Relapse to opioids facilitated by witnessing the distress of another is mediated by the activity of oxytocin:

a preclinical model of the neural substrates of the relationship between secondary trauma and addiction

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Dedication

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Abstract

Opioid abuse poses a grave danger to human life and has been declared a Public Health Emergency in the US. In 2018 alone, prescription opioids were the most commonly reported substance exposure to poison control centers, with cases soaring to near 300 000. 44% of cases were of children under the age of 5, with over 5000 exposures to heroin and fentanyl. These trends increased since 2018 and accounted for a reduced life expectancy in the US. Indeed, in the US alone, 2.5 million adults are afflicted by opioid addiction, which claims close to 50,000 deaths per year. Efforts to quit are frequently short-lived at best, while the incidence of relapse over the longer term is considerable.

Psychosocial stress and addiction liability

Severe stress plays a powerful role in the vulnerability to both addiction and relapse, perhaps contributing to the marked rates of relapse (75%) to opioid addiction. Indeed, it is well established in both preclinical and human studies that stress confers vulnerability to drug use and the perpetuation of abusing drugs, as well as relapse to addiction. Despite the abundance of data exposing stress as a critical factor in drug seeking and relapse to drug taking, the underlying neural circuitry implicated in the relationship between stress and relapse to drugs remains poorly understood.

Animal models of addiction are useful tools in attempting to elucidate mechanisms responsible for drug-seeking habits. Animal models are also widely used to examine the biological and physiological underpinnings of behavior in health and disease as it pertains

to humans. Critically, however, much of this research fails to capture a key stressor in humans: witnessing the trauma of another, especially of a familiar or close person. The significance of such forms of stress in humans is evident by the increased vulnerability to and prevalence of PTSD and drug addiction among those who have witnessed the suffering or death of others. Here, we propose a novel paradigm to attempt to model social stressors as contributing to reinstatement of drug seeking in mice .

A Novel paradigm for the study of Observational Distress and reinstatement of opioid-seeking

I propose that the susceptibility to PTSD, addiction, and addiction relapse conferred through observing another's distress is mediated by the assumption in the witness of an affective state that closely matches that of the one in distress. This tendency to match the emotional state of another, also called "Affective State Matching" (ASM) is central to human empathy. Although vicarious emotional experiences underlie prosocial behaviors and the development of compassion in humans (as well as prosocial and consolation behaviors in other mammals), they also enable the development of stress-induced disorders if the witnessed distress is sufficiently potent. Previous work in our lab has focused on understanding the neural underpinnings of vicarious fear using mouse dyads. We have demonstrated that fear-associated ASM can be elicited in experimental settings and shown that this phenomenon is dependent on the activity of oxytocin- a neuropeptide involved in sociality, empathy, and affiliation. Since vicarious distress is known to precipitate relapse to drug addiction, is a common precursor to PTSD in humans, and drug addiction and

PTSD are often comorbid, it is possible that oxytocin facilitates relapse to drug addiction following vicarious distress.

Does observational distress facilitate reinstatement of morphine-conditioned place preference? Effects of familiarity and sex.

To establish whether witnessing another's distress can reliable predict reinstatement to opioid seeking, I expose male and female mice to a biased design morphine-conditioned place preference CPP) task to first elicit the preference for morphine. Mice readily acquire a preference for the morphine-paired context relative to a compartment associated with the administration of a vehicle. Once CPP is established, mice undergo an extinction phase, the aim of which is to reverse the acquired morphine preference. This allows the testing of reinstatement of morphine seeking following observational distress. Again, male and female mice successfully extinguished mCPP.

Same-sex mice were then randomly assigned to one of two roles: Observer or Demonstrator. The Demonstrator was placed in a compartment adjacent to a natural predator, a large male rat, who had been administered cocaine (20mg.kg) to enhance locomotive activity. The Observer lacked visual access to the rat and was only exposed to the behaviors exhibited by the Demonstrator during this task. Since previous studies in our lab have revealed that mice show a familiarity bias which may affect behavior in response to the distressed Demonstrator, we included both familiar (cage mates) and unfamiliar (non-cage mates) Observer-Demonstrator dyads. Demonstrators and Observers exhibited

distinct, coordinated threat-associated behaviors, with Demonstrators showing increased territorial defensive aggression, attempts to escape, and threat assessment, while the Observer attempted approaching the distressed conspecific.

We found that exposure to predator threat was sufficient to produce reinstatement of CPP in Demonstrators. Male Observers, however, reinstated CPP only if they were familiar with the distressed Demonstrator. Such a familiarity bias was lacking in female mice.

Does Demonstrator distress predict magnitude of Observer reinstatement of CPP? Effects of familiarity and sex.

We found that behavioral manifestations of ethologically relevant threat responses in Demonstrators predicted the magnitude of reinstatement of CPP in familiar but not unfamiliar Observers. Female Observers reinstated CPP regardless of their familiarity with the Demonstrator-a finding consistent with our previous reports.

Does Oxytocin modulate observational distress and subsequent reinstatement?

To examine the aforementioned hypothesis that oxytocin is implicated in the social transmission of distress, we administered either intranasal oxytocin, or an oxytocin antagonist, to observer animals. Since we expected oxytocin to reverse the effect of familiarity, we administered oxytocin to Unfamiliar Observers (male). Similarly, to establish whether familiar Observers' distress behaviors were dependent on oxytocinergic activity, we administered the oxytocin antagonist in familiar Observers. Oxytocin administration reliably induced reinstatement in Unfamiliar male Observers, thus rescuing

the familiarity bias, while oxytocin antagonism blocked reinstatement of CPP in familiar male Observers. These effects of oxytocin activity on Observational Distress were reflected in reduced stress-related behaviors in the familiar Observer cohort. Control experiments are described.

Since female Observers reinstated the previously extinguished preference for morphine regardless of familiarity with the conspecific, we investigated whether administration of an oxytocin antagonist would rescue this effect. Indeed, both familiar and unfamiliar female Observers failed to reinstate morphine-CPP, lending further support to the hypothesis that oxytocin is implicated in mounting a stress response to the distress of a fellow conspecific. Similarly, behavioral indexes of stress-responsivity were altered in both Observer cohorts. Once again, control manipulations are described.

Does witnessing the distress of another impact social interactions between Observer and Demonstrator?

Bearing witness of the plight of another is associated with an elevated risk of PTSD in humans. In turn, PTSD following such exposure is frequently comorbid with altered, often aggressive, social interactions, especially against familiar individuals, such as family members or friends. To examine whether Observational Distress in our task was sufficient to alter social interactions in participating animals, we assessed social interaction between Observers, Demonstrators, and mice who were not included in the experiment. We find that familiar, but not unfamiliar male Observers exhibited consistent aggression toward the Demonstrator, indicated by numerous attacks, bites, and chasing. Interestingly, Observers

did not act aggressively toward the naïve conspecific in the home cage, indicating that aggression was related to the experience of observing distress in the Demonstrator and exhibiting defensive aggression as a result. Oxytocin antagonism eliminated social aggression in familiar Observer males, further confirming a role of oxytocin in the acquisition of and responding to social distress cues. Unlike male Observers, neither familiar nor unfamiliar female Observer mice acted aggressively following Observational Distress.

Does social aggression following observational distress coincide with social rank in males?

Cohabiting mice quickly form social hierarchies of dominance and subordination. Social dominance or subordination can affect the propensity of a mouse to act aggressively or flee to signal subordination and terminate attacks quickly. Thus, it is possible that mouse social aggression following Observational Distress coincided with dominant status in Familiar male Observers, and that the two are thereby confounded. To disambiguate social rank and aggression, we assessed social dominance status in a randomly selected group of males using a variant of the Tube Test. This task afforded the determination of rank in mice housed in groups of four relative to each member of the home cage. Mice were then assigned blindly to an Observer or Demonstrator role and subsequently assessed for social interaction. We found that Familiar Observers acted aggressively toward Demonstrators regardless of social rank and concluded that social aggression after witnessing a Demonstrator in distress was not confounded by social status. The implications, caveats,

and findings of these experiments are discussed, and directions for future work are proposed.

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List of Abbreviations

F/O	Familiar Observer
F/D	Familiar Demonstrator
U/O	Unfamiliar Observer
U/D	Unfamiliar Demonstrator
OXTA	Oxytocin Antagonist
OXT	Oxytocin
N/R O	No Rat Observer
N/R D	No Rat Demonstrator
N/D O	No Demonstrator Observer
FCbP	Fear Conditioning by Proxy
CPP	Conditioned Place Preference
mCPP	Morphine-conditioned Place Preference
HPA	Hypothalamic-Pituitary-Adrenal Axis

CHAPTER I

INTRODUCTION

I. The Addiction Epidemic: Data and At-risk Populations

Substance use disorders affect a significant proportion of the population in the US and worldwide. In the US alone, an estimated 32% of the population over the age of 12 reported ongoing illicit drug use in 2018. During the same year, 53 million Americans used either illegal drugs or misused prescription medications, including opioids, methamphetamines, and pain management therapies. These staggering statistics exclude tobacco and alcohol use; when accounted for, these substances increase the number of individuals in the US who use some form of drug of abuse to over 165 million (NCDAS, 2018). A considerable proportion reported formal substance use diagnoses, including alcohol use disorder, illegal drug disorder, and opioid use disorder (including prescription opioids and heroin). Substance use disorders (SUD) affect increasingly younger people, and predict substantial long-term cost due to disability, unemployment, or criminality. Indeed, it is estimated that around 50% of youth will have an experience with an illicit drug by the time they graduate high school, and 70% of those who try substance use before the age of 13 develop SUD within the next 7 years (NCDAS, 2018). In 2018, prescription opioids were the most commonly reported substance exposure to poison control centers, with cases soaring to near 300 000. 44% of cases were of children under the age of 5, with over 5000 exposures to heroin and fentanyl. Over the course of the decade leading up to 2018, prescription opioid exposure increased by 93%. Between 2012 and 2015, death due to synthetic opioid overdose increased by 264%, and in 2017, close to 70% of overdose-related deaths were caused by opioids, including fentanyl, prescription opioids, and heroin (NCDAS, 2018).

These trends continued in 2018 and 2019 and accounted for a reduced life expectancy in the US.

II. Psychological stress as a risk factor for substance use development

A variety of factors predict the prevalence of opioid abuse, including male gender, access to healthcare, treatment facilities, and support groups, social environment, and stressful life events, exposure to traumatic events, and prior exposure to drugs of abuse (Young et al., 2015; Hyman et al., 2010; Cottler et al., 1992). Epidemiological data suggest that, when compared to nicotine and cocaine users, individuals with problematic prescription opioid use had endured a higher number of traumatic events over the course of a lifetime and reported having their first traumatic exposure at a younger age than comparison groups (Lawson et al., 2013). Regardless of specific drug use, nearly all (96.5%) individuals surveyed in this study reported some kind of trauma. Similar findings regarding the relationship between elevated substance use and PTSD have been reported in recent literature (Dahby et al., 2020; Hawn et al., 2020; Pahl et al., 2020; Tripp et al., 2019). In a different study, Cottler and colleagues (1992) found that opiate and cocaine users were almost 3 times more likely to report having lived through a traumatic event, to indicate experiencing symptoms of trauma, and to meet diagnostic criteria for PTSD. While these reports fall short of substantiating a causal relationship between PTSD and drug use, it highlights the comorbidity between the two. Opioid dependence was associated with higher stress, greater incidence of maladaptive coping, and poor social support among individuals seeking treatment relative to healthy controls (Hyman et al., 2010). Indeed, families and friends can be ambivalent to the SUD treatment of a relative – a circumstance further

limiting positive treatment outcome as families are an important component of treatment success of individuals with addiction (Young et al., 2012). This likely contributes to the high incidence of relapse to opioids (Domino et al., Shah et al., 2006; Haastrup et al., 1984). The persistent nature of relapse to addiction has been demonstrated widely in pre-clinical models (Sinha et al., 2001; Kosten et al., 2000; McKay et al., 2009; Trask et al., 2017; Namba et al., 2018; Marchant et al., 2018). Considerable evidence indicates that psychosocial stressors, such as witnessing a harm to others in the aftermath of war, terrorist attacks, or shootings, constitutes a powerful risk factor for the development of PTSD in humans (Bromet et al., 2016; Liu et al., 2014; Perrin et al. 2007; Ozbay et al. 2013). Data gathered from first responders following 9/11, for example, indicate that a large proportion of non-traditional and traditional first responders reported symptoms consistent with PTSD (Ozbay et al. 2013; Pietrzak et al. 2014), and in many PTSD persisted for over a decade (Cone et al., 2015). Bearing witness to the death of colleagues and friends was linked to reports of experiencing symptoms of PTSD among World Trade Center (WTC) survivors (Yip et al., 2015). Indeed, such exposures predicted high perceived post-traumatic stress independent of other risk factors (Friedman et al., 2013). Despite profoundly affecting the quality of life of those meeting criteria for PTSD (Koenen et al., 2008; Stander et al., 2014), trauma and its effects on psychosocial well-being have received little attention (Bromet et al., 2016; Stellman et al., 2008; Schwarzer et al., 2014). Other forms of traumatic adversity have been identified in studies of early life trauma, including going through a divorce, living with a parent with a mental illness, witnessing violence) and also predict earlier onset of alcohol use as a strategy to cope with distress (Rothman et al., 2008). Witnessing and enduring sexual abuse and exposure to emotional

abuse was associated with substance use among youth joining the Marine Corps (Young et al., 2006). Bearing witness to the pain and suffering of others constitutes a predisposing factor for the development of compassion fatigue and comorbid substance abuse among health care professionals (Jarrad et al., 2018; Rauvola et al., 2019; Cocker et al., 2016). Recent data demonstrate a strong link between PTSD (including secondary trauma after witnessing the death or near-death experiences of another, see Bride et al., 2012) and SUD, and have motivated efforts to begin addressing PTSD and substance abuse simultaneously instead of separately, as they often coexist (RAND Corp, 2020; Blosnich et al., 2014). Recalling or being repeatedly exposed to aspects of the traumatic event have been found to negatively impact treatment efficacy (Lissek et al. 2015).

III. Pre-clinical models of the relationship between psychosocial stress and addiction are of limited precision and ethological application.

From the overview presented thus far, the function of psychosocial stress as a potent stressor in humans is evident. Yet, while the relationship between stress and addiction has been one of long-standing interest to clinicians and researchers alike, the neurophysiology of stress caused by observing the distress of another (hereby referred to as observational distress) remains elusive. While human studies have helped establish psychosocial stress as a prominent vulnerability-conferring aspect in the development of SUD, such research is limited in its capacity to unveil the mechanisms that link observational distress to addiction and relapse. As is true for much of the study of the nature of human disease, uncovering its neural underpinnings is the subject of animal models of vicarious distress. Most pre-clinical studies have focused on manipulating distress by using pharmacologic or physical stress stimuli. This remains true in the realm of social stress and its relevance to

drug use, as social stress stimuli are often confounded with physical stressors. For instance, vicarious distress is often elicited using the social defeat paradigm, in which the presence of an aggressive mouse produces distress in the attacked conspecific (Golden et al., 2011; Berton et al., 2006). While the presence of an aggressor undoubtedly adds a social component to the stressful experience, it is impossible in many cases to disambiguate the psychosocial and physical (pain) causes of distress (but see Miszek et al, 1996). This design limitation constrains the definitive interpretation of outcome measures as attributable to social distress and are an indication that alternative tests might serve as more appropriate tools in assessing the effects of psychosocial stress.

Additionally, existing pre-clinical models of psychosocial stress risk limited generalizability (Richter-Levin et al., 2019). Standard paradigms used in the assessment of stress rely on manipulations of limited ethological relevance and derive measures of distress from stimuli unlikely to be encountered by the subjects in a non-laboratory environment such as shock delivery and pharmacological interventions (Toth et al.,2012; Reber et al., 2016). Successfully measuring social distress behaviors requires a methodological approach that is at least two-prong: the stress stimulus and the test environment must possess ecological validity, and the outcome measures must involve the assessment of a behavioral repertoire consistent with distress behaviors elicited by naturally occurring distress stimuli. We note that while the methodologies mentioned above have been of marked utility in identifying circuits pertinent to stress, they do not measure a wide range of behavioral ethological indexes of distress behaviors in a species-specific manner. The objective of the present work is to demonstrate the utility of a novel paradigm designed to reliably elicit and measure observational distress in mice and

examine its ability to produce reinstatement of opioid-conditioned place preference. I proceed to examine the neurophysiological determinants of acquiring observational distress in mice.

In the following sections, I begin by briefly discussing stress and its relevance to addiction. I transition to a review of the existing literature on pre-clinical models of psychosocial stress. Then, I summarize caveats in these models and introduce a new paradigm to address these caveats. Next, I present in detail the experimental procedures and subjects, and describe the findings. I conclude with a discussion of the significance of my findings and a proposal of future directions.

