is typically low during early abstinence, particularly in females, but both features become progressively enhanced over a period of protracted abstinence. Molecular adaptations in response to chronic drug use and abstinence differ between males and females and may drive sex differences in anhedonia, craving, and relapse vulnerability during both early and late abstinence. Addiction-like features, including an enhanced motivation for the drug, compulsive drug use, and vulnerability to relapse, emerge sooner during abstinence and/or after less drug intake in females than males indicating that the telescoping effect is biological based. This effect is likely driven by interactions of estradiol and mGlu5 which cause an earlier recruitment of the glutamate system (i.e. AMPA receptors). Once addiction has developed, behavioral differences between males and females become subtle and often depend on estrous cycle phase (e.g., drug craving). The neuroadaptations that underlie addiction also differ between males and females (e.g., NMDA receptor signaling in the dorsomedial prefrontal cortex), even in the absence of behavioral differences. E2=estradiol. DA=Dopamine. AMPA=α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

2.3 Sex Differences in Animal Models of SUD

Much less is known regarding sex differences in vulnerability during later stages of the addiction process, and more specifically, following the development of an addiction-like phenotype. The use of extended-access drug self-administration appears to be critical to inducing this phenotype which has been defined by the development of one of more key addiction-like features, such as escalation of drug intake over time, binge/abstinence patterns of drug use, compulsive drug use despite negative consequences, the development of physical dependence, an increased preference for the drug over a non-drug reward, an enhanced motivation to obtain the drug, and enhanced drug-craving/vulnerability to relapse (Lynch 2018). While no one procedure captures all 11 diagnostic criteria listed in the DSM-5 (American Psychiatric Association, 2013), there are multiple extended-access procedures that induce two or more of these clinical features, the threshold for a diagnosis of SUD in humans. For example, with the most commonly used extended-access procedure, the long-access procedure (Ahmed and Koob, 1998), animals have unrestricted, fixed-ratio-1 access to infusions of a drug, such as cocaine, heroin, fentanyl, nicotine, methamphetamine, for 6-12 hours/day. Under these conditions, animals self-administer high levels of the drug and show an escalating pattern of use over time, which is believed to mimic the excessive drug use and loss of control features of SUD in humans. This loss of control feature is also observed in rats given extended-, intermittent-access to a drug using either a discrete trial (Fitch and Roberts, 1993) or a fixed-ratio 1 procedure (Zimmer et al. 2012), which results in a binge-abstinent of pattern of drug self-administration characterized by cycles of heavy/prolonged periods of drug use (binge intake) separated by periods of self-imposed abstinence. For example, rats given 24-hr/day intermittent-access to cocaine, heroin, or speedball using a discrete trial procedure (4, 10 min trials/hr), self-administer high levels of the drug in binge-abstinent patterns that are dysregulated from the normal diurnal cycle (i.e., responding occurs at high levels throughout the light-dark phase). Similar binge-abstinent patterns have been observed for cocaine and fentanyl under extended, intermittent-access conditions (2 5-min trials/hr) using a fixed-ratio 1 schedule.

Notably, extended-access drug self-administration using the long-access procedure or an intermittent-access procedure leads to the development of other core characteristics of SUD including compulsive drug use, as assessed by continued drug use despite punishment (e.g., foot shock), an enhanced motivation to use the drug, as assessed using a progressive-ratio schedule or

a threshold procedure, and enhanced drug-craving/vulnerability to relapse, as assessed using an extinction/reinstatement procedure (Ahmed and Koob, 1998; Allain et al. 2015; Balster and Woolverton, 1982; Fitch and Roberts, 1993; Lynch and Carroll, 2001; Lynch, 2018). Expression of each of these features emerges over abstinence following extended-access self-administration and increases, rather than decreases, in magnitude over time. This "incubation" effect is robust and has been described for cue-induced drug-craving in humans for nicotine (Bedi et al. 2011), methamphetamine (Wang et al. 2013), cocaine (Wang et al. 2013), and alcohol (Li et al. 2014; Bach et al. 2019) and in animals for these drugs along with opioids (see Pickens et al. 2011 and Li et al. 2015 for reviews). A similar incubation effect has also been reported for the expression of enhanced motivation with cocaine (Towers et al. 2021a) and for compulsive use with cocaine and heroin (Towers et al. 2021a; Gancarz-Kausch et al. 2014). Notably, as with humans, the development of some of these addiction-like features (e.g., an enhanced motivation for the drug) are expressed long-term and appear to reflect a relatively permanent shift to a higher motivational state (see Lynch et al. 2021). While it is possible to induce these addiction-like features using short-access drug self-administration procedures, it occurs in only a small minority of the rats (~30%; Belin and Deroche-Gamonet, 2012). The phenotype is also more robust following extended- versus short-access self-administration (e.g. Fischer et al. 2013; Pacchioni et al. 2011). Evidence also shows that molecular changes differ following extended- versus short-access self-administration.

Sex differences have been reported for both extended-access self-administration and the induction of an addiction-like phenotype following extended-access self-administration and abstinence (see **Figure 1**). During extended-access self-administration, studies have shown that female rodents self-administer higher levels of drugs including alcohol, opioids, such as heroin, fentanyl, oxycodone, and morphine, and psychostimulants, such as cocaine, methamphetamine, and nicotine, compared to male rodents (Becker and Koob, 2016; Carroll et al. 2005; George et al. 2021; Kawa and Robinson, 2019; Lynch and Taylor, 2004, 2005; Moore and Lynch, 2015; Nicolas et al. 2019; Reichel et al. 2012; Roth and Carroll, 2004; Sanchez et al. 2014; Smith et al. 2011; Towers et al. 2019; 2022). Female non-human primates also self-administer more phencyclidine than male non-human primates under long-access conditions (Carroll et al. 2005). Sex differences in intake are most apparent under low dose conditions and in procedures that do not limit total hourly or daily intake as such procedures increase the likelihood of individual

differences. There are also sex differences in patterns of extended-access drug self-administration under both high and low dose conditions with female rats and mice showing greater escalation of alcohol, opioids, and psychostimulant intake over time as compared to male rats and mice (Carroll et al. 2005; Reichel et al. 2012; Melon et al. 2013; Roth and Carroll, 2004; George et al. 2021). Female rats and mice also self-administer more heroin during the first hour of a long, continuous access session (fixed-ratio 1, 6-h session; Towers et al. 2019) and more fentanyl within active trials under the intermittent access procedure (Towers et al. 2022), have longer initial periods of "binge" cocaine intake (defined as continuous drug use with no breaks from drug self-administration greater than 1 hour) and greater dysregulation in diurnal patterns of cocaine intake under 24-hr/day discrete trial procedure (Lynch and Taylor, 2004), and have greater binge-like alcohol drinking under the "drinking-in-the-dark" procedure as compared to males (defined as the amount of ethanol consumed during the first 3-hours of the dark phase; e.g., Sneddon et al. 2019). These findings indicate that females are more vulnerable than males to excessive drug use and developing a loss of control over drug use. This sex difference also appears to be robust as it has been observed in several species and for multiple drugs.

Importantly, the sex differences observed for the development of an addiction-like phenotype mirror findings of a telescoping effect in women and indicate that this phenotype develops more readily in female as compared to male animals (**Table 3**; Lynch 2018; Lynch and Taylor 2004; Perry et al. 2013b; Ramôa et al. 2013; 2014). This work has focused on effects with cocaine with results from the initial study of sex differences showing that females, but not males, developed an enhanced motivation for cocaine under conditions predicted to be threshold for inducing this phenotype (**Figure 2;** 7 days of extended-access cocaine self-administration and 10 days of abstinence; Lynch and Taylor, 2004). We subsequently confirmed that this phenotype is absent in both females and males when assessed under sub-threshold self-administration and abstinence conditions (e.g., extended-access self-administration with no intervening period abstinence or following short-access self-administration with or without abstinence; Lynch and Taylor, 2005), and present in both sexes when the conditions are optimized for its development by lengthening the period of extended-access self-administration (i.e. 10 days) and/or the abstinence period (i.e. 14 days; Roberts et al. 2007; Ramôa et al. 2013; Kawa et al. 2019).

Table. 3 Summary of preclinical studies on the telescoping effect.

Source	Drug (dose/inf)	Rats	SA conditions	Addiction feature measured (procedure)	Vulnerability to developing addiction-like features
Kerstetter et al. 2012	Cocaine (0.4, 1.0 mg/kg)	39M/29F	**ShA** (FR1, up to 20 inf or food pellets, 5 days each)	**Preference for drug over other rewards (choice procedure):** Cocaine (0.4 or 1.0 mg/kg) vs food (45 mg pellet). Sessions began after acquisition and were run for 5 days.	**F>M**. Females were more likely than males to choose cocaine (low and high dose) over food (low dose, 59% vs 33%; high dose, 76% vs 68%)
Perry et al. 2013	Cocaine (0.4 mg/kg)	12M/12F	**ShA** (30-min each: pellet only, cocaine only, cocaine vs pellet choice; FR1, 1st 3 days then FR5 for 21 days)	**Preference for drug over other rewards (choice procedure):** Cocaine vs banana-flavored food pellet (45 mg pellet). Choice testing occurred daily after the pellet and cocaine only sessions.	**F>M.** Females were likelihood than males to develop a preference for cocaine over food (50% vs 17%)
Perry et al. 2015	Cocaine (0.4 mg/kg)	50M/50F	**ShA** (30-min each: pellet only, cocaine only, cocaine vs pellet choice; FR1, 1st 3 days then FR5 for 21 days)	**Preference for drug over other rewards (choice procedure):** Cocaine vs banana-flavored food pellet (45 mg pellet). Choice testing occurred daily after the pellet and cocaine only sessions.	**F>M**. Females were more likely than males to develop a preference for cocaine over food (42% vs 26%)
Kawa and Robinson, 2019	Cocaine (0.4 mg/kg)	28M/24F	**ShA** (intermittent-access: 2, 5-min trials/hr, 5-hr/day, 5 days/wk, 30 days)	**Enhanced motivation for the drug (threshold procedure):** Threshold tests (FR1, progressively decreasing doses of cocaine 1.28 to 0.004 mg/kg) were run following the tenth and 30th day of SA and again after 14 days of abstinence.	**F>M**: Females developed an enhanced motivation for cocaine after less abstinence than males (i.e. following ten days of SA vs following 30 days of SA and 14 days of abstinence)
Lynch and Taylor, 2004	Cocaine (1.5 mg/kg)	18M/20F	**ExA** (4, 10-min trials/hr, 24-hr/day, 7 days)	**Enhanced motivation for the drug (PR schedule):** PR testing with cocaine (0.5 mg/kg) was conducted prior to ExA SA and then again after ExA SA and 7 days of abstinence (3 sessions each)	**F>M**: Females, but not males, developed an enhanced motivation for cocaine under these threshold conditions.
Towers et al. 2021a	Cocaine (1.5 mg/kg)	39M/38F	**ExA** (4, 10-min trials/hr, 24-hr/day, 10 days)	**Enhanced motivation for the drug (PR schedule):** PR testing with cocaine (0.5 mg/kg) was conducted prior to ExA SA and then again after ExA SA and 7, 14, or 60 days of abstinence (3 sessions each). **Compulsive use (histamine-punishment):** Following the	**F>M**: Females develop an enhanced motivation for cocaine sooner during abstinence than males (7 vs 14 days) **F>M**: Females tested following 7 days of abstinence displayed greater compulsive use than males; males required more abstinence to reach the-female level of compulsivity (14 days).

				third PR session, histamine (0.4 mg/kg) was added to the cocaine solutions and three additional PR sessions were run.	
Townsend et al. 2021	Fentanyl (0.32, 1.0 3.2, 10.0 ug/kg)	18M/17F	**ExA** (FR5, 12-hr/day, 5-day/wk, 3 weeks)	**Preference for drug over other rewards (choice procedure).** Fentanyl vs Ensure. Tested at the end of each week of ExA SA 8hr after last ExA session	**F<M**: Males, but not females, showed withdrawal-induced increases in preference for fentanyl (at low doses), and methadone attenuated this effect.

M, male; F, female; FR, fixed-ratio; PR, progressive-ratio; ShA, short-access; ExA, extended-access, SA, self-administration.

We firmly established a telescoping effect with cocaine in our more recent studies by demonstrating that three key features of SUD, an enhanced motivation for the drug, compulsive drug use, and enhanced drug-craving/vulnerability to relapse, develop sooner during abstinence following extended-access self-administration in females than in males (Towers et al. 2021a; Towers et al. 2023a). Specifically, an enhanced motivation for cocaine was evident in females after 7 days of abstinence, whereas, in males it is not evident until after 14 days. Females tested after 7 days of abstinence also displayed greater compulsive cocaine use than males, and while males reached the same level of resistance to punishment as females, this did not occur until after 14 days of abstinence. We also found that in females, cocaine-craving, as defined by total drug-seeking during extinction and cue-induced reinstatement testing, was expressed at high levels during both early and late abstinence, whereas, in males, drug-craving progressively increased from early to later abstinence time-points (following 2 versus 14 days) (Towers et al. 2023a). Notably, once these addiction-like features develop, sex differences are subtle and some studies show greater effects in females than males (e.g., Towers et al. 2021a) while others show no differences (e.g., Ramôa et al. 2013). Estrous cycle effects still appear to be relevant though considering that numerous studies have shown that drug-craving following abstinence from extended-access self-administration is higher in females tested during estrus versus non-estrus phases (Corbett et al. 2021). Together, these findings show that an addiction-like phenotype with

cocaine develops at an accelerated rate in female rats compared to male rats and indicate that the parallel effect in women is biologically-based.

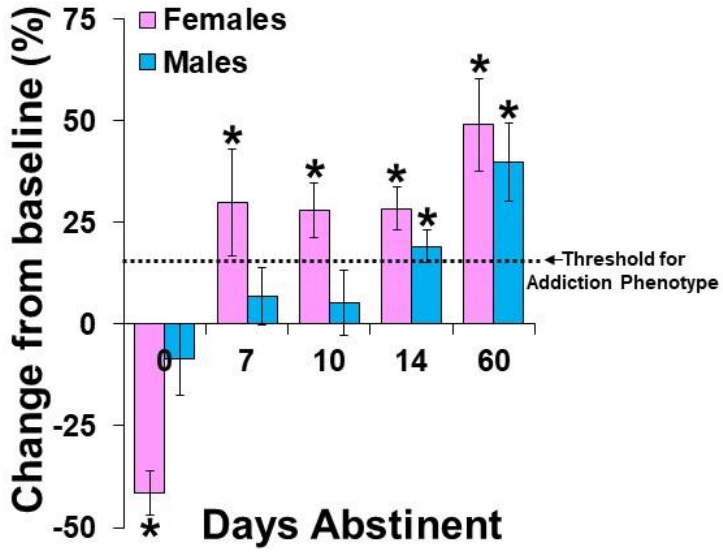

Figure 2. Rat model of the telescoping effect with cocaine. Data are plotted as mean percent change (±SEM) from the average number of infusions obtained during three baseline progressive-ratio sessions prior to extended-access cocaine self-administration (24-hr/day, 4 discrete trials/hr, 1.5 mg/kg/infusion, 7-10 days; refs) versus those obtained at retest following extended-access cocaine self-administration and 0, 7, 10, 14, or 60 days of abstinence. Motivation for cocaine increased progressively over abstinence following extended-access cocaine self-administration. Neither males or females showed an increase in motivation for cocaine when responding was assessed immediately following extended-access self-administration (0 days abstinent; Lynch and Taylor 2005); in fact, motivation was significantly decreased from baseline in females in the 0-day group. Females, but not males, showed an increase in motivation for cocaine when responding was assessed following 7 (Towers et al. 2021a) or 10 days of abstinence (Lynch and Taylor 2004). Both males and females showed an increase in motivation for cocaine when responding was assessed following 14 days of abstinence (Towers et al. 2021a) and motivation was highest in both males and females when responding was assessed following 60 days of abstinence (Towers et al. 2021a). The threshold for the development of an addiction-like phenotype, as defined by ≥15% increase from baseline and as represented by a dotted line, developed sooner during abstinence in females than males (following 7 versus 14 days of abstinence). Significant difference from baseline/no change (*). Data were redrawn, with permission, from the above cited references.

It is important to determine if similar sex differences occur for other drug classes. The initial findings with fentanyl suggest that both the time-course for the development of an addiction-like phenotype and the occurrence of sex difference may be different for opioids. Specifically, Townsend et al. (2021) found that males prefer low doses of fentanyl over a non-drug reinforcer

(i.e., Ensure) while females required a higher dose of fentanyl to shift their preference from Ensure. In males, preference for fentanyl increased progressively following repeated cycles of extended-access self-administration and withdrawal (8 hours); whereas, in females, preference for highest dose of fentanyl decreased during acute withdrawal. While these findings were interpreted to reflect a greater sensitivity in males than females to developing a preference for fentanyl over a non-drug reward, it is notable that even in males the preference observed for fentanyl following the third 1-week cycle of extended-access self-administration and withdrawal was not significantly greater than that observed for the non-drug reward (~50%), and in females, the non-drug reward was strongly preferred (~75%). Considering that both males and females showed a strong preference for high doses of fentanyl prior to extended-access self-administration, this phenotypic difference may be indicative of a sex difference of acute fentanyl withdrawal rather than a sex difference in the development of an addiction-like phenotype. In fact, a similar sex difference was observed with cocaine. Female rats tested following extended-access cocaine self-administration, without an intervening period of abstinence, showed a marked decrease in motivation for cocaine whereas, male rats did not show a change from baseline (i.e., prior to extended-access self-administration; Lynch and Taylor, 2005). One caveat to this interpretation, however, is that in males the behavioral phenotype was validated by showing that withdrawal-associated increases in heroin intake were blocked using methadone, an FDA-approved treatment for opioid use disorder (OUD). Further research comparing phenotypic changes in females versus males over a period of protracted abstinence following extended-access opioid is necessary to determine whether there are sex differences in the time-course for the development of addiction-like features with opioids.

Sex differences have also been observed for the expression of addiction-like features following short-access self-administration, particularly when behavior is examined following a prolonged period of self-administration (1-2 hr/day access for 30 or more days). For example, several studies have shown that the development of a preference for the drug (cocaine) over another competing reinforcer (food), another key characteristic of SUD in humans, occurs more readily in females than males tested over a prolonged period of short-access cocaine self-administration (~3-5 weeks; Kerstetter et al. 2012, Perry et al. 2013b, Perry et al. 2015); this preference also developed in a greater percentage of females than males (~50% versus 17%, respectively; Perry et al. 2015). The development of a preference for cocaine over food was also

associated with the development of two other key addiction-like features, an enhanced motivation for the drug and heightened drug-craving, indicating that females are more vulnerable than males to developing an addiction-like phenotype.

2.4 Summary and Integration of Preclinical and Clinical Findings

Together, these findings indicate that female laboratory animals display a greater vulnerability than male laboratory animals during the transition from initial drug use to the development of an addiction-like phenotype. Female animals take more psychostimulants, opioids, and alcohol and show greater escalation/binge intake under extended-access conditions than male animals. Female animals also develop an enhanced motivation for cocaine and a preference for cocaine over other reinforcers after less drug exposure and/or shorter periods of abstinence than male animals. It is important to emphasize, however, that the preclinical evidence demonstrating a faster time-course for the development of addiction-like features in females than males is based exclusively on findings with cocaine. To our knowledge, no studies have examined sex differences in the time-course for the development of addiction-like features following protracted abstinence from extended-access self-administration with other addictive drugs. While the preclinical findings with cocaine provide strong support for its biological basis, future research studies are necessary to determine if females also show an accelerated course for the development of addiction-like features in animal models of alcohol, opioid, and other psychostimulant use disorders. These studies are especially important considering that a telescoping effect has consistently been reported in women for cocaine use disorder (CoUD), in both treatment and non-treatment populations, which is in contrast to the findings for AUD and OUD. Future research is also necessary to address molecular mechanisms underlying the telescoping which, as discussed below, are currently unknown.

3 Biological Factors

3.1 Ovarian Hormones

Most of the work on potential mechanisms for sex differences in SUD has focused on the role of ovarian hormones. In clinical research, menstrual phase is often used as a proxy for ovarian hormones; several caveats to these studies need to be mentioned. First, it is essential that cycle stage is confirmed by hormone measurements. Without this confirmation it is likely that non-

ovulatory cycles and/or cycles with insufficient luteal phase will be included (Younis 2020). In addition, self-reported cycle lengths are often not accurate (Small et al, 2007). Women with polycystic ovarian disease and/or metabolic syndrome need to be excluded as do women on oral contraceptives since their cycles are anovulatory.

3.1.1 Human Studies – Ovarian Hormones and Substance Use

There is a large body of literature documenting fluctuations in the subjective and physiological effects of addictive drugs and patterns and motivation for drug use across the menstrual cycle phase (see Becker and Koob, 2016; Lynch et al. 2002 for reviews). Studies with psychostimulants have focused predominantly on the subjective and physiological effects of cocaine (in individuals with a cocaine use disorder) and amphetamine (in recreational users or "healthy controls"). These results indicate that the subjective/reinforcing effects of stimulants are higher in women during the late follicular phase, when levels of estradiol are high and progesterone levels are low, versus the mid-luteal phase, when levels of estradiol are moderate and progesterone levels are high (Lukas et al. 1996; Sofuoglu et al. 1999; Justice and de Wit, 1999; Evans et al. 2002; White et al. 2002). Similar conclusions of a faciliatory effect of estradiol and inhibitory effect of progesterone have been reached from clinical studies following exogenous hormone manipulation (Justice and deWit 2000; Lile et al. 2007; DeVito et al. 2014). For example, Lile and colleagues conducted a pilot study in 10 women without a SUD to determine the effects of exogenously administered estradiol on subjective ratings of d-amphetamine. They found that estradiol modestly increased the positive subjective effects (e.g., Like Drug) and discriminative stimulus effects of a low dose of d-amphetamine (Lile et al. 2007; also see Justice and deWit 2000). Conversely, administration of exogenous progesterone has been shown to decrease the positive subjective effects of psychostimulants in both normal controls and women with SUD (Peltier and Sofuoglu 2018; Evan and Foltin, 2006; Sofuoglu et al. 2002, 2004). Similar findings of enhanced positive subjective effects during the follicular versus luteal phase have been observed for nicotine in smokers (DeVito et al. 2014). While effects with opioids have focused on analgesic effects, these findings similarly show greater morphine analgesia in women during the follicular versus luteal phase (Ribeiro-Dasilva et al. 2011). These findings indicate that estradiol enhances, while progesterone reduces, the positive subjective effects of addictive drugs, particularly psychostimulants although future studies using

larger samples are needed to verify the effects of estradiol. Additional studies are also needed to determine if these effects also translate to other addictive drugs, such as opioids and cannabis.

There is also a large literature on alcohol documenting menstrual cycle effects in social drinkers and individuals with an AUD, but in contrast to literature on psychostimulants, most of these studies have focused on levels of use and craving (Terner and de Wit, 2006) rather than subjective effects (but see Evans and Levin 2011). The results have been less consistent than findings with stimulants. Some studies find greater intake and/or craving premenstrually (late luteal) and during menstruation (early follicular), whereas others show greater consumption/craving during the late follicular/ovulatory phase (see Joyce et al. 2021 for review). Affective state also fluctuates across the menstrual cycle and may overlap with changes in alcohol consumption and craving. For example, negative affect, including anxiety and depressive affect, peaks in the late luteal/premenstrual phase and early follicular/menstrual phase in response to progesterone withdrawal (Herzog 1995; Moran et al. 1998; Gallo and Smith 1993; Smith et al. 1998), and positive affect, including feelings of well-being and reward-processing, peak in the late follicular/ovulatory phase when levels of estradiol have are at their apex and progesterone levels are low (Aganoff and Boyle, 1994; Collins et al. 1985). Motivation for drinking similarly varies across the menstrual cycle with women reporting increases in drinking to combat negative affect during the late luteal/menstrual phase, and increases in drinking for social motives during late follicular/ovulatory phase (Joyce et al. 2018).

3.1.2 Human Studies – Ovarian Hormones and SUD

Motivation to use alcohol and other addictive drugs also likely differ between recreational users and individuals with a SUD given that once addiction has developed, the positive subjective/reinforcing effects of drugs diminish, and the negative reinforcing effects become the principal motivator for drug use (Koob 2021). This idea is also in line with findings showing that in healthy college women (without an AUD), social drinking and craving for alcohol are increased in the follicular phase (vs luteal phase) and associated with increased levels of estradiol (Warren et al. 2021; Martin et al. 1999), whereas, in women with an AUD and/or premenstrual dysphoric disorder, alcohol craving is highest during the late luteal/early follicular phases, when negative affect is highest and progesterone levels are low (Mello et al. 1990; Svikis et al. 2006; Evans and Levin, 2011; Kiesner, 2012). Higher levels of progesterone are also predictive of

lower levels of alcohol craving in postmenopausal women with AUD (Weinland et al. 2021). It is also notable that findings with psychostimulants similarly show that, in contrast to positive subjective responses, craving is predicted by progesterone levels. Craving is low when progesterone levels are high (versus when low or moderate; Sinha et al. 2007; Goletiani et al. 2015; Ethier et al. 2021) and can be offset by treatment with progesterone or its metabolite, allopregnolone (Peltier and Sofuoglu et al. 2018; Fox et al. 2013). It is also consistent with findings in smokers showing that nicotine withdrawal and depressive symptoms are increased during the late luteal phase, particularly in women who have premenstrual syndrome or premenstrual dysphoric disorder (Perkins et al. 2000; Mello et al. 1990; Svikis et al. 2006; Evans and Levin, 2011; Kiesner, 2012). Findings in daily cannabis users similarly show that cannabis use is higher in the late luteal phase (premenstrually) as compared to the follicular and ovulatory phases (Hanzal et la., 2019; Joyce et al. 2021) and preliminary evidence indicates that progesterone attenuates cannabis craving (Sherman et al. 2019). To our knowledge, no studies have examined the impact of ovarian hormones or menstrual cycle on craving or use of opioids highlighting an area for future research.

Together, these findings indicate that in women the role of ovarian hormones may vary in recreational users versus individuals with a SUD. In initial stages, or under conditions wherein the positive reinforcing actions of the drug are predominantly motivating drug use, estradiol enhances the subjective effects of drugs and likely enhances vulnerability to drug use. At these times, progesterone reduces the subjective effects of addictive drugs and likely reduces vulnerability to drug use. In contrast, progesterone appears to be more critical than estradiol in motivating drug use and craving for addictive drugs in individuals with a SUD and those using addictive drugs for their negative reinforcing effects. Evidence indicates that withdrawal from progesterone enhances drug craving and/or drug use to combat negative affect/craving whereas, high levels of progesterone either during the luteal phase or after exogenous administration reduces drug craving and/or use. These results further indicate that the telescoping effect in women may be driven by reward enhancing actions of estradiol as experienced during initial drug use. In turn, this increases the probability of additional recreational use and the subsequent development of a SUD. Additional research is necessary to determine the effects of ovarian hormones on the subjective effects, levels of use, and craving for opioids.

It is important to note that the relationship between ovarian hormones and drug use/SUD is reciprocal in that ovarian hormones both impact and are impacted by drug use and SUD. For example, during cocaine withdrawal, progesterone levels are elevated across the menstrual cycle resulting in significantly lower ratios of estradiol/progesterone as compared to healthy controls (Fox et al. 2007). This occurs in response to elevated cortisol levels and may indicate sub-fertile cycles (Dobson and Smith 1998). This response is also anxiolytic at first, but may lead to the later blunting of the stress response and increased anxiety, reduced tolerance to stress, and depression, which are all stress-related behaviors associated with relapse susceptibility in women with CUD (Fiad et al. 1996; Kaplan and Manuck 2004; Kampman et al. 2004; Sinha et al. 2006). Additionally, hypogonadism is common with chronic opioid use or opioid replacement therapy and is the result of suppression of the pulsatile release of gonadotropin-releasing hormone leading to deficiencies of luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol, and progesterone (Antony et al. 2018). Chronic alcohol use also causes hypothalamo-pituitary dysfunction and is associated with menstrual irregularities, such as anovulation, luteal-phase defects, recurrent amenorrhea, and early menopause (Hugues et al. 1980). As another example, nicotine reduces the aromatization of testosterone to estradiol, and as such, female smokers have higher testosterone levels and are more likely to experience estradiol deficiency and early menopause than nonsmoker females (Jandikova et al. 2017). Similar reciprocal effects of ovarian hormones and addictive drugs have also been observed in preclinical studies, but given that hormones can be more precisely manipulated in animals (e.g., using hormone replacement in ovariectomized, OVX, animals), these studies have been critical for establishing a causal role of ovarian hormones in substance use and SUD.

3.1.3 Animal Studies – Ovarian Hormones and Substance Use

Most of the support for a role of ovarian hormones on vulnerability to addiction has come from preclinical studies. These studies have shown that the reinforcing effects of addictive drugs vary in intact female rodents across the estrous cycle and in OVX females with and without hormone replacement. Studies in intact females have shown that following acquisition of drug self-administration, progressive-ratio responding for cocaine is markedly higher during estrus compared to other phases of the estrous cycle (Feltenstein and See, 2007; Roberts et al. 1989; Hecht et al. 1999; Lynch et al. 2008; Lacy et al. 2016). Findings in non-human primates similarly

show that progressive-ratio responding for cocaine varies across the menstrual cycle with the highest levels observed during the follicular phase (versus the late luteal phase); this effect is modest and only apparent at a low dose (Mello et al. 2007). Motivation for nicotine is also higher in female rats during estrus, but this effect is modest and has been observed in some studies (e.g., Lynch 2009), but not others (Donny et al. 2000).

Studies with alcohol have focused on consumption and have shown that consumption is lower in female rats during estrus and proestrus (versus metestrus and diestrus). However, these effects are modest and are only apparent when estrous cycles are synchronized such that each female is tested in the identical portion of each phase (Roberts et al. 1998; also see Forger and Morin, 1982). In contrast, no estrous cycle effects are observed for maintenance levels of alcohol consumption in free-cycling female rats indicating that the variability in hormone levels within different phases of the estrous cycle is enough to obscure the effects of estrous phase on drug intake. In female non-human primates, alcohol intake tends to be highest during mid-to-late follicular and the late luteal phase, which is similar to findings in humans (versus menses, Mello et al. 1984). Studies with opioids have also focused on maintenance levels of intake and have shown that intake is markedly lower during proestrus as compared to other phases of the estrous cycle (Lacy et al. 2016; Smith et al. 2021; Schmidt et al. 2021). This effect appears to be driven by estradiol given that it can be blocked using raloxifene, a selective estrogen receptor modulator/antagonist, and mimicked by administering supplementary estradiol treatments (Smith et al. 2021; Sharp et al. 2021).

Studies in OVX rats have consistently found a significant role for ovarian hormones in mediating the reinforcing effects of addictive drugs. For example, numerous studies have shown that OVX robustly attenuates the acquisition of cocaine self-administration (Hu et al. 2003; Jackson et al. 2006; Lynch and Carroll, 2001; Perry et al. 2013b; Hu and Becker, 2008; Zhao and Becker, 2010). It also decreases nicotine self-administration (Maher et al. 2022), the acquisition of methamphetamine and cannabinoid (WIN55,212-2, CB1 receptor agonist) self-administration (Kucerova et al. 2009; Fattore et al. 2009), and alcohol consumption and preference during acquisition and maintenance (Forger and Morin, 1982; Cailhol and Mormede, 2002). Studies with cocaine and methamphetamine further show that estradiol replacement in OVX females restores acquisition rates to those observed in ovary-intact females (Kucerova et al. 2009; Hu et al. 2003; Jackson et al. 2006; Lynch and Carroll, 2001; Perry et al. 2013a; Hu and Becker, 2008;

Zhao and Becker, 2010). Notably, concurrent administration of progesterone with estradiol inhibits the effect of estradiol on acquisition of cocaine self-administration (Jackson et al. 2006). Progesterone has also been shown to attenuate cocaine-induced conditioned place preference (Russo et al. 2008) and to decrease impulsive choice for cocaine in ovary-intact females (Smethells et al. 2016). Similar findings for the effects of OVX and hormone replacement have been observed for the rewarding effects of cocaine, alcohol, nicotine, methamphetamine, and amphetamine as assessed under the conditioned place preference paradigm (Hilderbrand et al. 2018; Mirbaha et al. 2009; Chen et al. 2003; Torres et al. 2009; Silverman et al. 2007; Fry and Rhodes, 2006). These findings indicate that estradiol enhances the reinforcing effects of psychostimulants and other drugs, while progesterone reduces it, similar to reports in humans.

There are also intriguing new data that suggest that in females, hormonal status at the time of initial drug exposure/conditioning impacts later vulnerability to drug use. Specifically, Johnson et al. (2019) showed that female rats that had undergone Pavlovian conditioning with cocaine (paired with a cue light) during estrus prior to cocaine self-administration were more motivated to obtain infusions of cocaine paired with the light cue as compared to males or to females that had been conditioned during diestrus. Levels of estradiol during the time of initial exposure/conditioning appears to drive this effect considering that in OVX females estradiol supplementation that occurs prior to acquisition, effectively restores drug intake to levels observed in intact females whereas, supplemental after acquisition does not impact intake (Maher et al. 2022). It is not yet known whether hormonal status during initial drug exposure would also impact vulnerability to developing addiction-like features. Future studies using animal models of SUD are necessary to determine this possibility and to determine if effects extended to other addictive drugs.

Finally, it is important to highlight a need for additional studies to examine the impact of ovarian hormones on the reinforcing effects of opioids considering that effects of OVX and estradiol on acquisition have been mixed with one study showing facilitation (Roth et al. 2002) and another finding no effect of estradiol replacement (Stewart et al. 1996). In contrast, Smith and colleagues have shown in a series of studies that ovarian hormones markedly impact maintenance levels of opioid self-administration. Specifically, they showed that heroin intake is markedly lower in females during proestrus (Lacy et al. 2016; Smith et al. 2021; Schmidt et al. 2021) and that this the effect could be mimicked by estradiol (in OVX and in ovary-intact

females) and blocked by an estrogen receptor antagonist (in ovary-intact females; Smith et al. 2021; Sharp et al. 2021). They also showed that progesterone treatment increased heroin self-administration compared to estradiol treatment in OVX females (Smith et al. 2021; but see Smith et al. 2022). These findings suggest that effects of ovarian hormones may be more robust for maintenance opioid use than initial opioid use, but additional studies are necessary to examine this possibility. Future studies are also necessary to determine the direction of effects of estradiol and progesterone on the reinforcing efficacy of opioids (e.g., using progressive-ratio schedules, the threshold procedure, or choice procedures).

3.1.4 Animal Studies – Ovarian Hormones and SUD

Ovarian hormones also appear to underlie the enhanced vulnerability in females to developing addiction-like features, and these effects are apparent during both the induction/extended-access drug self-administration phase, where high levels of drug intake lead to the subsequent development of an addiction-like phenotype, and again, with the development of an addiction-like phenotype. As with effects on maintenance levels of drug use, effects of ovarian hormones on extended-access intake have been subtle in intact females. For example, studies on extended-access alcohol self-administration in rats have failed to demonstrate an effect of estrous cycle on alcohol intake using an intermittent access paradigm (Priddy et al. 2017) or continuous access paradigm (Ford et al. 2002) although patterns of alcohol intake do differ by estrous cycle phase (e.g., greater bout frequency and size during diestrus versus proestrus; Ford et al. 2002). Similarly, there is no effect of estrous cycle phase on levels of cocaine intake under extended-access conditions (6-h a day, Corbett et al. 2021), but during estrus, females show a greater disruption in temporal patterns of cocaine self-administration (e.g., intake is more erratic/less tightly regulated) as compared to during non-estrus phases (Lynch et al. 2000). Cocaine intake also does not differ across the menstrual cycle in female non-human primates with an extensive history of cocaine self-administration (Cooper et al. 2013). These findings are in contrast to those reported by Mello et al. (2007) where menstrual cycle effects were observed for cocaine self-administration in female non-human primates that were drug-naïve prior to acquisition and progressive-ratio testing and suggest that the role of ovarian hormones may decrease from initial use to the development of an addiction-like phenotype. This conclusion is further supported by

findings showing that in female rats that develop a preference for cocaine over food pellets, the estrous cycle continues to modulate motivation for food pellets, but not cocaine.

Alternatively, it is possible that effects of ovarian hormones are obscured following chronic drug self-administration due to the reciprocal effects of addictive drugs and ovarian hormones, that is, the impact of chronic drug use on ovarian hormone levels. This conclusion is supported by multiple studies with cocaine showing that depletion of ovarian hormones by OVX robustly decreases extended-access cocaine self-administration whereas estradiol replacement robustly increases levels of self-administration (Larson et al. 2007; Ramoa et al. 2013; 2014; Martinex et. al., 2016). Similar findings have been observed for alcohol consumption under a 24-hr/day, two-bottle-choice, continuous-access paradigm (Ford et al. 2004; Rajasingh et al. 2007; Becker et al. 1985; Forger and Morin, 1982 but see Hilderbrand and Lasek, 2018) and nicotine intake under extended-access conditions (Flores et al. 2016). These findings also appear to extend to opioids given our recent findings showing that OVX females with estradiol self-administer markedly higher levels of fentanyl under extended- (24-hr/day) intermittent-access conditions (2, 5 min trials/hr, 10 days) than vehicle-treated OVX rats. OVX rats with estradiol replacement, but not vehicle treated OVX rats, also escalated their intake of fentanyl between the first and last extended-access sessions (Towers et al. 2022). Additionally, similar to the acquisition and conditioned place placement studies, progesterone attenuates the escalation of cocaine intake under extended-access conditions (Larson et al. 2007) and decreases alcohol consumption under a 24-hr/day, two-bottle-choice, continuous-access paradigm (Ford et al. 2002). Progesterone treatment has also been shown to decrease cocaine self-administration in intact and OVX female non-human primates with or without an extensive history of cocaine self-administration (Mello et al. 2011). Thus, estradiol appears to enhance, while progesterone protects against the transition from regular to escalated/dysregulated drug use. While additional studies are needed to determine if the protective effects of progesterone on escalation/dysregulation of cocaine and alcohol self-administration extend to other addictive drugs, such as opioids, there is strong evidence that estradiol similarly enhance vulnerability during extended-access self-administration for a number of addictive drugs, including cocaine, nicotine, opioids, and alcohol.

Findings with cocaine and opioids indicate that ovarian hormones also modulate the expression of addiction-like features following abstinence from extended-access self-administration. Most of this evidence is for drug craving and shows that levels of cocaine,

fentanyl, and heroin craving are highest in females tested during estrus (versus non-estrus phases; Corbett et al. 2021; Nicolas et al. 2019; Towers et al. 2022; Bakhti-Suroosh et al. 2021); estrus also prolongs the time-course for incubation of cocaine craving in females (Kerstetter et al. 2008). While surprisingly few studies have examined the role of estradiol in incubated drug craving/relapse vulnerability following extended-access self-administration, our recent findings with fentanyl indicates that it is critically involved. Specifically, we found that OVX females with versus without estradiol replacement had a greater a sensitivity to the reinstating effects of fentanyl-associated cues following extended, intermittent-access fentanyl self-administration and 14-days of abstinence (Towers et al. 2022). However, both the vehicle and estradiol treated groups showed an increase in responding following exposure to the cues indicating that while estradiol modulates the expression of this phenotype, it is not necessary for its development. Similar effects of OVX have been reported for cannabinoid-craving following short-access self-administration where OVX rats showed an attenuated response to drug-cues and drug-primes (CB_1 receptor agonist; Fattore et al. 2010). Progesterone may also be involved given that exogenous treatment has been shown to reduce cocaine-craving in intact females following short-access self-administration (Feltenstein et al. 2008 Anker et al. 2007).

In contrast to effects on drug craving, estradiol appears to be necessary for development of an enhanced motivation for the drug and an enhanced preference for the drug over other reward alternatives. Most of this work has focused on cocaine and has shown that depletion of ovarian hormones either surgically (OVX) or pharmacologically (tamoxifen treatment in ovary-intact females) prevents the development of an enhanced motivation for cocaine even under conditions optimized for its development (following extended-access self-administration and 14 days of abstinence; Bhakti-Soroush et al. 2019; Ramôa et al. 2013; 2014). In contrast, this phenotype is evident in both vehicle-treated intact females and in OVX females treated with estradiol (Bhakti-Soroush et al. 2019; Ramôa et al. 2013; 2014). Similar effects of OVX and estradiol have also been observed for the development of a preference for cocaine over food (Kerstetter et al. 2012). We also recently observed similar effects of OVX and estradiol for the development of an enhanced motivation for fentanyl (Towers et al. 2022) indicating that the role of estradiol on the development of this feature of SUD may be similar for both psychostimulants and opioids. However, further research is necessary to confirm its role with other psychostimulants (e.g., methamphetamine, nicotine) and other opioids (e.g., heroin, oxycodone). Additional studies are

also needed to confirm effects with fentanyl considering that the parameters for the development of an enhanced motivation for fentanyl are not yet known (i.e., when does the phenotype emerge, how much prior drug access is needed, and how long does phenotype persist).

Interestingly, pharmacological blockade of estrogen receptors with tamoxifen has been shown to similarly prevent the development of an enhanced motivation for cocaine in ovary-intact females, but, unlike the findings in the OVX model, tamoxifen did not decrease cocaine self-administration under extended-access conditions or relapse vulnerability (Bakhti-Suroosh et al. 2019). These findings indicate that differences in level of intake during the induction/ extended-access phase, which did not differ between tamoxifen- and control-treated females, is not critical for the development of motivational features of addiction (Bakhti-Suroosh et al. 2019). They also suggest other hormones, such as progesterone, may modulate the expression of certain addiction-like features, such as a loss of control over drug use and relapse vulnerability, but perhaps not critical for others, such as an enhanced motivation for the drug. For example, where estrogen receptors are antagonized, such as with chronic tamoxifen administration in ovary-intact females, there may be compensatory decreases in progesterone signaling which leads to increased extended-access drug intake and relapse vulnerability. However, it should be noted that, as a selective estrogen receptor modulator, tamoxifen can have both agonist and antagonist effects (Dutertre and Smith 2000); thus, it is possible that its antagonist effects at estrogen receptors were sufficient for preventing the development of an enhanced motivation for cocaine, but not for reducing extended-access intake or for attenuating relapse vulnerability. Future studies are needed to resolve these questions.

In summary, estradiol appears to enhance the expression of multiple features of SUD (loss of control over use, relapse vulnerability, preference for drug over other rewards, motivation for the drug) and to be necessary for the development an enhanced motivation for the drug and an enhanced preference for the drug over other rewards. Progesterone may attenuate the expression of addiction-like features and vulnerability maybe heightened when progesterone levels are low but additional studies are studies are necessary to confirm these possibilities.

3.2 Sex Chromosomes

One biological factor that few consider when they assess sex differences is the basic inequality in sex chromosome genes. In the mammals commonly used for preclinical studies, and in humans,

males have two different sex chromosomes, X and Y, whereas females have two copies of the X-chromosome. The X-chromosome is substantially larger (3x the physical size) and contains about 1,000 more coding genes than does the Y-chromosome (Balaton et al 2018). When the phenomenon of X-inactivation was discovered, we assumed it equalized this discrepancy. If, in fact, the entire second X-chromosome in each cell was inactivated in females, the sexes would still have differences in gene expression by virtue of unique genes on the male-only Y-chromosome. However, it is now clear that many (20% in humans) X-chromosome genes escape inactivation (Disteche and Berletch, 2015; Patrat et al. 2020).

To examine the actions of sex chromosome genes both independently of hormones and interactive effects, the Four Core Genotype (FCG) mouse is frequently used (DeVries et al 2002). The dam for this cross is a normal XX female but the sire carries a null mutation of the sex determining gene (Sry) on his Y-chromosome and a transgene for the Sry that has randomly incorporated into chromosome 3 (Lovell-Badge and Robertson 1990; Mahadevaiah et al. 1998; Itoh et al. 2015). The Y-chromosome and the Sry transgene segregate independently producing four genetic offspring from the cross: females with XX or XY chromosome, and males with XX or XY sex chromosomes. This model provides a way to disassociate the effects of hormones from the effects of sex chromosome complement. The FCG has been exploited for over 20 years for disease models, studies of neurobiology and behavior (Gatewood et al. 2006; Quinn et al. 2007; Smith-Bouvier et al. 2008; Cisternas et al. 2018; Arnold 2020).

3.2.1 Animal Studies – Sex Chromosomes and Substance Use

One study has used the FCG mouse model to determine how sex chromosomes influence vulnerability to drug use (Martini et al. 2020). This study found that females (XX and XY) acquired cocaine self-administration faster than males XY males; XXM also acquired faster than XY males and did not differ from XX or XY females. However, contrary to findings in rats, motivation for cocaine (as assessed under progressive-ratio schedule following acquisition) was highest in XY males as compared with all other groups. Together, these results suggest sex chromosomes may interact with gonadal hormones to impact initial vulnerability to drug use.

3.2.2 Animal Studies – Sex Chromosomes and SUD

Two studies have used the FCG mouse model to examine habit formation, which is believed to occur during the transition from recreational drug use to compulsive drug use and addiction. Barker and colleagues (2010) showed that chromosomal females are slower to develop habitual responding for alcohol reinforcement compared to chromosomal males, but gonadal females consumed more alcohol than gonadal males. Interestingly, the second study showed that chromosomal females are faster to form habitual responding for sucrose compared to chromosomal males, regardless of gonadal phenotype (Quinn et al. 2007). These findings suggest that sex chromosomes may differentially affect the formation of habitual drug versus non-drug reinforcers use. They also indicate that gonadal hormones, but not sex chromones, drive the enhanced vulnerability in females.

3.2.3 Summary and Integration of Preclinical and Clinical Findings

Together, these clinical and preclinical studies support an important role for estradiol in vulnerability during early phases of SUD, such as drug use initiation and the transition from use to SUD, thereby making estradiol a potential driver of the telescoping effect in women. These studies also highlight ovarian hormones as a potential target for intervention during initial periods of drug use and prior to the development of SUD. However, the role of ovarian hormones may be different with opioids, particularly during drug use initiation, and future studies are necessary to investigate this possibility. Finally, estradiol and progesterone have broad actions on many neural and non-neural tissues including breast, ovary and uterus. Any steroid treatments would have to take cancer risk into account.

It is important to consider that nearly all women use contraception in their lifetime (Daniels et al. 2013), and hormonal contraceptives composed of either a combination of estradiol and progesterone or progesterone alone are very popular (Cooper et al. 2022; Daniels and Joyce, 2020). Little is known about the influence of hormonal contraceptives on vulnerability to SUD in women or female laboratory animals and the clinical studies report mixed results with some showing that pill users have lower positive subjective ratings of nicotine (Hinderaker et al. 2015) and nicotine craving (Dickmann et al. 2009) than non-pill takers. However, current smokers are more likely that non-smokers to use hormonal contraceptives (Lee et al. 2013) and these women have increased ethanol intake, especially if contraceptive use begins at an early age (<20 years; Lund et al. 1990). These conflicting reports could be the result of the wide range of hormonal

contraceptives available to patients, which include high- versus low-doses of estradiol or just progesterone. Other characteristics of smokers versus non-smokers may also influence these results. Further studies need to determine the impact of these commonly used hormonal contraceptives as physicians could tailor their birth control recommendation if substance misuse is a concern or they could be repurposed as an adjunctive therapy for at risk adolescents since they have been proven to be safe in this patient population.

A final important consideration is pregnancy which appears to protect females. During pregnancy, progesterone and estradiol levels markedly increase and coincidentally, rates of drug use, including tobacco, alcohol, cannabis, and any drug not prescribed by a doctor to the individual, also markedly decline (Volkow et al. 2019; Kendler et al. 2017: Harrison et al. 2009). While there are obvious socio-cultural based factors that may also explain these decreased rates of drug use, studies in rats also show marked decreases in drug self-administration during pregnancy indicating that pregnancy decreases biological vulnerability to drug use in females. For example, Hecht et al. (1999) showed that in rats, motivation to obtain cocaine under short access conditions progressively declined from pre-pregnant levels over the course of pregnancy. One caution to note here, however, is that dose was not adjusted for weight changes during pregnancy, and thus motivation to obtain cocaine may be reduced by relatively low cocaine dose. However, because similar findings have also been observed for nicotine self-administration under extend-access conditions (23-hours a day; LeSage et al. 2007), where dose was adjusted for changes in body weight, and oral alcohol intake under continuous access conditions (Gene Forger and Morin, 1982) the results are likely to reflect a reduction in the reinforcing effects of addictive drugs as a result of pregnancy. These effects may differ for opioids, however, considering findings showing that under short-access conditions (1-hr/day), pregnant female rats self-administer similar levels of oxycodone as non-pregnant, female rats (Vassoler et al. 2018). While these findings in rats appear to contrast with epidemiological data showing that the prevalence of past month heroin use is markedly lower in pregnant than in non-pregnant women (0.05% versus 0.19 %, respectively, 15-44 years old; Vanderziel et al. 2020), data obtained during labor and delivery show that the prevalence of opioid use and OUD in pregnant women has quadrupled over the last decade and is present in approximately 3% of pregnancies in the US (Chang 2020). Thus, the rising levels of progesterone during pregnancy appears to be protective against drug use and possibly the transition to SUD, but this may be different for opioids.

4 Neurobiological Mechanisms

4.1 Mesolimbic Dopamine Signaling

Dopamine signaling in the mesolimbic pathway has been nearly the exclusive focus of studies on molecular mechanisms mediating sex differences in SUD. This pathway, which includes dopaminergic projection neurons from the VTA to the NAc, is well established based, mainly on studies conducted in males, to be a core component of the reward circuitry and critical for mediating the positive reinforcing effects of addictive drugs (for review see Pierce and Kumaresan, 2006; Koob and Volkow, 2016). Addictive drugs increase dopamine concentrations in the NAc, and antagonizing dopamine receptors, particularly dopamine D1-receptors (D1 and D5, referred to as D_1 hereafter), prevents the acquisition of drug self-administration and decreases short-access drug self-administration (see Volkow and Morales, 2015 for review).

Not surprisingly, the mesolimbic reward pathway is also implicated in the disease state of addiction. Again, these data are based predominantly on effects in men and male laboratory animals and show that neuroadaptations caused by chronic drug use leads to mesolimbic hypofunction, which in turn promotes drug use to combat negative affect/anhedonia induced by dopamine deficits during abstinence (Koob and Volkow, 2016). For example, it is well established based on positron emission tomography (PET) imaging studies in humans that individuals with a SUD have marked decreases in dopamine D2 receptor binding (D2, D3, and D4, referred to as D_2 hereafter) in the striatum. This molecular switch was first documented by Volkow and colleagues who showed that individuals with cocaine use disorder had lower D_2 receptor availability that corresponded to increased ratings of dysphoria which persisted months after abstinence (relative to healthy individuals, Volkow et al. 1990; 1993; 1996). These individuals also showed diminished dopamine release in the striatum and reported lower ratings of positive subjective effects (reduced liking, euphoria) and higher ratings of negative subjective effects (want more, craving) following psychostimulant administration as compared to healthy individuals (Volkow et al. 1996; 1997a). This phenomenon has been replicated in many subsequent studies and for multiple SUDs including methamphetamine, nicotine, heroin, and alcohol (alcohol Martinez et al. 2005; Volkow et al. 2001, 2014; van de Giessen et al. 2017; Martinez et al. 2004, 2005, 2011, 2012; Christoph Fehret al. 2008; Worhunsky et al. 2017; but see Casey et al. 2014; for review see Volkow et al. 2007). This is thought to reflect a shift from

positive reinforcing effects, a primary mechanism driving drug use during initial phases of SUD, to negative reinforcement, which drives drug use once addiction has developed to alleviate withdrawal, craving, or negative affect. A similar blunting of the dopaminergic response to psychostimulant drug administration is observed in cannabis use disorder (CaUD), and while this effect is also associated with increased relapse vulnerability, it is not accompanied by a downregulation of D_2 receptors (Volkow et al. 2014). A few studies have included both men and women (Martinez et al. 2012; Volkow et al. 2001), and some sex differences have been noted (as discussed below; Brown et al. 2012; Okita et al. 2016; Wiers et al. 2016a,b; Zakiniaeiz et al. 2019). Results from preclinical studies have revealed similar changes in D_2 receptor signaling with evidence to further indicate that mesolimbic D_2 receptor signaling contributes to both vulnerability to drug use and the development of key addiction-like features, such as a loss of control/escalation of drug use (for review see Everitt et al. 2008; Trifilieff et al. 2017a). There is also compelling evidence indicating the role of dopamine is minimized once SUD is established, and that other signaling pathways, particularly those involved in mediating the negative reinforcing effects due to craving, are recruited and drive the enhanced motivation for the drug (e.g., glutamatergic signaling; see Glutamate section below).

While most of the evidence for sex differences in dopaminergic signaling is focused on initial vulnerability, preliminary findings indicate that the mechanisms underlying SUD are different in males versus females and that molecular shifts that contribute to its development occur faster in females than males (as discussed below).

4.1.1 Human Studies – Dopamine and Substance Use

Clinical studies using healthy controls, report that men and women have similar D_2 receptor availability and densities in the striatum, but women have greater dopamine synthesis capacity and dopamine transporter availability in the striatum than men (for review see Woodcock et al. 2020). The net effect is that dopamine secretion and transport are more active in women than in men. Findings for evoked dopamine release in the striatum however, have been mixed, and tend to suggest greater effects in healthy men than women in response to psychostimulants and alcohol (Munro et al. 2006; Urban et al. 2010; Oswald et al. 2015; Smith et al. 2019; but see Riccardi et al. 2006). In contrast, Manza and colleagues (2022) reported more striatal dopamine release in women than men (as measured by displacement of [^{11}Craclopride]) in response to both

oral and intravenous administration of the psychostimulant methylphenidate. Women also reported higher ratings of "drug effects" than men (Manza et al. 2022), which is in contrast to the other studies reporting greater psychostimulant-evoked dopamine release and positive subjective effects in men than in women (Munro et al. 2006; Smith et al. 2019). Given that the positive subjective effects of addictive drugs are believed to be driven by mesolimbic dopamine signaling, this difference provides a plausible explanation for the differences between these results. Moreover, as with positive subjective ratings of addictive drugs, sex differences in evoked dopamine release are influenced by hormonal changes over the menstrual cycle. Cycle day and/or hormone data have not been included in some of the previous studies (Munro et al. 2006; Urban et al. 2010; Oswald et al. 2015) or testing was completed in women with low ovarian hormones (Munro et al. 2006; Oswald et al. 2015; Smith et al. 2019). As a specific example, in the Smith et al. (2019) study, the women included were in one of three low estradiol states; either postmenopausal, on hormonal contraceptives, or in the early follicular phase of their menstrual cycle. This is in contrast to the most recent study where hormone data were collected and at least some of the women included were tested during the mid-to-late follicular phase (Manza et al. 2022), when levels of estradiol are high and relatively unopposed by progesterone. However, even in this recent study, details are lacking regarding menstrual cycle status and estradiol levels are available for only 7 of the 11 female subjects. Future studies that measure, or manipulate, levels of estradiol and progesterone are necessary to resolve this issue.

4.1.2 Human Studies – Dopamine and SUD

Most of the studies on sex differences in dopamine signaling and SUD have been conducted among tobacco smokers. These studies have shown, that as with findings in individuals with CoUD, AUD, and OUD, individuals with a TUD have a blunted dopamine response to psychostimulant administration (Busto et al. 2009; Wiers et al. 2017; Calakos et al. 2022; Zakiniaeiz1 et al. 2019), with particularly robust effects in women (Cosgrove et al. 2014; Zakiniaeiz et al. 2019). There is also a sex difference in the mechanism underlying this effect. In male smokers, the mechanism appears to be similar to that observed for CoUD, OUD, and AUD, decreased striatal D_2 receptor binding (Brody et al. 2004; Fehr et al. 2008; Stokes et al. 2012; Albrecht et al. 2013a; Brown et al. 2012). This is not the case in female smokers, however, since striatal D_2 receptor binding does not differ between smokers and non-smokers (Brown et al.

2012; Zakiniaeiz et al. 2019). This is intriguing considering that in male smokers this molecular shift is thought to reflect greater addiction severity and poorer treatment outcomes (Volkow et al. 1999); yet this does not occur in women who show greater addiction severity and worse treatment outcome than males. It is similarly thought to reflect enhanced vulnerability in individuals with a CoUD, AUD, or OUD, but given that these studies have been conducted predominantly in men, it is possible that this molecular shift occurs in males but not females.

Sex differences and effects of smoking status have also been between reported for D_2 receptor availability in the midbrain, which includes the VTA (Okita et al. 2016). Female smokers have higher midbrain D_2 receptor availability than both female non-smokers and male smokers; however, no differences are seen between male smokers and non-smokers (Okita et al. 2016). This difference is thought to underlie the greater suppression of mesolimbic dopamine in women versus men smokers given that D_2 receptors are predominantly inhibitory. These differences also parallel sex differences in positive subjective ratings of nicotine and smoking, which have consistently been lower in women than in men with a TUD (for review see Perkins, 1999); whereas, among non-smokers, women are more sensitive than men to low doses of nicotine (MacLean et al. 2021). Taken together, these findings indicate that there are sex differences in the molecular mechanisms underlying tobacco use/smoking with the development of TUD. The different mechanisms likely lead to sex difference in motivation to use tobacco/nicotine and a greater shift in women than in men to negative reinforcement and to a diminished role of dopamine. This explanation is also consistent with data showing that smoking in women with a TUD does not produce ventral striatal activation, but does so in men with a TUD (Verplaetse et al. 2018). Nicotine replacement is also a less effective treatment strategy for TUD in women than men (Perkins et al. 2018). While future studies are necessary to determine whether similar sex differences exist for other SUDs, it is notable that sex differences in positive subjective drug effects are observed across multiple drug classes and parallel these effects with TUD and typically show greater effects in women than men among recreational drug users, particularly at low doses (Liechti et al. 2001; Mayo et al. 2019; Vandersickel et al. 2010; Fogel et al. 2017; Miller et al. 2009; Wright et al. 2021), but no difference, or a diminished response in women versus men among individuals with a SUDs (e.g., Lynch et al. 2008; McCane-Katz et al. 2008). Additionally, de Wit and colleagues (2012) showed that dopamine depletion using a dietary intervention biases women, but not men, towards habitual responding rather than goal-

directed behavior, indicating that women are prone to transition from recreational drug use, which is goal-directed, to compulsive use, which is habitual.

