ROLE OF SUBSTANCE P AND THE NEUROKININ-1 RECEPTOR IN STRESS, INFLAMMATION, AND ALCOHOL CONSUMPTION

by

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(Under the Direction of Jesse Schank)

ABSTRACT

Alcohol use disorder (AUD) negatively impacts the lives of millions of people in the United States each year. Major depressive disorder commonly co-occurs with AUD. Factors such as stress and inflammation may contribute to symptoms or development of these disorders. Substance P and its preferred receptor, the neurokinin-1 receptor (NK1R), are involved in both stress and inflammatory signaling. The following dissertation further characterizes the Substance P and the NK1R as a potential target for AUD. In an inflammatory model, lipopolysaccharide (LPS) administration induced significantly increased proinflammatory cytokine gene expression in both male and female mice, but only LPS-treated female mice had decreased social interaction measures, reflective of increased depressive-like behavior. LPS administration escalated alcohol consumption in both male and female mice. Systemic NK1R antagonism reduced alcohol consumption in the LPS-treated female mice but not the LPS-treated male mice. A model of vicarious defeat stress (VDS) in female mice induced increased depressive-like behaviors as measured by social interaction, expression of proinflammatory cytokine genes, and alcohol consumption. The alcohol consumption was significantly reduced with NK1R antagonist administration. To better understand the role of Substance P within the nucleus accumbens

(NAC), a region important for reward and motivated behaviors, tract tracing, neuronal activation analysis, and chemogenetic modulation of the SP inputs to the NAC were performed. Of the regions which project SP to the NAC, the paraventricular nucleus of the thalamus projections were significantly activated by social defeat stress. Inhibition either chronically during social defeat stress or acutely prior to the behavior reduced alcohol consumption. Activation of SP inputs to the NAC increased alcohol consumption. Neither activation nor inhibition impacted social interaction behavior. Overall, this dissertation further validated a LPS and VDS model to study inflammation or stress in alcohol consumption and depressive-like phenotypes in female mice. Furthermore, the characterization of the SP and NK1R involvement in these behaviors in the NAC will improve general understanding of mechanisms which drive AUD. These findings support NK1R as a promising therapeutic target for AUD.

INDEX WORDS: Alcohol, Stress, Neuroinflammation, Substance P, Neurokinin-1 Receptor, Social Defeat Stress, Vicarious Defeat Stress, Nucleus Accumbens

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Alcohol Use Disorder

Alcohol is the most commonly used drug with around half of all people over the age of 18 reporting past month use (U.S. Department of Health and Human Services, 2024b). Of substance use disorders, alcohol use disorder (AUD) is the most common, affecting 28.9 million or 10.2% of people in the U.S. in 2023 (U.S. Department of Health and Human Services, 2024b). AUD, as described within the DSM-V, is defined as "problematic pattern of alcohol use leading to clinically significant impairment or distress" and contains criteria which include an inability to curb or control amount of alcohol consumption, tolerance, withdrawal, and disruption of social, work, or school activities (*Diagnostic and statistical manual of mental disorders*, 2013).

One concern is that many with AUD do not seek treatment. The rate at which those with AUD or alcohol dependence seek treatment has drastically reduced within recent years, reducing from a peak of 37% in 2001-2003, to rates of less than 25% as of 2023 (U.S. Department of Health and Human Services, 2024b; Venegas et al., 2021). Interestingly, only around 2% of people with AUD had medication assisted treatment in recent years (2022-2023), which may be reflective of the lack of clinical efficacy for current treatments (U.S. Department of Health and Human Services, 2024a). Issues such as treatment access or stigma are commonly cited as reasons for why people do not seek treatment (Venegas et al., 2021). For people who do seek treatment, many treatment programs do not prescribe medication due to factors such as lacking sufficient physicians and staff and legal liability (Abraham et al., 2011; Kranzler, 2023; Roman

et al., 2011). However, medication-assisted treatment has been supported and recommended by professional societies and governmental agencies as a first-line treatment (Abraham et al., 2011; Kranzler, 2023; Roman et al., 2011).

There are currently three main FDA-approved drugs for AUD: Naltrexone, Acamprosate, and Disulfiram (Kranzler, 2023; Mar et al., 2023). Naltrexone is an opioid receptor antagonist, that can result in reduced craving and reduced heavy alcohol consumption with some mixed results for the extended release-injectable format (Kranzler, 2023; Kranzler et al., 2004). There is an oral and extended release format of the medication with more studies having been completed on the oral format to support its efficacy (Jonas et al., 2014). For the extended release injection, one study has indicated that higher doses of Naltrexone may be significantly more effective, but there are more adverse reactions (Garbutt et al., 2005). Acamprosate is an NMDA antagonist and glutamate metabotropic receptor 5 inhibitor which targets dysregulated excessive glutamatergic signaling induced by AUD (Kranzler, 2023; Plosker, 2015).Acamprosate has also been shown to reduce alcohol consumption, but some studies have found no significant effects (Kranzler, 2023; Mann et al., 2009; Morley et al., 2006). Disulfiram blocks the enzyme aldehyde dehydrogenase from breaking down the toxic byproduct of alcohol consumption acetaldehyde, which is responsible for negative symptoms associated with hangovers (Kranzler, 2023; Mar et al., 2023). This drug has also had mixed results, but a metanalysis found that the significant effects were only observable in cases where the study was open-label compared to blinded studies (Jonas et al., 2014; Skinner et al., 2014). Furthermore, one issue of this drug is that it does not impact cravings and functions to increase sickness effects only under the condition that the person consumes alcohol. Thus, compliance can be a larger issue with this drug than the others, as a

person can simply stop taking this drug to prevent the negative effects if they plan to consume alcohol (Mar et al., 2023; Skinner et al., 2014).

Importantly, it is necessary to consider treatment effectiveness on an individual level. Large percentages of people with AUD do relapse even with medicinal treatment. Although naltrexone and acamprosate have been shown to significantly increase abstinence, meta-analyses have found that 40-70% of people receiving acamprosate or naltrexone reported no measurable positive benefits (Jonas et al., 2014; Lohoff, 2022). One metanalysis has considered subgroups such as age, sex, ethnic background, and comorbid conditions, and it found that naltrexone and acamprosate worked relatively similarly between groups (Jonas et al., 2014). However, more research into additional factors such as genetic polymorphisms may yield better results. For example, some studies have found that polymorphisms in the OPMR1 gene, a gene which encodes the mu opioid receptor, may contribute to naltrexone effectiveness in this population (Anton et al., 2008; Kranzler et al., 2012; Oslin et al., 2003). Through research into additional factors and genetic polymorphisms, additional treatment options which could be personalized would likely improve treatment outcomes.

1.1.2 Major depressive disorder as a comorbid disorder of AUD

AUD is commonly comorbid with other conditions such as major depressive disorders, anxiety disorders, and post-traumatic stress disorder. However, major depressive disorder (MDD) which is the most common comorbid disorder of patients with AUD where approximately 1/3 of patients presenting with AUD will meet criteria for MDD (McHugh & Weiss, 2019). Interestingly, development of MDD or AUD have been shown to predict future development of the other disorder (Briere et al., 2014). Specifically, adolescent AUD predicted development of MDD in early adulthood, and early adult MDD predicted development of AUD

in adulthood (Briere et al., 2014). Given this relationship, a better understanding of the underlying causes and shared mechanisms of these disorders is critical. Notably, mechanisms involved in stress and inflammation which are discussed below may contribute to both of these disorders.

Like AUD, MDD affects millions of people in the U.S. In 2023, 21.9 million people over the age of 18 or 8.5 % of the U.S. population experienced a major depressive episode (U.S. Department of Health and Human Services, 2024b). MDD is defined by the DSM-5 as having five symptoms that represent a depressed mood or anhedonia for a period of at least two weeks (*Diagnostic and statistical manual of mental disorders*, 2013). These symptoms include feeling sad or helpless, suicidal ideation, lack of enjoyment in typical activities, changes in sleep, changes in weight, changes in psychomotor activity, and an inability to focus (*Diagnostic and statistical manual of mental disorders*, 2013). Historically, women have been found to be twice as likely to have MDD than men (Williams et al., 2022). Aligning with these previous statistics, approximately 10.5% of women and 6.3% of men experienced a major depressive episode in 2023 (U.S. Department of Health and Human Services, 2024a). In regards to treatment, approximately one third of people with MDD have treatment-resistant depression, typically defined as depression which does not respond to at least two different classes of antidepressants (Pandarakalam, 2018). This suggests that research into additional therapeutic targets may provide more beneficial options.

1.2 Role of Stress

1.2.1 Role of stress in alcohol consumption and depression

Stress is one risk factor that may contribute to both MDD and AUD. In humans, stress is a risk factor for both developing AUD as well as relapse, and alcohol consumption may be used

as avoidant-style coping for stress (Cooper et al., 1992; Lee et al., 2018; McGrath et al., 2016; Sinha, 2001; Slopen et al., 2011). Likewise, stress has been implicated in onset of depression episodes, and adverse childhood events, a measure of early life stressors, increase the likelihood of developing MDD even when controlling for genetic predispositions (Carter & Garber, 2011; Danielsdottir et al., 2024; Slopen et al., 2011). Models of stress in rodents have been used to induce depressive-like phenotypes of anhedonia, social withdrawal, and behavioral despair (Petkovic & Chaudhury, 2022). The most common preclinical models of stress used to study both depressive-like phenotypes and alcohol consumption are discussed further below (see section 1.2.3)

1.2.2 Neurobiological mechanisms of stress

Evolutionarily, the stress response should promote survival under life-or-death circumstances. However, physical as well as psychological stressors can result in dysregulation of neural circuitry involved in stress response as well as the limbic system and regions heavily involved in reward processing (Godoy et al., 2018). Dysregulation of this circuitry is believed to contribute to many conditions such as those described above. Following recognition of a stressor, several regions are activated, resulting in the formation of two stress responses, the sympathetic adreno-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis.

The SAM response, the quicker response to stress, enacts the sympathetic stress response (Godoy et al., 2018). The paraventricular nucleus of the hypothalamus (PVN), locus coeruleus (LC), and rostral ventrolateral medulla synapse onto preganglionic neurons within the spinal cord which synapse onto sympathetic postganglionic neurons or the adrenal medulla (Godoy et al., 2018). The SAM axis activates these circuits which result in the norepinephrine (NE) and epinephrine release from the adrenal medulla and acts on organs locally through the release of

NE from sympathetic nerves. This mechanism is primarily responsible for the 'fight or flight' response observed following a stressor. Within the brain, the LC is the predominant producer of NE, which it releases in several regions throughout the brain(Schwarz & Luo, 2015).

The longer mechanism of the hypothalamic-pituitary-adrenal (HPA) axis occurs due to a longer time for circulating hormones to act (Godoy et al., 2018). Activation of the PVN neurons which express CRH initiates the HPA axis, where CRH induces release of adrenocorticotropin hormone (ACTH) from the anterior pituitary gland (Godoy et al., 2018). ACTH is a hormone that acts within the adrenal gland to release cortisol in humans or corticosterone in mice. Cortisol also stimulates autonomic function like the effects of the SAM axis, including increased heart rate and blood pressure, but it also inhibits immune response and interacts with energy storage (Godoy et al., 2018; Knezevic et al., 2023).Cortisol also contributes to a negative feedback loop where it inhibits ACTH release from the anterior pituitary gland and CRH release from the PVN (Godoy et al., 2018). In this way, the body can resolve the stress response.

Importantly, there is crosstalk between the HPA and SAM axis were the PVN releases corticotropin releasing hormone (CRH) in the LC, and the LC releases NE in the PVN (Godoy et al., 2018; Hwang et al., 1998). This cross-talk encourages co-activation of the SAM axis and HPA axis as CRH activates neurons within the LC and the NE can activate neurons within the PVN through α1- adrenergic receptors (α1-AR) (Godoy et al., 2018; Hwang et al., 1998; Milanick et al., 2019).

Chronic stress can influence HPA axis activity and this negative feedback loop. Chronic stress has been shown to induce hyperactivity of the HPA axis which has been observed in many patients with MDD (Yang, 2015). Chronic stress has also been shown to dysregulate glucocorticoid receptors (GR) which are important in inhibiting CRH release from the PVN and

ACTH release from the pituitary gland (Han et al., 2017; Herman et al., 2016). This downregulation of GR expression as well as impaired nuclear translocation of the GR following chronic stress results in glucocorticoid resistance or a lack of response even to the high levels of cortisol (Herman et al., 2016; Tong et al., 2023). Chronic stressors in rodents such as social defeat stress have shown that increased HPA axis dysfunction, measured by continued increased corticosterone, increased CRH gene expression and reduced GR protein expression within the hypothalamus for four weeks following cessation of the stress only in the stress-susceptible mice (Han et al., 2017). This demonstrates that stress susceptibility may be reflective of a hyperactivated HPA axis with less negative feedback.

Importantly, both SAM and HPA axis mechanisms interact with several brain regions such as the prefrontal cortex, ventral striatum, and amygdala (Godoy et al., 2018). Dysregulated stress mechanisms can then dysregulate the reward system which may explain symptoms such as anhedonia as well as dopamine-seeking behaviors observed in MDD or AUD (McEwen, 2012; Yang, 2015). Meanwhile, stress can also induce inflammation which is another contributing factor discussed below.

1.2.3 Preclinical models of stress

Several models of stress in rodents have been developed to better understand stress mechanisms. The most common stress models in mice include chronic unpredictable mild stress (CUMS), social defeat stress (SDS), neonatal stress, and restraint stress. Although variations of these models and several others are also used as forms of acute stressors, acute stress does not typically induce the depressive-like phenotypes unless the mice are stress susceptible or have previously underwent a chronic stressor. The chronic variations of these stressors are discussed below in their ability to induce behavioral deficits and impact alcohol consumption.

CUMS involves a variety of different stressors in order to replicate the unpredictability of stress, and protocols take from 1-7 weeks typically (Petkovic & Chaudhury, 2022). Stressors used for CUMS include alteration of light/dark cycle, tilting cage, soiled bedding, and other stressors that are used as single stressors in other models such as restraint stress, foot shock, and forced swimming (Antoniuk et al., 2019). CUMS has been shown to impact a variety of depressive-like behavioral phenotypes such as anhedonia in the sucrose preference test, behavioral despair in the tail suspension test, social withdrawal in the social interaction test and anxiety-like phenotypes in the open field test and the elevated plus maze (Petkovic & Chaudhury, 2022; Sharma et al., 2024). Importantly, CUMS-induced behavioral phenotypes have been shown to last for several weeks (Sharma et al., 2024). 7 weeks of CUMS induced increased alcohol consumption in male but not female mice in one study (Quadir et al., 2019).

Social defeat stress (SDS) involves placing a mouse subject in a cage with a larger, territorially aggressive mouse which will attack the mouse subject for a short time period (typically 5-10 minutes) each day for 10+ days and housing the mouse subject across from the aggressor overnight (Golden et al., 2011). Like CUMS, SDS also induces durable behavioral effects where some effects such as reduced social interaction has been observed for 4 weeks following the last defeat (Golden et al., 2011; Pagliusi & Sartori, 2019; Petkovic & Chaudhury, 2022). Utilizing the social interaction ratio in order to divide the mice into susceptible and resilient is effective in determining different molecular differences, and susceptible mice also show other depressive-like phenotypes of anhedonia and increased immobility in the tail suspension test (Krishnan et al., 2007; Petkovic & Chaudhury, 2022). Although many researchers utilize SDS due to it being a natural stressor to mice, high face and construct validity, and consistent induction of depressive-like behaviors, this traditional form of SDS is largely

ineffective in female mice as aggressors will not defeat female mice under typical circumstances (Nestler & Russo, 2024; Petkovic & Chaudhury, 2022). Chronic SDS has been shown to significantly increase both continuous and intermittent alcohol consumption in male mice(Newman et al., 2018). This effect has been shown to last for 4 weeks (Albrecht et al., 2013; Newman et al., 2018).

Neonatal stressors include maternal separation and maternal deprivation such as limited bedding, and several variations of protocols exist (George et al., 2010; Mroue-Ruiz et al., 2024). Neonatal stressors occur during the first few weeks following birth to model early life stress (Petkovic & Chaudhury, 2022). Models of maternal separation can induce behavioral and molecular changes, but there have been extremely mixed and inconsistent results (Tractenberg et al., 2016). Although these protocols can induce depressive-like and anxiety-like phenotypes, some studies have found that these models reduce these phenotypes (Petkovic & Chaudhury, 2022; Tractenberg et al., 2016). Age, timing, and duration of these neonatal stress protocol variations can drastically influence the outcome of stress susceptibility or stress resilience (Petkovic & Chaudhury, 2022). Another concern with these models is that some studies have found it to have much greater effect in inducing depressive-like phenotypes in male rodents compared to female rodents which may be due to differences in development or due to a confound of maternal care where dams typically offer more care to male pups (Orso et al., 2019; Walker & Glasper, 2025).Similarly in alcohol use, maternal separation has only been shown to increase alcohol consumption and preference in male mice and rats who had underwent maternal separation but not in female mice or rats (Gustafsson & Nylander, 2006; Gustafsson et al., 2005; Roman & Nylander, 2009; Roman et al., 2004; Talani et al., 2025). Similarly, limited bedding

and nesting models of early life stress have found that it can increase alcohol consumption in male but not female mice (Okhuarobo et al., 2020).

For chronic restraint stress, mice are physically restrained in tubes or containers for 2-8 hours daily, most commonly for 1-4 weeks (Mao et al., 2022). However, inducing behavioral deficits of anhedonia and social withdrawal typically require a protocol of at least 2 weeks although some studies have found it to take up to 4 weeks (Mao et al., 2022; Petkovic & Chaudhury, 2022). Concurrent restraint stress and alcohol consumption can increase alcohol consumption in male rats compared to non-stressed counterparts (Gomez et al., 2012). However, alcohol consumption administered following stress cessation only significantly increased consumption during the 2nd session of alcohol access in adolescent male rats but not in adult male rats (Fernández et al., 2016). This short duration of this influence on alcohol consumption is similar to other behavioral studies which have found that this stressor does not induce depressive-like effects for long durations following cessation of stress as observed with the CUMS, neonatal stressors, and SDS models (Petkovic & Chaudhury, 2022).

1.3 Role of Inflammation

1.3.1 Inflammatory mechanisms and relation to stress

The main mechanisms of inflammation discussed below are through toll-like receptor signaling pathways. Toll-like receptors (TLRs) are located on several cell types including neurons, microglia, and several immune cell types, and make up a subset of pattern recognition receptors which are activated by individual pathogen-associated materials (Duan et al., 2022; Fitzgerald & Kagan, 2020; Jackson Hoffman et al., 2023). The TLR signaling pathway induces many transcription factors. Importantly, the majority of TLRs, specifically those discussed here, activate nuclear factor kappa B (NFkB) which is responsible for increased transcription of

proinflammatory cytokines(Duan et al., 2022). Particularly, NFkB induces production of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) which may be involved in driving symptoms of AUD and MDD (see section 1.3.2 and 1.3.3).

The main purpose of proinflammatory cytokines is to activate lymphocytes and endothelial cells and to act with the innate immune system in recruiting immune cells to target and clear the pathogen and debris (Wang et al., 2024). Interestingly, microglia, which act as the resident macrophages of the brain, can be activated by several cytokines, including Interleukin-1β (IL-1β), TNFα, and IL-6 (Kuno et al., 2005; Lee et al., 2023; Mendiola & Cardona, 2017). Furthermore, proinflammatory cytokines can directly activate the HPA axis locally in the PVN or anterior pituitary to increase CRH and ACTH release (Silverman et al., 2005). Proinflammatory cytokines can also increase NE release from sympathetic nerves as well as increase release of NE into the PVN (Pongratz & Straub, 2014; Silverman et al., 2005). In these ways, inflammatory mechanisms can influence stress systems.

Additionally, inflammation can be a consequence of stress. Acute stress has been shown to increase NFkB activation in both humans and mice (Koo et al., 2010; Wolf et al., 2009). Consequently, acute stressors have been shown to increase the production of proinflammatory cytokines (Godoy et al., 2018; Speakman et al., 2023). One direct mechanism which induces neuroinflammation is NE release from the locus coeruleus during the SAM axis activation which can activate microglia and promote the production of proinflammatory cytokines (Blandino et al., 2006; Johnson et al., 2005; Schramm & Waisman, 2022). Increased microglial activation is observed consistently across several brain regions such as the hippocampus (HPC), prefrontal cortex (PFC), and the nucleus accumbens (NAC), following both early life and adulthood stressors and in different stress models in rodents (Calcia et al., 2016).

Chronic stress can dysregulate inflammatory mechanisms, and elevated proinflammatory cytokines can further dysregulate stress responses (Alotiby, 2024; Mckay & Cidlowski, 1999; Raison et al., 2006). Chronic stress leads to dysregulation of these immune responses, resulting in chronic inflammation (Alotiby, 2024). This is despite cortisol having anti-inflammatory functions through the GR which inhibits NFkB (Mckay & Cidlowski, 1999). Chronic stress and excessive cortisol release can lead to glucocorticoid resistance and loss of anti-inflammatory function (Cohen et al., 2012; Walsh et al., 2021). Additionally, proinflammatory cytokines can also prevent GR translocation and thus prevents its ability to provide negative feedback to the HPA axis in the PVN or anterior pituitary (Mckay & Cidlowski, 1999; Raison et al., 2006; Wang et al., 2003).

In these ways, inflammatory mechanisms may be both a subcategory of stress-response and a direct influence on stress. However, discussed below, inflammatory mechanisms are discussed independently as how they influence and correlate to symptoms and behaviors of AUD and MDD.

1.3.2 Inflammatory mechanisms related to AUD

Many inflammatory effects of alcohol consumption occur through TLR-activation, predominantly TLR4, and chronic alcohol consumption has been found to significantly increase expression of several of the TLRs (Crews et al., 2017; Crews et al., 2013; Fernandez-Lizarbe et al., 2009; Flores-Bastias & Karahanian, 2018). Subsequently, proinflammatory cytokine expression is also elevated following chronic alcohol consumption both in humans and rodents (Baxter-Potter et al., 2017; Crews et al., 2017; Cruz et al., 2023; Garcia-Marchena et al., 2020; Moura et al., 2022; Walter & Crews, 2017). Interestingly, these elevated proinflammatory markers are still elevated in individuals abstinent for at least 4 weeks with history of alcohol use

disorder, indicating that AUD-induced alcohol consumption may induce chronic inflammation following cessation (Garcia-Marchena et al., 2020). Altogether, these findings indicate inflammation can be a consequence of alcohol consumption.