CHAPTER II

STRESS AND ADDICTION

I. Stress.

The term stress is commonly understood as the perception and appraisal of, and the response and adaptation to, harmful, threatening, or challenging events or stimuli (Lazarus and Folkman, 1984; Sinha, 2000). Inherent in this definition is the interplay of the separate constituents of stress, such as causal stimuli or events external to the organism (for example, life events, predators, bereavement, etc.), interoceptive signals (hunger, thirst, hypo- or hyperthermia, pain, etc.), cognitive and affective processes involved in the appraisal of the stressor and the resources available to cope and respond, biological adaptations, and behavioral, cognitive and emotional adaptations. Together, coordinated responses to stress can promote the restoration of homeostasis. Typically, stressful events produce conditioned or unconditioned emotional responses. Of note, however, while commonly associated with negative affect, not all stressors evoke negative affective states or constitute harmful stimuli (Selye, 1976). Thus, the range of affective states elicited by stressors can include fear, anxiety, anger, excitement, sadness, or hedonic experiences (Sinha, 2000). Given the plethora of physiological, perceptual, cognitive, affective, and behavioral changes produced by exposure to a stressor, it is evident that responding to stressful events implicates multiple, overlapping neural systems functionally involved in (to name a few) sensory, affective, and cognitive processing, motivation, and motor output. Previous work has identified the primary sensory and association cortexes as integral to the perception of external (environmental) and internally generated (cognitive and affective) stimuli (McEwen et al., 1993), while the appraisal of an event requires sensory inputs to the thalamus, insula, and structure implicated in sensory integration (Kunimatsu

et al., 2020). Additionally, corticolimbic circuits including the amygdala, prefrontal cortex, medial prefrontal and orbitofrontal areas are functionally relevant to the assessment of the "meaning" or significance of an event and are implicated in affective processing (Maier et al., 2019; Onaka et al., 2019; Denny et al., 2018; Fanselow et al., 1999; Lang et al., 2009). Inputs from thalamic and pre-frontal regions into the amygdala are thought critical in the preparation of a rapid survival responses, such as defense and avoidance (Gaffan et al., 1993; Lovallo, 1997). Importantly, these systems and structures involved in motivation, goal-directed behavior, action selection and attentional allocation (namely, the accumbens, see Lovallo, 1997; Robbins et al., 1996; Gaffan et al., 1993; Kunimatsu et al., 2020) act synergistically with changes in endocrine responses to produce behavioral adaptations to stressors.

Several tenets govern our understanding of coping with stressful events, from problem-focused (aimed at altering the cause of the stressor or one's relationship to it, and the development and consideration of behavioral alternatives and future planning, Lazarus et al., 1966), to emotion-focused (concerned with the management of one's affective distress in response to the stressful event or stimulus rather than with addressing the cause of the stress, Lazarus et al., 1984), to, finally, avoidance (understood as evading acknowledgment that the stressor has occurred or instantiated by forsaking any action to address the stressor, see Carver, 1989). More recent approaches to conceptualizing coping place it in the context of self-regulation, highlighting it as an attempt to re-establish homeostasis following its disruption by the stressful stimulus (Carver et al., 1999; Seeley et al., 2007). The notion of homeostatic dysregulation has been especially salient in the study of addiction, with the idea that difficulties in maintaining homeostasis are central to its intractability (Koob et al.,

1997). Thus, emerging is an increasingly well-substantiated link between stress, dysregulation, and addiction, and their shared neurobiological substrates, although the ability of different kinds of stressors to produce sustained drug-seeking and relapse remains incompletely understood.

The discussion of the heterogeneity of stressors was only vaguely hinted at above, with the acknowledgment that not all stressors need elicit negative affective states. Indeed, brief, mild, or controllable challenges can elicit hedonic states and excitement, yet implicate stress circuitry (Hennessey et al., 1979; D'Souza et al., 2018). Characteristics of stressful stimuli, such as duration, frequency, predictability, controllability, severity, and intensity, differentially impact the magnitude of the stress response, with less predictable, more uncontrollable stressors eliciting more intense affective states and associated with greater magnitude of responses to stress (Frankenhauser et al., 1980; Lovallo, 1997, D'Souza et al., 2018; Richter-Levin et al., 2019). These features of stressors, along with factors contributing to one's subjective vulnerability to maladaptive coping, are likely to uniquely contribute to the potential of a stimulus to trigger drug seeking (Sinha, 2000;).

II. Stress and susceptibility to drug seeking.

The relationship between stress and addiction has been the subject of multiple theories about addiction and addiction propensity. While most agree that a number of factors could predispose on to drug seeking and taking, such as parental drug use and positive experiences associated with the benefits of drug use, nearly every major theory of addiction postulates some link between altering an emotional state (be it by reducing negative affect, increasing positive affect, or both) and drug use, and links maladaptive coping with stress

to addiction liability (Tompkins, 1966; Russell et. Al., 1975; Leventhal et al., 1980; Koob et al., 1997). Within this conceptual framework, affective modulation, changes in motivation, and perceived restoration of homeostasis caused by drug consumption constitute viable, however maladjusted, coping strategies in response to stressful events (Shiffman et al., 1982; Wills et al., 1985). Drug consumption has been suggested as a particularly likely compensatory strategy for those with poor coping skills or struggling to regulate emotional distress (Marlatt et al., 1985; Conger, 1956; Sher et al., 1982; Khantzian, 1985). These hypotheses have been confirmed in human neuroimaging studies (Dedovic et al. 2009a; Li and Sinha 2008; Pruessner et al. 2010). Fundamental to these theories of addiction is the notion that acute and chronic stress potentiate the motivation to both alleviate distress and improve mood, and that the initial success in achieving these objectives by use of drugs reinforces, and therefore solidifies, the repeated intake of substances for either mood enhancement or relief of distress.

III. Chronic (social) stress, adversity, and vulnerability to addiction.

As mentioned previously, exposure to chronic stress has been deemed likely to contribute to drug seeking and abuse. Animal studies have substantiated the role of chronic stress at various stages of life in developing compulsive drug-seeking. Remarkably, both early and late throughout development, increased liability to greater consumption of morphine, cocaine, and alcohol later in life has been linked to socially relevant stressors, such as maternal neglect, isolation, or being reared by a peer (vs. biological) mother (Adler et al., 1975; Kostowski et al., 1977; Alexander et al., 1978; Schenk et al., 1987; Higley et al., 1991; Higley et al., 1993). In a series of primate studies, rearing by a peer during the first 6 months of life was associated with greater consumption of alcohol in adulthood, and

social isolation of primates reared by their biological mothers led to alcohol consumption comparable to that of peer-reared counterparts (Higley et al., 1991, 1993). These observations suggest that alcohol intake in adulthood is linked to socially relevant stressors, and that the effect of social distress on alcohol consumption is not bound to specific stages of development. Rodent studies of neonatal isolation show similar results of early life stress on enhanced acquisition of cocaine self-administration (Kosten et al., 2000), while studies of social instability and exposure to aggression and social defeat demonstrate a reliable elevation of corticosterone levels in female and male rats, respectively, as a function of these manipulations (Haller et al., 1999). Additional work has helped establish a link between chronic stress and physiological markers of distress present in several psychiatric disorders, such as anxiety and depression). The significance of life adversity and chronic stress as a risk factor for developing drug abuse in humans has been established in multiple cohorts. Both early physical and sexual abuse predict drug abuse and earlier onset of drug-taking (Dembo et al., 1988; Harrison et al., 1997; Widom et al., 1999). Among adolescents, poor coping, low parental support, and elevated stress are associated with an increased risk of using nicotine, alcohol, and marijuana (Newcomb et al., 1988, Kaplan et al., 1992; Kaplan et al, 1986; Wills et al., 1996; Sinha, 2001). Further, absence of social support and elevated stress have been linked to greater alcohol consumption, and coping with distress through drinking has been linked to drug dependence and compulsive drug taking (Aro 1981; Cronkite and Moos 1984; DeFrank et al. 1987; Chassin et al. 1988; Pohorecky 1991; Cooper et al. 1992; Laurent et al. 1997, but see also Conway et al. 1981; Rohsenow 1982; Allan and Cooke 1985). Psychopathology, especially mood and anxiety disorders among adolescents, confers vulnerability to abuse of nicotine, alcohol, and marijuana (King et al.

1996; Rohde et al. 1996; Kandel et al. 1997)- an association possibly indicative of resorting to drug use for self-regulation of affect and mood. Indeed, it has been suggested that psychopathologies of mood and affect constitute chronic aberrant manifestations of stress stemming from dysregulation of brain circuitry implicated in the processing of and responding to stress (Plotsky et al. 1995; Arborelius et al. 1999). Within this framework, increased and more persistent drug taking likely suggests heightened sensitivity to the reinforcing properties of drugs of abuse, thereby promoting the development of addiction- a notion validated in pre-clinical models of stress and compulsive drug intake (Covington et al., 2001). It is worth noting that in humans, elevated anxiety, depression, and reports of psychosocial stress have been linked to enhanced addictive behaviors, drug craving, and drug consumption (Childs et al., 2010), increased neural responses to drug-related cues (Dagher et al., 2009), and prolonged drug use among individuals with history of SUDs (Karlsogodt et al., 2003).

IV. Acute stress, drug seeking and relapse to drug taking.

Extensive evidence from work with a variety of substances (cocaine, morphine, and amphetamine) substantiates a relationship between acute stress and the motivation to administer a drug in pre-clinical models (Piazza et al. 1990; Piazza and Le Moal 1996; Alexander et al. 1978; Hadaway et al. 1979; Shaham and Stewart 1994; Ramsey and Van Ree 1993; Goeders and Guerin 1994; Haney et al. 1995; Miczek and Mutschler 1996). Stress-reinstatement tasks have revealed the potency of physical stressors (footshocks) to reinstate drug seeking behavior in animals whose prior preference for a drug (nicotine, cocaine, heroin, and alcohol) had been extinguished (Shaham and Stewart 1995; Erb et al.

1996; Ahmed and Koob 1997; Le et al. 1998; Mantsch and Goeders 1998; Shaham et al. 1998; Buczek et al. 1999).

Similar to these findings, evidence from human studies demonstrates that exposure to acute stress increases drug consumption. Psychosocial stress once again emerges as a potent form of stress for humans. Data reveal that fear from social evaluation, feedback after failing to solve an (unsolvable) task, and provocation all increased alcohol consumption in social drinkers in comparison to consumption in the absence of stressful stimuli (Higgins and Marlatt 1975; Marlatt et al. 1975; Hull and Young 1983). Both smoking and alcohol consumption increase following stress exposure (Miller et al. 1974; Pomerleau and Pomerleau 1987). In parents, exposure to aberrant externalizing behaviors children leads to increased consumption of alcohol compared to consumption in parents witnessing normal behaviors (Pomerleau and Pomerleau 1987). Acute psychosocial stress effectively triggered alcohol binging in non-treatment-seeking alcoholics (Thomas et al., 2011), increased cravings for alcohol among social drinkers (Clay et al., 2018) and cigarette craving (Buchmann et al., 2011), and has been found to precipitate relapse to nicotine use following attempts to quit smoking (Schultz et al., 2020; Shiffman et al., 2004;). Clinical trials (and pre-clinical models) have demonstrated the significant impact of stress on beginning to smoke, as well as on the maintenance of and relapse to smoking (al'Absi 2006; al'Absi et al. 2005; Shiffman et al. 1996; Sinha 2001). Similarly, stressful experiences, including traumatic events, have been linked to increased craving for opioids, while the number and gravity of such events predict the likelihood of relapse (Kosten et al., 1986; Krueger et al., 1981). Propensity for enhanced sensitivity to stress due to a history of lifetime traumatic experiences is thought to be implicated in opioid use and, in turn,

elevated stress responsivity in individuals who use opioids may be a risk factor (McCabe et al., 2016). Drug craving, or "wanting" is a key feature in the clinical conceptualization of addiction and is paramount to the maintenance of drug abuse (Dackis and Gold 1985; Tiffany 1990). It has been suggested that chronic use of addictive drugs results in sensitization the brain's reward circuitry, producing craving and seeking of drugs (Robinson et al., 1993, 2000). Within this framework, prolonged, repeated intake of drugs of abuse changes their incentive salience, and exposure to drugs or drug-related cues results in excessive "wanting" and can thereby lead to relapse (Sinha, 2001). Both external (environmental) and internally generated (stress, affect, arousal) stimuli can function as cues that elicit craving (Stewart et al. 1984; Rohsenow et al. 1991; Childress et al. 1993). Elevations in craving have been documented following experiences of negative affect, withdrawal-associated distress, and stress (Childress et al. 1994; Cooney et al. 1997; Sinha et al. 1999a, 2000a). Negative affect and psychological distress have been frequently linked to relapse (Killen et al. 1997; Doherty et al. 1996; Cooney et al. 1997). In clinical studies involving cocaine users, (Miller et al. 1987; McNeil et al. 1993; Sinha et al. 1992; Sinha and Parsons 1996; Sinha 2001) negative affect, including fear, sadness and anger elicited by mental imagery reliably induced drug craving (Sinha et al., 1999a). These findings add to the abundance of evidence linking persistent or recurrent negative affective states with problematic drug use. Further work has implicated opioid function in the modulation of the perceived hedonic value of stimuli and alters the incentive salience of reward-predicting cues (Kyle et al., 2011; Pecina, 2008; but see also Yager et al., 2015, Robinson et al., 2002).

V. Socially transmissible stress and levels of empathy

Stressful experiences are transmissible through social interactions. In human studies, the capacity to experience "empathic stress," or "resonance" with the distress of another (Engert et al., 2019; White et al., 2016) has been the object of growing scientific interest, though the general capacity for empathy has long been recognized as fundamental to human social relationships (Meyza et al., 2017). Yet, humans frequently experience stress in social settings and as part of participating in social life, even when we are not directly experiencing the stressful event but merely bear witness to it (Blanchard et al., 2004; Perlman et al., 2011; van Wingen et al., 2011; Engert et al., 2014; Dimitroff et al., 2017; Wethington et al., 2000; Waters et al., 2014). In animal models, such socially transmitted forms of stress are on occasion referred to as stress contagion and if sufficiently potent, can trigger debilitating sequelae of psychological and physiological symptoms (Carnevali et al., 2020). Thus, the affective attunement to the emotional states of others may act not exclusively as a means to facilitate and strengthen social bonds, but also as a vulnerability-conferring factor for the development of psychopathology, such as PTSD (Blanchard et al., 2004; Perlman et al., 2011). Empathy is widely regarded as a uniquely human capacity. While definitions of empathy may vary, the ability to understand and share feelings and cognitive states with others, and to respond to another's distress, are deemed essential to it (de Waal, 2008). It should be noted that responding to another's distress does not require sharing an effective state in some accounts (Decety et al., 2016). And although attributions of empathy to non-human animals may remain controversial, pro-social behaviors, including consolation behaviors and parental care, to name a few, have been recognized in a variety of species, including rodents, dogs, pigs, and primates (Palagi et al., 2014; Reimert et al., 2013; Huber et al., 2017; Atsak et al., 2011; Han et al., 2019; Han et al.,

2020; Sivaselvachandran et al., 2016; Sanders et al., 2013; de Waal et al., 2017; Burkett et al., 2016). This continuity of pro-social behaviors throughout evolution and their preservation in species phylogenetically older than humans strongly suggest that empathy exists along a continuum of social emotional experiences of varying complexity. This more inclusive perspective on the development of empathy has been most prominently proposed by de Waal (2008). In his multi-level conceptualization of empathy, de Waal defines emotional contagion as the simplest form of pro-social capacities, followed by the more complex sympathetic concern—the ability to experience concern for the state of another and respond appropriately (e.g. by consoling). Finally, empathic perspective taking-the ability to identify the affective state of another- is, within this framework, the most complex form of empathy. Notably, sympathetic concern and consolation behaviors have been recorded in non-human primates and domestic dogs (de Waal et al., 1997; Custance et al., 2012), and emotional contagion has long been observed in a variety of species (Panksepp et al., 2013). Perspective taking (as indicated by extending help appropriate to the recognition of the needs of another) has largely been reserved for humans and apes (Hirata, 2009; Hare et al., 2006), while aspects of cognitive empathy (involving understanding the cause of distress in a conspecific and terminating the distress-inducing behavior to reduce such emotions) have been reported in monkeys and rats (Church et al., 1959; Masserman et al., 1964) although such interpretations should be considered with caution.

VI. Empathy and oxytocin

neuropeptide synthesized in magnocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus and is heavily involved in the regulation of social behavior and cognition in humans and other mammals (Campbell et al., 2010). While some inter-species

differences exist in the specific behaviors OXT controls, its molecular structure is largely conserved across species (Johnoson et al., 2017; O'Connell et al., 2011; 2012). The effects of oxytocin are both peripheral and central. Peripherally, it regulates uterine contractions during labor and lactation; centrally OXT serves as a neuromodulator, and is implicated in maternal and sexual behavior, social bonds, pair bonding, and social recognition (Johnoson et al., 2017; O'Connell et al., 2011; 2012; Hoffman et al., 2014). OXT synthesizing parvocellular neurons in the hypothalamic PVN project to limbic areas implicated in memory, motivation, affect (fear), reward, and decision making (the hippocampus, amygdala, NAc, striatum) and the brain stem (Campbell, 2010). Perhaps most notably, oxytocin has received substantial attention in both scientific and popular press because of its implication in behaviors and affect central to the lives of social species: love, trust, and attachment (Young et al., 2009; Campbell et al., 2010). OXT is implicated in the enhancement of social memory, fear reduction, reciprocity (positive or negative (altruistic punishment)), and perspective taking (Campbell, 2010; Barraza et al., 2013; Batson et al., 1991; Davis et al., 1996; Singer et al., 2009; Davis et al., 2005; Batson et al., 2009; McDougall, 1926; Peterson et al., 2014). The involvement of OXT in pro-social behaviors and the facilitation of empathy has garnered strong scientific support (Campbell, 2010; Fisher et al., 2006), and this enhancement in "empathic identification" (Campbell, 2010) has often been attributed to increased trust and kinship. The behavioral consequences of OXT dysregulation manifest as deficits in social cognition, affect, and behavior. Rodent studies demonstrate that early-life deprivation predicts impairments in social motivation, heightened aggression, deficits in social learning (but intact performance on spatial learning tasks), and diminished affiliative behavior (Todeschin et al., 2009; Bales et al.,

2011; Donaldson et al., 2019; Diebec et al., 2014). Similar findings emerge from primate literature, which links early exposure to adversity to preference for social isolation later in life, as well as inability to harness social bonds when in distress (Winslow et al., 2003). Importantly, impoverished rearing environments can contribute to intergenerational transmission of deficient parental engagement in animals exposed to low levels of parental involvement (Ahern et al., 2009; Francis et al., 1999). These cross-generational effects of social deprivation implicate aberrant functioning of the OXT system, lending further support for the involvement of OXT in social cognition, affect, and behavior (Francis et al., 2000; Winslow et al., 2003; Francis et al., 2002; Kim et al., 2018).