Individuals with a CaUD also have blunted dopaminergic responses to psychostimulant administration (Volkow et al. 2014; van de Giessen et al. 2017), but unlike effects in CoUD, AUD, and OUD, this response is not associated with lower striatal D_2 receptor availability (Albrecht et al. 2013b; Sevy et al. 2008; Stokes et al. 2012; Urban et al. 2012). The mechanisms underlying the blunted responses also differ between men and women. Specifically, Wiers et al. (2016a) examined regional brain glucose metabolism in response to psychostimulant administration in men and women with CaUD versus healthy controls. They found decreased stimulant-induced metabolism in the midbrain and striatum as well as decreased glucose metabolism in the putamen and these correlated with addiction severity; however, all the effects were driven by changes in women. In men, no metabolic differences were observed between healthy controls and individuals with a CaUD. Women with a CaUD also had higher subjective ratings of craving in response to methylphenidate than healthy controls of either sex, whereas no difference was observed between healthy men and men with a CaUD (Wiers et al. 2016b). These results indicate that the neuroadaptations underlying SUD differ between men and women.

Sex differences in the time-course for these molecular shifts in dopamine signaling/receptor populations that are concurrent with the development of SUD have not been examined. However, data from young adult men and women at risk for an AUD indicate they are possible. Specifically, Oswald and colleagues (2010) showed that in young adults at high risk for an AUD based on levels of drinking (>10-15 drinks/week, 15 drinks/week for males, and 18 drinks/week for females), men had greater and more widespread increases in striatal dopamine release than women in response to alcohol administration. Subjective ratings of "activation" in response to alcohol were also positively correlated with dopamine release in the ventral striatum in men, whereas subjective ratings of alcohol were not correlated with dopamine release in women. These findings suggest that the shift toward a diminished role of dopamine signaling, believed to reflect greater addiction severity, may occur sooner in females than males. However, future studies are necessary to determine if this effect is reliable and consistent across SUDs.

4.1.3 Animal Studies – Dopamine and Substance Use

Results from preclinical studies also suggest that there are sex differences in the dopamine signaling pathway. Although there are divergent data on whether the density of D_1 receptors in the NAc differs between males and females (Festa et al. 2006; Andersen and Teicher 2000), markers of D_1-cAMP-PKA cell signaling, which is associated with greater vulnerability to drug use, are enhanced in drug naïve females compared to drug naïve males (Lynch et al. 2007). However, it is important to note that markers of vulnerability, which have been generated based predominantly on findings in males, may differ between males and females. For example, Morgan et al. (2002b) showed that dominant male cynomolgus monkeys have higher D2 receptor availability and are less vulnerable to the reinforcing effects of cocaine as compared to subordinate male monkeys. While dominant female cynomolgus monkeys also had higher D2 receptor availability than subordinate female cynomolgus monkeys, dominant females were more vulnerable to the reinforcing effects of cocaine as compared to subordinate females (Nader et al. 2012) indicating that the relationship between D2 receptor availability and vulnerability to cocaine is opposite in females versus males.

Preclinical findings also demonstrate that ovarian hormones modulate dopaminergic signaling in the reward pathway in females. Neuron firing rates in the VTA reach peak levels in females during estrus (versus diestrus; Calipari et al. 2017), and drug-induced dopamine release is greater during proestrus/estrus (versus metestrus/diestrus; Becker and Cha, 1989; Calipari et al. 2017). Results from non-human primates show that striatal D_2 dopamine receptor availability is lower during the follicular phase than the luteal phase (Czoty et al. 2009). OVX has also been shown to increase striatal D_2 receptors, reduces VTA firing rates, and drug-induced dopamine release in the NAc, while estradiol replacement restores each of these effects in female rats (Shams et al. 2016; Sham et al. 2018; Cummings et al. 2014; Zhang et al. 2008; Castner and Becker, 1993). Estradiol also increases tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis and production, decreases sensitivity of D_2 auto receptors, enhances D_1 receptor activation (Festa et al. 2006), and reduces the reuptake of DA, all of which enhance mesolimbic DA signaling (Calipari et al. 2017). Estradiol-induced changes in dopamine signaling have been linked to an increased sensitivity to the rewarding effects of cocaine, assessed using conditioned place-preference (Calipari et al. 2017), and the reinforcing effects of alcohol (Vandergrift et al. 2017).

Interestingly, progesterone can both potentiate and inhibit estradiol's effects on dopamine release with enhancement observed shortly after estradiol and progesterone administration in OVX rats and inhibition observed 24-hr after administration (Glaser et al. 1983). These differences likely explain estrus-induced enhancements of dopamine release given that both estradiol and progesterone peak prior to the beginning of estrus (Becker and Ramirez, 1981; Becker and Rudick, 1999; Dluzen and Ramirez, 1984; Becker et al. 1984; see Yoest et al. 2018 for review). Thus, estradiol enhancement of mesolimbic dopamine signaling appears to increase the rewarding and reinforcing properties of addictive drugs and likely drives the enhanced sensitivity in females during initiation/acquisition of drug use. While similar effects have been observed for cocaine, amphetamine, and alcohol, further research is necessary to determine if findings extend to other drug classes, including opioids and cannabis.

4.1.4 Animal Studies – Dopamine and SUD

While dopamine-estrogen interactions likely contribute to the enhanced sensitivity in females during initiation/acquisition of drug use (for review see Kokane and Perrotti 2020), it is not yet clear if similar mechanisms underlie vulnerability during later phases of SUD or the faster time-course to addiction in females versus males. Such effects are possible given findings from multiple studies showing that estradiol increases cocaine, alcohol, nicotine, and fentanyl intake under extended-access conditions (Larson et al. 2007; Ramoa et al. 2013; 2014; Martinex et. al., 2016; Ford et al. 2004; Flores et al. 2016; Towers et al. 2022). Findings with alcohol also show that estradiol potentiates alcohol-induced excitation of dopamine neurons in VTA and that targeted knockdown of estrogen receptors in the VTA reduces binge alcohol drinking in female, but not male mice (Vandergrift et al. 2017; Vandergrift et al. 2020). Thus, as with effects during drug use initiation, estradiol may enhance vulnerability to the development of addiction-like features by enhancing drug-induced dopamine signaling in the reward pathway.

Estradiol may also be necessary in females for the molecular switch to a diminished role of mesolimbic dopamine that accompanies the development of an addiction-like phenotype. Specifically, we showed that OVX prevents both the development of an enhanced motivation for cocaine and the corresponding molecular shift to a diminished role of NAc dopamine and that both effects can be restored by estradiol replacement (Ramôa et al. 2013; 2014). In our work, NAc D_1 receptors remained the critical mechanism motivating cocaine use in vehicle-treated

OVX rats that did not develop an addiction-like phenotype (Doyle et al. 2014; Ramôa et al. 2014). Similarly, Perry et al. (2015) showed that female rats that developed a preference for cocaine over a non-drug reward (i.e. palatable food pellets) also displayed attenuated cocaine-induced dopamine release in the NAc. In addition, the rats that developed a cocaine preference, the estrous cycle continued to modulate motivation for the palatable food pellets, but not cocaine (Perry et al. 2015) indicating that ovarian hormones may not be necessary for the expression of this feature of SUD. Thus, estradiol accelerates, and is necessary, for drug-induced changes in dopamine signaling that underlie the development of addiction, but it may not be necessary for the expression of the addiction-like behaviors once they have been established (although estradiol still modulates their expression as discussed in the next section for cocaine craving).

4.2 Corticomesolimbic Glutamate

Studies in animals have established that estradiol enhances mesolimbic dopaminergic signaling via interactions with metabotropic glutamate receptors (mGlu); this likely contributes to the enhanced sensitivity in females versus males to the reinforcing effects of addictive drugs (as detailed below). Glutamatergic signaling in corticomesolimbic regions, including projection neurons from the mPFC to the NAc, is also a strong candidate mechanism underlying the faster course to addiction in females. This pathway is critical for the development of multiple features of addiction including escalation of drug use, compulsive drug use, enhanced motivation for the drug, and enhanced craving/vulnerability to relapse (Koob and Volkow, 2016). Preclinical findings indicate that estradiol interacts with metabotropic glutamate receptors (mGlu) to enhance mesolimbic dopaminergic signaling, which may contribute to the enhanced sensitivity in females versus males to the reinforcing effects of addictive drugs. Glutamatergic signaling in corticomesolimbic regions, including projection neurons from the mPFC to the NAc, is also a strong candidate mechanism underlying the faster course to addiction in females. This pathway is critical for the development of multiple features of addiction including escalation of drug use, compulsive drug use, enhanced motivation for the drug, and enhanced craving/vulnerability to relapse. Glutamatergic projections from the mPFC to the NAc modulate the behavioral consequences of extended-access drug self-administration (Schmidt and Pierce 2010; Kalivas and Volkow, 2011; Quintero, 2013), and several studies have shown that extended-, but not short-access, self-administration produces long-lasting adaptations in glutamate NMDA and

AMPA receptors in the mPFC and NAc in humans, non-human primates, and rats (Hemby et al. 2005; Tang et al. 2004; Backes and Hemby 2003). This signaling pathway is known as the "final common pathway to relapse" since it is activated in response to relapse triggered by drug-associated cues, priming doses of the drug, and stress, and for multiple drug classes, including psychostimulants, nicotine, opioids, and alcohol (Peters et al. 2008; Kalivas and McFarland 2003; Knackstedt and Kalivas, 2009).

Corticomesolimbic glutamate pathways also underlie the progressive increase, or incubation, of drug-craving over abstinence. Glutamatergic signaling in this pathway changes dramatically during abstinence, from hypoglutamatergic during early abstinence, when levels of drug-craving are low (first 1-3 days), to hyperglutamatergic during protracted abstinence, when craving has incubated to high levels (after 7 or more days; Barry and McGinty, 2017; Ben-Shahar et al. 2009; Caffino et al. 2020; Chen et al. 2013; Funk et al. 2016; Hearing et al. 2018; Koob and Volkow, 2016; Roura-Martínez et al. 2020; Siemsen et al. 2019; Sun et al. 2014; Szumlinski et al. 2018). NMDA receptors are critically involved in both the early-withdrawal molecular cascade that triggers the incubation of craving (Barry and McGinty, 2017), as well as the enhanced cue-induced craving following protracted abstinence (Szumlinski et al. 2018; Barry and McGinty, 2017; Chen et al. 2013). These preclinical data are also consistent with pathophysiology of SUD in humans (Enoch et al. 2014; Hafenbreidel et al. 2017). AMPAR transmission is also critically involved, and this effect appears to be driven by Ca2+-permeable (CP) AMPAR, which accumulate in the synapses of neurons in the NAc core over a period of protracted abstinence following extended-, but not short-access drug self-administration (Caffino et al. 2021; Conrad et al. 2008; Murray et al. 2021; Purgianto et al. 2013; Wolf and Tseng 2012). While most of the work in this area has been conducted with cocaine, the role of the mPFC in the drug craving and relapse appears to be similar for other drug classes, including opioids, alcohol, and methamphetamine (Bauer et al. 2013; Hearing, 2018, Bossert et al. 2006; Doherty et al. 2013; Kuntz et al. 2008; Kuntz-Melcavage et al. 2009; Lalumiere et al. 2008; Rogers et al, 2008; Rubio et al. 2018; Schmidt et al. 2005; See et al. 2009; Shen et al. 2011; Mishra et al. 2017). Results from both clinical and preclinical studies also similarly show an association between heightened drug-craving/relapse and activation of the mPFC to NAc pathway (Bauer et al. 2013-alcohol; Grusser et al. 2004-alcohol, Goldstein and Volkow, 2011; See, 2009; Rubio et al. 2018; LaLumiere and Kalivas, 2008; Szumlinski and Shin, 2018; Shin et. al., 2018).

One major caveat is that the evidence implicating glutamatergic signaling in SUD is based almost entirely on findings in men and males. Data obtained from women and female animals are beginning to accumulate and they concur with the results from men and male laboratory animals indicating a critical role for glutamate in SUD. However, as detailed below, there is also preliminary evidence indicating that there are sex differences in corticomesolimbic glutamate signaling that may contribute to sex differences in vulnerability to drug use and the faster course to addiction in females.

4.2.1 Human Studies – Glutamate and Substance Use

Very few clinical studies have examined sex differences in glutamatergic signaling. In healthy controls, women had higher levels of glutamate (as assessed using magnetic resonance spectroscopy) than men in the striatum, which includes the NAc and dorsal striatum (Zahr et al. 2013). Sex differences were also seen among recreational drinkers in the activation of the corticomesolimbic regions, presumably due to glutamatergic signaling. Specifically, Seo et al. (2011) showed that exposure to alcohol-related cues increased activity in corticomesolimbic regions in both men and women, but women showed greater activation than men in the frontal gyrus (middle and superior; Seo et al. 2011).

4.2.2 Human Studies – Glutamate and SUD

To our knowledge, no studies have examined sex differences in glutamatergic signaling in individuals with a SUD. However, several studies have compared the effects of stress- or cue-induced craving on activity within corticomesolimbic regions in abstinent men and women with a SUD, typically CUD. These findings have been mixed, but generally show that this circuit, presumed to be glutamatergic, is activated in both men and women (Joseph et al. 2019; Grusser et al. 2004) although the regions activated, and degree of activation, varies by sex between studies. For example, Kilts et al. (2014) showed that corticomesolimbic activity increased (as measured using regional cerebral blow flow) in both men and women following exposure to cocaine-associated cues. In women, increased activation was observed in the precentral, middle frontal, and posterior cingulate gyri, whereas in men, increased activation occurred in the caudate, right postcentral gyrus, and left insula. Li et al. (2005) showed that both men and women show activation of the mPFC in response to stress-induced craving (using stress

imagery), but under these conditions, activation was greater in women than men. Similarly, Potenza et al. (2012) showed that subjective reports of craving positively correlated with corticomesolimbic activation in both men and women with a CUD (Potenza et al. 2012), but in women, corticomesolimbic activation occurred in response to stress, whereas in men, activation occurred in response to drug-associated cues. It is notable that in each of these studies, sex differences were apparent in brain regions activated in response to craving, yet subjective ratings of craving were similar between men and women. These findings add to a growing body of evidence indicating that, even in the absence of behavioral differences, the mechanisms underlying SUD in men and women may differ.

4.2.3 Animal Studies – Glutamate and Substance Use

Sex differences in mGlu signaling have been reported in drug naïve laboratory animals in several brain regions, and differences in the NAc are thought to underlie the enhanced vulnerability observed in females to initial drug use. Specifically, mGlu5 appears to be required for estradiol-evoked dopamine release in the NAc in females (Song et al. 2019). In OVX rats, either an mGlu5 antagonist (MPEP) or an estrogen receptor (ICI-182 780) antagonist can block estradiol's ability to enhance amphetamine-induced dopamine release in the NAc. Thus, mGlu5 likely contributes to sex differences in the reinforcing effects of psychostimulants and possibly other addictive drugs through an estradiol-dependent manner, which could translate to greater vulnerability to initial drug use.

4.2.4 Animal Studies – Glutamate and SUD

In OVX rats, mGlu5 activation is also necessary for estradiol-induced increases in extended-access cocaine self-administration (Martinez et al. 2016). In contrast to effects of estradiol-mGlu5 on dopamine release, which are likely mediated through rapid effects of membrane-associated estrogen receptors on neuronal excitability, effects of estradiol-mGlu5 on extended-access intake require repeated estradiol treatments over time indicating that changes are mediated through nuclear estrogen receptors that lead to altered synaptic plasticity. This idea is also in line with findings showing that estradiol mediates dendritic spine plasticity in the NAc through activation of mGlu5 in drug naïve control females (Peterson et al. 2015). Females also have greater increases in spine density of medium spiny neurons following chronic drug exposure

(Strong et al. 2017; Wissman et al. 2011), an effect also believed to be mediated via estradiol-mGlu5 interactions (for review see Eisinger et al. 2018). Differences are most apparent in the NAc core which is significant considering that dendritic spines on medium spiny neurons in this area integrate dopamine and glutamate signaling to mediate the reinforcing and motivational properties of addictive drugs. Thus, sex differences in mGlu5 signaling may contribute to sex differences during both initial exposure and the transition from use to addiction.

Notably, we showed that following the development of an addiction-like phenotype (i.e., an enhanced motivation for cocaine), the molecular mechanisms underlying drug use shifts from NAc dopamine to AMPA receptors in both males and females (Doyle et al. 2014). We further showed that estradiol is required in females for both the mechanistic shift to a diminished role of NAc dopamine and the development of an addiction-like phenotype (Ramôa et al. 2014). Considering that this mechanistic shift appears to accompany the development of an addiction-like phenotype, and considering that this phenotype develops sooner during abstinence in females than males, it is likely that estradiol is both necessary and accelerates the behavioral and molecular shifts (Ramôa et al. 2013). This idea is also supported by findings in drug naïve rats showing that females have enhanced glutamatergic input in the NAc compared to males (Forlano and Woolley 2010); as such, they may be "primed" for the recruitment of the glutamate system.

Enhanced NAc AMPA signaling also appears to underlie the development of drug craving/vulnerability to relapse in both males and females. However, in females, these mechanisms may be ovarian hormone dependent. Specifically, Bechard et al. (2018) showed that daily treatment with ceftriaxone, which offsets cocaine-induced deficits in the cystine-glutamate exchanger and the Na+ -dependent glial glutamate transporter (GLT-1), effectively decreases cue-induced reinstatement in both male and female rats. However, in female rats, ceftriaxone was only effective in reducing craving during non-estrus phases possibly because during estrus, the protective effects of ceftriaxone were countered by estradiol-induced increases in synaptic CP-AMPA receptors (as reflected by an increase in surface expression of GluA1 in the NAc). One caveat is that these effects were observed following short-access cocaine self-administration (2-hr/day) and extinction training (2–3 weeks), which may cause different molecular adaptations than those observed following abstinence from extended-access self-administration accompanied by development of an addiction-like phenotype. However, as females are more vulnerable than males to developing addiction-like features following short-access self-administration and we

observed similar results using extended-access conditions, these findings support the idea AMPA signaling is enhanced during estrus and with the development of an addiction-like phenotype.

There are also sex differences in glutamatergic signaling within mesocortical regions in drug naïve controls and following the development of an addiction-like phenotype. In drug naïve controls, females have less basal glutamatergic excitatory strength in the prelimbic region of the mPFC compared to males, but higher GluN1 subunit expression (which are ubiquitous to the NMDA receptor; Wange et al. 2015). Additionally, we recently showed that there are marked sex differences in molecular adaptations associated with the incubation of cocaine-craving. This study focused on effects in the dmPFC a region known to mediate the incubation of cocaine-craving in males. As with previous reports, in males, expression of brain-derived neurotrophic factor exon IV promoter, *Bdnf-IV*, a marker of epigenetic regulation, and NMDA receptor subunits, *Grin2a*, *Grin2b*, and *Grin1*, changed in response to abstinence and relapse testing; however, in females, only *Grin1* expression was impacted. The timeline for the change in *Grin1*, the gene that encodes the GluN1 subunit of the NMDA receptor, also differed between males and females. In males, as with previous studies, *Grin1* expression was increased following relapse testing during protracted abstinence (following 14 days), whereas, in females, *Grin1* expression was increased following relapse testing during intermediate abstinence (following 7 days). These effects also corresponded to differences in cocaine-craving in response to drug-associated cues which peaked during protracted abstinence in males and during intermediate abstinence in females (i.e., following 7 versus 14 days; Towers et al., 2023a) suggesting that glutamatergic signaling in the dmPFC is recruited earlier during abstinence in females compared to males. Similar sex differences have also been reported for the effects of extended-access methamphetamine self-administration on NMDA signaling in the dmPFC (Mishra et al. 2017; Pena-Bravo et al. 2019). Effects were first characterized in males only and showed that NMDA receptor currents were increased following abstinence (8-14 days) from extended-access self-administration and were associated with an increased GluN2B surface expression (Mishra et al. 2017). The effect was confirmed in females in a more recent study that included both males and females (Pena-Bravo et al. 2019); however, this study used a shorter period of extended-access self-administration, and under these "threshold" conditions, NMDA receptor currents were increased in females, but not males, providing further support for the idea that this molecular shift develops more rapidly in females. They also showed that the increase in NMDA receptor

currents in females was not affected by GluN2B antagonism in the dmPFC indicating that, in contrast to effects with males, this molecular shift is independent of GluN2B NMDA receptors in females (Pena-Bravo et al. 2019). These findings are similar to our observations with cocaine showing that *Grin2b*, the gene that encodes GluN2B subunit of the NMDA receptor, was changed in males, but not females, in response to abstinence and relapse testing. These findings are intriguing and suggest that some of the molecular changes associated with the development of an addiction-like phenotype are accelerated in females versus males (*Grin1*/GluN1), while others are qualitatively different between females and males (*Grin2b*/GluN2B).

There is also evidence indicating that sex differences in the molecular adaptations induced by substance use and SUD impact the efficacy of treatments for SUD. For example, the sex differences we recently observed for relapse-associated changes in NMDA receptor gene expression in the dmPFC likely explain findings of sex difference in the efficacy of exercise as an anti-relapse intervention. Specifically, in males, dmPFC expression levels of *Grin2a* and *Grin2b*, the genes encoding the GluN2a and GluN2b subunits of NMDA receptors, were decreased during early abstinence (day 2) after extended-access cocaine self-administration. In contrast, NMDA receptor-related mRNA levels (*Grin2a and Grin2b*) were not impacted by extended-access cocaine self-administration (versus saline) or abstinence in females. We have also shown that when exercise is available during early abstinence (days 1-7) it provides long-lasting protection against relapse during protracted abstinence (on abstinence day 15), but only in males (Beiter et al. 2016). In males, the efficacy of exercise appears to be mediated by upregulating NMDA signaling in the dmPFC during early withdrawal thereby preventing a cascade of molecular events that underlie the incubation drug-craving (Abel et al. 2019). In contrast, exercise restricted to early abstinence is not effective at reducing craving during protracted abstinence in females possibly because females do not have cocaine-induced deficits in dmPFC NMDA receptor signaling during early withdrawal and thus, there is not a deficit for exercise to offset. These findings highlight a need for further research on sex differences in both the neuroadaptations underlying addiction and the efficacy of potential interventions for addiction. This information is necessary to guide the development of prevention and treatment efforts for SUD in women and will also help shed light on the mechanisms underlying the telescoping effect.

4.3 Summary and Integration of Preclinical and Clinical Findings

A telescoping effect in females is supported by clinical and preclinical neurobiological evidence which indicates that in females, interactions of estradiol with dopamine and glutamate lead to an enhanced sensitivity in females to the reinforcing effects of addictive drugs and the faster course to addiction in females versus males (**Figure 1**). Enhanced reinforcing effects is evident in both women (among healthy controls) and female animals. Estradiol enhances mesolimbic dopamine signaling on its own and through interactions with mGlu5 which lead to greater dopamine release in response to addictive drugs in females versus males. This enhanced signaling may lead to a faster shift toward a diminished role for mesolimbic dopamine. This idea is supported by findings in humans showing a blunted dopaminergic response in women versus men in heavy drinkers and smokers and results showing that in women, but not men, dopamine depletion biases women toward habitual responding. Preclinical studies similarly show that in females, a shift toward a diminished role of dopamine accompanies the development of an addiction-like phenotype and requires estradiol. An addiction-like phenotype is also accompanied by a shift toward enhanced corticomesolimbic glutamatergic signaling in both males and females. AMPA signaling in the NAc is similarly enhanced in male and female animals, but one key difference is that this shift likely occurs sooner in females than males and underlies the faster course to addiction in females. This idea is supported by findings in both humans and animals indicating that women (healthy controls and recreational drinkers) and female rats (drug naïve) have enhanced glutamatergic input to the striatum and are thus "primed" for the recruitment of the glutamate system.

Finally, it is important to note that sex differences in vulnerability to drug use and addiction likely involved many more brain regions (e.g., amygdala, hippocampus) and neurotransmitter signaling pathways (opioids, norepinephrine, serotonin, GABA, and endocannabinoids). To take an illustrative example, clinical and preclinical studies have shown acute stress potentiates dopamine function in the striatum, similar to acute drug use (Bloomfield et al. 2019; Imperato et al. 1989; Wand and et al. 2007). This effect appears to be mediated by glucocorticoid in the mesolimbic dopamine reward pathway since adrenalectomy, which depletes glucocorticoid hormone levels, decreases dopamine release in the NAc following stress and glucocorticoid replacement prevents attenuation of this dopamine response (Piazza and Le Moal, 1996; Barrot et al. 2000). Glucocorticoids have also been shown to potentiate the reinforcing properties of

addictive drugs (Piazza et al. 1993; see review Berry et al. 2016; Piazza and Le Moal, 1997) and this effect is likely magnified in females considering psychostimulants, such as cocaine and methamphetamine, produced an even greater increase in brain glucocorticoid levels in females than in males (Kuhn and Francis, 1997; Zuloaga et al. 2014). Therefore, acute stress may prime the brain reward circuit for subsequent action of addictive drugs or act synergistically with addictive drugs to accelerate sensitization of the reward pathway.

Additionally, in contrast to acute stress, chronic stress and/or exposure to glucocorticoids has been shown to lead to anhedonia and blunting of striatal dopamine function and receptor availability (Bloomfield et al. 2019; Mangiavacchi et al. 2001; Pacak et al. 2002; Meaney et al. 2002; Brake et al. 2004; Chrapusta et al. 1997; Gresch et al. 1994), similar to the neurobiological changes induced by chronic substance use (as discussed in the dopamine section). Women may be biologically more vulnerable to this stress-induced neuroadaptation considering Oswald and colleagues (2014) showed that childhood trauma is negatively associated with D_2 receptor availability in striatum of women, whereas a positive relationship was observed for men. Additionally, women often initiate drug use as a form of self-medication to reduce stress or alleviate anxiety; whereas, men are more likely to initiate drug use for their rewarding effects in social settings (see reviews, Sinha, 2001; Sinha, 2008). Thus, the stress driving initial substance use likely disrupted the reward pathway prior to drug use and enhances vulnerability to transition to SUD. All of these effects could also contribute to the faster progression to addiction observed in females.

5 Conclusions and Future Directions

The data reviewed from human, animal, and neurobiological studies supports a telescoping effect in females. The evidence is particularly strong for CoUD considering that it has been consistently observed in both treatment and non-treatment populations (McCance, Griffin, White, Haas, Sofuoglu, O'Brien; but see Lewis); preclinical studies with cocaine also similarly indicate an accelerated course to addiction in females (Kerstetter et al. 2012; Perry et al. 2013b, 2015; Kawa and Robinson, 2019; Lynch and Taylor, 2004; Towers et al. 2021a). The neurobiological data, which has focused almost exclusively on cocaine and other psychostimulants, also supports its biological basis with findings from both human and animal studies indicating that in females, estradiol "primes" both the dopamine reward pathway and the

corticomesolimbic glutamatergic pathway thereby enhancing risk of addiction. The evidence for a telescoping effect with cannabis is also strong considering that it is observed in both treatment and non-treatment seeking populations although its biological basis has not yet been established in preclinical studies. Preclinical findings with cannabinoids do suggest that females have an enhanced sensitivity to their reinforcing effects although it is not yet clear if these differences would translate to a faster course to addiction. Future research is also necessary to determine sex differences in the neurobiological effects of cannabis/cannabinoids since these effects are virtually unexplored in women and female animals.

A telescoping effect is also evident with other addictive drugs including alcohol, opioids, methamphetamine, and tobacco, but in these cases, effects may be restricted treatment populations (e.g., vulnerable individuals that develop a severe SUD). This appears to contrast with effects in preclinical studies with these compounds which indicate an enhanced vulnerability in females for both use and the development of addiction-like features (excessive drug use and a loss of control over drug use under extended-access drug self-administration conditions). Neurobiological differences between males and females would also be presumed to impact psychostimulants and many of these drugs similarly; however, much less is known on sex differences in the neurobiological effects of alcohol, opioids, nicotine, and methamphetamine. Additionally, to date, no studies have examined sex differences in the time-course for the development of addiction-like phenotype with alcohol, opioids, methamphetamine, or tobacco. Such studies are necessary since they will determine whether females are biologically-biased to have an accelerated course to addiction with these drugs. Future epidemiological studies are also needed to determine gender differences in trajectories to addiction using models that control for known differences between men and women with regard to probabilities of drug use, SUD, and seeking treatment for SUD.

Future studies are necessary to identify intervention strategies for women to prevent the development of a SUD. In addition to the obvious need for additional research on hormone-based strategies, medications that target mGlu5 may have therapeutic potential in women considering that mGlu5 likely enhances both initial vulnerability to drug use and the development of addiction in females. mGlu5 is being considered as a therapeutic target for several disorders (addiction, bulimia nervosa, schizophrenia) and compounds are available for use in both humans and animals (e.g., Mihov et al. 2020). mGlu5 was recently shown to be

dysregulated in the striatum of individuals, mainly men (13 of 16), with SUD; normalization of these receptors over a period of protracted abstinence was also associated with decreased craving (Ceccarini et al. 2020). Preclinical studies have also noted sex differences in the effects of Glu5 manipulations on drug-related behaviors, including findings showing that Glu5 antagonism is more effective at decreasing binge alcohol drinking in females than males (Cozzoli et al. 2014). A better understanding of sex differences in the time-course for the disease progression and the underlying mechanisms is critical for the development of sex-specific personalized medicine approaches for the prevention and treatment of SUDs.

Chapter II

Females Develop Features of An Addiction-Like Phenotype Sooner During Withdrawal than Males

1 Introduction

Cocaine use disorder is a chronic, relapsing disease characterized by intense motivation for the drug along with compulsive use that occurs at the expense of other activities/obligations and despite negative consequences. Two million men and women in the United States use cocaine and unfortunately the rate of drug overdose deaths involving cocaine continues to increase being second only to opioids as a cause of overdose deaths (Substance Abuse and Mental Health Services Administration, 2019; Hedegaard et al., 2020). Despite higher rates of cocaine use and addiction among men, an accumulating body of evidence suggests that women are at greater risk than men on many different aspects of the disease process (Center for Substance Abuse Treatment, 2009; Greenfield et al., 2010; Becker and Koob, 2016). One of the most striking examples is "the telescoping effect" where following initial drug use, women meet criteria for substance use disorder and seek treatment for the disorder after fewer years of drug use as compared to men (Anglin et al., 1987; Brady and Randall, 1999; Griffin et al., 1989; Hernandez-Avila et al. 2004; McCance-Katz et al., 1999; Westermeyer et al., 2000). This phenomenon has been reported not only for cocaine but also other stimulants (methamphetamine; Mayo et al., 2019) and drug classes (e.g., alcohol, cannabis, and opioids; Back et al. 2011 a; b; Haas and Peters 2000; Hernandez-Avila et al. 2004; Hser et al. 1987a;b; Sartor et al. 2014). Additionally, once women develop a substance use disorder they have higher levels of drug craving, greater difficulty controlling their drug use, and experience more drug-related medical and psychological complications than men (Robbins et al., 1999; Elman et al., 2001; Center for Substance Abuse Treatment, 2009; Greenfield et al., 2010; Kennedy et al., 2013; Becker and Koob, 2016).

Results from preclinical studies using extended-access (ExA) cocaine self-administration, the "gold standard" for inducing features characteristic of addiction in humans, similarly indicate an enhanced vulnerability in females versus males (Lynch and Carroll, 2000; Lynch and Taylor, 2004; Kawa and Robinson, 2019; Nicolas et al., 2019). For example, female rats given ExA to cocaine (6-24 h/day) self-administer more drug, show greater escalation of drug intake over time, and a greater disruption of diurnal control over drug intake than males (Algallal et al. 2020; Kawa and Robinson, 2019; Lynch and Taylor, 2004; 2005; Smith et al. 2011; Roth and Carroll, 2004), which may indicate a greater vulnerability to transition from controlled to dysregulated drug use in females (Lynch et al., 2000; Becker and Koob, 2016). Even more notable, females develop an enhanced motivation for cocaine, as assessed under a progressive-ratio (PR)

reinforcement schedule, under conditions that do not induce this phenotype in males (e.g., following 7 days of ExA cocaine self-administration and 10 days of withdrawal; Lynch and Taylor, 2004). This phenotype is observed in both females and males when the conditions are optimized by lengthening the period of ExA self-administration (i.e. 10 days) and/or the withdrawal period (i.e. 14 days; Roberts et al., 2007; Ramôa et al., 2013). Together, these findings indicate that, like women, female rats have an enhanced vulnerability to developing key features characteristic of addiction; however, it is not yet known if these features develop faster in females versus males. Thus, the purpose of this study was to determine using a rat model whether there are sex differences in the time-course for the development of two key features of addiction in humans, an enhanced motivation for cocaine and compulsive use.

As with our previous studies, the development an enhanced motivation for cocaine was determined relative to levels of responding under progressive-ratio schedule of reinforcement prior to versus following ExA self-administration and withdrawal (Bakhti-Suroosh et al., 2019; Lynch and Taylor, 2004; also see Ramôa et al., 2013, 2014; Doyle et al., 2014;). We also included an assessment of compulsive use, or use despite negative consequences in this study, given that it is a defining feature of addiction in humans; there may also be gender/sex differences in compulsive use given results showing that women have a greater difficulty controlling drug use and/or a greater likelihood of continued use despite trying not to use (Kenney et al., 2013). Compulsive use was assessed using a histamine punishment procedure wherein histamine, an aversive stimulus that causes a delocalized itching or crawling sensation throughout the body (Lipman and Yosipovitch 2021), is added to the cocaine solution. Previous preclinical studies have shown that adding histamine (4.0 mg/kg/infusion) to the cocaine solution markedly decreases cocaine self-administration under short-access conditions with similar effects observed in females and males (Holtz et al., 2013). Effects were examined in this study following ExA self-administration and withdrawal once responding under the PR reinforcement schedule had stabilized. To our knowledge, sex differences have not yet been examined for measures of compulsive use following ExA self-administration. Based on previous findings indicating that addition-like features develop following ExA self-administration and increase, or "incubate", over withdrawal (e.g., drug-craving, compulsive use; Grimm et al., 2001; Gancarz-Kausch et al., 2014), effects were examined during early withdrawal (7 days), which was expected to be threshold for inducing addition-like features, versus late withdrawal (14 days),

which was expected to be optimal for inducing addition-like features. We also examined effects following protracted withdrawal (60 days) in order to assess the persistency of the behavioral phenotype and determine whether motivation for cocaine, like drug craving and compulsive use, incubates over withdrawal.

Based on reports of a telescoping effect in women, we hypothesized that female rats would develop an enhanced motivation for cocaine sooner during withdrawal than males (at 7 versus 14 days) and show a greater resistance to histamine punishment during early but not late withdrawal. We further predicted that like drug-craving and compulsive use, motivation for cocaine would incubate over withdrawal and remain elevated even after 60 days of withdrawal.

2 Methods and Materials

2.1 Subjects

Subjects were sexually mature female (N = 38) and male (N = 39) Sprague-Dawley rats (Charles River), weighing approximately 270 g (female) and 370 g (male) at the start of the study. Upon arrival, rats were individually housed in operant testing chambers (Med Associates, St. Albans, VT, USA). Rats had ad libitum access to water and food (Teklad LM-485 7912; except as noted below for some animals during cocaine self-administration training) and were maintained on a 12-h light/dark cycle (lights on at 7AM). Following a 2-day habituation period, in order to ensure rapid subsequent acquisition of cocaine self-administration, rats were pre-trained to lever-press for sucrose pellets (45 mg) using methods described previously (fixed-ratio 1, FR1; 24-h/day sessions; >50 sucrose pellets/session for 2 days; Lynch 2008). Rats were weighed three times/week and health was monitored daily. All procedures were conducted within animal care guidelines set by the National Institute of Health and were approved by The University of Virginia Animal Care and Use Committee.

2.2 Procedures

2.2.1 Surgery and Catheter Maintenance

Following lever pre-training, rats were anesthetized with ketamine/dexdomitor and implanted with an indwelling catheter (Silastic tubing; 0.51 and 0.94 mm o.d.; Dow Corning, Midland, MI, USA) into the right jugular vein using methods previously described (Lynch 2008). Catheters were flushed with heparinized saline 3 days/week to verify and help maintain patency. Patency

was also verified periodically by administering methohexital (1.5 mg/kg). If a catheter was no longer patent (i.e., the catheter was leaking, pressure prevented flushing, or the animal did not lose the righting reflex immediately after methohexital), data collected between this assessment and the last patency check were discarded and a new catheter was implanted into the left jugular vein with testing resuming following a 1-2-day recovery period.

2.2.2 Cocaine Self-Administration Training

Rats were initially trained to self-administer cocaine (1.5 mg/kg/infusion) under a FR1 schedule with a maximum of 20 infusions/day using methods previously described (**Figure 1**; Lynch et al., 2010). Acquisition was defined as 2 consecutive days wherein all 20 infusions were obtained. A relatively high dose of cocaine was used to encourage rapid rates of acquisition and moderate food restriction (20 g/day) was used briefly (2-3 days) when necessary (i.e. fewer than 15 infusions/day by training day 5). All groups acquired rapidly under these high dose conditions and rates of acquisition did not differ between groups. Responses on the right (non-active) lever were counted during self-administration sessions as a measure of general activity, but they did not have any programmed consequence.

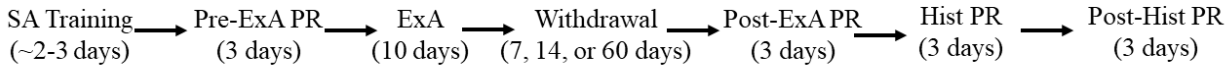

Figure. 1 Summary of experimental events. Female and male rats were trained to self-administer (SA) cocaine. Following acquisition of cocaine SA (2 consecutive sessions wherein all 20 infusions available were obtained), a baseline level of motivation for cocaine SA was established with a progressive-ratio (PR) reinforcement schedule (Pre-ExA PR). After achieving a stable PR baseline, rats were given 24-hr/day, extended access (ExA) to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure for 10 sessions. Following the last ExA session, rats started a 7-, 14-, or 60-day withdrawal period (Withdrawal). Following the withdrawal period, motivation for cocaine SA was retested (Post-ExA PR; also used as the Pre-histamine baseline PR). Then cocaine use despite negative consequences was assessed by addition of noxious agent histamine (4 mg/kg/infusion) to the cocaine solution. A stable PR (Hist PR) was established with the addition of histamine to the cocaine solution and then another stable PR was determined after removal of histamine from the cocaine solution (Post-Hist PR).

2.2.3 Motivation for Cocaine

Following acquisition, motivation for cocaine was assessed using a PR reinforcement schedule (Richardson and Roberts, 1996) wherein the response requirement to obtain a cocaine infusion

increased progressively throughout the session in the following steps: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, etc. Sessions were conducted as previously described (Ramôa et al., 2013; 22-hr/day, but responding typically ceased within 2-4 hours), and ran daily until a stable baseline was achieved (Pre-ExA PR; defined as no increasing or decreasing trend in the number of infusions obtained over 3 consecutive sessions; typically achieved within 3-4 sessions). The moderate dose of cocaine tested (0.5 mg/kg/infusion) has been shown to reveal motivational differences between females and males following ExA self-administration and withdrawal, while producing comparable levels of responding between the sexes at baseline (Ramôa et al., 2013).

2.2.4 ExA Cocaine Self-Administration

After achieving a stable PR baseline, rats were given extended 24-hr/day access to cocaine under a discrete trial procedure using methods previously described (1.5 mg/kg/infusion, 4-discrete trials/hr, 10 days; Ramôa et al., 2013, 2014; also see Fitch and Roberts, 1993). Briefly, 10-minute trials began every 15 minutes (up to 96 infusions/day) with the extension of the active-lever into the chamber; after either 10 minutes or a response on the active-lever the trial was terminated and the lever retracted. These conditions have been shown to induce high levels of cocaine intake and dysregulated patterns of self-administration (Ramôa et al., 2013; Lynch and Taylor 2004). After the last ExA session, responding was again assessed under a FR1 schedule with a maximum of 20 infusions in order to equate levels of cocaine intake between the groups before a 7-, 14-, or 60-day withdrawal period began following the second FR1 session, during which animals remained in their test chambers.

2.2.5 Enhanced Motivation for Cocaine

In order to determine the impact of sex on the development of enhanced motivation for cocaine, motivation for cocaine was reassessed following ExA self-administration and 7- (n=12 females/13 males), 14- (n=12 females/15 males), or 60-days (n=14 females/12 males) of withdrawal. Sessions occurred daily until responding was stable for 3 days (Post-ExA PR; typically, within 3-4 sessions) using the same PR reinforcement schedule and conditions as described above for the Pre-ExA PR baseline. One female and male in the 7-day withdrawal

group and two males from the 14-day withdrawal group were excluded from this analysis (due to either technical or patency issues) since these animals did not have a reliable PR baseline prior to ExA (Pre-ExA PR) to compare to the one obtained following ExA and withdrawal (Post-ExA PR; final n=11 females/12 males, 12 females/13 males, and 14 females/12 males for the 7-, 14-, and 60-day withdrawal groups, respectively).

2.2.6 Compulsive cocaine use

The impact of sex on compulsive cocaine use was determined within each of the withdrawal groups once a stable Post-ExA PR was obtained using a histamine punishment procedure. To do so, histamine, which induces an aversive response, was added to the cocaine solutions. We selected a moderate dose of histamine (4.0 mg/kg), which has previously been shown to similarly reduce cocaine self-administration in female and male rats following short access cocaine self-administration (Holtz et al. 2013). Responding was assessed for a total of 3 consecutive sessions using the same PR reinforcement schedule as described above (Hist PR). Following the third session, the cocaine-histamine solution was replaced with a cocaine-only solution and responding was assessed for an additional 3 days to assess recovery from punishment (Post-Hist PR).

The Post-ExA PR was the same as the Pre-Hist PR for all rats except 2 (one female in the 7-day group and one male in the 60-day group) which lost patency and required a new catheter implantation after the Post-ExA PR was obtained. For these rats, the Pre-Hist PR was obtained following recovery for surgery (their PR values were similar to their original Post-ExA PR and similar to the mean values observed within these groups). Seven rats were excluded from this component of the study due to patency issues (1 female in the 7-day group, 2 males in the 14-day group, and 2 females and 2 males in the 60-day group) and one rat due to illness (female in the 14-day withdrawal group). This analysis also included three rats that had been excluded from the enhanced motivation component of the study (due to issues with the Pre-ExA PR baseline) since we were able to establish a stable Pre-Hist PR following the ExA period (final n=11 females/13 males, 9 females/12 males, and 12 females/10 males for 7-, 14-, and 60-day withdrawal groups, respectively).

2.3 Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse and prepared in sterile saline (7 mg/ml). The mg/kg dose was adjusted for changes in body weight three times a week by adjusting the infusion duration. Histamine dihydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline (58.14 mg/ml) with the cocaine.

2.4 Data Analysis

Differences in cocaine intake over the 10-day ExA period were examined using repeated measure ANOVA with session as the repeated measure and sex and withdrawal group as the between-subject factors. The development of an addiction-like phenotype was defined as an enhanced motivation for cocaine (Pre-ExA PR versus Post-ExA PR) as well as compulsive use despite histamine punishment (Pre-Hist PR versus Hist PR). We also analyzed recovery from histamine punishment (Pre-Hist PR versus Post-Hist PR)). These analyses were conducted using linear mixed effects models and a nested design for session within each phase (the three Pre-ExA PR sessions versus the three Post-ExA PR sessions, the three Pre-Hist PR session versus the three Hist PR sessions, or the three Pre-Hist PR sessions versus the three Post-Hist PR sessions, respectively) and with sex, phase, and withdrawal group as fixed-factors. Session was also initially included as a fixed-factor in each of these analyses, but since no overall or interactive effects of session were observed, this factor was removed. Sex and withdrawal-dependent differences were further examined by comparing changes relative to baseline (percent difference from control) using similar linear mixed effects models with sex and withdrawal group as fixed-factors. In order to determine whether the percent change observed was significantly greater than zero, one sample t-tests were performed using the average of the 3 PR sessions. Pearson correlations were conducted to determine associations between average drug intake during ExA and the average number of infusions obtained during each of the PR testing phases (Pre-ExA, Post-ExA, Pre-Hist, Hist, and Post-Hist PR) as well as associations between average drug intake during ExA and the average percent change during Post-ExA (relative to Pre-ExA), Hist (relative to Pre-Hist), and Post-Hist (relative to Post-Hist). All post-hoc comparisons were corrected for multiple comparisons using the Bonferroni method. Statistical analyses were performed using SPSS (V26). Alpha was set at 0.05. Data are presented as the mean±SEM.

3 Results

3.1 ExA Self-Administration

Females self-administered more cocaine than males over the 10-day ExA period (effect of sex, $F_{1,71} = 16.403$, $P < 0.001$; **Figure 2**). Also, females and males decreased their cocaine intake over the 10 sessions (effect of session, $F_{9,639} = 20.038$, $P < 0.001$) taking significantly less cocaine in sessions 2-10 compared to session 1 (P's<0.05). There were no overall or interactive effects of withdrawal group for intake of cocaine (P>0.05). Thus, while females self-administered more cocaine under ExA conditions compared to males, there were no differences in intake between the different withdrawal groups prior to withdrawal and subsequent motivational testing.

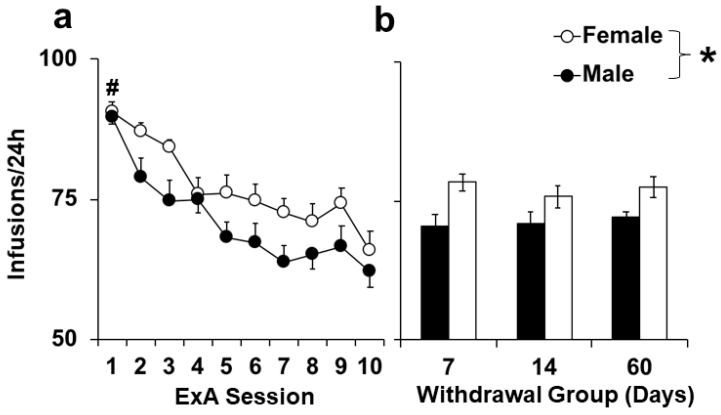

Figure. 2 Effect of sex on cocaine intake in female and male rats given ExA to cocaine under a discrete trial procedure. Mean (±SEM) number of infusions obtained during each of the 10 ExA sessions (**a**) and averaged across all ExA sessions (**b**) for females and males in the 7- (n = 12 and 13, respectively), 14- (n = 12 and 14, respectively), or 60- (n = 14 and 12, respectively) day withdrawal groups. Significantly higher cocaine intake in females compared to males (*). Significantly higher cocaine intake on day 1 compared to days 2-10 (#).

3.2 Incubation of Motivation

As predicted, the number of cocaine infusions obtained under a PR reinforcement schedule was higher Post-ExA versus Pre-ExA, in females versus males, and in groups tested following longer versus shorter periods of withdrawal with results revealing significant overall effects of phase ($F_{1,432} = 94.333$, $P< 0.001$; **Table 1**), sex ($F_{1,432} = 19.289$, $P < 0.001$), and group ($F_{2,432} = 4.290$, $P <0.05$) as well as significant interactions of group by phase ($F_{2,432} = 7.167$, $P<0.01$) and phase by sex ($F_{1,432} = 4.756$, $P < 0.05$). As mentioned above, there were no significant overall or

interactive effects of PR test session indicating that motivation was stable over the three test sessions at both phases (Pre- and Post-ExA PR) of the study. Given the significant interaction of phase and sex, we further analyzed effects within the Pre-ExA PR and Post-ExA PR phases. The analysis of number of infusions obtained within the Pre-ExA PR sessions revealed no significant overall or interactive effects of sex or group ($P>0.05$), indicating that the number of infusions obtained prior to the ExA period was similar between females and males and withdrawal groups. In contrast, analysis of the number of infusions obtained within the Post-ExA PR phase revealed a significant overall effect of group ($F_{2,216} = 12.336$, $P < 0.001$) indicating that the number of infusions obtained was higher following longer periods of withdrawal; post-hoc comparisons of the infusions obtained Post-ExA PR confirmed a significant difference between the 7- and 60-day withdrawal groups ($P<0.001$). Analysis within the Post-ExA PR phase of the study also revealed a significant effect of sex ($F_{1,216} = 23.728$, $P < 0.001$), but a non-significant interaction of sex by group ($P>0.05$), indicating that females had higher motivation for cocaine than males across each of the three-withdrawal time-points. This conclusion is further supported by results within females showing that while the number of infusions obtained increased significantly from Pre-ExA PR to Post-ExA PR (significant effect of phase, $F_{1,216} = 70.165$, $P<0.001$) it did so similarly across each of the withdrawal time-points (non-significant effect of withdrawal group, $P>0.05$). In contrast, the analysis within males revealed a significant overall effect of phase ($F_{1,216} = 28.591$, $P<0.001$) as well as a significant interaction of group and phase ($F_{2,216} = 5.851$, $P < 0.01$), with no difference in the number of infusions obtained between Pre-ExA PR and Post-ExA PR after 7 days of withdrawal ($P>0.05$), but an increase in the number of infusions obtained between Pre-ExA PR and Post-ExA PR after 14 days ($P<0.001$) and 60 days ($P< 0.001$) of withdrawal.

Table 1. Mean (±SEM) number of cocaine infusions obtained under the progressive-ratio reinforcement schedule prior to (Pre-ExA) and following (Post-ExA) extended access cocaine self-administration for females and males in the 7-, 14-, and 60-day withdrawal groups.

Withdrawal Group	7 Day		14 Day		60 Day^	
PR Testing Phase	**Pre-ExA**	**Post-ExA**	**Pre-ExA**	**Post-ExA**	**Pre-ExA**	**Post-ExA**
Female*	13±0.9	16±0.9+	13±0.9	17±0.8+	13±1.1	18±0.6+
Male	13±1.2	13±1.2	12±0.5	15±0.8+	12±0.8	16±0.9+

*Significant sex difference during Post-ExA (**P*<0.001); ^Significant difference between the 60-day versus the 7-day withdrawal group during Post-ExA (*P*<0.001). +Significant difference between Pre-ExA versus Post-ExA in females and males (*P*<0.001).

Similar differences were observed in the analysis of percent change in the number of infusions obtained during Post-ExA PR testing relative to Pre-ExA PR testing (**Figure 3**), with results revealing significant overall effects of sex ($F_{1,216}$ = 9.857, P < 0.01) and group ($F_{2,216}$ = 13.214, P < 0.001), but a non-significant interaction of group by sex (P> 0.05), indicating that females compared to males and groups tested following longer versus shorter periods of withdrawal had a larger increase in number of infusion obtained from Pre-ExA PR to Post-ExA PR. Further analysis of percent change in the number of infusions obtained during Post-ExA PR testing relative to Pre-ExA PR testing confirmed an increase at 60 days of withdrawal versus both 7 and 14 days (P's<0.001), but not at 14 days of withdrawal versus 7 days (P>0.05). Given our predicted hypothesis of a telescoping effect in females, sex differences were further examined within each of the withdrawal groups. These analyses revealed a significant effect of sex within the 7-day withdrawal group ($F_{1,67}$ = 7.286, P<0.05), but not the 14- or 60-day groups (P>0.05) indicating that motivation for cocaine was different between females and males after 7 days of withdrawal, but similar by 14 and 60 days of withdrawal. Similarly, analysis of percent change in the number of infusions obtained from Pre-ExA PR to Post-ExA PR within females revealed a non-significant trend for an effect of group ($F_{2,108}$= 3.589, P =0.062) indicating that motivation for cocaine increased similarly within each of the withdrawal groups. Additionally, post-hoc comparison revealed a significant increase in motivation for cocaine (versus no change, 0) across all the withdrawal groups in females (P<0.001). In contrast, this same analysis in males revealed a significant effect of group ($F_{2,108}$ = 14.453, P <0.001) with post-hoc comparison within each withdrawal group revealing a significant increase in motivation for cocaine (versus

no change, 0) within the 14 (P<0.001) and 60 (P<0.01) day withdrawal groups, but not within the 7-day withdrawal group (P>0.05). Together, these findings indicate that an enhanced motivation for cocaine develops sooner during withdrawal in females than males (7 days versus 14 days) and incubated to higher levels in females versus males. Level of motivation for cocaine also incubated over protracted withdrawal with motivation for cocaine being the highest in the 60-day withdrawal group.

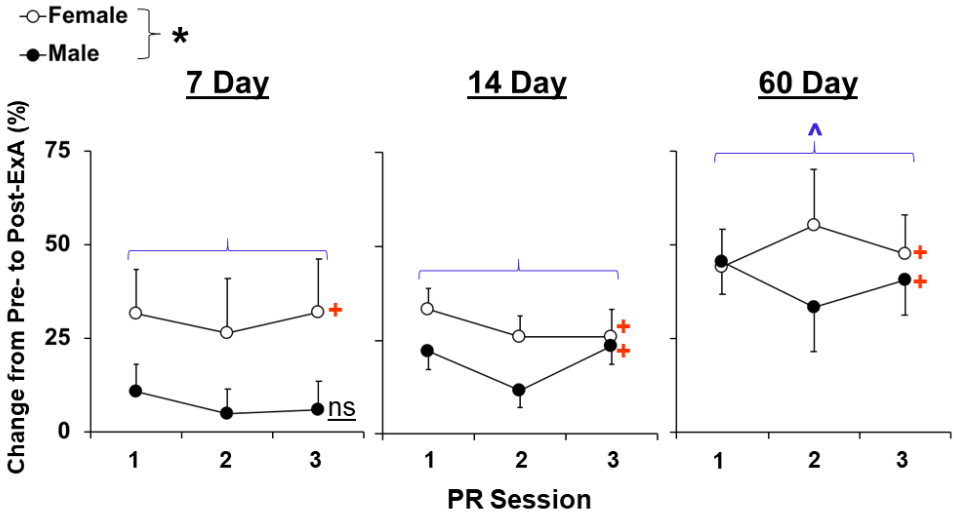

Figure. 3 Effect of sex on the development of enhanced motivation for cocaine. Percent change (±SEM) in the number of infusions obtained following ExA self-administration (Post-ExA) and withdrawal relative to baseline (Pre-ExA) for females and males in the 7- (n = 11 and 12, respectively), 14- (n = 12 and 13, respectively), or 60- (n = 14 and 12, respectively) day withdrawal groups. Significantly larger increase in the number of cocaine infusions obtained following ExA (Post-ExA) relative to baseline (Pre-ExA) in females compared to males (*) and in the 60-day withdrawal group compared to 7- and 14-day withdrawal groups (^). Significant increase (versus no change, 0) in the number of infusions obtained following ExA (Post-ExA) relative to the baseline (Pre-ExA) in females and males (+). ns, nonsignificant.

3.3 Compulsive Cocaine Use

Sex and withdrawal dependent effects were also observed in response to histamine punishment and recovery from histamine punishment (**Table 2**) with results from the overall analysis of PR testing during the three phases (pre-histamine baseline PR, Pre-Hist PR; histamine punishment PR, Hist PR; post-histamine recovery PR, Post-Hist PR) revealing significant overall effects of phase ($F_{3,579}$ = 33.295, P<0.001), sex ($F_{1,579}$ = 40.930, P<0.001), and withdrawal group ($F_{2,579}$ = 10.510, P < 0.001) as well as a trend for a significant interaction of group by sex ($F_{2,579}$ = 2.315,

P =0.10). Linear analysis within each of these phases also revealed significant effects of sex and group within the Pre-Hist PR (sex, $F_{1,195} = 15.753$, $P<0.001$; group, $F_{2,195} = 7.507$, $P<0.01$), Hist PR (sex, $F_{1,195} = 22.176$, $P<0.001$; group, $F_{2,195} = 6.200$, $P<0.01$), and Post-Hist PR (sex, $F_{1,195} = 17.517$, $P<0.001$; group, $F_{2,195} = 3.120$, $P<0.05$) phases. We also observed a trend for a significant interaction of group by sex ($F_{2,195} = 2.323$, $P=0.101$) within the Hist PR phase.

Table 2. Mean (±SEM) number of cocaine infusions obtained under the progressive-ratio reinforcement schedule prior to (Pre-Hist), during (HIST), and following (Post-Hist) histamine punishment for males and females in the 7-, 14-, and 60-day withdrawal groups.

Withdrawal^ Group	7 Day			14 Day			60 Day		
PR Testing+ Phase	**Pre-Hist**	**Hist**	**Post-Hist**	**Pre-Hist**	**Hist**	**Post-Hist**	**Pre-Hist**	**Hist**	**Post-Hist**
Female*	16±1.0	14±1.1	16±1.1	17±1.0	14±0.9	16±0.9	18±0.8	14±1.0	17±0.9
Male	14±1.1	10±1.0	13±1.2	15±0.7	11±0.8	15±0.8	16±1.0	13±0.7	14±0.9

*Significant overall effect of sex ($P<0.001$); ^Significant overall effect of withdrawal group ($P<0.001$); + Significant overall effect of phase ($P<0.001$).