In rodents, modulation of these mechanisms can have bidirectional influence on alcohol consumption. For example, TLR4 inhibition reduced alcohol consumption in male mice (Bajo et al., 2016). TLR3 knockdown within the dorsal striatum of male mice reduced alcohol consumption (Dilly et al., 2024). Interleukin-1 receptor antagonist treatment reduced alcohol consumption (Lowe et al., 2020). IL-6 knockout mice have reduced alcohol preference under 24 hour access compared to control counterparts although no effects were observed in drinking in the dark paradigm or in differences in alcohol preference (Blednov et al., 2012). Meanwhile, Blednov et al. (2011) demonstrated that two administrations of Lipopolysaccharide, which activates TLR4, significantly increased alcohol preference in male and female mice. Activation of TLR3 and TLR7 have also been able to increase alcohol consumption depending on various factors such as sex, dose, and dosing regimen (Grantham et al., 2020; Lovelock et al., 2022; Warden et al., 2019a, 2019b). In this manner, decreasing or increasing inflammatory mechanisms may reduce or drive alcohol consumption.

In humans, this inflammation may contribute to symptoms of AUD, though much more data is required. Changes in serum proinflammatory cytokine IL-6 expression to alcohol cue correlated with alcohol cravings and predicted future alcohol consumption in individuals who binge drink compared to social drinkers (Blaine et al., 2023). This study demonstrated that an alcohol cue can induce increased IL6 production, meaning that production of these proinflammatory cytokines may not only be a response to alcohol but a byproduct of other signaling mechanisms involved in alcohol seeking or craving. Furthermore, this suggests that

ongoing chronic inflammation observed in people with AUD may drive symptoms of craving and may promote relapse.

1.3.3 Inflammatory mechanisms related to MDD

Peripheral and neuroinflammation as measured by proinflammatory cytokines are increased in patients with MDD compared to control counterparts (Das et al., 2021; Elgellaie et al., 2023; Kohler et al., 2017; Liu et al., 2012). TLRs are also elevated in people with MDD (Hung et al., 2016; Hung et al., 2014; Keri et al., 2014). Specifically, proinflammatory cytokines such as IL-1β and TNFα measures have significantly correlated with measures of depression symptoms (Alcocer-Gomez et al., 2014; Elgellaie et al., 2023; Petersen et al., 2023). Meanwhile, reduction in symptoms following treatment has been associated with normalized measures of peripheral inflammation as well as toll-like receptor expression compared to control subjects (Beurel et al., 2020; Keri et al., 2014). IL-1β and TNFα are predictive of treatment resistant depression where elevated levels increased likelihood of treatment resistant depression (Benedetti et al., 2021). In people with elevated inflammatory markers with treatment resistant depression, administration of a TNFα antibody significantly improved symptoms compared to placebo (Raison et al., 2013). This suggests that treatment resistance may be due to an inability to reduce chronic inflammation which may contribute to their symptoms.

Conversely, inducing an inflammatory response has been shown to induce depressive-like symptoms in both humans and rodents (Eisenberger et al., 2010; Mello et al., 2018; Pitychoutis et al., 2009; Sens et al., 2017). In rodents, models of inflammation through activation of TLRs such as TLR4 have been particularly effective in inducing depressive-like phenotypes such as anhedonia (Bluthé et al., 1999; Bluthé et al., 1992; Bluthé et al., 2000; Mello et al., 2018; Pitychoutis et al., 2009; Sens et al., 2017). Similarly, activation of TLR4 in healthy humans

corresponds to significantly reduced ventral striatum activity in response to reward and decreased self-reported mood (Eisenberger et al., 2010). Administration of proinflammatory cytokines has been shown to induce depressive-like phenotypes in mice (Budni et al., 2021; Dunn & Swiergiel, 2005; Kaster et al., 2012; Koo & Duman, 2008; Manosso et al., 2013). This data suggests that immune responses and proinflammatory cytokines may have a causal relationship with some symptoms of depression.

On the other hand, inhibition or blocking these mechanisms may reduce some symptoms of depression or depressive-like behavior. For example, TLR4 knockout or pharmacological inhibition has been found to reduce symptoms of behavioral despair and anhedonia in mice (Cheng et al., 2016; Zhang et al., 2020). Inhibition of the proinflammatory cytokine signaling via antagonism of the TNFα receptor, administration of cytokine antibodies, or cytokine receptor knockout can also significantly reduce depressive-like phenotypes (Chourbaji et al., 2006; Kaster et al., 2012; Kong et al., 2015; Singhal et al., 2021). As discussed above, TNFα antagonism in patients with treatment resistant depression significantly reduced depression symptoms (Raison et al., 2013). Altogether, these findings demonstrate a bidirectional control of depressive-like phenotypes in humans and rodents through modulation of inflammatory mechanisms.

1.4 Neuroanatomical mediators of stress and inflammation

Stress and inflammation have been shown to impact various regions. As described above, it may be important to consider how stress and inflammation can induce physiological changes which may also drive symptoms of MDD and AUD. The following section highlights regions which are studied in subsequent chapters of the dissertation to detail some important neuroanatomical and neurophysiological mechanisms that are influenced by stress and inflammation.

Hippocampus

The hippocampus (HPC) is a region involved in cognition and memory and is associated with MDD and AUD (Anand & Dhikav, 2012; Griffin et al., 2023; Miskowiak et al., 2025). Long-term potentiation or long-term depression within the HPC can influence memory, and stress has been shown to shift the region toward increased long-term depression (Liu et al., 2017). Similarly, LPS-induced inflammation increased microglial activation and production of cytokines within this region and inhibition of long term potentiation (Hauss-Wegrzyniak et al., 1998; Hauss-Wegrzyniak et al., 2002; Rosi et al., 2003). As the HPC can have an inhibitory effect on the CRH neurons of the PVN, this suggests that inflammation within this region could result in disinhibition of the PVN neurons and the HPA axis (Cole et al., 2022). Stress and inflammation within this region can impair neurogenesis (Idunkova et al., 2023; Liu et al., 2017). Neurogenesis in the HPC was reduced in human subjects with MDD, but subjects currently on antidepressants had significantly increased neurogenesis (Boldrini et al., 2019; Boldrini et al., 2013). Alcohol use has been known to have detrimental effects on this region including neuronal loss, reduced volume, and reduced activity observed in people with alcohol dependence or AUD (Bengocheal & Gonzalo, 1990; Miskowiak et al., 2025; Suzuki et al., 2010).

Prefrontal Cortex

Unlike other regions such as the HPC, the PFC is particularly sensitive to stressors, requiring only acute stressors to induce marked changes such as significant loss of dendritic spines (Arnsten, 2009). Excess norepinephrine signaling through β-adrenergic receptors (β-AR) on pyramidal cells in this region impaired regulation of ion channels and subsequently resulted in reduced irregular action potential firing patterns (Arnsten, 2009). Dopaminergic release within the PFC is increased by local GR activation through corticosterone (Butts et al., 2011). This

demonstrates that stress can dysregulate the excitatory and inhibitory balance within the region through multiple mechanisms.

In regards to AUD, the prefrontal cortex (PFC) is involved in alcohol seeking, compulsive drinking, extinction and cue-induced consumption (Klenowski, 2018; Pahng et al., 2017). This region is known to have top-down inhibitory influence on alcohol consumption through inhibition of regions such as the NAC (Abernathy et al., 2010). Importantly, CRH within this region may be important in driving alcohol consumption. Aversion resistance, a type of compulsive-like drinking, in female mice was reduced by antagonizing the CRH receptor 1 in the medial PFC (Arnold et al., 2024). Furthermore, dysregulation of activity between subregions of the PFC and decreased functional connectivity with the ventral striatum have also been implicated in MDD (Felger et al., 2016; Pizzagalli & Roberts, 2022). Markers of inflammation and apoptosis were upregulated in patients with MDD in several subregions of the PFC (Shelton et al., 2011; Shinko et al., 2020). Altogether, these findings promote the PFC as a region which is important for AUD and MDD and can greatly be influenced by stress mechanisms and inflammation.

Ventral Striatum

The ventral striatum (VS) is involved in reward processing and reward-seeking behavior (Collins & Saunders, 2020). This region also receives major dopaminergic innervation which is significantly altered in response to stress or inflammation. In humans, daily life stress can reduce dopaminergic neurotransmission to the ventral striatum (Kasanova et al., 2018). Reduced functionality of this system is also seen in individuals with AUD (Heinz et al., 2004; Spitta et al., 2023). As discussed above (see section 1.3.3), inducing systemic inflammation in healthy humans can also reduce functional connectivity between the ventral tegmental area and the VS

and result in symptoms of anhedonia (Eisenberger et al., 2010). Regionally, microglia activation can reduce activation of medium spiny neurons in mice and NE administration to this region reduced excitatory post-synaptic currents specifically through the α2-AR (Klawonn et al., 2021; Kombian et al., 2006). In this way inflammatory and stress mechanisms may reduce activation of this region as seen in AUD and MDD. Meanwhile, deep brain stimulation of this region has been shown to improve cravings of AUD and symptoms of MDD (Bewernick et al., 2010; Ho et al., 2018).

Amygdala

The amygdala is involved in stress, fear response, and fear memory. The amygdala can become hyperactive due to stressors, and patients with post-traumatic stress disorders have been observed to have significantly increased activation of this region (Zhang et al., 2018). In rodents, knockdown of the GR in the central amygdala nuclei reduced fear response and knockdown in the basolateral amygdala reduced fear memory, demonstrating a role of the HPA axis in this region (Cuccovia et al., 2022; Wiktorowska et al., 2021). Furthermore, blocking α1-ARs in non-dependent rats or β-AR in dependent rats within the central amygdala decreased alcohol consumption, implicating the SAM axis and NE as direct regional mechanism that stress may influence alcohol consumption (Varodayan et al., 2022). Meanwhile, LPS administration in healthy human participants demonstrated that inflammation induced increased amygdala response to socially threatening images compared to placebo-treated participants (Inagaki et al., 2012). Altogether, these findings suggest that stress and inflammation can contribute to hyperactivity within the amygdala.

1.5 Known sex differences in stress and inflammatory mechanisms

1.5.1 Sex differences in prevalence and perception of stress

Interestingly, many studies support that women have increased susceptibility to stress-induced depression and alcohol use. Women are twice more likely to develop MDD and are likely to progress to AUD quicker than men (Randall et al., 1999; Williams et al., 2022). Women experiencing a depressive episode were more likely to have underwent a significant life stressor than men, indicating that stress may be more causal of depressive episodes in women (Sherrill et al., 1997). This is despite there being no significant difference in stressors between women and men as a whole in this study (Sherrill et al., 1997). Another study found that stressful life events were predictive of first onset of alcohol dependence and MDD for both sexes with no interaction between sex and stress (Slopen et al., 2011). However, other studies do suggest that women face greater number or severity of significant stressors or perceived stress compared to men, which may also contribute to this increased likelihood of developing these stress-related psychiatric conditions (Davis et al., 1999; Lutin et al., 2023; "Stress in America 2023: A nation recovering from collective trauma," 2023). Therefore, stress can be a significant contributing factor for both AUD and MDD in both sexes.

1.5.2 Sex differences in the neurobiological mechanisms of stress

Many significant sex differences exist within both the HPA axis as well as within the SAM axis. Within the HPA axis, there are sex differences in CRH neurons and receptor function. For example, females have greater CRH-expressing cells within the PVN (Handa et al., 2022). Interestingly, the CRH release from cells can be modulated by estrogen (Qi et al., 2020). Thus, estrous cycle and hormones may greatly influence HPA axis activation. Additionally, CRH receptors have increased expression in several brain regions in female mice (Bangasser &

Wiersielis, 2018; Handa et al., 2022). Furthermore, CRH receptor activation is biased more to G-protein activation in females while males have greater bias towards β-arrestin signaling, resulting in females having increased sensitivity to CRH activation compared to male mice (Bangasser & Wiersielis, 2018). The hormonal influence of cortisol also has decreased effect and has less negative feedback in female compared to males due to significantly less GR expression throughout several brain regions, importantly including the PVN (Handa et al., 2022). Altogether, these findings may explain why females have heightened HPA axis response to males under various stress conditions (Handa et al., 2022).

Meanwhile, sex differences within the SAM axis may also contribute to observed sex differences. For example, the LC, which supplies the majority of the NE release to the cortex, HPC, and forebrain, is larger in female rodents, and female rodents have a greater number of these norepinephrine-expressing cells in the LC (Bangasser et al., 2016). Cycling females have reduced α2-AR binding due to estrogen while greater β1-AR binding was observed in males or ovariectomized females (Bangasser et al., 2016; Karkanias et al., 1997; Wagner & Davies, 1980). Estrogen increases synthesis and decreases degradation of NE (Bangasser et al., 2016). The involvement of sex hormones in these systems may explain why stress-induced activation in several brain regions is greatly modulated by the hormonal cycle in women (Goldstein et al., 2010).

1.5.3 Sex differences in the neurobiological mechanisms of inflammation

Meanwhile, many sex differences have been observed within the immune response which may contribute to increased inflammation observed and increased rates of autoimmune disorders in humans (Klein & Flanagan, 2016). Several genes which encode important elements in immune response are on the X-chromosome, of which, TLR 7 has been shown to have increased

expression due to being able to escape X-inactivation in females (Klein & Flanagan, 2016). Other markers of inflammation, such as IL-1β and its upstream signaling molecules, are upregulated in female rodents (Cyr & de Rivero Vaccari, 2023). Stimulation of various TLRs have demonstrated that females have a greater or more prolonged response as measured by changes in inflammatory pathways, increased peripheral cytokine expression, or increased gene expression of cytokines within brain regions (Dockman et al., 2022; Klein & Flanagan, 2016; Sharma et al., 2018). Thus, observed elevated baseline inflammation as well as increased inflammatory responses may contribute to sex differences observed in AUD and MDD.

1.6 Substance P and Neurokinin-1 receptor system

The following dissertation focuses on substance P (SP) and its corresponding receptor, the neurokinin 1 receptor (NK1R). SP and the NK1R is a system involved both in stress and inflammatory responses. SP is a neuropeptide that is widely expressed within the central nervous system (Ebner & Singewald, 2006; Schank & Heilig, 2017). Tac1 (gene that encodes SP) knockout mice have been shown to have decreased depressive-like and stress-associated behavior such as increased social interaction, increased time in the center of the open field test, and decreased posturing behaviors such as stretches and rearing (Bilkei-Gorzo et al., 2002). Within the brain, several regions have been found to have increased SP release in response to stress, such as the medial amygdala, lateral septum, and the nucleus accumbens (Berton et al., 2007; Ebner et al., 2004; Ebner, Singewald, et al., 2008; Ebner & Singewald, 2006). These are regions associated with reward and affective behavior.

Furthermore, the NK1R is also widely distributed throughout the brain, particularly in regions which have been associated with stress(Ebner, Muigg, et al., 2008; Ebner & Singewald, 2006). The NK1R is a Gq-coupled receptor, but can also trigger activation of NFkB(Douglas &

Leeman, 2011). In this manner, this signaling can contribute to increased inflammation via increased transcription of proinflammatory cytokines(Douglas & Leeman, 2011; Li et al., 2022; Mashaghi et al., 2016). SP is released in many regions following stress, but SP also directly influences the SAM and HPA axis. SP can stimulate the LC and induce NE release throughout the CNS (Ebner & Singewald, 2007; Iftikhar et al., 2020). Interestingly, SP-NK1R has been shown to reduce ACTH and HPA axis activity under non-stressed and acute stress conditions (Culman et al., 2018; Iftikhar et al., 2020; Jessop et al., 2000). However, in the periphery, SP induced cortisol release from the adrenal cortex in bovine tissue or serum expression of cortisol in human subjects (Lieb et al., 2002; Yoshida et al., 1992). For these reasons, the SP/NK1R system may be at the intersection of stress and inflammatory mechanisms which may contribute to AUD or MDD.

Blocking signaling of SP to NK1R via a NK1R antagonist has been shown to alleviate stress-induced depressive-like phenotypes and alcohol consumption in mice. Systemic or local administration of an NK1R antagonist to the nucleus accumbens (NAC) reduced escape latency as a measure of depressive-like phenotype in mice (Berton et al., 2007). NK1R antagonism has been shown to reduce stress-induced alcohol consumption in yohimbine-escalated self-administration and reduce alcohol seeking in stress-induced reinstatement in male rats (Schank et al., 2015a; Sequeira et al., 2018). Additionally, NK1R antagonism was able to reduce stress-induced reinstatement Fos activation within the NAC (Schank et al., 2015a). These data support a role of the SP-NK1R system in stress-induced alcohol consumption and depressive-like phenotypes. Furthermore, systemic administration of a NK1R antagonist has also been shown to prevent inflammation-induced anhedonia in sucrose preference in male rats (Fulenwider et al.,

2018). This suggests that in addition to preventing or reducing stress-induced phenotypes, NK1R antagonism may also prevent inflammation-induced depressive-like phenotypes in rodents.

Clinically, NK1R antagonists have demonstrated some efficacy for AUD and MDD in clinical trials. In detoxified subjects with AUD selected for high trait anxiety, treatment with NK1R antagonist decreased cravings at a baseline as well as prevented stress-induced cravings compared to vehicle-treated counterparts (George et al., 2008). This indicates that NK1R antagonist treatment for AUD may be particularly effective in people with underlying anxiety. Subjects with depression had elevated serum levels of SP compared to controls that were not decreased with treatment with anti-depressant medications after 4 weeks (Bondy et al., 2003). However, change in SP level correlated with change in HAM-D score, where a decrease in SP correlated with a decrease in depression severity. In agreement with these findings, many clinical trials showed NK1R antagonists to be effective for treatment of MDD (Kramer et al., 1998; Rupniak & Kramer, 2017). However, following failed phase III clinical trials, testing NK1R antagonists for MDD ceased (Keller et al., 2006; Rupniak & Kramer, 2017). Recent analysis determined that these failed trials may be due to low receptor occupancy and that high receptor occupancy was required for the antidepressant effects (Rupniak & Kramer, 2017; Zamuner et al., 2012).

In humans, single nucleotide polymorphisms (SNPs) of TACR1, the gene for the NK1R, have been associated with increased risk of development of AUD and alcohol dependence and with response to alcohol cues (Blaine et al., 2013; Schank & Heilig, 2017; Seneviratne et al., 2009). For SNPs associated with AUD, the Rs6715729 is a synonymous mutation located on exon 1 which is at the 5' untranslated region (UTR) (Aspden et al., 2023; Seneviratne et al., 2009). Blaine et al. (2013) found that rs3755459, rs37771863, and rs1106855 SNPs significantly

correlated with Blood Oxygen Level Dependent (BOLD) response to alcohol cues. These SNPs were associated with greater activation within the caudate, cingulate, and pallidum. rs1106855 also significantly correlated with alcohol dependence symptoms. rs1106855 is located within a stop codon within an intron which could impact amount of mRNA expressed within the cell and may subsequently impact protein expression of the receptor. Rs3755459 is located within the 3' UTR, and rs377771863 is located within the 5' UTR. SNPs in these regions have been associated with less mRNA stability (Jia et al., 2020; Mayr, 2019). Although physiological differences caused by these SNPs is unknown, the association with alcohol use disorder and response to alcohol cues indicate that differences in receptor expression or physiological properties may contribute to AUD.

Furthermore, expression of the NK1R is associated with depressive-like behavior and alcohol consumption in mice. Increased TACR1 in the NAC is associated with reduced social interaction, and following social defeat stress (SDS), TACR1 expression is increased in the nucleus in stress-susceptible mice (Nelson et al., 2018). Alcohol consumption positively correlated with TACR1 gene expression in the amygdala (Nelson et al., 2018). SDS has also been shown to significantly increase protein expression within the nucleus accumbens shell(Solomon et al., 2024). A strain of alcohol preferring rats, P-rats, have significantly elevated TACR1 gene expression within the prefrontal cortex and the amygdala (Schank et al., 2013). Additionally, intermittent ethanol access, a model of escalated alcohol consumption, significantly increased the NK1R expression within the striatum compared to the water access-only or the continuous alcohol access groups (Sequeira et al., 2018). Together, this data suggests that expression of NK1R may drive alcohol consumption, but that elevated alcohol consumption may also increase expression of the receptor.

1.7 Objectives of Dissertation

This dissertation focuses on the role of SP and the NK1R across models of stress and inflammation to expand our understanding of this system. As discussed, the majority of the previous studies have included only male subjects which drastically limit the application of the models or mechanisms to the general population.

To combat the issue of only including male mice and expand on the role of NK1R antagonists in reducing alcohol consumption in a non-stress paradigm, chapter 2 will utilize a model of inflammation to study depressive-like phenotypes and alcohol consumption in both male and female mice. LPS, a component of gram-negative bacteria which activates TLR4, has been studied to induce both sickness-like behavior as well as subsequent depressive-like behavior in mice. Past studies have found similarities between sexes in LPS-induced anhedonia-like behavior in sucrose preference and behavioral despair in the forced swim test in rodents(Mello et al., 2018; Pitychoutis et al., 2009; Sens et al., 2017). One study which looked at the forced swim test as a measure of behavioral helplessness in both sexes found LPS resulted in increased immobility(Sens et al., 2017). However, some studies have found sex differences in these behaviors(Mello et al., 2018; Millett et al., 2019; Sens et al., 2017). Importantly, one past study had utilized a two-injection LPS regimen to induce elevated alcohol consumption in both male and female mice(Blednov et al., 2011). Chapter 2 utilizes this dose and strain of LPS to test the effects of a single-injection on depressive-like behavior of social interaction and proinflammatory gene expression 24 hours after treatment and LPS-induced alcohol consumption in both male and female mice. The NK1R antagonist was additionally utilized to test its effectiveness in reducing inflammation-induced alcohol consumption. A characterization of prior knowledge along with experimental objectives of chapter 2 are depicted in Figure 1.1.