Further, OXT release inhibits defensive aggressive behaviors against unfamiliar males and promotes lordosis in estrous females (Diebec, 2007; Pedersen et al., 2006). Research involving vicarious distress tasks as models of empathy have provided strong evidence that OXT enhances observational fear in mice, and that oxytocinergic function is required for the vicarious fear experiences (Pisansky et al., 2017). Intranasal oxytocin administration or chemogenetic enhancement of oxytocin transmission increase the sensitivity to the distress of a conspecific in unfamiliar male mice who show familiarity bias in empathy-related behaviors. Chronic oxytocin administration downregulates OXT receptor expression in the amygdala and causes facilitation of observational fear. Importantly, the aforementioned manipulations of OXT did not affect non-social forms of fear learning, suggesting that OXT preferentially modulates socially fear-associated cues (Pisansky et al., 2017).

VII. Oxytocin and social salience

Some studies suggest a role of OXT in the exacerbation of stress, the increase of aggressive and protective behaviors, elevation of anxiety in response to unpredictable or proximal

threat, and reveal that exogenous OXT administration facilitates maternal and territorial defensive aggression in rodents (Eckstein et al., 2014; Striepens et al., 2012; Ferris et al., 1992; Grillon et al., 2013). These data warrant the expansion of our thinking about OXT as a modulator of pro-social behaviors. Instead, it is useful to consider its role in enhancing the salience of social stimuli in a variety of contexts – those promoting cooperation, affiliation, altruistic acts, and group cohesion, as well as those evoking anxiety, fear from predation or threat, or trauma from witnessing severe distress or harm in others (Shamay-Tsoory et al., 2016). This broader notion of oxytocinergic function to encompass the processing and modulation of social stimuli in general provides a conceptual framework within which to integrate empathy and empathic pain through witnessing a traumatic event, and subsequent stress-associated coping such as substance use. These elements a frequently encountered as symptom clusters of secondary trauma and PTSD in humans.

It should be noted that substantial differences exist in the modulatory effects of oxytocin on behavior on the basis of sex. This renders the inclusion of both male and female animals in experimental testing critical and warrants a brief overview of the differential impact of oxytocin on male and female behavior.

VIII. A note on oxytocin and sex

The effects of OXT vary not only contextually, but also on the basis of individual characteristics. Differences in OXT availability and function were noted earlier in this work in relation to early life experiences, attachment, and nurturing. Here, I pay special attention to the differential impact of OXT on males and females with respect to social behavior,

which highlights the importance of examining the behavioral consequences of exogenous manipulation of oxytocin in both sexes.

While OXT receptors are present in males and females (Goodson et al., 2001), the synthesis of OXT and OXT receptors are regulated by estrogen (Lim et al. 2006; Patisaul et al., 2003, McCarthy et al., 1996). Thus, estrogen is vital for OXT receptor bindings and, as a consequence, central in eliciting OXT-related effects on affect, cognition, and behavior and varies across the estrous cycle (McCarthy et al., 1996; Campbell et al., 2012). The neuropeptide argentine vasopressin (AVP), which bears structural resemblance to OXT, is thought to have a greater impact on male behaviors (Campbell et al., 2012; it should be noted that OXT and AVP are regulated by gonadal steroids, see Choleris et al., 2009). Even so, OXT exerts socially-relevant behaviors in males, including sexual behaviors and partner selection (Cushing et al., 2005). It is unclear whether OXT and AVP receptors (central and peripheral) exclusively bind their respective neuropeptides, or whether the structural similarities between OXT and AVP can afford that each bind to both receptor types. Since both males and females possess OXT and AVP receptors, the answer to this question could elucidate the extent to which OXT and AVP differentially influence behavior in a sex-specific manner (Goodson et al., 2001).

To complicate matters further, animal studies of the relationship between OXT and its homologues in vertebrates and invertebrates to social behavior show divergent results on the basis of both sex and taxa (Campbell et al., 2012; Insel et al., 2000). Within mammalian species, sexually dimorphic effects frequently go hand in hand with particular OXT and AVP peptide lineages (Carmichael et al., 1987), with OXT lineages primarily influencing female social, sexual, and maternal behaviors, lactation and parturition. Vasopressin

lineages, on the other hand, have been linked to male reproductive behavior, aggression, territorial behaviors, and pair bonding (Donaldson et al., 2008; Gupta et al., 2008; Segara et al., 1998). Despite this, increasing evidence suggests that oxytocin modulates, albeit differentially, both male and female social behavior, including responses to threat, distress, approach, stress, and behavioral mimicry (Pisansky et al., 2017; Jiang et al., 2018).

Evidence suggests that the exogenous administration of AVP or OXT produces vastly different effects in males and females. In men, intranasal AVP administration was conducive to reduced perceived friendliness of same-sex strangers and encouraged antagonistic facial expressions. The same manipulation enhanced perceived friendliness and elicited corresponding facial expressions in women (Thompson et al., 2006). Intranasal OXT administration has been shown to reduce activity in the amygdala in men but have the opposite effect among women (Domes et al., 2007). Exogenous OXT administration eliminates differences in neural activity between male participants and female subjects receiving placebo (Rilling et al., 2014), suggesting higher baseline OXT concentration in females. This finding comports nicely with an enhanced empathic response in men administered OXT; indeed, male empathic behavior following intranasal OXT is similar to that of women who did not receive OXT (Hurlemann et al., 2010; Shamay-Tsoory et al., 2016). Additionally, OXT administration differentially affects male and female social perception, increasing the accuracy of perceived competition in males and kinship in women (Fischer-Shofty et al., 2013). Improvements in empathic accuracy (Shamay-Tsoory et al., 2016) have also been noted following OXT delivery in populations with social impairments, lending further support to the proposition that OXT modulates social

cognition, affect, and subsequently, behavior (Bartz et al., 2010; Feeser et al., 2015; Clark-elford et al., 2015; Smith et al., 2019; Duque-Wilkens et al., 2018).

IX. Concluding remarks

Oxytocin, a neural substrate of empathy and social behavior, has been implicated the processing of social stimuli, with differential effects on female and male animals. The recognition that forms of empathy can be observed in non-human animals has offered an avenue for the study of its neural bases. This is of great potential value for uncovering the underpinnings of trauma and addiction with the help of pre-clinical models of socially elicited stress-a potent form of stress in humans. In the following chapter, I provide an overview of animal models of psychosocial stress deemed translational to human experiences of trauma.

CHAPTER III

PRE-CLINICAL MODELS OF PSYCHOSOCIAL STRESS

I. Introduction

As mentioned in greater detail in the previous chapter, direct as well as vicarious exposure to stressful or traumatic events can precipitate the development of physiological and psychological symptoms consistent with post-traumatic stress. Importantly, not all individuals exposed to such stressors develop post-traumatic symptoms (Kessler et al., 2000). The latter encompass a range of severe disruptions in behavior, affect and cognition, such as intrusive thoughts or memories of the traumatic event, negative affect, avoidance of stimuli associated with the traumatic experience, altered activity of the hypothalamic-pituitary-adrenal (HPA) stress axis, and, often comorbid substance use disorder (Kessler et al., 1995; Blanco et al., 2013).

Animal models are critical for the understanding of the neurobiology of severe and lasting stress-related changes in affect and behavior. Generally, pre-clinical models of stress disorders, such as PTSD,can fall in one of three categories. The first category includes paradigms which use physical stimuli (for example, a foot shock, submersion, restraint stress) to elicit stress in the model animal. The second category introduces psychological stressors, such as housing instability, social isolation, social defeat stress, predator stress by way of a cue (odor, bedding, anesthetized predator), and others (Whitaker et al., 2014). Recently, models of vicarious stress, observational stress, social fear transmission, or stress contagion, have been added to the toolkit of pre-clinical paradigms with the recognition that vicarious psychosocial stressors constitute an important risk factor for the development of trauma in humans (Meyza et al., 2017).

For the purposes of this work, I will focus on pre-clinical models which incorporate a social stimulus to produce a distress state, specifically those that include the presence of a conspecific (as in social defeat stress), vicarious stress paradigms, emotional contagion of fear, vicarious fear, and socially transferred fear. As might become evident to the reader, the nomenclature used to describe a class of behavioral responses elicited by vicarious stress experiences varies on the basis of the stimulus used to induce a distress state in the animal directly subjected to it. These distinctions as they pertain to each paradigm will be discussed in the following sections.

II. Emotional fear contagion in animal models

Fear learning is an evolutionarily conserved process of adapting to environmental threats shared by a variety of species. In the past decade, Panksepp and colleagues (2011, 2013) have provided evidence suggesting that rats and mice are able to socially share fear states, thus making them pertinent model species for the study of the neural bases of empathy (Meyza et al., 2017). This has led to the development of models of fear contagion in rodents made distinct by the variability of threat imminence. Such paradigms utilize rodent dyads, with one animal, the Demonstrator, serving as the source of affective stimulation of its conspecific, the Observer. Threat imminence is achieved when the Demonstrator undergoes classical fear conditioning or retrieval of fear-associated memories within a small enclosure and without the possibility of escape. Conversely, remote threat manipulations require the transfer of the Demonstrator from the fear conditioning or fear memory retrieval context back into the home cage where social interaction with the

Observer follows. The objective of experiments on fear contagion is to determine the effect the distressed Demonstrator has on the behavior of the Observer.

II.1. Vicarious Fear

Observer behavior is substantially affected by the experience of witnessing a Demonstrator undergoing fear conditioning. In rats and mice alike, placing the Observer in a compartment immediately adjacent to the Demonstrator's reliably produces a fear responses (Atsak et al., 2011; Gonzalez-Liencres et al., 2014; Chen et al., 2009; Jeon et al., 2010; Pisansky et al., 2017), including heart rate deceleration and freezing (Chen et al., 2009; Atsak et al., 2011; Jeon et al., 2010). Several factors are known to modulate vicarious freezing, including prior foot shock experience, familiarity, rearing conditions, litter size, and genetic background (Atsak et al., 2011; Sanders et al., 2013; Jeon et al., 2010; Pisansky et al., 2017; Gonzalez-Liencres et al., 2014; Chen et al., 2009; Panksepp et al., 2013). Numerous exposures to a distressed Demonstrator have been associated with a decrease of freezing in some studies (Carrillo et al., 2015). Interestingly, vicarious fear learning has been used as a measure of empathy in humans (Kleberg et al., 2015), suggesting that the models described here are of translational utility to understanding of the basis of vicarious emotional experiences across species (Meyza et al., 2017). A limitation of vicarious fear paradigms is that the disambiguation of an Observer's response to the Demonstrator's pain as well as fear is challenging. One way to address this problem has been to eliminate witnessing the Demonstrator's experience of physical pain. Such "fear conditioning by proxy" tasks are discussed next.

II.2. Fear Conditioning by Proxy (FCbP)

In this model, the Demonstrator animal undergoes fear conditioning in the absence of the Observer. Observers are allowed to freely interact with the previously fear-conditioned demonstrator during fear memory retrieval. This design confers the advantage of decoupling the source of vicarious fear learning in the Observer, who bears witness of fear-associated behaviors in the Demonstrator in the absence of physical pain (Bruchey et al., 2010; Jones et al., 2014; Jones et al., 2016). This clever design involves 3 co-habiting rats and takes place over the course of three consecutive days. On Day 1, one rat undergoes conditioning to a cue (tone) paired with a shock. On day 2, the fear conditioned rat and a cage mate are placed in the fear conditioning apparatus and exposed to the tone in the absence of a foot shock. During day 2, the third rat is left in the home cage. On day 3, all three animals are placed in the conditioning chamber (individually) and tested for freezing in response to the cue. This task is deemed advantageous because it allows free interaction among the conspecifics during the fear learning portion of the experiment in addition to affording assessment of fear learning both individually (on day 3) and within a pair (on day 2). Of note, conducting testing when the Demonstrator is not present is deemed essential in eliminating possible confounds due to changes in motivation or social facilitation effects of sharing the conditioning chamber with the Demonstrator (Meyza et. al., 2017). Also of interest is the observation that FCbP prior to the direct experience of the foot shock-tone pairing leads to small increases in freezing when exposed to the cue on the following day relative to exposure to conditioned and unconditioned stimuli alone (Bruchey et al., 2010). This suggests that FCbP might tap into neural circuits implicated in direct fear conditioning (Meyza et al., 2017).

II.3 Social transmission of fear

Unlike vicarious fear learning tasks, paradigms assessing the social transmission of fear in mice and rats (Knapska et al., 2010; Knapska et al., 2006; Meyza et al., 2015) test the social transfer of the Demonstrator's affective state in the absence of other distress cues, such as vocalizations, urination, defecation, or bradycardia. The task is conducted on dyads of co-housed animals and entails the removal of the Demonstrator from the home cage for fear conditioning. Following conditioning, the Demonstrator is returned to the home cage and interacts with the naïve conspecific. Demonstrators in control dyads are placed in the fear conditioning chamber but do not undergo fear conditioning. Such studies have demonstrated that the Demonstrator's affective state can be transferred to the cage mate, who shows signs of elevated affective arousal indicated by increased exploratory and risk-assessment behaviors and demonstrates enhanced acoustic startle response (Knapska et al., 2006).

II.4 Social defeat stress and its variants

A shortcoming shared by all of the aforementioned models of emotional contagion regards the use of foot shocks as a means to elicit distress in the Demonstrator. The rationale behind criticisms of this approach is that such forms of stress bear little resemblance to the stressors experienced by humans and are therefore of limited translational utility (Bjorkqvist, 2001; Rohde, 2001). Another criticism of the paradigms described above is that they do not include assessments of other adverse affective consequences (depression

and anxiety-like states) commonly occurring after a stressful or traumatic experience (Carnevali et al., 2020).

The social defeat test (or resident-intruder test, see Miczek, 1979) offers a more ethologically relevant variant of socially related stress based in interactions between conspecifics who differ in social rank (dominance). Social defeat constitutes a severe form of stress in mice, rats, and other social animals, and entails the introduction of an animal (intruder) into the home cage of an older and aggressive conspecific (resident) who then engages in threatening behavior and physical attacks to elicit social defeat (submission) in the intruder (Hollis et al., 2014). Once social defeat is achieved, the intruder is separated by a partition from the aggressive resident to limit continued physical harm while not precluding continued exposure to aggressive threats. This model has been employed in the study of the neurobiology, behavioral, and physiological changes following single or repeated social defeat stress in an effort to elucidate aspects of human psychopathology in pre-clinical disease models (Carnevali et al., 2017b; Hollis et al., 2014; Padurariu et al., 2017; Schoner et al., 2017; Sgofio et al., 2014).

The social defeat stress paradigm, however, suffers from a previously noted design limitation: the confounding of physical and psychosocial stress. This has encouraged the development of variations of the task to include an observational component.

II.4.1 Vicarious social defeat stress paradigm

The vicarious social defeat stress task, also referred to as the “trauma witness model” and “social defeat witness model” (Carnevali et al., 2020; Patki et al., 2014; Sial et al., 2016; Warren et al., 2013) introduces an element of vicarious distress by the inclusion of an

Observer separated from a resident/intruder pair by a divider and forced to witness the social defeat of the intruder. In the first study adopting the vicarious variant of the social defeat stress adult male mice witnessed the repeated social defeat of a conspecific by a CD-1 male (a particularly aggressive phenotype) over the course of 10 consecutive days (Warren et al., 2013). 24 hours after the last vicarious exposure to social defeat, the witnessing males showed social avoidance of a novel CD-1 mouse relative to controls. This effect of even stronger at follow up after one month without further experiences of witnessing social defeat. These findings are of particular interest to the study of human trauma, as avoidance of trauma-related cues (in this case, a CD-1 mouse) is a core feature of PTSD and certain depression subtypes (Carnevali et al. 2020; Foa et al., 2006; Nemeroff et al., 2006). The lasting impact of vicarious social defeat stress on avoidance behaviors has demonstrated the capacity of potent vicarious social stressors to elicit lasting aversion to stress-related cues. The utility of this approach is further highlighted by the fact that mere exposure to a CD-1 male placed in a separate compartment did not produce avoidance behaviors in the absence of vicarious social defeat stress, indicating that observing another's distress is uniquely salient in the development of social avoidance. Physiological signs of elevated stress responsivity, such as elevated plasma corticosterone at 24 hrs and 1 month after the last defeat, as well as reduced sucrose preference (a measure of anhedonia in rodents), stunted weight gain, and passive coping in the forced swim test, and reduced time spent in the open arms of the Elevated Plus Maze were also recorded in Observer mice. Together, these behavioral and affective deficits demonstrate the potency of vicarious social stress as a contextual odulator of social behavior. Similar deficits have been reported in subsequent studies of female adult mice (Iniguez et al., 2018), and were

rescued by administration of pharmacologic agents used in the treatment of depression in humans (Parise et al., 2013). The vicarious social defeat task has also been used in rat studies (Patki et al., 2014), and once again elicited depression and anxiety-like phenotypes, changes in resting blood pressure, and HPA-axis hyperactivity (Finnel et al., 2017) as well as striking similarities in cardiovascular changes between witness rats and intruders (Finnel et al., 2017). Evidence of physiological, affective, and behavioral changes resulting from vicarious social defeat stress have been extended to female rats (Finnel et al., 2018). Remarkably, overectomized, but not intact, female witness rats showed increased physiological markers of distress when re-exposed to the social defeat context in the absence of an intruder, potentially implicating sex-related differences governing the behavioral, affective, and physiological effects of vicarious social defeat stress.