However, given that the sex and withdrawal group differences were apparent during the Pre-Hist PR baseline, effects were further explored as percent change from Pre-Hist PR baseline with separate analyses conducted for the Hist PR and Post-Hist PR phases. Results from the Hist PR phase revealed a significant effect of sex (**Figure 4**; $F_{1,195} = 5.173$, $P<0.05$) and group by sex interaction ($F_{2,195} = 5.331$, $P<0.01$), with females showing less of a response to histamine than males, particularly within the 7-day withdrawal group ($F_{1,70} = 11.243$, $P<0.01$). Indeed, subsequent linear analysis within each of the withdrawal groups revealed a significant effect of sex within the 7-day ($P<0.01$), but not the 14-day or 60-day withdrawal groups (P's>0.05) indicating that males reached the female-level of resistance to histamine punishment after 14 days of withdrawal. This is further supported by results from the analysis within females, which revealed a non-significant effect of group ($P >0.05$) indicating that motivation for cocaine was similarly impacted by histamine punishment across each of the three-withdrawal time-points. Additionally, post-hoc comparison revealed a significant decrease in motivation for cocaine (versus no change, 0) as a result of histamine punishment within females across all withdrawal groups ($P<0.001$). In contrast, this same analysis in males revealed a significant effect of group

($F_{2,102}$ = 5.089, P <0.05) with post-hoc comparison between each withdrawal group revealing a significant reduction in the effect of histamine punishment in the 60-day withdrawal group as compared to the 7-day withdrawal group (P <0.01). Like findings in females, however, post-hoc comparison within males revealed a significant decrease in motivation for cocaine (versus no change, 0) as a result of histamine punishment within each of the withdrawal groups (P<0.01). Together, these findings demonstrate that females have a greater resistance to histamine punishment during early withdrawal, but by 14 days of withdrawal, males reach the female-level of resistance to punishment.

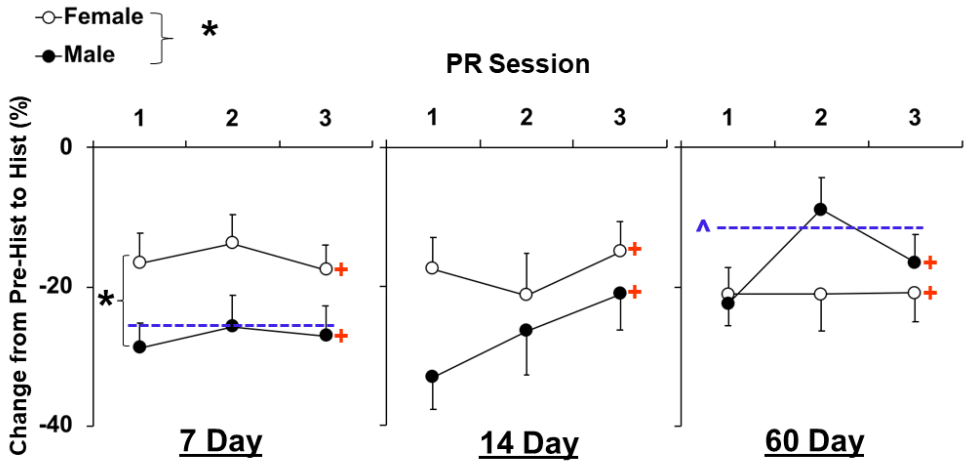

Figure. 4 Effect of sex on the development of compulsive cocaine use. Percent change (±SEM) in the number of infusions obtained during histamine punishment (Hist) relative to the pre-histamine baseline (Pre-Hist) for females and males in the 7- (n = 11 and 13, respectively), 14- (n = 9 and 12, respectively), or 60- (n = 12 and 10, respectively) day withdrawal groups. Significantly larger decrease in the number of cocaine infusions obtained during histamine punishment relative to pre-histamine baseline in males compared to females (*). Significant increase in the number of cocaine infusions obtained during histamine punishment relative to baseline (Pre-Hist) in the 60-day withdrawal group compared to the 7-day withdrawal group in males (^). Significant decrease (versus no change, 0) in the number of infusions obtained during histamine punishment (Hist) relative to the baseline (Pre-Hist) in females and males (+).

3.4 Recovery from Punishment

We also observed withdrawal-dependent changes during recovery from histamine punishment (Post-Hist PR; **Figure 5**). However, unlike the Hist-PR phase, the analysis of effects within the Post-Hist PR phase (relative to the Pre-Hist PR baseline) revealed a significant effect of group

($F_{1,195}$ = 4.220, P<0.05), but non-significant effects of sex (P>0.05) and sex by group (P>0.05) indicating that females and males recovered similarly from the histamine punishment. Subsequent linear analysis within each of the withdrawal groups revealed a significant difference between the 14- and 60-day withdrawal groups (P<0.01), but not the 7- and 14-day withdrawal groups (P>0.05) or 7- and 60-day withdrawal groups (P>0.05). Post-hoc comparison within each withdrawal group revealed a significant decrease (versus no change, 0) in the number of infusions obtained during Post- Hist PR relative to the Pre- Hist PR baseline (prior to histamine) within the 60-day withdrawal group (P <0.05), a trend for a significant difference in the 7-day withdrawal group (P=0.06), but a non-significant difference in the 14-day withdrawal group (P <0.05). Thus, motivation for cocaine recovered similarly in females and males, and rats tested following 60 days of withdrawal showed less recovery from punishment as compared to those tested following 14 days of withdrawal.

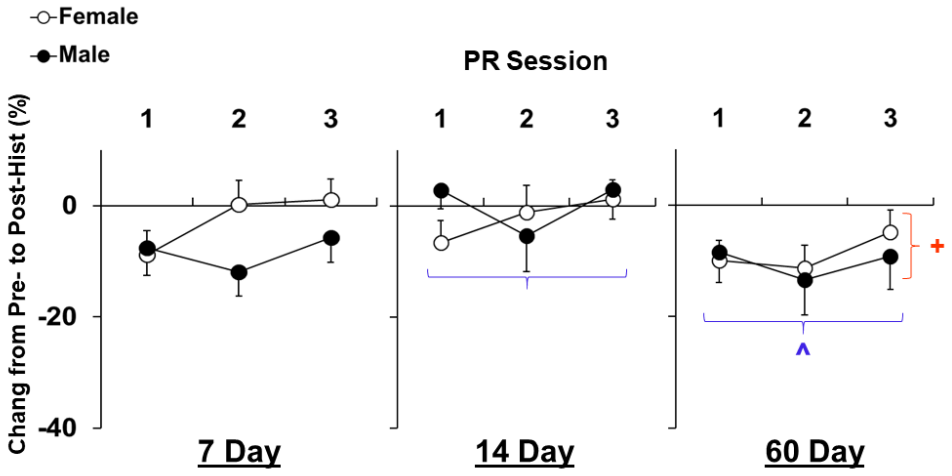

Figure. 5 No effect of sex on recovery from histamine punishment. Percent change (±SEM) in the number of infusions obtained following histamine punishment (Post-Hist) relative to the pre-histamine baseline (Pre-Hist) for females and males in the 7- (n = 11 and 13, respectively), 14- (n = 9 and 12, respectively), or 60- (n = 12 and 10, respectively) day withdrawal groups. Significant decrease in the number of cocaine infusions obtained following histamine punishment relative to baseline (Pre-Hist) in the 60-day withdrawal group compared to the 14-day withdrawal group in males (^). Significant decrease (versus no change, 0) in the number of infusions obtained during recovery from histamine punishment (Post-Hist) relative to baseline (Pre-Hist) in 60-day withdrawal group (+).

3.5 No Associations Between ExA Intake and Development of Addiction-Like Features

Given sex differences in cocaine intake during the 10-day ExA period, we also determined whether ExA cocaine intake was predictive of the development of an enhanced motivation for

cocaine or compulsive cocaine use during the histamine punishment phase (**Figure 6**). While ExA intake was not associated with the development of an enhanced motivation for cocaine (percent increase from Pre-ExA to Post-ExA), resistance to histamine punishment (percent decrease from Pre-Hist to Hist), or recovery from histamine punishment (percent change from Pre-Hist to Post-Hist; data not shown), it was significantly associated with levels of motivation for cocaine (average number of infusions) during each of the phases of the study including Pre-ExA PR testing (r=0.320; P<0.01), Post-ExA PR testing (r=0.389; P=0.001), Pre-Hist PR testing (r=0.320; P<0.01), Hist PR testing (r=0.342; P<0.01), and Post-Hist PR testing (r=0.349; P<0.01; data not shown). Together, these findings indicate that while average ExA intake was predictive of overall level of motivation for cocaine, it was not predictive of the development of enhanced motivation for cocaine or compulsive cocaine use.

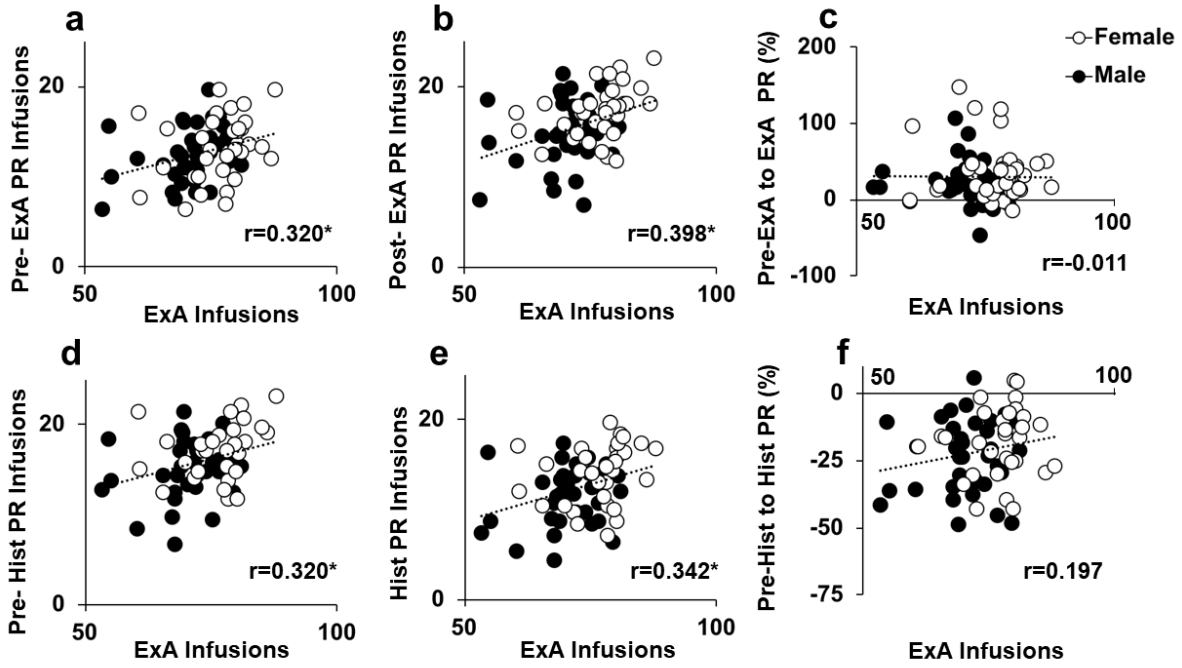

Figure. 6 No associations between ExA intake and development of addiction-like features. While average ExA intake was significantly correlated with motivation for cocaine (number of infusion) prior to ExA (**a**, Pre-ExA PR; n=37 females, n=37 males) and following ExA (**b**, Post-ExA PR; n=37 females, n=37 males), it was not associated with the development of an enhanced motivation for cocaine (**c**, percent change from Pre-ExA to Post-ExA PR; n=37 females, n=37 males). Average ExA intake was also significantly correlated with motivation for cocaine (number of infusion) prior to histamine punishment (**d**, Pre-Hist PR; n=32 females, n=35 males) and during histamine punishment (**e**, Post-Hist PR; n=32 females, n=35 males), but it was not associated with the development of compulsive use of cocaine (**f**, percent change from Pre-Hist to Hist PR; n=32 females, n=35 males). r, Pearson correlation; *Significant association.

4 Discussion

The goal of this study was to determine whether females show an accelerated time-course for the development of two key features of addiction in humans, an enhanced motivation for cocaine and compulsive use, using a rat model. As predicted, females developed an enhanced motivation for cocaine sooner during withdrawal than males (7 vs 14 days). Females also showed greater resistance to histamine punishment than males during early withdrawal (7 days); notably, males reached the female-level of resistance to histamine punishment by late withdrawal (14 days). These sex differences are unlikely to be due to higher cocaine intake in females than males given that no association was observed between intake and the development of an enhanced motivation for cocaine, resistance to histamine punishment, or recovery from histamine punishment. As with previous findings for drug-craving (Grimm et al., 2001), we found that motivation for cocaine incubated over withdrawal following ExA self-administration and was highest in female and male rats tested following 60 days of withdrawal. We also saw incubation of compulsive use, or resistance to histamine punishment, in males; whereas, in females this phenotype was already robustly observed following 7 days of withdrawal and was maintained at a similar level following late and protracted withdrawal. Overall, these findings show that features of the addiction-like phenotype develop sooner during withdrawal in females than males. This study also shows that, like drug-craving and compulsive use, motivation for cocaine incubates over a period of protracted withdrawal.

As predicted, females developed an enhanced motivation for cocaine following ExA sooner during withdrawal compared to males. These results are consistent with our previous findings showing that females develop an enhanced motivation for cocaine under conditions that do not induce this phenotype in males (e.g., following 7 days of ExA cocaine self-administration and 10 days of withdrawal; Lynch and Taylor, 2004). Also consistent with our previous findings (Roberts et al., 2007; Ramôa et al., 2013), we observed this phenotype in both females and males when the conditions were optimized by lengthening the withdrawal period (i.e. the 14-day withdrawal group). Up until now, there were no studies with females and males investigating sex differences in the time-course for the development of an enhanced motivation for cocaine; therefore, it remained unknown whether females developed an enhanced motivation for cocaine following less drug exposure and/or over a shorter time course as compared to males. These

results expand on the previous studies and provide clear support for an accelerated time-course for the development of an addiction-like phenotype in females versus males.

We also expanded the investigation of sex differences in the development of an addiction-like phenotype to include a measure of compulsive use despite negative consequences. We showed that females were more resistant to histamine punishment than males during early withdrawal (7 days). Similar to the development of enhanced motivation, males developed a similar level of resistance to punishment as females following 14 days of withdrawal which was maintained at 60 days of withdrawal. The histamine punishment procedure we used has been shown to decreases cocaine use similarly in females and males under short access conditions, suggesting there is no sex difference in the effect of histamine punishment on cocaine use prior to the development of an addiction-like features (Holtz et al., 2013). To our knowledge, sex differences have not been examined for measures of compulsive use following ExA and during withdrawal when addiction-like features are known to emerge. Thus, our results are the first to show sex differences in the development of compulsive use despite negative consequences with evidence supporting a faster time-course in females.

We also observed withdrawal-dependent differences in recovery from histamine punishment with responding tending to remain decreased from pre-histamine punishment levels in the 7-day withdrawal group, but returning to pre-histamine punishment levels in the 14-day withdrawal group. However, in contrast to effects observed for motivation for cocaine and resistance to punishment, recovery from punishment was optimally observed in the 14-day group, whereas in the 60-day withdrawal group, responding continued to be significantly lower than levels observed prior to histamine punishment. Also, in contrast to effects observed for motivation for cocaine and resistance to punishment, females and males within each of the groups recovered similarly from histamine punishment. These data are consistent with previous findings showing that despite greater effects of electric shock punishment in rats given short versus ExA to cocaine, both groups showed similar recovery of responding for cocaine (Ducret et al., 2016), and together suggest that recovery from punishment may not be a measure of compulsive use. Recovery from punishment may, however, be an important measure of vulnerability to initial cocaine use based on findings under short-access conditions showing that rats with a low saccharin preference took longer to acquire cocaine self-administration and recover from histamine punishment compared to rats with a high saccharin preference. (Carroll et al., 2008;

Holtz et al., 2013). Future studies are necessary to determine the significance of our current findings showing withdrawal-dependent differences in recovery from histamine punishment and to determine if similar effects occur during recovery from other forms of punishment (e.g., electric shock).

Our data also show that motivation for cocaine incubates over prolonged periods of withdrawal with the highest levels observed within the 60-day withdrawal group. These findings are similar to a large body of work on cue-induced drug craving (Grimm et al., 2001; see review, Li et al., 2015) and indicate that a similar phenomenon occurs for both the primary and secondary reinforcing effects of cocaine. As expected and described above, there was a robust sex difference observed in the 7-day withdrawal group with females (and not males) developing an enhanced motivation for cocaine following ExA, which was then maintained at a similar level during late and protracted withdrawal. In contrast, motivation for cocaine was not increased from Pre-ExA levels in males until after 14 days of withdrawal and levels of motivation progressively increased over withdrawal. While these findings seem to suggest that motivation for cocaine incubates in males, but not females, based on our previous work showing that motivation for cocaine is unchanged or even decreased in females and males tested following 1-day of withdrawal (Morgan et al., 2002a; Lynch and Taylor, 2005), we argue that incubation of motivation for cocaine occurs in both sexes, but in females, the phenotype is already maximally expressed following 7 days of withdrawal.

To our surprise, motivation for cocaine was higher in females than males following ExA self-administration and withdrawal. This is surprising because we had previously showed that motivation is similar between the sexes following ExA self-administration and 14 days of withdrawal (Ramôa et al., 2013; Doyle et al., 2014). However, our previous studies focused on effects at just one withdrawal time-point (14 days) and thus, just one group of males versus females tested following ExA self-administration. In this study, the sample size was approximately three times higher than our previous studies since effects were examined at three withdrawal time-points following ExA self-administration. As such, we believe that the overall effect of sex observed during Post-ExA PR testing is fairly modest. This idea is also consistent with our current and previous findings showing that males and females tested following ExA self-administration and 14 or more days of withdrawal show a similar increase in motivation for cocaine (Ramôa et al., 2013; Doyle et al., 2014). We also observed here that females and males

are similarly resistant to histamine punishment when they are tested following ExA self-administration and 14 or 60 days of withdrawal. Together, these findings suggest that once the addiction-like phenotype develops, females and males are fairly similarly motivated to obtain cocaine and show similar levels of compulsive use. Similar conclusions have also been made for cue-induced cocaine craving (Lynch et al. 2005; Doyle et al. 2014; Peterson et al. 2014; Sanchez et al. 2014, but see Reichel et al. 2012).

To our knowledge, the incubation of compulsive cocaine use following ExA has been examined in only one previous study, which established continued cocaine use despite histamine punishment as a model of compulsive drug use in rodents (Gancarz-Kausch et al., 2014). This study focused on behavior in males only and showed that they become more resistant to histamine punishment over withdrawal with rats tested following 30 days of withdrawal obtaining more cocaine/histamine infusions under a fixed-ratio schedule as compared to those tested following 1-day of withdrawal (Gancarz-Kausch et al., 2014). Here, we observed a similar effect in males with results showing that histamine was less effective at decreasing PR responding for cocaine in males tested following 60 days of withdrawal as compared to males tested following 7 days of withdrawal. In contrast, females in the 7-, 14-, and 60-day withdrawal groups showed roughly the same response to histamine. Thus, the sex difference in the time-course for incubation of compulsive use despite negative consequences appears to be similar to the sex difference observed for the incubation of motivation for cocaine. However, since no previous studies have examined sex differences in compulsive use at earlier time-points during withdrawal, it is not yet known if compulsive use increases from a lower level in females such that, like motivation for cocaine, by 7 days of withdrawal, compulsive use is already maximally expressed in females. Future research that includes an earlier time-point during withdrawal will be necessary to determine whether compulsive use incubates in females.

In summary, we showed that female rats develop key features characteristic of addiction sooner during withdrawal than male rats. These findings provide further support for the idea that the telescoping effect observed in women (Anglin et al., 1987; Brady and Randall, 1999; Griffin et al., 1989; Hernandez-Avila et al. 2004; McCance-Katz et al., 1999; Westermeyer et al., 2000) has a biological basis. These parallel findings also support the utility of our self-administration procedure as model of the telescoping effect. Future work will need to determine whether this model of the telescoping effect can be used to study other drug classes known to be associated

with the telescoping effect in humans. Additionally, this model may be useful for identifying mechanisms underlying the faster time-course in females, including the role of ovarian hormones, as well as interventions to prevent its occurrence. Lastly, the incubation phenomenon appears to occur for a multitude of key features characteristic of addiction including craving, compulsive use and now motivation to obtain the drug. Future research is needed to determine the underlying molecular changes driving the incubation of these behavioral phenotypes as they could provide targets for novel sex-specific treatment methods for cocaine use disorder. This is of importance because prolonged withdrawal periods appear to be risk factor for cocaine-related overdose deaths (Binswanger et al. 2012; Binswanger et al. 2007; Seymour et al. 2000).

Chapter III

Sex Differences in the Neuroadaptations Associated with Incubated Cocaine-Craving: A Focus on the Dorsomedial Prefrontal Cortex

1 Introduction

Overdose deaths involving cocaine have been steadily increasing since 2014 (CDC WONDER, 2022). Despite men having higher rates of cocaine use disorder than women (SAMHSA, 2020), woman are more vulnerable to many aspects of the disease. For example, women have a shorter time period from initial cocaine use to meeting the criteria for cocaine use disorder and/or seeking treatment for the disorder as compared to men (Griffin et al., 1989; McCance-Katz et al., 1999; White et al., 1996; Sofuoglu et al., 1999; O'Brien and Anthony, 2005; Hernandez-Avila et al., 2004; Haas and Peters, 2000). This phenomenon has been termed the "telescoping effect" and is a consistent effect that has been reported across multiple drug classes including psychostimulants, such as cocaine and methamphetamine (Griffin et al., 1989; McCance-Katz et al., 1999; White et al., 1996; Sofuoglu et al., 1999; O'Brien and Anthony, 2005; Hernandez-Avila et al., 2004; Haas and Peters, 2000; Brecht et al., 2004) as well as other addictive drugs, including alcohol, opioid, tobacco, and cannabis (Hernandez-Alvila et al., 2004; Lewis et al., 2014; Peltier et al., 2021; Anglin et al., 1987; Adelson et al., 2018; Hser et al., 1987a,b; Back et al., 2011a,b; DiFranza et al., 2002; Hernandez-Avila et al., 2004; Lewis and Nixon, 2014; Khan et al., 2013; Ehlers et al., 2010; Haas and Peters, 2000) and non-pharmacological addictions, such as gambling (Tavares et al., 2003; Ladd and Petry, 2002; Ibanez et al., 2003; Grant et al., 2012). Women with a cocaine use disorder also experience more drug-related medical and psychological complications, report greater stress-induced cravings, and longer periods of use after relapse compared to their male counterparts (White et al., 1996; Luchansky et al., 2000; Gallop et al., 2007; Center for Substance Abuse Treatment, 2009; Greenfield et al., 2010; Potenza et al., 2012; Becker and Koob, 2016).

While this increased risk in women may be due to socio-cultural factors (Becker et al., 2016), results from preclinical studies indicate that biological factors also contribute given that female animals develop addiction-like features more readily than male animals (Kerstetter et al., 2012; Kawa and Robinson, 2019; Perry et al., 2013b, 2015; Lynch and Taylor, 2004; Towers et al., 2021a). For example, female rats self-administer higher levels of cocaine, show greater escalation of cocaine intake over time, and have a more significant disruption of diurnal control over drug intake than males under extended-access cocaine self-administration conditions, which supports an enhanced vulnerability to transition from controlled to dysregulated cocaine use in females (Algallal et al. 2020; Kawa and Robinson 2019; Lynch and Taylor 2004; 2005; Smith

et al. 2011; Roth and Carroll 2004). Additionally, our more recent study showed that two addiction-like features, an enhanced motivation for cocaine and compulsive cocaine use despite negative consequences, developed sooner during withdrawal from extended-access cocaine self-administration in females compared to males (following 7 versus 14 days; Towers et al., 2021a). Our previous study and work from others has also shown that these addiction-like features increase, rather than decrease, in magnitude over withdrawal (Towers et al., 2021a; Gancarz-Kausch et al., 2014). This incubation effect has been extensively described for cue-induced cocaine-craving, which increases progressively, or incubates, over protracted withdrawal in both humans and animals tested following extended-access drug self-administration (Grimm et al., 2001; Li et al., 2015). This incubation effect also occurs in both males and females (Nicolas et al., 2022) and is particularly robust in females tested during estrus (versus non-estrus phases and males; Corbett et al., 2021; Nicolas et al., 2019). However, it remains unknown whether there are sex differences in the time-course for the incubation of cue-induced cocaine-craving.

The molecular mechanisms underlying incubated cocaine-craving are also largely unknown in females since the vast majority of the work has been conducted in male animals only. Determining sex differences in the underlying neuroadaptations of key features of cocaine use disorder, such as the incubation of cocaine-craving, will be imperative as the effort to develop the first FDA-approved treatment for cocaine use disorder continues. While not yet examined, glutamatergic signaling is a strong candidate as a mechanism underlying the telescoping effect given the critical role this pathway plays in mediating drug-craving/relapse in both humans and animal models (Rebec and Sun, 2005; Kalivas and Volkow 2011; Goldstein and Volkow 2011; Szumlinski and Shin 2018). Findings from male animals further indicates a causal role for glutamate receptor signaling in the dorsomedial prefrontal cortex (dmPFC) in drug-craving and its incubation over withdrawal (McClure et al. 2014; Szumlinski and Shin 2018). Specifically, glutamatergic signaling in this pathway changes dramatically during withdrawal, from hypoglutamatergic during early withdrawal, when levels of drug-craving are low, to hyperglutamatergic during late withdrawal, when craving has increased to high levels (after 7 or more days; Barry and McGinty, 2017; Ben-Shahar et al. 2009, 2012, 2013; Caffino et al. 2020; Chen et al. 2013; Funk et al. 2016; Hearing et al. 2018; Koob and Volkow, 2016; Roura-Martínez et al. 2020; Siemsen et al. 2019; Sun et al. 2014; Szumlinski and Shin 2018). NMDA receptors have been shown to be critically involved in the early-withdrawal molecular

cascade that triggers the incubation of craving (Barry and McGinty, 2017), as well as the enhanced cue-induced craving following late withdrawal (Szumlinski et al. 2018; Barry and McGinty, 2017; Chen et al. 2013). Brain derived neurotropic factor (BDNF) signaling, which requires coincident activation by glutamate and dopamine, also increases in the dmPFC following relapse testing (Peterson et. al., 2014b, Abel et. al., 2019) and clinical studies have similarly shown elevated BDNF serum levels are predictive of cocaine craving and vulnerability to relapse in abstinent cocaine-dependent individuals (D'Sa et al. 2011; Corominas-Roso et al. 2013). Although glutamatergic regulation of cocaine-craving has not yet been examined in females, recent findings with methamphetamine show that extended-access self-administration differently impacts excitatory signaling in the PFC of females versus males (Pena-Bravo et al. 2019). Further support is provided by data showing that estradiol induces glutamate release (Le Saux ert al., 2006), mediates synaptic plasticity in brain through a glutamate-dependent mechanism (Peterson et al. 2014a), and earlier data showing sex-specific differences in NMDA receptor functioning (Cyr et al. 2001).

The purpose of the present study was to expand on our previous study on the telescoping effect and determine whether cocaine-craving, as assessed using an extinction/cue-induced reinstatement procedure, also incubates sooner during withdrawal in females than males. We also determined whether the molecular mechanisms associated with incubated cocaine-craving differed between males and females and from early to later periods of withdrawal focusing on markers known to mediate cue-induced cocaine-craving in males (brain-derived neurotrophic factor exon-IV, *Bdnf-IV*, and NMDA receptor subunit-related gene expression, *Grin1*, *Grin2a*, *Grin2b*, in the dorsomedial prefrontal cortex, dmPFC; Peterson et. al., 2014b, Abel et. al., 2019; Szumlinski et al. 2016). Based on previous findings indicating that addiction-like features, including cocaine-craving, develop following extended-access self-administration and increase, or incubate, over withdrawal (Towers et al., 2021a; Gancarz-Kaush et al., 2014; Grimm et al., 2001), effects were examined during early withdrawal (following 2 days; W2), which was expected to be sub-threshold for inducing an addiction-like phenotype, intermediate withdrawal (following 7 days; W7), which was expected to be threshold for inducing an addiction-like phenotype, and late withdrawal (following 14 days; W14), which was expected to be optimal for inducing an addiction-like phenotype. Given our previous findings (Towers et al., 2021a) and reports of a telescoping effect in women, we hypothesized that cocaine-craving (extinction and

cue-induced reinstatement) would incubate to high levels sooner during withdrawal in females versus males. Additionally, we predicted that BDNF and NMDA receptor gene expression in the dmPFC would correspond to withdrawal- and sex-dependent differences in cocaine-craving.

2 Methods and Materials

2.1 Subjects

Sexually mature female (N=52) and male (N=41) Sprague-Dawley rats (Charles River) were used as subjects in this study. Rats were approximately 11-weeks-old and weighed roughly 260 g (females) and 380 g (males) at the start of the study. Upon arrival at the facility, rats were individually housed in operant conditioning chambers (Med Associates Inc., St. Albans, VT) with ad libitum access to water and food (Teklad LM-485 7912; except as noted below for some animals during cocaine self-administration training) and maintained on a 12-h light/dark cycle (lights on at 7AM). Over the course of the study, the rats were weighed three times a week and their health were monitored daily. All procedures were conducted within animal care guidelines set by the National Institution of Health and were approved by The University of Virginia Animal Care and Use Committee.

2.2 Procedures

2.2.1 Lever Pre-training

Following a 2-day habituation period to the operant chamber, rats were pre-trained to lever press for sucrose pellets (45 mg) in order to ensure rapid subsequent acquisition of cocaine self-administration using methods previously described (Lynch, 2008; fixed-ratio 1 access, 24-hr/day). Briefly, pellets were available 24hr/day under a fixed ratio 1 (FR1) schedule; no stimulus was paired with sucrose pellet delivery. Acquisition was defined as 2 consecutive days wherein >50 pellets were obtained. Rates of acquisition of lever-pretraining did not differ between males and females (days ± SEM, 2 ± 0.06 versus 2 ± 0.11, respectively).

2.2.2 Surgery and Catheter Maintenance

After lever pre-training, an indwelling catheter (Silastic tubing; 0.51 and 0.94 mm o.d.; Dow Corning, Midland, MI, USA) was implanted into the right jugular vein of the rats using methods previously described (Lynch 2008). Catheter patency was maintained and verified throughout the study by flushing with heparinized saline 3 days/week. If the patency of a catheter was

questionable, it was verified by administering sodium brevital (1.5 mg/kg). Any catheter that was no longer patent (i.e., the catheter was leaking, pressure prevented flushing, or the animal did not lose the righting reflex immediately after sodium brevital) was replaced by a new catheter that was implanted into the left jugular vein with testing resuming following recovery from surgery (1-2-days). Data collected between this assessment and the last patency check were discarded.

2.2.3 Cocaine Self-Administration Training

Following surgery, rats were trained to self-administer cocaine (**Figure 1A**; 1.5 mg/kg/infusion, a relatively high dose shown to encourage rapid rats of acquisition) under a FR1 schedule with a maximum of 20 infusions/day using methods previously described (Lynch, et al. 2010). Each session began with the introduction of the active lever (cocaine-associated lever) into the left side of the operant chamber and remained extended until all 20 infusions were obtained or until the session ended at 11:50 AM the next day. Responses on the active lever were reinforced with an infusion of cocaine under a fixed-ratio 1 schedule; infusions were paired with the sound of the infusion pump located inside the sound attenuated chamber and a stimulus light located above the active lever. The active lever retracted following the 20th infusion (or at 11:50AM) and remained retracted until the next session. Responses on the non-active (right) lever were counted during the session to measure general activity, but did not have any programmed consequence. Acquisition was defined as two consecutive days wherein 20 infusions were obtained. Moderate food restriction (85% of its free-feeding body weight, roughly 20g/day and 15g/day for males and females, respectively) was used briefly (2-3 days) when necessary (i.e. fewer than 15 infusions/day by training day 5; one male in the W2 group, one male and one female in the W7 group, and two males in the W14 group). All groups acquired cocaine self-administration rapidly under these high dose conditions (typically within 2-3 days) and rates of acquisition did not differ between males and females or withdrawal groups.

2.2.4 Extended-Access Cocaine Self-Administration and Withdrawal

Once rats acquired cocaine self-administration, they were given 24-h/day, intermittent-access to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure using methods previously described (Ramôa et al., 2013, 2014). Briefly, there were 4, 10-minute discrete trials each hour that began every 15 minutes (up to 96 infusions/day) with the extension of the active-lever into the

chamber. Cocaine was available during each of the trials under a fixed-ratio 1 schedule; each infusion was paired with the sound of the infusion pump and a stimulus light located above the active lever. Trials terminated, and the lever retracted, after an infusion was obtained or after 10 min. The inactive (right) lever remained extended the entire duration of the session and responses on the lever were recorded but had no consequence. Sessions ran continuously for 10 days. These extended-, intermittent-access conditions have been shown to induce 'binge/abstinent' patterns of intake and lead to the development an addiction-like phenotype when assessed following protracted withdrawal (Towers et al., 2021a; Ramôa et al., 2013). After the last extended-access session, cocaine was again available for two additional sessions under fixed-ratio 1 session (up to 20 infusions) in order to equate intake between the groups prior to withdrawal. Based on our previous findings showing that females develop an addiction-like phenotype sooner during withdrawal than males (7 versus 14 days; Towers et al., 2021a), we assigned males and females randomly to one of three different withdrawal conditions: early (2 days; n=14 and 10, respectively), intermediate (7 days; n=14 and 10, respectively), or late (14 days; n=14 and 11, respectively) withdrawal. The withdrawal period began following the second FR1 session. Rats remained in their operant chambers during withdrawal. Additional male and female rats were also randomly assigned to an early (n=4 and 5, respectively), intermediate (n=4 and 4, respectively), or late (n=6 and 2, respectively) withdrawal groups; they underwent the same procedures as those described for the rats given extended-access to cocaine except they had access to saline instead of cocaine.

2.2.5 Extinction/Reinstatement Testing

Extinction/reinstatement testing was conducted in two consecutive sessions after 2, 7, or 14 days of withdrawal. First, extinction responding was examined in a minimum of 6, 1-hr sessions using methods previously described (Sanchez et al. 2014; Peterson et al. 2014b; Beiter et al. 2016). These sessions started between 9 and 10:00 AM with the re-introduction of the active-lever into the operant chamber; responses on this lever and the inactive lever were recorded but had no programmed consequence. Sessions continued until responding on the active lever met an extinction criterion of less than 15 responses/hr. This criterion was typically met within 6 sessions and all rats extinguished within 8 sessions. Cue-induced reinstatement responding was assessed the following day in a 1-hr session. This session also started between 9 and 10:00 AM

with the re-introduction of the active lever into the operant chamber as well as a presentation of the cues formerly associated with cocaine (sound of infusion pump and the light above the active-lever; roughly 3-5 seconds based on body weight). Each response on the left-lever produced these same cues. Female rats were also swabbed 3 days prior to the extinction and reinstatement test days and on the behavioral test days as described previously (Lynch et al. 2019) to determine the phase of the estrous cycle. Male rats underwent similar handling. Unfortunately, the phase of the estrous cycle could not be determined because the slides were contaminated during staining and unable to be interpreted. Therefore, the phase of the estrous cycle was not included in the data analysis. Additionally, one female in the early withdrawal group, one female in the intermediate withdrawal group and one female and one male in the late withdrawal group were excluded from the study and all analyses due to patency issues during extended-access self-administration or technical issues during withdrawal/relapse testing. The final group size for females and males in the cocaine groups was 13 and 10 for the early withdrawal group, 13 and 10 for the intermediate withdrawal group, and 12 and 10 for the late withdrawal group. For the saline groups, since there were no behavioral differences between rats tested following early, intermediate, or late withdrawal, data were collapsed into one group (n=14 females and 11 males).

2.2.6 Gene Expression

Immediately following the reinstatement session, rats were anesthetized using isoflurane and then euthanized by rapid decapitation. Tissue from the dmPFC was rapidly dissected from ice-chilled 2-mm-thick coronal brain slices based on boundaries defined previously (**Figure 1B**; coordinates: Bregma 3.2 mm; Paxinos and Watson, 2007). The dmPFC, which includes the prelimbic region and anterior cingulate, was selected since this region is known to be critical for the incubation of cocaine-craving in males (Ishikawa et al. 2008; Kalivas and McFarland 2003; Shin et al. 2018). The tissue was immediately frozen using liquid nitrogen and stored at -80°C until further processing.

RNA extraction, cDNA transcription, and RT-qPCR were performed using methods previously described (Smith et al. 2018). Briefly, total RNA was isolated using a RNeasy (R) Lipid Tissue Mini Kit (Qiagen, Valencia, CA) and the quantity and quality was determined using a NanoDrop^{TM} Spectrophotometer. cDNA templates were prepared using High-Capacity cDNA

Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Carlsbad, CA). RT-qPCR was preformed using the Applied Biosystem StepOnePlus™ real-time PCR system and Applied Biosystems TaqMan™ Gene Expression assays using oligonucleotide primers chosen from prior publications. We analyzed the relative expression *of Bdnf-IV*, *Grin1*, *Grin2a*, and *Grin2b* (**Supplemental Table 1**). One housekeeping gene (*Gapdh* for Bdnf-associated genes or *B2m* for glutamate genes) was used for normalization. Since multiple qPCR plates were required for a plate study analysis for each gene, males and females, matched for group, were run on the same plate to eliminate potential sex differences due to qPCR plate run. A small number of samples were outliers and excluded based on the Grubb's test, which included 8 of the 320 samples. The final group sizes of females and males for *Bdnf-IV* were 13 and 9 for the early withdrawal group, 12 and 10 for the intermediate withdrawal group, 12 and 10 for the late withdrawal group, and 14 and 11 for the saline controls, for *Grin1* were 13 and 10 for the early withdrawal group, 13 and 10 for the intermediate withdrawal group, and 12 and 10 for the late withdrawal group and 14 and 10 for the saline controls, for *Grin2a* were 13 and 9 for the early withdrawal group, 13 and 9 for the intermediate withdrawal group, and 12 and 9 for the late withdrawal group and 14 and 11 for the saline controls, and for *Grin2b* were 13 and 9 for the early withdrawal group, 13 and 10 for the intermediate withdrawal group, and 12 and 9 for the late withdrawal group and 14 and 11 for the saline controls.

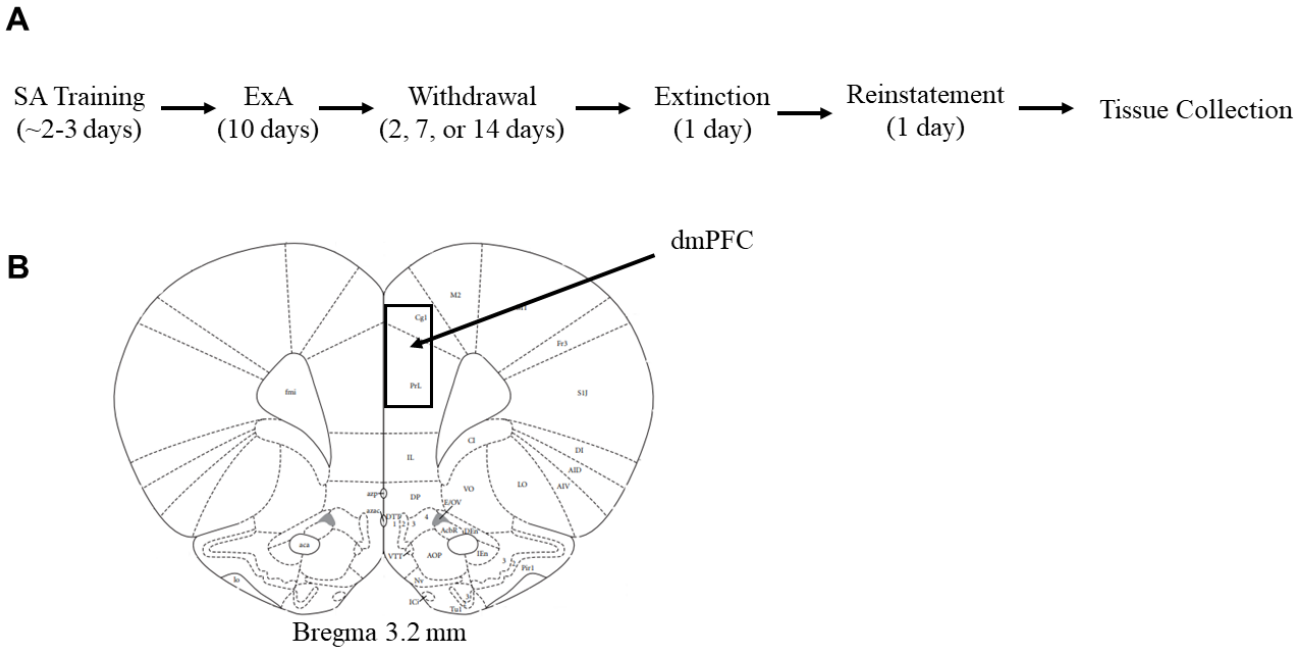

Figure 1. Summary of experimental events and brain region dissected for the gene expression analyses. (**A**) Timeline of experimental events: Following self-administration training (SA), rats were given 24-h/day extended (ExA) access to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure for 10 days (4 trials/hr). After the last cocaine self-administration session, rats were assigned to an early (n = 13 females,10 males), intermediate (n = 13 females,10 males), or late (n = 13 females,10 males) withdrawal groups. Additional rats were given access to saline and assigned to an early (n = 4 females, 5 males), intermediate (n = 4 females, 4 males), or late (n = 6 females, 2 males) withdrawal groups. Following the appropriate days of withdrawal, rats underwent extinction testing in a minimum of 6, 1-hr sessions and then the next day reinstatement testing in a 1-hr session. Tissue was collected immediately after the reinstatement session. (**B**) Schematic illustration of the brain region dissected for the dmPFC.

2.3 Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC) and dissolved in 0.9% sterile saline (7 mg/ml), filtered (0.22μm; Millipore, Billerica, MA), and stored at 4°C. The infusion duration was adjusted three times/week based on body weight to maintain a constant mg/kg dose (2 s/100 g).

2.4 Data Analysis

Data were first examined to verify that the assumptions of parametric analyses were met (i.e., normal distribution, equal variances). Sex differences were examined using both between sex

analyses, to address quantitative differences, and using separate analyses within each sex, to address qualitative sex differences. This approach is analogous to the one we used recently to determine sex differences in the development of other addiction-like features (Towers et al., 2021a) and has been recommended by other experts in the addiction field for detecting quantitative versus qualitative sex differences (Beltz et al., 2019). Specifically, quantitative differences in cocaine intake over the 10-day extended-access period were addressed using a repeated measures ANOVA with session as the repeated measure and sex and withdrawal group as the between-subject factors. Post hoc comparisons of the first and later sessions (averaged across sessions 2-10) were made using the paired t test. Sex and withdrawal group were also included as between subject factors in the analyses of total extinction responses (univariant ANOVA), extinction responses during the first six extinction sessions run (repeated measures ANOVA), and responses during the last extinction session versus the reinstatement session (repeated measures ANOVA). Post hoc comparison of the first extinction session versus later sessions (averaged across sessions 2-6) and of the last extinction session versus the reinstatement session were made using the paired t-test. Withdrawal-dependent changes in extinction and reinstatement responding were also examined within females and males separately to address expected qualitative differences (e.g., ovarian hormones contribute to cocaine-craving in females, but not males; Jackson et al., 2006; Becker, 2009). Pearson correlations were also conducted to determine associations between average cocaine intake during extended-access and the total number of extinction and cue-induced reinstatement responses.

Similar analyses were used to determine quantitative and qualitative sex differences in gene expression with separate univariate ANOVA analyses conducted for each gene. Sex and withdrawal group differences were determined using percent difference from saline controls. Changes from baseline/saline expression levels were determined using a one-sample t-test (versus no change), and one-tailed t-tests were used for all *a priori* predicted hypotheses (i.e., higher cocaine-craving and *Bdnf-IV* and *Grin1* expression in the W7 and W14 withdrawal groups compared to the W2 withdrawal group*;* Abel et al., 2019). Partial eta squared (η_P^2) was used as a measure of effect size and Tukey correction was used to control for multiple comparisons. Statistical analyses were performed using SPSS (V26).

3 Results

3.1 Behavioral Results

3.1.1 Extended-Access Self-Administration. During the 10-day extended-access period, female rats self-administered more cocaine than males (**Figure 2A-B**; effect of sex, $F_{1,62}$=15.16, P<0.001). However, there were no overall or interactive effects of group (P>0.05) indicating that levels of cocaine intake were similar between groups within females and males prior to withdrawal. Patterns of intake were also similar between the groups (group by session, P>0.05) and sexes (sex by session, P>0.05) with males and females in each of the groups having the highest intake during the first session (effect of session, $F_{9,558}$=8.07, P<0.001; session 1 versus average intake of sessions 2-10, t(92)=8.56, P<0.05). Thus, while females had higher cocaine intake during the extended-access phase, there were no significant group differences in levels or patterns of cocaine intake within males or females prior to withdrawal and subsequent extinction/reinstatement testing.

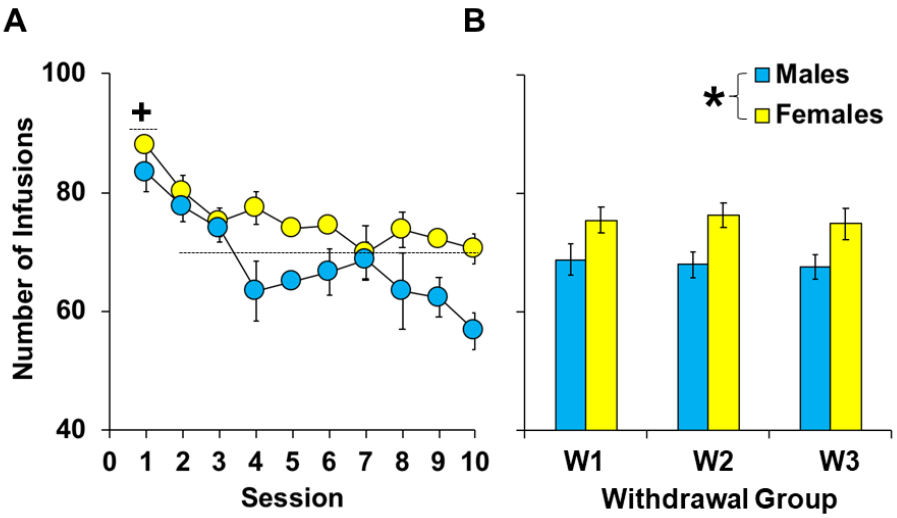

Figure. 2 Effect of sex on cocaine intake during 10 extended-access session under discrete trial procedure. Mean (±SEM) number of infusions obtained during each of the ten extended-access sessions for (**A**) males, (**B**) females, and (**C**) average across all extended-access sessions in the early (W2; n= 13 females,10 males), intermediate (W7; n= 13 females,10 males), and late (W14; n= 13 females,10 males) withdrawal groups. *Significantly higher cocaine intake in females compared to males. +Significantly higher cocaine intake during the first extended-access session compared to later ones (averaged across sessions 2-10) for both sexes.

3.1.2 Extinction. As predicted, cocaine-craving was significantly affected by length of withdrawal (effect of group, $F_{2,62}$=4.5, P<0.05) and the highest levels of extinction responding were observed in the late withdrawal group (versus the early withdrawal group, 170.2±17.0

versus 95.5±21.4, respectively; $P<0.05$). Although no overall or interactive effects of sex were observed (P's> 0.05, **Supplemental Figure 1A**), planned analyses within males and females separately indicate that the withdrawal-dependent increase in extinction responding was driven primarily by effects in males (**Figure 3A-D**). Specifically, within males there was a significant effect of withdrawal group for both hourly extinction responses (**Figure 3A;** $F_{2,27}=7.6$, $P<0.01$; η_P^2, 0.36) and total extinction responses across all extinction sessions run (**Figure 3B;** $F_{2,27}=7.6$, $P<0.01$; η_P^2, 0.36). Post-hoc comparison also confirmed higher total extinction responses in the intermediate and late withdrawal groups versus the early withdrawal group (P's<0.01). The analysis of hourly extinction responding in males also revealed a significant effect of session ($F_{5,135}=40.2$, $P<0.001$), which reflects higher responding in the first session versus later ones (session 1 versus average of sessions 2-6, $t(29)= 7.70$, $P<0.001$), as well as an interaction of group by session ($F_{10,135}=3.4$, $P<0.001$) which reflects a group difference ($F_{2,27}=6.8$, $P<0.01$) and higher responding in the intermediate and late withdrawal groups as compared to the early withdrawal group (P's<0.05) during the first extinction session, but not later ones (P's>0.05). In contrast, within females, the effect of group was non-significant for both hourly extinction responses (**Figure 3C;** $F_{2,35}=1.7$, $P>0.05$) and total extinction responses across all extinction sessions run (**Figure 3D;** $F_{2,35}=1.8$, $P>0.05$) indicting that extinction responding was similarly increased at early versus later withdrawal time-points. However, similar to males, the analysis of hourly extinction response within females revealed a significant effect of session ($F_{5,175}=35.34$, P<0.001), which reflects higher responding in the first session versus later ones (sessions 2-6, $t(37)= 9.45$, $P<0.001$), as well as an interaction of group by session ($F_{10,175}=4.63$, $P<0.001$) which reflects a group difference ($F_{2,35}=5.1$, $P<0.05$) and higher responding in the late withdrawal group compared to the early withdrawal group ($P<0.05$) during the first extinction session. For both males and females, responding decreased to similarly low levels in each of the groups by session 6 (effect of group, *P's*>0.05). These findings indicate that in males, extinction responding increases over withdrawal and peaks during intermediate withdrawal (i.e., following 7 days of withdrawal). While a similar effect also occurred in females for responding during the first extinction session, total extinction responses were already elevated in females during early withdrawal (i.e., following 2 days of withdrawal) and did not further increase over withdrawal.

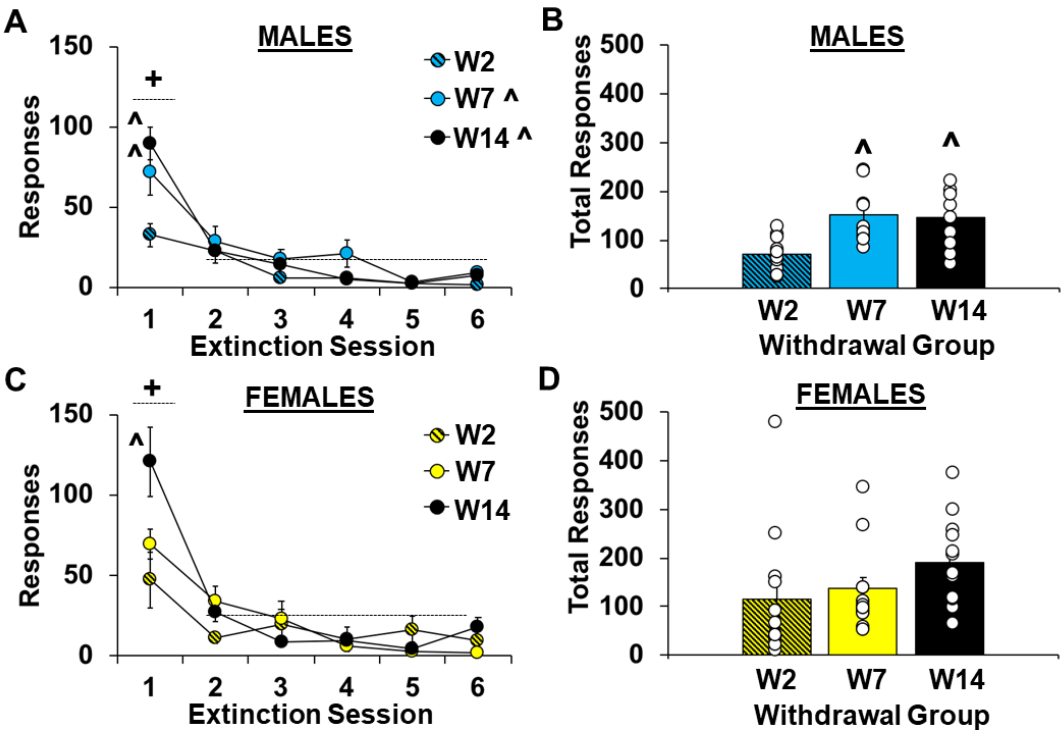

Figure. 3. Sex- and withdrawal-specific changes in extinction responding. Mean (±SEM) number of responses on the lever formerly associated with cocaine during the first six 1-h extinction sessions (**A, C**) and across all extinction sessions completed (**B, D**) for females (**C, D**) and males (**A, B**) in the early (W2; n= 13 females,10 males), intermediate (W7; n= 13 females,10 males), and late (W14; n= 13 females,10 males) withdrawal groups. White circles indicate individual data points. +Significantly higher responding in the first extinction session versus later ones (averaged across sessions 2-6). ^Significantly higher than the early withdrawal group (overall and/or during the first extinction session).

3.1.3 Reinstatement. As with extinction responding, cue-induced reinstatement of cocaine-craving was significantly affected by the length of withdrawal (effect of group, $F_{2,62}$=4.6, P<0.05) and the highest levels of responding were observed in the intermediate and late withdrawal groups (versus the early withdrawal group; 80.3±17.6 and 86.5±8.8 versus 38.0±6.2, respectively; P's<0.05). Although no overall or interactive effects of sex were observed in the overall analysis **(Supplemental Figure 1B)**, planned analyses within males and females separately indicate that, like extinction responding, withdrawal-dependent increases in reinstatement responding were driven primarily by effects in males (**Figure 4A**). Specifically, for males responding was significantly reinstated by the cues in each of the withdrawal groups (versus the last extinction session; effect of session, $F_{1,27}$= 102.4, P<0.001; within the early, t(9)= -4.40, P<0.001, intermediate, t(12)= -5.95, P<0.001, and late, t(12)= -5.55, P<0.001, withdrawal

groups) and this reinstatement effect was significantly affected by length of withdrawal (effect of group, $F_{2,27}$= 6.4, P<0.01; session by group interaction $F_{2,27}$= 5.9, P<0.01). Although no group difference was observed within the last extinction session (P<0.05), there was a significant effect of group within the reinstatement session ($F_{2,27}$= 6.1, P<0.01; η_P^2, 0.13) with subsequent post-hoc comparisons revealing a significant difference between the early and the late (P<0.01) withdrawal groups. In contrast, the same analysis within females revealed a significant effect of session (**Figure 4B**; $F_{1,35}$= 40.5, P<0.001), but non-significant trends for group ($F_{1,35}$= 2.47, P=0.10) and session by group ($F_{1,35}$= 2.63, P=0.09). Post-hoc analyses also confirmed non-significant group differences within both the last extinction session (P>0.05) and the reinstatement session (P>0.05). Together, these findings indicate that in males, cue-induced cocaine-craving progressively increases from early to late withdrawal (following 2 versus 14 days) whereas in females, cocaine-craving is already elevated during early withdrawal (following 2 days) and does not further increase over withdrawal.

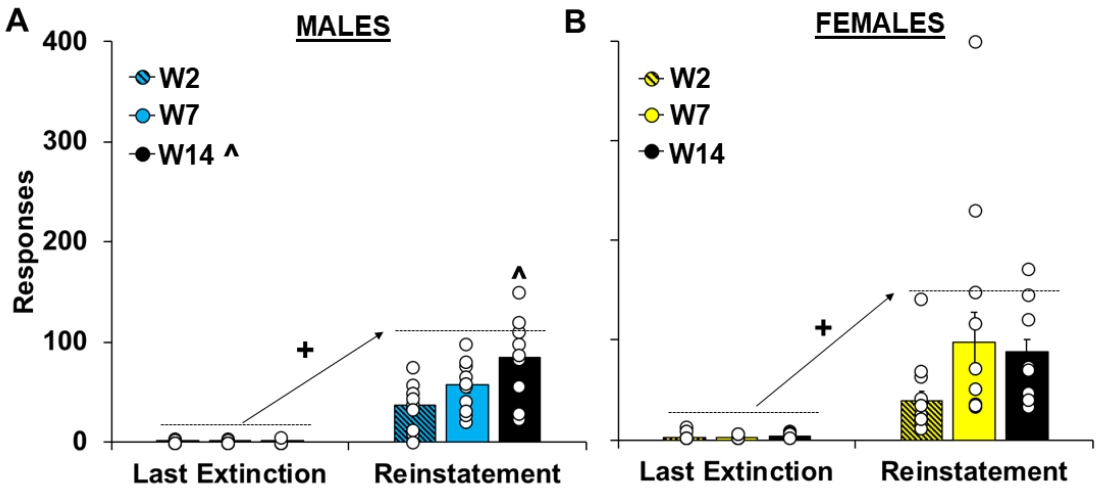

Figure. 4 Sex- and withdrawal-specific changes in cue-induced reinstatement responding. Mean (±SEM) number of responses made on the lever formerly associated with cocaine during the last extinction session versus the reinstatement session for (**A**) males and (**B**) females the early (W2; n= 13 females,10 males), intermediate (W7; n= 13 females,10 males), and late (W14; n= 13 females, 10 males) withdrawal groups. White circles indicate individual data points. +Significant increase from the last extinction session. ^Significantly higher than the early withdrawal group.

Given the sex difference in cocaine intake during the extended-access period, we ran correlational analyses to determine whether cocaine intake was predictive of later cocaine-craving during extinction and reinstatement testing (total responses for each; data not shown). These associations were non-significant for both extinction (r=0.04) and cue-induced reinstatement (r=0.13) in the overall sample and within females (r= -0.08 and 0.11, respectively) and males (r= 0.10 and 0.04, respectively) separately. These findings indicate that prior cocaine intake was not predictive of later cocaine-craving.