SDS is a commonly used stress paradigm which has been shown to escalate alcohol consumption in male mice. However, under typical settings an aggressor mouse will not defeat a female mouse, making it an ineffective model of stress for female rodents. An alternative paradigm or vicarious social defeat stress (VDS) has been created to better study the natural social stress in which the female mouse acts as a witness to the social defeat from across a clear, perforated barrier. This model has been successful in inducing depressive-like phenotypes and elevated cortisol in male mice, however has not been well studied in female mice(Parise et al., 2022; Sial et al., 2016; Warren et al., 2020). The objective of chapter 3 was to further validate this model of VDS in female mice to support its use as a model of stress that is effective in female rodents. The social interaction test was utilized as a measure of depressive-like behavior, followed by alcohol consumption. One cohort of mice was also sacrificed for gene expression analysis of the proinflammatory cytokine IL6 following VDS in the ventral striatum, dorsal striatum, prefrontal cortex, hippocampus and the amygdala. NK1R antagonism was utilized to determine effectiveness in reducing alcohol consumption both in this model as well as within female mice. The existing research gaps and objectives of chapter 3 are further illustrated in figure 1.2.

Finally, NK1R has been particularly studied to be involved in stress within the nucleus accumbens(Schank et al., 2015a; Solomon et al., 2024). Thus, chapter 4 builds onto this work by determining regions which project to the NAC and the role of these SP-expressing neurons in SDS-induced behavior and alcohol consumption. Utilizing methods of chemogenetics, the experiments will activate these SP inputs to the NAC or inhibit these inputs either during SDS or prior to post-stress behavioral measures. Therefore, chapter 4 will improve understanding of the

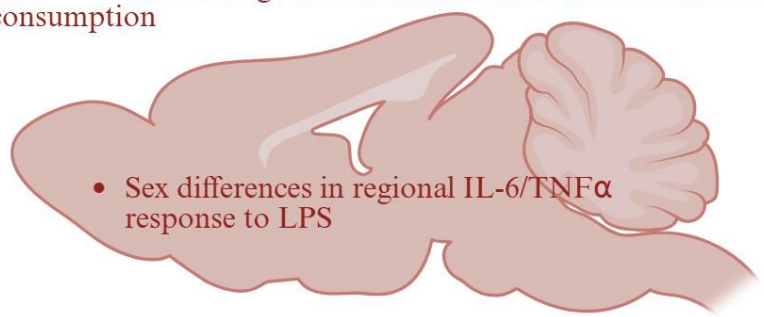
role of SP within the NAC as a stress-associated mechanism involved in alcohol consumption. Figure 1.3 depicts the current knowledge and objectives of chapter 4.

LPS: Model of inflammation/immune response

- ↑ immune response in females
- Two doses of LPS ↑alcohol in both sexes
- LPS ↑depressive-like phenotypes in both sexes

Unknown:

- Effect on SI
- Effect of NK1R antagonist on inflammation-induced alcohol consumption



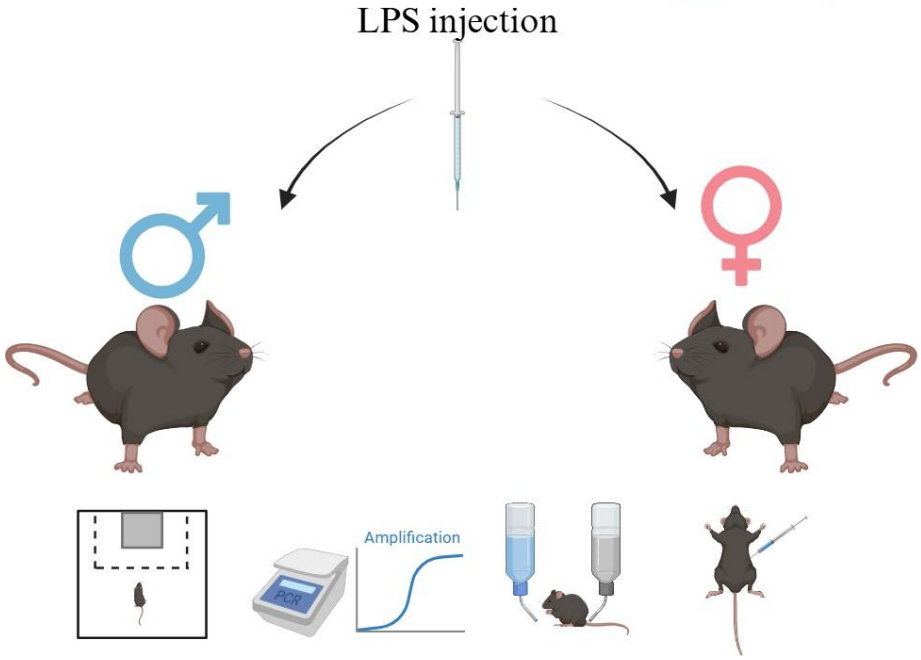


Figure 1.1. Overview of chapter 2. LPS has been used as a model of inflammation-induced depressive-like phenotypes and alcohol preference. The current study aims to directly compare sex differences across variables of social interaction (SI), regional gene expression of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα), and alcohol consumption to bridge the research gap. Furthermore, the neurokinin-1 receptor (NK1R) antagonist treatment will determine a role for NK1R in inflammation-associated alcohol consumption.

VDS: novel stress model for psychosocial stress

- ↑ depressive-like phenotypes in male mice
- IL-6 plays role in SDS and depressive-like phenotypes

Unknown:

- Effects in female mice
  - SI
  - Alcohol consumption
  - NK1R antagonist treatment on alcohol consumption
- Change in regional IL-6 gene expression

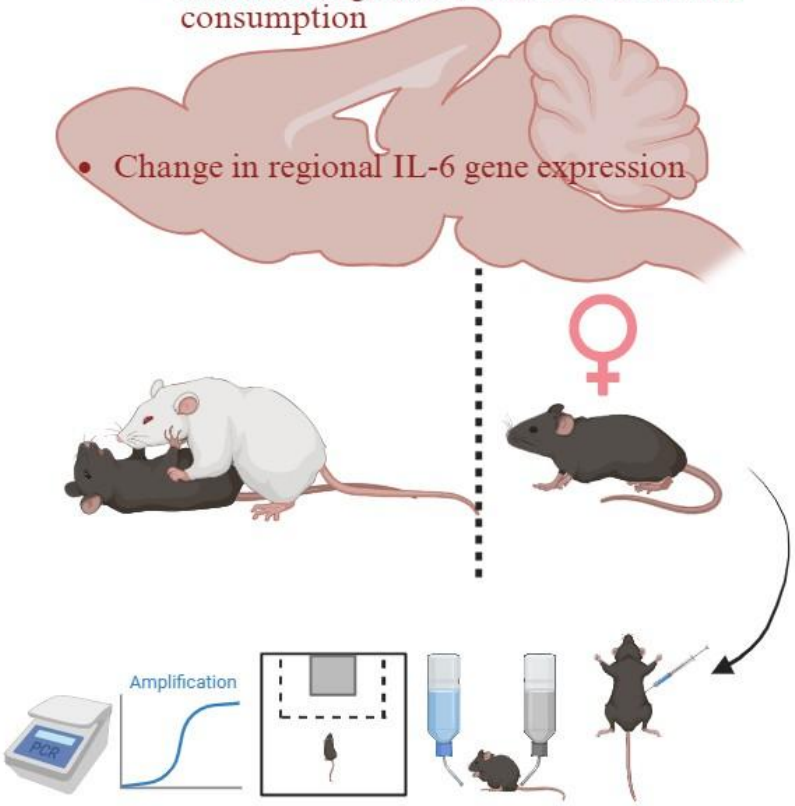


Figure 1.2 Overview of chapter 3. Vicarious social defeat stress (VDS) has been used as a model to study psychosocial stress. Past research into the social defeat stress (SDS) model of stress had found that interleukin-6 (IL-6) plays a role in SDS-induced depressive-like phenotypes. The current study aims to expand past findings of VDS-induced depressive-like phenotypes in females and determine if this model may be an effective study of stress-induced alcohol consumption. Following completion of VDS, quantitative polymerase chain reaction (qpcr) analysis of regional IL-6 expression, social interaction (SI) test, and alcohol consumption were measured in female mice. Additionally, neurokinin-1 receptor (NK1R) antagonist treatment was utilized to further test the efficacy of NK1R antagonism in females.

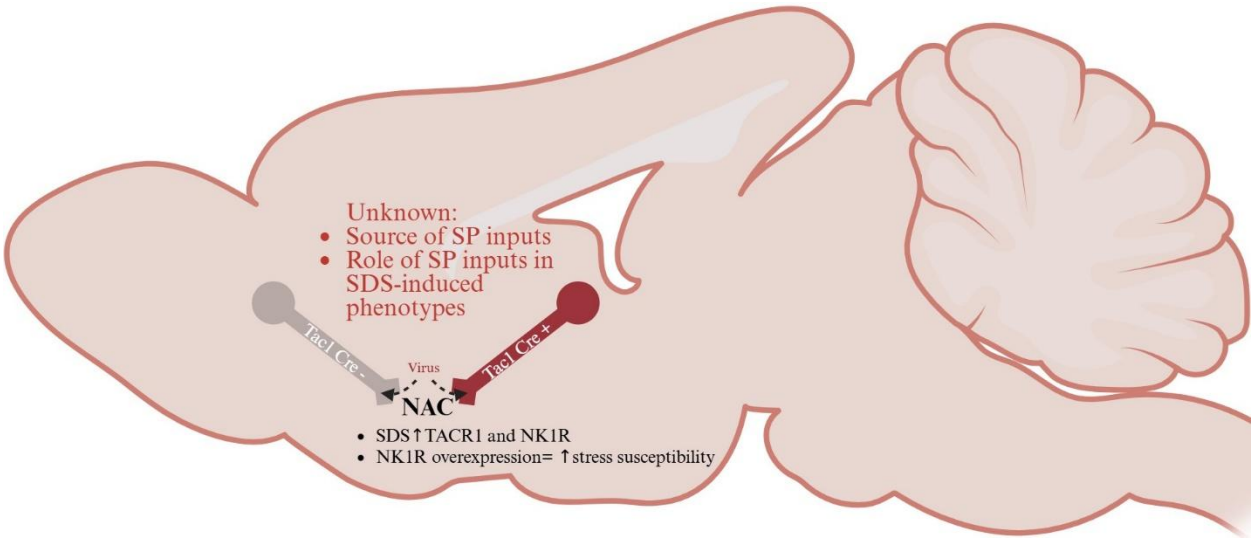

Unknown:
• Source of SP inputs
• Role of SP inputs in SDS-induced phenotypes
Tac1 Cre -
Virus
Tac1 Cre +
NAC
• SDS↑TACR1 and NK1R
• NK1R overexpression= ↑stress susceptibility

Figure 1.3 Overview of chapter 4. Social defeat stress (SDS) has been found to influence neurokinin-1 receptor (NK1R) expression in the nucleus accumbens (NAC), and NK1R expression can further induce stress susceptibility. The current source of SP is unknown. To better characterize this system, a retrograde, cre-dependent virus was infused into the NAC of Tac1 Cre mice to allow for expression of virus in substance P (SP) neurons, or Tac1 positive neurons, which project to the nucleus accumbens. Utilizing DREADD viral strategies, SP neuronal inputs to the NAC can be activated or inhibited to further determine the role of SP in stress, behaviors, and alcohol consumption.

CHAPTER 2

THE EFFECTS OF LIPOPOLYSACCHARIDE ON SOCIAL INTERACTION, CYTOKINE EXPRESSION, AND ALCOHOL CONSUMPTION IN MALE AND FEMALE MICE[1]

[1]Decker Ramirez, E. B., Arnold, M. E., McConnell, K. T., Solomon, M. G., Amico, K. N., & Schank, J. R. 2023. *Physiology and Behavior*. Reprinted here with permission of publisher.

2.1 Abstract

Much recent research has demonstrated a role of inflammatory pathways in depressive-like behavior and excess alcohol consumption. Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria that can be used to trigger a strong inflammatory response in rodents in a preclinical research setting to study the mechanisms behind this relationship. In our study, we exposed male and female mice to LPS and assessed depressive-like behavior using the social interaction (SI) test, alcohol consumption in the two-bottle choice procedure, and expression of inflammatory mediators using quantitative PCR. We found that LPS administration decreased SI in female mice but had no significant impact on male mice when assessed 24 hours after injection. LPS resulted in increased proinflammatory cytokine expression in both male and female mice; however, some aspects of the cytokine upregulation observed was greater in female mice as compared to males. A separate cohort of male and female mice underwent drinking for 12 days before receiving a saline or LPS injection, which we found to increase alcohol intake in both males and females. We have previously observed a role of the neurokinin-1 receptor (NK1R) in escalated alcohol intake, and in the inflammatory and behavioral response to LPS. The NK1R is the endogenous target of the neuropeptide SP, and this system has wide ranging roles in depression, anxiety, drug/alcohol seeking, pain, and inflammation. Thus, we administered a NK1R antagonist prior to alcohol access. This treatment reduced escalated alcohol consumption in female mice treated with LPS but did not affect drinking in males. Taken together, these results indicate that females are more sensitive to some physiological and behavioral effects of LPS administration, but that LPS escalates alcohol consumption in both sexes. Furthermore, NK1R antagonism can reduce alcohol consumption that is escalated by LPS treatment, in line with our previous findings.

2.2 Introduction

Much recent work has shown a critical role of inflammatory processes in the development of psychiatric disorders such as depression (Kim et al., 2016; Miller et al., 2009; Troubat et al., 2021). For example, higher levels of peripheral proinflammatory cytokines and elevated central nervous system inflammatory markers are observed in patients with depression and associate with depression severity (Dowlati et al., 2010; Felger, 2018; Holmes et al., 2018; Richards et al., 2018). Accordingly, drugs that reduce inflammatory signaling have shown efficacy in reducing depressive symptoms (Kappelmann et al., 2018). Preclinically, inflammatory mediators and their downstream signaling mechanisms influence the behavioral, cellular, and molecular phenotypes induced by models of depression-like behavior in rodents (Christoffel et al., 2011; Christoffel et al., 2012; Hodes et al., 2014; Koo & Duman, 2008; Koo et al., 2010).

Alcohol use disorder (AUD) is often expressed comorbidly with major depression, and approximately one third of people in treatment for AUD meet criteria for major depressive disorder (McHugh & Weiss, 2019). One longitudinal study found that AUD in adolescent participants predicted MDD in early adulthood, and MDD in early adulthood predicted later AUD (Briere et al., 2014). Interestingly, women are more likely than men to have the co-occurrence of AUD and MDD (McHugh & Weiss 2019). As with depression, inflammation plays a role in the development of AUD (Crews et al., 2017). Preclinically, the effects of chronic alcohol heighten the response of inflammatory pathways, and neuroimmune signaling systems mediate alcohol consumption (Harris & Blednov, 2013; Qin & Crews, 2012; Qin et al., 2008; Robinson et al., 2014). For example, the inflammation-associated toll like receptor 4 (TLR4) mediates alcohol responses, and manipulating downstream signaling processes of this receptor,

such as MyD88 and IKKbeta can influence alcohol consumption (Blednov et al., 2017; Truitt et al., 2016). IKKbeta is upstream regulator of a transcription factor known as nuclear factor kappa-light-chain-enhancer of activated B (NF-kB), which regulates many genes related to inflammation and addiction, and plays a role in alcohol reward (Nennig et al., 2017; Nennig & Schank, 2017).

Inflammatory processes and their role in complex behaviors are often studied in the lab by exposing animals to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria that induces a major inflammatory response and upregulation of cytokines and other inflammatory mediators. Endogenously, LPS can enter the bloodstream from the gut due to stress and binds to TLR4, resulting in an inflammatory response (de Punder & Pruimboom, 2015). Exogenous LPS administration has been shown to induce depressive-like behavior in human and rodent models (Lasselin et al., 2020). For example, rodents injected with LPS show behavioral responses such as social withdrawal and anhedonia (Bluthé et al., 1999; Bluthé et al., 1992; Bluthé et al., 2000; Frenois et al., 2007; Fulenwider et al., 2018; Haba et al., 2012; Henry et al., 2008; Jangra et al., 2014; Orlandi et al., 2015; Reis et al., 2022). Similar effects on depressive-like behaviors have been observed in human studies as well, with low dose endotoxin administration resulting in decreased ventral striatum activation to reward cues, and increased observer-rated depressed mood (Eisenberger et al., 2010). Important for our particular study is the fact that LPS exposure also induces a long lasting increase in alcohol consumption in mice (Blednov et al., 2011). Although much research suggests that TLR4 signaling is a driver in escalated alcohol consumption following the stimulation of inflammatory signaling by LPS, Harris et al. found that TLR4 signaling was more involved in the acute effects rather than a cause of excess alcohol consumption (2017). However, the experiment in this study that examined

LPS-induced escalation in alcohol intake utilized TLR4 knockout rats that self-administered alcohol in an operant task. Thus, this study differed from much of the earlier work in the species and drinking model used, which have generally studied mice in models of voluntary homecage consumption. Also, it is possible that additional signaling mechanisms are recruited either directly or indirectly by LPS to trigger escalation in voluntary alcohol intake.

Many of the studies referenced above included only male subjects. However, some studies suggest that females show greater inflammatory responses than their male counterparts. For example, females may show prolonged LPS-induced cytokine expression compared to males (Sharma et al., 2018). Increased sensitivity to LPS in females has also been observed in aged mice, with LPS resulting in greater proinflammatory cytokine levels in plasma and more significant depressive-like phenotypes compared to male mice (Dockman et al., 2022). In humans, administration of endotoxin decreased activity in the ventral striatum during reward anticipation in female but not male subjects, and cytokine levels negatively correlated with striatal activity (Moieni et al., 2019). For preclinical models of alcohol consumption, some studies have demonstrated that certain inflammatory mechanisms preferentially effect drinking in female mice. For example, manipulations of inflammatory mediators including the cytokine IL6, chemokine receptors Ccr2 and Ccl2, and the NLRP3 inflammasome influenced drinking in female mice but not male mice (Blednov et al., 2005; Harris & Blednov, 2013; Lowe et al., 2020). Taken together, these findings suggest that females may be more sensitive to the behavioral effects induced by immune stimulation.

In our previous work, we have found that both excessive alcohol intake and the neuroinflammatory response to LPS are mediated by the neurokinin-1 receptor (Fulenwider et al., 2018; Nelson et al., 2019; Nelson et al., 2017; Schank et al., 2013; Sequeira et al., 2018). The

NK1R is the primary endogenous target of the neuropeptide substance P, and this system mediates complex behaviors such as anxiety, stress responses, pain processing, and drug/alcohol seeking (Ebner & Singewald, 2006; Schank, 2014, 2020; Schank et al., 2014; Schank et al., 2012). For alcohol specifically, our group and others have shown that the NK1R mediates escalated alcohol consumption induced by multiple interventions, as well as stress-induced reinstatement of seeking to several classes of drugs (Fulenwider et al., 2019; Nelson et al., 2019; Nelson et al., 2017; Schank et al., 2014; Schank et al., 2015b; Schank et al., 2011; Schank et al., 2013; Sequeira et al., 2018). In regards to LPS effects specifically, we have shown that inhibiting the NK1R prevented the effects of LPS on sucrose preference and proinflammatory cytokine levels in the hippocampus(Fulenwider et al., 2018). In that study, LPS increased NF-kb DNA binding activity, but an NK1R antagonist pretreatment impeded this effect. This suggests that NF-kb activation is at least partially dependent on NK1R activation. Taken together, this provides strong evidence to suggest that NK1R inhibition may attenuate LPS-induced escalation in alcohol intake.

In the current study we used LPS exposure as a model to examine sex differences in inflammatory signaling, and its effect on social behavior and alcohol consumption. Additionally, we assessed the ability of a NK1R antagonist to reduce LPS-induced escalation in alcohol intake. We hypothesized that female mice would have a stronger inflammatory and behavioral response to LPS and that NK1R antagonism could attenuate LPS-induced escalation in alcohol consumption.

2.3 Methods

Animals

Male and female C57BL6/J mice arrived from Jackson Laboratories at 8-10 weeks of age and were allowed to habituate for 7 days. Mice were housed 4-5 per cage in standard rodent

cages with food and water provided ad libitum. The housing room was on a 12:12 light/dark cycle (lights off at 11:00 am). The social interaction test and bottle measurements were conducted during the dark cycle. All experiments were approved by the University of Georgia Institutional Animal Care and Use Committee. The experiments proceeded as depicted by Figure 2.1. The experiments were performed in separate cohorts of mice with one cohort used for behavioral and cytokine expression analysis (Fig. 2.1 a) and another cohort for alcohol consumption (Fig. 2.1 b). Mice in drinking experiments were individually housed, which is necessary for two bottle choice consumption models, so that intake of individual mice can be tracked.

LPS Treatment

Lipopolysaccharides from Escherichia coli O111:B4 from Sigma-Aldrich (St. Louis, MO; product #2630), was diluted in 0.9% sterile saline, then was administered via i.p. injection at a dose of 1 mg/kg and volume of 10 mL/kg. This dose was selected based on the work of Blednov and colleagues, who used this treatment dose to induce escalated alcohol consumption (2011). Mice were randomly assigned to saline or LPS treatment groups.

Social Interaction Test

The Social Interaction (SI) test was conducted during the dark cycle approximately 24 hours after LPS injection (n=16/group). The test took place in a plexiglass testing box with a perforated metal cage which allowed for sensory contact in the presence of a target mouse. The dimensions of the testing box and the marked corner and target zones followed the dimensions previously reported by our group and others (Golden et al., 2011; Nelson et al., 2017).

Each SI test consisted of a pre-test habituation period and test period with a social target mouse present. For the pre-test, the mouse was placed into the middle of the testing container to

habituate for 150 seconds with the empty cage in the target zone. The test mouse was placed in its home cage for 30 seconds to place the target mouse into the cage. Then, the test mouse is placed into the arena with the target mouse present in the enclosure for another 150 seconds. An adult C57BL6/J target mouse which corresponded to the same sex and approximate age as the test mouse was used as the social target during the test. The testing box was cleaned and disinfected in between each test. Behavioral tests were recorded and scored by an experimenter that was blind to treatment group. The dependent variable used for analysis was the amount of time spent in the interaction zone that included the social target mouse enclosure.