III. Summary and remaining problems in existing pre-clinical models of psychosocial stress

Severe stress is a risk factor for the development of psychopathology in humans, including addiction, PTSD, and disorders of mood and affect. Substance use is often comorbid with disorders of stress, making relapse to drug taking likely as a way of stress regulation. This contributes to the treatment resistance of addiction and to prolonged disability and diminished quality of life.

In everyday life, most stressors relevant to humans are social in nature, and an ethologically valid model of social stressors requires stimuli specifically eliciting social distress in animal paradigms. This has encouraged the use of social stressors in preclinical models aiming to elucidate the biological underpinnings of trauma and its comorbid conditions. While commonly used paradigms have informed much of our current understanding of the

physiological, behavioral, and affective consequences of exposure to social stress, many continue to present challenges that constrain their ethological relevance. For instance, has been difficult to disambiguate the response to pain in Demonstrators from their fear-related behaviors as sources of vicarious fear in the Observer. In social defeat paradigms, the social relevance of the aggressor is confounded by the subjection to physical attacks in the intruder, once again making a purely social (and not nociceptive) stress stimulus elusive. This problem, while ameliorated, is not entirely eliminated in vicarious social defeat tests. In such paradigms, witnessing the attack of a conspecific precludes the direct experience of physical assault, yet it is possible that other cues of intruder pain, such as the emission of distress vocalizations, influence the behavior of Observers. Finally, most of the aforementioned experimental set ups rely on equipment that hardly allows for the assessment of a wide range of ecologically valid behavioral measures of vicarious distress or proximal threat stress in Observer/Demonstrator dyads. Thus, novel observational distress paradigms combining flexible environments while eliminating direct exposure to physical harm for both Observer and Demonstrator, on the one hand, and incorporating an ecologically plausible fear-eliciting stimulus would benefit our understanding of the isolated impact of purely social stressors on affect, behavior, and vulnerability to drug seeking.

CHAPTER IV

OBSERVATIONAL DISTRESS INDUCED REINSTATEMENT (ODIR) OF MORPHINE SEEKING BEHAVIOR IS MEDIATED BY OXYTOCIN: A NOVEL PRE-CLINICAL MODEL OF PTSD

I. Introduction

To help fill the advance an ecologically sound approach to the study of vicarious distress and its relationship to drug-seeking behavior, while remedying the shortcomings of currently used models of vicarious social stress discussed previously, I propose a novel, ethologically relevant observational distress paradigm. It harnesses the notion of Observer-Demonstrator dyads to accommodate observational distress, yet obviates the need to rely on physical stressors to influence the behavior of the demonstrator animal. I achieve this by introducing a proximal (but not physically imminent), ecologically plausible threat to the Demonstrator—a predator, which is not visually accessible to the Observer, but to which the Demonstrator has visual and safe tactile access. Animals are placed within a novel apparatus, which permits the recording and assessment of a range of species-specific behaviors in the Observer and Demonstrator and allows the examination of social transmission of distress from Demonstrator to Observer. Prior to inclusion in this task, mouse dyads have undergone conditioned place preference for morphine, followed by extinction of the acquired morphine place preference. The observational distress task is thus embedded within a context of opioid preference and its extinction, allowing me to test the capacity of observational distress in this paradigm to elicit reinstatement of the previously extinguished preference for morphine. Effectively, this design is the first to capture a form of traumatic stress particularly relevant to humans—witnessing the distress of another (Bryant et al., 2019), and link it to a painfully salient and frequently comorbid threat to human health: relapse to drug seeking behavior.

While offering a methodological contribution to current behavioral tests of social stress and a means to measure the potency of socially transmitted distress to reinstate drug preference, this design offers opportunities to address important questions. One obvious inquiry regards the neural underpinnings mediating the effects of social stress on drug seeking behavior. Utilizing existing knowledge of the role of OXT in social and empathy-like behaviors in mice, we pharmacologically manipulated OXT and assessed its impact on reinstatement of the extinguished place preference for morphine. Additionally, prior studies have demonstrated that familiarity with the distressed conspecific uniquely influence observer behavior. To account for the possibility of such familiarity biases and examine their significance in the context of socially transmitted fear and relapse to opioids, we tested dyads of familiar and unfamiliar observer and demonstrator mice. Mindful of the ample literature documenting the differential OXT-mediated effects of OXT and in an attempt to remedy some of the paucity of data on female animals in pre-clinical models, we conducted these experiments in cohorts of familiar and unfamiliar male and female mice. Aside from the obvious benefit of expanding what remains a limited, and therefore non-generalizable, pool to include females, testing well-powered cohorts of both sexes enables between and within-sex comparisons for each of our outcome measures. Finally, we tested the interaction between treatment and familiarity with regard to post-stress aggression between conspecifics. We included this measure because of the frequently observed comorbidity between secondary trauma, drug-taking, and aggression in humans. In the following sections, I describe the methodological approach and discuss the findings of this novel experimental approach.

II. Materials and methods:

Subjects. 194 male and 58 female C57/BL6 mice (age 9-12 weeks) were used in these experiments. All animals were housed in groups of 2-4 in a 12/12 light/dark cycle. Conditioned place preference (CPP) training, test, and extinction phases were conducted between noon and 2pm each day. The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota.

III. Experimental Overview

The experimental timeline is shown in Figure 1A. Mice first underwent acquisition and extinction of morphine-CPP. Experimental groups consisting of same-sex Observer (O)-Demonstrator (D) dyads were then exposed to a predator (rat) so as to induce fear in the Demonstrator mouse and observational distress in the Observer. Mice were then returned to their home cages for 5 minutes where their behavior was monitored, prior to testing for ODIR in the CPP apparatus. The abbreviations used throughout this text to refer to the various conditions and treatment groups and their controls are summarized in Table 1.

Abbreviation	Role	Familiar?	Oxytocin?	OXTA?	Aggression test?	CPP
F/O	Observer	Yes	No	No	Yes	Yes
F/D	Demonstrator	Yes	No	No	Yes	Yes
U/O	Observer	No	No	No	Yes	Yes
U/D	Demonstrator	No	No	No	Yes	Yes
F/O + OXTA	Observer	Yes	No	Yes	Yes	Yes
F/D + OXTA	Demonstrator	Yes	No	Yes	Yes	Yes
U/O + Oxytocin	Observer	No	Yes	No	Yes	Yes
U/D + Oxytocin	Demonstrator	No	Yes	No	Yes	Yes
U/O + OXTA	Observer	No	No	No	Yes	Yes
U/D + OXTA	Demonstrator	No	No	No	Yes	Yes
N/D	Observer	--	No	No	Yes	Yes
N/R O	Observer, No Rat	Yes	No	No	Yes	Yes
N/R D	Demonstrator, No Rat	Yes	No	No	Yes	Yes

Table 1. Summary of the primary groups of animals included in the battery of tasks described here. "Role" denotes whether an animal served as an Observer or a Demonstrator. "Familiar?" indicates whether an animal was a cage mate of the conspecific (Yes/No). "Oxytocin?" indicates whether an animal received intranasal oxytocin prior to the Observational Distress paradigm (Yes/No). "OXTA?" indicates whether an animal received a systemic oxytocin antagonist prior to the Observational Distress paradigm (Yes/No). "Aggression test?" indicates whether animals' social interaction was assessed following the Observational Distress paradigm (Yes/No). "CPP" indicates whether animals went through morphine-conditioned place preference (and extinction) prior to the Observational Distress paradigm. "Abbreviations" are used throughout the text to refer to animals within their respective roles, treatments, and conditions. N/R denotes the absence of a rat (a predator threat) in a control group of familiar dyads. N/D denotes an observer who did not have a corresponding demonstrator during Observational Distress to control for potential confounding effects of rat cues on observer behavior and disambiguate observer behavior in response to demonstrator distress from that elicited by odor or auditory predator cues.

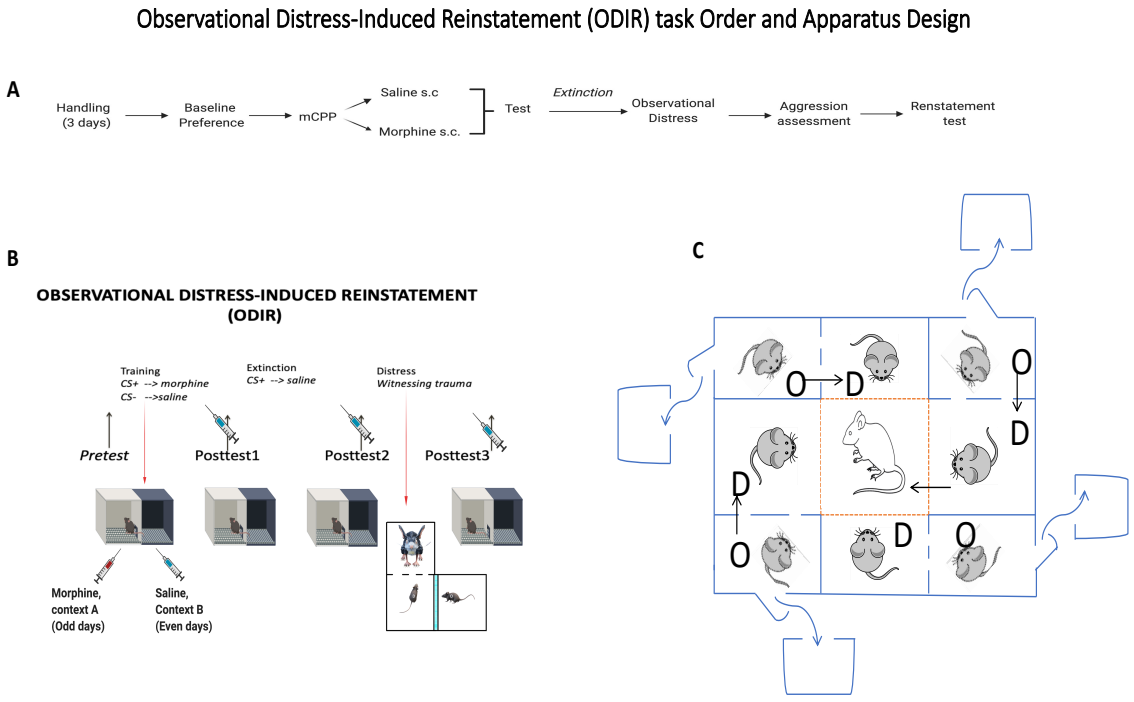

Figure 1. Panel A-B: Task order for the paradigms included in ODIR. Animals undergo handling and baseline testing in the CPP apparatus. Mice then undergo morphine conditioning, with morphine administered in the compartment of the CPP chamber less preferred by them at baseline. Saline was administered in the opposite compartment. Treatment was alternated, so that each animal received morphine and saline on alternate days for a total of 8 conditioning days. On Day 9, mice were tested for morphine-CPP (mCPP) and began extinction of mCPP no the following day. Panel C: Following extinction, mice Observer (O)-Demonstrator (D) dyads were introduced into the Observational Distress apparatus, with the D directly facing the predator (rat) through a mesh divider, while the O could see the D but not the R.

III.1 Social Dominance assessment: The Tube Test

To account for possible confounds of social dominance status in the assessment of social aggression following observational distress, we tested a subset of mice using the tube test (Fan et al., 2019). Originally, this task was developed to assess dominance tendencies in various mouse strains (Bernstein 1981), though it has since also been used for behavioral testing of genetically modified mice (Williamson et al., 2017). The task takes advantage of

the natural tendency of mice to move within a confined space. Pairs of animals are placed at the opposite ends of a narrow tube and are thereby confronted with each other's opposing motivations to walk through and exit the tube. As a result, "winner" – the mouse who successfully forces its conspecific out-emerges and is deemed dominant. The robustness of the tube test as a measurement of social dominance (Fan et al., 2019) rendered it well-suited for the purposes of our experiment.

The test apparatus consisted of two separate compartments (40cm x 25cm x 25 cm) connected by a plexiglass tube (25cm in length, 3cm in diameter) extending into each compartment by 3 cm. The experiment consisted of three phases: a habituation phase (3 days), a training phase (2 days), and a testing phase (4 days). The habituation phase consists of handling mice to reduce potential stress caused by interacting with the experimenter. During the training phase, mice are acclimated to the testing apparatus and encouraged to walk through each end of the plexiglass tube 10 times/daily, 5 entries from each side of the tube (Fan et al., 2019). The purpose of this procedure is to minimize any aversion to the testing environment. The total duration of training per animal ranged between 12 and 15 minutes/day. In the testing stage, dyads of mice were placed in the apparatus, one in each of the two compartments, and promptly entered the tube consistently with mice's natural tendency to seek shelter (Latham et al., 2004). A typical trial duration was <25s, which is consistent with existing literature (Fan et al., 2019). Dominance scores were calculated by assigning a value of zero or one to each mouse within an experimental dyad. A score of 0 was assigned to the mouse that was first to exit the tube and place two paws on the compartment floor. A score of 1 was assigned to the mouse that remained in the tube. Each dyad was tested four times to ensure reliability of scoring. Since mice were housed in cages

of 4 maximum, it was useful to derive a ranking score for each animal relative to its cage mates. Thus, a mouse could be given a rank of 0, 1, 2, or 3. Stable ranks were considered once each animal's rank remained constant for 4 days. Mice who underwent the tube test were blindly assigned to either an Observer or a Demonstrator role, and their social interactions following Observational Distress was subsequently assessed. After social interaction assessment, we examined the relationship between rank, role, and propensity for aggression. In all animals, rank was not predictive of type of social interaction with a conspecific following Observational Distress. Role (Observer or Demonstrator) regardless of rank correlated with aggressive behavior (See Supplementary Figure 2).

III.2. Conditioned Place Preference (CPP)

Conditioned place preference is a commonly used paradigm in which a stimulus (for example, a drug of abuse) is administered within a distinct context to assess its reinforcing properties. The motivational significance of the stimulus is determined by evaluating an animal's subsequent preference for the stimulus-paired context. Variations of the task involve biased and unbiased designs. In the former, an animal's baseline tendency to spend more time in one chamber (context) of the testing apparatus is paired with a vehicle during conditioning. The less preferred context is, in turn, paired with the reinforcing stimulus, and a reversal of the baseline context preference is indicative of the reinforcing properties of the stimulus. Unbiased design assign stimulus to the specific context randomly. Additionally, variations exist with regard to the testing apparatus, which may contain two, three, or four compartments, and the relative merits of each have been discussed at length elsewhere (Prus et al., 2009). Here, we use a biased procedure in order to secure that the

reinforcing properties of the conditioning stimulus (morphine) were potent enough to reverse the animals' original context preference (Figure 1B). Mice were acclimated to the testing room for 30 minutes prior to saline injection (10ml). The mice were then placed within an arena (20x25x45 cm) with two different types of flooring (a metal mesh covering one half, and metal rods covering the other half of the floor) and allowed to explore freely for 15 minutes. Time spent in each half of the apparatus was recorded using Any-MAZE (Stoelting Co; IL, USA). On each day of the conditioning phase, animals were injected either with morphine (15 mg/kg s.c.) and confined to the drug-paired context for 15 minutes, or with saline and confined to the opposite side of the arena for the same period of time. The side preferred less by the animal at baseline served as the drug-paired side, and the order of morphine and saline injections across days was counterbalanced across animals. All subjects underwent 8 days of conditioning. On the ninth (Test) day, animals received a saline injection and were allowed to explore the arena freely for 15 minutes.

III.3. Extinction of CPP

During this phase, animals were confined to the previously drug-paired context of the CPP apparatus and given an injection of saline (s.c.). The preference for each side of the conditioning chamber was then tested. Extinction of CPP was based on a criterion of 3 consecutive days of reversal of morphine-CPP defined by at least indifference (50% preference) between the two compartments of the apparatus.

III.4. Predator/Observational Distress

Mice are a prey species, with a multitude of natural predators, including larger rodents (Blanchard et al., 2005). While the specific predator-related sensory cues which induce defensive responding in prey animals may vary (Blanchard et al., 2005), predator threat consistently elicits unconditioned defensive and fear-associated behaviors. It is therefore unsurprising that predator cues (odor, bedding, fur, or an anesthetized predator) have been utilized in experimental settings in pre-clinical models of disorders of fear (Lezak et al., 2017). Perhaps one of the greatest advantages to using predator exposure tasks is their ability to capture a wide range of defensive and fear-related behaviors approximating the natural repertoire of a species when confronted with a predator. Other classically used tasks for the assessment of fear in mice rely on their propensity for reduced locomotion, thigmotaxis, or preference for areas with low illumination (Lezak et al., 2017). Unfortunately, however, these measures capture only a small number of the diversity of behaviors indicating defensiveness, fear, and alertness in the presence of danger that can be evaluated. Indeed, ecologically sound defensive, territorial, threat-investigative, and alertness phenotypes include a host of behaviors, most commonly stretch attend posture (a state of assessing danger), tail rattling (a display of territorial defensive aggression), freezing, and escape (Blanchard et al., 2005; Kaesermann et al., 1986; Rodgers et al., 1997; Henriques-Alves et al., 2016). Of note, social species benefit from the display of such responses to danger since they can be observed and in turn elicit a reaction in the observing conspecific while sparing direct exposure to the threatening stimulus. Importantly, however, experimental settings must be spacious enough to afford the proper positioning and mobility of the predator, the prey animal, and the observer. To our knowledge, no currently used protocols fully meet these conditions (Blanchard et al., 2005). We therefore

constructed a novel testing environment to accommodate the objective of evaluating the social transmission of a wide range of naturally occurring distress-related cues in an ecologically sound setting.