3.2 Molecular Results

3.2.1 Bdnf-IV Gene Expression. Sex- and withdrawal-dependent effects were observed for *Bdnf-IV* expression within the dmPFC (**Figure 5A**) with results from the analysis of percent difference from saline controls, which normalizes baseline sex differences, revealing that males had a larger increase than females in *Bndf-IV* expression (effect of sex, $F_{1,60}$= 27.7, P<0.001; η_P^2, 0.30). Effects were most pronounced in males in the early withdrawal group (effect of group, $F_{2,60}$= 6.9, P<0.01; early withdrawal group versus 0, P<0.05), and while the interaction of sex and withdrawal group was not statistically significant (P>0.05), results from the planned analysis within each sex confirmed an effect of withdrawal group within males ($F_{2,4.4}$=4.4, P<0.05). Within males, the increase in *Bdnf-IV* expression was also significantly greater than zero for both the early and late withdrawal groups (P<0.05's). In contrast, the effect of withdrawal group was not significant in females (P>0.05), and collapsed across withdrawal groups, expression levels were not significantly different from zero (P>0.05). Thus, *Bdnf-IV* expression was increased following relapse testing in males, but not females, and while effects were most pronounced following testing during early withdrawal, expression remained elevated persistently (i.e., following relapse testing and 14 days of withdrawal).

3.2.2 Grin1 Gene Expression. Sex- and withdrawal-dependent effects were observed for *Grin1* expression within the dmPFC (**Figure 5B**) with results from the analysis of percent difference from saline controls revealing non-significant overall effects of sex and withdrawal group but a trend for a significant interaction of sex by withdrawal group (**Figure 5D**; $F_{2,62}$=3.1, P=0.053; η_P^2, 0.09). Further analysis within each sex revealed non-significant effects of withdrawal group for both males and females (P's>0.05) although within females there was a trend for increased

Grin1 expression (>0, relative to saline controls, P=0.06) which appears to be driven by effects within the intermediate withdrawal group given that expression was significantly higher than saline controls in the intermediate withdrawal group (P<0.05), but not in the early or late groups. The same analysis within males showed that *Grin1* expression was also increased relative to saline controls, but in contrast to effects in females, this occurred in the late withdrawal group in males (P<0.05), not the intermediate group (or early group). Thus, females appear to have higher *Grin1* expression following relapse testing during intermediate withdrawal, while, males showed an increase in *Grin1* expression during late withdrawal.

3.2.3 Grin2a Gene Expression. Sex-dependent effects were observed for *Grin2a* expression within the dmPFC (**Figure 5C**) with results from the analysis of percent difference from saline controls revealing a significant overall effect of sex ($F_{1,59}$=10.8, P<0.01; η_P^2, 0.15) indicating that males show a greater decrease in *Grin2a* expression than females following relapse testing (P<0.01). Further analysis in males revealed a non-significant effect of withdrawal group (P>0.05) and a significant difference from zero for percent of control expression when collapsed across withdrawal groups (P<0.001), indicating that the decrease in *Grin2a* expression was consistent across each of the withdrawal time-points. In females, the overall effect of withdrawal group was also non-significant (P>0.05), but in contrast to males, in females, percent of control *Grin2a* expression was not significantly different from zero (P>0.05). Thus, *Grin2a* expression was decreased following relapse testing during early, intermediate, and late withdrawal in males but was unchanged at these withdrawal time-points in females.

3.2.4 Grin2b Gene Expression. Sex- and withdrawal-dependent effects were observed for *Grin2b* expression within the dmPFC (**Figure 5D**) with results from the analysis of percent difference from saline controls revealing a significant effect of sex ($F_{1,60}$=4.4, P<0.05; η_P^2, 0.07) indicating that males had a greater decrease in *Grin2b* expression (relative to saline controls) than females following relapse testing. There was also a significant effect of withdrawal group ($F_{2,60}$=3.2, P<0.05) that again appears to be driven by changes in males given that follow-up analysis within each sex revealed a significant effect of withdrawal group for males ($F_{2,25=}$5.2, P<0.05), but not females (P>0.05). Further analysis within males also showed that the decrease in *Grin2b* expression (relative to saline controls) was significantly lower than zero for both the early

(P<0.01) and late (P<0.05) withdrawal groups. Within females, the change in percent of control *Grin2b* expression was not significantly different from zero (P>0.05). Thus, *Grin2b* expression in the dmPFC decreased following relapse testing in males, but not females, and while effects were most pronounced following testing during early withdrawal, modest decreases were also observed during late withdrawal.

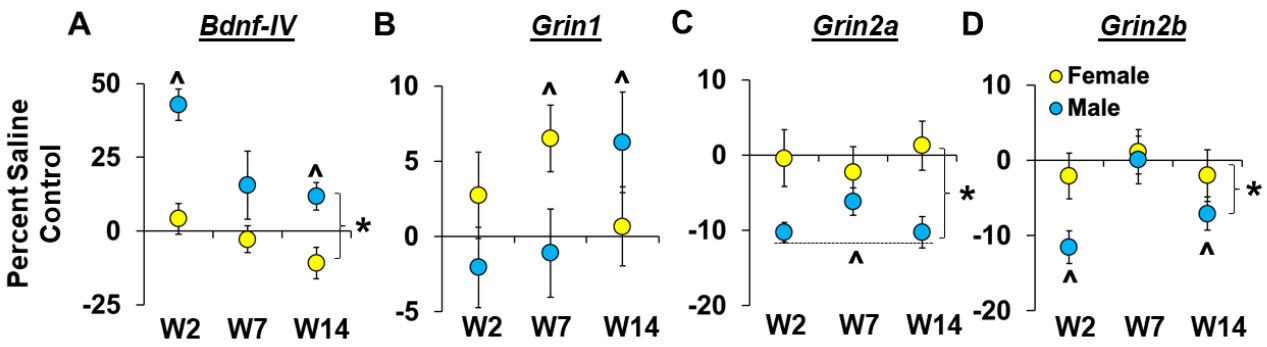

Figure. 5 Sex- and withdrawal-specific changes in *Bdnf-IV*, *Grin1*, *Grin2a*, and *Grin2b* expression in the dmPFC. Mean (±SEM) percent difference from saline controls for *Bdnf-IV* (**A**), *Grin1* (**B**), *Grin2a* (**C**), *Grin2b* (**D**) in the dmPFC for males and females in the early (W2; n = 13 females, 10 males), intermediate (W7; n = 13 females,10 males), and late (W14; n = 13 females,10 males) withdrawal groups. *Significant difference between females and males. ^Significant difference from zero in females and males.

4 Discussion

Women have a shorter duration of cocaine use prior to developing problematic cocaine use and show an enhanced vulnerability to cocaine-related medical consequences including a shorter time interval between onset of cocaine use and a fatal outcome compared to men (White et al., 1996; Sofuoglu et al., 1999; O'Brien and Anthony, 2005; Haas and Peters, 2000; Origer et al., 2014; see Agabio et al., 2016 for review). These sex difference appear to be driven by biological differences considering our previous findings showing that a parallel phenomenon also occurs in female rats with the development of two key features of cocaine use disorder, an enhanced motivation for cocaine and compulsive cocaine use despite negative consequences (Towers et al., 2021a). Here, we expanded on the investigation of the telescoping effect by determining whether the time-course for the incubation of cocaine-craving, another key feature of cocaine use disorder in humans, also develops sooner during withdrawal in females than males. We found that in males, cocaine-craving (total extinction and cue-induced reinstatement responding) progressively

increased from early to later periods of withdrawal whereas in females, cocaine-craving was already elevated during early withdrawal (following 2 days of withdrawal) and did not further increase over withdrawal (i.e., non-significant effect of withdrawal group). While these findings are consistent with our hypothesis for a faster course for the incubation of cocaine-craving in females, this effect was modest considering it was only apparent in the sex-specific time-course analyses (i.e., there were no overall or interactive effects of sex on levels of cocaine-craving). Cocaine-craving during the first hour of extinction testing also similarly increased from early to later periods of withdrawal in both males and females. Despite these modest sex differences in behavior, we observed marked sex differences in the molecular adaptations associated with the incubation of cocaine-craving. Specifically, we showed that *Bdnf-IV*, *Grin2a*, *Grin2b*, and *Grin1* gene expression changed in response to withdrawal and relapse testing in males, while females only showed a modest increase in the expression of *Grin1* at the intermediate withdrawal time-point. Together, these findings provide evidence for a modestly faster time-course for the development of incubated cocaine-craving in females versus males, and indicate that the underlying molecular adaptations differ in females versus males. Each of these molecular findings are discussed further below.

A large body of work conducted in males has shown that cue-induced drug-craving increases, or incubates, from early (days 1-2) to later periods of withdrawal (days 14-60; Grimm et al., 2001; Li et al., 2015). A similar effect has been confirmed to occur in females with findings showing that males and females have similarly low levels of cue-induced cocaine-craving during early withdrawal (on withdrawal day 1); high levels of cue-induced cocaine-craving are reported in males and females during later withdrawal time-points (following 14 and 48 days of withdrawal), with the highest levels observed in females tested during estrus (versus males and females tested during non-estrus phases; Nicolas et al., 2019; Corbett et al., 2019). Our current results build on this body of literature and show that there is also a modest sex difference on the ascending limb of the incubation of cocaine-craving curve, with elevated cue-induced cocaine-craving already evident in females during early withdrawal (following 2 days of withdrawal). This conclusion is also consistent with findings from Nicolas and colleagues (2019) showing that in females, levels of cue-induced cocaine-craving during late withdrawal (day 29) was not significantly higher than those observed during early withdrawal (day 2) indicating that levels of cocaine-craving were already elevated in females during early withdrawal. However, as

with our effects here, the effect in this previous study was modest and observed in only one of the two cohorts tested suggesting that the 2-day withdrawal time-point is a threshold condition for females to start expressing high levels of cocaine-craving. This idea is also consistent with findings showing that cocaine-craving is low in both males and females immediately following extended-access self-administration (day 1 of withdrawal), but then increases in both sexes by withdrawal day 15 (Corbett et al., 2021). It is also consistent with our findings showing that motivation for cocaine, as assessed under a progressive-ratio schedule, is either unchanged or decreased from baseline in females and males tested immediately following extended-access self-administration (day 1 of withdrawal), but then increases in both males and females tested following 14 or more days of withdrawal (Lynch and Taylor, 2005; Towers et al., 2021a). Together, these findings suggest that cocaine-craving incubates over withdrawal in both sexes, but that in females, incubation occurs sooner such that by withdrawal day 3, cocaine-craving is already increased from low levels. Future studies are necessary to confirm this possibility.

Our findings on incubation of cocaine-craving are also consistent with previous results from our group and others showing that females develop other features of an addiction-like phenotype, including an enhanced motivation for cocaine, compulsive cocaine use despite punishment, and cocaine preference over other non-drug rewards, sooner during withdrawal or after less drug exposure than males (Kawa et al. 2019; Lynch and Taylor 2004; Towers et al., 2021a; Kerstetter et al., 2012; Perry et al., 2013b; Perry et al., 2015; Lynch and Taylor, 2004). This effect with cocaine-craving does appear to be less robust as compared to effects reported for the development of other addiction-like features. For example, with the development of an enhanced motivation for the cocaine, females, but not males, develop this phenotype under threshold conditions (following extended-access self-administration and 7 days of withdrawal), with females responding at roughly 25% higher levels than males to obtain infusions of cocaine. In contrast, the effect here with cocaine-craving was observed for total extinction and cue-induced reinstatement responding, but not for the first hour of extinction responding which increased progressively in both sexes from early to later periods of withdrawal.

These sex differences in the incubation of cocaine-craving do not appear to be driven by females having great cocaine intake during the extended-access period considering that cocaine intake was not predictive of subsequent cocaine-craving, which is consistent with our previous findings for the development of enhanced motivation for cocaine and compulsive cocaine use

despite negative consequences (Towers et al., 2021a). This idea is also supported by findings from the two previous studies on sex differences in cue-induced cocaine-craving (Corbett et al., 2021; Nicolas et al., 2019) which showed that females had higher levels of cue-induced craving during late withdrawal despite self-administering similar levels of cocaine during the extended-access phase. It is notable that intake did not differ between the sexes in the previous studies given that these studies also used extended-access conditions (i.e., 6-hr/day with the long-access procedure, Corbett et al., 2021; 8-hr/day using a different intermittent-access procedure, Nicolas et al., 2019). Both studies also used low to moderate doses of cocaine (i.e., 0.5 or 0.75 mg/kg/infusion) which are typically more sensitive to individual differences, such as sex, than high doses like the one used here (1.5 mg/kg). None-the-less, we have consistently observed higher intake in females than males under these extended-access conditions (e.g. Towers et al., 2021a; Doyle et al., 2014; Peterson et al., 2014b) suggesting that our procedure is sensitive to sex differences in intake.

One limitation to our findings is that we were not able to consider the impact of the estrous cycle on the time-course for the incubation of cocaine-craving in females. Estrous cycle phase is known to impact drug-craving during later (day 15 and 48), but not early (day 1) withdrawal time-points with estrus females having higher levels of cocaine-craving than males, but no difference between non-estrus females and males (Corbett et al., 2021; Nicolas et al., 2019). Considering that overall levels of craving were similar between males and females in the current study, it is likely that the majority of our females were tested during a non-estrus phase of their cycle. However, future studies are necessary to determine whether the phase of the estrous cycle impacts cocaine-craving following 2 days of withdrawal and whether ovarian hormones contribute to the modestly accelerated time-course for the incubation of cocaine craving in females. This question is important considering evidence indicating that estradiol underlies the accelerated course in females for the development of other key addiction-like features, such as an enhanced motivation for cocaine (Ramôa et al., 2013; 2014; Bakhti-Suroosh et al., 2019).

Our molecular findings are suggestive of an earlier recruitment of glutamatergic signaling in the dmPFC of females than males, which is critical for the incubation of cocaine-craving in both humans and animal models (Rebec and Sun, 2005; Kalivas and Volkow 2011; Goldstein and Volkow 2011; Szumlinski and Shin 2018). Specially, *Grin1*, the gene that encodes the ubiquitous subunit of NMDA receptor (i.e. GluN1), was increased sooner in withdrawal in

females than males (during intermediate versus late withdrawal). While this effect was modest and only apparent in the within-sex analyses, it does mirror our behavioral findings of an accelerated course to incubated craving in females; however, the molecular shift is delayed compared to the behavior, which is likely the result of the early withdrawal timepoint being threshold for females to start expressing high levels of cocaine-craving. This idea is further supported by findings in males where total extinction response reaches peak-levels following 7 days of withdrawal, but the molecular shift in *Grin1* does not occur until after 14 days of withdrawal. Our findings are also consistent with previous findings with methamphetamine showing that a molecular shift toward increased NMDA receptor currents in the PFC occurs after less extended-access self-administration in females than males (Mishra et al., 2017; Pena-Bravo et al., 2019). These previous studies further showed that the increase in NMDA receptor currents that developed following withdrawal from extended-access methamphetamine self-administration was likely due to changes in GluN2B signaling in the PFC males, whereas, it was not affected by GluN2B antagonism in the PFC in females (Mishra et al., 2017; Pena-Bravo et al., 2019). These findings are similar to our observations here that *Grin2b*, the gene that encodes GluN2B subunit of the NMDA receptor, was decreased in males during both early and late withdrawal, but unchanged across withdrawal in females. These findings are intriguing because they suggest that some of the molecular adaptations associated with development of substance use disorder may be accelerated (*Grin1*), while others are qualitatively different in females compared to males (*Grin2a* and *Grin2b*).

Although *Bdnf-IV*, *Grin2a*, and *Grin2b* expression did not change in response to withdrawal and relapse testing in females, our molecular results in males are consistent with previous findings indicating that in males, BDNF and NMDA receptor subunits play a critical role in the incubation of cocaine-craving. More specifically, for *Bdnf-IV*, we observed increase expression in the dmPFC of males following relapse testing during early and late withdrawal. These findings are consistent with our previous findings from two separate studies showing that *Bdnf-IV* expression in the dmPFC is elevated in males following withdrawal from extended-access cocaine self-administration and cue-induced relapse testing (day 15; Peterson et. al., 2014b, Abel et. al., 2019). We also previously showed that exercise (i.e. wheel running) during withdrawal dose dependently attenuated both relapse responding and *Bdnf-IV* expression (Peterson et. al., 2014b), which provides further support for dmPFC BDNF-IV contributing to

the incubation of cocaine-craving/relapse vulnerability in males. It is notable that the greatest increase in *Bdnf-IV* expression observed in males in the current study was following relapse testing during early withdrawal, when levels of cocaine-craving were low. This is surprising considering that *Bdnf-IV* expression presumably increases progressively over withdrawal. Our current findings suggest that this is not the case; this idea is also consistent with clinical findings in individuals with cocaine use disorder showing that high levels of serum BDNF during early withdrawal were predictive of early relapse following discharge from an inpatient abstinence program (within ~4 days; Corominas-Roso, 2015). Individuals with high levels of BDNF during early withdrawal also did not show withdrawal-dependent increases in serum BDNF (Corominas-Roso, 2015) which is in contrast to individuals with lower levels of serum BDNF during early withdrawal who did show an increase serum levels of BNDF over withdrawal (Corominas-Roso, 2015). Together, these findings suggest that increased Bdnf-IV expression in the dmPFC can precede the development of incubated cocaine-craving; BDNF is also likely a clinically relevant marker for early relapse. However, considering that the clinical data were obtained from a predominantly male sample (only 2 of the 40 patients were women; Corominas-Roso et al. 2015), along with our current findings showing that *Bdnf-IV* expression did not change in response to withdrawal and relapse testing in females, it is possible that findings with BDNF apply to males, but not females. Future research is necessary to address this possibility.

Similarly, our findings in males for NMDA receptor subunit expression are consistent with a large body of work indicating that NMDA receptors are causally involved in the incubation of cocaine-craving in males (Szumlinski and Shin, 2017). Here, we confirmed that expression levels of *Grin1*, *Grin2a*, and *Grin2b* are low following cue-induced relapse testing during early withdrawal (after 2 days), when cocaine-craving was the lowest in males (also see Barry and McGinty, 2017; Ben-Shahar et al., 2009; Szumlinski et al., 2016), and that expression of *Grin1* was increased following cue-induced relapse testing during late withdrawal (after 14 days), when cocaine-craving was the highest in males (also see Abel et al. 2019). These findings are also consistent with the pathophysiology of substance use disorder in humans, which is associated with increased levels of GluN1 expression, the protein encoded by *Grin1* (Daneshparvar et al. 2019; Enoch et al. 2014). However, it is surprising that *Grin2b* expression levels continued to be decreased during late withdrawal considering findings showing that GluN2B protein levels in the dmPFC become increased in response to drug-associated cues as

early as day 3 of withdrawal and remain increased up to 30 days of withdrawal (Szumlinski et al., 2016). The reason for the discrepancy between *Grin2b* mRNA expression and the protein it encodes, GluN2B, is unknown but may be the result of procedural differences, or more likely, a difference in gene versus protein expression. GluN2A protein levels also increase from low levels within the first week of withdrawal from extended-access self-administration, but do not become elevated until 60 days of withdrawal (Ben-Shahar et al., 2009; Szumlinski et al., 2016). A limitation to this study is that we only investigated differences at the mRNA level; thus, future studies will need to determine differences at the protein level.

In conclusion, our findings indicate that the incubation of cocaine-craving develops more rapidly in females than males, although this effect is modest and overall levels of cocaine-craving were similar between the sexes. Despite the modest sex difference in behavior, there were marked differences between males and females in the molecular adaptations known to mediate cocaine-craving in males, and some of these differences (i.e., *Grin1*) parallel the behavioral differences. These findings highlight a need for further research on sex differences in mechanism underlying cocaine use disorder as this information is needed to develop sex-specific prevention and treatment strategies.

5 Supplemental Material

Table 1. Rat RT-qPCR Primers

Gene ID	Gene Name	Forward primer nucleotide sequence (5'-3')	Reverse primer nucleotide sequence (5'-3')	Reference
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	GTGGACCTCATGGCCTACAT	TGTGAGGGAGATGCTCAGTG	Koo *et al.* 2015
B2m	Beta-2 microglobulin	CGAGACCGATGTATATGCTTGC	GTCCAGATGATTCAGAGCTCCA	Walder *et al* 2014
Bdnf-IV	Brain-Derived Neurotrophic Factor exon IV	TTCCACTATCAATAATTTAACTTCTTTGC	CTCTTACTATATATTTCCCCTTCTCTTCAGT	Schmidt *et al.* 2012
Grin1	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 1	CACAGGAGCGGGTAAACAACA	TGAGTAGCTCGCCCATCATTC	Wingo *et al.* 2016
Grin2a	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 2a	GCATCTGCCACAACGAGAAG	CCCGCCATGTTATCGATGTC	Wingo *et al.* 2016
Grin2b	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 2b	CTGTCCGCCTAGAGGTTTGG	TGCGCTGGGCTTCATCTT	Wingo *et al.* 2016

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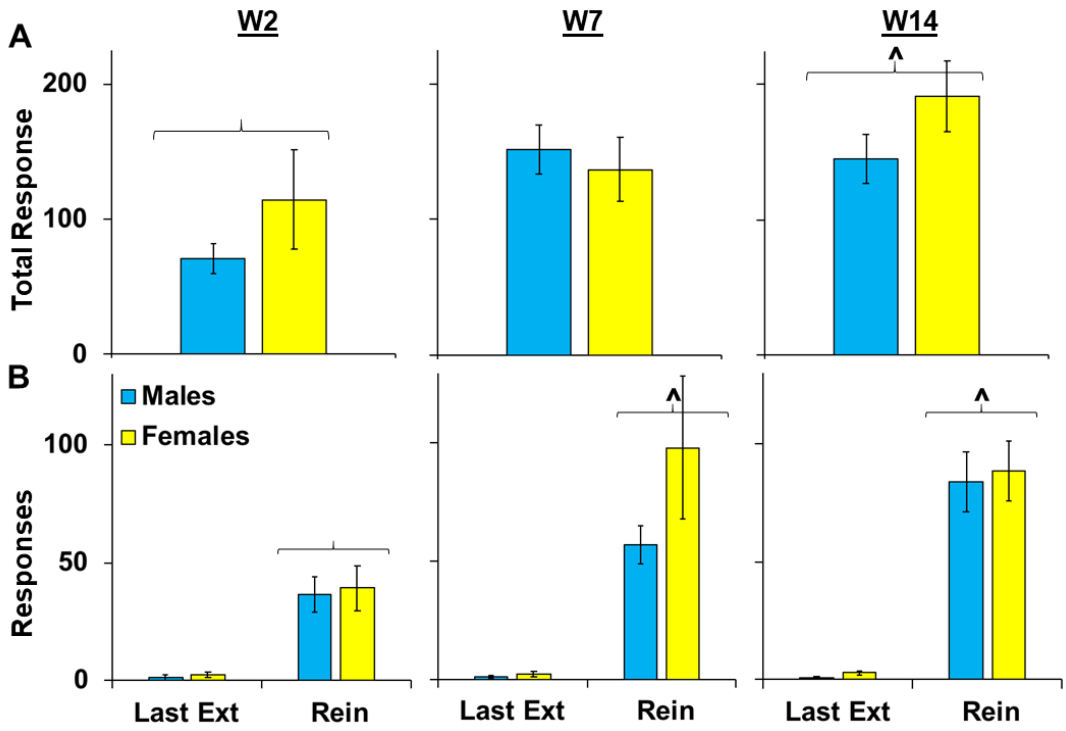

Supplemental Figure. 1 Withdrawal-specific changes in cocaine craving. Mean (±SEM) number of responses on the lever formerly associated with cocaine across all extinction sessions completed (**A**) and during the last extinction session versus the reinstatement session (**B**) in females and males in the early (W2; n= 13 females,10 males), intermediate (W7; n= 13 females,10 males), and late (W14; n= 13 females,10 males) withdrawal groups. ^Significant difference from early withdrawal group.

Chapter IV

Role of Nucleus Accumbens Dopamine D2 Receptors in Motivating Cocaine Use in Male and Female Rats Prior to and Following the Development of An Addiction-Like Phenotype

1 Introduction

A hallmark of cocaine use disorder (CUD) is dysfunction of dopamine signaling in the mesolimbic pathway, including impaired dopamine 2 (D2) receptor signaling. This was first reported in humans by Volkow and colleagues (1990) using positron emission tomography (PET) and showed that individuals with CUD had lower striatal D2 receptor availability compared to healthy controls. Since the original report with cocaine (Volkow et al. 1990), this neuroadaptation has been observed in many subsequent studies for cocaine and other drugs such as methamphetamine, nicotine, opioids, and alcohol (Volkow et al., 1993; 2001; Fehr et al., 2008; Martinez et al., 2004; 2005; 2012). Low levels of striatal D2 receptor availability has also been shown to be associated with greater drug craving and relapse vulnerability in individuals with substances use disorders (Wang et al., 2011; Heinz et al., 2004). Moreover, high levels of striatal D2 receptor availability has been observed in unaffected members of families with a history of alcohol use disorder, suggesting that higher striatal D2 receptor availability is protective against the development of substance use disorder (Volkow et al., 2006).

In parallel, preclinical studies have shown that subordinate male cynomolgus monkeys have lower D2 receptor availability in the basal ganglia, which includes the ventral striatum (nucleus accumbens; NAc), and are more sensitive to the reinforcing effects of cocaine as compared to dominant monkeys (Morgan et al., 2002a). Studies in male rats further indicate that changes in D2 receptors occur as the result of chronic self-administration. For example, Conrad and colleagues (2010) showed that surface expression of D2 receptors in the NAc decreased following prolonged cocaine self-administration (4hr/day for 3 weeks). Similarly, Mateo et al., 2005 showed that NAc dopamine terminals and presynaptic D2 receptors (autoreceptors) are less sensitive to cocaine following extended-access (ExA; 24-hr/d for 10 days) cocaine self-administration versus acute cocaine administration (1.5 mg/kg, i.v.). Our recent findings in male rats further indicate that there is a functional shift in D2 receptor signaling in the reward pathway following the development of an addiction-like phenotype, which develops over a period of withdrawal following extended-access (24hr/day for 10 days), but not ShA (20 infusions/day for 5 days), cocaine self-administration. In males, the development of this phenotype corresponded to a decreased sensitivity to NAc D2 receptor antagonism (relative to ShA controls), indicating that the role of D2 receptor signaling in motivating cocaine use becomes diminished with the development addiction (Lynch et al., 2021).

One major caveat is that the evidence for changes in D2 receptor signaling with the development of addiction has been derived almost entirely from men and male animals. This is important particularly considering an accumulating body of evidence indicating that the mechanisms that underlie addiction are different in males and females. For example, while both male and female subordinate cynomolgus monkeys are less vulnerable to the reinforcing effects of cocaine as compared to their dominant counterparts, in females, this protection corresponds to lower D2 receptor availability (Nader et al. 2012), indicating that the relationship between D2 receptor availability and vulnerability to cocaine is opposite in females versus males. Even more notable for this study are data from human smokers showing that males have reduced striatal D2 receptor availability compared to healthy controls, but within females, striatal D2 receptor availability is comparable between smokers and health controls (Brown et al. 2012). Similar sex differences have also been reported for cortical D2 receptor availability (Zakiniaeiz et al. 2019). These findings are intriguing, particularly considering that this neuroadaptation is thought to reflect greater addiction severity and poorer treatment outcomes (Volkow et al. 1999), yet it is not observed in females who show greater addiction severity and worse treatment outcome than males (for review see Towers et al., 2023b). Therefore, sex differences in the molecular shifts that occur with D2 receptor signaling with the development of addiction needs to be further investigated.

Thus, the purpose of this study was to determine whether there are sex differences in the role of NAc D2 receptors in motivating cocaine use prior to and following the development of an addiction-like phenotype. As in our previous study in males, shifts in D2 receptor signaling were determined by comparing effects of intra-NAc D2 receptor antagonism on progressive-ratio (PR) responding for cocaine following withdrawal from either ShA cocaine self-administration (prior to the development of an addiction-like phenotype) or ExA cocaine self-administration (following the development of an addiction-like phenotype). Based on the clinical finding in smokers, we predicted that the role of NAc-D2 receptors would become diminished with the development of an addiction-like phenotype in males, but not females.

2 Methods

2.1 Subjects

Sexually mature male (*N* = 39) and female (*N* = 36) Sprague-Dawley rats (Charles River Laboratories, ME) were used as subjects. Behavioral data from a subset of the male rats (N = 28) has previously published (Lynch et al. 2021) and was included here for comparison to the female behavioral data. The male and female subjects included in the present study were run contemporaneously. Upon arrival, rats were individually housed in operant test chambers (Med Associates Inc, VT) in a temperature (20-22^{o} C) and humidity (40-70%) controlled vivarium that was maintained on a 12-hr light/dark cycle (room/house lights on at 7AM) with *ad libitum* access to food and water (Teklad LM-485 7912; except as noted below for some rats during cocaine self-administration training). To accelerate acquisition of cocaine self-administration, rats were pre-trained to lever-press for sucrose pellets (45 mg) under a fixed-ratio 1 (FR1) schedule using methods previously described (Doyle et al. 2014; Ramôa et al. 2014). The health of the rats was monitored daily over the course of the study, which included daily observation and weighing the rats at least three times a week. All the procedures were approved by the University of Virginia Animal Care and Use Committee and were conducted within the guidelines set by the National Institutes of Health.

2.2 Procedures

2.2.1 Surgeries and Catheter Maintenance

Following lever pre-training, rats underwent a jugular catheterization surgery using methods previously described (Doyle et al. 2014; Ramôa et al. 2014). The catheters were maintained by flushing with heparinized saline three days a week, which also helped verify patency throughout the study. Methohexital (1.5 mg/kg) was used to confirm patency when necessary. If a right catheter failed, a new catheter was implanted into the left jugular vein with testing resuming after 2-days of recovery. Rats were also implanted with a bilateral infusion cannula aimed at the NAc core (+1.2 mm anterior-posterior, ±1.5 mm mediolateral, -5.7 mm dorsoventral; Doyle et al. 2014; Ramôa et al. 2014; Lynch et al., 2021). This region was targeted because it is known to integrate dopamine and glutamate signaling and to mediate the reinforcing and motivational properties of addictive drugs (Doyle et al. 2014; Ramôa et al. 2014; Lynch et al. 2021; Kalivas and McFarland 2003).

2.2.2 Cocaine Self-Administration

After recovering from surgery, rats were trained to self-administer cocaine (1.5 mg/kg/infusion) as previously described (**Figure 1a**; Doyle et al. 2014; Ramôa et al. 2014). Following acquisition, rats were randomly assigned to either a ShA (*N* = 19 males/20 females) or ExA (*N* = 20 males/16 females) group. Rats in the ShA group were given three additional fixed-ratio 1 (FR1) training sessions and obtained the maximum number of infusions in each session (20 infusions). Rats in the ExA group were given extended, 24-hr/day access to cocaine for 10 consecutive days using a discrete trial procedure (4 trials/hour; up to 96 infusions/day; Doyle et al. 2014; Ramôa et al. 2014) that was designed to mimic the binge-abstinence pattern of drug use observed in humans with CUD. Following the last ExA session, rats were given two additional FR1 sessions to confirm catheter patency and to minimize differences in cocaine levels between the ShA and ExA groups before withdrawal. A 14-day withdrawal period began after the last FR1 session for both ShA and ExA rats wherein rats remained in their chambers with the active lever retracted. Following the 14th day of withdrawal, motivation for cocaine (0.5 mg/kg/infusion) was examined under a progressive ratio (PR) schedule and using methods previously described (Doyle et al. 2014; Ramôa et al. 2014; Towers et al. 2021a). These sessions continued daily until a stable baseline was achieved, which was defined as no increasing or decreasing trend in the number of infusions obtained over three consecutive sessions (typically 3 to 4 sessions), then intra-NAc infusions of eticlopride were administered as detailed below.

2.2.3 Effect of Intra-NAc Infusion of Eticlopride on Motivation for Cocaine

Once a stable baseline level of motivation for cocaine was established, effects of intra-NAc infusion of the D2 receptor antagonist eticlopride (0.1, 0.3, 1, 3-µg/side) were examined using a within-subject design similar to our previous studies (Doyle et al. 2014; Ramôa et al. 2014; Lynch et al., 2021). Briefly, infusions were administered immediately before the PR test session (0.5 µl/side; 2-min), a minimum of three stable PR sessions separated each test/intra-NAc session, and dose order was counter-balanced between subjects. The eticlopride doses selected were based on our previous intra-NAc infusion study in males showing that these doses either minimally or moderately, but selectively (i.e., no effects on locomotor behavior), impact cocaine self-administration (Lynch et al., 2021). Cannula placement was confirmed using methods previously described (Doyle et al. 2014; Ramôa et al. 2014). Placement was within the NAc core (**Figure. 1b-e**) for all but 4 male and 6 female rats who were excluded from the study. The final group sizes were 17 for ShA males, 17 for ShA females, 18 for ExA males,

and 13 for ExA females. However, due to catheter patency issues, not all rats received all the doses. The final group sizes per treatment (0, 0.1, 0.3, 1, and 3-µg/side) were as follows: 11, 8, 14, 11, and 10 for ShA males, 12, 10, 12, 10, and 12 for ShA females, 9, 9, 10, 8, and 10 for ExA males, and 12, 9, 11, 8, and 9 for ExA females, respectively.

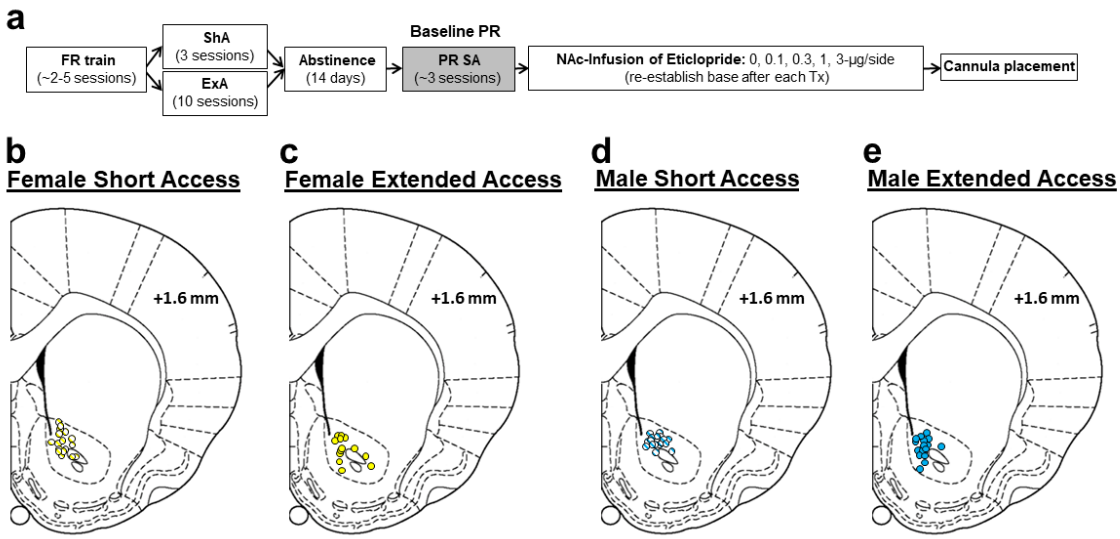

Figure 1. Summary of experimental design and cannulae placement. (**a**) Following acquisition under a fixed ratio (FR) 1 schedule (FR train), male and female rats were given either short-access (ShA; FR1, maximum of 20 infusions/day, 3 days) or extended-access (ExA; 4 discrete trials/h, maximum of 96 infusions/day, 10 days) to cocaine self-administration. Then, rats in the ExA group were given access to cocaine in an additional FR1 session (maximum of 20 infusions/day) to verify patency and equate cocaine intake between the ShA and ExA groups prior to withdrawal. Following a 14-day withdrawal period, motivation to obtain cocaine was assessed using a progressive-ratio (PR) schedule (PR SA). Once a baseline level of motivation was established (3 days), the effects of nucleus accumbens (NAc) infused eticlopride (0-3 µg/side) were examined. A minimum of three stable PR sessions separated each eticlopride test session. Following study completion, brain tissue was collected for cannulation placement verification. Histological cannulae placements in the NAc are shown for (**b**) ShA females, (**c**) ExA females, (**d**) ShA males, and (**e**) ExA males. Schematics adapted from the atlas of Paxinos and Watson.

2.3 Drugs

Cocaine ([-]-cocaine hydrochloride) was obtained from the National Institute on Drug Abuse (NIDA; Research Triangle Park, NC) and dissolved in sterile saline, filtered, and stored at 4ºC. The infusion duration of cocaine was adjusted three times per week based on body weight (2-sec/100

g/kg). Eticlopride was purchased from Sigma (St. Louis, MO) and dissolved in sterile water (vehicle).

2.4 Data Analysis

Sex differences in cocaine intake over the 10-day ExA period were examined using a mixed effects model with sex as the between-subject factor. A mixed effect model was also used ~~a~~ to assess the development of an addiction-like phenotype in the ExA group, which was defined by an enhanced motivation for cocaine relative to the ShA group. This analysis focused on the group differences (i.e., ShA females, ShA males, ExA females, ExA males) in the number of infusions obtained during the three PR baseline sessions that preceded the first treatment. Given baseline differences in the number of cocaine infusions obtained between the ExA and ShA groups and males and females, group differences in the effects of NAc-eticlopride infusion were analyzed as percent change from baseline using group and treatment (saline versus D2 receptor antagonism) as fixed factors and dose (0-3.0 µg/side) as a covariant. Post hoc comparisons to baseline (0) were made using two-tailed Bonferroni-corrected one sample t-tests. Statistical analyses were performed using SPSS (V26). Alpha was set at 0.05. Data are presented as the mean ± SEM.

3 Results

3.1 Cocaine Self-Administration and Subsequent Motivation for Cocaine

Females self-administered significantly more cocaine infusions under ExA conditions than males (**Figure 2a**; overall effect of sex: $F_{1,300}$= 16.2, P < 0.001); however, females and males had a similar pattern of cocaine self-administration over the 10-day ExA period (no session by sex interaction) with both sexes obtaining more infusions during early sessions compared to later sessions (overall effect of session: $F_{9,300}$= 5.7, P < 0.001; session 1 versus 10, P < 0.001). Additionally, the average number of infusions obtained by ExA males and females over the 10-day ExA period (75 ± 1 infusions/session and 67± 2 infusions/session, respectively) was significantly higher than the number of infusions obtained by ShA males and females which were limited to 20 infusions per session (data not shown). Thus, while ExA females had higher

cocaine intake than ExA males, both sexes had a similar pattern of cocaine intake over the ExA period and had higher intake than ShA controls.

As expected, following the 14-day withdrawal period, ExA rats obtained more cocaine infusions during the three PR baseline sessions that preceded the first eticlopride infusion than ShA controls (**Figure 2b** overall effect of group: $F_{3,183}$= 19.5, P < 0.001). Post-hoc comparison within each sex confirmed that ExA rats obtained more cocaine infusions than ShA rats for both males (P < 0.05) and females (P < 0.05). ShA females also obtained more cocaine infusions than ShA males (P < 0.05); however, there was no difference in the number of infusions obtained between ExA females and males (P < 0.05). There were also no overall or interactive effects of session, indicating that baseline levels of motivation were stable within each group. Thus, while females had higher motivation for cocaine than males following ShA cocaine self-administration, both males and females showed a stable increase in motivation for cocaine following withdrawal from ExA self-administration (relative to ShA controls), which confirms the development of an addiction-like phenotype in both sexes.

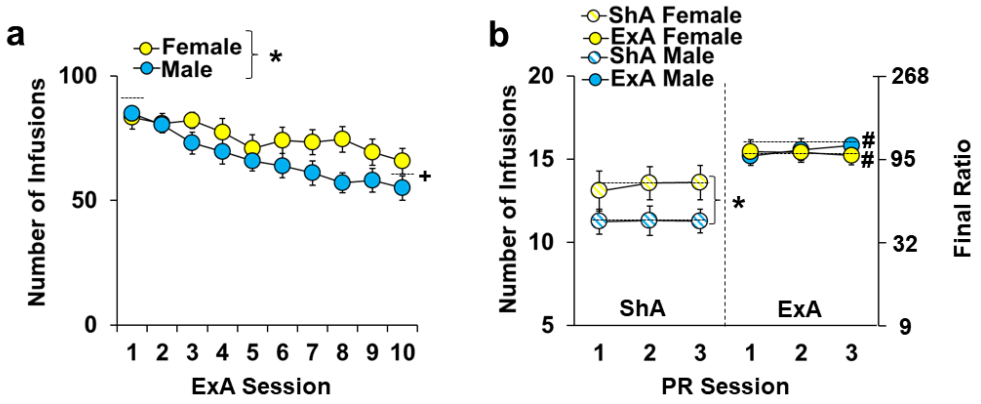

Figure 2. Sex differences in the impact of extended-access cocaine self-administration and withdrawal on motivation for cocaine. Mean (±) number of infusions obtained during the 10 extended-access (ExA) cocaine self-administration sessions (**a**) and the first 3 stable progressive ratio sessions (**b**) for ExA males (*n*= 18) and females (*n*= 13) and short-access (ShA) males (*n*= 17) and females (*n*= 17). * significant difference between males and females. # significant difference between the ShA and ExA group.

3.2 Effects of NAc-D_2 Antagonism on Motivation for Cocaine

Similar group differences were also observed for the number of infusions obtained under the PR schedule in the larger analysis that included the three baseline sessions that preceded each treatment session (overall effect group: $F_{3,558}$= 41.7, P < 0.001; **Figure 3a-b**). As with the

analysis above, this difference was driven by higher cocaine infusions in the ExA versus ShA group ($P < 0.001$'s) and in ShA females compared to ShA males ($P < 0.001$). Additionally, this group difference was consistent and maintained across the study (no overall or interaction of dose: $P > 0.05$'s).

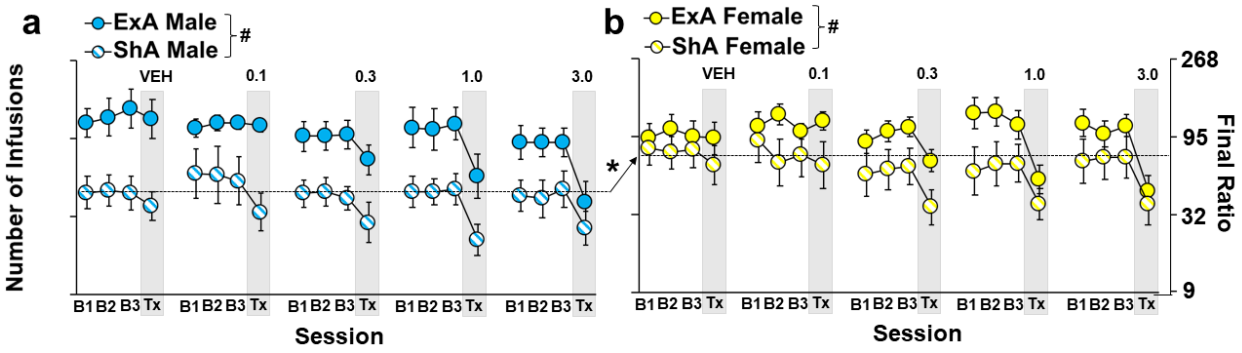

Figure 3. Group differences in baseline levels of motivation for cocaine were maintained throughout the study. Mean (±) number of infusion obtained during the three stable progressive ratio (PR) sessions prior to treatment (B1, B2, B3) and the day of treatment (Tx; grey box) as a function of eticlopride dose (0, 0.1, 0.3, 1.0, and 3.0 -µg/side) in short-access (ShA) (*n*=11, 8, 14, 11, 10) and extended-access (ExA *n*= 9, 9, 10, 8, and 10) males (**a**) and ShA (*n*= 12, 10, 12, 10, 12) and ExA (*n*=12, 9, 11, 8, 9) females (**b**). * significant difference between males and females. # significant difference between the ShA and ExA

Given these baseline group differences in PR responding, effects of D_2 receptor antagonism were determined as percent change from the average number of cocaine infusions obtained during the three baseline PR sessions that preceded each treatment (**Figure 4a-b**). This analysis indicated that greater effects were obtained at higher versus lower eticlopride doses (overall effect of dose: $F_{1,194}= 32.6$, $P < 0.001$) and in the ShA versus ExA group (overall effect of group: $F_{3,194}= 3.7$, $P < 0.05$; posthocs versus ShA males), particularly in males at low doses of eticlopride (interaction of group and dose: $F_{3,194}= 2.7$, $P < 0.05$). Further analysis within males confirmed significant effects of group ($F_{1,95}= 8.0$, $P < 0.01$) and group by dose ($F_{1,95}= 6.0$, $P < 0.05$), which was driven by greater decreases in the ShA versus ExA group, particularly at lower doses (0.1 and 0.3 µg/side; $P < 0.01$). There was also a significant effect of dose ($F_{1,95}= 13.5$, $P < 0.001$) indicating that, as with the larger group, higher doses induced larger decreases. Additionally, post-hoc comparisons to baseline (0) within each group of males confirmed a significant decrease in motivation for cocaine use in the ShA group at both the low doses (0.1 and 0.3 µg/side: $P < 0.001$) and high doses (1.0 and 3.0 µg/side: $P < 0.001$), but not vehicle (P

> 0.05); whereas, in the ExA group, only the effect at the high doses reached statistical significance (P > 0.001); vehicle was not significant (P > 0.05), and the low doses only tended to decrease motivation for cocaine (P = 0.06).

In contrast to effects in males, the analysis within females revealed only a trend for greater effects in the ShA versus ExA group (overall effect of group: $F_{1,99}$= 2.8, P = 0.10) and no significant interaction of group and dose (P > 0.05). Additionally, further analysis within the two low doses (0.1 and 0.3 µg/side) and two high doses (1.0 and 3.0 µg/side) of eticlopride revealed a similar decrease in motivation for cocaine use in ShA and ExA females (no group effect: P's > 0.05). There was also a significant effect of dose ($F_{1,95}$= 13.5, P < 0.001), indicating that, as with the larger group and findings in males, higher doses induced larger decreases in motivation for cocaine use. Post-hoc comparisons to baseline (0) within each group of females confirmed the only significant decrease in motivation for cocaine use was at the two high doses in ShA and ExA females (P's < 0.001); whereas, vehicle was not significant in ShA and ExA females and the low doses tended to decrease motivation for cocaine use in ShA females (P = 0.054), similar to ExA males. Thus, while NAc-infused eticlopride dose-dependently decreased motivation of cocaine use prior to and following the development of an addiction-like phenotype in males and females, the effect was most robust in ShA males, particularly at low doses.

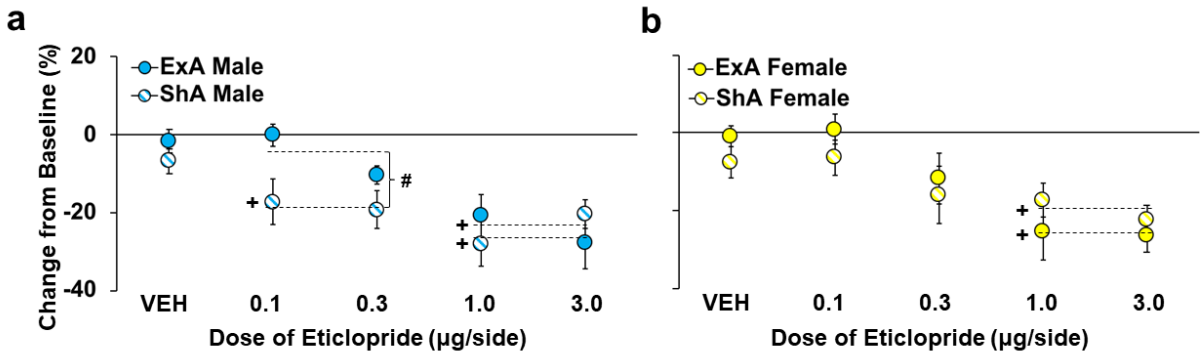

Figure 4. Sex differences in effects of NAc-D2 receptor antagonism on motivation for cocaine prior to and following the development of an addiction-like phenotype. Mean (±) percent change from the baseline number of infusions obtained by short-access (ShA) and extended-access (ExA) groups following treatment with eticlopride (0, 0.1, 0.3, 1.0, and 3.0 - µg/side) in males (**a**; ShA, *n*=11, 8, 14, 11, 10; ExA *n*= 9, 9, 10, 8, and 10) and females (**b**; ShA, *n*= 12, 10, 12, 10, 12; ExA *n*=12, 9, 11, 8, 9). # significant difference between the ShA and ExA group. + significant decrease from baseline (0).

4 Discussion

We previously showed that an addiction-like phenotype, defined as the development of enhanced motivation for cocaine, develops over withdrawal following ExA cocaine self-administration and, in males, corresponds with a diminished role of NAc-D2 receptor signaling (Lynch et al., 2021). In the present study, we expanded the investigation of D2 receptor mechanisms motivating cocaine use to include females, and based on findings in human smokers, predicted that the role of NAc-D2 receptor signaling would vary between males and females and in females, would not become diminished with the development of an addiction-like phenotype. Our findings support this hypothesis as we found that eticlopride was less effective at decreasing motivation for cocaine in ExA males versus ShA males, particularly at low doses, whereas, in females, there were no differences in the effectiveness of the eticlopride between ExA and ShA rats. These findings indicate that males, but not females, become less sensitive to NAc-D2 receptor antagonism with the development of an addition-like phenotype.

Decreased striatal D2 receptor binding in addiction is one of the most replicated findings in human imagining research (see Trifilieff et al., 2017b for review); however, this molecular shift may not translate to females (Zakiniaeiz et al., 2019; Brown et al., 2012). Our findings in the present study build on this possibility by showing that while low doses of eticlopride were less effective at decreasing motivation for cocaine in ExA versus ShA males, there was no difference in its efficacy between ExA and ShA females. Based on the assumption that low doses are more selective and induce fewer off target effects than high doses, our findings indicated that males, but not females, develop a decrease in sensitively to D2 receptor antagonism with the development of an addiction-like phenotype. One alternative possibility, however, is that this shift does occur in females, but perhaps sooner/more readily, such that it was already present in females tested following ShA self-administration. Females are known to transition from initial drug use to meeting the criteria for CUD/seeking treatment faster than males (for review see Towers et al., 2023b), and in our present study, ShA females were more motivated to obtain cocaine than ShA males. Thus, it is possible that at least some of the females in the ShA group had already developed an addition-like phenotype and a shift toward a diminished role of D2 receptors and this explains why we did not see a difference between ShA and ExA females. It is notable that no differences were observed for the effects of eticlopride between ExA males and females. Further research is necessary to resolve this question.

It is also important to note that findings in this study along with ours and others previous studies provide support for similarities in the mechanisms underlying addiction in males and females. More specifically, here we showed NAc-D2 receptor blockade with eticlopride dose-dependably decreased motivation for cocaine in both ShA and ExA males and females, indicating that D2 receptors remain critical for motivating cocaine use prior to and following the development of an addiction-like phenotype in both sexes. This is in contrast to our previous findings for NAc dopamine 1 (D1) receptor signaling, which showed NAc-D1 receptor blockade with SCH-23390 dose-dependably decreased motivation for cocaine in ShA, but not ExA, males and females, indicating that D1 receptors are critical for motivating cocaine use prior to, but not following, the development of an addiction-like phenotype in both sexes (Doyle et al. 2014; Ramôa et al. 2014). Preclinical studies in non-human primates have also shown stimulant-induced dopamine release from presynaptic terminals is decreased in males and females with repeated drug use (Castner et al., 2000; Henry et al., 2009). Thus, blunted dopamine transmission in the striatum likely renders males and females vulnerable to continued drug use, and there are likely similarities (D1R signaling) and differences (D2 receptor signaling) in the mechanisms underlying these striatal dopamine deficits associated with addiction in males and females.

There is an urgent need to disentangle the differences and similarities in the mechanisms underlying CUD in males and females because although males having higher rates of cocaine use than females, the number of females using cocaine and other addictive drugs in their lifetime is on the rise, and females are more vulnerable to many aspects of CUD compared to males. For example, females develop CUD and/or seeking treatment for the disorder after few years of cocaine use, report longer periods of cocaine use after relapse, have greater stress-induced cravings, and show an enhanced vulnerability to cocaine-related medical consequences compared to males (McCance-Katz et al., 1999; Griffin et al., 1989; White et al., 1996; Haas and Peters, 2000; Waldrop et al., 2010; Moran-Santa Maria et al., 2018; Gallop et al., 2007). Preclinical studies have similarly shown females have an enhanced sensitivity to reinforcing effects of cocaine, display a greater vulnerability during the transition from initial cocaine use to the development of an addiction-like phenotype, self-administer more cocaine under ExA conditions, and develop an enhanced motivation for cocaine and a preference for cocaine over other reinforcers after less drug exposure and/or shorter periods of withdrawal than males (see Towers et al., 2023b for review). Although the present study was not designed to assess

behavioral sex differences, similar to the findings mentioned above, we observed higher levels of cocaine intake under ExA conditions and motivation for cocaine use following ShA cocaine self-administration in females than males. Thus, females and males also differ in several key features of addiction, and understanding sex differences in the mechanisms underlying these features will hopefully lead to the development of sex-specific treatments and the first FDA approved treatment for CUD.

In summary, despite the severity of addiction in females and an accumulating body of evidence indicating that females and males differ in several features of addiction, clinical and preclinical studies have historically used male subjects, resulting in a male-centric neurobiological basis of addiction. The current study builds on the literature showing that findings in males do not necessarily translate to females. Future studies will need to determine whether striatal D2 receptor signaling becomes diminished in females with the development of other substance use disorders such as opioid use disorder and alcohol use disorder. It will be important to disentangle the similarities and differences in the mechanisms underlying addiction to promote the health of both males and females with substance use disorder.

Chapter V

Effect of SMAASH-C, A Novel Nutritional Supplement, on Drug-Seeking and Toxicity in a Rat Model of Cocaine Use Disorder

1 Introduction

Cocaine use disorder (CUD) is a serious public health concern with approximately 5.1 million regular cocaine users over the age of 12, and 1.3 million individuals with CUD in the Unites States in 2020 (NSDUH, 2021). Despite decades of study and the allocation of a significant amount of resources, no medication has been approved for the treatment of CUD. The lack of medications for CUD is largely due to candidate medications failing in clinical trials despite promising preclinical results. The challenge of failed translation is not unique to CUD research (Strickland et al., 2022); however, we argue that in order to produce a more representative and translationally relevant body of knowledge on the neurobiological basis and potential treatments for addiction, studies need to consider biological sex as a variable and use animal models validated to induce addiction-like features like those observed in women and men with CUD (Towers and Lynch, 2021b).

Extended-access (ExA) drug self-administration procedures (i.e., ≥ 6-hr/day access to the drug) are the gold-standard for inducing addiction-like features in animals (Lynch, 2018). We use an intermittent, ExA procedure (4 trials/hr; 24-hr/day for 10 days) that was developed to approximate the use patterns observed in humans (e.g., binge/abstinent patterns; Fitch and Roberts, 1993). We have confirmed that this procedure induces a binge/abstinent patterns of drug intake in both males and females (Roberts et al., 2002), as well as other key features of addiction, including an enhanced vulnerability to relapse assessed using an extinction/cue-induced reinstatement procedure (Towers et al., 2023a; Peterson et al., 2014; Abel et al., 2014).

Here, we used this rat model of CUD to determine the efficacy of oral treatment during abstinence with a novel nutritional supplement, SMAASH-C, at reducing relapse vulnerability and markers of cocaine-induced toxicity. We developed (Ajibike Salako-Akande) SMAASH-C as part of a nutritional approach for managing substance use disorder in our clinical practice. This formulation contains a combination of vitamins, minerals, omega-3 fatty acids, and tyrosine and other amino acids that are known to be depleted in both humans and animals following chronic cocaine use/exposure (Cerretani et al., 2012; Jacobsen et al., 2001; Trulson et al., 1987; Virmani et al., 2006, 2007; Volkow et al., 1997a,b). Dr. Ajibike Salako-Akande then patented this formulation (Patent number: US 2022/0339204; Getwele Natureceuticals, LLC; Halethorpe, MD) based on anecdotal reports and open label observational data in humans indicating that oral SMAASH-C (0.1-0.4 g/kg/day; 6-12 months) treatment during abstinence improves markers of

general health (e.g., one patient with co-morbid AIDS-related herpes simplex virus infection reporting marked improvement in viral symptoms) and reduces drug-craving, frequency of relapse, and withdrawal symptoms. Results from subsequent preclinical studies were also promising and showed that chronic SMAASH-C treatment (oral, 0.0408-0.123 g/kg, 8 weeks) markedly and selectively reduced cocaine- and amphetamine-induced conditioned place preference and markers of drug-induced toxicity in male and female rats (Webber-Waugh et al., 2017; Gardner et al., 2015).

In the present study, we expanded on these findings by evaluating the efficacy of SMAASH-C at reducing relapse vulnerability and markers of cocaine-induced toxicity. Effects were examined in both males and females and following a two-week treatment regime at a moderate and moderate-to-high dose (0.4 and 0.8 g/kg/day; Experiment 1) and a 6-week regime at the moderate dose (0.4 g/kg/day; Experiment 2). Based on the preliminary clinical and preclinical findings, we predicted both the two- and six-week SMAASH-C treatment regimens would decrease relapse vulnerability and cocaine-induced toxicity in male and female rats.

2 Material and Methods

2.1 Subjects

Adult male (N=49) and female (N=42) Sprague-Dawley rats (Charles River Laboratories) were used as subjects in this study. As previously described (Lynch, 2008), rats were housed in individual Med-Associates operant and pre-trained to lever-press for sucrose pellets to expedite subsequent cocaine self-administration training. Rats were also pre-exposed to a jar with ground food and some rats were given sticks to prevent teeth overgrowth. All procedures were approved by the University of Virginia Animal Care and Use Committee and were conducted in accordance with NIH guidelines.