Quantitative Polymerase-chain reaction

To confirm neuroimmune activation by LPS, and to examine any potential sex differences in the magnitude of response to LPS administration at this dose and time point, we assessed the expression of IL6 and TNFα following LPS injection and SI testing. We selected these specific transcripts because TNFα shows some of the strongest activation in rodent brain following LPS injection in our hands (Fulenwider et al., 2018) and IL6 has been shown to play a critical role in stress-induced effects on social interaction (Hodes et al., 2014). Here, one cohort of the mice exposed to injections and SI testing were immediately sacrificed following the SI test (n=8/group) to analyze gene expression. Brain tissue punches measuring 1.5mm were taken from the ventral striatum (VS), hippocampus (HPC), and prefrontal cortex (PFC), flash frozen with isopentane, then transferred to dry ice. Samples were homogenized with a mechanical homogenizer and pestle and passed through an 18-gauge needle, then RNA extracted using the Purelink™ RNA Mini Kit (Invitrogen™) following the manufacturer's instructions. RNA concentration was measured with the Nanodrop 1000 and calculations used to ensure cDNA samples of each brain region consisted of the same concentration. RNA was synthesized into

cDNA using the Maxima First Strand cDNA synthesis kit for RT-PCR (Thermo Scientific™) according to the manufacturer's instructions. IL-6 (Mm00416190_m1) and TNFα (Mm00443258_m1) FAM-labeled TaqMan™ primers from Applied Biosystems™ were used to analyze gene expression with Gapdh (Mm99999915_g1) used as the housekeeping gene. Samples were run in triplicate and measured in a Biosystems Quantstudio 6 Flex machine. Samples for a particular brain region were excluded if they had too low of an RNA concentration as measured by Nanodrop. Additionally, four samples were removed as outliers in the measure of fold change in expression as detected by Grubb's test (one in the male saline group for TNFα in the PFC, and two from the female saline group for IL6 and TNF in the VS, and one from the female LPS group for TNF in the VS). Every group in every brain region had at least 6 samples. Data were expressed as fold change from saline treated controls and calculated using the $2^{-\Delta\Delta CT}$ method.

Two-Bottle Choice

Mice (n=8/group) were single housed with two water bottles for three days before switching one bottle out with a 20% alcohol bottle. Bottles were weighed and sides were switched daily to prevent effects of side preference. Alcohol consumption (g/kg) was calculated based on amount consumed and individual animal weight. Drinking continued for 12 days until the alcohol consumption stabilized with less than 15% variability over a three-day period. Mice were then randomly assigned to receive saline vehicle or LPS injections. Alcohol bottles were taken off 24 hours prior to the LPS injection and returned 72 hours after the injection. This 72 hour delay was to ensure that alcohol access was not given in the first few days after LPS injection, when sickness behavior may suppress food/fluid consumption. First, we aimed to determine the effect of NK1R antagonism on LPS-induced escalation in alcohol consumption.

After 5 days of post-LPS drinking, mice were injected with vehicle or L-733060 hydrochloride prior to alcohol availability. L-733060 was diluted in Milli-Q ultrapure water and administered via i.p. injection at a dose of 15 mg/kg and volume of 10 mL/kg. Each mouse received both treatments in a repeated measures design with 1 day of drinking without pretreatment in between test days. Next, we aimed to determine the effect on NK1R antagonism under conditions where drinking had not been escalated by LPS injected. To accomplish this objective, we gave saline pretreated mice 14 days of two bottle choice, after which mice were treated with L-733060 (15 mg/kg) or vehicle, as above.

Statistical Analysis

Data were analyzed using GraphPad prism 9 (San Diego, California). Two way ANOVAs were performed to analyze treatment and sex differences, followed by Bonferroni multiple comparison test for posthoc analysis. Data was considered significant when the p value was less than 0.05. Graphs are shown as mean ± SEM.

2.4 Results

Social Interaction (SI)

The SI test indicated that LPS had a greater impact on female mice (Figure 2.2). Mice (n=16/group) were tested in the SI task approximately 24 hours after LPS or vehicle injection. Two way ANOVA analysis revealed a main effect of treatment ($F_{1,60}$=10.6, p=0.002) and a sex by treatment interaction ($F_{1,60}$=4.2, p=0.04). The main effect of sex did not reach statistical significance ($F_{1, 60}$= 0.018, p=0.89). Bonferroni's posthoc test indicated that female mice treated with LPS spent significantly less time in the interaction zone when compared to saline treated controls (p=0.002). Time spent in the interaction zone was not affected by LPS treatment in male mice (p>0.99).

Cytokine expression

Brain tissue was extracted immediately following SI testing and mRNA transcripts for the cytokines TNFα and IL-6 were assessed in the VS, HPC, and PFC (Figure 2.3). For IL6 expression, two way ANOVA revealed a main effect of LPS treatment in the HPC ($F_{1,22}$=22.6, p<0.0001; Figure 3A), PFC ($F_{1,23}$=10.8, p=0.003; Figure 2.3B), and VS ($F_{1,24}$=7.3, p=0.013; Figure 3C), with expression levels in the LPS treated animals being significantly higher. No main effect of sex was observed in any of these regions (HPC: $F_{1,22}$=0.09, p=0.77; PFC: $F_{1,23}$=3.0, p=0.097; VS: $F_{1,24}$=3.8, p=0.064). No significant sex by treatment interaction was detected for the HPC ($F_{1,22}$=0.005, p=0.94) or PFC ($F_{1,23}$=3.2, p=0.086). However, a nearly significant interaction effect was observed for the VS ($F_{1,24}$=4.1, p=0.054). Posthoc tests comparing males and females treated with LPS indicated significantly higher expression of IL6 in the VS of female mice (p=0.046).

For TNFα expression two way ANOVA revealed a main effect of LPS treatment in the HPC ($F_{1,21}$=12.8, p=0.0018; Figure 3D), PFC ($F_{1,23}$=29.9, p<0.0001; Figure 2.3E), and VS ($F_{1,22}$=31.1, p<0.0001; Figure 2.3F), with expression levels in the LPS treated animals being significantly higher. No main effect of sex was observed in the PFC ($F_{1,23}$=2.1, p=0.16) or VS ($F_{1,22}$=0.42, p=0.52), but this effect nearly reached significance for the HPC ($F_{1,21}$=4.2, p=0.052). No significant sex by treatment interaction was detected for the PFC ($F_{1,23}$=2.1, p=0.16) or VS ($F_{1,22}$=0.46, p=0.50). However, a nearly significant interaction effect was observed for the HPC ($F_{1,21}$=4.2, p=0.052). Posthoc tests comparing males and females treated with LPS indicated higher expression of TNFα in the HPC of female mice that nearly reached signficance (p=0.057). Taken together, these data suggest that LPS increases the proinflammatory cytokines very

strongly, with the effect being greater in female mice for IL6 expression in the VS and perhaps also for TNFα in the HPC.

Bodyweight Change

After 12 days of baseline drinking, mice (n=8/group) were injected with LPS. After treatment with LPS, three-way repeated measures ANOVA revealed a main effect of treatment ($F_{1,28}$=227.2, p<0.0001), day post-LPS administration ($F_{7,196}$=75.9, p<0.0001), and sex ($F_{1,28}$=8.65, p=0.0065) on percent change of bodyweight (Figure 2.4). Three way ANOVA revealed significant interactions between day and sex ($F_{7,196}$=5.42, p<0.0001), day and treatment ($F_{7,196}$=71.2, p<0.0001), sex and treatment ($F_{1,28}$=6.16, p=0.019), and a three-way interaction between day, sex, and treatment ($F_{7,196}$=2.24, p=0.032). Posthoc test showed that on days 1-4 post injection both the male and female LPS groups had a significantly decreased weight compared to their respective saline group (P<0.001 for all). The male and female mice lost a similar amount of weight due to LPS treatment, however the female mice were able to regain body weight slightly faster. The female LPS group's percent weight change was not significantly different from the female saline group by day five. Male mice which received LPS had lower weights than their counterparts for days 5-7 (p<0.001 for days 5-6, p<0.01 for day 7).

Alcohol consumption

To assess LPS-induced escalation in consumption we compared the average of the last 3 days of alcohol consumption prior to LPS to the average of the first 3 days after LPS treatment. After treatment with LPS, drinking was significantly increased in both sexes with two way ANOVA revealing an effect of treatment ($F_{1,28}$=27.7, p<0.0001) and sex (F1,28=21.5, p<0.0001), but no significant treatment by sex interaction ($F_{1,28}$=0.0037, p=0.95; Figure 2.5). Overall, female mice consumed more alcohol than male mice (saline treated males versus saline

treated females p=0.02, LPS treated males versus LPS treated females p=0.02) and LPS greatly increased alcohol intake (saline treated males versus LPS treated males p=0.006, saline treated females versus LPS treated females p=0.005), as has been reported previously. Daily averages in alcohol consumption over the 12 days prior to LPS injection and the 5 days following injection are shown in figure 2.6. We do not think that LPS-induced escalation in g/kg alcohol intake is the result of body weight change, as the absolute grams of alcohol consumed (not corrected for body weight) is higher in LPS treated mice as compared to their pretreatment baseline, but this is not observed in saline treated mice. Specifically, two way ANOVA revealed a main effects of drinking phase ($F_{1,28}$=11.8, p = 0.0019) and LPS treatment ($F_{1,28}$=5.9, p = 0.022), as well as a phase by treatment interaction ($F_{1,28}$=19.8, p = 0.0001; Figure 2.7). Posthoc tests indicated that mice treated with LPS show increases in grams consumed compared to their pre-injection baseline (male LPS: p = 0.0056, female LPS: p = 0.033).

Next, mice were pretreated with L-703060 or vehicle prior to alcohol drinking and intake over the next 24-hour period was analyzed. In mice treated with LPS, we observed a main effect of antagonist treatment ($F_{1,13}$=5.7, p=0.03) and sex ($F_{1,14}$=12.4, p=0.003), as well as an interaction effect ($F_{1,13}$=5.5, p=0.04; Figure 2.8A). Post hoc analysis showed a significant difference between the vehicle and antagonist treated females (p=0.009), and between the male and female vehicle group (p=0.0004). Antagonist treated male mice did not differ from vehicle treated controls. For saline treated mice, two-way repeated measures ANOVA revealed main effects of treatment ($F_{1,13}$=21.0, p=0.0005) and sex ($F_{1,14}$=10.9, p=0.005), but no interaction effect ($F_{1,13}$=1.1, p=0.31; Figure 2.8B). Posthoc tests indicated a significant difference between the female vehicle and antagonist groups (p=0.002), and between males and females following

treatment with vehicle ($p=0.004$) or antagonist ($p=0.02$). Antagonist treated male mice did not differ from vehicle treated controls.

2.5 Discussion

LPS administration triggers a rapid and intense immune response that induces cytokine expression, weight loss, and depression-like behaviors. In line with this literature, we found that LPS induced social avoidance and increased expression of the inflammatory mediators IL6 and TNFα. Interestingly, these effects were more pronounced in female mice. We also found that LPS injection induced increased alcohol consumption, as has been shown previously (Blednov et al., 2011). When pretreating mice with an NK1R antagonist, we observed an interesting pattern of effects in that this intervention reduced drinking in females treated with LPS as well as those treated with saline, and did not seem to have any effect on alcohol intake in male mice under either condition. Taken together, these findings suggest that LPS can induce inflammation and increased alcohol consumption, with some of these effects being more pronounced and more strongly influenced by the NK1R in female animals.

It is well known that LPS can induce sickness behavior that includes depression-like symptoms such as anhedonia and social avoidance. We found that LPS treatment had this effect in female mice. We were a bit surprised that this effect was not observed in males. However, it is important to note that our behavioral measure was taken 24 hours after LPS injection. Most groups that have examined post-LPS social behavior in adult male rodents observed these effects at earlier timepoints after injection (typically 2 to 6 hours), and that this behavior was mostly normalized after 24 hours (Bluthé et al., 1999; Bluthé et al., 1992; Fishkin & Winslow, 1997; Henry et al., 2008; Jangra et al., 2014; Konsman et al., 2008; Orlandi et al., 2015; Reis et al., 2022). However, some groups have reported longer effects of LPS on SI lasting up to 24 hours

in some mouse strains (Haba et al., 2012). Notably, all of the studies referenced above assessed this response in male rodents. Some studies have assessed the effect of LPS at early timepoints on SI in female rodents, but it is unclear how long this response persists (Painsipp et al., 2008). Based on our data, we would suggest that this pattern would persist for up to 24 hours. An primary motivation for testing at this later timepoint was to ensure that SI behavior was not confounded by general locomotor suppression that occurs in the immediate response to LPS exposure. Taken together, these data suggest that female mice have a longer lasting effect of LPS exposure on SI behavior as compared to males.

We also observed a strong activation of cytokine mRNA expression in both male and female mice. Some aspects of this response seemed to be stronger in females as compared to males. Specifically, IL6 expression was more strongly stimulated in female mice in the VS. This is interesting in light of the fact that IL6 has been shown to play a strong role in social stress and subsequent interaction behavior (Hodes et al., 2014), and the nucleus accumbens, a core region of the VS, it a critical mediator of depression-like behavior following stress (Chaudhury et al., 2013; Christoffel et al., 2011; Christoffel et al., 2012; Fox et al., 2020; Francis et al., 2015). One limitation of our cytokine measures is that they were performed in animals that were alcohol-naïve, and do not directly examine possible interactions between alcohol exposure history and LPS-induced effects. This interesting question will be addressed in future experiments. In line with our findings, other groups have found a similar increase in sensitivity to LPS in females in regards to expression of inflammatory mediators and behavioral effects. For example, Dockman and colleagues (Dockman et al., 2022), showed elevated sickness behavior and cytokine activation in aged female mice relative to males. In support of this increased sensitivity to LPS in female rodents, Tonelli and colleagues (Tonelli et al., 2008) show

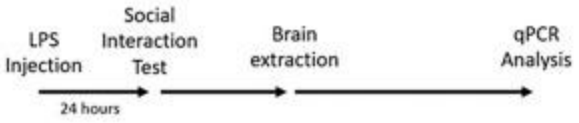
increased sensitivity to intranasally administered LPS in adult female rats as measured by cytokine expression, glucocorticoid release, and depressive-like behavior. Taken together, these findings demonstrate that female rodents may respond more strongly to multiple effects of LPS.

In agreement with the work of Blednov and colleagues (Blednov et al., 2011), we observed significantly increased alcohol consumption following injection of LPS. This was observed in both sexes and to similar degrees. However, when we treated mice with an NK1R antagonist, we found that this significantly reduced LPS-induced escalation in alcohol intake in females only. A similar pattern was observed in saline treated mice. Together this shows that NK1R antagonism can reduce alcohol consumption under conditions of high intake. We were a bit surprised to see no effect of the NK1R antagonist in male mice, given prior reports (Thorsell et al., 2010). However, the current study differed from those prior studies in terms of alcohol concentration (20% versus a range of concentrations with a maximum of 15%), antagonist used (L733060 versus L703606), and duration of access (17 days versus several weeks).

In summary, we show an ability of LPS administration to increase cytokine expression, social avoidance, and alcohol intake, with increased sensitivity to some aspects of this response in female mice. Escalated alcohol consumption could be reversed in females treated with NK1R antagonist, but this effect was not observed in males. Taken together, this suggests an increased sensitivity to inflammatory stress in females that is partially mediated by the NK1R.

Figures

a


LPS Injection
Social Interaction Test
Brain extraction
qPCR Analysis
24 hours

b


Two-bottle Choice
Alcohol removed
LPS Injection
Two-bottle Choice
12 days
24 hours
3 days

Figure 2.1 Timeline of experiments.

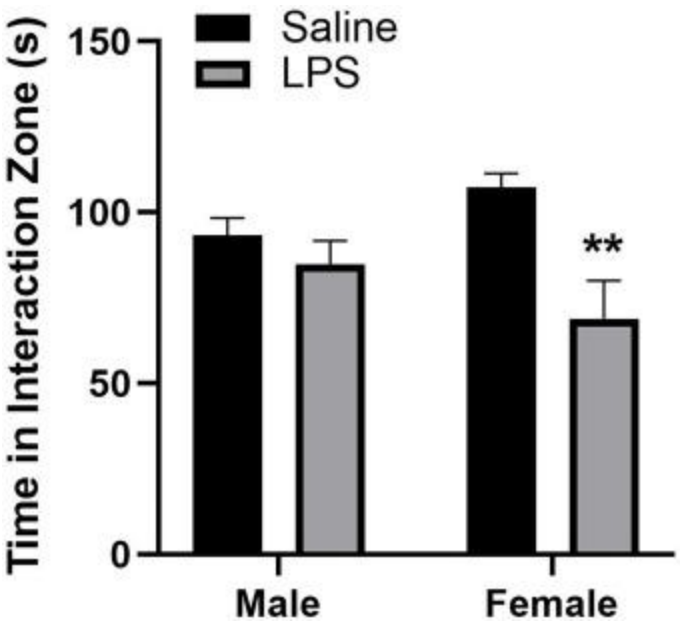

Saline
LPS
Time in Interaction Zone (s)
150
100
50
0
**
Male
Female

Figure 2.2 LPS reduces social interaction in female mice. Shown is the time (in seconds) that male and female mice spent in the interaction zone during the SI test. Main effects of treatment ($p=0.002$), and interaction effect ($p=0.04$) were observed. **$p<0.01$ compared to saline treated females.

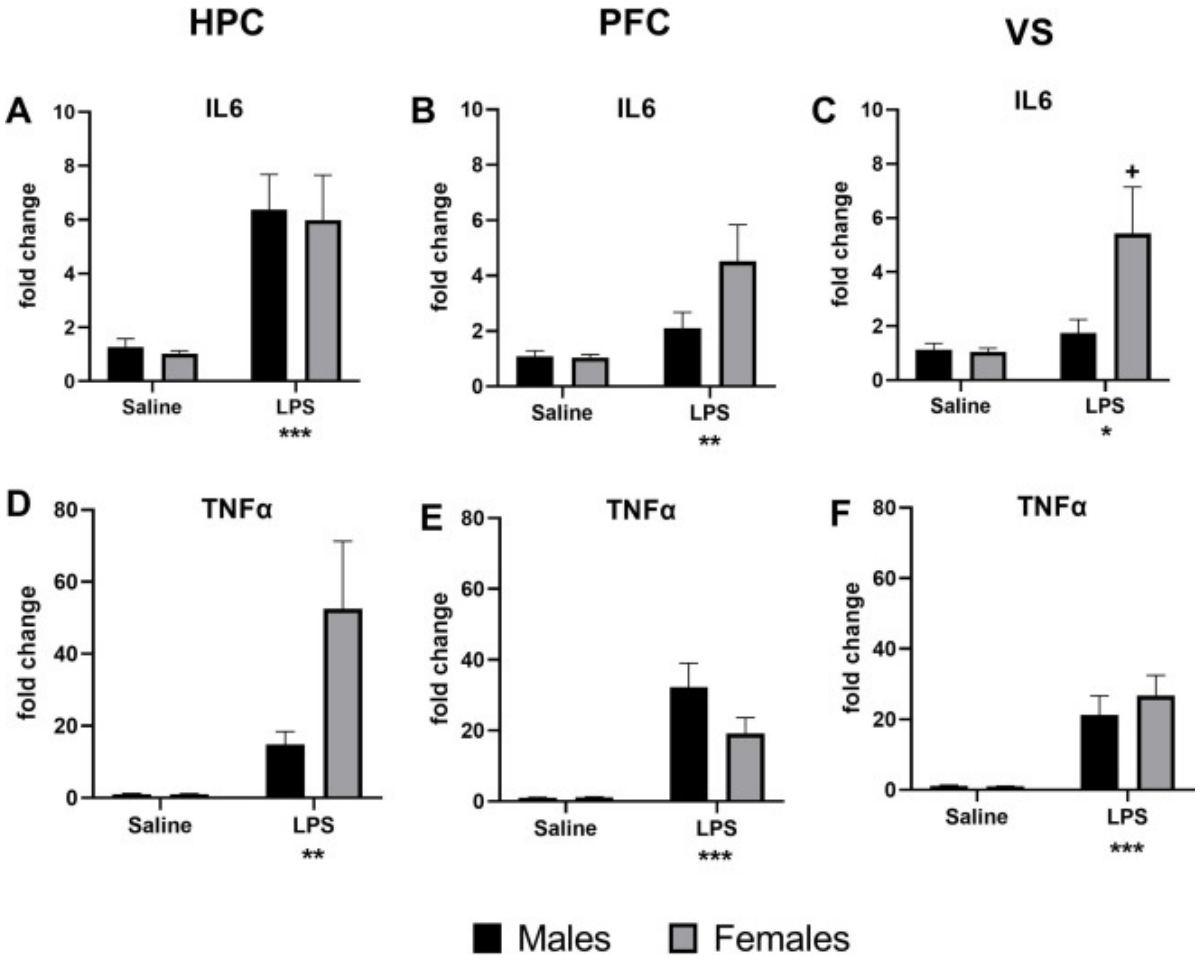

HPC
PFC
VS
A
IL6
fold change
Saline
LPS
***
B
IL6
fold change
Saline
LPS
**
C
IL6
fold change
+
Saline
LPS
*
D
TNFα
fold change
Saline
LPS
**
E
TNFα
fold change
Saline
LPS
***
F
TNFα
fold change
Saline
LPS
***
Males
Females

Figure 2.3 TNFα and IL6 expression following LPS injection. Expression of IL-6 and TNFα proinflammatory cytokines in the hippocampus (HPC), prefrontal cortex (PFC), and ventral striatum (VS). **a-c** Expression of IL-6 in the HPC, PFC, and VS. **d-f** Expression of TNF in the HPC, PFC, and VS. Data expressed as fold change in expression compared to saline treated mice of same sex. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to saline treatment. + $p<0.05$ compared to male mice treated with LPS.