Within cages containing 2-4mice, we randomly assigned two of them to the roles of Observer or Demonstrator. An adult male Sprague-Dawley rat (520g) served as the anxiogenic stimulus for the Demonstrator (Blanchard et al., 2001). The rat was placed in the central compartment of a 1mx1m square apparatus consisting of 10 chambers (3 along each wall, and one located in the center of the square box). The rat was injected with cocaine (20mg/kg s.c.) 10 minutes prior to the experiment to produce locomotor activation. Four Demonstrator mice were placed into each of the four chambers adjacent to the central chamber. The four mice were separated from the rat by a wire mesh, i.e., there was no opportunity for physical contact with the rat. The Observer was placed in the compartment next to the Demonstrator's compartment. These compartments were separated by a plexiglass wall containing evenly spaced holes. The Observers could not see the rat. Each Observer only see its own dyadic partner, while the Demonstrator also had visual access to the rat. Mice remained in the apparatus for 10 minutes. Behavior was recorded and categorized by trained researchers (Figure 1C).

A number of different groups of mice underwent the behavioral procedures described above (for a summary of baseline and control groups, see Table 1). In light of previous findings of sex differences in observational distress as a function of dyadic familiarity (Pisansky et al., 2017), we ran groups of familiar (i.e., siblings/cage mates) and unfamiliar dyads. To control for the possible effects of rat odor on the observers' behavior, we included a control group ("No Rat", N/R) of male dyads that were tested in the absence of

a rat. To account for the possible effects of olfactory and/or auditory cues from the predator on the observers' behavior, we ran an additional group of male Observers in which the Demonstrator cage remained empty ("N/D" or No Dem Observers).

III.5. Social interaction

Immediately following observational distress, animals were returned their home cages for 5 minutes. Behavior was video-recorded and assessed for social interaction behaviors by trained experimenters. Since social interaction (and, in particular, aggressive behavior, defined as attacks and bites, see Results) could affect the reinstatement of CPP, we included a control ("No Interaction") group of dyads for whom this phase was omitted.

III.6. Observational Distress-Induced Reinstatement (ODIR)

Immediately after the assessment of social interaction, mice were placed back in the CPP apparatus and allowed to explore freely for 15 minutes. Their preference for the previously morphine- or saline-paired sides was assessed. Reinstatement was defined as spending significantly more time in the compartment in which morphine was administered during conditioning.

III.7. Oxytocinergic drug administration

To examine the role of oxytocinergic neurotransmission in ODIR, Observer male mice were administered the selective oxytocin antagonist L-368,899 hydrochloride (10 mg/kg IP; Tocris Bioscience) or oxytocin itself (20 μg/kg IN, 5 μL per nostril; Sigma-Aldrich) 30 minutes prior to stress exposure. The determination for the use of these doses was made following procedures outlined in Pisansky and colleagues, 2017). Since we expected

oxytocin to enhance observational distress and ODIR and the antagonist to block these phenomena, oxytocin was administered to *Unfamiliar* Observers, which otherwise showed little or no observational distress (see Results). Since we expected the antagonist to block Observational Distress and ODIR, we administered it to *Familiar* Observers.

IV. Results

IV.1. Morphine Administration Reliably Produces Conditioned Place Preference in mice in the Conditioned Place preference Test.

Male and female groups were first trained and extinguished in a morphine-CPP paradigm so that the effects of observational distress on reinstatement of drug-seeking behavior could be assessed subsequently (see below). All male ($t(96) = 19.17$, $p < 0.0001$) and female ($t(57)= 17.82$, $p < 0.0001$) mice showed a significant preference for the morphine-paired compartment at test followed by a reversal of this preference after extinction of CPP (male ($t(96) = 18.33$, $p < 0.0001$); female ($t(57)=16.38$, $p < 0.0001$) (Figure 2A-2B).

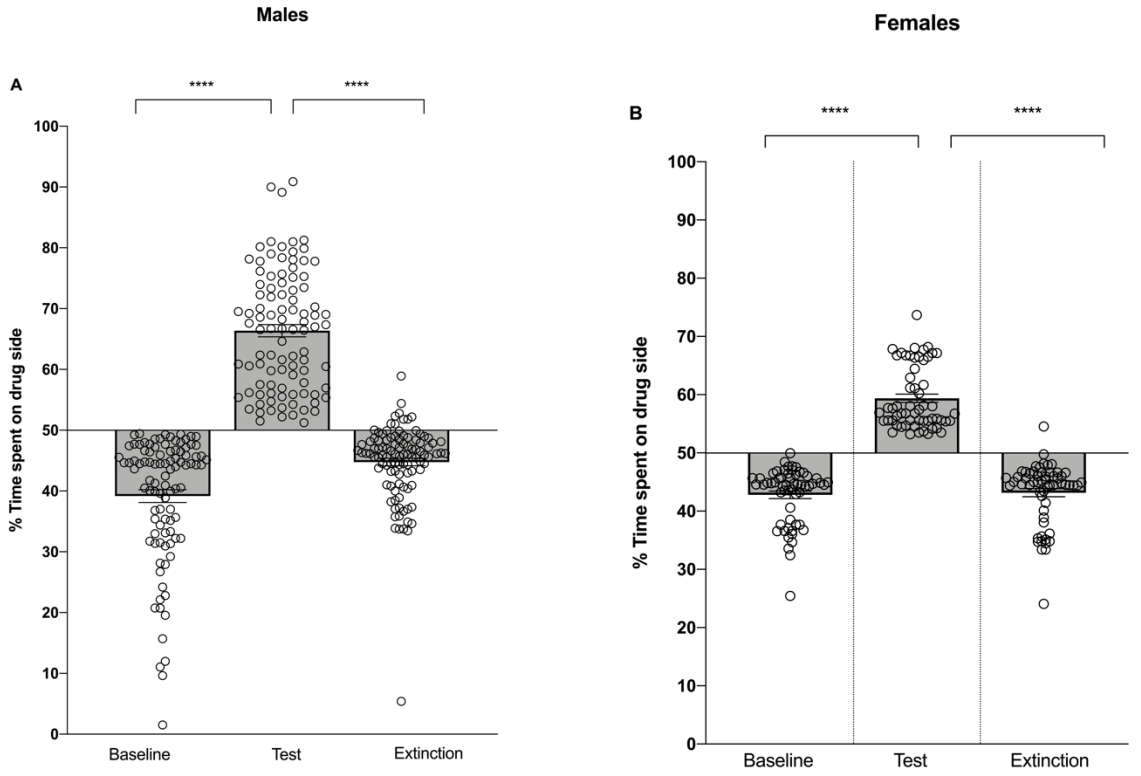

Figure 2. Male (Panel A) and **Female (Panel B)** mice behavior throughout the phases (Baseline, Test, Extinction) of the mCPP task. Preferences are calculated as % time (s) spent in the morphine-paired context. For baseline, this corresponds to the less preferred compartment of the CPP apparatus. All male ($t(96) = 19.17$, $p < 0.0001$) and female ($t(57) = 17.82$, $p < 0.0001$) mice showed a significant preference for the morphine-paired compartment at test followed by a reversal of this preference after extinction of CPP (male ($t(96) = 18.33$, $p < 0.0001$); female ($t(57) = 16.38$, $p < 0.0001$) Bars represent SEM. **p*<0.05*;* ***p*<0.01; ****p*<0.001; *****p*<0.0001. Means +/- SEM.

IV.2. Predator/Observational Distress Produces Distinct Coordinated Distress-Related Behaviors in Mouse Dyads

To induce observational distress, we then exposed pairs of mice to a novel, and ethologically relevant stress paradigm, in which the Demonstrator mouse was placed in a compartment adjacent to a much larger rat, and the Observer was placed in a compartment adjacent to the Demonstrator. Demonstrator/Observer dyads were either familiar (F/D and F/O, respectively) or unfamiliar (U/D and U/O, respectively) to one another. Compared to

controls, in which no rat was present, male F/D mice exposed to the rat most frequently exhibited stretch-attend posture (SAP), tail-rattling, and efforts at escape (classified as instances of rapid movement toward the wall opposite the rat) ($t(6)=3.113$, $p = 0.0208$, one-sample t-test) (Figure 3A). In naturalistic settings these behaviors are typically associated with displays of defensive aggression in the presence of a proximal threat (Blanchard et al., 2005; Rodgers et al., 1997). Consistent with this, Demonstrators exposed to an empty rat enclosure (No-rat Demonstrators–NR/D) did not show these behaviors. The presence of a distressed conspecific elicited efforts at escape in F/O ($t(6)=17.00$, $p =0.0374$) compared to No-Rat familiar observers (NR/O) ($t(7)=1.000$, $p = 0.5$). Similar behavior was not displayed by a further Observer control group, in which the rat was present but the Demonstrator cage remained empty (No-Demonstrator Observers, ND/O) ($t(5)=1$, $p = 0.05$, one sample t-test). This indicates that Familiar Observers were not simply responding to nonvisual (e.g., auditory, tactile, odor) cues emanating from the rat. Rather, their behavior likely reflected active "coping" responses displayed when mice are exposed to threat cues that are neither imminent nor localizable (Blanchard et al. (2001); de Boer & Koolhaas, 2003).

Unlike male Familiar Observers, Unfamiliar Observers did not display increased escape or ($t(1)=1$, $p = 0.05$, one sample t-test), consistent with previous findings that the social transmission of fear in male mice is dependent on their familiarity (Pisansky et al., 2017) (Figure 3A).

Familiar and unfamiliar female Demonstrators behaved similarly to males, producing SAP, escape, and tail rattling, (Figure 3B). However, female Observers more consistently exhibited observational fear than males, in that Unfamiliar, as well as Familiar Observers

expressed fear-related behavior. This difference between male and female mice observing an unfamiliar conspecific responding to predator threat replicates our previous finding in the context of Pavlovian fear conditioning (Pisansky et al., 2017).

IV.3. Social aggression in home cage

After the stress task, we returned mice to their home cages. Since mice were housed in groups of three, this meant that Familiar Demonstrator and Observer dyads were reunited with a third, non-stressed cage-mate, whereas Unfamiliar Demonstrator and Observer were reunited in their respective home cages with three non-stressed cage-mates. The behavior of male Familiar Observers was notable in that these mice directed frequent attacks and biting behavior towards the Demonstrator but not the non-stressed cage mates. Such behavioral indices of social aggression were not displayed by the other groups of male mice (Figure 4A). Aggressive behavior by Familiar Observers was seen regardless of their dominance status assessed in the tube test (Garner et al., 2004; Tan et al., 2018; see Supplementary Figure 2). In contrast, both Familiar and Unfamiliar female Observers did not display aggressive behavior upon return to their home cages.

IV.4. Observer Distress-Induced Reinstatement (ODIR)

When reintroduced to the CPP apparatus, male Demonstrator and Observer mice showed reinstatement of opioid seeking. A two-way ANOVA to examine the possible effects of familiarity and role (Demonstrator versus Observer) revealed a main effect of familiarity ($F(1,24)=43.61$, $p<0.0001$), a significant role x familiarity interaction $F(1,24)=29.41$, $p<0.0001$), but no effect of role ($F(1,24)= 0.0052$, $p=0.8213$) (Figure 5). Post hoc analyses

indicated that, whereas Demonstrators showed reinstatement regardless of their familiarity among Observers, only those Observers in familiar dyads showed this effect. Neither of the two control groups (no rat (NR/D and NR/O) showed any reinstatement (Supplemental Figure 3).

Following the same test and the same analysis in female mice, our data revealed a significant main effect of Role (Observer or Demonstrator) ($F(1,26)=8.676$, $p=0.0067$), a significant main effect of Familiarity ($F(1, 26) = 8.676$, $p=0.0067$), and a significant Role x Familiarity interaction ($F(1, 26) = 8.050$), $p=0.0087$). Further tests revealed that female Observers reinstated morphine-conditioned place preference regardless of familiarity with the Demonstrator, in stark contrast with our male findings (Figure 6).

To further examine the differential impact of Familiarity and Role on distress-induced reinstatement in males and females, we conducted a 3-way ANOVA (Sex x Familiarity x Role). We found a significant main effect of Sex ($F(1,50)=10.03$, $p = 0.026$), a significant main effect of Familiarity ($F1,50)=51.43$, $p<0.0001$ but no effect of Role ($F(1,50)=2.65$, $p=0.1156$), interactions of Sex x Familiarity ($F(1,50)=12.12$, $p=0.001$), Role x Familiarity ($F(1,50)=7.044$, $p=0.0106$), and a Sex x Role x Familiarity ($F(1,50)=37.14$, $p<0.0001$) were also significant. Thus, the differences between male and female Unfamiliar Observers in observational fear was recapitulated in the ODIR test.

IV.5. Role of oxytocinergic system in observational distress

IV.5.1. Males

Given the putative involvement of the neuropeptide oxytocin in processing the salience of socioemotional cues,we investigated whether pharmacologically modulating oxytocinergic neurotransmission during observational stress affected reinstatement. To this end, male

Observer mice in familiar dyads were administered an oxytocin antagonist L-368,899 (10 mg/kg, I.P.), whereas Observers in unfamiliar dyads were administered oxytocin itself intranasally (5 μL per nostril, see Pisansky et al., 2017). Oxytocin antagonist treatment eliminated escape behavior in Familiar Observers, while substantially increasing the frequency of approach towards the Demonstrator's enclosure. (Figure 3C). Conversely, Unfamiliar Observers administered oxytocin displayed escape, a behavior not otherwise shown by this group. Hence, oxytocin administration caused Unfamiliar Observers to behave similarly to Familiar Observers, whereas the oxytocin receptor antagonist caused Familiar Observers to behave similarly to Unfamiliar Observers. In contrast, the oxytocin antagonist had no effect when administered to a separate group of Demonstrators (paired with drug-free Observers). These data suggest that oxytocin positively modulates stress induced through observation of a conspecific under predatory threat but not the stress associated with direct exposure to the predator itself.

We found a similar directionality of drug effects when mice were returned to their home cage. That is, the oxytocin antagonist blocked the expression of aggression towards cage-mates in Familiar Observers, whereas oxytocin itself induced aggressive behavior in Unfamiliar Observers (Figure 4B). In contrast, neither oxytocin nor the antagonist affected the behavior of Demonstrators. This indicates, once again, that manipulation of oxytocin neurotransmission affected observational distress but not predator distress.

Finally, a between-group comparison of reinstatement in Familiar Observers, F/O treated with OXTA, Unfamiliar Observers treated with oxytocin, and Unfamiliar Observers who received saline I.N. was significant ($F(3, 21)=34.00$, $p<0.0001$). Familiar Observers showed significantly greater reinstatement than both Unfamiliar Observers in the saline

condition (M=63.27., SD=9.557, p=0.0008) and familiar observers treated with OXTA (M=63.27., SD=9.557, p<0.05). Unfamiliar Observers treated with oxytocin showed greater reinstatement than Unfamiliar Observers who were treated with saline (M=81.75, SD=5.668, p<0.0001) (Figure 8). Summarizing across all three test phases, the administration of an oxytocin antagonist blocked observational fear, aggression, and reinstatement in male Familiar Observer mice, whereas oxytocin induced these behaviors in male Unfamiliar Observer mice. Once again, these findings add further support to those of Pisansky and colleagues (2017).

IV.5.2. Females

Unlike males Observers, both Familiar and Unfamiliar female Observers in the No Treatment condition showed signs of distress when witnessing Demonstrator behaviors, as indicated by similar approach and escape behaviors (Figure 3B). Familiar and Unfamiliar Demonstrators exhibited behaviors consistent with the presence of a proximal threat, including signs of territorial defensive aggression (tail rattling), SAP (threat assessment), and escape attempts.

Administration of the oxytocin antagonist reduced freezing and increased conspecific Approach in female Familiar and Unfamiliar Observers, suggesting that blockade of oxytocin systemically altered Observer distress behaviors regardless of familiarity (Figure 3D). The robustness of these findings is reflected in the relative lack of variability in in the patterns of behavior within individual pairs of animals (Supplementary Figure 1). It was possible, however, that having undergone mCPP alone was altering female behavior during Observational Distress. To investigate whether this was the case, we conducted the

Observational Distress experiment in a group of female mice who did not receive morphine conditioning. The behavioral patterns between Observer and Demonstrator dyads in the basic condition (mCPP, not oxytocinergic manipulation) did not differ from that of dyads who did were not subjected to morphine-conditioning. We concluded, therefore, that morphine-CPP and extinction did not account for our observations during this task. Relative to individual Observers who received oxytocin antagonist, Observers who did not receive such treatment, as well as those who did not undergo conditioning showed greater freezing and fewer attempts to approach the Demonstrator (Supplementary Figure 1).