2.2 Procedures

2.2.1 Surgery and catheter maintenance

An indwelling catheter (Dow Corning, Midland, MI, USA) was implanted into the right jugular vein of the rat using methods previously described (Lynch, 2008). Catheters were flushed with heparinized saline 3 days/week; patency was occasionally verified using methohexital (1.5 mg/kg).

If catheter patency was lost, a left jugular vein catheter was implanted and testing resumed after a 1-2-day recovery period.

2.2.2 Cocaine Self-Administration

Rats were trained to self-administer cocaine (1.5 mg/kg/infusion) under a fixed-ratio 1 schedule (FR1; Lynch et al., 2010), and once acquired (i.e., 2 consecutive days wherein all 20 infusions were obtained), they were given ExA to cocaine for 10 days using a discrete trial procedure (4, 10-minute trials/hr; up to 96 infusions/day; Roberts et al., 2002). After the last ExA session, cocaine was available for one additional session under a FR1 schedule (maximum of 20 infusions) to equate intake between prior to abstinence. Then, a 2- (Experiment 1) or 6-week (Experiment 2) abstinence period began wherein rats remained in their chambers with the active lever retracted. Prior to the abstinence period, three males and one female had to be removed from the study because of health or patency issues.

2.2.3 Experiment 1

For experiment 1 (**Figure 1A**), on the first day of abstinence, rats were randomly assigned to control-chow (14 males, 11 females), a moderate SMAASH-C dose (0.4 g/kg; 11 males, 11 females), or a moderate-to-high SMAASH-C dose (0.8 g/kg; 8 males, 8 females). These doses were selected because they are analogous to the g/kg doses used clinically (after equating for rat-human differences in body surface area using a conversion factor of 6.2; U.S. Food and Drug Administration, 2005). SMAASH-C was mixed with ground chow (Teklad LM-485 7912) and water (2:1 ratio), placed in a jar, and given to the rats daily throughout abstinence and relapse testing. Control rats received the same ground chow/water mash, but without SMAASH-C.

Drug-seeking was examined on abstinence day 15 using an extinction/cue-induced reinstatement procedure (Peterson et al., 2014; Sanchez et al., 2014; Beiter et al., 2016). Estrous cyclicity and the estrous cycle phase on the test day was determined in females as previously described (Lynch et al., 2019). One male and two females in the control-chow group and one female in the 0.4 and 0.8 g/kg SMAASH-C groups were removed from the study because of health issues. One male and one female in the control-chow group and one male and one female in the 0.4 g/kg SMAASH-C group were removed from the study due to patency or technical issues. One non-estrus female in the 0.8 g/kg SMAASH-C group was a significant Grub's outlier on all

measures of drug-seeking (total extinction and reinstatement responses and overall seeking as defined by hour-1 extinction responses plus reinstatement responses) and was excluded from all analyses. This resulted in a final group size of 12 males and 8 females in the control-chow group, 10 males and 9 females in the 0.4 g/kg SMAASH-C group, and 8 males and 6 females in the 0.8 g/kg SMAASH-C group.

Trunk blood was collected from a subset of rats in the control-chow (4 males, 5 females), 0.4 g/kg SMAASH-C (4 males, 5 females) and 0.8 g/kg SMAASH-C groups (7 males, 5 females) the morning following the extinction/reinstatement test (between 10AM and 12PM) and separated into plasma and serum (Lynch 2008; Lynch 2009). Serum was stored at -80 C until a small animal chemistry profile examining markers for liver (aspartate transaminase, AST; alanine transaminase, ALT), pancreases (amylase), and kidney (urea nitrogen) function was completed by Michigan State University's Veterinary Diagnostics Lab (https://cvm.msu.edu/vdl). An additional cohort of saline rats (5 males, 6 females) were also run and included as healthy, drug-naïve controls. One female in the saline group was a significant Grubb's outlier for AST and one female in the saline group and one male in the 0.4 g/kg SMAASH-C group were significant Grubb's outliers for ALT; these rats were excluded from that particular analysis

2.2.4 Experiment 2

The same procedure as Experiment 1 was followed for Experiment 2, except that rats were randomly assigned to receive control-chow (6 males, 5 females) or 0.4 g/kg SMAASH-C (7 males, 6 females) and relapse testing occurred after a 6-week treatment regimen (day 43; **Figure 3A**). Truck blood was collected the day after the relapse test from a subset of rats in the control-chow (6 males and 4 females) and SMAASH-C groups (5 males and 3 females). Serum markers of toxicity were compared to those observed in drug-naïve controls using the same saline rats as Experiment 1. One male in the 0.4 g/kg SMAASH-C group was a significant Grubb's outlier for amylase and urea nitrogen and was excluded from these analyses.

2.3 Drug

Cocaine hydrochloride (National Institute of Drug Abuse; Research Triangle Park, NC) was dissolved in sterile saline, stored at 4°C, and sterile filtered prior to use. The infusion duration (2-

sec/100g/kg) and SMAASH-C dose was adjusted three times/week based on body weight. The purity of each component of SMAASH-C (see US 2022/0339204) was verified by AEON Technologies (Frostburg, MD) using a combination of Inductively Coupled Plasma Mass Spectrometry, High Performance Liquid Chromatography with a Diode Array Detector, and Gas Chromatography Mass Spectrometry. SMAASH-C was then compounded by Kydes Pharamaceuticals (Halethorpe, MD) and Getwele Natureceuticals (Dr. Salako-Akande) provided the verified samples tested here.

2.4 Data Analysis

Group (SMAASH-C versus control-chow) and sex differences in average cocaine intake over the ExA period were examined using a univariant ANOVA. Group and sex differences in cocaine-seeking, responses during extinction and reinstatement testing, were determined using repeated measures ANOVA by comparing the number of responses made during the first 6 extinction sessions and during the last extinction session versus the reinstatement session. Dose (0.4 and 0.8 g/kg) was considered as a covariant (Experiment 1), but since there were no significant effects of dose on any measure of cocaine-seeking (total and hourly responses during extinction, total responses during reinstatement, and overall seeking as defined by hour one extinction responses plus reinstatement responses), effects were collapsed across dose (note: Data are presented for each dose separately for clarity). A univariate analysis was used to examine effects of SMAASH-C (versus control-chow) on cocaine-seeking within females tested during estrus versus non-estrus phases in Experiment 1 only since Experiment 2 was not powered to address estrous cycle effects (i.e., n=3 estrus and 2 non-estrus, control-chow; n=5 estrus and 1 non-estrus, SMAASH-C). Univariate analyses were used to determine effects of SMAASH-C on cocaine-induced toxicity focusing on serum markers for liver (AST, ALT), pancreases (amylase), and kidney (urea nitrogen) function; effects were examined as both relative levels and percent difference from saline controls. Since Experiments 1 and 2 were run sequentially, rather than contemporaneously, effects of 2 versus 6 weeks of SMAASH-c treatment were not made statistically. The one exception was for the behavioral summary of percent change in cocaine-seeking from 2- to 6-weeks; this was determined within the control-chow group in order to verify the incubation of cocaine-seeking over abstinence. Post-hoc comparisons were made using two-tailed Bonferroni-corrected t-tests or

two-tailed one-sample t-tests. Statistical analyses were performed using SPSS (V26). Alpha was set at 0.05. Data are presented as the mean ± SEM.

3 Results

3.1 Experiment 1

3.1.1 Cocaine Intake

Males and females self-administered high levels of cocaine during the 10-day ExA period and average intake did not differ between sex or within males and females later given control-chow (75±3, female, 71±2, males), 0.4 g/kg SMAASH-C (72±4, female, 72±1, males), 0.8 g/kg SMAASH-C (71±2, female, 70±2, males) or during abstinence. Thus, intake was similar between males and females and treatment groups prior to the start of SMAASH-C or control treatment.

3.1.2 Relapse Vulnerability

Extinction responding over the first six extinction session was similar in females versus males and the control-chow versus SMAASH-C groups (**Figure 1B**), with results revealing an overall effect of session ($F_{5,24}$ =72.6, p<0.001) and higher responding in session 1 vs sessions 2-6 (p<0.001), but no overall or interactive effects of treatment or sex indicating that SMAASH-C did not impact extinction responding in males or females.

In contrast, significant effects of sex and SMAASH-C treatment were observed for reinstatement responding (**Figure 1C**), with results revealing an overall effect of session ($F_{1,49}$=111.7, p<0.001) and an interaction of session, sex, and treatment ($F_{1,49}$=4.6, p<0.05). Subsequent within-sex analyses revealed significant effects of session within both males ($F_{1,28}$=49.8, p<0.001) and females ($F_{1,21}$=60.0, p<0.001) indicating that responding was reinstated by the cues formerly associated with cocaine in both sexes. A significant interaction of session by treatment group was also observed but only in females ($F_{1,21}$=6.0, p<0.05) indicating that SMAASH-C treatment reduced reinstatement responding in females, but not males. Subsequent analyses within the control-chow and SMAASH-C treatment groups also revealed no differences between males and females during the last extinction session for either the control-chow or SMAASH-C groups, but a significant effect of sex within the reinstatement session

within the control-chow (F>M, $p<0.05$), but not SMAASH-C group, indicating that SMAASH-C reduced reinstatement responding in females down to the male level.

3.1.3 Effect of Estrous Cycle Phase

Interestingly, the efficacy of SMAASH-C at decreasing relapse vulnerability varied within females across the estrous cycle (**Figure 1D-E**). As with the analysis in males and females, for extinction (**Figure 1D**), there was an overall effect of session ($F_{5,95}=38.1$, $p<0.001$), with responding being higher in session 1 versus 2-6 (p's<0.001), but no significant effects of treatment group, indicating that SMAASH-C did not impact extinction responding in estrus or non-estrus females. For reinstatement (**Figure 1E**), there was an interaction of treatment group and estrous cycle phase ($F_{1,19}=9.0$, $p<0.01$), as well as significant overall effects of treatment group ($F_{1,19}=12.7$, $p<0.01$) and estrous cycle phase ($F_{1,19}=10.4$, $p<0.01$), which appear to be driven by control-chow females tested during estrous given that responding was significantly higher in this group as compared to all other groups (p's<0.05). There were also no significant differences between any of the other groups. Together, these findings indicate that SMAASH-C is most effective in females tested during estrus, when levels of relapse vulnerability is heightened.

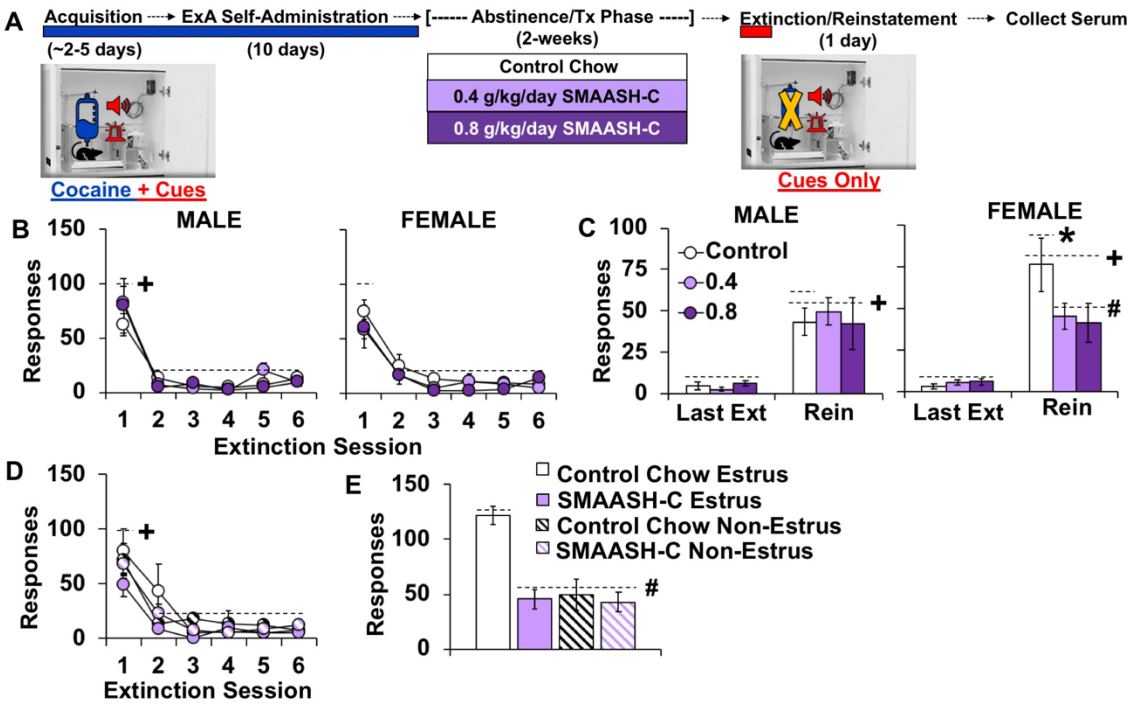

Figure 1. SMAASH-C blunts cue-induced cocaine-seeking following a two-week treatment regime over abstinence in females. Male and female rats were trained to self-administer cocaine under a fixed ratio (FR) 1 schedule (acquisition). Once they reach the acquisition requirements (2 days, 20 infusions), they were given extended-access (ExA; 4 discrete trials/h, maximum of 96 infusions/day, 10 days) to cocaine self-administration. Then, rats were given access to cocaine in one additional FR1 session (maximum of 20 infusions/day) to verify patency prior to the start of abstinence. On abstinence day one, rats were randomly assigned to one of three daily SMAASH-C treatment conditions: control-chow (13 males, 10 females), a moderate SMAASH- C dose (0.4 g/kg; 11 males, 11 females), or a high SMAASH-C dose (0.8 g/kg; 8 males, 8 females). The SMAASH-C was administered daily over a two-week abstinence period by mixing it in fresh, ground chow (Teklad LM-485 7912). On abstinence day 15, rats underwent extinction testing in a minimum of 6, 1-hr sessions and once cocaine-seeking was extinguished (<15 responses), cue-induced reinstatement testing in a 1-hr session. Serum was collected the next morning (**A**). The data are plotted as mean (±SEM) number of responses on the lever formerly associated with cocaine during the first six 1-h extinction sessions (**B**) and during the last extinction session versus the reinstatement session (**C**) in males (left) and females (right). Mean (±SEM) number of responses on the lever formerly associated with cocaine during the first six 1-h extinction sessions (**D**) and during the reinstatement session (**E**) in control-chow and SMAASH-C estrus (n = 3 and 5, respectively) and non-estrus females (n = 7 and 8, respectively). +Significant effect of session (**B**, **C, D**). #Significant effect of SMAASH-C (**C, E**). *Significantly effect of sex (**C**).

3.1.4 Cocaine-Induced Toxicity

In contrast to the behavioral findings, two-weeks of SMAASH-C treatment over abstinence reduced serum makers of cocaine-induced toxicity in males, but not females. Results from the analysis of the liver enzyme AST revealed an effect of treatment group (**Figure 2A**; $F_{2,33}$=11.7, p<0.001) and a sex by treatment group interaction ($F_{2,33}$=5.7, p<0.01). Subsequent within-sex analyses, revealed significant effects of treatment group in both males ($F_{2,17}$=11.9, p<0.001) and females ($F_{2,16}$=6.2, p<0.01). In males, this was driven by higher AST levels in the control-chow group compared to both the saline (p<0.001) and SMAASH-C groups (p<0.001). The SMAASH-C group was not different from saline controls indicating that this effect of cocaine was normalized by SMAASH-C treatment during abstinence. In contrast, in females, both the control-chow and SMAASH-C groups had higher AST levels compared to saline controls (*p*'s<0.01) indicating that SMAASH-C treatment did not normalize AST levels in females. Interestingly, for the liver enzyme ALT, there was no overall or interactive effects of sex or treatment group (**Figure 2B)**. For the pancreas enzyme amylase, there was an overall sex effect (**Figure 2C**; $F_{1,34}$=10.8, p<0.01) and sex by treatment group interaction ($F_{2,34}$=5.9, p<0.01), which appears to be driven by higher amylase in males versus females, particularly in the SMAASH-C group (p<0.001). Subsequent within-sex analyses revealed a trend for an effect of treatment group in both males ($F_{2,17}$=2.9, p=0.08) and females ($F_{2,17}$=3.3, p=0.06), but within males, none of the post-hoc comparisons were significant, and within females, there was only a trend for lower amylase levels in the SMAASH-C group versus saline (*p*=0.06). For urea nitrogen, which is a marker of kidney function, there were no overall or interactive effects of sex or treatment group (**Figure 2D**). Thus, following ExA cocaine self-administration and 14 days of abstinence, levels of the liver enzyme AST were increased in both males and females and normalized within males, but not females, by SMAASH-C treatment during abstinence. ALT, amylase, and urea nitrogen levels were unchanged following ExA cocaine self-administration and 14 days of abstinence although a sex difference was apparent for levels of the pancreas enzyme, amylase, with SMAASH-C treated females having lower levels than their male counterparts.

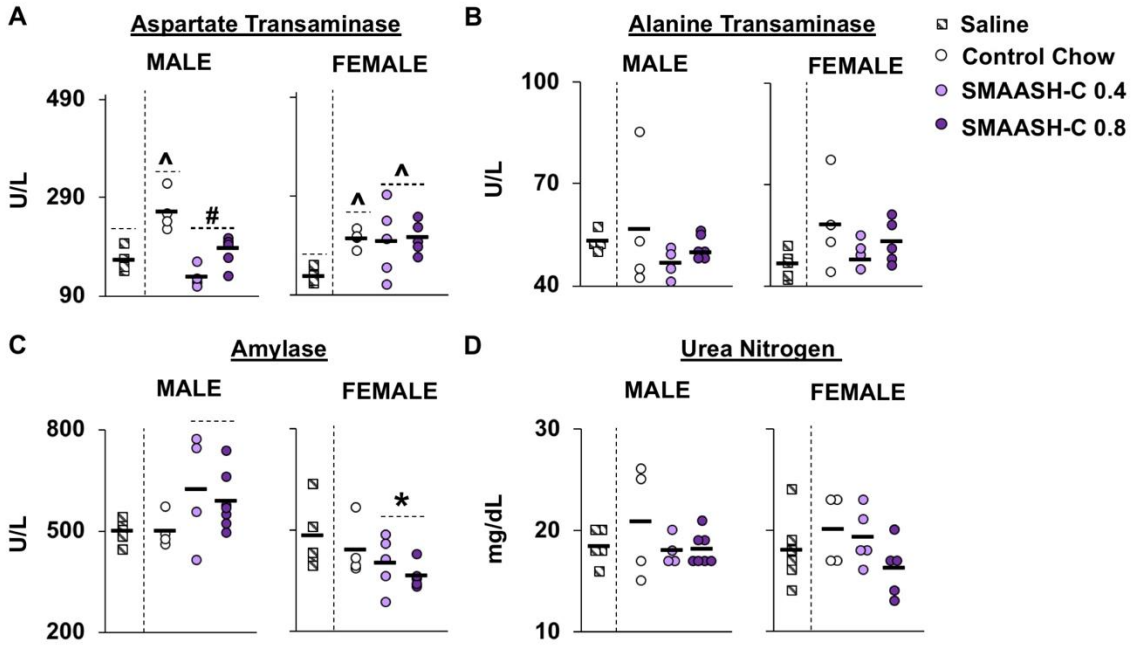

Figure 2. SMAASH-C blunts cocaine-induced toxicity following a two-week treatment regime over abstinence in males. Mean (±SEM) serum concentration of aspartate transaminase (AST; **A**), alanine transaminase (ALT, **B**), amylase (**C**), and urea nitrogen (**D**) in saline (n=5 males and n=6 females), control-chow (n=4 males and 5 females), 0.4 g/kg SMAASH-C (n=4 males and 5 females) and 0.8 g/kg SMAASH-C (n=7 males and 5 females) rats. #Significant effect of SMAASH-C (**A**). ^Significant difference from baseline (control-chow saline) (**A**). *Significant effect of sex (**C**).

3.2 Experiment 2

3.2.1 Cocaine Intake

Males and females self-administered high levels of cocaine during the 10-day ExA period and average intake did not differ between sexes or within males and females later given control-chow (76±3, female, 70±4, males) or 0.4 g/kg SMAASH-C (75±1, female, 75±1, males) during abstinence. Thus, intake was similar between sexes and treatment groups prior to SMAASH-C or control treatment.

3.2.2 Relapse Vulnerability

In contrast to findings in Experiment 1, in this Experiment, significant sex/group differences were observed for extinction responding following 6-weeks of SMAASH-C treatment (**Figure 3B**) with results revealing an effect of session ($F_{5,100}$=27.7, p<0.001), session by treatment group

interaction ($F_{5,100}$=2.6, p<0.05), and session by sex by treatment group interaction ($F_{5,100}$=4.0, p<0.05). Subsequent analysis within males, revealed an overall effect of session ($F_{5,55}$=13.6, p<0.001) with responding being higher in session 1 versus 2-6 (p's<0.001), but no overall or interactive effects of treatment group, indicating that SMAASH-C did not impact extinction responding in males. Whereas, the same analysis in females revealed an effect of session ($F_{5,45}$=14.5, p<0.001) and an interaction of session and treatment group ($F_{5,45}$=6.2, p<0.001) which appears to be driven by lower responding in SMAASH-C versus control-chow females in session one (p<0.05). Further analysis within the control-chow females confirmed higher responding in session 1 versus 2-6 (p<0.05); whereas no effect of session was observed within SMAASH-C females. Thus, SMAASH-C reduced responding on the lever associated formerly-associated during extinction testing in females, but not males, particularly during the first extinction session.

In contrast to Experiment 1 and effects in this experiment during extinction, reinstatement responding was similar between the SMAASH-C and control-chow groups within both males and females (**Figure 3C**). Specifically, results revealed an effect of session ($F_{1,20}$=59.2, p<0.001), but no overall or interactive effects of sex or treatment group indicating that SMAASH-C did not impact cue-induced reinstatement responding in males or females.

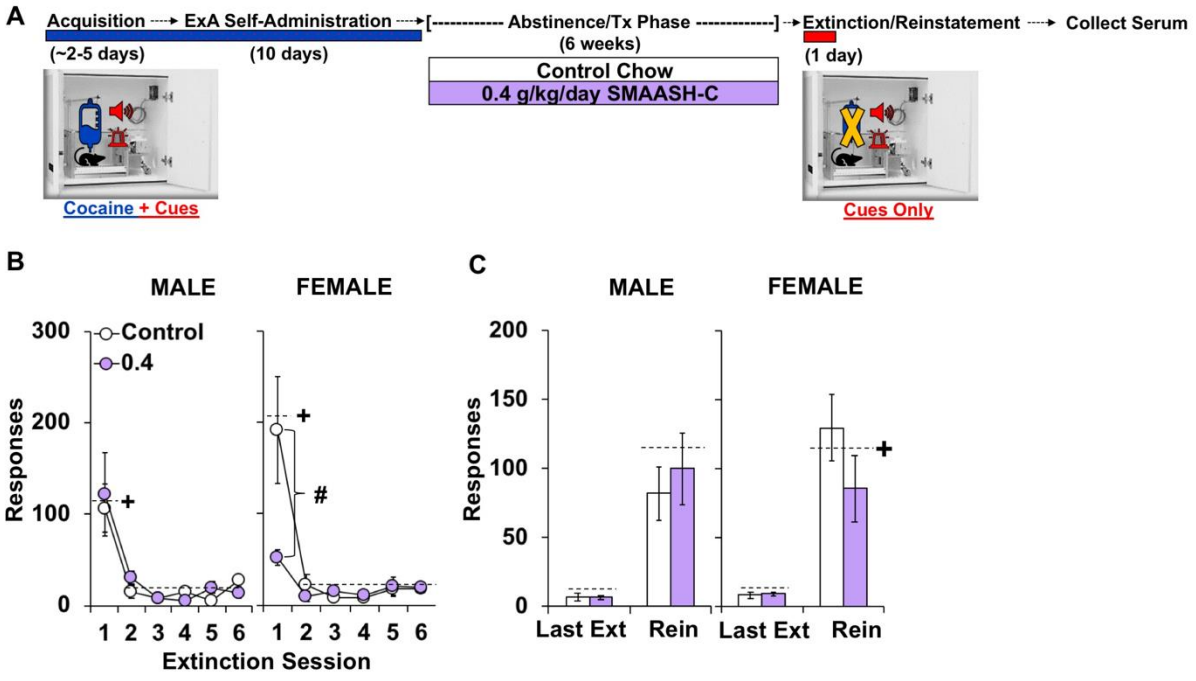

Figure 3. SMAASH-C blunts cocaine-seeking in response to the lever associated with drug availability following a six-week treatment regime over abstinence in females. Male and female rats were trained to self-administer cocaine under a fixed ratio (FR) 1 schedule (acquisition). Once they reach the acquisition requirements (2 days, 20 infusions), they were given extended-access (ExA; 4 discrete trials/h, maximum of 96 infusions/day, 10 days) to cocaine self-administration. Then, rats were given access to cocaine in one additional FR1 session (maximum of 20 infusions/day) to verify patency prior to the start of abstinence. On abstinence day one, rats were randomly assigned to one of two daily SMAASH-C treatment conditions: control-chow (6 males, 5 females) or 0.4 g/kg SMAASH-C (7 males, 6 females). The SMAASH-C was administered daily over a six-week abstinence period by mixing it in fresh, ground chow (Teklad LM-485 7912). On abstinence day 43, rats underwent extinction testing in a minimum of 6, 1-hr sessions and once cocaine-seeking was extinguished (<15 responses), cue-induced reinstatement testing in a 1-hr session. Serum was collected the next morning (**A**). The data are plotted as mean (±SEM) number of responses on the lever formerly associated with cocaine during the first six 1-h extinction sessions (**B**) and during the last extinction session versus the reinstatement session (**C**) in males (left) and females (right). +Significantly effect of session (**B**, **C**). #Significantly effect of SMAASH-C (**C**).

3.2.3 Cocaine-Induced Toxicity

As in Experiment 1, six-weeks of SMAASH-C treatment over abstinence improved markers of cocaine-induced toxicity in males, but not females. However, in contrast to Experiment 1, in this experiment AST levels were increased in both the SMAASH-C and control-chow groups in both

males and females (**Figure 4A**). Results from the univariant analysis of AST levels revealed an effect of treatment group ($F_{2,22}$=6.2, p<0.01) and a trend for an effect of sex ($F_{1,22}$=3.9, p=0.06) due to higher levels of AST in males versus females (242.8±20 versus 183.3±23, respectively), but a non-significant interaction of sex by treatment. Post-hoc comparison to saline across males and females revealed significantly higher AST levels in the control-chow group (p<0.05) and a trend for higher levels in the SMAASH-C group (p=0.072). For ALT, there was a trend for an overall effect of treatment group (**Figure 4B**; $F_{2,20}$=3.4, p=0.053), which was driven by higher ALT levels in the control-chow and SMAASH-C groups compared to the saline group (p<0.05). For amylase, there was an interaction of sex and treatment group (**Figure 4C**; $F_{2,21}$=3.8, p<0.05) and subsequent within-sex analyses revealed a significant effect of treatment group in males only ($F_{2,12}$=15.5, p<0.001) due to higher amylase levels in the control-chow group as compared to both the saline (p<0.05) and SMAASH-C (p<0.05) groups. Notably, there was no difference in amylase levels between the saline and SMAASH-C groups indicating that in males SMAASH-C treatment normalized cocaine-induced increases in amylase. For urea nitrogen (**Figure 4D**), there were no overall or interactive effects of sex or treatment group. Thus, following chronic cocaine self-administration and 6 weeks of abstinence, levels of the liver enzymes AST and ALT were increased in both males and females and the pancreatic enzyme amylase was increased in males. While AST and ALT were not normalized in either sex by SMAASH-C, the cocaine-induced increase in amylase levels observed in males was normalized by SMAASH-C treatment during abstinence.

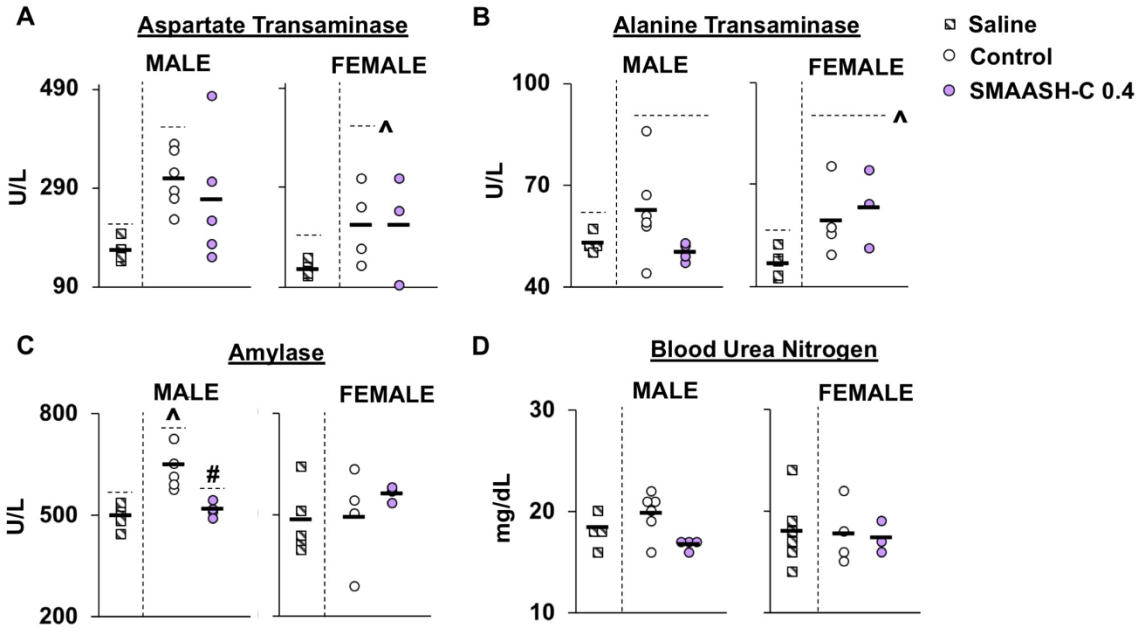

Figure 4. SMAASH-C blunts cocaine-induced toxicity following a six-week treatment regime over abstinence in males. Mean (±SEM) serum concentration of aspartate transaminase (AST; **A**), alanine transaminase (ALT, **B**), amylase (**C**), and urea nitrogen (**D**) in saline (n=5 males and n=6 females), control-chow (n=6 males and n=4 females) and 0.4 g/kg SMAASH-C (n=5 males and n=3 females) rats. ^Significant difference from baseline (control-chow saline) (**A**, **B**, **C**). #Significant effect of SMAASH-C (**C**).

3.3 Summary

A summary of the behavioral and toxicity findings across both experiments is shown in **Figure 5** with data presented as percent difference from the 2-week (**A**), control-chow (**B**), and saline control groups (**C-E**). Our behavioral findings show that cocaine-seeking (i.e., total responses during reinstatement plus the first extinction session) was higher in both males and females tested following six-versus two-weeks of abstinence (or 0; **Figure 5A**; *p*'s<0.05), and that in females, but not males, both the 2- and the 6-week SMAASH-C treatment regimens decreased cocaine-seeking relative to control-chow females (or 0; **Figure 5B**; *p*'s<0.01).

Our toxicity findings for percent difference from control AST levels (**Figure 5C**) were similar to the previous analysis and within the 2-week groups and revealed an effect of treatment group ($F_{1,25}$=6.3, p<0.05) and trends for a sex ($F_{1,25}$=3.4, p=0.076) and a sex and treatment group interaction ($F_{1,25}$=3.1, p=0.089). Subsequent within-sex analyses revealed a significant effect of treatment group within males ($F_{2,13}$=18.1, p<0.001), but not females. AST levels also did not differ from saline control values for males in the SMAASH-C group, whereas they were significantly

higher than saline control values (versus 0) for both males and females in the control chow group (*p's*<0.05) and for females in the SMAASH-C group (*p*<0.01). While no significant overall or interactive effects of sex or treatment group were observed for percent difference in AST levels within the 6-week condition, subsequent comparison across groups/sex confirmed that AST levels were significantly higher than saline control values (or 0; *p*<0.001). For ALT (**Figure 5D**), findings within the 2-week condition revealed an effect of treatment group ($F_{1,25}$=4.3, p<0.05control-chow) and a trend for a sex effect ($F_{1,25}$=3.5, p = 0.072). However, subsequent comparison within control-chow males and females confirmed that ALT levels were not significantly increased from saline control values indicating that, similar to the previous analysis, ALT is not elevated following two weeks of cocaine abstinence. By 6-weeks, however, ALT levels were significantly increased in control-chow males and females relative to saline control values (or 0; *p*<0.05). Additionally, as with the previous analysis, the analysis of percent change in ALT levels within the 6-week condition revealed an effect of sex ($F_{1,25}$=4.9, p<0.05) which is likely due to increases in ALT levels in both control-chow and SMAASH-C females. However, similar to the original analysis, no overall or interactive effects of treatment group were observed in this analysis. Finally, findings within the 2-week condition for amylase revealed an effect of sex ($F_{1,25}$=8.5, p< 0.01) and a trend for a sex by treatment group interaction ($F_{1,25}$=3.2, p=0.085) which appears to be driven by a sex difference with the SMAASH-C group (p<0.001) with females showing decreases in amylase and males showing increases (relative to saline controls or 0; *p*<0.01 and *p*<0.05, respectively). The analysis following 6 weeks of cocaine abstinence revealed a significant interaction of treatment group by sex ($F_{1,13}$ =5.8, p<0.05) which was driven by control-chow males having a larger increase in amylase than control-chow females (p<0.05) and SMAASH-C males (p<0.05). Indeed, subsequent within-sex analyses revealed a significant effect of treatment group within males ($F_{1,8}$ =14.3, p<0.01), but not females, with the follow-up comparison within males confirming an increase in amylase (relative to saline control values or 0) within control-chow males (*p*<0.01), but not SMAASH-C males.

Thus, cocaine-seeking incubated in both males and females over abstinence, and in females, SMAASH-C effectively reduced cocaine-seeking at both time-points. Both males and females also showed elevated markers of organ toxicity following abstinence and these effects persisted even after 6-weeks of abstinence. In males, SMAASH-C effectively reduced markers of

cocaine-induced toxicity, with normalization observed for AST following 2-weeks of SMAASH-C treatment and for amylase following the 6-week treatment.

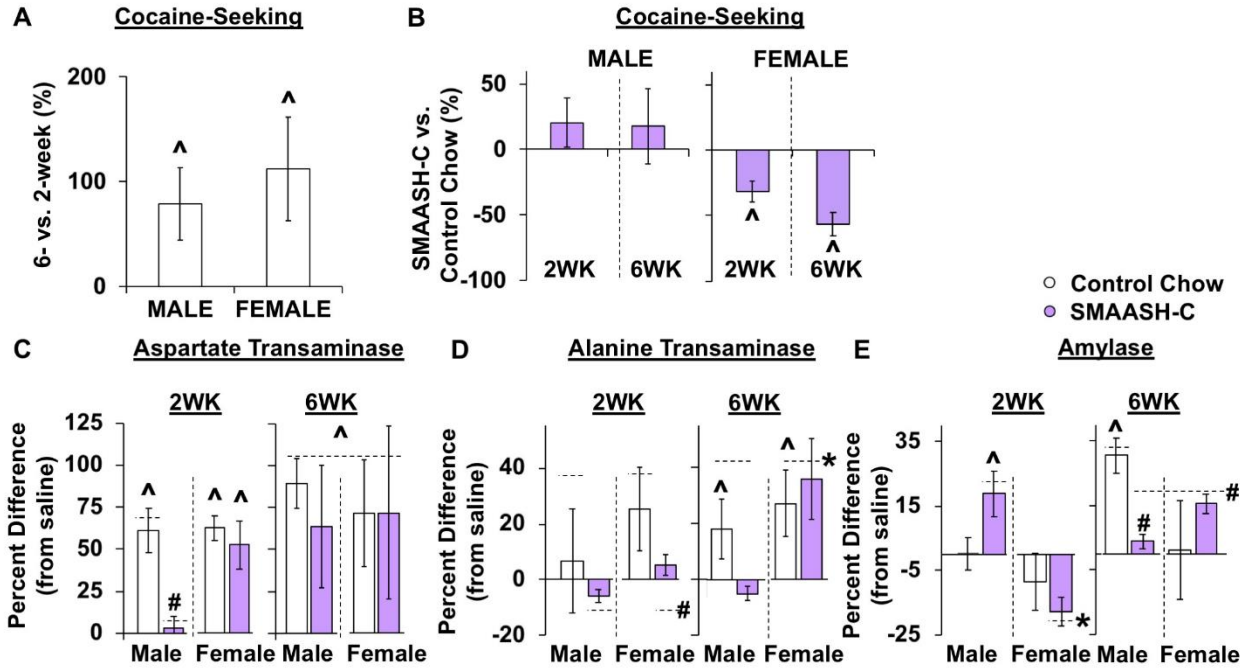

Figure 5. A summary of the behavioral and toxicity findings across both experiments. Mean (±SEM) percent difference from the 2-week (**A**), control-chow (**B**), and saline control groups (**C-E**). ^Significant difference from baseline (or 0; **A-E)**. #Significant effect of SMAASH-C (**C, D, E**). *Significantly effect of sex (**D, E**).

4 Discussion

The goal of the present study was to determine the efficacy of oral treatment during abstinence with a novel nutritional supplement, SMAASH-C, on markers of relapse vulnerability and cocaine-induced toxicity. We examined effects in both males and females and found that SMAASH-C treatment during abstinence reduced drug-seeking in females and markers of cocaine-induced toxicity in males. In females, SMAASH-C treatment over a two-week abstinence period reduced drug-seeking in response to cocaine-associated cues (stimulus light and sound of the pump), particularly when females were tested during estrus, when levels of responding are heightened; whereas, treatment over a six-week abstinence period reduced drug-seeking in response to the lever associated with drug availability during extinction testing, particularly during the first extinction session. Despite the lack of efficacy of SMAASH-C to reduce drug-seeking in males, two-weeks of SMAASH-C treatment normalized cocaine-induced

increases in AST, a marker of liver functioning, and six-weeks of SMAASH-C treatment normalized cocaine-induced increases in amylase, a marker of pancreatic functioning. Thus, the beneficial effects of oral SMAASH-C treatment over abstinence following chronic cocaine self-administration appears to be sex-specific.

To our surprise, following ExA cocaine self-administration, SMAASH-C treatment over abstinence reduced cocaine-seeking in females, but not males. The sex-specific efficacy of SMAASH-C appears to be rather robust considering that these effects were observed following both two- and six-weeks of treatment. SMAASH-C was also most effective in females tested during estrus, when levels of relapse vulnerability is the highest. Notably, multiple studies with cocaine and other addictive drugs (opioids) have shown that, following ExA drug self-administration, females have an enhanced vulnerability to drug-associated cues during late (14 days) and protracted abstinence (47 days) as compared to males, and that these effects are most evident when females were tested during estrus versus non-estrus phases of their estrous cycle (Corbett et al., 2021; Nicolas et al., 2019; Bakhti-Suroosh et al., 2019; Towers et al., 2022). In fact, this sex difference can be missed when estrous cycle phase is not considered or the sample size is small since non-estrus females and males tend respond similarly (Nicolas et al., 2019; Corbett et al., 2021; Towers et al., 2022). This likely explains the lack of sex difference in relapse vulnerability within control-chow group from Experiment 2. Thus, SMAASH-C treatment during cocaine abstinence appears to blunt relapse vulnerability when females are most vulnerable (during estrus). The mechanism underlying the efficacy of SMAASH-C in females is unknown and is difficult to determine considering the mechanisms underlying sex- and estrous cycle-dependent changes in addiction liability have been historically understudied.

Notably, over the 2- and 6-week cocaine abstinence periods we observed signs of extreme toxicity with one male and two females in the control-chow group and two females in the SMAASH-C group spontaneously developing unexplainable, life-threating illnesses that resulted in their removal from the study (or death). We also observed blood in the urine of a female and male in the control-chow group that had been withdrawn from cocaine for 6 and 37 days, respectively. The follow-up urinalysis for the male indicated kidney failure. These observations lead us to assess serum markers for organ health. Surprisingly, the results revealed no difference in the marker for kidney toxicity (urea nitrogen); however, urea nitrogen is not the preferred marker for renal function and, unfortunately, the small animal chemistry panel was not

sensitive enough to detect individual differences in creatinine levels. Notably, the panel did reveal marked differences in makers of liver toxicity (AST and ALT) in both females and males following two- and six-weeks of cocaine abstinence as well as signs of pancreatic toxicity in males following six-weeks of cocaine abstinence (**Figure 6**). These results align with findings in humans which have shown that cocaine use can induce extreme hepatoxicity accompanied with high level of aminotransferases (AST and ALT), which can be fatal and is often accompanied with other major organ involvement (Dix et al., 2021).

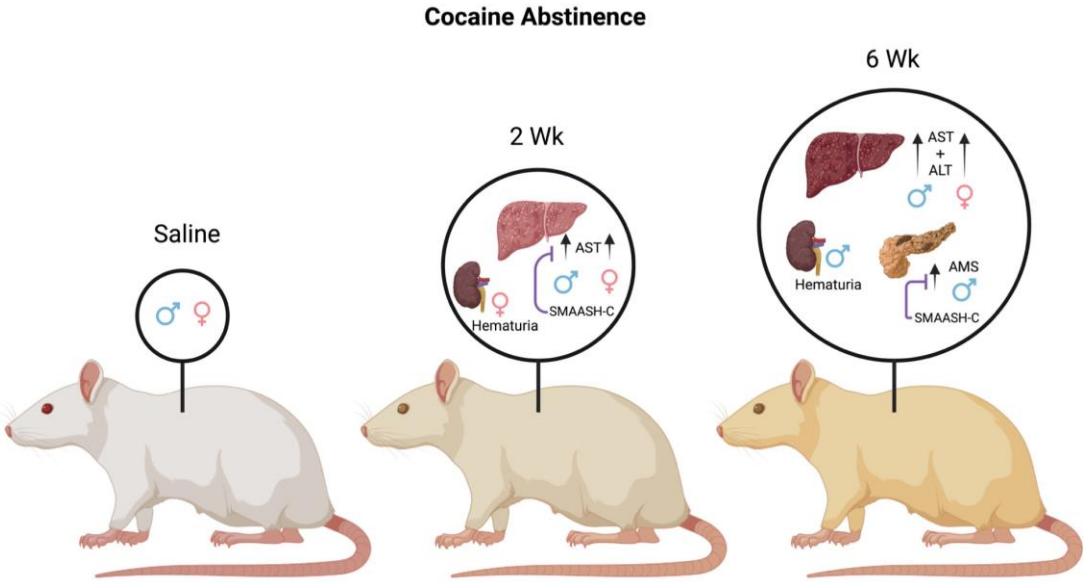

Figure 6. A graphical summary of toxicity changes over abstinence. In drug-naïve saline controls no organ toxicity would be expected. In contrast, AST serum levels, which is a marker of liver damage, was elevated in both males (n=4) and females (n=4) 2-weeks following extended-access cocaine self-administration. SMAASH-C blocked the effect in males (n=11). Also, in two-week abstinence cohort, hematuria was observed in one female. In the 6-week group, AST and ALT serum levels (ALT is another marker of liver damage) was elevated in males (n=10 and 11, respectively) and females (n=7). AMS serum levels (a marker for pancreatic dysfunction) increased in males (n=6), with SMAASH-C blocking the effect (n=4). Also found in 6-week group, one male (n=1) with hematuria.

Surprisingly, the beneficial effects of SMAASH-C treatment on cocaine-induced toxicity were only apparent in males with two weeks of SMAASH-C treatment improving a marker of liver toxicity (AST) and six weeks of SMAASH-C treatment improving a marker of pancreas toxicity (amylase). More specifically, while both males and females had increases in AST

following two weeks of cocaine abstinence, only males showed improvement with SMAASH-C treatment. We also observed a similar sex difference in the development of severe health complications over cocaine abstinence with both males and females in the control-chow group having to be removed from the study due to severe illness (or death); whereas, among SMAASH-C treated rats, only females became ill indicating that SMAASH-C treatment may improve the overall health of males, but not females, during cocaine abstinence. A previous study reported that SMAASH-C offers protective effects on organ health by increasing plasma clearance of cocaine and its toxic metabolites through increasing the activity of the liver enzyme CYP450 (Gardner et al., 2015). One caveat is that while both males and females were included in this previous study; sex was not considered so it is still possible that effects on liver activity were driven by males. Future research is needed to address this question and to determine the mechanisms underlying these sex-specific health benefits of SMAASH-C treatment. One possibility worth consideration is that since the course for development of drug-related health consequences is accelerated in females (see Towers et al., 2023b and Shalev et al., 2002 for reviews), it is possible that cocaine-induced liver damage may have been more severe/occurred earlier and thus less responsive to treatment over abstinence. This idea is also supported by the fact that at 6 weeks SMAASH-C is no longer effective at reducing abstinence-induced increases in AST in males along with the earlier observation of blood in the urine of the control female versus male.

In summary, the present findings validate anecdotal reports and open label observational data in humans indicating that SMAASH-C reduces relapse vulnerability and improves the health of patients with CUD. SMAASH-C is in an oral, non-prescription treatment, and as such, it would be relatively easy to administer at home and have few barriers to access for patients with CUD. Future studies are needed to determine whether SMAASH-C also reduces vulnerability to other aspect of the disease process, such as motivation for cocaine and compulsive use. It would also be helpful to understand the mechanisms underlying the sex-specific benefits; however, there are many treatments used in practice today that we do not understand the mechanism of action. Therefore, this lack of understanding should not delay further exploration of the beneficial application of SMAASH-C in patients with CUD and potentially other substance use disorders.

Chapter VI

Sex- and Dose-Dependent Differences in the Development of an Addiction-Like Phenotype Following Extended-Access Fentanyl Self-Administration

1 Introduction

Opioid use disorder (OUD) is a major epidemic in the United States. The epidemic is intensifying with opioid-involved overdose deaths reaching the highest number ever recorded in the 12-month period leading up to April 2021, which was primarily driven by fentanyl, a synthetic opioid (Centers for Disease Control and Prevention, 2021). The National Institute on Drug Abuse (NIDA) has highlighted an urgent need for research on risks and outcomes of OUD specifically with fentanyl (NIDA, 2021). The influence of biological factors, such as sex, on risks and outcomes of OUD are important to consider given that the opioid epidemic has been particularly impactful on women. For example, although men have higher rates of OUD and opioid-induced overdose deaths than women, differences have narrowed in the current opioid epidemic (i.e., 2.3-1 male-to-female ratio in 2002 versus a 1.8-1 ratio in 2018; National Survey on Drug Use and Health, 2018) with women showing a sharper increase in opioid use in the past decade than men (e.g., 283% versus 108% increase in heroin use from 2007 to 2014; Marsh et al., 2018) and being more likely than men to be prescribed opioids and to misuse prescription opioids (Mazure et al., 2018). Women are also more sensitive to the reinforcing effects of opioids, develop OUD more rapidly, and have higher craving in response to drug cues compared to men (Lynch and Carroll, 2001; Aldelson et al., 2018; Hernandez-Avila et al., 2004; Hser 1987a,b; Back et al., 2011a; Anglin et al., 1987; Kennedy et al., 2013; Moran et al., 2018; Yu et al., 2007).

In response to this need to understand the impact of biological sex on risks and outcomes of OUD with fentanyl, we recently developed an extended-access fentanyl self-administration procedure that readily induces addiction-like features, including binge-abstinent patterns of use and an enhanced vulnerability to relapse, in both male and female rats (Bakhti-Suroosh et al., 2021). This latter feature, the enhanced vulnerability to relapse, emerged following extended-access self-administration and protracted withdrawal and was blocked in both males and females by administering buprenorphine, an FDA-approved treatment for OUD, during abstinence thus validating our relapse model. Importantly, our preclinical findings were also similar to reports of sex/gender differences in humans and showed that females self-administered higher levels of fentanyl during the extended-access phase and responded at higher levels than males during subsequent relapse testing, especially when they were tested during estrus versus non-estrus phases of their cycles. These findings demonstrated that both males and females developed an

addiction-like phenotype when given extended-access to fentanyl and demonstrated that sex is an important risk factor for both intake and the development of expression of relapse vulnerability with fentanyl.

Now in this study, our goal was to determine sex-differences across a broad dose range, including high doses expected to maximize the expression of addiction-like features (e.g., vulnerability to relapse and physical dependence). This is important because in our original study we focused on effects of a low dose of fentanyl (0.25 ug/kg/infusion) since low doses engender greater individual differences and are thus more sensitive to sex differences. However, low doses may not maximally induce an addiction-like phenotype considering that higher drug intake and/or frequency of use is predictive of an enhanced vulnerability to relapse in both humans with an OUD (Grau-Lopez et al., 2012; Smyth et al., 2010; Gossop et al., 2002) and in animal models with other addictive drugs (Mantsch et al., 2004). Thus, in the current study we examined fentanyl self-administration across a broad range of fentanyl doses (0.25, 0.75, 1.5, and 3.0 µg/kg/infusion) and hypothesized that relapse vulnerability would be highest following high dose fentanyl self-administration. We also expanded our model to include an additional key feature of OUD in humans, physical dependence (American Psychiatric Association, 2013), as assessed by spontaneous weight loss during early withdrawal, a highly predictive single factor of withdrawal (Townsend et al., 2021; Seaman and Collins, 2021; Bobzean et al., 2019; Houshyar et al., 2004; Maldonado et al., 1992; Navarro-Zaragoza et al., 2010; Pinter-Kubler et al., 2013; Cicero and Meyer et al., 1973; Gellert and Holtzman, 1978; Nickel and Aledter, 1987). Physical dependence is a defining feature of OUD in humans (American Psychiatric Association, 2013) and women experience a more severe withdrawal syndrome than men (Huhn and Dunn 2020). Given that higher drug intake/frequency of use is also predictive of greater physical dependence, we hypothesized that weight loss would be greatest following high dose fentanyl self-administration. Based on findings in humans and our previous results with fentanyl, we further hypothesized that the expression of enhanced vulnerability to relapse would be greater in females than males.

2 Materials and Methods

2.1 Subjects

Sexually mature male (N =29) and female (N =29) Sprague-Dawley rats (Charles River) that weighed approximately 250 g (female) and 340 g (male) upon arrival were used as subjects in

this study. At the start of the study, rats were individually housed in operant test chambers (Med Associates, St. Albans, VT, USA) with ad libitum access to water and food (Teklad LM-485 7912; except as noted below for some animals during fentanyl self-administration training) and maintained on a 12-h light/dark cycle (lights on at 7AM). After a 2-day acclimation period, rats were pre-trained to lever-press for sucrose pellets (45 mg) in 24-hr/day sessions under a fixed-ratio 1 schedule to ensure rapid subsequent acquisition of fentanyl self-administration. Sessions continued daily until lever-press responding was acquired (2 consecutive days wherein >50 pellets were obtained, typically 2-3 sessions; Lynch, 2008). Rats were weighed three times a week and health was monitored daily throughout the study. Body weight was used as an assessment of overall health during extended-access and as a measure of physical dependence to fentanyl. Physical dependence has been assessed previously by expression of opioid withdrawal syndrome upon cessation of chronic opioid exposure and spontaneous loss of body weight has long been used as a highly predictive single factor of withdrawal (Houshyar et al., 2004; Maldonado et al., 1992; Navarro-Zaragoza et al., 2010; Pinter-Kubler et al., 2013; Cicero and Meyer et al., 1973; Gellert and Holtzman, 1978; Nickel and Aledter, 1987). All procedures were conducted within animal care guidelines set by the National Institute of Health and were approved by The University of Virginia Animal Care and Use Committee.

2.2 Procedure

2.2.1 Surgery and Catheter Maintenance

After lever pre-training, rats underwent jugular catheterization surgery using methods previously described (Lynch 2008). Briefly, rats were anesthetized with ketamine/dexdomitor and implanted with an indwelling catheter (Silastic tubing; 0.51 and 0.94 mm o.d.; Dow Corning, Midland, MI, USA) into the right jugular vein. Catheters were flushed with heparinized saline three days a week to help verify and help maintain patency. If the patency of a catheter was questionable, patency was verified by administering methohexital (1.5 mg/kg). Any catheter that was no longer patent (i.e., the catheter was leaking, pressure prevented flushing, or the animal did not lose the righting reflex immediately after methohexital) was replaced with a new catheter implanted into the left jugular vein with testing resuming following recovery from surgery (1-2-days).

2.2.2 Fentanyl Self-Administration Training

Following recovery from surgery, rats were randomly assigned to self-administer one of four fentanyl doses (µg/kg/infusion): 0.25 (9 females, 8 males), 0.75 (9 females, 8 males), 1.5 (8 females, 8 males), or 3.0 (7 females, 8 males). These doses were selected because the majority of studies using fentanyl self-administration procedures in rodents use a dose of fentanyl ranging from 0.25 to 2.5 ug/kg (Morgan et al., 2002c; Wade et al., 2015; Martin et al., 2021; Bakhti-Suroosh et al., 2021; Dao et la., 2021; Malone et al., 2021; Fragale et al., 2021; Hammerslag et al., 2021); therefore, we selected a dose range that included both low (0.25 and 0.75 µg/kg/infusion) and moderate-to-high doses (1.5 and 3.0 µg/kg/infusion; Morgan et al., 2002c; Wade et al., 2015; Martin et al., 2021; Dao et la., 2021; Malone et al., 2021; Hammerslag et al., 2021) in order to maximize the likelihood of sex and group differences in levels and patterns of fentanyl self-administration and subsequent effects on relapse vulnerability. Rats were trained to self-administer their assigned dose of fentanyl under a fixed-ratio 1 schedule with a one second time out following each infusion and a maximum of 40 infusions/day (Bakhti-Suroosh et al., 2021). At the beginning of each session, the left-lever was extended into the chamber and remained extended until the session ended once all 40 infusions were obtained or until 11:50 AM the next day. Each response on the left lever produced an infusion of fentanyl which was paired with the sound of the pump and the illumination of a stimulus light above the lever. The right lever remained extended throughout the session and responses on this lever (inactive) were recorded but had no consequence. Sessions were conducted daily until acquisition occurred (i.e. 5 consecutive days wherein all 40 infusions were obtained). Moderate food restriction (85% of its free-feeding body weight) was used briefly (2-3 days) when necessary (i.e. fewer than 15 infusions/day by training day 5). All groups acquired fentanyl self-administration rapidly under these conditions and rates of acquisition did not differ between groups.

2.2.3 Extended-Access Fentanyl Self-Administration

Once rats acquired fentanyl self-administration, they were given extended, 24-hr/day access to fentanyl for ten consecutive days under an intermittent-access procedure shown to induce an addiction-like phenotype in both males and females (Bakhti-Suroosh et al., 2021). With this procedure, rats have unrestricted, fixed-ratio 1 access (no time-out after infusions) to rapidly delivered infusions of fentanyl (within 1-2 seconds) during 5-min trials that initiated every 30-min around the clock. Each trial began with the extension of the left-lever into the operant

chamber; each response on this lever resulted in an infusion of fentanyl paired with the sound of the pump and the illumination of the stimulus light above the active lever. The 5-min trial ended with left-lever being retracted from the operant chamber. The right lever remained extended the entire duration of the session; responses on this lever were recorded but had no consequence. Two females in the 0.25 ug/kg group, one female and one male in the 0.75 ug/kg group, and one female and one male in the 1.5 ug/kg group were excluded from the study and all analyses due to patency, toxicity, or technical issues during acquisition or extended-access self-administration. The final group size for females and males was 7 and 7 for the 0.25 ug/kg group, 8 and 7 for the 0.75 ug/kg group, 7 and 7 for the 1.5 ug/kg group, and 7 and 8 for the 3.0 ug/kg group, respectively.

2.2.4 Extinction and Reinstatement Testing

Vulnerability to relapse was assessed on withdrawal day 15 using an extinction/cue-induced reinstatement procedure (Bakhti-Suroosh et al., 2021). Testing began between 12PM and 1PM with extinction responding being examined in a minimum of 6, 1-hr sessions (Sanchez et al. 2014; Peterson et al. 2014; Beiter et al. 2016). Each session began with the introduction of the left-lever into the operant chamber; responses on this lever, as well as the right lever, were recorded but did not have a consequence. Sessions continued until responding extinguished (≤15 responses/hr). This extinction criterion was typically meet within 6-9 sessions, and with the exception of 2 males and 3 females (as detailed below). Cue-induced reinstatement responding was assessed 5-min after the final extinction session in a 1-hr session. This session began with the introduction of the left-lever into the operant chamber and the presentation of the cues formerly associated with fentanyl (sound of pump activation and the light above the left-lever, 1-2 sec). Each response on the lever-lever produced these same cues under a fixed-ratio 1 schedule. For the 2 males (1 each in the 0.75 and the 1.5 ug/kg dose groups) and 3 females (2 in the 0.25 ug/kg dose group and one in 1.5 ug/kg dose group) that did not extinguish within the 9 extinction sessions run, their sessions terminated following the ninth extinction sessions, and then the next day, a second day of extinction testing was conducted the using the same procedures (i.e., 6-9 1-hr sessions) to ensure that responding had extinguished prior to reinstatement testing and reinstatement testing was conducted during a similar time in the light cycle. The reinstatement test session began once responding had extinguished using the same procedure as described

above. Data from the first day of extinction testing were used in the analyses of hourly extinction responses, whereas the second day was used for the last extinction session (versus reinstatement).