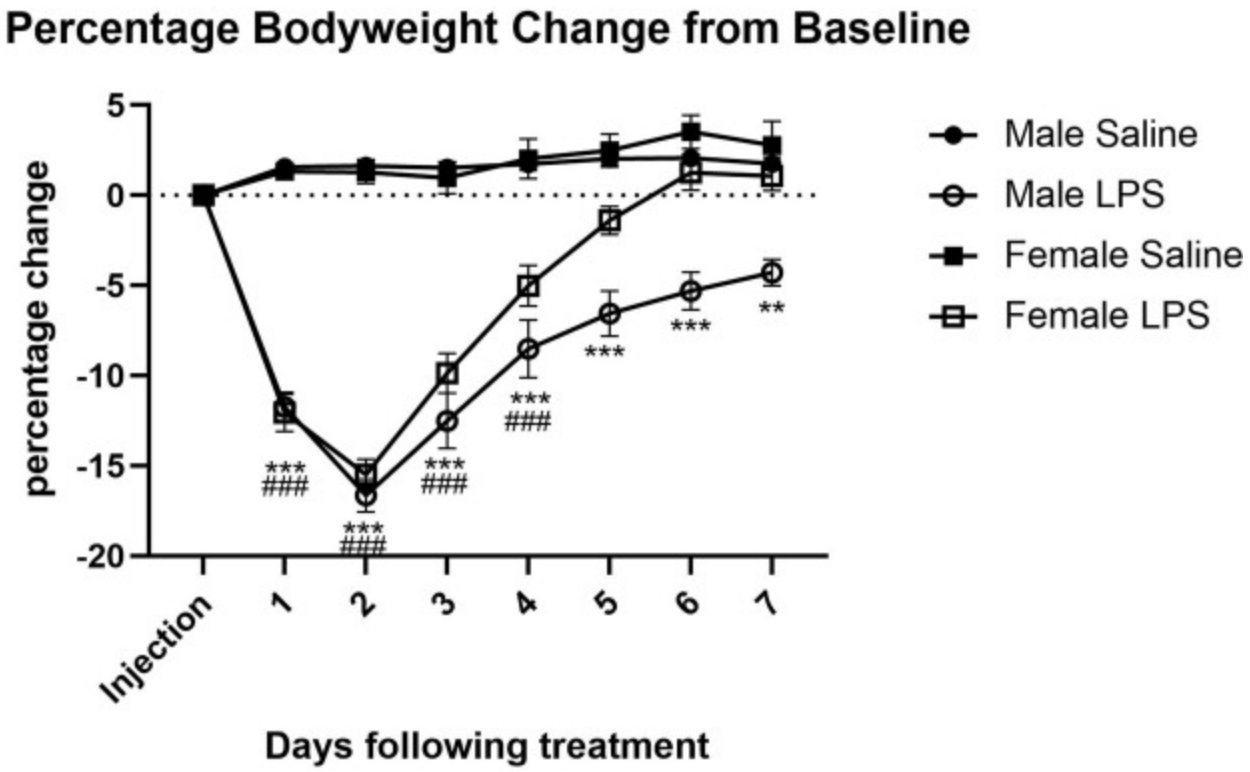

Percentage Bodyweight Change from Baseline
percentage change
5
0
-5
-10
-15
-20
Injection
1
2
3
4
5
6
7
Days following treatment
Male Saline
Male LPS
Female Saline
Female LPS
***
###
**

Figure 2.4 Body Weights after LPS treatment. Body weights were tracked for 7 days following injection of LPS. **p<0.01, ***p<0.001 male saline versus male LPS; ###p<0.001 female saline versus female LPS.

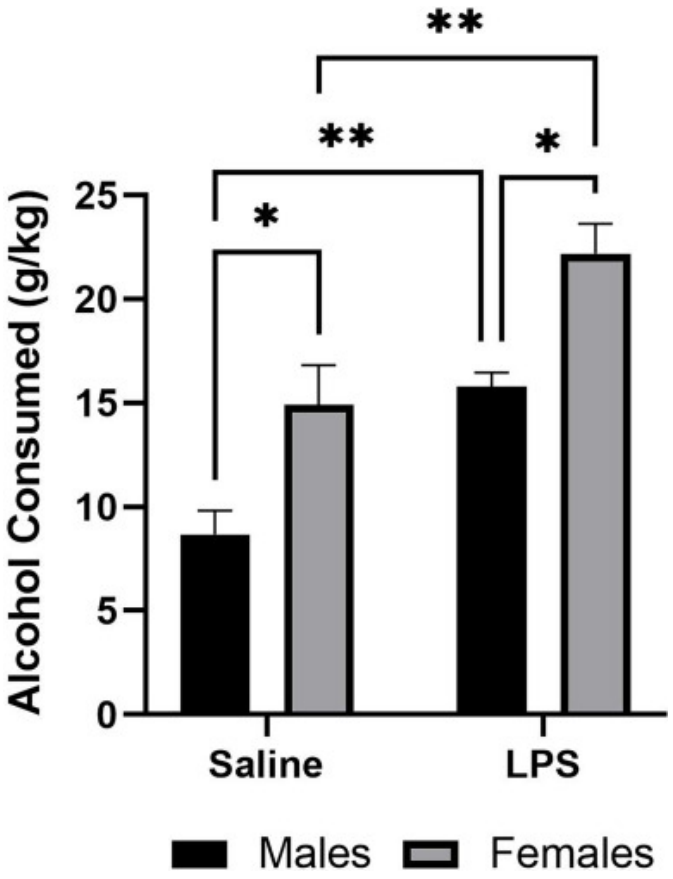

**
**
*
*
Alcohol Consumed (g/kg)
25
20
15
10
5
0
Saline
LPS
Males
Females

Figure 2.5 Alcohol consumption following LPS injection. Ethanol consumption, averaged over the three-day periods immediately before and after LPS injection, were compared in male and female mice with a treatment of saline or LPS (main effects of treatment $p<0.0001$ and sex: $p<0.0001$). *$p<0.05$, **$p<0.01$.

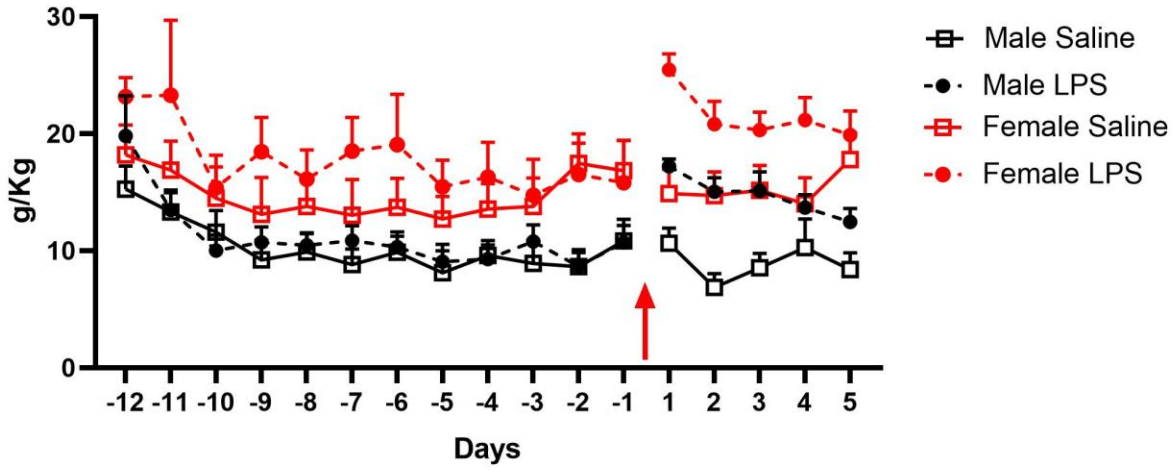

30
20
10
0
g/Kg
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 1 2 3 4 5
Days
Male Saline
Male LPS
Female Saline
Female LPS

Figure 2.6. Daily alcohol consumption in g/kg over 12 days prior to saline or LPS injection, and 5 days following this treatment. Red arrow indicates time of experimental treatment.

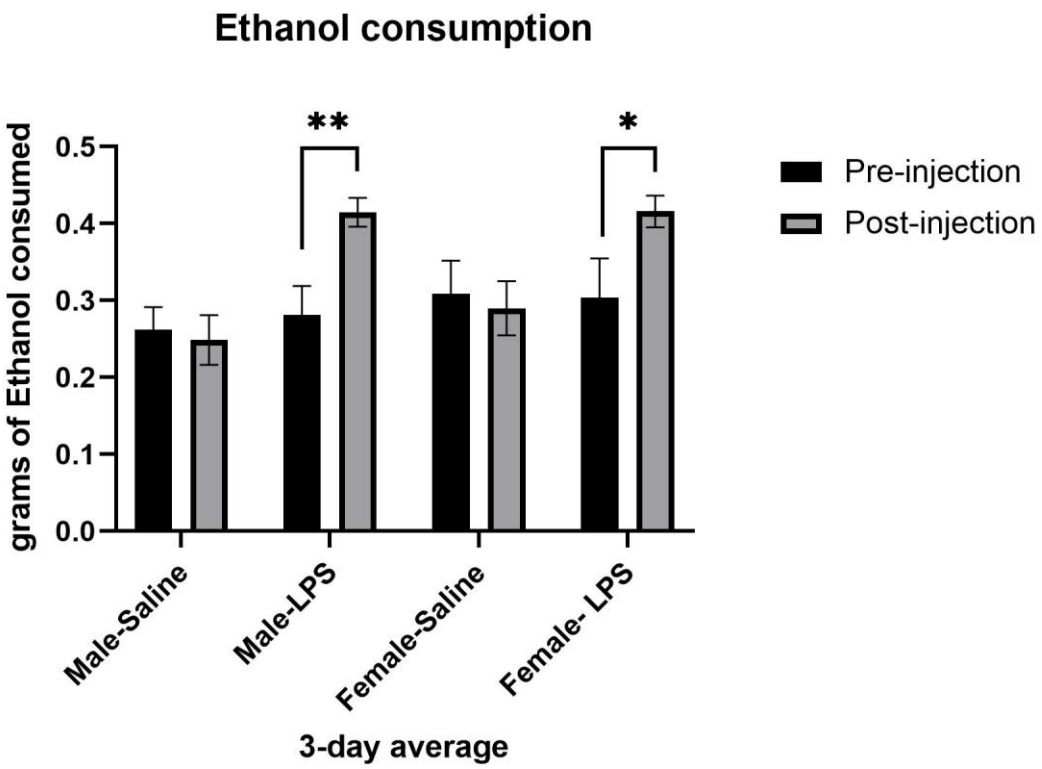
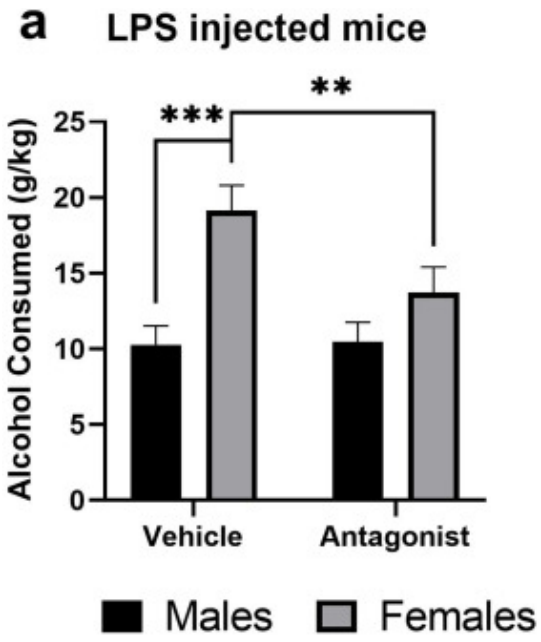

Ethanol consumption
grams of Ethanol consumed
0.5
0.4
0.3
0.2
0.1
0.0
**
*
Pre-injection
Post-injection
Male-Saline
Male-LPS
Female-Saline
Female- LPS
3-day average

Figure 2.7 Grams of alcohol consumed before and after LPS injection, not corrected for body weight. *$p < 0.05$, **$p < 0.01$.


a
LPS injected mice
***
**
Alcohol Consumed (g/kg)
25
20
15
10
5
0
Vehicle
Antagonist
Males
Females

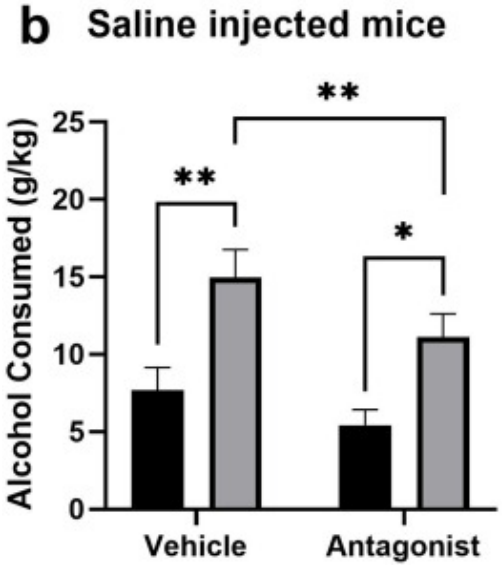

b
Saline injected mice
**
**
*
Alcohol Consumed (g/kg)
25
20
15
10
5
0
Vehicle
Antagonist

Figure 2.8 Effects of NK1R antagonist on alcohol consumption. **a** LPS treated mice were given pretreatment injections with vehicle or NK1R antagonist (main effects of antagonist treatment: $p=0.03$, sex: $p=0.003$, interaction: $p=0.04$). **b** Saline treated mice were given pretreatment injections with vehicle or NK1R antagonist (main effects of antagonist treatment $p=0.0005$, sex: $p=0.005$). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CHAPTER 3

VICARIOUS DEFEAT STRESS INDUCES INCREASED ALCOHOL CONSUMPTION IN FEMALE MICE: ROLE OF NEUROKININ-1 RECEPTOR AND INTERLEUKIN-6[2]

[2] Decker Ramirez, E. B., Arnold, M. E., & Schank, J. R. 2024. *Addiction Biology*. Reprinted here with permission of publisher.

3.1 Abstract

There is a high frequency of comorbidity of alcohol use disorder (AUD) and depression in human populations. We have studied this relationship in our lab using the social defeat stress (SDS) model, which results in both depression-like behaviors and increased alcohol consumption in male mice. However, standard SDS procedures are difficult to use in female mice due to a lack of territorial aggression. In the experiments presented here, we used vicarious defeat stress (VDS) to assess social withdrawal and alcohol consumption in female C57BL6/J mice. We also assessed the expression of interleukin-6 (IL6), which is a proinflammatory cytokine that is associated with depression in humans and sensitivity to SDS in mice. In these experiments, C57BL/6 female mice underwent 10 days of VDS where they witnessed the physical defeat of a male conspecific by an aggressive CD1 mouse. After the end of VDS, mice were either given access to alcohol or sacrificed for the measurement of IL6 expression. We found that VDS increased alcohol consumption and IL6 expression in the frontal cortex and hippocampus. Given that the neurokinin-1 receptor (NK1R) can mediate both stress-induced alcohol consumption and IL6 expression, we tested the ability of NK1R antagonism to reduce VDS-induced alcohol consumption and found that this treatment reduced alcohol intake in both VDS-exposed mice and in unstressed controls. The observed increase in alcohol consumption suggests that VDS is a model that can be utilized to study stress-induced alcohol consumption in female mice, and that this is sensitive to NK1R antagonism.

3.2 Introduction

There is a high comorbidity of alcohol use disorder (AUD) and depression in human populations. Stress is a risk factor for developing both AUD and depression, and past research has demonstrated that stress alters circuitry in the reward system. In preclinical rodent models,

various modalities of stress exposure increase alcohol intake including forced swim, restraint stress, chronic variable stress, and yohimbine injection (Anderson et al., 2016; Boyce-Rustay et al., 2008; Le et al., 2005; Lynch et al., 1999; McCarthy et al., 2022; Sequeira et al., 2018; Yang et al., 2008). A particularly effective stressor for increasing alcohol intake in rodents is chronic social defeat stress (SDS), which also induces a constellation of behavioral phenotypes that are associated with depression- and anxiety-like behavior (Berton et al., 2006b; Golden et al., 2011; Nelson et al., 2017). This co-expression of depressive-like behaviors, anxiety-like behavior, and escalated alcohol consumption makes SDS an ideal model for the study of depression and AUD comorbidity.

A key aspect of the SDS procedure is the physical defeat of the experimental mouse by a larger, aggressive mouse. Unfortunately, SDS models are difficult to utilize in female mice due to a lack of territorial aggression in this species under standard housing conditions. However, it is important to note that some labs have devised ways to induce aggressive bouts toward female mice using modifications to the original protocol developed by the Nestler group (see, for example Harris et al., 2018; Newman et al., 2019; Takahashi et al., 2017). It is of vital importance to continue to develop models that can effectively study AUD and depression comorbidity in females, as depression is more common in women than men, and the gender gap in AUD diagnosis is rapidly shrinking. For example, while the ratio of men to women diagnosed with AUD was roughly 5:1 in the 1980s, it is at 2:1 today (Keyes et al., 2008; Keyes et al., 2010)

Our lab has attempted to address the challege of assessing these behaviors in female subjects through the use of vicarious defeat stress (VDS) (Iniguez et al., 2017), which uses the emotional stressor of witnessing an aggressive bout. In this model, the experimental mouse observes the physical defeat of a conspecific, but is protected from any physical interaction with

the agressor or defeated mouse. VDS induces depressive-like and anxious phenotypes, and elevates corticosterone serum levels up to a month post-exposure (Nakatake et al., 2020; Sial et al., 2016; Warren et al., 2013). Most studies have observed that behavioral phenotypes caused by VDS in mice are significantly different from controls but are milder than those caused by SDS (Iniguez et al., 2017; Nakatake et al., 2020; Sial et al., 2016). Because the VDS exposed mouse is not the target of an aggressive bout, this protocol can be effectively utilized with female mice. In addition to its utility on studying social stress effects in female mice, using a psychological stressor may be a more translational model for the many people who experience stresses that have a primarily emotional quality.

The ability of VDS to cause stress-induced increase in alcohol consumption has yet to be studied in depth. Notably, one very recent study did find that VDS increases alcohol self-administration rats in operant models (Rodenas-Gonzalez et al., 2023). Also, Cooper and colleagues found that VDS increased morphine consumption and had similar effects on neuronal activation in the mesocorticolimbic cortex as did SDS (Cooper et al., 2017). Additionally, dopaminergic projections from the ventral tegmental area to the nucleus accumbens (NAC) were strongly activated by emotional stress (Qi et al., 2022). These findings indicate that VDS, like other models of physical/social stress, affects reward circuitry and may be a beneficial model to study AUD, but there is still considerable work to be done in this realm.

Neuroinflammation has been implicated as a mediator of depressive-like phenotypes in mice and neuroimmune markers have been used as indicators of depression in humans. (Felger, 2018; Harsanyi et al., 2022; Kitaoka, 2022; Miller et al., 2009). Neuroinflammation also plays a role in the neurophysiological and behavioral consequences of chronic alcohol exposure (Crews et al., 2015; Crews & Vetreno, 2016; Crews et al., 2011), and the comorbidity of major

depressive disorder with AUD may be related neuroinflammatory processes (McHugh & Weiss, 2019; Neupane, 2016). In preclinical rodent models, neuroinflammatory responses in specific brain regions, including the hippocampus and frontal cortex, have been implicated in depressive-like behaviors, including those induced by stress (Koo et al., 2010; Tabassum et al., 2022; Wang et al., 2018; Weng et al., 2019). Interleukin-6 is a specific proinflammatory cytokine that correlates with depression symptoms in humans and depression-like behavior in mice (Hodes et al., 2016; Hodes et al., 2014; Sukoff Rizzo et al., 2012). Thus, we assessedtranscriptional changes in IL6 in brain samples of VDS exposed mice in our study.

There is a considerable literature demonstrating a role of the neurokinin-1 receptor (NK1R) in escalated alcohol consumption, including that which is induced by social stress (Ayanwuyi et al., 2015a; Nelson et al., 2019; Nelson et al., 2017; Schank et al., 2013; Sequeira et al., 2018). The NK1R is the preferred endogenous target of the neuropeptide substance P, and has been shown to mediate complex behaviors such as drug/alcohol seeking, anxiety, depression, and stress responses (Ebner, Muigg, et al., 2008; Schank, 2020; Schank & Heilig, 2017). The NK1R is located throughout the brain and influences neurocircuity that mediates the response to stress (Mantyh, 2002; Yip & Chahl, 2000). For example, SP is released in the amygdala and lateral septum in response to stressors (Ebner, Singewald, et al., 2008), and NK1R antagonism attenuates release of monoamines in the frontal cortex after stress (Ebner & Singewald, 2007; Hutson et al., 2004). Additionally, the NK1R can mediate the activity of monoaminergic nuceli of the brainstem (Gobbi et al., 2007; Guiard et al., 2007; Ma & Bleasdale, 2002). The brain regions where the NK1R influences escalated alcohol consumption and stress-induced alcohol seeking are varied. For example, we have found that NK1Rs in the amygdala, dorsal striatum, and NAC contribute to escalated alcohol consumption that is induced by genetic selection,

intermittent access schedules, or stress exposure, respectively (Ayanwuyi et al., 2015b; Nelson et al., 2019; Nelson et al., 2018; Schank et al., 2013; Sequeira et al., 2018). For stress-induced reinstatement of alcohol seeking, NK1Rs in the amygala and NAC appear to mediate this response (Nelson et al., 2019; Schank et al., 2015b). Linking the effects of NK1R with neuroinflammation, we have observed that NK1R antagonism can attenuate LPS-induced anhedonia, escalation in alcohol consumption, and the expression of inflammatory cytokines including IL6 in the hippocampus (Decker Ramirez et al., 2023; Fulenwider et al., 2018). Taken together, we hypothesized that NK1R antagonism would be an effective intervention for reducing alcohol consumption in mice exposed to VDS, perhaps through its ability to reduce neuroinflammatory activation in multiple brain regions..