Observational Distress Behaviors in Male and Female Mice

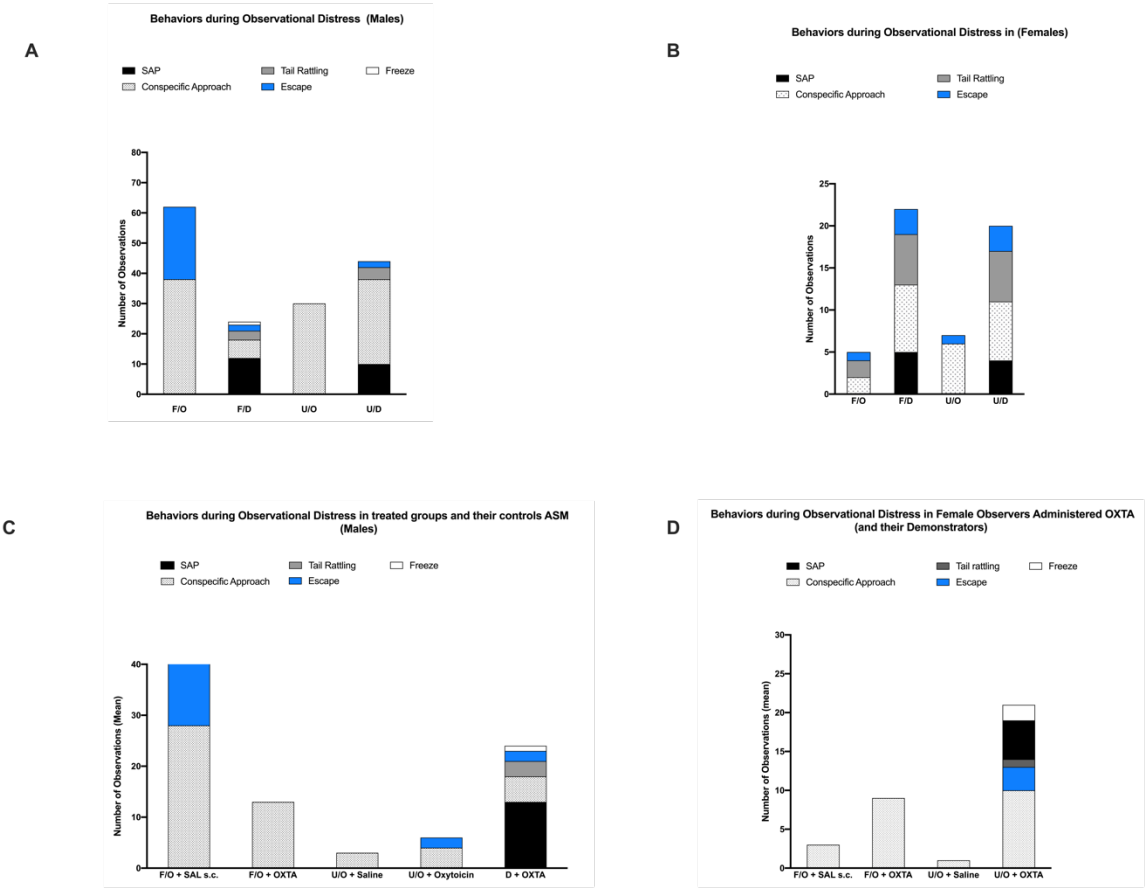

Figure 3. Observational Distress behaviors in Male and Female mice. **Panel A:** Demonstrator/Observer dyads were either familiar (F/D and F/O, respectively) or unfamiliar (U/D and U/O, respectively) to one another. Compared to no rat controls, male F/D mice exposed to the rat most frequently exhibited stretch-attend posture (SAP), tail-rattling, and efforts at escape (classified as instances of rapid movement toward the wall opposite the rat) ($t(6)=3.113$, $p = 0.0208$, one-sample t-test) Demonstrators exposed to an empty rat enclosure (No-rat Demonstrators–NR/D) did not show these behaviors. The presence of a Demonstrator elicited escape in F/O ($t(1)=17.00$, $p =0.0374$) compared to No-Rat familiar observers (NR/O) ($t(1)=1.000$, $p = 0.5$). Similar behavior was not displayed by a further Observer control group, in which the rat was present but the Demonstrator cage remained empty (No-Demonstrator Observers, ND/O) ($t(1)=1$, $p = 0.05$, one sample t-test). **Panel B:** Female Familiar and Unfamiliar Observers showed similar levels of escape and approach behaviors. Both Familiar and Unfamiliar female Demonstrators behaved consistently with the presence of a proximal threat (rat), including SAP, tail rattling, and elevated freezing. **Panel C:** Oxytocin antagonism (OXTA) rescued escape behaviors in familiar Observer males, while increasing approach behaviors. OXTA thus made Familiar Observers behave similarly to Unfamiliar ones who received either saline or no treatment. Conversely, intranasal oxytocin administration produced freezing behavior in Unfamiliar Observers, causing them to behave similarly to untreated Familiar Observers. Intranasal saline did not alter freezing in Unfamiliar Observer controls. Demonstrator behavior was unchanged by oxytocin manipulation. **Panel D:** OXTA administration among Familiar and Unfamiliar female Observers increased conspecific approach and slightly reduced escape behavior.

Aggressive behavior is differentially exhibited by Familiar and Unfamiliar Male Observers: effects of Familiarity and Treatment

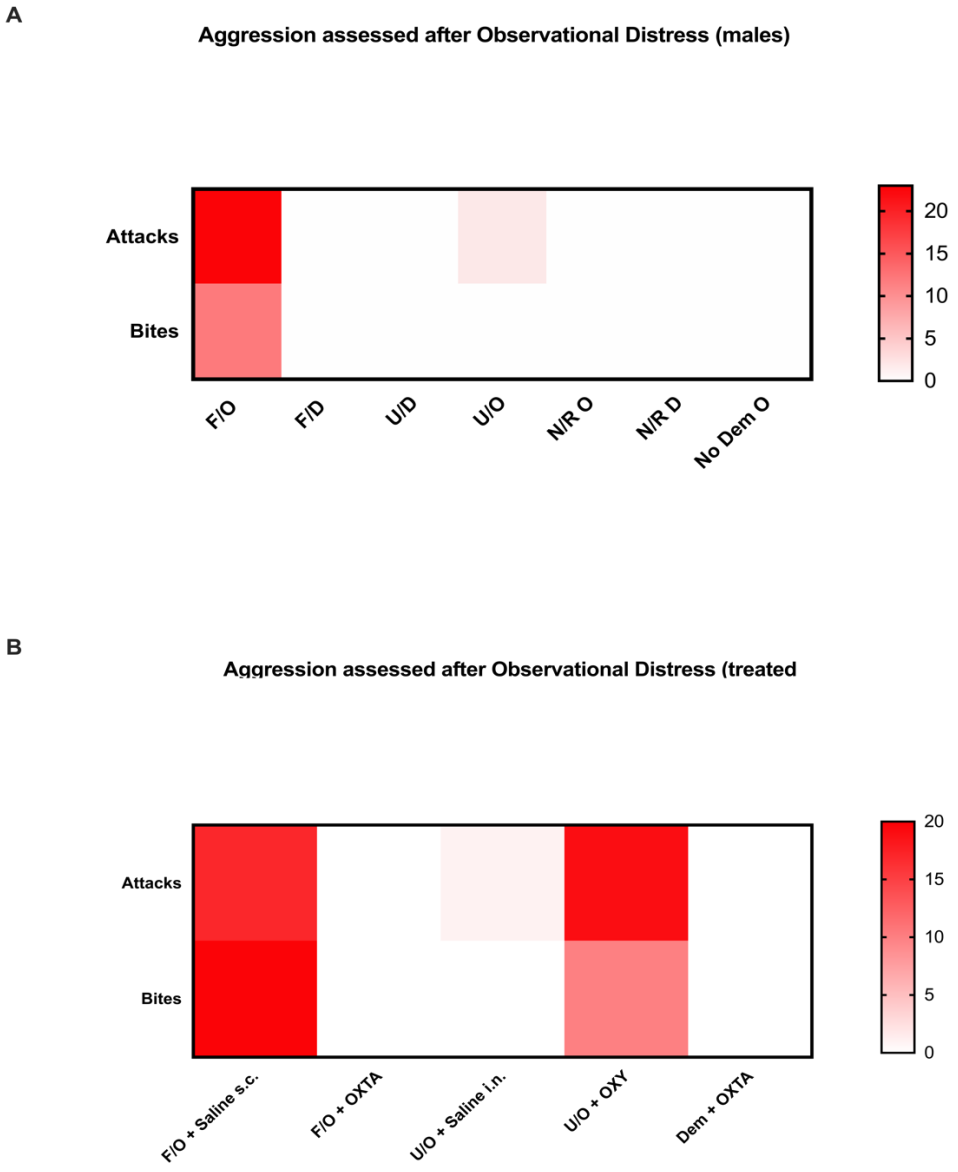

Figure 4. Social aggression in treated (by either intranasal oxytocin or by systemic antagonism of oxytocin) and untreated males. **Panel A:** Familiar Observers who received saline exhibited aggression comparable to this of Unfamiliar Observers given I.N. oxytocin and Familiar Observers who received no treatment. **Panel B:** Familiar Observers exhibited aggression toward Demonstrator mice following Observational Distress. Observers who

were not assigned a Demonstrator ("No Dem"), as well as those for whom the predator was not present ("No threat,"), and their respective Demonstrators did not attack or bite conspecifics during social interaction assessment. The bias of Familiarity is thus rescued in Unfamiliar Observers by oxytocin administration and is reversed in Familiar Observers by oxytocin antagonism.

IV.6. Relationship between Demonstrator and Observer behavior

To further explore the role of oxytocin on ODIR, we assessed the relationship between distress behavior elicited by Demonstrator-Observer pairs and subsequent levels of reinstatement. We found that reinstatement in Familiar Observers was strongly predicted by SAP ($r(7)=0.8552$, $p = 0.0141$), tail rattling (($r(7)=0.8535$, $p = 0.0146$), and window contact($r(7)=0.9150$, $p = 0.0039$) by the Demonstrators in each pair, as well as by a composite measure of Demonstrator distress behaviors ($r(7)=0.8988$, $p = 0.0059$). The same was true for Unfamiliar Observers administered intranasal oxytocin (SAP: $r(7)=0.9533$, $p = 0.0009$; tail rattling: $r(7)=0.8430$, $p = 0.0172$; conspecific approach: $r(7)=0.8818$, $p = 0.0087$; composite measure: $r(7) = 0.9522$, $p = 0.0009$). Conversely, no such relationships were seen between Demonstrator behavior and reinstatement of CPP in Familiar Observers that received the oxytocin antagonist or Unfamiliar Observers administered saline (Figure 7). These findings further support the conclusion that OXTA administration rendered familiar male mice similar to their unfamiliar counterparts, whereas oxytocin administration rendered unfamiliar males similar to their familiar counterparts.

The same correspondence between the behavior of Demonstrators and Observers and subsequent reinstatement was not apparent in female mice, likely due to the narrow range of reinstatement scores in females.

Observational Distress-Induced reinstatement of mCPP in Males: Effects of Familiarity and Role

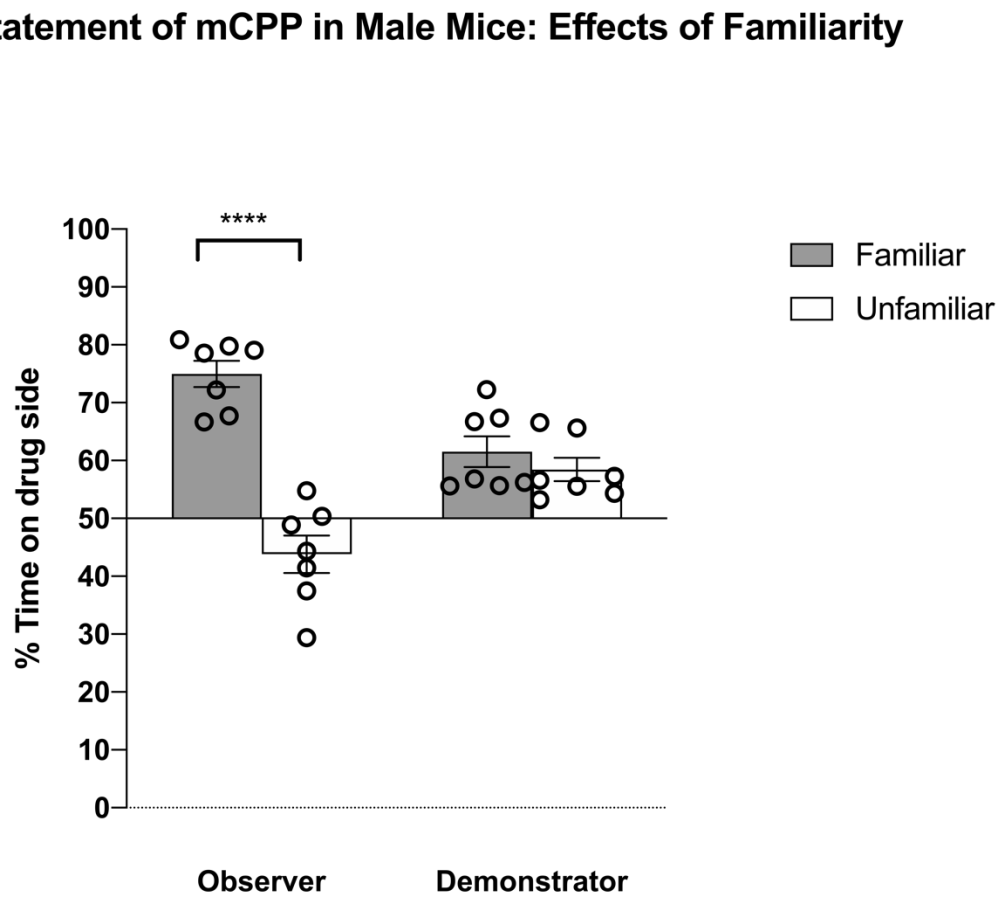

Figure 5. Two-way ANOVA of reinstatement behaviors in male Familiar and Unfamiliar Observer and Demonstrator mice to examine the effects Familiarity and Role (Demonstrator versus Observer) revealed a main effect of Familiarity ($F(1,24)=43.61$, $p<0.0001$), a significant Role x Familiarity interaction ($F1,24)=29.41$, $p<0.0001$), but no effect of role ($F(1,24)= 0.0052$, $p=0.8213$). *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. Means +/- SEM.

Observational Distress-Induced reinstatement of mCPP in Females: Effects of Familiarity and Role

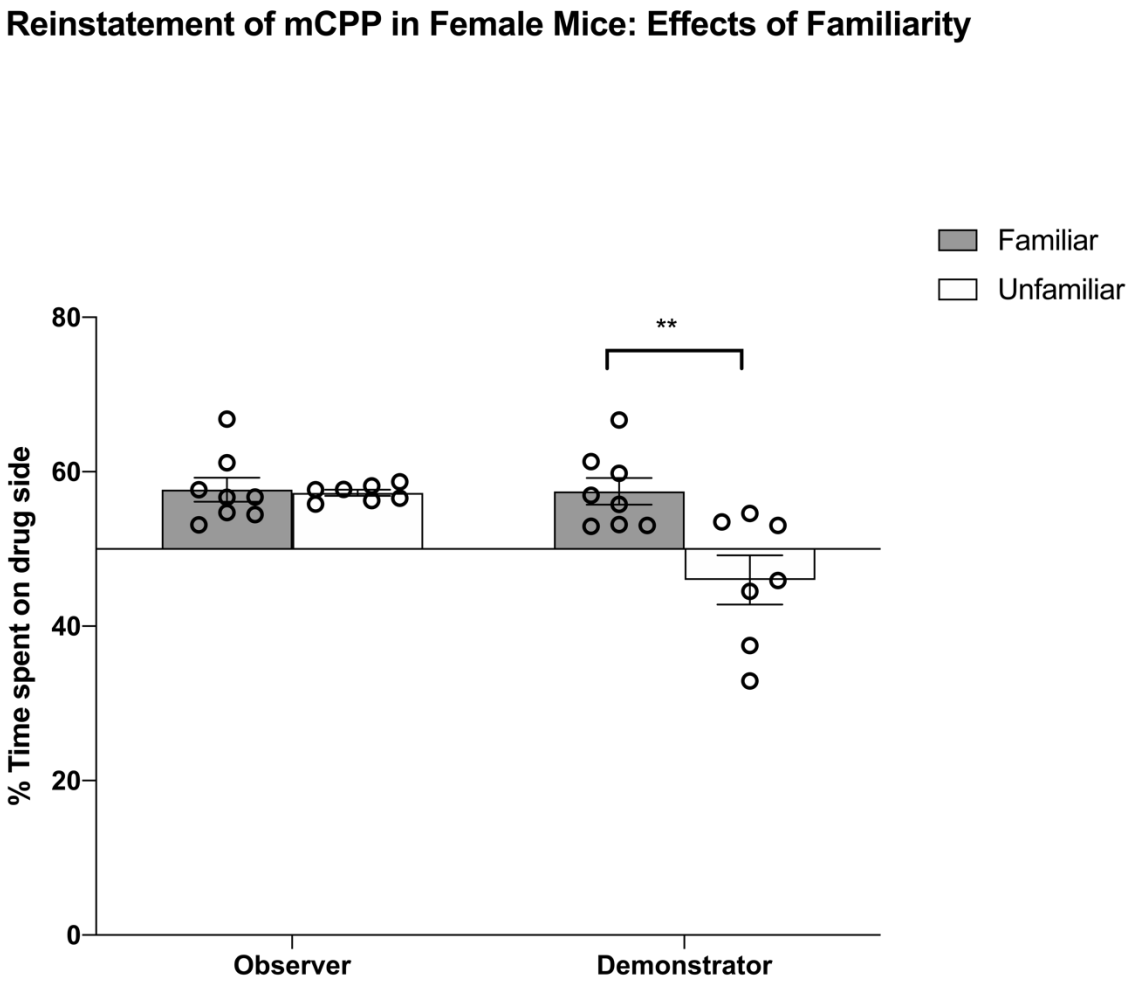

Figure 6. Two-way ANOVA of reinstatement behaviors in female Familiar and Unfamiliar Observer and Demonstrator mice to examine the effects of Role and Familiarity main effect of Role (Observer or Demonstrator) ($F(1,26)=8.676$, $p=0.0067$), a significant main effect of Familiarity ($F(1, 26) = 8.676$, $p=0.0067$), and a significant Role x Familiarity interaction ($F(1, 26) = 8.050$), $p=0.0087$).
*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. Means +/- SEM.