2.2.5 Estrous Cycle Phase Determination

In order to track the pattern of the estrous cycle leading up to relapse testing and to habituate rats to the procedure, the phase of the estrous cycle was determined daily over a five-day period beginning 3 days prior to extinction/reinstatement testing. The swabs of the vaginal epithelium cells were collected between 11AM and 12PM; male rats underwent similar handling by brushing their rear end with the cotton swab as described previously (Lynch et al. 2019). The phase of the estrous cycle was determined based on the proportion of three vaginal cell types: leukocytes, nucleated epithelial cells, and cornified epithelial cells. The rat was considered to be in estrus if there were an abundant number of cornified epithelial cells with no leucocytes, metestrus or diestrus if leukocytes were present, and proestrus if there were numerous, uniform in size round nucleated cells and no or few leucocytes. Swabs obtained on the day of extinction/reinstatement test were further categorized as either estrus (n =13) or non-estrus (n =17) based on findings from our group (Peterson et al. 2014; Lynch et al. 2019) and others (Kerstetter et al. 2008) showing that relapse vulnerability, including opioid-seeking, is highest during estrus, but not different between metestrus, diestrus, and proestrus (Bakhti-Suroosh et al., 2021; Nicolas et al., 2019; Corbett et al., 2021; Lynch et al. 2019; Lacy et al. 2020).

2.3 Drugs

Fentanyl hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC) and dissolved in sterile saline at a concentration of 6.25, 18.75, 37.5, or 75 µg/ml for the 0.25, 0.75, 1.5, and 3.0 µg/kg dose conditions, respectively. Fentanyl solutions were sterile filtered (0.22 µm; Millipore, Billerica, MA) and stored at 4 °C. The duration of infusions was adjusted for changes in body weight on Monday, Wednesday, and Friday to ensure that the mg/kg dose was consistent throughout the study.

2.4 Data Analysis

We first determined whether there was an effect of fentanyl dose on levels (ug/kg/day) and patterns of intake over the 10-day extended-access self-administration period. Patterns of intake included frequency of use (total number of infusions/day and total intake/day in μg/kg), number of active trials within each extended-access session, and "binge" intake (average intake/day in μg/kg within each of the "active" trials that had 1 or more infusions). Group differences were assessed using repeated measures ANOVA with sex and fentanyl dose as between subject factors and extended-access session as a within subject factor; separate analyses were used for each of the dependent measures. Given that sex differences have previously been shown to be most robust under low versus high drug dose conditions (Towers et al., 2019; Carroll et al., 2004; Lynch and Carroll 2001; Torres et al., 2014), following a significant interaction of sex by dose, we examined sex differences within the two low fentanyl dose groups (0.25 and 0.75 ug/kg) and the two high dose groups (1.5 and 3.0 ug/kg). Repeated measures ANOVA was also used to examine sex and group differences in body weight over arrival, training (day 3-5), extended access (ExA; ExA 1, days 4-6; ExA 2, days 9, 10, first day of withdrawal at the time of discontinuation of drug self-administration), and withdrawal (W1, days 2-4; W2, days 6-8; W3, days 12-14). To equate baseline sex differences in body weight, the same analysis was run for percent change in body weight over ExA-1 and ExA-2 relative to prior to the beginning of extend-access (training) as an assessment of overall health, and percent change in body weight over W1, W2, and W3 relative to ExA-2 as a measure of physical dependence to fentanyl. This measure (e.g., weight loss during spontaneous withdrawal) is a known proxy of physical dependence in animals and has long been used as a highly predictive, single factor of withdrawal (Houshyar et al., 2004; Maldonado et al., 1992; Navarro-Zaragoza et al., 2010; Pinter-Kubler et al., 2013; Cicero and Meyer et al., 1973; Gellert and Holtzman, 1978; Nickel and Aledter, 1987). A one-sample t-test was used to confirm significant decreased or increases in changes of body weight relative to baseline (or 0).

Effects on extinction and reinstatement were compared between the sexes and each of the dose groups as well as between males and females tested during estrus versus non-estrus phases. For extinction, we compared hourly responses on the formerly active lever within the first six extinction sessions run using repeated measures. The total number of responses on the formerly active lever were also using univariate ANOVA. For reinstatement, we compared responses on the formerly active lever during the last extinction session versus the cue-induced reinstatement

session. Associations between fentanyl intake/infusions and total extinction and reinstatement responding/ weight loss during withdrawal were assessed using the Pearson correlation coefficient. The analysis was performed collapsed across sex unless the univariant ANOVA determined there was a significant difference in the correlation coefficients for males and females. All post hoc comparisons were corrected for multiple comparisons using Tukey method. One tailed tests were used for all a priori predicted differences (higher intake/infusions in females than males, greater fentanyl-seeking in estrus females versus males and non-estrus females, positive association between fentanyl intake/infusions and extinction/reinstatement responding); all other tests were two-tailed. Statistical analyses were performed using SPSS (V26) with alpha set at 0.05.

3 Results

3.1 Extended-Access Fentanyl Self-Administration

Sex and dose-dependent effects were observed for the number of infusions self-administered over the 10-day extended-access period (**Figure 1A)** with results revealing significant overall effects of sex ($F_{1, 50}$= 8.5, P<0.01), dose ($F_{3, 50}$= 11.4, P<0.001), and session ($F_{9,450}$= 2.6, P<0.01) as well as significant interactions of sex by dose ($F_{3, 50}$= 3.2, P<0.05) and session by dose ($F_{27, 450}$= 1.6, P<0.05). While the overall effect of sex indicates higher infusions in females than males, this difference appears to be driven primarily by effects at the lower doses given the sex by dose interaction as well as results from the subsequent analyses within the low (0.25 and 0.75 ug/kg) and high (1.5 and 3.0 ug/kg) doses which revealed a significant effect of sex within the low ($F_{1, 27}$= 9.7, P<0.01), but not high doses (P>0.05). While the overall effect of dose reflects higher infusions at the lower versus higher doses (0.25 versus 1.5 and 3.0 ug/kg, P's<0.05; 0.75 versus 1.5 and 3.0 ug/kg; P's<0.05), this difference appears to be driven primarily by effects in females given the significant interaction of sex by dose as well as results from the follow up comparisons within females and males which revealed a significant effect of dose within females ($F_{3, 25}$= 11.4, P<0.001), but only a trend for an effect of dose within males (P=0.076). Further comparison within females revealed significant differences between the two lower doses and the two higher ones (0.25 versus 1.5 and 3.0 ug/kg, P's<0.001; 0.75 versus 1.5 and 3.0 ug/kg, P's<0.05). Finally, the overall effect of session appears to be attributable to a decrease in infusions from session 1 to 2 (t_{57} = 4.2, P<0.001) as well as an increase in infusions from session

2 to 10 (t_{57} = 2.3, P<0.05). However, both effects were apparent for the two highest doses, but not the two lowest doses, which likely accounts for the significant interaction observed between dose and session. Indeed, subsequent analysis within each of the doses revealed a significant overall effect of session within the 1.5 and 3.0 ug/kg doses ($F_{9, 108}$= 4.1, P<0.001 and $F_{9, 117}$= 3.7, P<0.001, respectively), but not the 0.25 or 0.75 ug/kg doses (P's>0.05); subsequent analyses within the 1.5 and 3.0 ug/kg doses also confirmed a significant decrease in infusions from session 1 to 2 and a significant increase in infusions from sessions 2 to 10 for both doses (P's <0.01). Thus, females self-administered more fentanyl infusions than males, particularly at low doses.

Average daily fentanyl intake (μg/kg) over the extended-access period was greatest in rats given access to higher versus lower doses of fentanyl (**Figure 1B**; effect of dose, $F_{3, 50}$= 23.4, P<0.001) with the 3.0 ug/kg group having the highest intake (versus 0.25, 0.75, and 1.5 ug/kg, P<0.05) and the 0.25 ug/kg group having the lowest intake (versus 0.75 and 1.5 ug/kg, P's <0.05). In contrast to effects with infusions, dose-dependent effects on intake were apparent for both males and females (non-significant interaction of dose by sex). Although no overall or interactive effects of sex were observed for intake (P's>0.05), a planned comparison of males and females in the low dose groups (0.25 and 0.75 ug/kg) confirmed that, similar to effects with infusions, females had higher fentanyl intake than males (t_{27}= 1.7, P<0.05). Thus, fentanyl intake dose-dependently increased in both males and females with increases in fentanyl dose with females taking more fentanyl than males at low fentanyl doses.

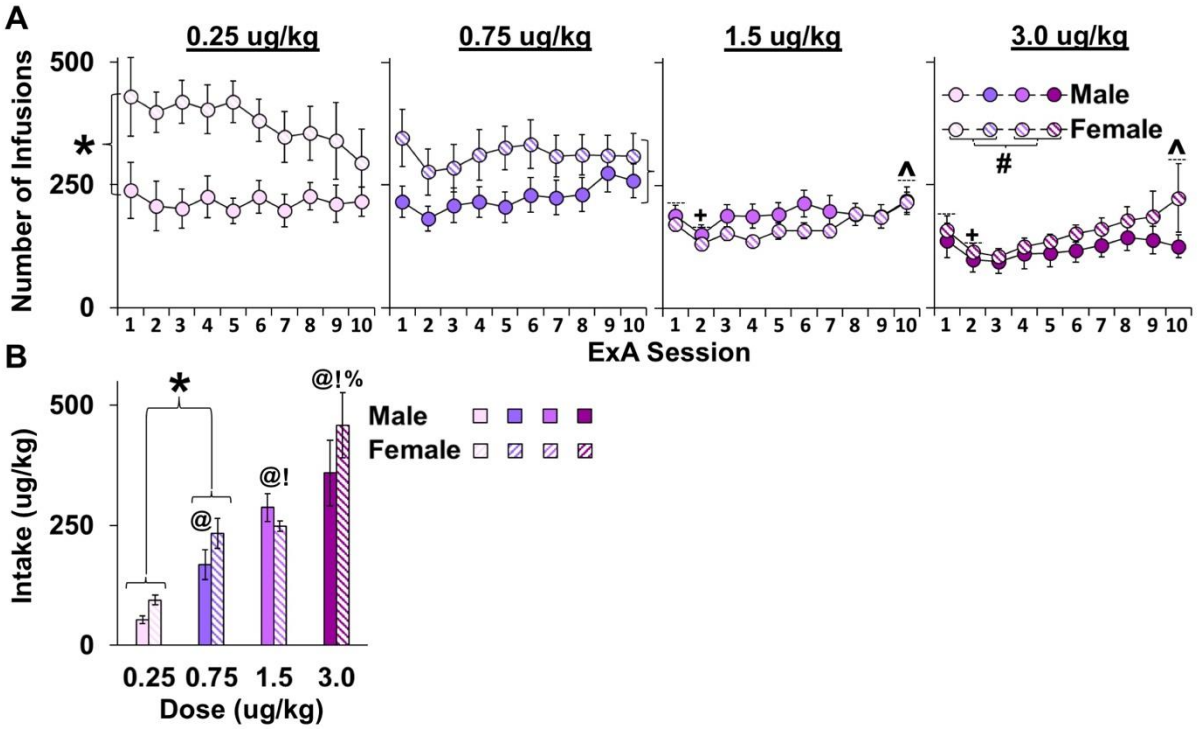

Figure 1. **Effect of sex and dose on number of infusions and fentanyl intake in female and male rats under extended-access conditions.** Mean (±SEM) number of infusions for each of the ten extended-access sessions (**A**) and fentanyl intake averaged across the extended-access period (ug/kg; **B**) for females and males in the 0.25 (n= 8 males, n= 9 females), 0.75 (n= 8 males, n= 9 females), 1.5 (n= 8 males, n= 8 females), and 3.0 (n= 8 males, n= 7 females) dose (ug/kg) conditions. (*) Significant sex difference within the 2 lower doses. (+) Significant decrease from session 1. (^) Significant increase from session 2. (#) Significant difference between the 2 lowest and 2 highest doses in females. Significant difference from (@) 0.25, (!) 0.75, and (%) 1.5 ug/kg.

To further explore sex and dose-dependent differences in patterns of fentanyl self-administration, we also analyzed the number of active trials during each of the 10 extended-access sessions. This analysis revealed an overall effect of dose (**Figure 2A**; $F_{3, 50}$= 3.7, P<0.05) and session ($F_{9, 450}$= 21.0, P<0.001) and a trend for an interaction of sex by dose (P=0.069) and session by dose (P=0.085). The overall effect of dose appears to be attributable to rats in the 0.75 ug/kg dose having significantly more active trials compared to the lowest and highest dose conditions (0.75 versus 0.25 and 3.0 ug/kg, P's<0.05). As with findings for daily intake, the session effect in this analysis appears to be due to a decrease in the number of active trials from session 1 to 2 (t_{57} = 6.4, P<0.001) and an increase from session 2 to 10 (t_{57} = 8.4, P<0.001). However, these session effects appear to be more robust at higher versus lower doses, which

likely accounts for the trend for an interaction between dose and session. Although no overall effect of sex was observed ($P>0.05$), given the trend for an interaction of sex by dose ($P=0.069$) and our hypothesis that sex differences would be most apparent under low dose conditions, we examined sex differences within the low versus high dose groups. This analysis showed, that as with daily intake, females had more active fentanyl trials than males under low ($F_{1, 27}= 7.9$, $P<0.01$), but not high dose ($P>0.05$) conditions. Thus, rats in the 0.75 ug/kg group had more active trials than rats in the other dose groups and females in the low dose groups had more active trials than males in these groups.

Average binge fentanyl intake (µg/kg) within active trials across the 10 extended-access sessions was greater in females compare to males (**Figure 2B**; overall effect of sex, $F_{1, 50}= 4.8$, $P<0.05$) and in higher versus lower dose conditions ($F_{3, 50}= 61.4$, $P<0.001$; 3.0 versus 0.25, 0.75, and 1.5 ug/kg, P's<0.010; 1.5 versus 0.25 and 0.75, P's <0.01; 0.75 versus 0.25, $P<0.01$). Given that there were no significant overall or interactive effects of session (P's>0.05), these data are presented as average binge intake/day across the extended-access period to highlight the overall effects of sex and dose. There were no interactions of dose or sex (P's>0.05). Thus, in contrast to daily intake, the higher binge intake in females was similarly maintained across low and high fentanyl doses. However, similar to daily fentanyl intake, binge intake increased in both sexes with increases in fentanyl dose.

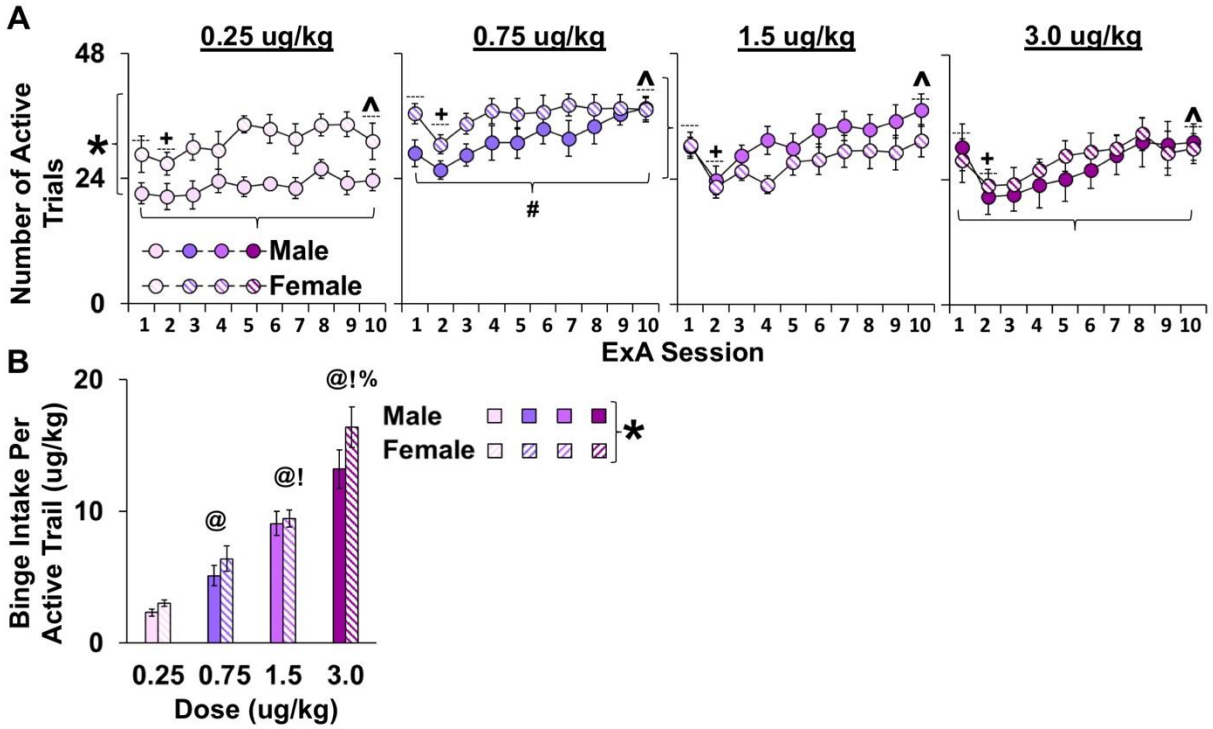

Figure 2. **Effect of sex and dose on binge fentanyl intake in female and male rats under extended-access conditions.** Mean (±SEM) number of active trials for each of the ten extended-access sessions (**A**) and binge fentanyl intake (ug/kg) in active trials across all extended-access session (**B**) for females and males within the 0.25 (n= 8 males, n= 9 females), 0.75 (n= 8 males, n= 9 females), 1.5 (n= 8 males, n= 8 females), and 3.0 (n= 8 males, n= 7 females) dose (ug/kg) conditions. (*) Significant sex difference within the 2 lower doses. (+) Significant decrease from session 1. (^) Significant increase from session 2. (#) Significant increase compared to 0.25 and 3.0 ug/kg. Significant difference from (@) 0.25, (!) 0.75, and (%) 1.5 ug/kg.

3.2 Changes in Body Weight During Extended-Access Self-Administration and Over Withdrawal

As expected, there was a marked sex difference in body weight across the study (**Figure 3A**; overall effect of sex, $F_{1, 50}$= 1338.6, P<0.001) with males weighing more than females. There were also significant overall effects of dose ($F_{1, 50}$= 4.2, P=0.01) and time ($F_{6, 300}$= 508.2, P<0.001), and interactions of time by sex ($F_{6, 300}$= 95.7, P<0.001), time by dose ($F_{18, 300}$= 3.3, P<0.001), and time by sex by fentanyl dose ($F_{18, 300}$= 4.3, P<0.001). Further analysis of body weight at the end of fentanyl self-administration training (days-3-5) just prior to the start of extended-access confirmed an overall effect of sex ($F_{1, 50}$= 906.7, P<0.001) with males weighing more than females (P<0.001), but no overall or interactive effects of dose (P's>0.05); therefore, to determine sex- and dose-dependent effects of extended-access fentanyl self-administration on body weight, we analyzed percent change in body weight from just prior to extended-access self-

administration (training) to after approximately 5 (ExA-1) or 10 (ExA-2) days of access (**Figure 3B).** Results from this analysis revealed an overall effect of dose ($F_{3, 50}$= 4.0, P<0.05) as well as significant interactions of sex by dose ($F_{3, 50}$= 5.5, P<0.01) and sex by day by dose ($F_{3, 50}$= 3.9, P<0.05). While the overall effect of dose reflects lower percent body weight gain in the 3.0 ug/kg group compared to the 0.75 ug/kg group (P<0.05), this difference appears to be driven by males given the significant interaction between dose and sex and the follow-up comparisons within males and females which revealed a significant effect of dose within males ($F_{3, 25}$= 7.7, P<0.001), but only a trend for an effect of dose within females (P=0.084). The analysis within males also revealed a significant interaction of day by dose ($F_{3, 25}$= 32.7, P<0.05) with follow-up comparisons within ExA-1 and 2 revealing that males in the 3.0 ug/kg dose gained significantly less weight compared to males in the 0.25, 0.75, and 1.5 ug/kg doses at ExA-1 and that males in both the 1.5 and 3.0 ug/kg doses gained significantly less weight than males in the 0.25 and 0.75 ug/kg dose at ExA-2 (P's<0.05). Given the significant interactions of sex by dose and sex by dose by day, we also examined sex differences within each of the dose groups. This analysis revealed a significant effect of sex within the 0.25 ug/kg dose group ($F_{1, 12}$= 12.0, P<0.01), wherein females had less percent body weight gain than males, as well as a trend for an effect of sex in the 3.0 dose group (P=0.052), wherein males tended to have less percent body weight gain than females. Thus, in males, fentanyl dose-dependently decreased percent body weight gain and the highest dose tended to have a greater anorectic effect in males than females. In contrast, females showed an enhanced sensitivity to the anorectic effect of the low dose of fentanyl compared to males, and although this effect may be the result of greater fentanyl intake in females than males at the lower doses, it was not further enhanced in females with increases in fentanyl dose.

We also analyzed percent change in body weight during early (W1, withdraw days 2-4), intermediate (W2, withdrawal days 6-8), and late withdrawal (W3, withdrawal days 13-15) relative to the end of extended-access self-administration (ExA-2) as a measure of physical dependence to fentanyl (**Figure 3C**). This analysis revealed significant overall effects of sex ($F_{2, 50}$= 33.2, P<0.001) and withdrawal time-point (early, immediate, late; $F_{2, 100}$= 341.4, P<0.001), which reflect greater increases in percent body weight in males versus females and at later versus earlier time-points during withdrawal (early versus intermediate and late, P's<0.001; intermediate versus late, P<0.05) as well as significant interactions of sex by withdrawal time-

point ($F_{2, 100}$= 50.5, P<0.001) and sex by withdrawal time-point by dose ($F_{6, 100}$= 2.7, P<0.05). The overall sex effect appears to be driven by changes during intermediate and late withdrawal given the significant interaction of sex by withdrawal time-point and the results from analysis within each withdrawal time-point which revealed significant effects of sex within the intermediate and late withdrawal time-points (P's<0.001), but not within the early withdrawal time-point (P>0.05). Further analysis within the early withdrawal time-point revealed a significant effect of dose ($F_{3, 50}$= 7.9, P<0.001) and significant differences between the 0.25 ug/kg dose and all other doses (P's<0.05). The decreases in percent body weights during early withdrawal were also significantly different from body weights at the end of the extended-access (ExA-2; versus 0) for each of the doses except the 0.25 ug/kg dose (P's<0.001). Further analysis within intermediate and late withdrawal revealed non-significant overall and interactive effects of dose indicating that the sex differences were due to greater percent body weight gain in males than females in each of the dose groups with males, but not females, surpassing their previous body weight at ExA2 by intermediate withdrawal (P<0.001); by late withdrawal, both males and females had surpassed their previous body weight at ExA-2 (versus 0; P's<0.001). Thus, rats in the three highest dose groups lost body weight during early withdrawal and this weight loss was similar between males and females indicating that physical dependence was expressed similarly in males and females following fentanyl self-administration at 0.75 ug/kg doses and higher. Despite the similar weight loss between males and females during early withdrawal, weight loss persisted longer in females compared to males.

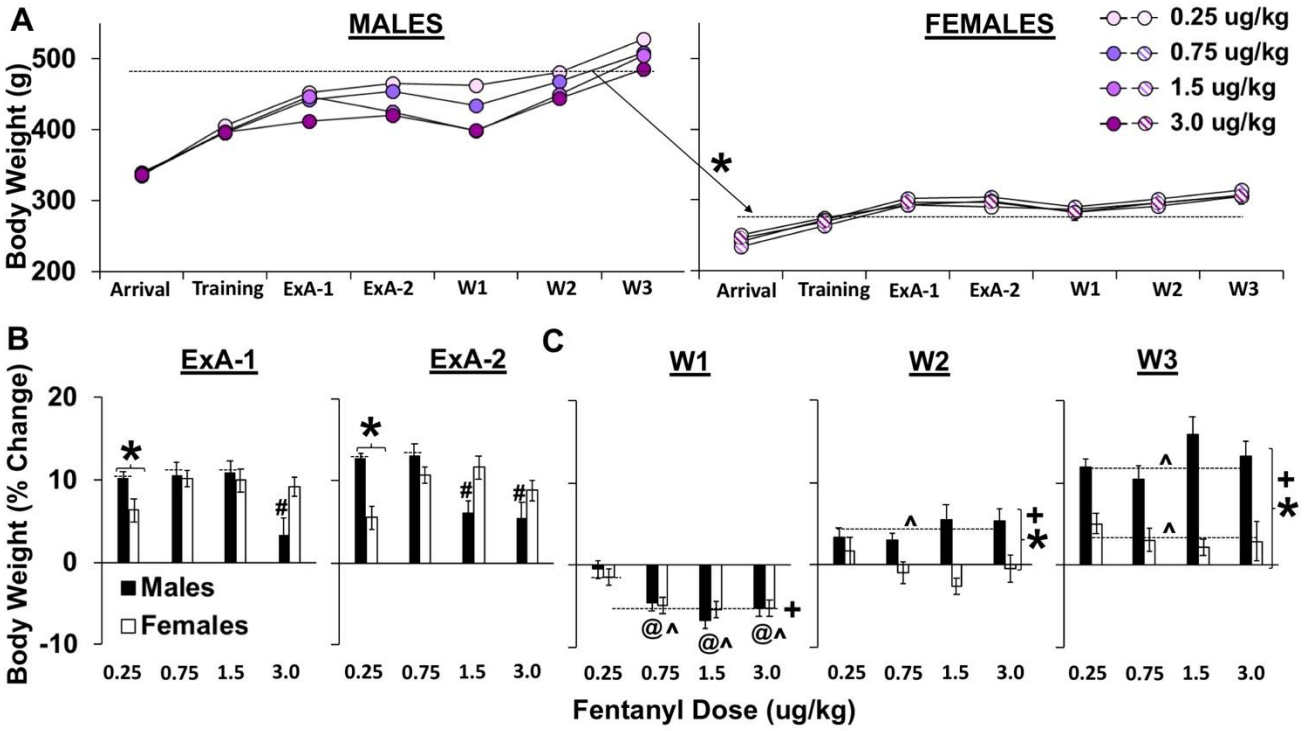

Figure 3. **Effect of sex and dose on body weight (g) in female and male rats during extended-access self-administration and withdrawal.** Mean (±SEM) body weight (g) at arrival, the end of self-administration training (training, days 3-5) just prior to extended-access self-administration, following approximately 5 days (ExA-1, days 4-6) and 10 days (ExA-2, days 9-10 or at the time of discontinuation of drug self-administration on withdrawal day 1) of extended-access self-administration, and during early (W1, days 2-4), intermediate (W2, days 6-9), and late withdrawal (W3, days 12, 13, 14) for males and females in the 0.25 (n= 8 males, n= 9 females), 0.75 (n= 8 males, n= 9 females), 1.5 (n= 8 males, n= 8 females), and 3.0 (n= 8 males, n= 7 females) dose (ug/kg) conditions (**A**). These data are also plotted as percent change in body weight after approximately 5 (ExA-1) and 10 (ExA-2) days of extended-access self-administration relative to the end of training (training) just prior to extended-access self-administration (**B**) and during early, intermediate, and late withdrawal relative to the end of extended-access self-administration (ExA-2) just prior to withdrawal (**C**). (*) Significant effect of sex. (#) Significant difference from higher doses in males. (+) Significant difference between each of the withdrawal phases. (@) Significant difference from 0.25 ug/kg. (^) Significant difference from pre-withdrawal body weight (versus 0 or no change).

3.3 Extinction and Reinstatement of Fentanyl-Seeking

To our surprise the dose of fentanyl self-administered during the extended-access period did not impact extinction responding over the first six extinction sessions (**Figure 4A**; no overall or interactive effects of dose, P's>0.05). As expected, however, females responded at higher levels

than males (effect of sex, $F_{1, 250}$= 4.9, P<0.05). Responding was also highest during the first extinction session compared to later ones (effect of session, $F_{5, 250}$= 40.7, P<0.001; session 1 compared to session 2-6, P<0.001). Analysis of total extinction responding confirmed the non-significant overall and interactive effect of dose (P's>0.05) and higher responses in females than males ($F_{1, 50}$= 5.3, P<0.05; **Figure 4B**). Extinction responding also differed between males and females tested during estrus versus non-estrus phases ($F_{1, 46}$= 6.3, P<0.01) with estrus females responding at higher levels compared to both males (P<0.001) and non-estrus females (P<0.05).

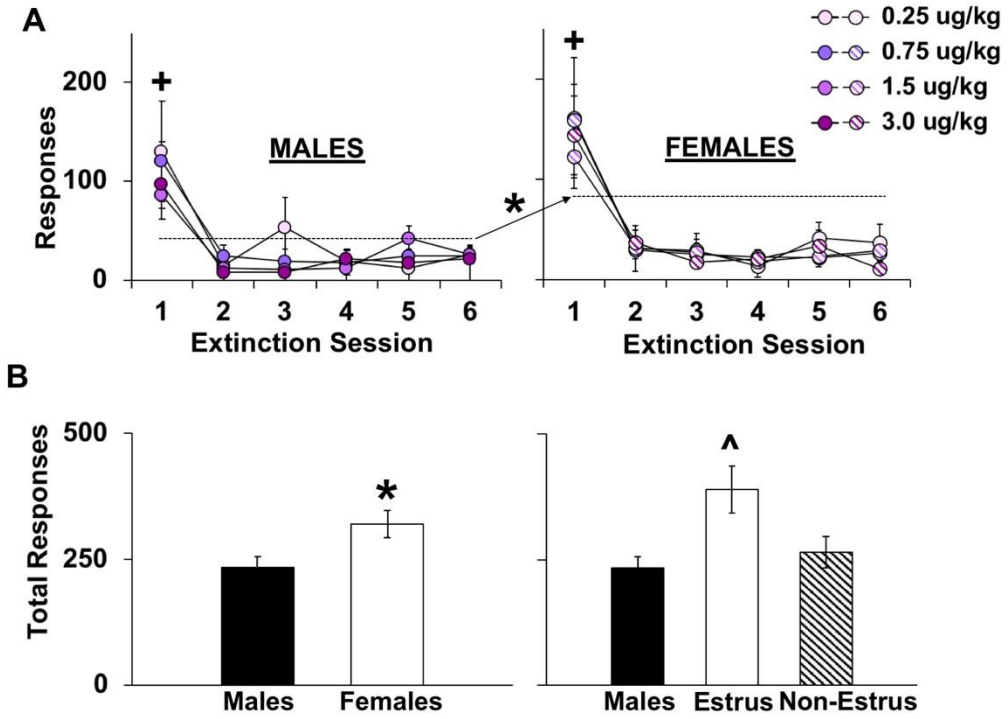

Figure 4. **Effect of sex, estrous cycle phase, and dose on responding during extinction testing in female and male rats.** Mean (±SEM) number of responses made on the lever formerly associated with fentanyl during the first six 1-h extinction sessions for males (**A**) and females (**B**) within the 0.25 (n= 8 males, n= 9 females), 0.75 (n= 8 males, n= 9 females), 1.5 (n= 8 males, n= 8 females), and 3.0 (n= 8 males, n= 7 females) dose (ug/kg) conditions and across all doses and extinction sessions run (**C, D**, n= 29 males, n= 29 females). (*) Significantly higher responding in females compared to males. (+) Significantly higher responding in session 1 compared to sessions 2-6. (^) Significantly higher responding in estrus females compared to non-estrus females and males.

Similar to the effects during extinction, the dose of fentanyl self-administered during the extended-access period did not impact cue-induced reinstatement responding (**Figure 5A**). Specifically, results from the repeated measures ANOVA comparing responding during the last extinction session to the reinstatement session revealed a significant overall effect of session ($F_{1,}$

$_{50}$= 100.1, P<0.001), but non-significant overall or interactive effects of dose (P's>0.05) indicating that fentanyl-seeking was similarly reinstated within each of the fentanyl groups. In contrast to the extinction findings, there was also no overall or interactive effect of sex (**Figure 5B**; P>0.05). However, as predicted, reinstatement responding was higher in females tested during estrus versus non-estrus phases (P<0.05); estrus females also tended to respond at higher levels than males (P=0.08). Inactive lever responses during extinction and reinstatement were minimal and analysis of inactive lever responses revealed no overall or interactive effects of sex or groups (P>0.05). Thus, the dose of fentanyl self-administered during extended-access did not have a significant effect on subsequent relapse vulnerability; relapse vulnerability was most pronounced in females during estrus.

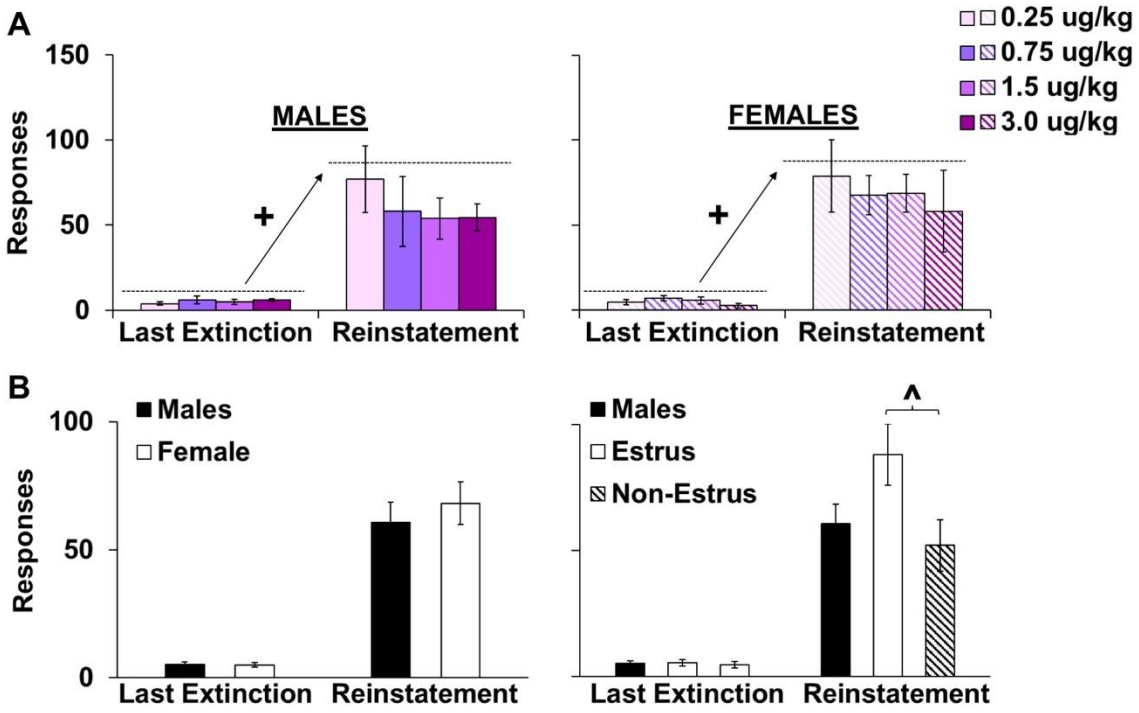

Figure 5. **Effect of sex, estrous cycle phase, and dose on responding during reinstatement testing in female and male rats.** Mean (±SEM) number of responses made on the lever formerly associated with fentanyl during the last extinction session versus the reinstatement session for males (**A**) and females (**B**) within the 0.25 (n= 8 males, n= 9 females), 0.75 (n= 8 males, n= 9 females), 1.5 (n= 8 males, n= 8 females), and 3.0 (n= 8 males, n= 7 females) dose (ug/kg) conditions and across all doses (**C, D**, n= 29 males, n= 29 females). (+) Significantly higher responding in the reinstatement session compared to the last extinction session. (^) Significantly higher responding in estrus females compared to non-estrus females.

3.4 Associations Between Frequency or Amount of Intake and Vulnerability to Relapse or Physical Dependence

As predicted, frequency of fentanyl use, as defined by the average number of fentanyl infusions obtained during the extended-access period, was predictive of later relapse vulnerability during extinction and reinstatement testing (total responding; **Figure 6A**; $r=0.45$, $P<0.001$); this relationship was also similar between males and females (non-significant interaction of sex, $P>0.05$). However, to our surprise, fentanyl intake (averaged across the extended-access period) was not significantly associated with relapse vulnerability in males or females (**Figure 6B**; P's>0.05). Although, as expected, amount of fentanyl use was predictive of the development of physical dependence (**Figure 6C**), as defined by percent decrease in body weight during early withdrawal; however, this correlation was significant in males ($r= -0.72$, $P<0.001$), but not females (interaction of sex, $F_{1, 54}= 5.5$, $P<0.05$). Importantly, this effect was specific to fentanyl intake, and not frequency of fentanyl use, given that the relationship between infusions and percent change in body weight during early withdrawal was non-significant for both males and females (**Figure 6D**; P's>0.05). Thus, frequency of opioid use, but not opioid intake, was predictive of relapse vulnerability in both males and females; whereas, opioid intake, but not frequency of use, was predictive of physical dependence in males, but not females.

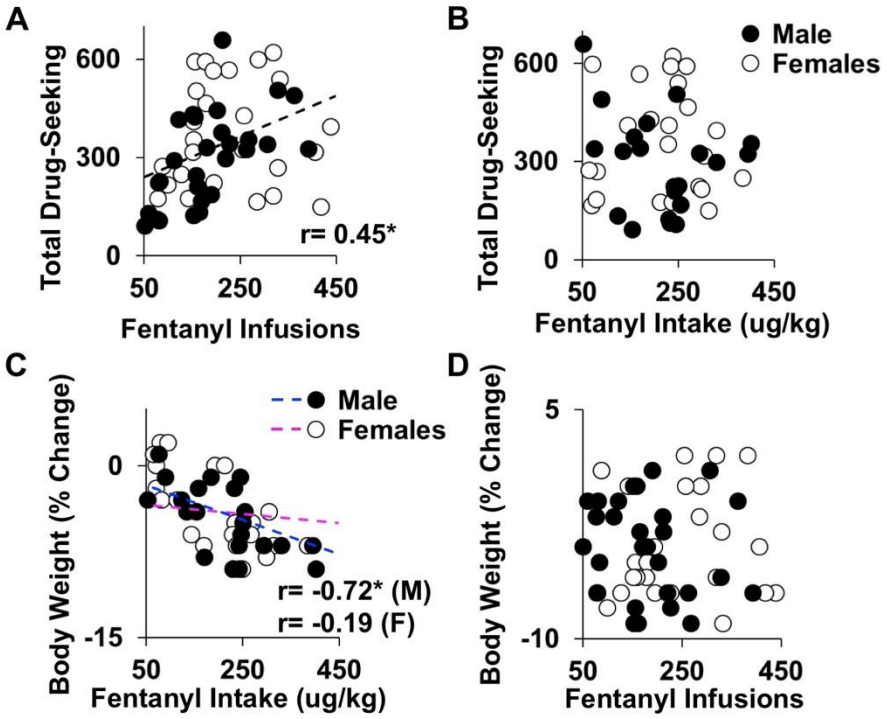

Figure 6. **Associations between frequency of fentanyl use and relapse vulnerability and fentanyl intake and the development of physical dependence.** Frequency of fentanyl use (**A**), or average number of fentanyl infusions, but not, amount of fentanyl use (**B**), or average fentanyl intake, was positively correlated with relapse vulnerability during extinction and reinstatement testing (total responses) in both males and females (, n= 29 males, n= 29 females). However, amount of fentanyl use (**C**), but not frequency of fentanyl use (**D**), was significantly correlated with development of physical dependence, or percent decrease in body weight during early withdrawal in males, but not females (**C**, n= 29 males, n= 29 females) r, Pearson correlation, (*) significant association.

4 Discussion

The goals of this study were to determine the fentanyl dose conditions that maximize the expression of an addiction-like phenotype in both males and females and to expand our model to include an additional key feature of OUD in humans, physical dependence. Surprisingly, despite markedly higher intake in groups given access to higher versus lower doses of fentanyl, each of the groups responded at similar levels during relapse testing (extinction and cue-induced reinstatement). We did observe a positive association between relapse vulnerability and number of infusions, but not fentanyl intake, indicating that frequency of use, but not total intake, impacts vulnerability to relapse. However, total intake was associated with the development of physical dependence given that weight loss was apparent following discontinuation of fentanyl self-administration at the three highest doses, but not following discontinuation of self-

administration at the lowest dose. While this effect was similar between both males and females, the association between intake and weight loss was significant in males, but not females. Other notable sex differences were that both frequency of fentanyl use and intake were greater in females than males, with particularly robust differences at lower doses, and that the time course for recovery of body weight loss during withdrawal was prolonged in females versus males. As with our previous findings, females also had exhibited higher relapse vulnerability than males, particularly when they were tested during the estrus phase of their estrous cycle. Together, these findings indicate that sex is an important risk factor for pattern and levels of fentanyl intake, physical dependence, and relapse vulnerability, and while fentanyl intake predicts physical dependence, frequency of use predicts relapse vulnerability.

Contrary to our prediction, the dose of fentanyl self-administered during extended-access did not impact subsequent vulnerability to relapse. This is surprising considering that fentanyl intake priori to withdrawal and relapse testing was markedly higher in the groups given access to higher versus lower doses of fentanyl. Our prediction that vulnerability to relapse would be enhanced in groups with higher versus lower intake of fentanyl (i.e., groups given access to higher versus lower doses of fentanyl) was based on reports in humans showing that risk of relapse following treatment for OUD is higher in individuals who report higher levels of opioid use prior to treatment as compared to those reporting lower levels (Grau-Lopez et al., 2021; Smyth et al., 2010; Gossop et al., 2002). While our findings appear to be in contrast to these results, it is important to note that these same studies also identified frequency of opioid use as a risk factor for relapse following treatment. It is also important to note that it is difficult in humans to determine levels of opioid use, since the dose is often unknown. For example, in one study (Smyth et al., 2010) the amount of heroin use prior to treatment was estimated based on amount of the substance relative to a quarter. In this preclinical study, where levels and frequency of use were precisely measured, we found that frequency of use, but not levels of use, was predictive of vulnerability to relapse. Specifically, like findings in humans, we observed significant associations between frequency of use and relapse responses; rats in the lower fentanyl dose groups also obtained more infusions than rats in the higher dose groups and the highest relapse responses were observed in the groups given access to the lowest dose of fentanyl, not the highest dose (although this difference was not significant). Our interpretation of the association is also consistent with other studies with fentanyl and other addictive drugs

showing that patterns of intake, but not amount of intake, are predictive of an enhanced vulnerability to relapse (Belin et al. 2009; Martin-Garcia et al. 2014; Allain et al., 2019) as well as the development of an enhanced motivation for the drug (Fragale et al., 2021; Martin et al. 2021; Allain et al., 2018; Zimmer et al. 2012). The translational implication is that additional anti-relapse interventions should be targeted toward individuals reporting high frequencies of opioid use and questions such as "how often" rather than "how much" may be more beneficial when trying to identify high risk patients with OUD.

Intake was predictive of the development of physical dependence to fentanyl, and while the correlation between intake and weight loss was only significant in males, it is notable that in both sexes, physical dependence only developed following fentanyl self-administration at the three highest doses and neither males nor females showed weight loss during withdrawal from the 0.25 ug/kg dose. The significant correlation between intake and weight loss in males was expected based on multiple previous studies showing rodents with greater opioid intake have more signs of physical dependence compared to rodents with less opioid intake (Towers et al., 2019; Vendruscolo et al., 2018; Moussawi et al., 2020). Clinical studies also report greater physical dependence in individuals reporting higher levels of opioid use (O'Malley and O'Malley, 2020; Rodríguez-Espinosa, 2021). However, it is somewhat surprising to observe it here considering that the weight loss observed during early withdrawal in males was similar between each of the three highest dose groups and fentanyl intake increased significantly with increases in the dose of fentanyl self-administered during extended-access. Although, there was considerable variability in intake within dose groups, which likely accounts for this association in males. Interestingly, despite intake being greater in females compared to males, weight loss during early withdrawal was the same in females and males, which is similar to findings in mice where females had greater heroin intake over extended-access self-administration, but displayed a similar number of naloxone-precipitated withdrawal signs (Towers et al., 2019). This along with weight loss not being significantly associated with physical dependence in females is curious and suggests that physical dependence develops in females once a certain threshold of intake is achieved, but that, unlike effects in males, further increases in intake do not further enhance physical dependence in females.

Our findings showing that physical dependence developed in the three highest dose groups, but not the lowest dose group, yet each of the groups, including the lowest dose group,

responded at similar levels during relapse testing suggest that the development of physical dependence is not necessary for the development of other addiction-like behaviors. This conclusion is supported by findings in humans showing that a substantial subgroup of people with OUD have relatively low levels of physical dependence (Kanof et al., 1991). Interestingly, a recent preclinical study (Townsend et al., 2021) also showed that despite similarities between males and females for both escalation of fentanyl intake and physical dependence, only males developed an enhanced preference for fentanyl over a non-drug alternative reinforcer (Ensure). This is important because this shift is believed to represent another key feature of OUD in humans, an enhanced preference for drug to the exclusion of other reinforcing stimuli and activities and future supports the notion that features of the addiction-like phenotype develop independently of one another. It also suggests that sex differences in vulnerability to OUD vary between different features of the disease (relapse, preference for the drug over other rewards, compulsive use, motivation for the drug). This idea is also support by our current findings showing that females were more vulnerable than males during relapse testing but did not differ from males for the expression of physical dependence during early withdrawal. It should be noted, however, that the conditions necessary for inducing addiction-like features with fentanyl have yet to be fully established. For example, we and others have shown that extended-access fentanyl self-administration induces an enhanced vulnerability to relapse when assessed following protracted abstinence; we also showed that this phenotype can be blocked by buprenorphine treatment during withdrawal. However, it is not clear if these effects differ from those observed following short-access self-administration and the time-course for such changes during withdrawal have yet to be fully explored. Few studies have explored effects with other addiction-like features although the Townsend study did show that the preference for the drug over a non-drug reinforcer was attenuated following treatment with methadone in males, but not females, which further indicates that their model induced this feature of an addiction-like phenotype in males, but not females. Further research is necessary to explore the fentanyl self-administration and withdrawal conditions necessary to induce addiction-like features in males and females.

Our findings also confirmed biological sex as an important vulnerability factor across the disease process. Specifically, during the extended-access phase, females self-administered more infusions and had higher levels of fentanyl intake than males, and as expected (Towers et al.,

2019), these differences were most apparent under low dose conditions. These findings are consistent with our previous study with fentanyl self-administration under these extended-access conditions (Bakhti-Suroosh et al., 2021) and previous work with heroin showing robust sex differences in intake during extended-access self-administration under lower dose conditions (30, 60 ug/kg/infusion; Towers et al., 2019), but not higher dose conditions (250 ug/kg/infusion; Zhang et al., 2015). These findings also provide insight as to why a few studies using higher doses of opioids have reported no sex differences in levels of opioid intake under extended access conditions with heroin (100 ug/kg/infusion; Venniro et al. 2017, 2019) and fentanyl (3.2 ug/kg/infusion, Townsend et al., 2021; 2.5 ug/kg/infusion, Hammerslag et al., 2021, Reiner et al. 2020). One exception was for binge intake where females had higher intake than males regardless of dose. This is interesting because it suggests that even under high dose conditions where intake is similar between males and females (Hammerslag et al., 2021; Reiner et al., 2020; Townsend et al., 2021; Venniro et al. 2017; 2019), there is likely a sex difference in the pattern of self-administration. Indeed, numerous studies have shown that there are sex differences in patterns of extended-access drug self-administration under both high and low dose conditions (e.g., Lynch and Taylor, 2004; Towers et al., 2019) with female rodents self-administering more heroin during the first hour of a long, continuous access session (fixed-ratio 1, 6-h session; Towers et al., 2019), having a longer initial period of "binge" cocaine before taking a break and showing a more diurnal dysregulation pattern of cocaine use during 24-h/day sessions (Lynch and Taylor, 2004), and having greater binge-like alcohol drinking using a 2-bottle, limited-access "drinking-in-the-dark" procedure (Sneddon et al., 2019) compared to males.

Females also showed an enhanced sensitivity to the anorectic effect of the low dose of fentanyl during extended-access self-administration. Most preclinical self-administration studies use 6- or 12-h extended-access sessions which result in daily cycles of intake and withdrawal that are long enough to induce weight changes due to physical dependence (Townsend et al., 2021). This is the first study, to our knowledge, that has monitored changes in body weight in males and females over a period of 24-hr/day opioid access followed by prolonged withdrawal. Under the lowest dose condition, females gained less percent body weight than males during both the first and last 5 days of extended-access self-administration. Although this effect may be driven by females having greater fentanyl intake than males under the lowest dose condition, weight gain in females was not further impacted with increases in fentanyl dose/intake like it was

in males. In fact, males tended to have a smaller percent of body weight gained in the high dose conditions compared to females even though fentanyl intake was similar under these self-administration conditions. These anorectic effects of fentanyl are consistent with the side effects reported with use of fentanyl patches in cancer patients (Wiffen et al., 2014), and while sex/gender differences have not been examined in humans, based on our findings, we would expect that this side-effect might be more apparent in women at low doses and more apparent in men at high doses. Further research is necessary to examine these possibilities as dose-dependent side effects of opioid analgesics may have important sex differences that could impact patient care.

Although fentanyl intake was not correlated with weight loss during early withdrawal in females, there was an overall effect of dose with females self-administering the three highest doses of fentanyl, but not the lowest dose, losing a significant amount of their body weight during early withdrawal, similar to the findings in males. This finding, in addition to previous findings showing that weight loss occurs during early withdrawal (~12 hours) in females, similar to males (Townsend et al., 2021), and that the recovery of this weight loss follows a similar pattern as other somatic signs of opioid withdrawal in females (Bobzean et al., 2019), provides support for the use of weight loss as a measure of physical dependence in females. Interestingly, despite similar weight loss in males and females during early withdrawal from the three highest doses of fentanyl, females took longer to regain their body weight and continued to gain less body weight than males even during late withdrawal. These findings are consistent with another study that showed weight loss and somatic withdrawal symptoms, including stomach writhing symptoms, persisted longer in females compared to males following discontinuation of morphine administration (Bobzean et al., 2019) and findings in humans indicating that women experience a more severe withdrawal syndrome than men (Dunn et al., 2020; Huhn et al., 2019b; Back et al., 2011b). These findings indicate that the physiological effects of opioid withdrawal may be prolonged in females compared to males. However, it is important to note that there are sex differences in weight gain under normal conditions and these differences may contribute to the effects observed here during withdrawal. Future research that includes additional measures of physical dependence/opioid withdrawal is necessary to examine this possibility especially considering that the current withdrawal scales were developed using male animals. To our

knowledge a detailed withdrawal syndrome following extended-access opioid self-administration remains unknown for females.

Finally, females showed an enhanced vulnerability during relapse testing as compared to males, and these effects were particularly robust during extinction testing and when females were tested during estrus versus non-estrus phases of their estrous cycle. These results were expected based on our previous findings with fentanyl (Bakhti-Suroosh et al., 2021). They are also consistent with results with other addictive drugs showing greater cue-induced reinstatement responding in estrus females compared to non-estrus females and males during late and protracted withdrawal (day 15, 30, and 48; Nicolas et al., 2019: Corbett et al., 2021); although, here, there was only a trend for estrus females having higher reinstatement males, extinction responding was significantly higher in estrus females compared to non-estrus females and males. This is important considering that a number of recent studies have reported that sex differences are not relevant for cue-induced relapse/reinstatement with opioids (Venniro et al., 2017; 2019; Cooper et al., 2007; Fredriksson et al., 2020; Reiner et al., 2020), yet differences were likely due to inclusion of a greater percentage of non-estrus versus estrus females. Importantly, our preclinical findings are consentient with clinical findings showing that women have greater opioid (Kennedy et al., 2013; Moran et al., 2018; Yu et al., 2007), alcohol (Seo et al., 2011; Willner et al., 1998), and cocaine (Robbins et al., 1999) craving in the presence of drug-associated cues compared to men (but see Volkow et al., 2011; Avants et al., 1995). These sex/gender differences in humans have also attributed, at least in part, to hormonal changes over the menstrual cycle given that the positive subjective effects of cocaine tend to increase with higher levels of estradiol during the follicular phase and to be reduced when progesterone is higher during the luteal phase (Fox et al., 2008). Although similar effects have not been observed in women with OUD, the menstrual cycle is disrupted by opioid use making it difficult to determine the impact of gonadal hormones on drug craving and use (Santen et al., 1975). Our findings indicate that gonadal hormones likely impact relapse vulnerability with opioids given that extinction and reinstatement responding was higher in females tested during estrus, when the ratio of estradiol to progesterone is relatively high, versus non-estrus females. Future studies investigating relapse vulnerability in females, and particularly those that exam effects within one test session, should consider hormonal status prior to making conclusions regarding sex

differences (or lack thereof) as the proportion of females tested during estrus versus non-estrus phases (or luteal versus follicular phases) could drastically change the results and conclusions.

In summary, our findings indicate that patterns of use, rather than absolute levels of use, impact vulnerability to relapse. Females also showed an enhanced vulnerability to relapse compared to males, particularly when they were tested during estrus. The translational implications of our findings are that additional anti-relapse intervention may be necessary for females and individuals reporting high frequencies of opioid use. These findings also have implications for studies on sex/gender differences in substance use disorder as they further support the idea that sex differences are most apparent under low dose conditions that induce inter-subject variability. They also indicate that sex/gender differences may be apparent for patterns of intake even in the absence of a sex difference for overall levels of use; this is important considering that the pattern of use, but not intake, was predictive of relapse vulnerability. Finally, they indicate that a lack of a sex/gender difference for relapse vulnerability during extinction/reinstatement testing may be due to the distribution of females tested at different menstrual/estrus cycle. Thus, phase of menstrual/estrous cycle should be considered in studies of sex differences in relapse.

Chapter VII

Estradiol Enhances the Development of Addition-Like Features in a Female Rat Model of Opioid Use Disorder

1 Introduction

Opioid use disorder (OUD) is a major epidemic in the United States with opioid-involved overdose deaths reaching the highest number ever recorded in the 12-month period ending in March 2022, which was primarily driven by fentanyl, a synthetic opioid (Center for Disease Control and Prevention, 2021). Although men have historically had higher rates of OUD than women, these differences have been narrowing and in 2020 a greater number of women (18 years and older) misused opioids than men (United States Department of Health and Human Services, 2020). Additionally, women appear to be more vulnerable than men on many aspects of the disease process including meeting the criteria and/or seeking treatment for OUD more rapidly after initial use [i.e. the telescoping effect; Hernandez-Avila et al., 2004; Lewis et al., 2014; Peltier et al., 2021; Anglin et al., 1987; Adelson et al., 2018; Hser et al., 1987a,b; Back et al., 2011a,b), exhibiting more negative affective symptoms during attempts to stop using opioids (Back et al., 2011,a,b; Giacomuzzi et al., 2005; Huhn et al., 2019b), experiencing higher levels of cue- and stress-induced opioid craving (Moran et al., 2018; Yu et al., 2007), and suffering more serious drug-related medical and psychological consequences than men (Chatham et al., 1999; McHugh et al., 2013; Vo et al., 2016; Vigna-Taglianti et al., 2016; Weschberg et al., 1998).

Despite the severity of this problem in women, the vast majority of preclinical studies on OUD have only included males. However, recent studies have started to consider sex as a biological variable in OUD and, as with clinical findings, have reported notable sex differences in the development and expression of an addiction-like phenotype. For example, females self-administer higher levels of opioids, including heroin, fentanyl, and oxycodone, under extended-access conditions compared to males (George et al., 2021; Murphy et al., 2021; Kimbrough et al., 2020; Towers et al., 2019, 2022). There are also sex differences in the pattern of opioid use under extended-access conditions with females showing greater escalation of heroin intake under the long-access procedure (i.e., fixed-ratio 1 access to the drug for 6 or more hours/day; George et al., 2021; Towers et al., 2019) and having greater fentanyl intake within active trials (or binge intake) under the intermittent-access (IntA) procedure (fixed-ratio 1 access during 2, 5-min trials/hr, 24-hr/day; Towers et al., 2022) than males. Sex differences in levels and patterns of opioid intake are most apparent under conditions that maximize individual differences, such as under low doses and using procedures that do not limit the number of infusions/hour or day. Additionally, following extended-access self-administration, females show a prolonged course of

physical dependence during withdrawal (Towers et al., 2022; Bobzean et al., 2019) and a greater vulnerability to cue-induced relapse, especially when tested in the estrus phase of their estrous cycle (Bakhti-Suroosh et al., 2021; D'Ottavio et al., 2022).

A major theory of sex differences in substance use disorders is that differences are due to ovarian hormones with estradiol enhancing vulnerability in females. This hypothesis is supported by numerous studies showing that in females, ovariectomy (OVX), which depletes ovarian hormones, decreases extended-access drug self-administration for multiple drugs including cocaine, nicotine, and alcohol and, that estradiol replacement restores levels of drug self-administration (Larson et al., 2007; Ramoa et al., 2013, 2014; Martinez et al., 2016; Ford et al., 2004; Rasjasing et al., 2007; Beck et al., 1985; Forger et al., 1982). Additionally, estradiol is critical for the development of key features of an addiction-like phenotype such as an enhanced motivation for the drug and a preference for the drug over other reward alternatives. For example, our previous studies with cocaine show that both OVX and pharmacological blockade of estradiol via treatment with the selective estrogen receptor modular tamoxifen in ovary-intact females effectively block the development of an enhanced motivation for cocaine following extended-access self-administration and 14 days of withdrawal, which are optimal conditions for inducing this addiction-like phenotype (Ramoa et al., 2013, 2014; Bakhti-Suroosh et al., 2019). Additionally, as with effects on drug intake, this phenotype can be rescued in OVX females with estradiol replacement (Ramoa et al., 2013, 2014). OVX has also been reported to prevent the development of a preference for cocaine over food which can be enhanced by estradiol replacement (Kerstetter et al., 2012). Thus, estradiol enhances both drug use and vulnerability to developing an addiction-like phenotype.