3.3 Methods

Animals

Male and Female C57BL6/J mice aged 8-10 weeks arrived from Jackson Laboratories and were allowed to habituate for one week before handling. Male CD1 mice (retired breeder, 3-6 months of age) used as aggressors were ordered from Charles River. C57BL6/J females were single housed in standard rodent cages during habituation and for pre- and post-VDS drinking. During VDS exposure, female mice were housed adjacent to the CD1 aggressor mouse in a hamster cage separated by a clear perforated divider. Male C57BL6/J mice were singly housed and introduced into the CD1 side of the hamster cage during defeats before being returned to their home cage (see below). Throughout the study, mice had ad libitum access to food and water. Light cycle was maintained on a 12:12 light/dark cycle with lights off at 11 am.

VDS

The VDS model is a modified version of SDS, allowing for a witness mouse. Male CD1 retired breeder mice aged 3-6 months were first screened for aggressive behavior by the introduction of a novel C57BL6/J mouse into the home cage of the CD1. For inclusion as an experimental aggressor, CD1 mice had to demonstrate a latency to fight of less than 60 seconds for 2 consecutive days, as described by Sial et al. (2016). CD1s that met these criteria were used for the VDS procedure, and were placed onto one side of the divided hamster cage for 3 days prior to defeats to encourage territorial aggression. On the first day of VDS exposure, female mice were placed in the hamster cage on the opposite side of the divider from a CD1 aggressor mouse. The dividers were made of clear acrylic and contained small holes, thus allowing for sensory exposure, but no physical contact. A novel male C57BL6/J mouse was then placed in the side of the cage with the CD1 aggressor, and the female mouse observed the physical defeat of the male for 5 minutes. Once the defeat was over and the defeated mouse was removed, the female mouse remained overnight across from the aggressor until she was moved the following day to a new aggressor cage to witness another defeat. Control female mice were placed into hamster cages with one on each side of the divider, and were rotated every day to mimic handling and caging alterations in VDS-exposed mice. After the 10^{th} defeat session, the female mice were singly housed.

Social interaction (SI)

Female mice underwent the social interaction (SI) test the day following the final VDS exposure. Tests were performed during the dark cycle. The SI test took place in a plexiglass box which has a metal perforated enclosure at one end, allowing for sensory contact between the test mouse and a social target. The interaction and corner zones are marked and follow the

dimensions previously reported (Golden et al., 2011). The SI test consisted of a pre-test habituation period where the mouse is able to explore the box for 150 seconds, followed by a test period of 150 seconds where a social target is placed in the enclosure within the interaction zone. An age matched female C57BL6/J mouse was used as the social target. Between each test, the test box was cleaned and disinfected. Tests were recorded and scored for time spent in the social target interaction zone during the test phase of the behavioral assay.

Two bottle choice (2BC) Alcohol Consumption

Mice were allowed to drink for 10 days with free access to a bottle containing water and a bottle containing 20% alcohol (n=10 control, n=19 VDS). Bottles were weighed every 24 hours and the side of the alcohol bottle was switched daily. Mice were given a 2 day washout period before the VDS protocol began. VDS and control groups were matched for alcohol consumption prior to assignment. Specifically, control mice consumed 18.6 ± 1.3 g/kg (mean ± SEM), and mice to be exposed to VDS consumed 20.1 ± 1.6 g/kg, which did not differ statistically ($t(27)=0.6$, $p=0.5$). Alcohol access was resumed 3 days after the final day of VDS and continued for 12 days prior to treatment with NK1R antagonist. The average of the last 3 days of drinking prior to the antagonist treatment phase were averaged to calculate post-VDS stable intake levels. For experimental timeline, see Figure 3.1. NK1R antagonist or vehicle treatment was administered to groups on the 13th and 16th day, with 2 days of alcohol access without pretreatment in between treatment days. Within each treatment group (control versus VDS) injection assignment was matched based on baseline consumption prior to injection. All mice received both treatments in a counterbalanced order and these data were used for analysis. The NK1R antagonist, L-733060 hydrochloride (Tocris), was dissolved in Milli-Q ultrapure water and administered i.p. at a dose of 15 mg/kg with an injection volume of 10 ml/kg, conditions

which we have used in prior studies (Decker Ramirez et al., 2023). Bottles were weighed at 2 and 24 hours after treatment injections.

Quantitative Polymerase Chain Reaction (qPCR)

A separate group of mice (n=6/group) were sacrificed 1 day following VDS exposure or control conditions to determine the effects of VDS on transcriptional changes in IL6 levels. Brains were dissected with samples taken from the hippocampus (HPC), prefrontal cortex (PFC), dorsal striatum (DS), ventral striatum (VS), and amygdala (AMG) and flash frozen in isopentane. For dissection, whole brains were placed in a brain matrix (Roboz) and sectioned with stainless steel razor blades at a thickness of 1 mm. Specific regions were dissected by hand with guidance from landmarks and reference to the Paxinos and Franklin Mouse Brain Atlas.

For RNA extraction, samples were first homogenized with a mechanical homogenizer and pestle, then passed through an 18-gauge needle. Using the Purelink RNA Mini Kit (Invitrogen), RNA was extracted per the manufacturer's instructions. Nanodrop 1000 was used to measure RNA concentration which was used to calculate the quantity necessary for equal cDNA concentration for all samples. cDNA was synthesized from RNA with the Maxima First Strand cDNA synthesis kit for RT-PCR (Thermo Scientific), following the manufacturer's instructions. Transcript levels were analyzed for IL6 (Mm00416190_m1) and the house-keeping reference gene Gapdh (Mm99999915_g1) using FAM-labeled TaqMan primers from Applied Biosystems. Samples were run in triplicate in a Biosystems Quantstudio 6 Flex machine. Difference in cycle threshold (ΔCT) was calculated by subtracting the CT for IL6 from CT for Gapdh. Fold change in expression relative to control group was calculated using the $2^{-\Delta\Delta CT}$ method. Data was tested for outliers using Grubb's test and resulted in the removal of 1 sample from analysis (sample from control group for AMG).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism. T-tests were used to compare the control and VDS groups in average alcohol consumption and IL6 expression. A repeated measures two-way ANOVA was used to analyze the effects of the NK1R antagonist treatment, and daily alcohol intake in the post-VDS period. P-values less than 0.05 were considered significant. All graphs are presented as mean ± SEM.

3.4 Results

SI

Female VDS and control mice were tested for SI behavior 1 day after the end of VDS. VDS-exposed mice spent significantly less time in the interaction zone during the test compared to corresponding control mice ($t(27)=2.4$, $p=0.02$, Figure 3.2).

IL6 Expression

In a separate cohort of female mice, we assessed IL6 transcript levels in multiple brain regions 1 day following the end of VDS. We found that IL6 expression was increased in VDS exposed mice relative to controls in multiple brain regions. Specifically, IL6 expression was higher in females exposed to VDS in the HPC ($t(10)=4.27$, $p=0.002$) and PFC ($t(10)=5.11$, $p=0.0005$; Figure 3.3). The increase in IL6 expression was nearly significant for the AMG ($t(9)=2.13$, $p=0.06$) and the DS ($t(10)=2.14$, $p=0.06$). For the VS, IL6 expression levels did not differ between treatment groups ($t(10)=0.96$, $p=0.36$).

Alcohol Consumption

Alcohol consumption was tracked in the 2BC procedure for 12 days following VDS exposure. Mixed-effects analysis of daily intake revealed a significant effect of stress on alcohol consumption ($F(1,27)=11.3$, $p=0.002$, Figure 3.4a), with VDS exposed female mice showing

higher levels of consumption across all days. Neither the effect of day ($F(11, 291)=1.3$, $p=0.21$) nor the interaction effect ($F(11,291)=0.29$, $p=0.99$) reached statistical significance. To assess stable consumption, we compared the average of the last 3 days of alcohol consumption between groups, and found that mice which experienced VDS consumed significantly more alcohol than control mice ($t(27)=2.70$, $p=0.01$; Figure 3.4b). Alcohol preference was also increased in VDS exposed mice ($t(27)=3.4$, $p=0.002$; Figure 4C). To further examine the relationship between SI behavior and alcohol consumption, we calculated the change in consumption from pre- to post-VDS exposure and found that this measure negatively correlated with SI time ($R^2=0.2$, $p=0.02$; Figure 4D). In other words, lower SI time following VDS exposure correlated with larger increases in alcohol intake.

NK1R antagonist

We have previously found that NK1R antagonism reduces escalated alcohol consumption and cytokine expression. Thus, we tested whether pretreatment with an NK1R antagonist could reduce alcohol consumption in VDS-exposed female mice. A two-way repeated measures ANOVA revealed that NK1R antagonist treatment attenuated alcohol consumption at the 2-hour timepoint ($F(1,27)=38.8$, $p<0.0001$; Figure 3.5 A). The main effect of stress exposure did not reach significance ($F(1,27)=0.008$, $p=0.93$), nor did the interaction effect ($F(1,27)=0.86$, $p=0.36$). NK1R antagonist treatment also attenuated alcohol consumption at the 24 hour time point ($F(1,27)=20.7$, $p=0.0001$; Figure 3.5 B). At this time point, the main effect of stress exposure did not reach significance ($F(1,27)=1.97$, $p=0.17$), nor did the stress x antagonist interaction effect ($F(1,27)=0.044$, $p=0.83$). Antagonist administration unexpectedly reduced water consumption when considering this measure at 2 hours after injection (main effect of antagonist $F(1,26)=5.1$, $P=0.03$; Figure 3.5C). However, this effect was no longer present at 24 hours (main effect of

antagonist F(1,27)=0.05, P=0.83; Figure 3.5D). Taken together, this suggests that NK1R antagonism reduces alcohol consumption in female mice under both stressed and unstressed conditions.

3.5 Discussion

The objective of the current study was to determine the effect of VDS, an emotional stressor that is known to induce depression-like phenotypes in female mice, on subsequent alcohol intake and cytokine expression. We found that VDS exposure reduced SI behavior, which is indicative of depression-like behavior, and is consistent with prior reports (Iniguez et al., 2017). VDS exposure was also associated with an increase in voluntary alcohol consumption and IL6 expression. Furthermore, VDS-induced escalated alcohol consumption was sensitive to NK1R antagonism. However, this intervention also reduced alcohol consumption in unstressed controls. Taken together, these findings indicate that, like SDS in males, VDS exposure in females can sensitize neuroinflammatory processes and trigger increased alcohol intake.

Consistent with prior reports, VDS reduced social interaction behavior in female mice, similar to the behavioral effect of SDS in males. This supports the utility of VDS as a viable model for the study of depression in female subjects in preclinical rodent studies. VDS also increased IL6 expression in the PFC and HPC, which partially aligns with previous studies indicating increased IL6 in the HPC and VS following SDS exposure in males (Deng et al., 2019; Hodes et al., 2014). While we were surprised to see no changes in IL6 expression in the ventral striatum, this agrees with a recent study that indicated no effect of VDS on this measure (Rodenas-Gonzalez et al., 2023). While we did not assess effect of NK1R antagonism on IL6 expression, we have previously shown that this intervention reduces LPS-induced expression of IL6 and other cytokines (Fulenwider et al., 2018).

Importantly, VDS also induced an increase in alcohol consumption in female mice, similar to what is observed following SDS in males. This is in agreement with very recent findings by Rodenas-Gonzalez and colleagues, which showed increased operant alcohol self-administration following VDS exposure (Rodenas-Gonzalez et al., 2023). One interesting divergence in the effects of SDS and VDS on alcohol intake in our lab is the time course of escalation. Specifically, we reliably observe an increase in alcohol intake following SDS after a 2 week incubation period following the final SDS exposure in male mice, and not at earlier timepoints. For VDS in females, however, we observed this increase when alcohol access was restored 2 days following the final VDS session. This difference could be due to the relative intensities of these stressors, as SDS has more potent effects on depressive-like phenotypes compared to VDS in males.

We suspected that NK1R signaling would be a potential mechanism for VDS-induced escalation in alcohol intake, as this receptor mediates escalated alcohol intake that is induced by multiple models including genetic selection for high preference, stress exposure, intermittent access schedules, and yohimbine injection (Nelson et al., 2019; Nelson et al., 2017; Schank et al., 2013; Sequeira et al., 2018). In line with this hypothesis, we observed that pretreatment with a NK1R antagonist reduced intake in VDS exposed mice. However, this intervention reduced alcohol intake in unstressed controls as well. The effect at early time points (2 hours following antagonist treatment) may be confounded by the fact that water intake was also reduced. However, this effect on water consumption was no longer present at 24 hours, and the reduction in intake was specific for alcohol. It is important to note that while 24 hour drinking is numerically higher in VDS exposed mice treated with vehicle compared to control mice treated with vehicle, there is no longer a statistical significance in this analysis. This may be due to the

effect of VDS normalizing over time. Another possibility is that VDS mice reduced their drinking somewhat after vehicle injection. Even though habituation injections were provided prior to treatment, VDS exposed mice may have found the injection procedure more stressful, as they would be expected to have heightened stress reactivity. Overall, however, this does not impact the general conclusion that NK1R antagonism reduces intake in both controls and mice previously exposed to VDS. While unexpected, this does agree with the findings of another recent study from our lab that showed NK1R antagonist reduction in alcohol intake following LPS-induced escalation in female mice, but also in vehicle controls (Decker Ramirez et al., 2023). It is important to note that C57BL6/J mice show some of the highest levels of alcohol consumption relative to other laboratory strains, and female mice consistently drink more than males across multiple models of consumption. Thus, female C57BL6/J mice may serve as a model of escalated intake even in the absence of stress. Another point to consider is that IL6 levels increased in VDS mice specifically, but NK1R antagonism suppressed drinking in both controls and VDS exposed subjects. Thus, it seems as though NK1R effects on drinking may not act specifically through IL6 signaling. This does, however, raise the interesting question of if IL6 specific inhibitors could reduce alcohol intake following VDS exposure. Yet another possibility is that NK1R influences alcohol consumption via multiple mechanisms, including IL6, some of which affect baseline intake and some of which affect the escalated component.

Taken together, the findings of our study support the further use of the VDS model to assess comorbidity of AUD and depression in female rodents in preclinical studies. It is unclear if similar results would be obtained with male rodents. However, traditional SDS increases alcohol intake and reduces social interaction in males (Nelson et al., 2018), and male mice show similar behavioral responses to VDS (Iniguez et al., 2018). Thus, one would predict that VDS

would also increase alcohol intake and reduce social interaction in male rodents. Future studies in the lab will examine this as well as additional depression-like phenotypes induced by VDS, including anhedonia and anxiogenesis. VDS is a highly valuable model, as it allows for examining the mechanisms that contribute to the development of depression in females, which have a higher prevalence of depression in clinical populations, and also because the gender gap in AUD diagnosis is rapidly shrinking. Many outstanding questions remain in regards to the mechanisms that mediate the co-expression of escalated alcohol intake and depressive-like behavior in females such as the neurocircuitry, pharmacology, and molecular mechanisms of this relationship.

Figures

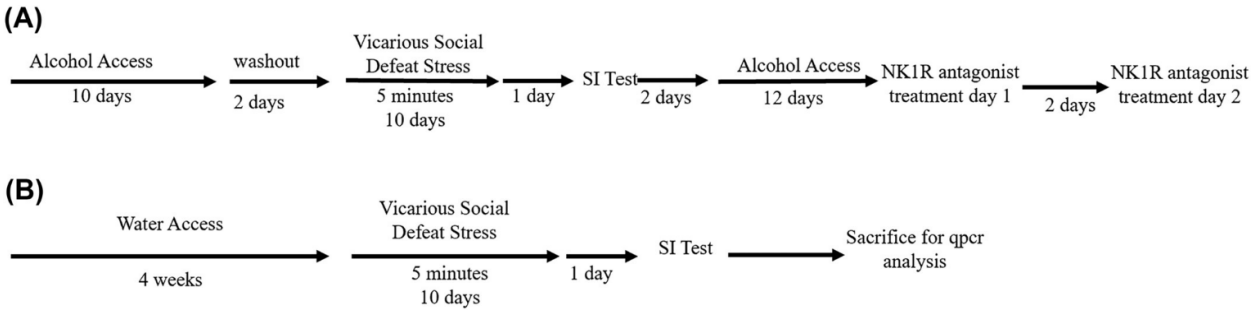

(A)
Alcohol Access
10 days
washout
2 days
Vicarious Social Defeat Stress
5 minutes
10 days
1 day
SI Test
2 days
Alcohol Access
12 days
NK1R antagonist treatment day 1
2 days
NK1R antagonist treatment day 2
(B)
Water Access
4 weeks
Vicarious Social Defeat Stress
5 minutes
10 days
1 day
SI Test
Sacrifice for qpcr analysis

Figure 3.1 Timeline of Experiments.

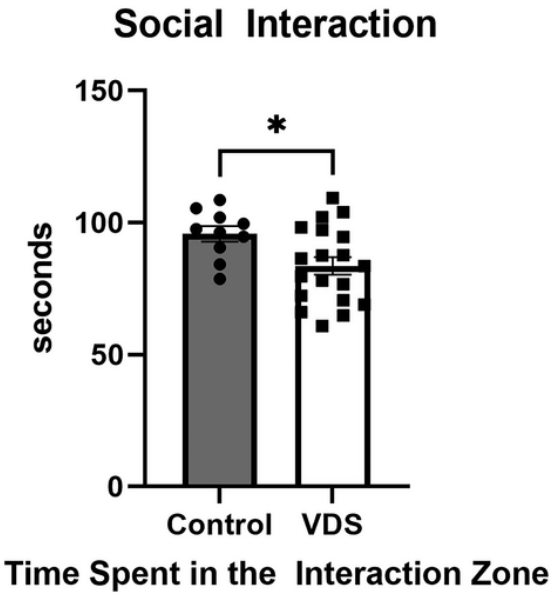

Social Interaction
150
100
50
0
seconds
*
Control
VDS
Time Spent in the Interaction Zone

Figure 3.2 VDS decreases social interaction. Time (in seconds) spent in the interaction zone was measured in the SI test. A t-test revealed that the VDS-stressed mice spent significantly less time in the interaction zone compared to the control counterparts ($t(27)=2.4$, $p=0.02$). * $p<0.05$

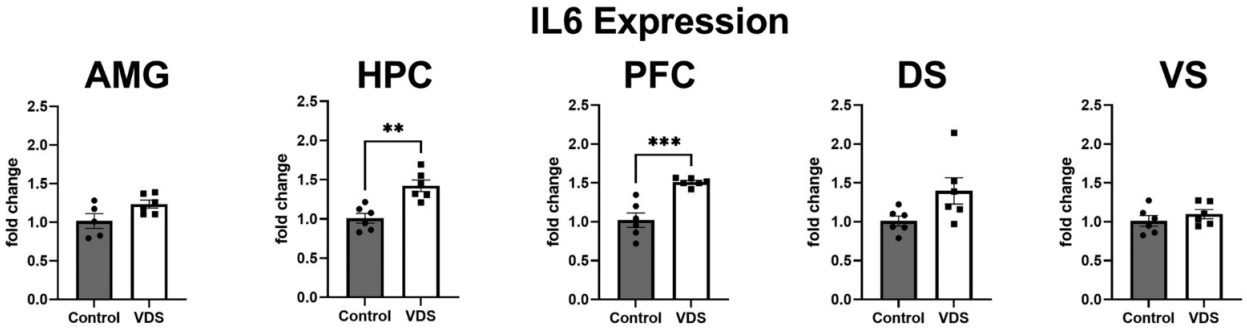

IL6 Expression
AMG
HPC
PFC
DS
VS
fold change
2.5
2.0
1.5
1.0
0.5
0.0
**
***
Control
VDS

Figure 3.3 Changes in IL6 expression following VDS. Expression of the proinflammatory cytokine IL6 in the AMG, HPC, PFC, DS, and VS. A significant effect of stress was found for the HPC ($t(10)=4.27$, $p=0.002$) and PFC ($t(10)=5.11$, $p=0.0005$). ** $p<0.01$, ***$p<0.001$.