Demonstrator behavior during the Observational Distress Task predicts Observer Reinstatement in Familiar, but not Unfamiliar males and varies with oxytocin manipulation

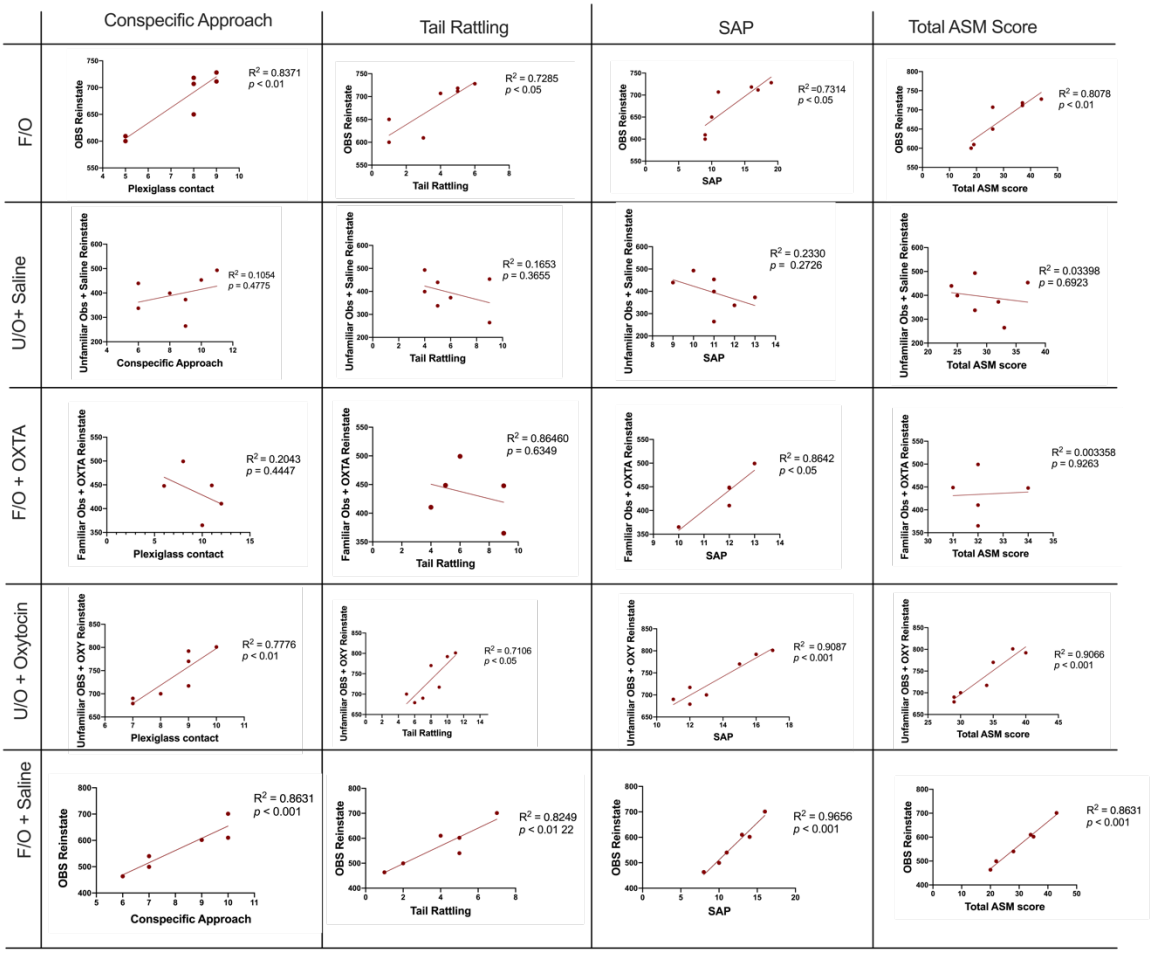

Figure 7. Reinstatement in Familiar Observers was predicted by SAP (r(7)=0.8552, p = 0.0141), tail rattling ((r(7)=0.8535, p = 0.0146), and window approach (r(7)=0.9150, p = 0.0039) by the Demonstrators in each pair, and by a composite measure of Demonstrator distress behaviors (r(7)=0.8988, p = 0.0059). The same was true for Unfamiliar Observers administered intranasal oxytocin (SAP: r(7)=0.9533, p = 0.0009; tail rattling: r(7)=0.8430, p = 0.0172; conspecific approach: r(7)=0.8818, p = 0.0087; composite measure: r(7) = 0.9522, p = 0.0009).

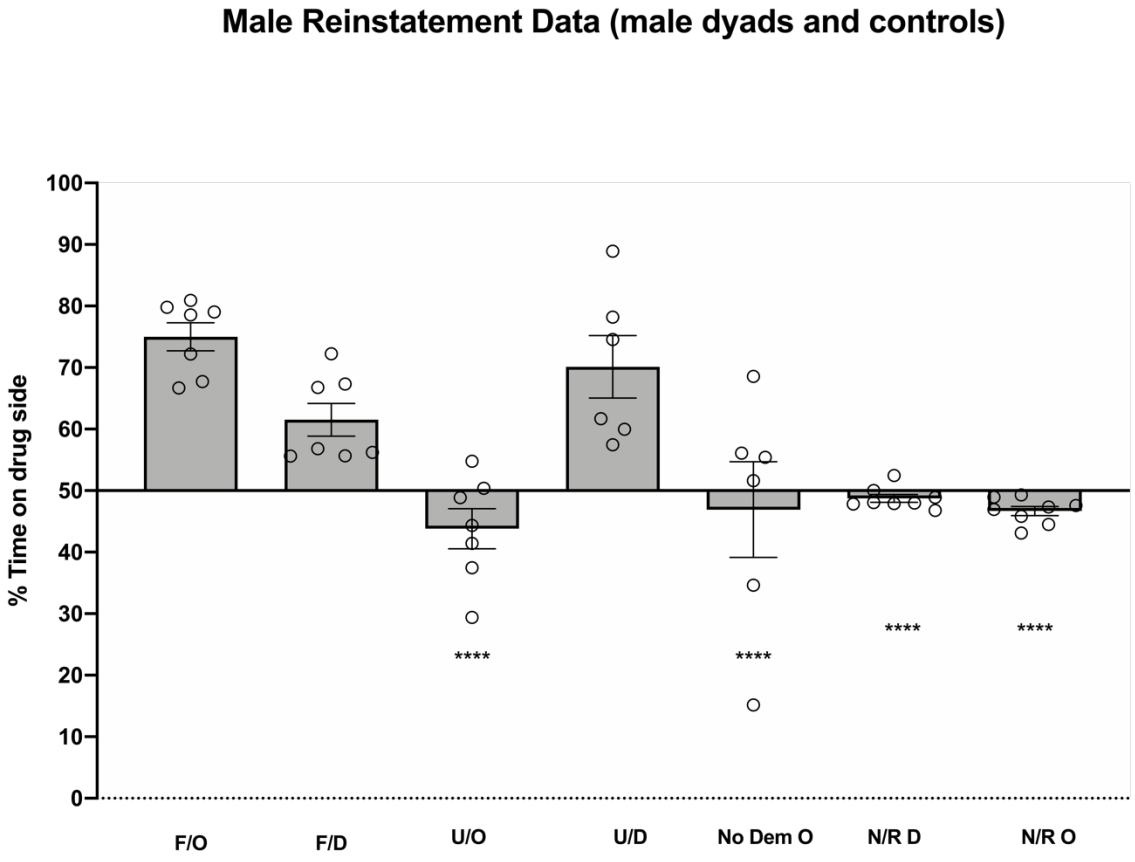

Figure 8. One-Way ANOVA between-group comparison. Familiar Observers, F/O treated with OXTA, Unfamiliar Observers treated with oxytocin, and Unfamiliar Observers who received saline I.N. differed significantly in degree of reinstatement ($F(3, 21)=34.00$, $p<0.0001$). Familiar Observers showed greater reinstatement than both Unfamiliar Observers in the saline condition ($M=63.27.$, $SD=9.557$, $p=0.0008$) and familiar observers treated with OXTA ($M=63.27.$, $SD=9.557$, $p<0.05$). Unfamiliar Observers treated with oxytocin showed greater reinstatement than Unfamiliar Observers who were treated with saline ($M=81.75$, $SD=5.668$, $p<0.0001$).

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

V.1. Psychosocial stress and reinstatement

Perhaps the broadest distinction among sources of stress is that between those that are physical (e.g., assault, injury) and those that are psychosocial (e.g., economic, bullying). When severe, both contribute to human psychopathology (Sontag et al., 2011; Livingston et al., 2019). In preclinical models, extensive research into the interactions between stress and drug addiction has revealed neural mechanisms underlying relapse to drug-seeking after exposure to physical stressors such as footshock or the anxiogenic drug yohimbine (Mantch & Shaham, 2016; Manvitch et al. 2016). Studies addressing mechanisms underlying reinstatement in response to psychosocial stressors typically involve exposure of male mice to a socially dominant conspecific — an encounter that has a clear physical dimension. In the current experiments, we assessed whether reinstatement of opioid seeking would occur in conditions under which there was no physical contact with the stress-inducing conspecific. We found that observation of a conspecific mouse responding to the threat of predation precipitated reinstatement of opioid-seeking, even though the Observer could not see the source of the threat to the Demonstrator. Such Observational Distress-induced Reinstatement (ODIR), was found in both male and female dyads. In common with other behavioral sequelae of observational distress, ODIR was dependent on activity of the neuropeptide oxytocin. Similar effects of pharmacological manipulation of oxytocinergic neurotransmission were not seen in the mice exposed to predator stress itself. Thus, the ODIR paradigm has high face validity as a novel approach to studying the neurobiology of psychosocial stress and its interactions with opioid seeking.

One of the most striking features of this paradigm is how the behavior of the two members of the Observer-Demonstrator dyad differed both during and after stress exposure.

Whereas the mouse confronted by a predator displayed defensive aggression, observation of such behavior induced efforts at escape, behaviors typically provoked by more distal threats (Blanchard et al., 2012). Male Observer mice initiated bouts of aggression towards the familiar Demonstrator when returned to their home cage and subsequently exhibited a degree of ODIR which was correlated with the intensity of defensive aggression previously observed. These findings suggest that reinstatement in the Observer is at least as strong as reinstatement in the Demonstrator after its exposure to predatory threat and stems directly from witnessing that encounter.

Consistent with previous studies using Pavlovian fear conditioning paradigms, the most straightforward explanation of the Observer's distress is that it results from the social transmission of fear, between the two (Mikosz et al., 2015; Bredy et al., 2009; Knapska et al., 2006; Nowak et al., 2013).

However, the behavior of male mice when returned to their home cage does not easily fit with this explanation, in that the Observer was the sole aggressor, and the Demonstrator was the sole target of its attacks. This may suggest that the Observer became fearful by perceiving the Demonstrator's behavior as threatening, even though the Demonstrator's defensive aggression was not directed towards the Observer itself. In the resident-intruder paradigm, aggression is similarly instigated by introduction of an inaccessible "intruder" mouse into the "resident" mouse's cage (Mizcek, 1979). Territorial incursion is unlikely to lie at the core of male Observer's behavior in the current paradigm since the stressful interaction occurred in a novel environment, and distress occurred only when the Demonstrator was familiar. The Observer's distress may, instead, have resulted from a dramatic alteration in the behavior of an inaccessible cage-mate that could not be attributed

to an obvious change in circumstances (e.g., the presence of a threatening predator). In other words, distress may have occurred through observing a familiar mouse, with which the Observer could not directly interact, acting in a distinctly unfamiliar or unpredictable – and therefore potentially threatening – way. Notwithstanding whether induced by social transmission of the Demonstrator's fear or by the perception of the Demonstrator as a direct threat, this experience clearly represented a significant source of psychosocial stress to the Observer mouse. The possibility that Observer aggression toward the familiar Demonstrator may somehow be influenced by social rank was explored in a small sample of male cage mates who subsequently underwent our experimental procedures. We found that rank had no relevance to the expression of aggression by Familiar Observers, suggesting that observing the distress behaviors of the Demonstrator modulates social behavior independently from social hierarchy. The replication of observations should be pursued further in larger samples to determine whether vicarious distress can alter the social structures of species with the tendency to form hierarchical groups.

V.2. Sex differences in observational distress and subsequent reinstatement

This paradigm may offer a means of further investigating sex differences in the induction of psychosocial stress and in stress-induced reinstatement in mice. Female mice responded similarly to males when confronted with a distressed conspecific inasmuch as they exhibited escape behavior and, subsequently, reinstatement of opioid seeking. Female Observers, however, differed from males in that they became distressed irrespective of their familiarity with their dyadic partner, a finding consistent with other studies of observational distress (Pisansky et al., 2017) Furthermore, female Observers did not

behave aggressively when returned to their home cage. Such an absence of aggressive behavior is typical of nonlactating female mice (Svare & Gandelman, 1968).

V.3. Effects of Oxytocin on Observational Distress-Induced Reinstatement (ODIR)

The neuropeptide oxytocin has been implicated in rodent models of addiction-related behavior, anxiety, and social transmission of affective states, as well as in human socio-emotional interaction and emotional empathy (Young et al., 2009; Campbell et al., 2010). In this study, administration of oxytocin and an oxytocin receptor antagonist respectively enhanced and blocked the induction of observational distress in males, as well as two sequelae: intraspecies aggression and reinstatement of opioid seeking. Neither drug impacted the behavior of Demonstrator mice that were directly confronted with a predator. Notably, oxytocin administration in Unfamiliar Observers enhanced sensitivity to the distress of the Demonstrator, while blockade of oxytocin reversed observational distress in Familiar Observers. This provides confirmatory evidence to prior work conducted inn our lab, whereby male Observer showed a familiarity bias which was effectively rescued by oxytocinergic manipulation (Pisansky et al., 2017). Our data extended these findings to female mice, in whom the administration of an oxytocin antagonist blocked reinstatement of morphine CPP following observational distress.

Our results support the predictions of the Social Salience hypothesis, whereby oxytocin modulates the affective salience of social cues but not of nonsocial cues, as represented, for example, by footshock or the presence of a heterospecific predator (Guzman et al., 2013;, Shamay-Tsoory & Abu-Akel, 2015). In contrast, the fact that oxytocin positively modulated fear and anxiety in Observer mice, is inconsistent with a second major

hypothesis about oxytocin's psychoactive properties; namely, that it is anxiolytic (Neuman & Slattery, 2015). In fact, the effects of oxytocin on anxiety in humans and on anxiety-like behavior in rodents have been mixed, with a number of studies showing anxiolytic effects but others showing either null, or even anxiogenic effects (Missig et al., 2010; Eckstein et al., 2015, but also see Eckstein 2014). Recent evidence offers a possible resolution to these apparently contradictory findings, suggesting that oxytocin can both negatively modulate fear and positively modulate the salience of fearful cues, depending on contextual factors (Anpilov, 2020). Further research may help to define the boundary conditions under which oxytocin neurotransmission exerts these opposing effects.

Similar complexity surrounds the role of oxytocin in stress-induced reinstatement. We found that exogenous oxytocin prior to stress exposure increased reinstatement of opioid seeking. In contrast, administration of the oxytocin analog carbetocin has been found to ameliorate stress-induced reinstatement of morphine CPP (Zanos et al., 2014) as well as stress-induced reinstatement of alcohol seeking in a doze-dependent manner (King et al., 2019). Similar treatments ameliorate forms of reinstatement to drug seeking that do not involve stress (i.e., drug- and cue-induced, (Baracz et al., 2016) as well as other measures of drug-seeking and drug-taking (Kovacs et al., 1985; Kovacks et al., 1998). This suggests that oxytocin may target reinforcement pathways to reduce the efficacy of addictive drugs, including opioids, countering a promotive effect on reinstatement induced by psychosocial stress. Thus, the effects of manipulations of the oxytocinergic system appear to be multifactorial, affecting to varying degrees fear and anxiety, drug reinforcement efficacy and social salience.

Notwithstanding these complexities, our findings indicate that the oxytocin antagonist L-368,899 ameliorates aggressive behavior and relapse to opioid seeking in response to socially mediated distress among male Familiar Observers. This, together with evidence that the same drug reduces stress-induced social avoidance, at least in female mice (Duque-Wilckens et al., 2017) suggest there may be therapeutic potential for the use of oxytocin antagonists to treat the social dimensions of anxiety and mood disorders. Since, depending on the circumstances, some of these effects may countervail one another, the therapeutic efficacy of oxytocin analogs and/or antagonists may depend on nuances of the contexts in which they are administered and may therefore be difficult to predict. This may go some way towards explaining the inconsistency of findings regarding oxytocin's efficacy in human studies (Tabak et al., 2019).