One major caveat, however, is that most of the evidence implicating estradiol in substance use disorders is for psychostimulants and alcohol. Evidence with opioids is sparse and restricted to effects on drug use under short-access self-administration conditions (<2 hr/day access), which likely reflects vulnerability to drug use/reinforcement, but not necessarily vulnerability to developing OUD. To our knowledge, no studies have examined the impact of estradiol in a rat model of OUD that has been optimized/validated for inducing an addiction-like phenotype similar to that observed in humans with an OUD (e.g., compulsive use, an enhanced motivation to use the drug, vulnerability to relapse). Thus, the goals of this study were to determine the impact of estradiol on fentanyl self-administration under extended-access

conditions and the subsequent development of three key addiction-like features including physical dependence, as defined by the magnitude and time-course of weight loss during withdrawal, an enhanced motivation for fentanyl, as assessed using a progressive-ratio schedule, and vulnerability to relapse, as assessed using an extinction/cue-induced reinstatement procedure. These later two characteristics were examined following 14 days of withdrawal, when levels of motivation for the drug and drug-seeking are high. We also used an extended (24h/day), IntA fentanyl self-administration procedure (2, 5 min trials/hr, 10 days) that mimics patterns of drug use observed in humans (i.e. binge-abstinent patterns of use and repeated spiking drug levels; Zimmer et al., 2021) and has been buprenorphine-validated to induce an addiction-like phenotype in both females and males [i.e., vulnerability to relapse is attenuated following buprenorphine treatment during a 14-day withdrawal period; Bakhti-Suroosh et al., 2021). Based on previous reports of estradiol increasing extended-access drug self-administration and the subsequent development of an addiction-like phenotype with psychostimulants and alcohol (Larson et al., 2007; Ramoa et al., 2013, 2014; Martinez et al., 2016; Ford et al., 2004; Rasjasing et al., 2007; Beck et al., 1985; Forger et al., 1982; Bakhti-Suroosh et al., 2019; Kerstetter et al., 2012), we predicted that estradiol would increase fentanyl intake and subsequent vulnerability to developing addiction-like features with fentanyl.

2 Methods

2.1 Subjects

Sexually mature OVX female Sprague-Dawley rats (N = 53; Charles River) were purchased from Charles River and arrived at the vivarium within one week of surgery. Rats were individually housed in operant test chambers (Med Associates, St. Albans, VT, USA) for the duration of the experiment and maintained on a 12-h light/dark cycle with lights on at 7 AM and *ad libitum* access to water and food (Teklad LM-485 7912). To accelerate the acquisition of fentanyl self-administration, rats were pre-trained to lever-press for sucrose pellets (45 mg) under a fixed-ratio 1 schedule using methods previously described (Lynch, 2008). The health of the rats was monitored daily throughout the study and the rats were weighed at least three times a week. Body weight was used as an indicator of overall health throughout the study and as a measure of physical dependence to fentanyl during opioid withdrawal as previously described (Towers et al., 2022). All procedures were conducted within animal care guidelines set by the

National Institute of Health and were approved by The University of Virginia Animal Care and Use Committee.

2.2 Procedures

2.2.1 Drugs

Fentanyl hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC) and dissolved in sterile saline at a concentration of 6.25 µg/ml. Fentanyl solutions were sterile filtered (0.22 µm; Millipore, Billerica, MA) and stored at 4 °C. Rats were weighed Monday, Wednesday, and Friday and the duration of infusion was adjusted for changes in body weight to ensure that the mg/kg dose was consistent throughout the study. 17B-estradiol was purchased from Sigma-Aldrich (St Louis, MO) and dissolved in corn oil (administered as 5 µg/0.1 ml).

2.2.2 Estradiol Replacement and Vaginal Cytology

Upon arrival, rats were randomly assigned to the estradiol or vehicle group and treatment was started the next day. Rats received either a subcutaneous injection of estradiol (5 µg/day; OVX+E; *N*=28) or an equal volume of corn oil (0.1 ml; OVX+V; *N*=25) at 11 AM 5 days a week (Monday-Friday). We have previously shown that this dose of estradiol enhances extended-access cocaine self-administration and restores the development of an enhanced motivation for cocaine in OVX females (Ramoa et al., 2013; 2014). In order to confirm successful OVX and estradiol replacement, at the start of the study daily vaginal swabs were performed at noon for a minimum of 5 days as described previously (Ramoa et al., 2013; 2014).

2.2.3 Surgery and Catheter Maintenance

Rats underwent catheterization surgery after lever pre-training using methods previously described (Lynch, 2008). The catheters were flushed with heparinized saline three days a week, which helped to maintain and verify patency throughout the study. Methohexital (1.5 mg/kg) was used to confirm patency when necessary. Any right jugular catheter that was no longer patent was replaced with a new catheter implanted into the left, external jugular vein and behavioral testing re-started following recovery.

2.2.4 Fentanyl Self-Administration Training

Following recovery from surgery, rats were trained to self-administer fentanyl (0.25 μg/kg/infusion) as previously described (Bakhti-Suroosh et al., 2021). Sessions were conducted daily until the acquisition requirement was met, which was defined as 5 consecutive days wherein all 40 infusions were obtained. Moderate food restriction (85% of free-feeding body weight) was used when necessary to encourage acquisition. Two OVX+V females failed to meet the acquisition requirements within 28 days and one OVX+V female developed an issue with patency resulting in their removal from the study and a final group size of 28 OVX+E and 22 OVX+V females.

2.2.5 Motivation for Fentanyl

Following acquisition, in a subset of OVX rats (OVX+E, *N*=17 and OVX+V, *N*=14), a baseline level of motivation for fentanyl was established prior to IntA fentanyl self-administration (Pre-IntA PR) using a progressive-ratio schedule as previously described (Lynch et al., 2022). Briefly, the response requirement to obtain a fentanyl infusion increased throughout the session in the following steps: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, etc. These sessions continued daily until a stable baseline was achieved, which was defined as no increasing or decreasing trend in the number of infusions obtained over three consecutive sessions (typically 3 to 4 sessions).

2.2.6 Extended, IntA Fentanyl Self-Administration

Once the acquisition criteria were met or a stable, baseline level of motivation for fentanyl was established, rats were given extended, 24-hr/day access to fentanyl (0.25 μg/kg/infusion) for ten consecutive days under an IntA procedure that mimics patterns of drug use observed in humans by inducing a binge-abstinent pattern of drug use with repeated spiking drug levels as previously described (Bakhti-Suroosh, 2021). One OVX+E female developed an issue with patency resulting in their removal from the study and a final group size of 27 OVX+E and 22 OVX+V females.

2.2.7 Assessing the Development of Addiction-Like Features

Physical Dependence to Fentanyl. Following IntA fentanyl self-administration, physical dependence to fentanyl was evaluated over the 14-day withdrawal period by measuring the magnitude and time-course of weight loss during early (W1, 24-hr after IntA self-administration), intermediate (W7, days 6-8), and protracted (W14, days 14 or 15) withdrawal relative to the end of IntA fentanyl self-administration (IntA10, at the start or end of the last day of IntA self-administration) as previously described (Towers et al., 2022). Four OVX+E females developed severe health complications in response to spontaneous fentanyl withdrawal resulting in removal from the study and a final group size of 23 OVX+E and 22 OVX+V females.

An Enhanced Motivation for Fentanyl. Following IntA fentanyl self-administration and 14 days of withdrawal, motivation for fentanyl was reassessed in a subset of rats screened prior to IntA self-administration (**Figure 1A**; 13 OVX+E and 12 OVX+V) using the same progressive-ratio schedule and conditions as those used for the Pre-IntA PR test except that, due to health and patency complications, only one progressive-ratio session was conducted.

Vulnerability to Relapse. Following IntA fentanyl self-administration and 14 days of withdrawal, fentanyl-seeking was assessed in a different subset of rats (**Figure 1B**; 10 OVX+E and 10 OVX+V females) using a within-session extinction/cue-induced reinstatement procedure as previously described (Bakhti-Suroosh et al., 2021). If responding did not extinguish within 9 sessions (two OVX+E rats), the session terminated and extinction responding was re-assessed the next day. For the two OVX+E females that did not extinguish within 9 extinction sessions, data from the first day of extinction testing were used in the analyses of hourly extinction responses, whereas the second day was used for the last extinction session and reinstatement. Technical issues during relapse testing prevented the inclusion of the reinstatement data from one OVX+E rat and it should also be noted that one of the OVX+E and two of the OVX+V females underwent progressive-ratio testing prior to IntA fentanyl self-administration; these animals underwent relapse testing following withdrawal rather than progressive-ratio testing because patency issues during withdrawal prevented Post-IntA PR testing. These rats did not

differ from the other rats within their groups in levels of fentanyl intake during the extended-access period or in responding during the extinction/reinstatement test.

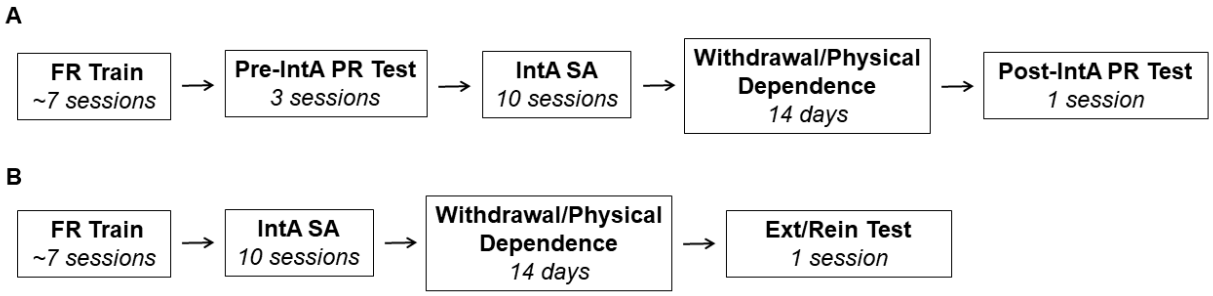

Figure 1. Summary of experimental events. Ovariectomize female rats with and without estradiol replacement were trained to self-administer fentanyl (FR train; 0.25 μg/kg/infusion). Following acquisition, a baseline level of motivation for fentanyl was established using a progressive-ratio schedule (Pre-IntA PR). Then, rats were given extended, intermittent-access to fentanyl (IntA SA; 0.25 μg/kg/infusion; 24h/day, 2, 5 min trials/h) for 10 days. Following the last fentanyl self-administration session, physical dependence was evaluated over a 14-day withdrawal period and on withdrawal day 15 motivation for fentanyl was reassessed using the same progressive-ratio schedule (Post-IntA PR Test; **A**). A different subset of the rats underwent the same procedure except vulnerability to relapse was only assessed on withdrawal day 15 using an extinction/reinstatement procedure (**B**).

2.3 Data Analysis

We first determined whether there was an effect of estradiol on the acquisition of fentanyl self-administration using the non-parametric Mann-Whitney U t-test to compare the average number of days to acquire self-administration between OVX+E and OVX+V females that met the acquisition criteria. We also compared percent group acquisition between OVX+E and OVX+V females within the first 5 training sessions and by the end of the 28-day training period using a Chi-square test. Then, repeated measures ANOVA was used to analyze group differences in levels and patterns of fentanyl intake, including the number of infusions per session and per active trial or binge as defined by trials with one or more infusions, and the number of active trials or binges per day, using session as the repeated measure and treatment group as the between-subject factor. Post-IntA Tests, including the Post-IntA PR test or extinction/reinstatement test, was included as an additional factor in the acquisition and IntA analyses; however, since no significant overall effects of Test were observed, the data were collapsed across Test and presented as OVX+E versus OVX+V.

Next, repeated measures ANOVAs were used to determine the impact of estradiol on the development of an opioid addiction-like phenotype with separate analyses used for each feature (physical dependence to fentanyl, an enhanced motivation for fentanyl, and vulnerability to relapse). Specifically, group differences in the magnitude and time-course of physical dependence were determined by comparing percent change in body weight during early, intermediate, and protracted withdrawal relative to the end of IntA fentanyl self-administration. Group differences in the development of an enhanced motivation for fentanyl were also determined using repeated measures ANOVA by comparing the number of infusions obtained during the Pre- versus Post-IntA PR testing phase. We also used a univariate ANOVA to examine the percent change in the number of infusions obtained during the Post-IntA PR test relative to group averages for the OVX+V and OVX+E rats during the Pre-IntA PR test. Both analyses focused on the first day of PR testing within each phase. Additionally, group differences in vulnerability to relapse were determined using repeated measures ANOVA by comparing the number of responses during each of the 6 extinction sessions as well as during the last extinction session versus the reinstatement session. We also examined the effects of estradiol on total extinction responding across all extinction sessions run (1-9) using a univariate ANOVA and effects on the likelihood of reinstatement in response to cues were determined by comparing the percentage of rats within each group that had higher responses during the reinstatement session as compared to last extinction session using a Chi-square test.

Lastly, the effects of estradiol on body weights were also determined at each of the major phases of the study including arrival, training (days 3-5), IntA fentanyl self-administration (early, days 1-3, IntA1; intermediate, days 4-6, IntA5; late, days 14-15, IntA10), and withdrawal (early, 24-hr after IntA self-administration, W1; intermediate, days 6-8, W7; protracted, days 14-15, W14) using repeated measures ANOVA. In order to determine whether the incidence of severe health complications, defined as labored breathing or death, in OVX+E was significantly higher than that observed in OVX+V, we analyzed percent group survival as a function of phase of study using a Kaplan-Meier survival analysis and the Log-rank (Mantel-Cox) statistic. Pearson correlations were also conducted to determine the association between the frequency of drug use (number of infusions or active trials) and the expression of addiction-like features (development of enhanced motivation, relapse vulnerability, and physical dependence). The analysis was performed collapsed across group since the univariant ANOVA determined there was not a

significant difference in the correlation coefficients for OVX+E and OVX+V females. A one-tailed test was used for a priori predicted differences (i.e. OVX+E females would have a higher level of motivation for fentanyl than OVX+V females following protracted withdrawal from IntA fentanyl self-administering and there would be a significant correlation between frequency of drug use and the expression of addiction-like features), all other tests were two-tailed. All *post hoc* comparisons were corrected for multiple comparisons using Tukey's method. Statistical analyses were performed using SPSS (V26) with alpha set at 0.05.

3 Results

3.1 Effect of Estradiol on Acquisition

Both OVX+E and OVX+V females acquired fentanyl self-administration quickly under these training conditions with the number of days to acquire not differing between the groups for the rats that met the acquisition criteria (**Figure 2A**). However, a greater percentage of OVX+E females met the acquisition criteria within the first five training sessions than OVX+V females ($U=4.0$, $p < 0.05$; **Figure 2B**). While a greater percentage of OVX+E versus OVX+V females also acquired self-administration by the end of the acquisition testing period (within a maximum of 28 sessions), this difference was not statistically significant. Thus, although both OVX+E and OVX+V rats acquired fentanyl self-administration relatively quickly under these conditions, OVX+E females are more likely to acquire fentanyl self-administration rapidly than OVX+V females.

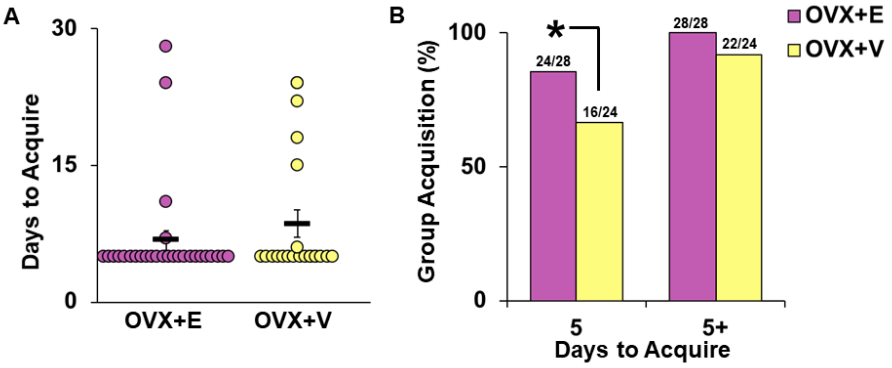

Figure 2. Effect of estradiol on fentanyl acquisition. Mean (±SEM) number of days to meet the acquisition criteria for OVX+E (n=28) and OVX+V (n=22) females that acquired fentanyl self-administration (A) and percent of OVX females that reached the fentanyl acquisition criteria within the first five training sessions and the end of the acquisition training period for OVX+E (n=28) and OVX+V (n=24) females (B). * $p < 0.05$, versus OVX+V (B).

3.2 Effect of Estradiol on Extended, IntA Fentanyl Self-Administration

OVX+E females self-administered markedly more fentanyl than OVX+V females during IntA self-administration (1.6 times more fentanyl intake than OVX+V females; overall effect of group, $F_{1,47} = 9.8$, $p < 0.01$; **Figure 3A**). This difference is most pronounced during later IntA sessions (interaction of session and group, $F_{9,423} = 2.3$, $p < 0.05$) and appears to be driven by a greater increase, or escalation, of intake from the initial sessions to later ones in OVX+E females (65% increase from session 1 versus 10, $p < 0.05$), but not in OVX+V females, which maintained a similar level of fentanyl intake across the IntA period. Further analysis confirmed that fentanyl intake was similar between OVX+E and OVX+V females in session one, but significantly greater in OVX+E compared to OVX+V females by session 10 ($p < 0.01$). Thus, OVX+E females escalated their fentanyl intake over the IntA period resulting in significantly greater fentanyl intake compared to OVX+V females.

To further explore differences in patterns of fentanyl self-administration between OVX+E and OVX+V females, we analyzed the number of active trials (or binges) per session across the IntA period. OVX+E females had more binges per session than OVX+V females during the IntA period (**Figure 3B**; overall effect of group, $F_{1,47}=18.438$, $p<0.001$). Similar to the effects observed with infusions, this difference is more pronounced during later IntA sessions (interaction of session and group, $F_{9,423}=2.8$, $p<0.05$) and appears to be driven by a greater increase in binges per session from initial sessions to later ones in OVX+E versus OVX+V females. While both groups showed an increase in binges/session from day 1 to 10 of the IntA period (P's < 0.001), analysis within days 1 and 10 confirmed that on day 1 the number of binges per session was similar between OVX+E and OVX+V females, but on session 10, it was significantly greater in OVX+E compared to OVX+V females ($p<0.001$). Fentanyl intake within each binge/active trial also tended to be higher in OVX+E versus OVX+V rats (**Figure 3C**; $F_{1,47}=3.0$, $p = 0.09$). In contrast to the effects observed for the number of binges/session (or active trials), intake within each binge, or active trial, decreased from initial to later sessions (overall effect of session, $F_{1,47} = 2.9$, $p < 0.01$; session 2 vs. 10, $p < 0.05$). Thus, compared to OVX+V females, OVX+E females had a higher frequency of binges, particularly during later sessions, and tended to take more fentanyl per active binge.

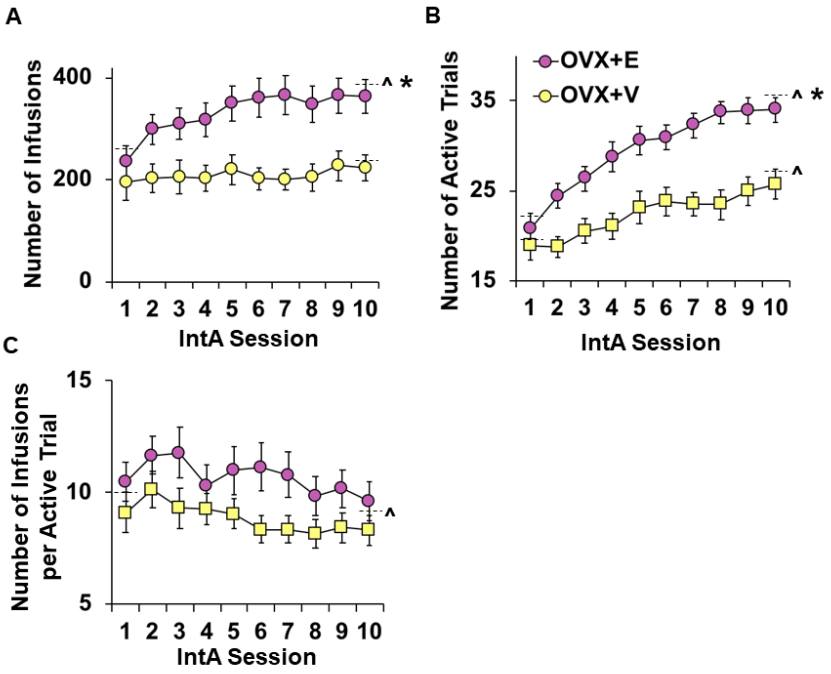

Figure 3. **Effect of estradiol on extended-, intermittent-access (IntA) fentanyl self-administration.** Mean (±SEM) number of infusions (A), active trials (B), and number of infusions per active trials (C) for each of the 10 IntA sessions for OVX+E (*n*=27) and OVX+V (*n*=22) females. * $p < 0.01$, versus OVX+V (A, B). ^ $p < 0.05$, versus session 10 (A, B, C).

3.3 Effect of Estradiol on the Development of Features of an Addiction-Like Phenotype

3.3.1 Physical Dependence to Fentanyl. OVX+E females lost a greater percentage of their bodyweight during withdrawal compared to OVX+V females (**Figure 4A**; overall effect of group, $F_{2, 43} = 16.8$, $p < 0.001$); this effect appears to be due to OVX+E females having a prolonged course of weight loss (overall effect of withdrawal timepoint, $F_{2, 86} = 30.3$, $p < 0.001$; interaction of withdrawal time point and group, $F_{2, 86} = 9.2$, $p < 0.001$). Post-hoc analysis during early withdrawal confirmed that both OVX+E and OVX+V females lost a significant percentage of their body weight relative to the end of the IntA period (or 0; $p < 0.05$). During intermediate withdrawal, the body weight of OVX+E females remained significantly below their pre-withdrawal baseline (relative to the end of the IntA period, or 0; $p < 0.05$); whereas, OVX+V females had not only regained their lost weight, they also gained additional body weight relative to the end of the IntA period (or 0; $p < 0.05$) resulting in a significant difference in the percent change in body weight between the OVX+E and OVX+V females during intermediate withdrawal ($p < 0.05$). By protracted withdrawal, the body weight of OVX+E females returned

to baseline relative to the end of the IntA period (or 0; $p < 0.05$) and the body weight of OVX+V females remained increased from baseline relative to the end of the IntA period (or 0; $p < 0.05$) resulting in a significant difference in the percent change of body weight between the OVX+E and OVX+V females. Thus, OVX+E and OVX+V females lost a similar percentage of body weight during early withdrawal indicating that physical dependence was expressed similarly following fentanyl self-administration; however, this weight loss persisted longer in OVX+E females compared to OVX+V females indicating that estradiol may prolong the course of physical dependence with fentanyl.

3.3.2 An Enhanced Motivation for Fentanyl. As predicted, OVX+E and OVX+V females obtained a similar number of infusions under the PR schedule prior to IntA fentanyl self-administration, but after IntA self-administration and withdrawal, OVX+E females obtained more infusions than OVX+V females (**Figure 4B**). Results from the repeated-measures ANOVA revealed significant effects of phase ($F_{1,23} = 67.3$, $p < 0.001$) and phase by group ($F_{1,23} = 4.1$, $p < 0.05$). Post-hoc comparisons within each phase confirmed a group difference in the number of infusions obtained during Post-IntA PR testing ($p < 0.05$), but not Pre-IntA PR testing. However, within group comparisons of infusions obtained during Pre-IntA versus Post-IntA PR testing revealed significant increases in both OVX+E and OVX-V females (p's < 0.01) indicating that both groups developed an enhanced motivation for fentanyl following extended-access self-administration and protracted withdrawal. The analysis of the percent change in the number of infusions obtained during Post-IntA PR testing (relative to the average number of infusions obtained by the OVX+E and OVX+V females during Pre-IntA PR testing) also confirmed an overall effect of group with OVX+E females having a larger increase in motivation for fentanyl following IntA fentanyl self-administration and protracted withdrawal than the OVX+V females ($F_{1,23} = 5.7$, $p < 0.05$; **Figure 4C**). Thus, both OVX+E and OVX+V females developed an enhanced motivation for fentanyl following IntA fentanyl self-administration and protracted withdrawal, but its expression was more robust in the OVX+E females than the OVX+V females.

3.3.3 Vulnerability to Relapse. OVX+E and OVX+V females had similar levels of responding during the first six extinction sessions (**Figure 4D**) with results from the repeated measures

ANOVA comparing responding during the first six extinction sessions revealing an overall effect of session ($F_{5,90} = 18.0$, $p < 0.001$), but no overall or interactive effect of group. Post-hoc analysis revealed that responding was highest in both groups during the first extinction session compared to later sessions (session 1 vs session 2-6, p's < 0.05). Analysis of total extinction responding confirmed no significant overall and interactive effect of group (data not shown).

Both OVX+E and OVX+V females reinstated fentanyl-seeking upon the presentation of fentanyl-associated cues with results from the repeated measures ANOVA comparing responding during the last extinction session to the reinstatement session revealing a significant overall effect of session (**Figure 4E**; $F_{1,17} = 26.2$, $p < 0.001$). There was no overall or interactive effect of group; however, analysis of percent group reinstatement using Chi-square revealed that a greater percentage of OVX+E females (100%) reinstated compared to OVX+V females (60%; x2= 48.4, $p < 0.001$). Thus, both the OVX+E and OVX+V females developed an enhanced vulnerability to relapse following IntA fentanyl self-administration and protracted withdrawal, but the OVX+E females showed an enhanced sensitivity to the reinstating effects of fentanyl-associated cues compared to OVX+V females.

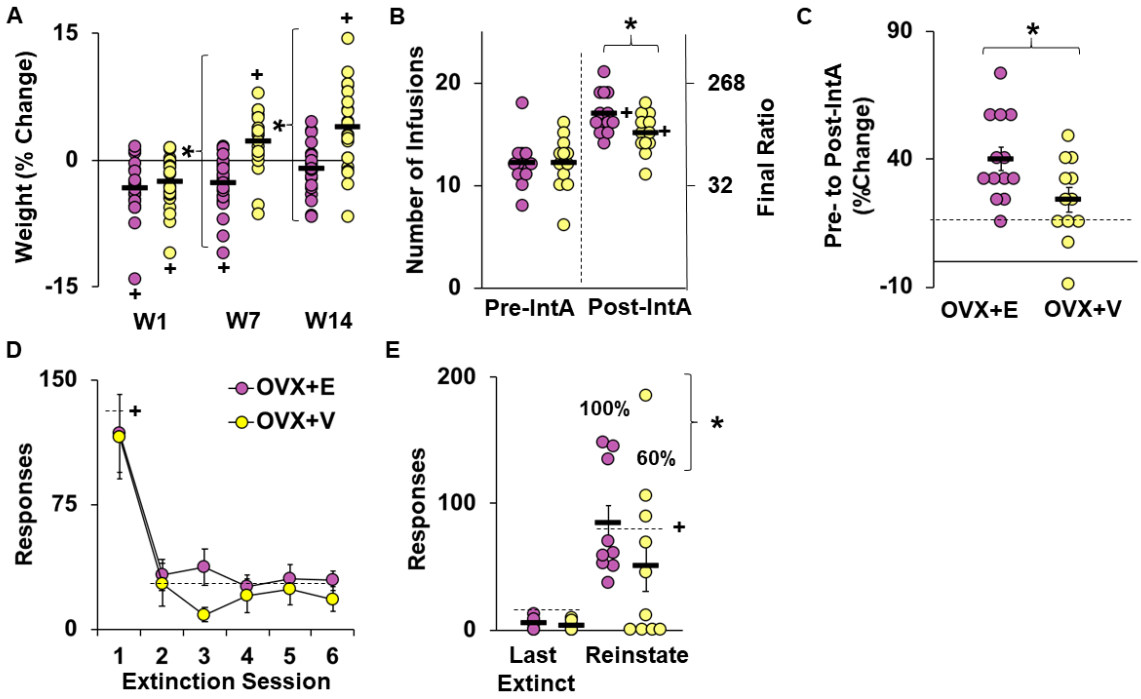

Figure 4. Effect of estradiol on the development of addiction-like features. *Physical Dependence:* Mean (±SEM) percent change in body weight (g) during early (W1), intermediate (W7), and protracted (W14) withdrawal relative to end of IntA fentanyl self-administration (IntA10) for OVX+E (*n*=23) and OVX+V (*n*=22) females (A). *An Enhanced Motivation:* Mean (±SEM) number of infusions obtained under a progressive-ratio schedule prior to (Pre-IntA) and following IntA fentanyl self-administration and protracted withdrawal (Post-IntA; B) and percent change in number of infusions obtained under a progressive-ratio schedule Post-IntA relative to Pre-IntA (C) for OVX+E (*n*=13) and OVX+V (*n*=12) females. *Vulnerability to Relapse:* Mean (±SEM) number of responses made on the lever formerly associated with fentanyl during the first six 1-h extinction sessions (D) and the number of responses made on the lever formerly associated with fentanyl during the last extinction session versus the reinstatement session (E) for OVX+E (*n*=10) and OVX+V (*n*=10) females. * **p** < 0.05, versus OVX+V (A, B, C, E). + **p** < 0.05, versus baseline (or 0; A), Pre-IntA (B), hours 2-6 (D), and the last extinction session (E). The dashed lines in C represents the threshold for the development of an enhanced motivation for fentanyl (15% increase) and dashed line and 100% and 60% values in E represent the percentage of animals within each group to show an increase in responses from the last extinction session to the reinstatement session.

3.4 Effect of Estradiol on Physical Health (Body Weight) and Probability of Survival.

There were also significant differences in general health effects with the analysis on body weight revealing that OVX+E females weighed less than OVX+V females (**Figure 5A**; overall effect of group, $F_{1, 43} = 72.5$, $p < 0.001$). This difference appears to be driven by less weight gain in the OVX+E females during later phases of the study, including during IntA fentanyl self-

administration and withdrawal (overall effect of phase, $F_{7, 301} = 100.7$, $p < 0.001$; interaction of phase and group, $F_{7, 301} = 47.8$, $p < 0.001$). Post-hoc analysis within the different phases of the study confirmed there was no difference in body weight upon arrival, but that OVX+E females weighed significantly less than OVX+V females during each subsequent phase (P's<0.05). Notably, a greater proportion of OVX+E females developed severe adverse health effects, including labored breathing (3 of 27) and death (4 of 27) during withdrawal, than OVX+V females where neither issue was observed (**Figure 5B**; $x^2 = 6.2$, $p < 0.05$). Thus, estradiol appears to blunt weight gain and exacerbate opioid withdrawal-related adverse health consequences. These life-threating health complications also appear to be most pronounced during later withdrawal time points when weight differences are the largest.

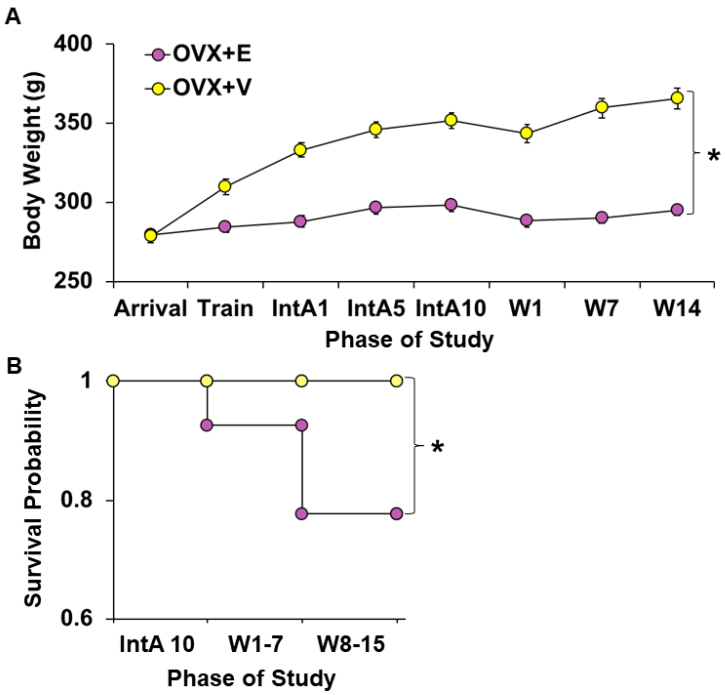

Figure 5. Effect of estradiol on physical health and probability of survival. Mean (±SEM) body weight (g) at arrival, training (train), early (IntA1), intermediate (IntA 5) and late (IntA10) phases of intermittent-access (IntA) fentanyl self-administration, and early (W1), intermediate (W7), and protracted phases of withdrawal (W14; A) for OVX+E (*n*=23) and OVX+V (*n*=22) females and percent survival prior to withdrawal at the end of IntA self-administration (IntA 10), early withdrawal (days 1-7), and protracted withdrawal (days 8-15) for OVX+E (*n*=27) and OVX+V (*n*=22) females (B). * $p < 0.05$, versus OVX+V (A, B).

3.5 Association Between Frequency of Fentanyl Intake and the Expression of Features of an Addiction-Like Phenotype.

As with our previous study (23), the frequency of fentanyl use, as defined by the average number of fentanyl infusions obtained during the IntA period, was predictive of the subsequent development of physical dependence (**Figure 6A**; r= -0.41, $p<0.001$), as defined by the percent body weight loss during early withdrawal (or W1) relative to the end of the IntA period (or IntA10), development of an enhanced motivation for fentanyl (**Figure 6B**; r=0.40, $p<0.05$), as defined by an increase in the number of infusions obtained under the PR schedule Post-IntA and withdrawal relative to Pre-IntA, and development of enhanced relapse vulnerability (**Figure 6C**; r=0.45, $p<0.05$), as defined by reinstatement responses; these relationships were similar between OVX+E and OVX+V females (non-significant interaction of group). Notably, the frequency of binge-intake, as defined by the average number of active trials during the IntA period, was also predictive of the development of physical dependence to fentanyl (**Figure 6D**; r= -0.34, $p<0.05$), the development of enhanced motivation for fentanyl (**Figure 6E**; r=0.50, $p<0.05$), and relapse vulnerability (**Figure 6F**; r=0.40, $p<0.05$); these relationships were also similar between OVX+E and OVX+V females (non-significant interaction of sex). Thus, the frequency of opioid use appears to be a relatively strong predictor for the severity of OUD in females.

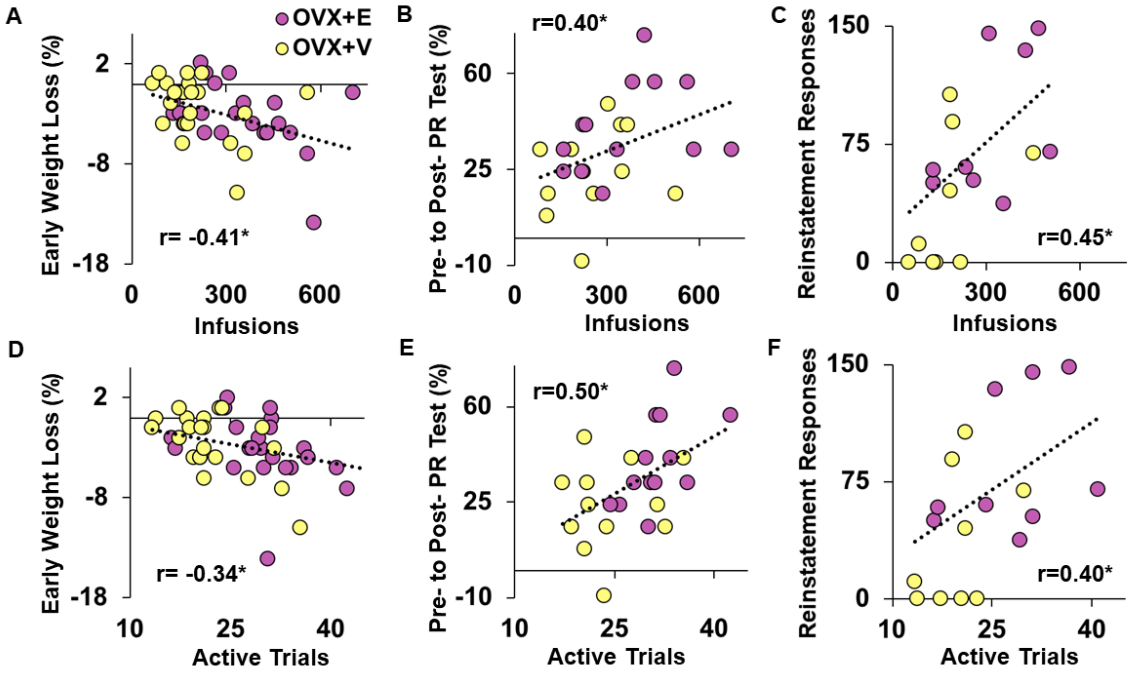

Figure 6. Association between frequency of fentanyl use and the development of addiction-like features. Number of infusions self-administered during the intermittent-access (IntA) period was predictive of the development of physical dependence, defined as the percent change in body weight during early withdrawal relative to the end of IntA (A), an enhanced motivation, defined as percent change in number of infusions obtained under a progressive-ratio schedule following IntA fentanyl self-administration and protractive withdrawal (Post-IntA) relative to prior to IntA fentanyl self-administration (Pre-IntA; B), and vulnerability to relapse, defined as the number of response on the formerly active lever upon the presentation of fentanyl-associated cues (reinstatement responses; C), and for OVX+E (*n*=13, 10, and 23, respectively) and OVX+V (*n*=12, 10, and 22, respectively) females. Notably, the number of active trials (or binges) during the IntA period was also predictive of the development of physical dependence (D), enhanced motivation (E), and vulnerability to relapse (F), and for OVX+E (*n*=13, 10, and 23, respectively) and OVX+V (*n*=12, 10, and 21, respectively) females. * $p < 0.05$, significant association (A-F).

4 Discussion

The purpose of this study was to determine the impact of estradiol on fentanyl intake and the development of an opioid addiction-like phenotype in females. Similar to findings with psychostimulants and alcohol (for review see, Towers et al., 2023b), we found that estradiol replacement in OVX females increased fentanyl use and the expression of several key features of an addiction-like phenotype. More specifically, compared to OVX+V, OVX+E females were more likely to acquire fentanyl self-administration rapidly and to escalate their fentanyl use over time under extended, IntA conditions, indicating that estradiol may accelerate transitions from

initial to regular opioid use and from regular opioid use to escalated/dysregulated opioid use. Following IntA fentanyl self-administration, physical dependence (weight loss) was similarly expressed in OVX+E and OVX+V females; however, the weight loss persisted significantly longer in OVX+E females indicating that estradiol prolongs the course of physical dependence in response to fentanyl. Additionally, following protracted withdrawal, OVX+E females expressed a greater increase in motivation for fentanyl and were more sensitive to the reinstating effects of fentanyl-associated cues than OVX+V females. A significant percentage of OVX+E females also developed severe health complications over withdrawal (27%) and 4 of the 7 sick rats succumbed to illness which is striking considering that signs of severe illness were not observed in OVX+V females. Together, these results indicate that estradiol enhances vulnerability in females to developing opioid addiction-like features and serious opioid-related health complications.

The most striking difference between OVX females with and without estradiol replacement was the markedly higher levels of fentanyl intake in OVX+E females during the extended, IntA period. This is significant because these extended-access conditions are thought to model the "binge-abstinence" pattern of opioid use observed in humans with OUD (Zimmer et al., 2012). We also saw evidence of loss of control of fentanyl intake in OVX+E, but not OVX+V, females, which is another key feature of OUD in humans that has been defined in animal models as an increase, or escalation, of drug intake over time. More specifically, OVX+E females increased their fentanyl intake by an average of 65% from the first to the last session; whereas, OVX+V females maintained relatively constant levels of intake across the IntA period. The escalation effect observed in OVX+E females appears to be attributable to an increase in the frequency of fentanyl use (or the number of infusions and active trials) which is significant since we know from our previous work (Towers et al., 2022) and from findings in this study that frequency of use is predictive of an enhanced vulnerability for the development of addiction-like features. Notably, these effects of estradiol on drug intake are consistent with findings with other addictive drugs, including cocaine (Larson et al., 2007; Ramoa et al., 2013; 2014; Martinez et al., 2016), nicotine (Flores et al., 2016), and alcohol (Ford et al., 2004; Rajasingh et al., 2007; Becker et al., 1985; Forger et al., 1982; Hilderbrand et al., 2018), showing that while OVX robustly decreases drug intake under extended-access condition, estradiol replacement increases drug intake to levels similar to intact females. That similar findings were observed here with

opioids provides support for the hypothesis that estradiol's effect on vulnerability in females is similar for opioids as compared to psychostimulants and alcohol. These findings also indicate that estradiol may accelerate the transition from regular use to escalated/dysregulated opioid use; therefore, this effect of estradiol may also explain the clinical phenomenon termed the telescoping effect where women progress faster from initial opioid use to meeting the criteria for OUD and/or seeking treatment for OUD than men Hernandez-Avila et al., 2004; Lewis et al., 2014; Peltier et al., 2021; Anglin et al., 1987; Adelson et al., 2018; Hser et al., 1987a,b; Back et al., 2011a,b).

Since the main goal of this study was to determine the effects of estradiol on the development of addiction-like features following extended-access drug self-administration, the training conditions used were designed to induce rapid and maximal rates of acquisition rather than to maximize individual differences in rates of acquisition. None-the-less, group differences were apparent with OVX+E females showing a greater likelihood of acquisition during the first five training sessions as compared to OVX+V females (86% versus 67%, respectively). Our low fentanyl dose may have contributed to this finding considering that sex and other individual differences during the acquisition of drug self-administration are more likely to be observed under low dose conditions. Indeed, a previous study also found that estradiol replacement in OVX females facilitated the acquisition of heroin self-administration at a relatively low dose (7.5 µg/kg/infusion; Roth et al., 2002). This is also consistent with a large body of work with psychostimulants and alcohol indicating that estradiol enhances initial vulnerability to drug use as defined by faster rates of acquisition and greater percent group acquisition (for review see, Towers et al., 2023b). However, it is possible that the effects of estradiol differ for rates of acquisition versus initial levels of use considering that several studies have reported no differences between OVX+E and OVX+V females in initial levels of heroin self-administration under short-access conditions (2-3h/day, fixed ratio 1 schedule; Smith et al., 2021; Stewart et al., 1996) even when assessed across a broad range of doses (0.3-50 µg/kg/infusion). Notably, this provides additional evidence for estradiol accelerating transitions between the stages of addiction in females with OVX+E females transitioning faster from initial to regular (or stable) opioid use than OVX+V females, similar to the transition from regular to escalated/uncontrolled opioid use.

As predicted, estradiol also increased vulnerability to developing addiction-like features with fentanyl; however, in contrast to effects with cocaine (Ramoa et al., 2013) where OVX+E, but

not OVX+V, females developed an addiction-like phenotype, defined as an enhanced motivation for the drug, both OVX+V and OVX+E females developed features of an addiction-like phenotype with fentanyl. More specifically, despite OVX+E and OVX+V females expressing a similar degree of physical dependence (percentage of body weight lost) following fentanyl self-administration, OVX+V females recovered their body weight quickly over the first week of withdrawal. In contrast, weight loss persisted well into the second week of withdrawal for OVX+E females indicating that estradiol may prolong the course of physical dependence with fentanyl, similar to ours and others previous findings in intact females compared to males (Towers et al., 2022; Bobzean et al., 2019). Additionally, both OVX+E and OVX+V females developed an enhanced motivation for fentanyl following protracted withdrawal from IntA fentanyl self-administration, but this phenotype was more robustly expressed (1.7 times higher) and more likely to occur in OVX+E versus OVX+V females given that 100% of OVX+E females developed this phenotype (≥15% increase relative to baseline; Ramoa et al., 2013; 2014) versus 83% of OVX+V females. Similarly, both OVX+E and OVX+V females expressed high levels of fentanyl-seeking during extinction and reinstatement testing following IntA fentanyl self-administration and protracted withdrawal; however, responding was reinstated by fentanyl-associated cues in a higher percentage of OVX+E females (100%) compared to OVX+V females (60%). These findings indicate that OVX+E females also have an enhanced sensitivity to the reinstating effects of fentanyl-associated cues compared to OVX+V females. Taken together, these findings suggest that while estradiol enhances the expression of opioid addiction-like features in females, it is not necessary for their development. The development of a loss of control over drug use may be one exception considering that only females with estradiol showed an escalation of fentanyl intake over the 10-day extended, IntA self-administration phase.

One of the most important findings of this study from a translational perspective is that risk of life-threating health complications was markedly higher in OVX+E versus OVX+V females. It is notable that this risk was observed exclusively during withdrawal, and not during extended, IntA fentanyl self-administration. The most obvious explanation for this effect of estradiol is that the lower body weights of OVX+E females may have made them more susceptible to adverse health effects associated with fentanyl withdrawal. This effect of estradiol on body weight is well known [53], and it was apparent in this study prior to fentanyl self-administration and withdrawal. As such, body weight loss during early withdrawal, even though

at a similar percentage, would be expected to have a bigger impact in OVX+E females. Indeed, unlike OVX+V females, which quickly recovered their body weight over the first week of withdrawal, OVX+E females showed a prolonged course of weight loss that lingered into the second week of withdrawal, as discussed above. Another possibility, however, is that the greater intake of fentanyl in OVX+E females during the extended-access period may have contributed to both the adverse health effects and the prolonged course of physical dependence in OVX+E versus OVX+V females. It is also possible that estradiol enhances the risk of adverse health effects during fentanyl withdrawal by exacerbating physical dependence/opioid withdrawal severity considering findings in humans with OUD or alcohol use disorder showing that women experience greater adverse withdrawal effects and require more support to manage withdrawal effects in response to extended-release naltrexone, a long-acting opioid antagonist, presumably due to effects of ovarian hormones in women (Herbeck et al., 2016; Roche et al., 2015). Additionally, high levels of estradiol may enhance immunosuppression induced by opioid withdrawal (see Shepherd et al., 2021 for review) and result in both a prolonged course of physical dependence (as observed in OVX+E females) and an increased vulnerability to infections and/or sepsis (Eisenstein et al., 2006). Finally, it is possible that the enhanced risk of adverse health effects in OVX+E females was mediated independent of withdrawal considering that both intravenous drug use and estrogen replacement therapy are risk factors for health complications such as deep vein thrombus which can lead to life-threatening sequela including pulmonary embolisms (Jain et al., 2021; Masoomi et al., 2010; Miller et al., 2002; Cosman et al., 2005). While future research is necessary to determine the mechanism for the enhanced vulnerability in the OVX+E females to the adverse health effects that developed over fentanyl withdrawal, it is notable that women with substance use disorders have also been reported to experience greater adverse health effects and/or an accelerated course of drug-related medical consequences than men with substance use disorders, including opioids (Arfken et al., 2001; Fernandez-Montalvo et al., 2014; Agabio et al., 2016; Geddes et al., 2020).

In summary, as with the effects with psychostimulants and alcohol, our findings indicate that estradiol enhances vulnerability in females to opioid use and the development of an opioid addiction-like phenotype. Our findings also indicate that estradiol prolongs the course of physical dependence and increases the likelihood of developing life-threating health complications during withdrawal. These effects are very concerning and future studies are

needed to understand the mechanism for these adverse health effects. Further research is also needed to understand the neurobiological mechanism underlying the behavioral differences found here.

Chapter VIII

Incubation of Fentanyl-Craving Following Intermittent-Assess Self-Administration: Sex Differences and the Efficacy of R-ketamine as an Anti-Craving Intervention

1 Introduction

In 2017, opioid use disorder (OUD) was declared a public health emergency in the United States (US Department of Health and Human Services, 2017). This disease has spared no demographic and has cost the nation a record $1.5 trillion, which represents a 37% increase from 2017 when the Center for Disease Control and Prevention last measured its economic impact (Joint Economic Committee Democrats, 2022). Opioid-involved overdose deaths also remain a problem of epidemic proportions in the United States with fentanyl being the major contributor (Centers for Disease Control and Prevention, 2022; 2023). Fentanyl is a synthetic opioid that when used intravenously is 150 times more potent than morphine (Torralva and Janowsky, 2019). The surge in fentanyl-involved overdose deaths led the National Institute on Drug Abuse to highlight an urgent need for research focused on fentanyl use in the context of OUD (NIDA, 2021). In response, we developed an extended-, intermittent-access (IntA; 2, 5-minute trials, or binges, an hour; 24-hr/day) fentanyl self-administration procedure in rats that readily induces a binge-abstinence pattern of drug use, similar to the pattern of use observed in individuals with OUD, and an enhanced vulnerability to cue-induced relapse in males and females (Bakhti-Suroosh et al., 2021). In the original study (Bakhti-Suroosh et al., 2021), we showed that the enhanced vulnerability to cue-induced relapse emerges following prolonged withdrawal from IntA fentanyl self-administration (15 days) and can be blocked with buprenorphine treatment during withdrawal, an FDA-approved treatment for OUD; thereby validating our rat model of cue-induced relapse.

In this study, our goal was to use our rat model of OUD to evaluate the time-course for the development of cue-induced relapse vulnerability (or fentanyl-craving) during withdrawal in males and females. We chose to characterize withdrawal time-point dependent changes in vulnerability to relapse because drug-craving and relapse are major challenges in treating patients with OUD and a better understanding of how these risk factors change over withdrawal could help advance treatment strategies. More specifically, clinical studies have shown that exposure to cues previously associated with drug use can trigger intense drug-craving and that cue-induced drug-craving is one of the strongest predictors of relapse during and after treatment (see Vafaie and Kober, 2022 for review). There is also a large body of clinical and preclinical evidence with psychostimulants, such as cocaine, and opioids, such as heroin and oxycodone, that further shows that drug-craving progressively increases from low levels during early

withdrawal to increasingly higher levels over a period of protracted withdrawal (see Li et al. 2015 for review). This phenomenon has been termed the incubation of craving; however, the time-course for the incubation of craving has not been studied with fentanyl. In addition to examining effects specific to fentanyl, there is also a need to examine sex differences considering that a majority of the clinical and preclinical studies on incubation of drug craving is based on findings in males even though there are known sex differences, including a faster course for the development of addiction in women as compared to men (see Towers et al., 2023 for review). Therefore, in the present study, we assessed vulnerability to cue-induced relapse in males and females following IntA fentanyl self-administration and 0, 1, or 14 days of withdrawal based on findings with other addictive drugs showing that drug-seeking increases from low levels on withdrawal days 0-3 and reach peak levels after 2-3 weeks of withdrawal (Towers et al., 2023). We have also shown that 14 days of withdrawal is optimal for inducing high levels of cue-induced fentanyl-craving in males and females (Bakhti-Suroosh et al., 2021).

An additional goal of the study was to evaluate R-ketamine, a candidate medication for OUD treatment, using our rat model with fentanyl. Specifically, our goal was to determine whether R-ketamine can attenuate the incubation of fentanyl craving, as assessed using a cue-relapse procedure. Notably, racemic ketamine, which contains an equal mixture of S- and R-enantiomers, has shown great promise as an anti-relapse intervention given that it markedly and persistently reduces drug-craving and improves abstinence rates in humans with a substance use disorder (Dakwar et al., 2019, 2014; Jones et al., 2018; Krupitsky et al., 2007) and counters the rewarding effects of addictive drugs, including opioids such as morphine, in animal models (McKendrick et al., 2020). While concerns over its addiction potential and neurotoxic effects have hindered its development, evidence indicates that these effects are mediated by the S-enantiomer (Bonaventura et al., 2021). Importantly, its R- enantiomer (R-ketamine) recapitulates the pharmacological effects of racemic ketamine, including anti-reward and withdrawal effects, but is devoid of or has a reduced side-effect and safety risk profile (Bonaventura et al., 2021; Witkin et al., 2020). To our knowledge, R-ketamine has not yet been studied in clinical or preclinical studies in the context of OUD and based on the previous preclinical study in males and clinical studies with other addictive drugs, we predicted that R-ketamine administration immediately following fentanyl self-administration would significantly attenuate the incubation of opioid-craving as observed during protracted withdrawal.

2 Methods and Materials

2.1 Subjects

Sexually mature male (*N* =35) and female (*N* =29) Sprague-Dawley rats (Charles River) were used as subjects in this study. Upon arrival, rats were individually housed in operant test chambers (Med Associates, St. Albans, VT, USA) with *ad libitum* access to food (Teklad LM-485 7912; except as noted below for some animals during fentanyl self-administration training) and water and maintained on a 12-h light/dark cycle (lights on at 7AM). After an acclimation period (2 days), rats were pre-trained to lever-press for sucrose pellets (45 mg) using methods previously described (24-hr/day sessions under a fixed-ratio 1 schedule; Towers et al., 2022). Sessions continued daily until lever-press responding was acquired (2 consecutive days wherein >50 pellets were obtained, typically 2-3 sessions) to ensure rapid subsequent acquisition of fentanyl self-administration. Health was monitored daily throughout the study and rats were weighed at least three times a week. Changes in bodyweight were used as an indicator of overall health throughout the study and as a measure of physical dependence to fentanyl during withdrawal as previously described (Towers et al., 2022). All procedures were conducted within animal care guidelines set by the National Institute of Health and were approved by The University of Virginia Animal Care and Use Committee.

2.2 Procedure

2.2.1 Surgery and Catheter Maintenance

Following lever pre-training, rats underwent right jugular catheterization surgery using methods previously described (Lynch 2008). Catheters were flushed with heparinized saline three days a week to help verify and help maintain patency. Occasionally, patency was also verified by administering methohexital (1.5 mg/kg) and monitoring for immediate loss of the righting reflex. Any catheter that was no longer patent (i.e., the catheter was leaking, pressure prevented flushing, or failed the methohexital test) was replaced with a new catheter in the left jugular vein and testing resumed following recovery from surgery (1-2-days).

2.2.2 Fentanyl Self-Administration

Following recovery from surgery, rats were trained to self-administer fentanyl as described previously (0.25 ug/kg/infusion; under a fixed-ratio 1 schedule with a one second time out

following each infusion and a maximum of 40 infusions/day; Bakhti-Suroosh et al., 2021). Sessions were conducted daily until the acquisition criterion was met (i.e. 5 consecutive days wherein the rat obtained all 40 infusions). When necessary (i.e. fewer than 15 infusions/day by training day 5), moderate food restriction (85% of its free-feeding body weight) was used briefly (2-3 days) to encourage acquisition of fentanyl self-administration. As with our previous studies (Bakhti-Suroosh et al., 2021; Towers et al., 2022), males and females acquired fentanyl self-administration rapidly under these conditions.

Following acquisition of fentanyl self-administration, rats were given extended, 24-hr/day access to fentanyl (0.25 ug/kg/infusion) under an IntA procedure for ten days as previously described (Bakhti-Suroosh et al., 2021). Some rats underwent additional cycles of extended-, IntA self-administration as detailed below. Phenotypic behavioral assessment (cue-induced relapse testing or progressive-ratio responding for fentanyl) was conducted between these cycles as part of a different experiment. However, prior to inclusion here, we first verified that intake and responding for fentanyl during the subsequent cycle of IntA self-administration had reached the same level observed at the end of the initial IntA period (within 10%; also see **Figure 2**). The average total number of sessions run for females and males in this study were 14.6 ± 0.7 and 14.3 ± 0.5, respectively, with each additional IntA cycle being 6 ± 0.4 sessions for females and 6 ± 0.3 sessions for males. We also confirmed that relapse testing did not differ between rats tested after one versus repeated cycles of IntA self-administration or rats that had previously undergone relapse testing (see **Figure 3E**) along with one versus repeated relapse tests (see **Figure 3F**). Two females and two males were given access to a 1.5 ug/kg fentanyl dose during acquisition and IntA self-administration. These rats were included here because they underwent the same procedures, obtained a similar number of infusions during IntA self-administration, and responded at similar levels during relapse testing as compared to the rats in the 0.25 ug/kg dose condition. We have also previously shown that the dose of fentanyl self-administered during the IntA phase does not impact subsequent relapse behavior (Towers et al., 2022). Following the last IntA session, rats were assigned to Experiment 1 (**Figure 1A**) and one of three withdrawal groups, a 0-, 1-, or 14- day or Experiment 2 (**Figure 1B**) and one of two treatment conditions, R-ketamine (20 mg/kg) or control (water) as detailed below. For both

studies, rats remained in their operant chambers with the active lever retracted during the withdrawal period.

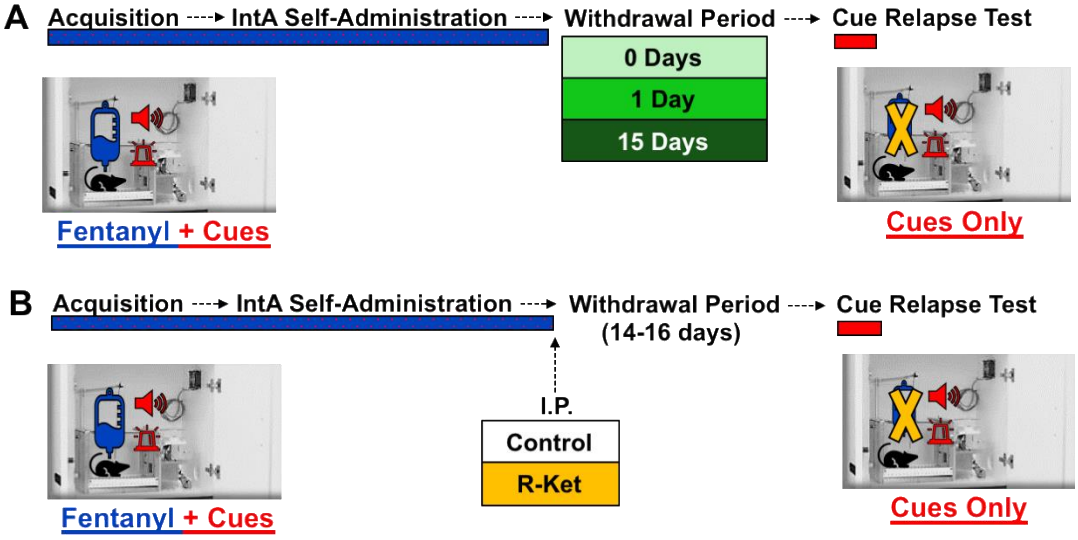

Figure 1. **Summary of experimental events.** In Experiment 1, males and females were trained to self-administer fentanyl (acquisition) and then given extended, intermittent-access to fentanyl (IntA Self-Administration; 24h/day, 2, 5 min trials/h). On withdrawal day 0, 1, 14, vulnerability to fentanyl-associated cues were assessed using a cue-relapse test (**A**). In Experiment 2, the same procedure was followed except upon fentanyl discontinuous R-ketamine (20 mg/kg) or water was administered via an interparental injection and vulnerability to fentanyl-associated cues was only assessed on withdrawal day 14-16 (**B**).