(A)
Alcohol Consumption
VDS
control
**
g/Kg
Day
(B) 3-day drinking average
*
control
VDS
(C)
**
Alcohol Preference (%)
Control
VDS
(D)
R2=0.2
p=0.02
Change g/kg
SI time

Figure 3.4 Alcohol Consumption following VDS. (A) The alcohol consumption monitored daily revealed a significant main effect of stress ($p=0.002$). (B) Average of the final 3 days of alcohol consumption prior to NK1R antagonist treatment indicated a significant effect of VDS on alcohol consumption ($t(27)=2.70$, $p=0.01$). (C) Alcohol preference was also affected by VDS exposure $t(27)=3.4$, $p=0.002$. (D) The change in alcohol consumption following VDS negatively correlated with social interaction time ($R^2=0.2$, $p=0.02$). * $p<0.05$, ** $p<0.01$

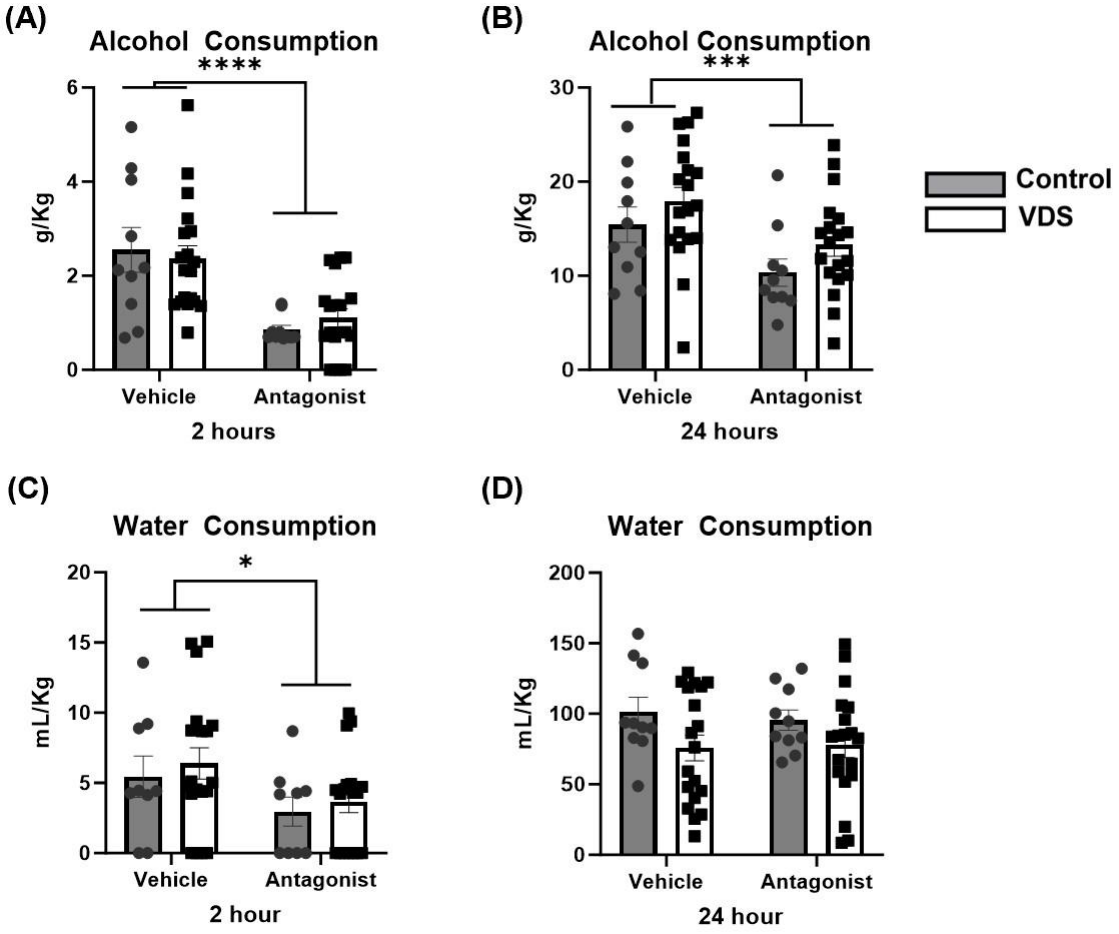

(A)
Alcohol Consumption
****
g/Kg
6
4
2
0
Vehicle
Antagonist
2 hours
(B)
Alcohol Consumption
***
g/Kg
30
20
10
0
Vehicle
Antagonist
24 hours
Control
VDS
(C)
Water Consumption
*
mL/Kg
20
15
10
5
0
Vehicle
Antagonist
2 hour
(D)
Water Consumption
mL/Kg
200
150
100
50
0
Vehicle
Antagonist
24 hour

Figure 3.5 Effect of NK1R Antagonist Treatment. Alcohol consumption following L-733060 treatment was assessed and a main effect of antagonist was observed at both 2 hour ($F(1,27)=38.8$, $p<0.0001$) and 24 hour ($F_{1,27}=0.044$, $p=0.0001$) time points, suggesting that NK1R antagonism attenuates alcohol consumption in stressed and unstressed mice. Antagonist administration unexpectedly reduced water consumption at 2 hours ($F(1,26)=5.1$, $P=0.03$), but this effect was no longer present at 24 hours ($F(1,27)=0.05$, $P=0.83$). *$p<0.05$, ***$p<0.001$, **** $p<0.0001$

CHAPTER 4

SUBSTANCE P INNERVATION OF THE NUCLEUS ACCUMBENS MEDIATES ALCOHOL CONSUMPTION FOLLOWING CHRONIC SOCIAL STRESS[3]

[3]Ellie B. Decker Ramirez, Lauren A. Beugelsdyk, Miranda E. Arnold-Tolbert, Danielle Q. Jiang, Komal Patel, Cecilia E. Harber, Andrew Nguyen, Swetha Pendela, & Jesse R. Schank. To be submitted to a peer-reviewed journal.

4.1 Abstract

Social defeat stress (SDS) is a rodent model used to assess the effect of chronic stress on depressive-like behavior and alcohol consumption. Our previous studies have indicated that the neurokinin-1 receptor (NK1R) mediates the behavioral responses to this stressor, especially through its actions in the nucleus accumbens (NAC). The NK1R is the high affinity, endogenous target of the neuropeptide substance P (SP). In the experiments presented here, we first infused a cre-dependent, retrogradely transported virus into the NAC of Tac1-cre mice (Tac1 is the gene for SP) to identify brain regions that send SP-expressing inputs to the NAC. We found that significant SP projections originated in the paraventricular nucleus of the thalamus (PVT). Next, we used this same tracing strategy, exposed mice to SDS or control conditions, and assessed Fos expression in the PVT. This experiment confirmed that SP projections from the PVT to the NAC are activated by SDS. To chemogenetically manipulate SP innervation of the NAC, we bilaterally infused a cre-dependent, retrogradely transported virus that expresses an inhibitory DREADD receptor into the NAC of Tac1-cre mice and delivered the DREADD actuator clozapine-n-oxide (CNO) prior to each defeat exposure. We found that this treatment had no effect on SDS-induced social avoidance or anxiety-like behavior, but did reduce alcohol consumption after stress. In a following experiment, CNO was administered just prior to behavioral testing, as opposed to during stress. In line with the previous experiment, chemogenetic inhibition affected post-stress drinking, but not social interaction or anxiety-like behavior. Conversely, chemogenetic activation of these inputs acutely increased alcohol consumption without affecting social behavior. Together, these results suggest that SP innervation of the NAC, likely from the PVT, mediates stress-induced alcohol consumption.

4.2 Introduction

Alcohol use disorder (AUD) is a chronic disorder affecting approximately 1 in 10 people in the United States (U.S. Department of Health and Human Services, 2024b). There is a high level of comorbidity between AUD and depression, where major depressive disorder is the most commonly occurring comorbid psychiatric disorder with AUD (McHugh & Weiss, 2019). Specifically, approximately one third of people diagnosed with AUD met criteria for MDD (McHugh & Weiss, 2019). Stress is considered a risk factor for developing AUD, anxiety disorders, and depression. Many studies have found that stress-induced changes to physiology, epigenetics, and gene expression may contribute to subsequent alcohol consumption as well as relapse (Lee et al., 2018; McEwen, 2012; Spanagel et al., 2014). Given that many individuals suffering from AUD and depression remain insensitive to available, FDA-approved medications, additional research is critical to examine novel targets for therapeutic development.

Substance P (SP) is an 11 amino acid neuropeptide that preferentially binds to the neurokinin-1 receptor (NK1R) and is found throughout the central nervous system, with high levels of expression in the extended amygdala stress circuitry (Commons, 2010; Ebner, Muigg, et al., 2008; Schank, 2020; Schank & Heilig, 2017). SP and the NK1R have been implicated in both alcohol consumption and stress. Work by our group and others has demonstrated that NK1R antagonism can attenuate stress-induced escalation and reinstatement of alcohol seeking in rodents. For example, NK1R antagonists prevent both yohimbine- and footshock-induced reinstatement of alcohol seeking in rats (Ayanwuyi et al., 2015b; Nelson et al., 2019; Schank et al., 2015a; Schank et al., 2013), an effect that extends to other drug classes as well (Fulenwider et al., 2020; Schank, 2020). Additionally, NK1R activity mediates escalated alcohol consumption that is induced by multiple procedures, including genetic selection, yohimbine injection,

intermittent access schedules, and stress exposure (Nelson et al., 2018; Schank et al., 2013; Sequeira et al., 2018). In human populations, polymorphisms in TACR1, the gene for NK1R, has been associated with risk for AUD, as well as fMRI response to alcohol-associated cues (Blaine et al., 2013; Seneviratne et al., 2009; Sharp et al., 2014). Additionally, NK1R antagonists reduce craving in treatment seeking, alcohol dependent individuals with high-trait anxiety (George et al., 2008).

In our studies, we have examined the brain regions where the NK1R acts to mediate the effect of stress on alcohol consumption/seeking. We have found specific roles of this receptor in the central nucleus of the amygdala (CeA) and the nucleus accumbens (NAC) shell. Specifically, fos activation in the NAC shell during stress-induced reinstatement is reduced following NK1R antagonist treatment, and direct infusion of a NK1R antagonist to this region attenuates reinstatement (Schank et al., 2015a). Also, alcohol preferring P rats, which self-administer increased amounts of alcohol relative to control strains and have increased sensitivity to yohimbine-induced reinstatement, show increased NK1R levels in the CeA, and manipulation of NK1R signaling in this region bidirectionally influences alcohol intake/seeking (Nelson et al., 2019; Schank et al., 2015a). Other groups have also shown neuroadaptations to NK1R signaling in the CeA following exposure to chronic intermittent alcohol vapor (Khom et al., 2020).

In our lab, we use the social defeat stress (SDS) model to examine the impacts of chronic stress on alcohol consumption and depressive-like behavior (Berton et al., 2006a; Golden et al., 2011). This model induces depressive-like symptoms including social avoidance and anhedonia, as well as increased alcohol consumption in mice (Berton et al., 2006a; Croft et al., 2005; Karlsson et al., 2017; Krishnan et al., 2007; Macedo et al., 2018; Nelson et al., 2018; Nestler & Russo, 2024). We have found that SDS increases TACR1 expression and NK1R levels within the

NAC specifically (Nelson et al., 2018; Solomon et al., 2024). In line with this observation, overexpression of the NK1R in the NAC results in increased sensitivity to defeat stress exposure (Solomon et al., 2024). These findings suggest that NK1Rs in the NAC play a significant role in stress sensitivity and stress-induced alcohol consumption. However, little is known in regards to the origin of substance P innervation to this critical region. The current study aims to determine which brain regions send SP-positive innervation to the NAC and will examine their role in SDS-induced behavior and post-stress alcohol consumption.

4.3 Methods

Animals

Heterozygous Tac1-Cre mice (8-11 weeks of age) were utilized for all experiments. These mice were bred in house by crossing homozygous Tac1-Cre mice (Jackson Labs #021877) and C57BL6/J wildtype mice (Jackson Labs). Mice were weaned at 3-4 weeks and housed in groups of 3-5 until after the surgeries were performed. At the time of surgery, mice were 8-11 weeks old. For the acute CNO control experiments where mice did not undergo surgeries, mice were individually housed at the age of 9-11 weeks and allowed to habituate for at least 2 days prior to any experimental procedures. All mice were housed on a 12:12 reverse light cycle (11:00 am lights off: 11:00 pm lights on) with food and water provided *ab libitum* throughout experiments. All experimental measures occurred during the dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Georgia and adhered to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

Intracranial Infusions

Mice were anesthetized with ketamine/xylazine (100 mg/kg, zoetis, 10004027; 20 mg/kg, Dechra Veterinary Products, NC1646100, i.p.), treated with 1% lidocaine (Hospira, 206605) at site of incision, and administered carprofen (Norbrook Laboratories, RXCARPRIEVE-INJ; 5 mg/kg, s.c.) as an analgesic. The scalp was shaved and cleaned with alcohol swabs and iodine solution. A small incision was made to reveal the skull. To target the nucleus accumbens, the following coordinates were used: AP +1.6mm, ML +/- 0.9mm, DV -4.6mm. These infusions were targeted to the ventromedial NAC, which included the medial shell and core. After locating bregma, calculating the location of infusion, and drilling holes in the skull, Hamilton 1702 syringe and needle (65460-10) were utilized for the infusion. Mice were bilaterally infused with 0.3 µl of retrograde AAV virus, which included mCherry control virus (AAV-hSyn-DIO-mCherry; Addgene 50459-AAVrg), Gi DREADD (AAV-hsyn-DIO-hM4D(GI)-mCherry; Addgene 44362-AAVrg), or Gq DREADD (rAAV-hSyn-DIO-hM3D(Gq)-mCherry; Addgene 4361-AAVrg). Because these viruses were retrogradely transported and we were unsure if we would be able to determine location of viral infusion with confidence using this virus alone, 0.2 µl of an AAV1 virus that drove local eGFP expression(AAV1-hsyn-EGFP; 50465-AAV or AAV1-CMV-MCS-IRES-eGFP) was also infused, for a total of 0.5 µl infused per side. The infusion rate was 0.05-0.1 µl/minute and was delivered using a Quintessential Stereotaxic Injector (Stoelting, #53311). Following infusion, there was a 5 minute hold prior to removal of infusion needle to prevent aspiration along the needle tract. Following removal of needle, the incision was closed with a wound clip (BD biosciences, AUTOCLIP 427631) and yohimbine (Sigma Life science Y-3125-1G, s.c.) was administered as anesthesia reversal. Mice were monitored until recovery. Carprofen (5 mg/kg, s.c.) was administered for the next 2 days

following surgery. Mice were monitored daily until the incision healed and the wound clips were removed under brief isoflurane anesthesia.

Clozapine-N-oxide (CNO) treatments

Clozapine-N-oxide (CNO) was made daily by dissolving CNO (Tocris, product #6329) in 0.9% sterile saline (Teknova, product #S5815) to a concentration of 0.3 mg/mL. CNO (3 mg/kg) or saline was administered i.p. at a volume of 10 ml/kg. Treatments occurred 30 minutes prior to behavior, SDS, or alcohol consumption. For the counterbalanced CNO treatments during alcohol consumption, there were 3 days between each treatment.

Aggressor Screening

Upon arrival, 3-4 month old retired CD1 breeders from Charles River Labs were given at least 1 week to acclimate to the facility prior to screening. Initial screening occurred over 4 days and consisted of the CD1 aggressor being in their home cage and placement of a novel C57 mouse in their home cage for 3 minutes. CD1 aggressors which attacked within the first minute of the 3 minute screening for 2 days in a row were marked as aggressive to be utilized for social defeat stress. Subsequent screenings to ensure the mice were aggressive between different defeat experiments consisted of a single day of one novel mouse encounter in their home cage. If they still attacked within 1 minute, the CD1 aggressor was considered aggressive to be utilized for another social defeat stress experiment.

Social Defeat Stress (SDS)

A modified version of the standard SDS procedure was utilized to permit co-occurring vicarious social defeat stress in female mice. In this model, a novel female mouse is housed in a hamster cage across from a retired breeder CD1 (Charles River Labs) aggressor mouse (3-12 months of age, screened for aggressive behavior prior to experiments). Tac1-cre test mice are

individually housed overnight. On each day of defeats, the test mice are placed into the hamster cage on the side of the barrier with a novel aggressor, and novel female mouse on the opposite side of the barrier. The female mice are removed from SDS prior to the 11th day of the defeat, so the 11th day of defeat occurs with no female witness mouse. The physical defeat occurred for 5 minutes or until an injury is observed on the test mouse. All test mice were monitored and any observed injuries reported to the veterinary staff. Control mice were placed in a hamster cage across the barrier from an age-matched counterpart for 5 minutes each day. Each day following the defeat or the control procedure, all test mice were immediately returned to their individual home cage.

Social Interaction (SI) Test

During the social interaction (SI) test, mice were placed into an arena measuring 42 x 42 cm for 2.5 minutes. The designated interaction zone surrounds the social target enclosure at one edge of the box. Meanwhile, the corner zones encompass the two corners furthest from the enclosure and interaction zone. This behavioral assay and apparatus is described in detail by Golden and colleagues(Golden et al., 2011). Following the 2.5 minute habituation period to the arena with no social target present, the test mouse was removed and the social target was placed in an enclosure in the interaction zone. Then, the test mouse was returned for another 2.5 minutes to measure SI. Following the test, the mice were returned to their homecage and the SI arena was thoroughly cleaned with peroxigard (Virox Technologies, 29305) diluted 1:16, a disinfectant and deodorizer. For the chemogenetic activation experiment, the mice had an age-, strain- and sex-matched social target (because this experiment included female mice, and the animals had no prior experience with social defeat). For the chemogenetic inhibition experiments, a novel aggressor was utilized as the social target. The SI tests were scored from video recordings by

investigators blinded to treatment group. Measures included the time spent in the interaction zone and the corner zone during the pre-test habituation time and during the time with the social target present. The SI ratio is calculated as the time spent in the interaction zone with the social target present divided by the time spent in the interaction zone during the pre-test.

Elevated Plus Maze

The elevated plus maze (EPM) test used an EPM apparatus (Stoelting #60140) which has two open arms and two closed arms which face opposite of each other. Each test consisted of the test mouse being placed in the center of the EPM, facing an open arm. Mice were allowed to explore the EPM for 5 minutes. Between each mouse, the EPM was cleaned with peroxigaurd. For the mice that underwent chemogenetic activation experiments and the acute CNO control experiments, the EPM took place in a separate behavioral room. Mice were habituated to this behavior room the day prior for approximately one hour and habituated to the room prior to the test for 45 minutes. For the mice which underwent SDS, the EPM occurred in their vivarium to prevent the additional stress of switching rooms. The mice were scored for time spent in the open and closed arms, number of entries into the open and closed arms, and number of stretches into the open arm. A mouse was considered to be in an arm when all four feet were inside an open or closed arm. Stretching into the open arm was measured as placing 2-3 feet into the open arm. Open arm time and open arm entries are calculated as a percentage out of total time in all arms or total arm entries in order to control for possible differences in locomotion. Open arm stretches and total arm entries are presented as the total number.

Alcohol Access

During alcohol access, mice were presented ab libitum access to water and alcohol via a two-bottle choice paradigm where one bottle contained water and the other contained 20%

ethanol, made by diluting 95% ethanol (190 proof ethanol, Decon Laboratories Inc., 2805HC) in water. Bottle sides were switched on a daily basis. Weight was monitored to calculate g/kg consumption of alcohol. Specific timelines for alcohol access are provided in the description of each experiment.

For Fos activation, and chemogenetic inhibition experiments, mice were allowed 10 days baseline alcohol consumption prior to undergoing the stressor of SDS. Mice had 2-3 days without alcohol prior to starting SDS. Following SDS, the mice had alcohol access returned 2 weeks following the last day of SDS. For the mice with chemogenetic inhibition during SDS, alcohol access occurred for 14 days of which the last 9 days were averaged to determine the post-SDS average alcohol consumption. To calculate percent change from baseline, the last 7 days of baseline, pre-defeat alcohol consumption was averaged. For the mice undergoing acute CNO treatments during behavior, all mice had 12-14 days of alcohol access prior to counterbalanced CNO treatments. A 3-day period occurred between the counterbalanced CNO treatments during alcohol consumption for all experiments. Additionally, to account for the onset and half-life of CNO, a 2-hour measure of alcohol consumption was measured during these counterbalanced treatments.

Immunohistochemistry

To confirm viral infusion sites and perform tract tracing of substance P inputs into the NAC, immunohistochemistry (IHC) was performed to stain for either mCherry or GFP as described. In order to perform IHC, all brains were fixed by transcardially perfusing the mice with 4% paraformaldehyde under deep anesthesia. Brains were post-fixed in 4% paraformaldehyde at 4°C, then transferred to a 10% sucrose solution for one hour at 4°C, then transferred to 30% sucrose solution until the brain sunk. Brains are then flash frozen and stored

at -80°C until sectioning. Brains were sectioned at 30 µm with a Leica CM1950 cryostat, and sections were placed in well plates in cryopreservant and stored at -20°C until the IHC process. The cryopreservant consisted of 20% glycerol (Fisher Scientific, BP229-1), 2% DMSO (Fisher Scientific, BP231-1), and sodium azide (Acros Organics, 190380050) in 1X PBS.

For IHC, sections were washed in 1X PBS (diluted from 10X PBS from Omnipur 10X liquid concentrate phosphate buffered saline, MilliporeSigma 6507, Fisher Scientific, Waltham, MA, USA) three times for 10 minutes each at room temperature, blocked in 5% normal goat serum (Invitrogen, #331873) with 1% Triton X-100 (BIO-RAD, Hercules, CA, USA, 161-0407) in 1X PBS at 4 °C for 1 hour, then placed in primary antibody diluted in 5% normal goat serum (1:1000) overnight at 4°C. When staining for GFP, Rabbit primary antibody to GFP (Abcam, Ab290) was used and when staining for mCherry, rabbit mCherry polyclonal antibody (Invitrogen, PA5-141089) was used. The next day, sections were again washed in 1X PBS the placed in the secondary antibody diluted in 5% normal goat serum (1:500), covered, and incubated at room temperature for 2 hours. For mCherry secondary antibody, Alexa Fluor 633 goat anti-rabbit IgG (Invitrogen, A21070) was used and for GFP secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11008) was used. Sections were again washed, mounted on slides, and coverslipped using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, H-1500-10). All tissue was then imaged on a Carl Zeiss Imaging system with a Axiocam MRc5 digital camera.

RNAscope

In order to determine neuronal activity via Fos expression, mice were sacrificed 45-60 minutes after the final physical defeat exposure. Brains were extracted and flash frozen in dry ice. Brains were sectioned at 15 µm on a Leica CM1950 cryostat, and sections from the regions

of interest (OLF and PVT) were placed on fisherbrand Superfrost Plus microscope slides (Fisher Scientific, 1255015). Slides were stored at -80°C until processing. Slides underwent a fixing process in 4% PFA at 4°C for 1 hour, then rinsed in 1X PBS two times. Slides then underwent a dehydration series at room temperature where they were placed in 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, then 100% ethanol for 5 minutes two times. Slides were then stored in 100% ethanol at -20°C for up to 1 week until performing RNAscope.

Upon removal from the ethanol, slides were permitted to air dry, and a hydrophobic barrier was made with a ImmEdge pen (Vector Laboratories, H-4000). Hydrogen Peroxide (Advanced Cell Diagnostics, #322335) was then applied for 10 minutes at room temperature and slides washed two times with deionized (DI) water. Next, Protease IV treatment (Advanced Cell Diagnostics, #322337) followed to further permeabilize the tissue, and slides were subsequently washed with DI water 2 times.

Probes and opal dyes were used to fluorescently tag target transcripts. Specifically, for Fos activation experiment Mm-Fos (316921), mCherry-C2 (431201-C2), Mm-Tac1-C3 (410351-C3) were used (Advanced Cell Diagnostics). For placement confirmation, eGFP-C3 (400281-C3) was used. These probes were combined to make a master probe at ratio of 50:1:1 of channel 1, channel 2, and channel 3 probes. In the case of staining for GFP for placement of virus only Channel 3 was utilized, and a probe dilution was used at a ratio of 50:1 of Probe diluent to Channel 3. Opal dyes from Akoya biosciences were used as the fluorophores: Opal 520 Reagent (OP-001001) for channel 1, Opal 570 reagent (OP-001003) for channel 2, and Opal 690 Reagent (OP-001006) for channel 3. The opal dyes were used at a dilution factor of 1:1000 – 5:1000 of fluorophore in TSA buffer (Advanced Cell Diagnostics, #322809).