V. Conclusions

Witnessing the distress of others is a common form of psychosocial stress that, when traumatic or chronic, can precipitate or exacerbate mental illness (Kowalchyk et al., 2020; Bromet et al., 2018). While neural mechanisms underlying the deleterious effects of stress have been identified through animal models (Takahashi et al., 2018; Mcewen et al., 1993; Misczek et al., 2008), little is known regarding mechanisms underlying the deleterious effects of the vicarious or "secondary" experience of trauma. Similarly, while there have been advances in our understanding of the biology underlying multiple interactions between stress responses and addictive behavior (Koob, 2008) the behavioral and biological effects of witnessing the fear, injury, or death of others on the development or severity of addiction are less well understood. The experiments described here indicate

how witnessing the distress of a conspecific can negatively impact a mouse's social behavior and drive relapse to seeking an opioid drug. As in other psychosocial distress paradigms, these effects were sensitive to pharmacological manipulation of the oxytocinergic system (Burkett et al., 2016; Pisansky et al., 2017). Hence, this behavioral paradigm may offer a useful framework for further investigating the relationship between severe psychosocial stress and opioid addiction. Given the known interactions between oxytocinergic and opioidergic neurotransmitter systems (Quintana et al., 2019; Dal Monte et al., 2018), and evidence for the effects of oxytocin on a range of other measures of opioid dependence and addiction (Kovacs et al., 1985; Zanos et al., 2014) it will be important to ascertain whether relapse to the seeking of other classes of addictive drugs, such as stimulants, is similarly affected by manipulations of the oxytocinergic system.

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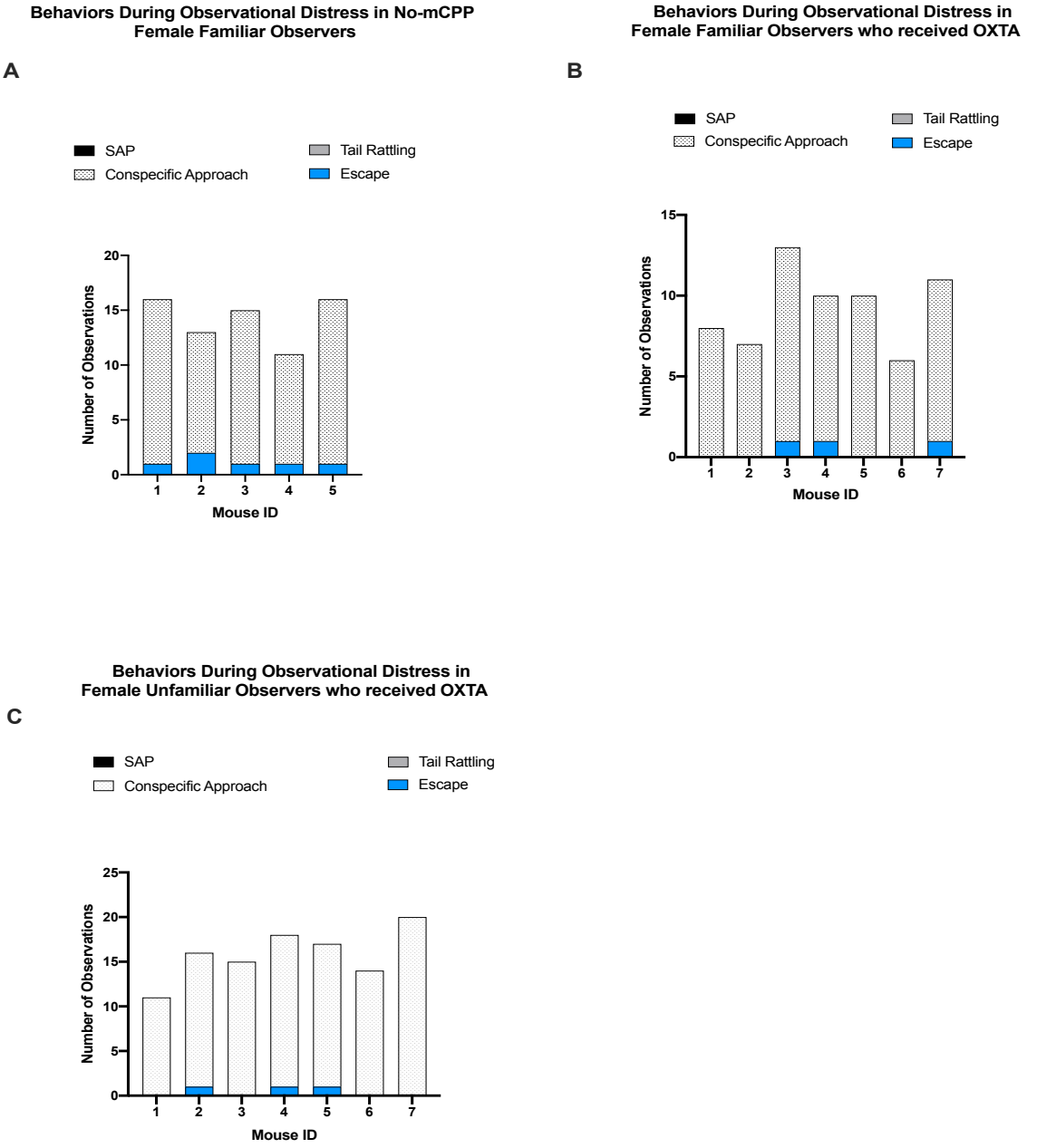
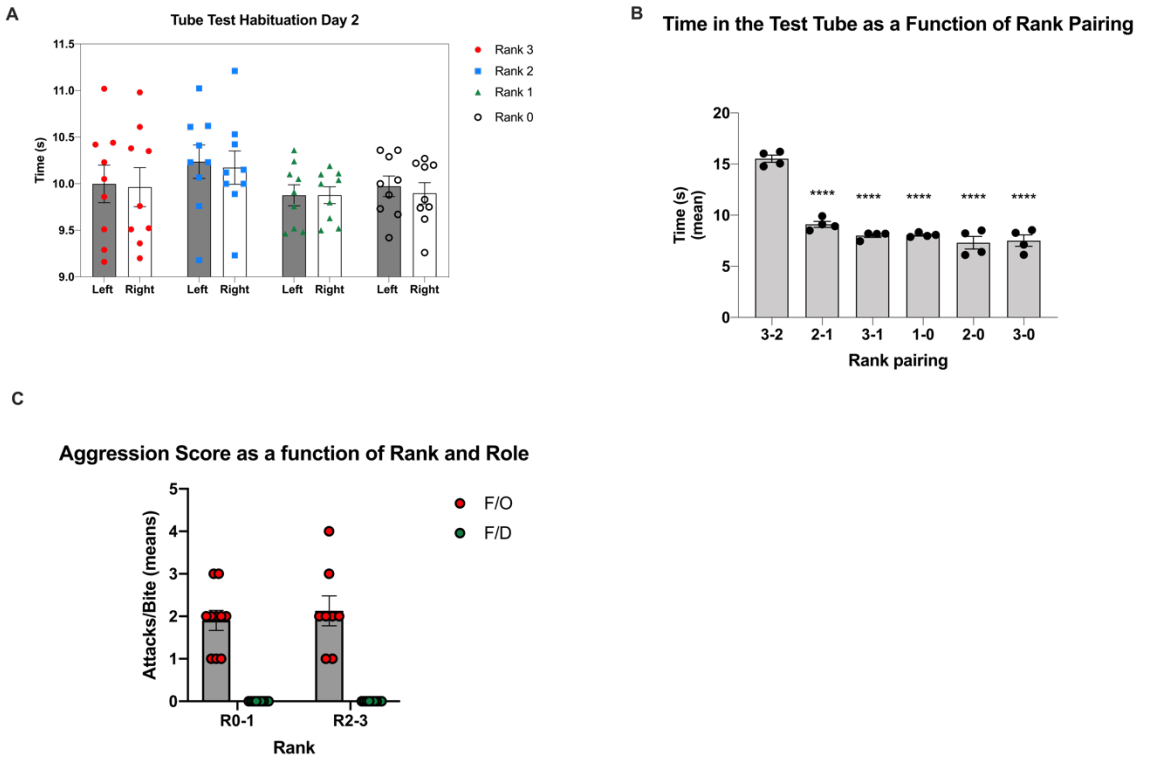
APPENDIX

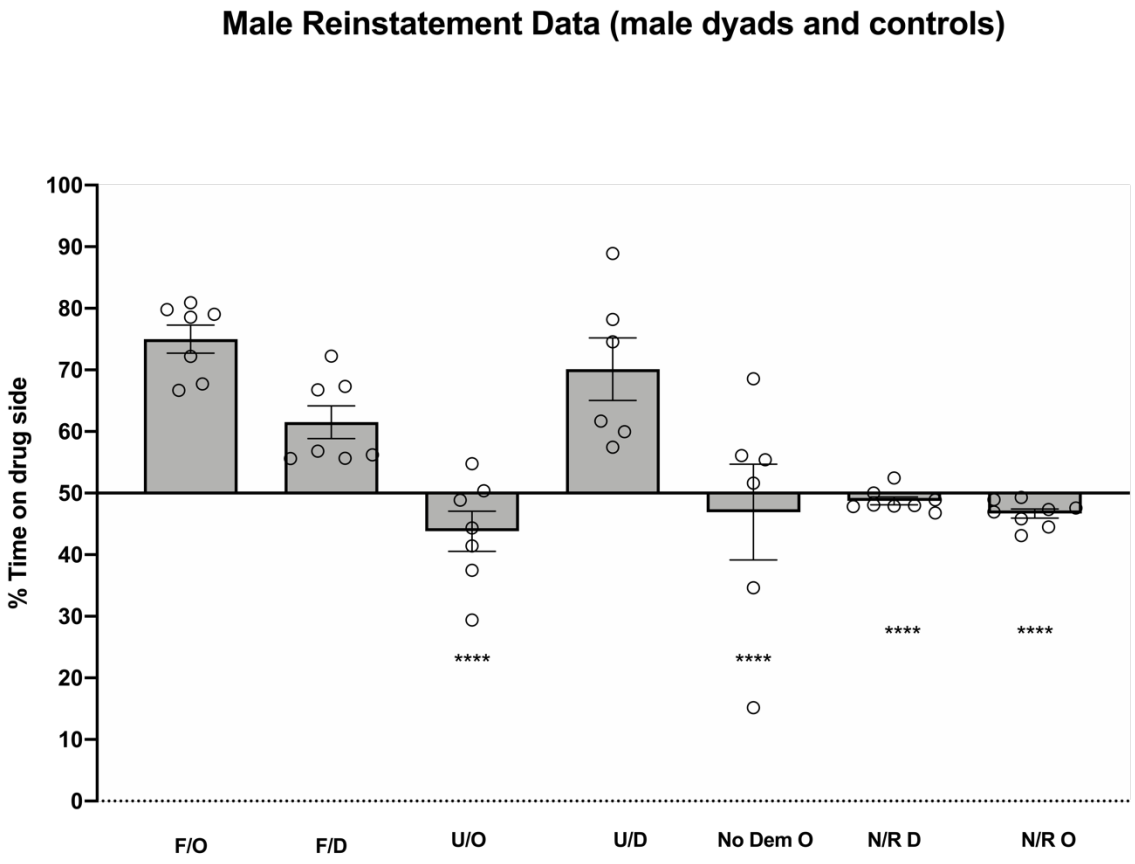


Supplementary Figure 1. Individual female mouse behaviors during Observational Distress in Familiar Observers who did not undergo mCPP **(Panel A)**, Unfamiliar Observers who were administered an oxytocin antagonist (OXTA) intranasally **(Panel B)**, and in Familiar Observers who received OXTA **(Panel C).** OXTA-treated Observers showed greater variability in freezing behaviors than non-CPP and mCPP mice regardless of familiarity.

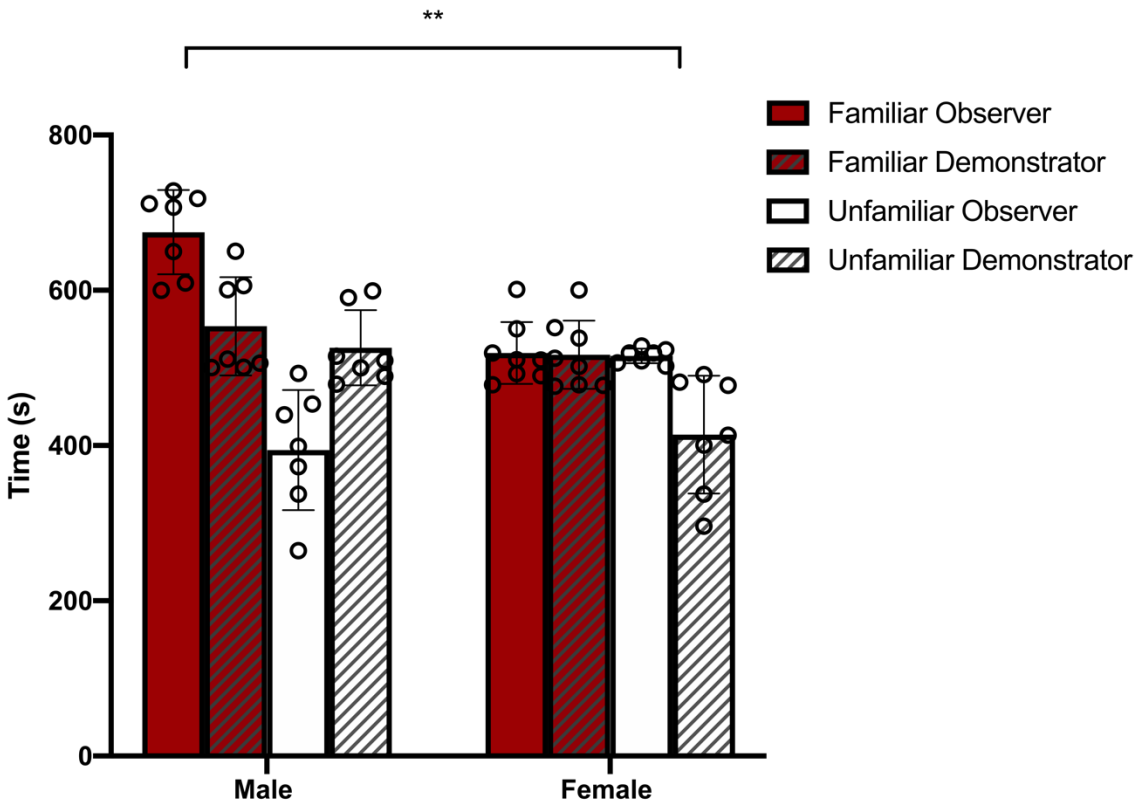


Supplementary Figure 2. Panel A: Left or right entry into the test tube did not affect time spent in the tube during habituation ($F(1,64)=0.1539$, $p=0.6962$). In all animals, rank was not predictive of time spent in the tube ($F(3,64)=0.1692$, $p=0.1776$). **Panel B:** Time spent in the test tube as a function of rank (from 0 to 3) and pairing. Pairs are indicated on the x axis, so that, for example, an animal of rank 3 paired with an animal of rank 2 is denoted by "3-2." The 3-2 pairing differed significantly from all other pairings in length of time spent in the test tube ($F(5,18)=62.36$, $p<0.0001$). **Panel C:** Role (Observer or Demonstrator) regardless of rank was associated with aggressive behavior ($F(1,32)=98.45$, $p < 0.0001$).

Cage mate rank (0-lowest, 3, highest) determined by rank scores on the Tube Test. During the test, 2 mice entered the tube from each side. A score of 1 was assigned to the mouse that remained in the tube, while the mouse pushed out of the tube received a score of 0. Each dyad was tested four times to ensure reliability of scoring. Since mice were housed in cages of 4 maximum, it was useful to derive a ranking score for each animal relative to its cage mates. Thus, a mouse could be given a rank of 0, 1, 2, or 3. Stable ranks were considered once each animal's rank remained constant for 4 days. Mice were grouped in two categories (dominant and subordinate) based on this tanking score. Rank 0-1 (R0-1) reflects the subordinate category and Rank 2-3 (R2-3) denotes the dominant category. Mice who underwent the tube test were blindly assigned to either an Observer or a Demonstrator role, and their social interactions following Observational Distress was subsequently assessed.



Supplementary Figure 3. Male Familiar Observers and Demonstrators reinstate morphine-CPP, but not in the absence of a predator threat (No Rat, N/R Observer (N/R O) and N/R Demonstrator (N/R/D). To examine the contributions of Role and Familiarity, a 2-way ANOVA was conducted. Familiar Observers (F/O) and Familiar Demonstrators (F/D) differed significantly from Observers and Demonstrators in the absence of a predator threat but not when a predator threat was absent (N/R O and N/R D) or, in the case of Observer mice, when the Demonstrators were absent (No Dem O).



Supplementary Figure 4. 3-way ANOVA (Sex x Familiarity x Role). There was a significant main effect of Sex ($F(1,50)=10.03$, $p = 0.026$), a significant main effect of Familiarity ($F1,50)=51.43$, $p<0.0001$ but no effect of Role ($F(1,50)=2.65$, $p=0.1156$), interactions of Sex x Familiarity ($F(1,50)=12.12$, $p=0.001$), Role x Familiarity ($F(1,50)=7.044$, $p=0.0106$), and a Sex x Role x Familiarity ($F(1,50)=37.14$, $p<0.0001$) were also significant.