2.2 3 Experiment 1. Vulnerability to Relapse during Early versus Protracted Withdrawal.

Cue-relapse testing was conducted on withdrawal day 0, 1, or 14 in a two-hour session that began at the onset of the dark phase at 7PM. The session began with the introduction of the lever formerly associated with fentanyl and one presentation of the fentanyl associated cues (sound of the pump, stimulus light above formerly active lever). Subsequent responses on the active lever resulted in the presentation of these cues under a fixed ratio 1 schedule. Responses on the active and inactive levers were recorded over a 2-hour period. Female rats were swabbed the day before, the day of, and the day after the cue-induced relapse test session as described previously (Lynch et al., 2019). One female in the 14 -day withdrawal group had to be removed from the experiment due to illness. This resulted in a final group of 8 females and 8 males in the 0-day, 7 females and 10 males in the 1-day, and 10 females and 12 males in the 14-day group.

2.2.4 Experiment 2: R-Ketamine as an Anti-Relapse Intervention.

Immediately after the last IntA session, rats were administered an intraperitoneal injection of R-ketamine (20 mg/kg) or water and began a 14-16 day withdrawal period. This dose was selected based on a previous preclinical study showing it effectively blocked morphine-induced place preference and attenuated morphine withdrawal signs, but did not induce dysphoria in rodents (Witkin et al., 2020). On withdrawal days 14-16, rats were tested under the same cue-relapse procedure described above. We also included five male and four female controls that underwent the same experimental conditions as the other control rats, but did not receive an intraperitoneal injection of water. Importantly, these animals did not differ on any behavioral measure (average number of fentanyl infusions in the last 5 IntA sessions and responding to fentanyl-associated cues during the cue-relapse test) as compared to the male and female controls that received the intraperitoneal injection of sterile water at the start of the withdrawal period. The final group sizes were as follows: 8 males and 8 females in the control group and 8 males and 5 females in the R-ketamine group.

2.3 Drugs

Fentanyl hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC), dissolved in sterile saline at a concentration of 6.25 (or 37.5 μg/ml for the 4 rats run at the 1.5 μg/kg dose), sterile filtered (0.22 μm; Millipore, Billerica, MA), and stored at 4 °C. To ensure that mg/kg dose was consistent for each rat throughout the study, the duration of the infusions was adjusted for changes in body weight three times a week. R-ketamine was provided as a gift from Perception Neuroscience and dissolved in sterile water at a concentration of 20mg/ml.

2.4 Data Analysis

Sex differences in the number of fentanyl infusions obtained over the 10-day IntA period and the last five days of the additional IntA cycle were examined using a repeated measures ANOVA. In experiment 1, a univariant analysis was also used to examine the effect of sex and withdrawal group on the average number of fentanyl infusion obtained in the five IntA session that immediately precede the initiation of the withdrawal period. The same analysis was used in Experiment 2 to examine the effect of sex and treatment group (R-ketamine or Control) on the

average number of fentanyl infusion obtained in the five IntA session that immediately precede the administration of treatment and initiation of the withdrawal period.

Sex and withdrawal group (Experiment 1) / treatment group (Experiment 2) differences in fentanyl-seeking were determined using a repeated measures ANOVA comparing the number of responses over the 4, 30-minute segments of the cue-relapse test. A univariate analysis was also used to examine effects of sex and withdrawal/treatment group effects on total fentanyl-seeking during the cue-relapse test. In Experiment 1, the same analysis was also used to examine the effect of estrous cycle phase (in females), repeated cycles of IntA self-administration, and repeated cue-relapse tests on total fentanyl-seeking. In Experiment 2, sex and treatment group differences in the magnitude and time-course of physical dependence were also determined using a repeated measures ANOVA and comparing percent change in body weight during early (24 hours), intermediate (5-7days), and protracted (14-16 days) withdrawal relative to the end of IntA fentanyl self-administration (IntA 10). Post-hoc comparisons were made using two-tailed Bonferroni-corrected t-tests. Statistical analyses were performed using SPSS (V26). Alpha was set at 0.05. Data are presented as the mean ± SEM.

3 Results

3. 1 Fentanyl Intake:

As with our previous study (Towers et al., 2022), females obtained more fentanyl infusions than males during the initial 10-day IntA period with results from repeated measures ANOVA revealing an effect of sex ($F_{1,61}$=4.8, P<0.05; **Figure 2, left**). This difference was most pronounced at the beginning of the IntA period (interaction of session and sex: $F_{9,549}$=2.8, P<0.01) with females obtaining more fentanyl infusion than males in sessions 1-4 (*P's*<0.05), but not session 5-10. There was also a trend for an effect of session ($F_{9,486}$=1.8, P=0.075) that appears to be driven by males obtaining more fentanyl infusions in later IntA sessions compared to earlier sessions (session 1 versus 10, P<0.01). In contrast, in females, there was no difference in the number of fentanyl infusions obtained in session 1 and 10. Thus, females obtained more fentanyl infusions during the initial IntA sessions than males and maintained this high level of fentanyl self-administration across the entire IntA period. In contrast, males increased the number of fentanyl infusions obtained per session over the IntA period and reached the female-level of fentanyl intake by the later sessions (6-10).

A similar, but more modest, sex different was observed for the pattern of fentanyl self-administration in the subset of rats that underwent additional cycles of IntA fentanyl self-administration (**Figure 2A, right**). This analysis revealed an effect of session ($F_{4,176}$=6.5, P<0.001) and a trend for an interaction of session and sex ($F_{4,176}$=2.1, P=0.079) that was again driven by males, but not females, increasing the number of fentanyl infusions obtained in later sessions compared to earlier sessions during the re-escalation period (session 1 vs. 5; P<0.001). Additionally, as with the initial cycle of IntA self-administration, there were no differences between males and females in number of infusions obtained during the last five sessions of the subsequent cycle of IntA self-administration. Thus, males and females obtained a similar number of fentanyl infusions during subsequent cycles of IntA fentanyl self-administration.

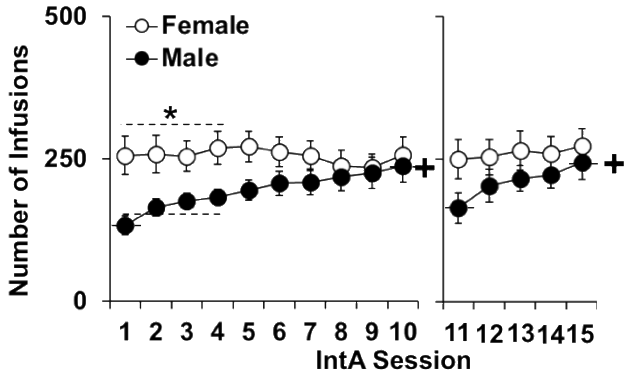

Figure 2. **Effect of sex on the number of fentanyl infusions obtained during the initial and additional cycle of intermittent-access (IntA) self-administration in females and males.** Mean (±SEM) number of infusions obtained during the initial 10-day IntA period and last five days of the addiction cycle of IntA for females (n=29) and males (n=35). (*) Significant difference between males and females. (+) Significant difference between the first and last session.

3.2 Experiment 1:

3.2.1 Fentanyl Intake

Prior to evaluating sex- and withdrawal-dependent differences in cue-induced relapse vulnerability, we analyzed the average number of fentanyl infusion obtained in the five IntA session that immediately preceded the initiation of the withdrawal period (**Figure 3A**). This analysis showed no effects of sex or withdrawal group. Thus, fentanyl self-administration was similar between sexes and withdrawal groups prior to the start of the withdrawal period and the subsequent cue-relapse test.

3.2.2 The Time-Course for the Development of an Enhanced Relapse Vulnerability

To our surprise, following IntA fentanyl self-administration, males and females had high levels of responding to fentanyl-associated cues (or fentanyl-seeking) during both early and protracted withdrawal (**Figure 3B**) with the repeated measure ANOVA comparing cue-induced responding over the 4, 30-minute segments revealing an effect of session ($F_{3,138}$=38.5, P<0.001), but no overall effect of withdrawal group or sex. Subsequent analysis showed that responding was highest in the first 30-minute segment (*P's*<0.001) and then decreased to a similar level for the last three segments of the session. Analysis of total responding during cue-relapse testing confirmed no effects of sex and withdrawal group (**Figure 3C**). Cue-induced relapse responding also did not differ between estrus versus non-estrus females (no effect of estrous cycle; **Figure 2D**) or between rats with a history of one cycle versus repeated cycles of IntA fentanyl self-administration (**Figure 2E**) or one versus two relapse tests (**Figure 3F**). Thus, vulnerability to fentanyl-associated cues was high regardless of withdrawal time point, sex, or estrous cycle phase (in females). Additionally, vulnerability to cues remained high following repeated cycles of fentanyl self-administration, withdrawal, and relapse testing.

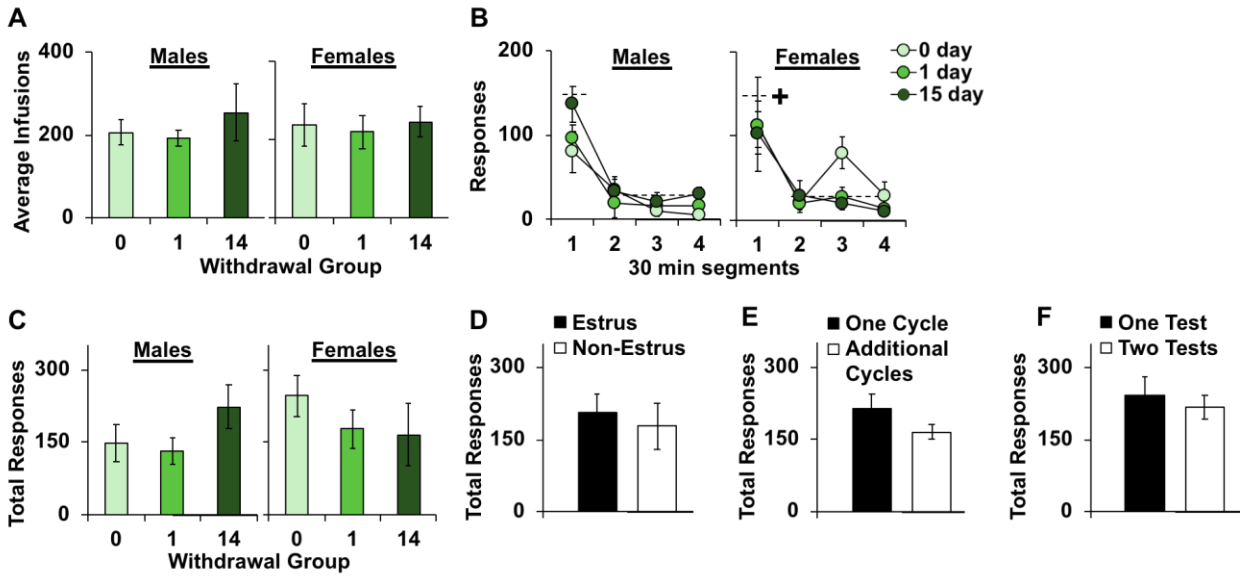

Figure 3. Effect of withdrawal time-point, sex, estrous cycle phase, and additional cycles of fentanyl self-administration, withdrawal, and relapse testing on responding during cue-relapse testing. Mean (±SEM) number of fentanyl infusions obtained in the five IntA session that immediately preceded the initiation of the withdrawal period (**A**), number of responses made on the lever formerly associated with fentanyl during the four-thirty minute segments of the cue-relapse test (**B**), and total number of responses made on the lever formerly associated with fentanyl during the cue-relapse test (**C**) in males and females in the 0- (female: 8, male: 8), 1- (female:7, male: 10), and 14- (female: 10, male: 12) day withdrawal groups. Additionally, mean (±SEM) number of total number of responses made on the lever formerly associated with fentanyl during the cue-relapse test in estrus (n=15) and non-estrus (n=10) females (n=15 and 10, respectively; **D)**, rats that underwent one and additional cycles of IntA self-administration (n= 28 and 35, respectively; **E**), and one and additional cue-relapse tests (n=11 and 11, respectively; **F**). (+) Significant difference between the first and last segments (**B**).

3.3 Experiment 2:

3.3.1 Fentanyl Intake

Males and females in the control (water) and R-ketamine (20 mg/kg) treatment groups had high levels of fentanyl self-administration prior to the administration of treatment and the initiation of withdrawal (**Figure 4A**). An univariant ANOVA comparing the average number of fentanyl infusions obtained during the five IntA sessions that preceded the treatment and withdrawal period revealed no effects of sex or treatment group. Thus, fentanyl intake was similar between sexes and treatment groups prior to the administration of treatment and the subsequent cue-relapse test.

3.3.2 Effect of R-ketamine on Opioid Withdrawal in males and females

R-ketamine administered at the time of fentanyl discontinuation did not prevent weight loss during early withdrawal in fentanyl dependent males and females (**Figure 4B**) with results from the repeated measure ANOVA comparing percent of bodyweight loss during early (24 hours; W1), intermediate (withdrawal day 6-8; W7), and protracted (withdrawal day 12-14; W14) withdrawal relative to the end of IntA fentanyl self-administration (IntA day 10) revealing no effects of treatment group. There was an effect of time ($F_{2,48}$=36.7, P<0.001) and sex ($F_{1,24}$=11.2, P<0.01) and a trend for an interaction of time and sex ($F_{2,50}$=3.1, P=0.056) which appears to be driven by males, but not females, regaining and surpassing the bodyweight lost during early withdrawal faster over the protracted withdrawal period than females. Subsequent analysis within each withdrawal timepoint revealed no sex difference in the percentage of bodyweight lost during early withdrawal, but significant differences within the intermediate ($F_{1,26}$=10.0, P<0.01) and the protracted withdrawal time-points ($F_{1,26}$=8.1, P<0.01). Post-hoc comparisons to bodyweights at the end of IntA (or 0) within each of these withdrawal time-points also confirmed these differences. Specifically, within early withdrawal, both males and females lost a significant percentage of their bodyweight (P's<0.01), indicating that both sexes developed opioid dependence. Within intermediate withdrawal, both females and males had regained the bodyweight lost during early withdrawal, but only males had surpassed their previous weight (P<0.05). This sex difference persisted during protracted withdrawal with males (P<0.001), but not females, showing a significant increase in their pre-withdrawal body weights. Thus, males and females developed physical dependence to fentanyl and R-ketamine did not prevent opioid withdrawal upon discontinuation of fentanyl use. Additionally, similar to our previous work (Towers et al., 2022), opioid withdrawal persisted longer in females than males.

3.3.3 Efficacy of R-ketamine as an Anti-Relapse Treatment for OUD in males and females.

R-ketamine (20 mg/kg) administered at the time of fentanyl discontinuation persistently attenuated vulnerability to fentanyl-associated cues (or fentanyl-seeking) during withdrawal in males, but not females (**Figure 4C-D**). Results from the repeated measures ANOVA comparing responding over the 4, 30 minutes segments showed an effect of session (**Figure 4C**; $F_{3,72}$=36.0, P<0.001) as well as significant interactions of session, treatment group, and sex ($F_{3,72}$= 3.0, P<0.05) and sex and treatment group ($F_{1,24}$=5.7, P<0.05). Subsequent within-sex analyses

showed that, in males, there was an effect of session ($F_{3,42}$= 29.0, P<0.001) and treatment group ($F_{1,14}$=5.7, P<0.05) and an interaction of session by treatment group ($F_{3,42}$=6.6, P<0.001). Post-hoc analysis within each 30-minute segment revealed these effects were driven by fentanyl-seeking being reduced in the R-ketamine group compared to the control group within the first 30-minute segment (P<0.01), but no difference between the treatment groups in segments 2-4. Additionally, subsequent analysis within each treatment condition showed that fentanyl-seeking was the higher in the first segment versus segments 2-4 in the control group (*P's*<0.001) and segment 2 (P<0.05) and 4 (P<0.05) in the R-ketamine group in males. The same analysis within females revealed an overall effect of session only ($F_{3,30}$=11.6, P<0.001) with post-hoc analysis showing that similar to males, fentanyl-seeking was higher in the first segment versus later segments (*P's*<0.05). Notably, in contrast to the findings in males, there were no significant effects of treatment group.

Similar results were observed for total responding during cue-relapse testing (**Figure 4D**) with results from an univariant analysis revealing an interaction of sex and treatment group ($F_{1,24}$=5.7, P<0.05). Post-hoc analysis within each sex confirmed that total fentanyl-seeking was lower in the R-ketamine versus control group in males (P<0.05), but not females (P>0.05). Thus, R-ketamine administered at the time of discontinuation of fentanyl use attenuated subsequent cue-induce relapse vulnerability during protracted withdrawal in males, but not females.

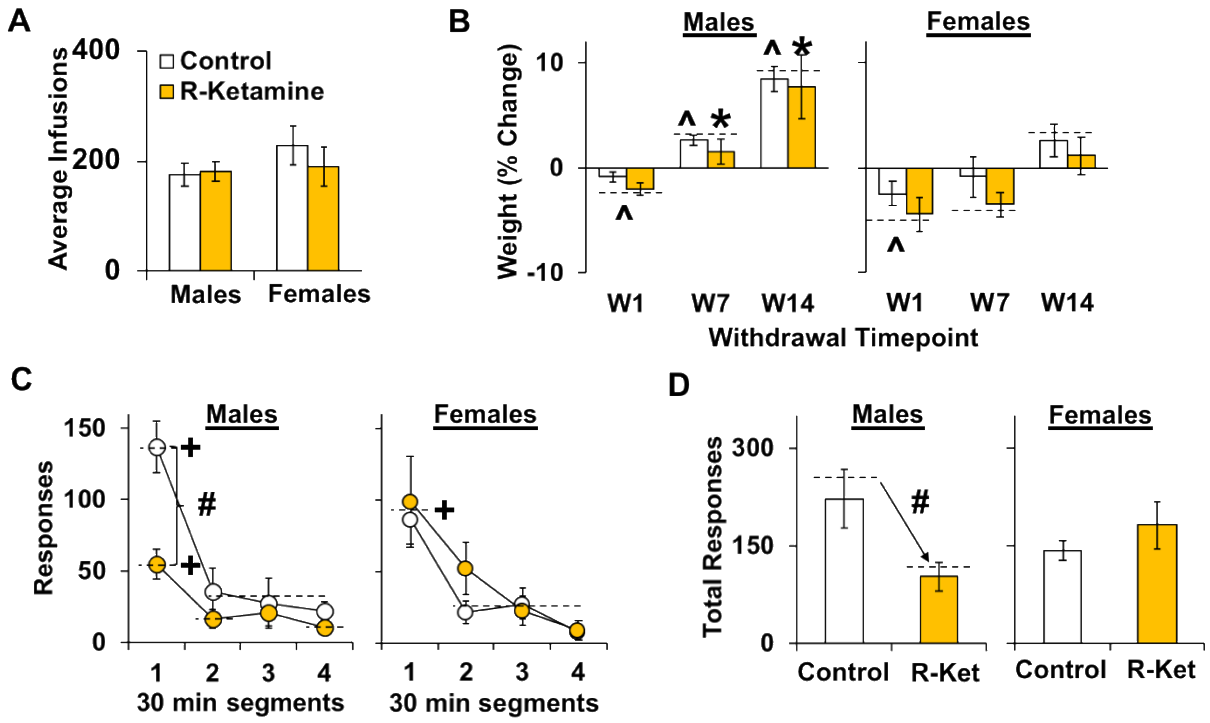

Figure 4. **Effect of R-ketamine on physical dependence and responding during cue-relapse testing.** Mean (±SEM) number of fentanyl infusions obtained in the five IntA session that immediately preceded the initiation of the withdrawal period (**A**), percent change in body weight during early (W1), intermediate (W7), and protracted (W14) withdrawal relative to the end of IntA period just prior to withdrawal (**B**), number of responses made on the lever formerly associated with fentanyl during the four, thirty-minute segments of the cue-relapse test (**C**), and total number of responses made on the lever formerly associated with fentanyl during the cue-relapse test (**D**) in males and females in R-ketamine (female: 5, male: 8) and control (female: 7, male: 8) groups. (*) Significant difference between females and males (**B**). (^) Significant difference from baseline (or 0; **B**). (+) Significant difference between the first and last segments (**C**). (#) Significant differences between the treatment and control group (**C, D)**.

4 Discussion

The current study evaluated sex differences in the time-course for the development of an enhanced vulnerability to relapse as measured by levels of drug-seeking in response to fentanyl associated cues during early (withdrawal day 0 and 1) versus protracted withdrawal (withdrawal day 14) when levels are expected to be low versus high, respectively. In contrast to ours and others previous findings with other addictive drugs (see Li et al., 2015 for review), we found vulnerability to fentanyl-associated cues was high following discontinuous of fentanyl use (early withdrawal, day 0) and these high levels persisted through protracted withdrawal (day 14) in males and females. This was a rather robust effect as cue-induce fentanyl-craving remained high regardless of withdrawal time point and was not impacted by sex, estrous cycle phase (in females), or repeated cycles of fentanyl self-administration, withdrawal, and relapse testing. As a second goal of this study, we determined the efficacy of R-ketamine, a novel potential treatment to substance use disorder, at reducing vulnerability to relapse. To our excitement, despite the development of persistently, high levels of relapse vulnerability in this animal model, R-ketamine administered at the time of fentanyl discontinuation greatly attenuated subsequent vulnerability to fentanyl-associated cues in males as assessed during protracted withdrawal. However, there was no effect of R-ketamine on fentanyl-craving in females. Thus, the beneficial effects of R-ketamine treatment following IntA fentanyl self-administration appears to be sex-specific.

The most unexpected finding in the present study was that males and females had high levels of responding to fentanyl-associated cues during early withdrawal (day 0) and this level of

responding was maintained through protracted withdrawal (day 14). One likely explanation for this discrepancy is our self-administration conditions given the majority of the preclinical studies on the incubation effect have used continuous-access rather than IntA opioid self-administration conditions (e.g., Fanous et al., 2012; Shalev et al., 2001; Theberge et al., 2012; Venniro et al., 2019; Altshuler et al., 2021; Wong et al., 2022). Indeed, the only preclinical study to our knowledge that has compared the incubation of cue-induced opioid-craving following continuous and IntA self-administration showed that heroin-craving only incubated following withdrawal from continuous-access self-administration (D'Ottavio et al., 2023). One caveat, however, is that preclinical studies with stimulants, such as cocaine, have previously shown an incubation effect following withdrawal from continuous and IntA self-administration (e.g. Nicolas et al., 2019; Corbett et al., 2021). Therefore, this lack of an incubation effect following IntA self-administration may only apply to opioids and be the result of IntA opioid self-administration inducing a more sever phenotype in animals that results in a high level of craving during early withdrawal that is maintained through protracted withdrawal. This explanation would also align with clinical studies that have shown opioid craving remains persistently high in patients with severe OUD, such as treatment refractory active users and treatment-seeking individuals (Blaken et al., 2012; Wang et al., 2012; Childress et al., 1986).

Notably, the findings presented here in combination with the work by D'Ottavio and colleagues (2023) can be used to help optimized current animal models of opioid addiction. More specifically D'Ottavio et al. (2023) showed under continuous-access conditions, males and females require a prolonged withdrawal period (21 days) to express an addiction-like phenotype (or incubation of heroin-seeking); whereas, under IntA self-administration conditions, males and females expressed a similar level of heroin-seeking on withdrawal day 1 as withdrawal day 21 following continuous-access self-administration, and these high levels of heroin-craving were maintained through withdrawal day 21. In the present study, we expanded these findings to fentanyl and similarly showed males and females have high levels of fentanyl-seeking on withdrawal day 0, which was maintained through withdrawal day 14. Together, these findings indicated an acquisition period paired with a 10-day IntA opioid self-administration period is sufficient to induce a severe opioid-addiction like phenotype in animals and withdrawal is not necessary. This conclusion is further supported by our findings showing these high levels of fentanyl-seeking on withdrawal day 0 are also maintained with repeated cycles of fentanyl self-

administration, withdrawal, and relapse testing, which more closely models the chronic, relapsing pattern of opioid use described in the human condition (NIDA, 2019). Additionally, we have previously shown and show here that these conditions induce other addiction like-features such as physical and an enhanced motivation for fentanyl (Towers et al., 2022, 2023), which also speaks to the severity of the phenotype; thereby making this procedure a strong model for studying the neurobiological basis of opioid addiction and tool for screening possible treatment strategies.

The lack of a sex and estrous cycle effect on vulnerability to cue-induced relapse was also surprising considering our previous findings with fentanyl showing females, particularly when tested in the estrus phase of the estrous cycle, have higher levels of responding to fentanyl-associated cues than males (Bakhti-Suroosh et al., 2021; Towers et al., 2022). The reason for these conflicting results is not clear but sex differences, and other individual differences, are most easily detected during transition points in the disease process and/or under threshold conditions that allow for individual variability (Towers et al., 2021; Lynch, 2018). As described above, the conditions used here are optimal for inducing an addiction-like phenotype and appear to induce a severe phenotype in males and females. We have previously shown under optimal conditions once the shift to addiction occurs the expression of the phenotype is more similar between males and females, which is also true for humans (see Towers et al., 2023 for review). Another explanation for the conflicting results is procedural differences since in our previous studies with fentanyl we used an extinction/reinstatement procedure whereas here we used a cue-replace procedure that has previously been used by others with opioids and other addictive drugs. Notably, these other studies have produced mixed results with some reporting higher levels in females compared to males (e.g., D'Ottavio et al., 2023; Corbett et al., 2021) and other reporting no effect of sex and/or estrous cycle phase (e.g., Venniro et al., 2017, 2019). Thus, this procedure may also be less sensitive to detecting sex and ovarian hormone differences.

However, during the initial, 10-day self-administration period, we did observe a sex difference in fentanyl intake with females self-administering more fentanyl than males under the IntA procedure, particularly during the initial sessions. These results are consistent with our previous findings with fentanyl and others with heroin showing sex differences in opioid intake are most apparent under lower dose conditions (or threshold conditions), but not higher dose conditions (or optimal conditions; e.g., Towers et al., 2019, 2022; Zhang et al., 2015). In the

present study, we also expanded on our previous work and show that with additional cycles of IntA fentanyl self-administration this sex difference in fentanyl intake dissipates and females and males self-administer a similarly high levels of fentanyl. This is another example of sex difference being most apparent at a transition point in the disease process (i.e., from acquisition of drug self-administration to establishing maintenance levels of drug intake); whereas, once an addiction-like feature has developed the sex difference becomes less apparent. Interestingly, during the initial IntA period and additional IntA cycles, males showed an escalation of fentanyl intake and obtained more fentanyl infusions during the later sessions compared to the earlier sessions, similar to our previous study (Towers et al., 2022). However, unlike our previous study (Towers et al., 2022), in the present study, females maintained a high level of fentanyl intake across the IntA periods. The reasons for these different results are unknown but could be attributed to females tending to have a longer initial period of "binge" drug intake than males as previously described for opioids, cocaine, and alcohol (Towers et al., 2019; Towers et al., 2022; Lynch and Taylor, 2004; Sneddon et al., 2019).

Lastly, and probably of most importance, despite the robust vulnerability to fentanyl-associated cues across the prolonged withdrawal period, R-ketamine administered at the time of fentanyl discontinuation attenuated subsequent vulnerability to cue-induced fentanyl-craving in males as assessed during protracted withdrawal. We decided to administer R-ketamine on withdrawal 0 based on our findings in Experiment 1 which showed fentanyl-seeking was already robustly expressed during early withdrawal. To us, these findings indicated to have translational significance, as an anti-relapse intervention, the treatment needed to be implemented at the time of stopping fentanyl use. Additionally, a study on depression in rodents previously showed that R-ketamine can have long-lasting (7-days) anti-anhedonic and anti-apathic effects and we wanted to test the durability of this effect in an addiction model (Zang et al., 2017; Rafało-Ulińska and Pałucha-Poniewiera, 2022). Notably, our findings presented here with R-ketamine in males are similar to those previously reported for the racemic ketamine in humans and animals (Dakwar et al., 2019, 2014; Jones et al., 2018; Krupitsky et al., 2007). This is particularly exciting because other studies have shown the R-enantiomer of ketamine is devoid of the unwanted side effects of racemic ketamine, which have hindered its development as a treatment for substance use disorders (Bonaventura et al., 2021). However, these beneficial effects of R-ketamine appear to be sex-specific considering that R-ketamine did not attenuate opioid-craving

in females. Considering the previous clinical and preclinical studies with ketamine were predominately preformed in males, it was difficult to predict the effect in females. Although, these findings add to the accumulating evidence that there are sex differences in the neuroadaptations underlying addiction and the incubation of drug-craving. Future studies are needed to determine the mechanisms underlying the sex-specific benefits of R-ketamine as it will likely shed light on these mechanistic sex differences in addiction.

In summary, this study builds on pervious findings with heroin and shows that cue-induced fentanyl-seeking is also high during early withdrawal from IntA self-administration and these high levels persist through protracted withdrawal in both males and females. As described above, these finding can be used to help optimize current animal model of opioid addiction. Additionally, these findings indicate that implementing treatment strategies at the time of stopping fentanyl use, such as in emergency room settings, could help patients with severe OUD. We used this strategy with R-Ketamine and provide the first evidence for its use as a potential anti-opioid craving medication in males. The fact that the beneficial effects of R-ketamine do not translate to females highlights the urgent need for the investigation of sex-specific treatment strategies so that equitability treatments can be provided to both males and females.

Chapter IX

Dissertation Summary and Future Directions

Dissertation Summary and Future Directions

This dissertation provides strong evidence for sex being an important biological factor in addiction. Below I will highlight some of the most exciting findings and provide preliminary data on the directions we are taking to follow up on these exciting advances in addiction research.

The evidence presented in this dissertation is particularly strong for the faster progression to addiction in females versus males (or the telescoping effect). In Chapters II and III, we firmly established a telescoping effect with cocaine by demonstrating that three key features of SUDs in humans, an enhanced motivation for the drug, as assessed under a progressive ratio schedule, compulsive drug use, as assessed under a histamine punishment procedure, and enhanced vulnerability to cue-induced relapse, as assessed using an extinction/reinstatement procedure, develop (or peak) sooner during withdrawal from extended-access cocaine self-administration in female versus male rats. Notably, as discussed in Chapter I, earlier preclinical studies in the Lynch laboratory showed that females, but not males, develop an enhanced motivation for cocaine under conditions predicted to be threshold for inducing this phenotype (7 days of extended-access cocaine self-administration and 10 days of abstinence; Lynch and Taylor, 2004). The phenotype was also subsequently confirmed to be absent in both females and males when assessed under sub-threshold self-administration and abstinence conditions (e.g., 7 days of extended-access self-administration with no intervening period abstinence or following short-access self-administration with or without abstinence; Lynch and Taylor, 2005), and present in both sexes when the conditions are optimized for its development by lengthening the period of extended-access self-administration (i.e. 10 days) and/or the abstinence period (i.e. 14 days; Roberts et al. 2007; Ramôa et al. 2013). Additionally, others have shown that females develop an enhanced motivation for cocaine following fewer cycles of intermittent-access cocaine self-administration than males (10 days of intermittent-access versus 30 days intermittent-access and 14 days of withdrawal; Kawa and Robinson, 2019) and several studies have shown a greater percentage of females than males develop a preference for the drug (cocaine) over another competing reinforcer (food), another key characteristic of SUDs in humans, following a prolonged period of short-access cocaine self-administration (~3-5 weeks; ~50% versus 17%, respectively; Kerstetter et al. 2012, Perry et al. 2013, Perry et al. 2015). Together, these findings show that an addiction-like phenotype with cocaine develops at an accelerated rate in female rats

compared to male rats and indicate that the parallel effect in women is based at least in part on biological factors.

Another point worth highlighting is that the molecular findings presented in this dissertation provide modest support for females having a faster course than males for the development of key neuroadaptations that are thought to underlie the development of an addiction-like phenotype. For example, in Chapter III, we show the timeline for the change in *Grin1* expression, the gene that encodes the GluN1 subunit of the NMDA receptor, in the dorsomedial prefrontal cortex (dmPFC) differs between males and females. In males, as with previous studies, *Grin1* expression was increased following relapse testing during protracted abstinence (following 14 days), whereas, in females, *Grin1* expression was increased following relapse testing during intermediate abstinence (following 7 days). These molecular effects correspond to differences in cocaine-craving in response to drug-associated cues which peaked during protracted abstinence in males and during intermediate abstinence in females (i.e., following 14 versus 7 days; Towers et al., 2023a) suggesting that glutamatergic signaling in the dmPFC is recruited earlier during abstinence in females compared to males. Similar sex differences have also been reported for the effects of extended-access methamphetamine self-administration on NMDA signaling in the dmPFC (Mishra et al. 2017; Pena-Bravo et al. 2019). In this series of studies, effects were first characterized in males only and showed that NMDA receptor currents were increased following abstinence (8-14 days) from extended-access self-administration (Mishra et al. 2017). Then, the effect was confirmed in females in a more recent study that included both males and females (Pena-Bravo et al. 2019); however, this study used a shorter period of extended-access self-administration, and under these "threshold" conditions, NMDA receptor currents were increased in females, but not males, providing further support for the idea that this molecular shift to glutamate signaling develops more readily in females.

Notably, the mechanisms underlying the telescoping effect is an area of research that we are highly interested in and currently starting to investigate further. One of the major theories that we are currently exploring is that the accumulation of synaptic, Ca^{2+}-permeable AMPA- type glutamate receptors (CP-AMPAR) in the nucleus accumbens core (NAc) mediates the development of enhanced motivation for cocaine and occurs sooner during withdrawal in females thereby underlying the telescoping effect. We are choosing to focus on AMPA receptors because the Lynch laboratory has previously demonstrated that AMPA signaling in the NAc,

though not necessary for motivating initial cocaine use, becomes critical once an addiction-like phenotype develops (Doyle et al., 2014). Additionally, there is a large body of work showing the incubation, or progressively increasing levels, of drug-seeking over withdrawal from extended-access drug self-administration with multiple addictive drugs (cocaine, methamphetamine, and oxycodone) is mediated by the accumulation of synaptic, CP-AMPAR. Therefore, in this on-going study, motivation for cocaine is being assessed under a progressive-ratio schedule before and after extended-access cocaine self-administration (24 h/day, 96 infusions/day, 10 days) and a 7-day withdrawal period, which are threshold conditions that induce an addiction-like phenotype (defined as a greater than 15% increase in motivation) in females, but not males. During withdrawal, we are using pharmacological approaches that either prevent the recruitment of CP-AMPA (intraperitoneal injection of the mGluR1 positive allosteric modulator SYN119, 0 or10 mg/kg; Loweth et al., 2013) or accelerate the recruitment (intraperitoneal injection of the mGluR1 antagonist JNJ16259685, 0 or 5 mg/kg; Loweth et al., 2013). We also used a neuropharmacological approach to site-specifically target CP-AMPA receptors in the NAc (intra-NAc infusions of the CP-AMPA receptor antagonist naspm (40 µg/side; Loweth et al., 2013) to verify their recruitment (or lack thereof). Preliminary findings indicate that similar to our previous studies, motivation for cocaine is increased from baseline (or prior to extended-access cocaine self-administration) in vehicle-treated females, but not males, following extended-access cocaine self-administration and a 7-day withdrawal period. Additionally, as expected, this motivational shift is blocked in females by systemic administration of SYN119 and induced in males by systemic administration of JNJ16259685 (**Figure 1A, C**). NAc-infused naspm also appears to reduce motivation for cocaine in males that developed an enhanced motivation for cocaine, but not females where this phenotype was blocked from being expressed (**Figure 1B, D**). Together, these preliminary results support our hypothesis that CP-AMPA receptors in the NAc contribute the development of an enhanced motivation for the cocaine and the faster time-course for its development in females versus males.

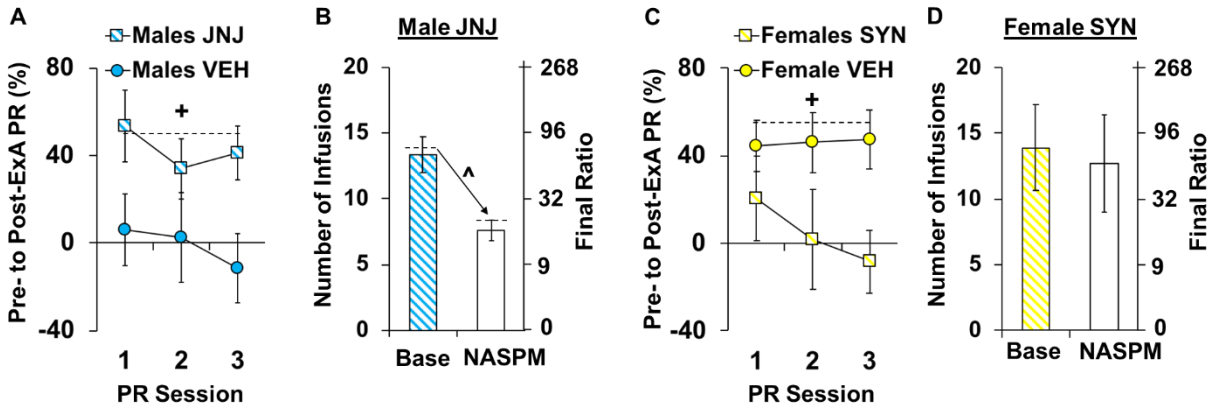

Figure 1. The role of Ca^{2+}-permeable AMPA- type glutamate receptors the development of an enhanced motivation for cocaine and the telescoping effect. Percent change (±SEM) in the number of infusions obtained following ExA self-administration (Post-ExA) and withdrawal relative to baseline (Pre-ExA) for JNJ16259685 males (JNJ, n = 8), vehicle males (VEH, n= 7), SYN119 females (n = 7), and vehicle females (n = 10; **A, C**). Mean (± SEM) number of infusions obtained during the three-progressive ratio (PR) sessions prior to treatment (Base) and the day of treatment (NASPM) in JNJ16259685 males (JNJ, n= 5) and SYN119 females (n = 3; **B, D**). Significant increase (versus no change, 0) from baseline (**A, C**; +). Significant effect of CP-AMPA antagonist, NASMP (**B**; ^).

To investigated the mechanisms underlying the telescoping effect more broadly, we have also replicated our previous studies showing a faster progression to an addiction-like phenotype in females compared to males (Lynch and Taylor, 2005; Towers et al., 2021a) and collected whole brains for molecular analyses (relative to saline controls run contemporaneously). As with our previous studies, in this new cohort we show following 10-days of extended-access cocaine self-administration, motivation for cocaine is decreased or unchanged during early withdrawal in males and females (day 0), increased in females, but not males, during intermediate withdrawal (day 7), and increased in females and males during prolonged withdrawal (day 14) relative to the baseline levels of motivation established prior to extended-access self-administration (**Figure 2)** We intend to dissect tissue from the NAc and its primary glutamatergic projection source, the dmPFC and use RNA- and ChIP- sequencing to identify novel genes and epigenetic mechanism that underlie the telescoping effect focusing on the role of the estrogen receptor.

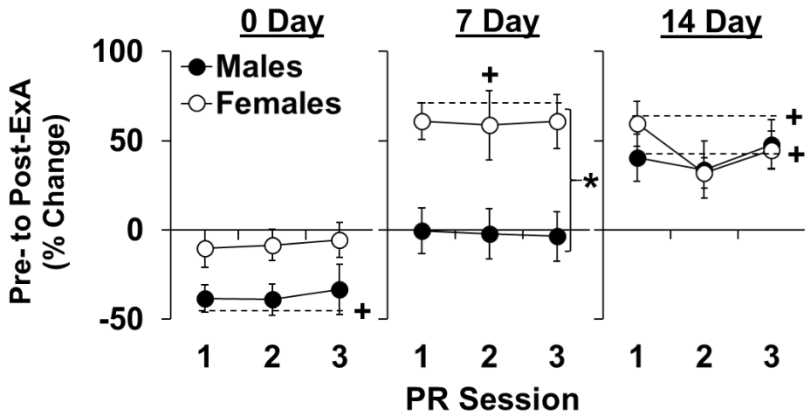

Figure 2. Effect of sex on the time-course for the development of enhanced motivation for cocaine. Percent change (±SEM) in the number of infusions obtained following ExA self-administration (Post-ExA) and withdrawal relative to baseline (Pre-ExA) for females and males in the 0- (n = 6 and 12, respectively), 7- (n = 10 and 7, respectively), or 14- (n = 11 and 9, respectively) day withdrawal groups. Significant difference between females and males (*). Significant increase (versus no change, 0) from baseline (+).

Considering a major theory of sex differences in SUDs is that differences are due to ovarian hormones with estradiol enhancing vulnerability in females, we are also currently investigating the role of estradiol in the telescoping effect in females. For this study, we are using ovariectomized females with (OVX+E) and without estradiol (OVX+V) replacement and assessing the development of enhanced motivation for cocaine using a progressive-ratio schedule prior to and following 7- and 14-day of withdrawal from 10-days of extended-access cocaine self-administration. As we predicated, OVX+E females, but not OVX+V females, showed a significant increase in motivation for cocaine following 7- and 14-day of withdrawal (relative to baseline levels established prior to extended-access cocaine self-administration; **Figure 3A**). However, to our surprise, a subset of the OVX+V females (12 out of 24; 50%) also met the criteria for the development of enhanced motivation (i.e., greater than 15% increase in motivation for cocaine; **Figure 3B**). The likelihood is just greatly increased in the OVX+E females (20 out of 25; 80%), which explains the overall findings described above. Additionally, within the OVX+E an OVX+V females, there was no difference in the proportion of rats that met the criteria for the development of enhanced motivation in the 7-day (OVX+E: 79%; OVX+V: 50%) and 14-day (OVX+E: 83%; OVX+V: 50%) withdrawal groups. Therefore, estradiol appears to enhance vulnerability to, but is not necessary for, the development an addiction-like phenotype and the telescoping effect in females. This experiment was run alongside the intact

males and females described above and a representative sample of OVX+E and OVX+V females that developed an addiction-like phenotype (i.e. OVX+E addicted and OVX+V addicted, respectively) and OVX+V females that did not develop an addiction-like phenotype (i.e. OVX+V non-addicted) have been selected to be included in the molecular analyses to help more clearly identify estrogen-dependent and non-dependent mechanisms underlying the addiction-like phenotype in females (**Figure 3C**).

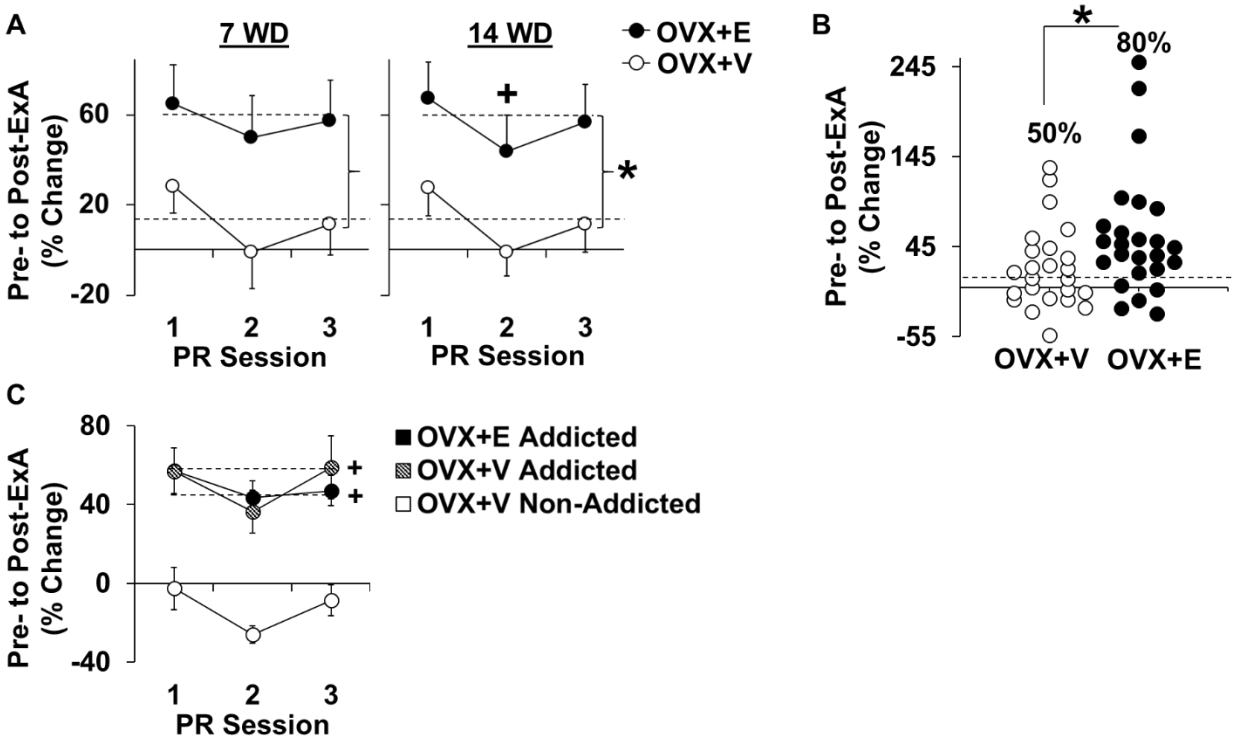

Figure 3. Effect of estradiol on the time-course for the development of enhanced motivation for cocaine. Percent change (±SEM) in the number of infusions obtained following ExA self-administration (Post-ExA) and withdrawal relative to baseline (Pre-ExA) for OVX+E and OVX+V in the 7- (n = 16 and 19, respectively) and 14- (n = 8 and 6, respectively; **A, B**) and a representative sample of OVX+E and OVX+V females that developed an addiction-like phenotype (i.e. OVX+E addicted, n= 6, and OVX+V addicted, n=6, respectively) and OVX+V females that did not develop an addiction-like phenotype (i.e. OVX+V non-addicted, n =6; **C**). Significant difference between OVX+E and OVX+E females (**A, B**; *****). Significant increase (versus no change, 0) from baseline (**A, C**; +).

To date, all of the behavioral and molecular preclinical work on the telescoping effect has focused only on cocaine. Although we do not provide evidence for the telescoping effect being biologically-based with other addictive drugs, such as opioids, in this dissertation, this is also an area of research we are currently investigating. We are using our extended-, intermittent-access

fentanyl self-administration procedure (2, 5 min trials/hr, 10 days) that has been validated to induce multiple addiction-like features including a binge-abstinent patterns of use (Towers et al., 2022), physical dependence (Towers et al., 2022), an enhanced motivation for fentanyl (Towers et al., 2023c), and an enhanced vulnerability to relapse (Bakhti-Suroosh et al., 2021; Towers et al., 2022). These latter features, the enhanced motivation for fentanyl and vulnerability to relapse, have been shown to emerge following intermittent-access self-administration and protracted withdrawal (day 15). We have also shown relapse vulnerability can be attenuated in both males and females by administering buprenorphine during withdrawal, an FDA-approved treatment for OUD; thus, validating our rat model of OUD. In the ongoing study, we chose to focus on the time-course for the development of an enhanced motivation for fentanyl considering the majority of the studies with cocaine have used this feature to determine the time-course for the development of an addiction-like phenotype in rats (Towers et al., 2021; Lynch and Taylor, 2005; Ramôa et al. 2013; Perry et al. 2015). Similar to our previous studies, we defined an enhanced motivation for fentanyl as a greater than 15% increase in motivation relative to baseline levels established after acquisition (Towers et al., 2023c; Ramoa et al.,2013, 2014). We have assessed this phenotype following short-access fentanyl self-administration (or acquisition) and 14 days of withdrawal, an abbreviated, 3-day intermittent-access fentanyl self-administration period and 14-days of withdrawal, and an optimal, 10-day intermittent-access fentanyl self-administration period and 0-, 1-, or 14-day of withdrawal. Our preliminary findings indicated that similar to cocaine (e.g., Lynch and Taylor, 2005; Towers et al., 2023d), a short-access self-administration period paired with a 14-days of withdrawal is not sufficient to induce an increase in motivation for fentanyl in females and males (**Figure 4A**). However, as predicted, females, but not males, showed a transient increase in motivation for fentanyl following an abbreviated intermittent-access period paired with a 14-day withdrawal period (**Figure 4B**). And, following an optimal intermittent-access period, males and females show a stable increase in motivation for fentanyl that was maintained across the withdrawal period (14 days; **Figure 4C**). These preliminary findings provide the first evidence for the telescoping effect observed in humans with OUD also being biologically-based.

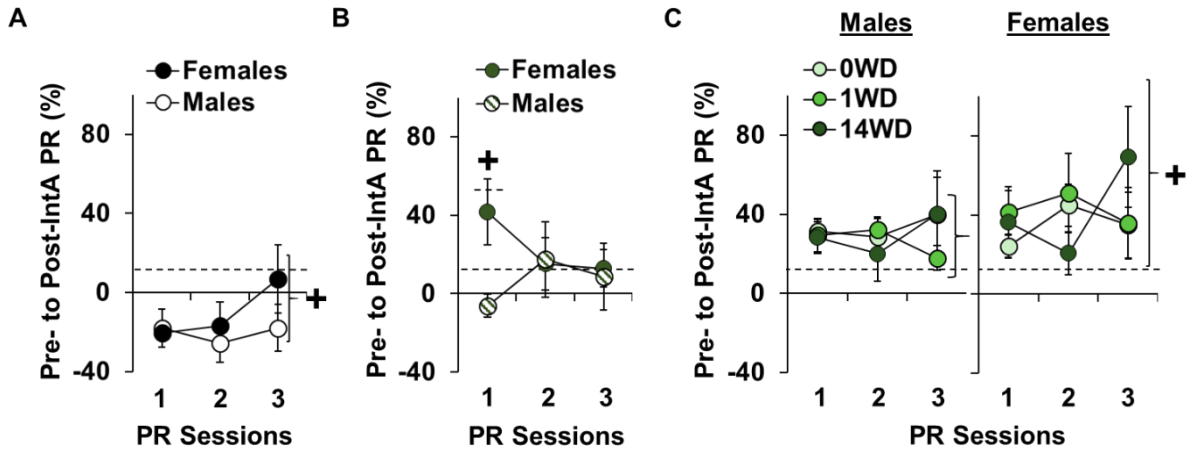

Figure 4. Effect of sex on the time-course for the development of enhanced motivation for fentanyl. Percent change (±SEM) in the number of infusions obtained following short-access self-administration and 14-days of withdrawal (**A**), a 3-day intermittent-access period of fentanyl self-administration and 14-days of withdrawal (**B**), and a 10-day intermittent-access period of fentanyl self-administration and 0-, 1-, or 14-days of withdrawal (**C**) relative to baseline levels assessed after acquisition in male (n = 7, 8, 11, 13, and 11, respectively) and females (n= 8, 9,12, 9, and 9, respectively). Significant change (versus 0) from baseline (**A**, **B**, **C**; +).

To further support this conclusion, we also went back and analyzed sex differences in the onset of adverse health events that resulted in the removal from studies using our OUD rat model for roughly the past three years (February 2020- September 2023). We predicted the females would also be more vulnerable than males to experiencing adverse health events since clinical studies have shown females have an accelerate time-course and/or enhanced sensitivity to developing drug-related health consequences compared to males. Although the original clinical reports were primarily focused on alcohol and showed females develop alcohol-associated cirrhosis, brain atrophy, and cardiomyopathy more readily than men (Loft et al. 1987; Hommer et al. 1996, 2001; Mann et al. 1992, 2005; Fernandez-Sola et al. 1997; Urbano-Marquez et al. 1995), more recent studies have focused other drugs, including opioids, and found a similar pattern (Iversen et al. 2010; Des Jarlaise et al. 2012; see Towers et al., 2023 for review). As predicted, we found a robust sex difference with females having a greater probability of experiencing adverse health events that males, particular during the initial fentanyl self-administration and withdrawal period (**Figure 5**). Preliminary results from the necropsies performed by the veterinarians at the University of Virginia indicate that these adverse health events may be the result of fentanyl-induced acute liver injury that progresses to multi-organ

failure, which is similar to the findings report in Chapter V for cocaine. The mechanisms underlying these sex differences in drug-related health consequences are unknown making this a high priority area of research for the lab going forward.

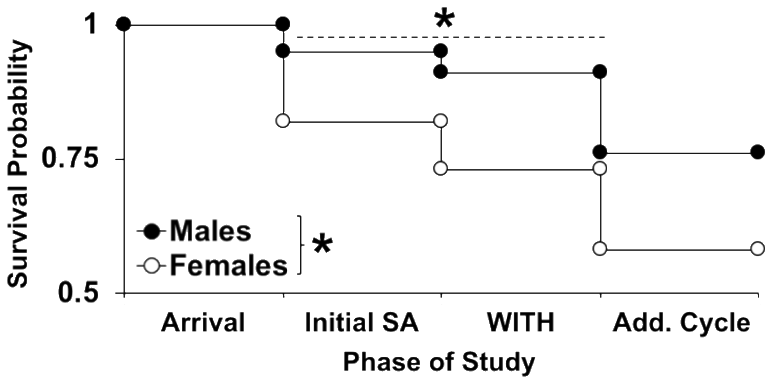

Figure 5. Effect of sex on the probability of the development of a fentanyl-related adverse health event.
Survival probability during arrival, initial fentanyl self-administration period (initial SA), intial withdrawal period (WITH), and additional cycles of fentanyl self-administration and withdrawal (add. cyle) for females (*n*=208) and males (*n*=181). Significant differences between females and males (*).

It is not surprising that similar results are being found with opioids and cocaine considering in this dissertation we show multiple sex difference previously reported with cocaine and other addictive drugs translate to fentanyl. More specifically, in Chapter VI, we show females are more vulnerable than male to expressing multiple addiction-like features including binge intake of fentanyl, physical dependence, and cue-induced relapse, particularly during the estrus phase of the estrous cycle. Notably, these findings align with previous extended-access self-administration studies that have shown female self-administer higher levels of alcohol, opioids, including heroin, oxycodone, and morphine, and psychostimulants including, cocaine, methamphetamine, and nicotine, compared to males (Becker and Koob, 2016; Carroll et al. 2005; George et al. 2021; Kawa and Robinson, 2019; Lynch and Taylor, 2004, 2005; Moore and Lynch, 2015; Nicolas et al. 2019; Reichel et al. 2012; Roth and Carroll, 2004; Sanchez et al. 2014; Smith et al. 2011; Towers et al. 2019). Preclinical studies have also shown females self-administer more heroin during the first hour of a long, continuous access session (or the loading phase; Towers et al., 2019), have a longer initial periods of "binge" cocaine intake (defined as continuous drug use with no breaks from drug self-administration greater than 1 hour) and greater dysregulation in

diurnal patterns of cocaine intake under 24-hr/day discrete trial procedure (Lynch and Taylor, 2004), and have greater binge-like alcohol drinking under the "drinking-in-the-dark" procedure as compared to males (defined as the amount of ethanol consumed during the first 3-hours of the dark phase; e.g., Sneddon et al. 2019). Additionally, multiple preclinical studies with cocaine have shown estrus females have higher levels of cue-induced relapse vulnerability than non-estrus females and males (Corbett et al. 2021; Nicolas et al., 2019). Therefore, these sex differences that appear across the disease process of addiction are rather robust considering they are consistent across different categories of addictive drugs (e.g., opioids, psychostimulants, and alcohol).

This dissertation also provides evidence indicating the overlap in sex differences between addictive drugs is not only in regards to the expression of addiction-like features, but also the biological factors contributing to the enhanced vulnerability to addiction in females. More specifically, in Chapter VII, we show estradiol is critical for the enhanced vulnerability in females to developing opioid addiction-like features including escalation of fentanyl intake, prolonged physical dependence, enhanced motivation for fentanyl, and enhanced vulnerability cue-induced relapse. These findings of estradiol on drug intake are consistent with findings with cocaine, nicotine, and alcohol showing ovariectomy robustly decreases drug intake under extended-access condition and estradiol replacement increases drug intake to levels similar to intact females (Larson et al., 2007; Ramoa et al., 2013; 2014; Martinez et al., 2016; Flores et al., 2016; Ford et al., 2004; Rajasingh et al., 2007; Becker et al., 1985; Forger et al., 1982; Hilderbrand et al., 2018). Our findings with opioids are also consistent with findings with cocaine showing estradiol is critical for the development of other addiction-like features including an enhanced motivation (Bakhti-Suroosh et al. 2019; Ramôa et al. 2013; 2014) and preference for cocaine over non-drug rewards (Kerstetter et al. 2012). Thus, estradiol's effect on vulnerability in females appears to be similar for opioids as compared to other addictive drugs such as psychostimulants and alcohol.

In summary, biological sex and ovarian hormones, such as estradiol, matter in addiction and need to be considered as factors in research on the development, neurobiological basis, and treatment of SUD. We believe the studies included in this dissertation have advanced our understanding of the disease process in males and females, but there is still a lot of research that needs to be completed considering we do not have an FDA approved treatment for CUD and

opioid-involved overdose deaths remain above 100,000 annually. We hope the on-going projects in the lab will lead to a better understanding of the molecular mechanisms underlying sex differences in addiction, such as the telescoping effect, and help identify novel targets for the prevention and treatment of SUD.

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