The multiplex V2 RNAscope manual assay kit (Advanced Cell Diagnostics, #323110) was used per the manufacturer's instructions. The probe was hybridized for 2 hours at 40°C, then washed two times with RNAscope wash buffer (Advanced Cell Diagnostics, #322000). Following this, the AMPs, a mix of pre-amplifier and amplifier molecules, were hybridized at 40°C, 30 minutes for AMP 1 and 2 and 15 minutes for AMP 3. After each step, the slides were washed with RNAscope wash buffer two times. Next the channel signals were developed. First, the sections were treated with the multiplex HRP-C for 15 minutes at 40°C then slides were washed and the fluorophore was added and sections were treated for 30 minutes at 40°C. Following a wash step, the HRP blocker was added to prevent compounding signals. After washing, the sections underwent the subsequent HRP-Channel development. Following the final channel, the slides were washed a final time, then were counterstained with DAPI for 30 seconds. After removing the DAPI, ProLong Gold Antifade Mountant (Invitrogen, #P36930) and a 24 mmm x 50 mm globe scientific (# 1415-15) cover glass was used to coverslip. The slides were covered and dried overnight, then stored at 4°C until imaging. A Nikon A1R confocal microscope was used to image the tissue within 2 weeks of staining. Images were taken at 20X. One image was taken per brain of the PVT and 4 images were taken per brain of the OLF region as indicated in figures.

Images were scored by blinded scorers using ImageJ software. Individual counts were made for each target as well as colocalizations of the targets. For the OLF, the counts from the 4 images were summed prior to calculating percentage of the activation. For the activation of TAC1 neurons, the colocalization of mCherry, Tac1, and Fos was divided by the colocalization of mCherry and Tac1 and multiplied by 100 to represent the percentage of neurons activated.

Statistical Analysis

All data were analyzed using GraphPad Prism. Unpaired t-Tests or paired t-Tests were used for experiments where there were two treatment groups or counterbalanced treatments, respectively. For experiments with four groups, a one-way ANOVA analysis was utilized and if significant, Bonferroni's post hoc analysis was performed. In the case of significant results in the Bartlett's test, indicating a significant difference in standard deviation between groups, Kruskal-Wallis test were utilized followed by Dunn's multiple comparison test for the post-hoc analysis comparing each group to control. For cases where the F-statistical test of variances was significant during t-test analysis, the Mann-Whittney nonparametric test was utilized. For chemogenetic activation and CNO control experiments, the data of male and female mice were combined if there was no observed sex or interaction effect. Only male mice were used in experiments with SDS exposure.

After placement of virus was confirmed, mice who did not have at least one infusion hitting the medial shell and core were removed from the behavioral data. For tract tracing, mice were included only if both infusions hit the target area. For analysis of RNAscope data, outliers were removed which were over 2 standard deviations from the mean (removed 1 data point from PVT control group). One mouse was removed from alcohol consumption analysis in the experiment utilizing chemogenetic inhibiton during stress due to abnormally low daily alcohol consumption during pre-defeat alcohol consumption (<2.5 g/kg each day). No mice from any other experiment consumed below a 3 mg/kg threshold. One female mouse treated with CNO in the acute CNO administration control experiment was removed from statistical analysis on the EPM measures due to spending 100% of the time in the open arm of the EPM. One mouse was removed from the acute CNO administration control measure for alcohol preference due to a water leak of >200 mL/Kg within the 2 hour measure.

4.4 Results

SP innervation of NAC

Following retrograde tract tracing, we identified mCherry positive cell bodies in several brain regions. As the virus infused into the heterozygous Tac1-Cre mice (N=6) was retrogradely transported and cre-dependent, mCherry expression indicates a neuron which expresses SP and projects to the NAC. As shown in Figure 1 (NAC infusion shown in Figure 1A), regions where we detected mCherry positive cell bodies included the olfactory bulb (OLF; Figure 4.1B-C), paraventricular nucleus of the thalamus (PVT; Figure 4.1D-E), piriform cortex (PIR; Figure 4.1F-G), and midbrain regions located near the ventral tegmental area including the supramammilary nucleus of the hypothalamus (SuM), the rostral linear nucleus (RLi) and interfascicular nucleus (IF; Figure 1 H-I).

The PVT and the OLF were of particular interest due to the dense expression of mCherry and their known involvement in stress responses. To determine activation of SP positive PVT→NAC projections and OLF→NAC projections, heterozygous Tac1-Cre mice were infused with the same retrogradely transported, cre-dependent mCherry virus as above, completed baseline alcohol consumption and either underwent SDS (n=8) or control (n=9) conditions, as depicted in the timeline (Figure 4.2A). Following SDS, brains were extracted 45-60 minutes following the final defeat to assess Fos mRNA expression in cells that colocalized with Tac1 and mCherry (Figure 4.2C,F). Analysis of percent of the Tac1/mCherry neurons in the PVT that co-express Fos, indicated significantly more of these cells in the PVT of the SDS group compared to unstressed control (t_{14}=2.2, p=0.04; Figure 2D), indicating that SDS significantly activated SP expressing PVT→NAC projections. However, SDS did not alter activation of the SP positive OLF→NAC projections (t_{14}=0.52, p=0.61; Figure 4.2G)

Chronic chemogenetic inhibition during SDS

To determine the effect of inhibition of the SP innervation of the NAC, heterozygous Tac1-Cre mice were infused with retrograde cre-dependent Gi DREADD virus or an mCherry control virus. This results in inhibitory DREADD expression specifically in SP neurons that project to the NAC, and enables inhibition of these neurons following CNO administration. The control group was infused with the Gi DREADD expressing virus, was unstressed, and received saline treatments (Gi/Control/Saline, n=14). Of the defeated groups, two received the Gi DREADD virus. One group received saline injections (Gi/SDS/Saline, n=11) and was expected to exhibit SDS-induced behavior, whereas the other group received CNO treatments prior to each SDS exposure (Gi/SDS/CNO, n=9) which should inhibit the SP neuronal input of the NAC. The remaining defeated group was infused with mCherry (non-DREADD expressing) virus and treated with CNO (mCherry/SDS/CNO, n=10), to control for potential off target effects of CNO treatment. The experiment followed the timeline as depicted in Figure 4.3A, and treatment groups are shown in Figure 4.3B. Following recovery from viral infusion surgery, mice received 10 days of alcohol access, and 3 days later were exposed to SDS. Following SDS, the mice completed the SI test. A one-way ANOVA revealed a significant effect of group ($F_{3,40}$=15.9, $p<0.0001$) and Bonferroni's multiple comparison post-hoc tests indicated that each defeated group had a significantly reduced SI ratio compared to the Gi/Control/Saline group ($p<0.0001$; Figure 4.3C). To determine anxiety-like behavior the elevated plus maze was administered the following day. There was no significant effect on percent open arm time (Bartlett's test <0.0001, Kruskal-Wallis test p=0.52; Figure 4.3D) or percent open arm entry ($F_{3,40}$=1.021, p=0.39; Figure 4.3E). One-way ANOVA analysis was significant for total number of entries ($F_{3,40}$=8.86, p=0.0001) and open arm stretches ($F_{3,40}$=5.36, p=0.0034). Bonferonni's posthoc analysis

revealed that each defeated group had significantly fewer total arm entries compared to the Control/SDS/Saline group (Gi/SDS/Saline p=0.0003; Gi/SDS/CNO p=0.006; mCherry/SDS/CNO p=0.0004; Figure 4.3F) and fewer open arm stretches compared to the Control/SDS/Saline group (Gi/SDS/Saline p=0.002; Gi/SDS/CNO p=0.036; mCherry/SDS/CNO=0.038; Figure 4.3G). Two weeks following the last defeat, alcohol access was returned for two weeks. This is in line with our prior experiments, which suggest that a delay is required following SDS exposure to observe elevated alcohol consumption relative to unstressed controls (Nelson et al., 2018). We calculated a change in alcohol intake from prestress baseline and used nonparametric Kruskal-Wallis analysis (due to Bartlett's test p=0.02), which indicated a significant effect of treatment (p=0.008; Figure 4.3H). Posthoc comparisons indicated that both Gi/SDS/Saline (p=0.01) and mCherry/SDS/CNO (p=0.03) differed from the Gi/Control/Saline group, indicating elevated alcohol consumption in these groups. Meanwhile, the Gi/Control/Saline did not differ significantly from the Gi/SDS/CNO group (p>0.99), suggesting that chemogenetic inhibition of SP inputs to the NAC attenuated alcohol consumption following SDS.

Acute chemogenetic inhibition prior to post-stress behavioral measures

To determine if inhibition of SP innervation to the NAC during the behavioral test (as opposed to during each stress exposure, as above) attenuates the effects of SDS, heterozygous Tac1-Cre mice were infused with the retrograde Gi DREADD virus (n=12) and were exposed to SDS (timeline shown in Figure 4.4A). Following stress exposure, mice were treated with saline (n=7) or CNO (n=5) prior to the SI test, and there was no effect of treatment (t_{10}=0.51, p=0.62; Figure 4.4B). The following day, counterbalanced treatment of CNO 30 minutes prior to the elevated plus maze test (saline n=5, CNO n=7) had no effect on percent open arm time (t_{10}=0.46,

p=0.66; Figure 4.4C), percent open arm entry (t_{10}=0.47, p=0.65; Figure 4.4D), number of open arm stretches (t_{10}=0.41, p=0.69; Figure 4.4E), or total arm entries (t_{10}=0.43, p=0.68; Figure 4.4F). Two weeks following SDS, alcohol access was returned, as above. Mice had two weeks of continuous access prior to counterbalanced CNO treatments. Drinking rates were measured 2 and 24 hours after treatment. CNO significantly reduced alcohol consumption at 2 hours following treatment (t_{11}=2.7, p=0.02; Figure 4.4G), but this was normalized by the 24 hour measurement (t_{11}=0.45, p=0.66; Figure 4.4H). At the two hour measure, there was a trending effect of CNO treatment on alcohol preference (t_{11}=1.8, p=0.10; Figure 4.4I).

Acute chemogenetic activation of SP innervation to NAC

To determine if acute activation of SP inputs to the NAC induces phenotypes consistent with stress exposure, stress-naïve Tac1-Cre mice were infused with a cre-dependent, retrogradely transported, Gq DREADD virus, which stimulates neuronal activation following CNO administration. Female mice were included in this experiment because the SDS exposure, which works most effectively in male mice on C57BL6/J background, was not required. Following a 3-week recovery for viral expression, mice underwent the SI test (Figure 4D). CNO or saline vehicle was injected 30 minutes prior to the SI test (male: saline n=8, CNO n=5), (female: saline n=7, CNO n=8). There were no significant sex ($F_{1,24}$=1.1, p=0.31) or interaction ($F_{1,24}$ = 0.08, p=0.78) effects, so males and females were combined for analysis. Unpaired t-test indicated that CNO treatment did not significantly affect SI Ratio (t_{26}=1.4, p=0.18; Figure 5.5B). The following day, the EPM took place where mice received counterbalanced Saline/CNO treatments, receiving the opposite of what they received from the previous test (male: saline n=5, CNO=8), (female: saline n=8, CNO=7).There were significant or trending interaction effects on the elevated plus maze in several of the measures (percent open arm time: $F_{1,24}$=5.15, p=0.03;

percent open arm entry: $F_{1,24}$=4.15, p=0.05; Open arm stretches: $F_{1,24}$=3.19, p=0.09) and the open arm stretches had a significant sex effect as well ($F_{1,24}$=5.09, p=0.03). No effects of sex were observed in the percent open arm time ($F_{1,24}$=1.032, p=0.3199) or the percent open entry ($F_{1,24}$=0.5715, p=0.4570). Due to significant interaction effects, these measures were analyzed by sex with t-tests. CNO significantly increased percent open arm time in male mice ($F_{7,4}$=18.56, p=0.013, Mann-Whitney test p=0.04;Figure 4.5C) whereas there was no effect observed in female mice ($F_{7,6}$=0.006, Mann-Whitney test p=0.61; Figure 4.5D). Similarly, the CNO administration increased the percentage of open arm entries in male mice (t_{11}=2.36, p=0.03; Figure 4.5E) with no effect observed in female mice (t_{13}=0.53, p=0.60; Figure 4.5F). CNO administration significantly increased number of stretches into the open arm in male mice (t_{11}=2.85, p=0.02; Figure 4.5G), but had no effect in female mice (t_{13}=0.34, p=0.74; Figure 4.5 H). There was no significant sex or interaction effect in analysis of total arm entries (Interaction: $F_{1,24}$=1.64, p=0.21, Sex: $F_{1,23}$=0.052, p=0.82), but sexes were kept separate due to all other measures requiring separation. CNO resulted in a significant increase in total arm entries in male mice (t_{11}=3.579, p=0.0043; Figure 4.5I) with no significant effect observed in female mice (t_{13}=1.347, p=0.2011; Figure 4.5J).

The mice then had 12-14 days of alcohol access prior to counterbalanced CNO and vehicle treatments. There was no observed sex effect ($F_{1,26}$=0.63, p=0.44) or interaction ($F_{1,26}$=0.04, p=0.84) in analysis of the 2 hour measure of alcohol consumption, so this data was combined for analysis (n=28). Paired t-test indicated that CNO treatments significantly increased alcohol consumption at the 2 hour measure (t_{27}=3.4, p=0.002; Figure 4.5K). When analyzing alcohol preference at the 2 hour measure using repeated measure two-way ANOVA, there was a trend toward an interaction effect ($F_{1,26}$=3.9, p=0.06). This appeared to be the result of a strong

increase in alcohol preference in male mice with minimal change in female mice (see Figure 4.5 L, M). At the 24 hour measure, there was a sex effect ($F_{1,26}$=13.01, p=0.0013) where female mice consumed more alcohol than male mice, so sexes were separated for analysis. There were no significant effects of CNO treatment on alcohol consumption in male (t_{12}=0.32, p=0.75) or female (t_{14}=0.018, p=0.88) mice (Figure 4.5 N, O).

Control for off-target effects of acute CNO administration

To control for off-target CNO effects, CNO was administered in heterozygous Tac1-Cre mice without DREADD expression (n=10-16 per sex). For SI ratio, there was no effect of sex ($F_{1,22}$=1.1, p=0.30) nor an interaction ($F_{1,22}$=0.04, p=0.84), so sexes were combined for analysis. Unpaired t-test indicated that CNO had no effect on SI Ratio (t_{24}=0.63, p=0.54; Figure 4.6B). For the elevated plus maze, the open arm time measures had no sex($F_{1,21}$=0.43, p=0.52) or interaction effects($F_{1,21}$=0.36, p=0.55), so they were combined. CNO had no significant effects on percent of time on the open arms (t_{23}=0.35, p=0.72; Figure 4.6C). Similarly, there was no sex difference ($F_{1,21}$=0.48, p=0.49) or interaction effect ($F_{1,21}$=0.62, p=0.44) in percent of open arm entry, so the sexes were again combined. There was no significant effect observed of CNO on percent time spent in the open arms (t_{23}=0.26, p=0.80; Figure 4.6D). There was a trending sex effect observed in the number of stretches onto the open arm ($F_{1,21}$=4.240, p=0.521), where male mice had a greater number of stretches than female mice. When analyzing by sex, there was no significant effect of CNO on open arm stretches in male (t_{8}=0.76, p=0.47; Figure 4.6E) or female mice (t_{13}=1.25, p=0.23; Figure 4.6F). Total arm entries had a significant effect of sex ($F_{1,21}$=5.19, p=0.03) When analyzing by sex, CNO had no significant effect on total arm entry in male mice (t_{8}=0.72, p=0.49; Figure 4.6G) or female mice (F-test to compare variances $F_{6,7}$=6.69, p=0.02; Mann-whitney test p>0.9999; Figure 4.6H)

In a separate cohort of mice, the mice underwent 14 days of alcohol consumption prior to CNO treatments (n=10-16 per sex). No sex ($F_{1,24}$=0.51, p=0.48) or interaction effects ($F_{1,24}$=0.0074, p=0.93) were observed for alcohol consumption at the 2 hour measurement, so data for males and females was combined. Paired t-test indicated that CNO did not impact alcohol consumption at the 2 hour timepoint (t_{25}=0.34, p=0.74; Figure 4.6J). There was no significant sex ($F_{1,23}$=1.80, p=0.19) or interaction ($F_{1,23}$=0.16, p=0.70) effects on alcohol preference at the 2 hour measure, so sexes were combined. There was no observed effect of CNO on alcohol preference at the two-hour measure (t_{24}=0.29, p=0.78; Figure 4.6K). At the 24 hour measure of alcohol consumption, there was again an effect of sex ($F_{1,24}$=16.8, p=0.0004) in the repeated measure two-way ANOVA where female mice consumed more alcohol than their male counterparts. Upon paired t-test analysis of each sex, there was no effect of CNO treatment for male (t_9=0.70, p=0.50) or female (t_{15}=0.13, p=0.90) mice (Figure 4.6L,M).

4.5 Discussion

The above experiments demonstrate that SP innervation to the NAC is activated by chronic SDS, and that these projections mediate the impact of this stress exposure on subsequent alcohol consumption. This effect was observed with both chronic chemogenetic inhibition during stress exposure and following acute inhibition during drinking. There appeared to be no effect of these interventions on social avoidance induced by SDS. Conversely, acute chemogenetic activation of these inputs resulted in increased alcohol consumption with no effect on SI. Based on tract tracing studies and assessments of neuronal activation using Fos labeling, we suspect that the critical source of SP innervation to the NAC is the PVT.

Our anatomical tract tracing studies outline the SP neurons that project to the NAC. Although the NAC has previously been found to have high levels of NK1R expression, to our

knowledge no studies have completed unbiased tract tracing to determine the source of SP innervation to this region. We identified SP-positive cells that send projections to the NAC in the OLF, PVT, PIR, SuM, RLi, and IF. Previous studies have shown that repeated SDS can induce activation of each of these nuclei, but did not assess specific cell types (Matsuda et al., 1996). The PVT has been extensively studied in this regard, and neuronal subpopulations in this region are involved in stress, social avoidance, and alcohol consumption, and thus was a region of primary focus in our studies (Barson et al., 2020; Barson et al., 2017; Beas et al., 2024; Dong et al., 2020; Gao et al., 2020; Robinson et al., 2020). The OLF and PIR have been implicated in both olfactory processing and stress (Athanassi et al., 2023; Kondoh et al., 2016; Shin et al., 2023). The OLF was also assessed in our circuit/Fos experiment, but SDS did not significantly activate the SP neuronal population from this region projecting to the NAC. This is unexpected but suggests that perhaps not all SP neuronal populations that innervate the NAC are involved in stress. Alternatively, these SP projections may be activated on a differential time scale. For example, perhaps SP expressing OLF→NAC neurons are activated with initial stress exposure, but habituate following chronic stress. Also, the additional regions where we observed SP innervation to the NAC were not directly assessed in our Fos study and may contribute in part to the stress response. Future experiments using circuit specific manipulations with local infusion of CNO to specific source regions will begin to assess these effects.

Our previous findings have indicated that the NK1R is involved in stress-induced alcohol seeking and escalated alcohol consumption under multiple experimental conditions in rodents. The experiments presented here agree with these findings and add to this literature. The fact that inhibition of SP neuronal inputs into the NAC either during SDS or during drinking behavior significantly reduced alcohol consumption indicates that inhibition of these inputs can both

prevent as well as rescue stress-induced behavior. Furthermore, chemogenetic activation of these SP neuronal inputs into the NAC increased alcohol consumption in both sexes, although this was observed as a significant increase in preference in males with no change in preference in females. This suggests that there may be a sex difference in the role of SP innervation of the NAC where it drives overall consumption in females but drives preferential consumption of alcohol in males.

Unexpectedly, chemogenetic inhibition of SP inputs to the NAC did not affect SI behavior following stress. This is somewhat incongruent with our previous findings that show that overexpression of the NK1R in the NAC induced increased sensitivity to SDS, as measured by the SI ratio (Solomon et al., 2024). It is important to note, however, that our prior work utilized a standard SDS design, with 10 days of defeat, no female witness mouse, and overnight housing of defeated mice with the aggressor. In this model, approximately 70% of defeated mice exhibit susceptibility, as indicated by SI ratio less than 1. In our modified model that we used here (female witness, isolated housing of defeated mouse overnight between physical defeats, 11 days of defeat total), almost 100% of mice would meet this criterion for susceptibility. Thus, while this major stress is sufficient to induce significant social avoidance and escalated alcohol consumption, the results of exposure to this stressor on social behavior may be more difficult to experimentally manipulate due to its strength. Another interpretation is that SP inputs to the NAC mediate the effects of chronic SDS on alcohol consumption, but not on social behavior, which is instead mediated by SP inputs to a different region, or a different mechanism altogether. Finally, perhaps SP projections to the NAC from a region such as the PIR, which we identified but did not assess in our Fos experiments, could contribute to the effects of SDS on SI.

The effects of our elevated plus maze had inconsistent results. First, control males (Gi/SDS/Control and saline-treated in acute activation experiment) had lower baseline values for

percent time and percent entry into the open arm. This may have contributed to a lack of effect observed in the SDS chemogenetic inhibition experiments, where we did not observe any effect of SDS on EPM. Other researchers have found that SDS increases anxiety and have observed reduced time or entry into open arms as a measure of increased anxiety in stressed mice (Krishnan et al., 2007; Morel et al., 2022; Vialou et al., 2014). However, our experiment may have been influenced by a floor effect where our control mice did not exhibit a high enough time to see a reduction. Regardless, no effects were observed between the chronically administered CNO or the acute administration of CNO in impacting these anxiety measures. Chemogenetic activation resulted in significantly increased time spent in open arms, percentage of entries into the open arm and number of stretches into the open arms only in the male mice. However, the saline-treated male mice had unusually low percent time into open entry which may be driving this effect. One additional concern is that activation of these SP inputs into the NAC significantly increased the number of total entries. This suggests that there may be the confound of increased locomotor activity. Furthermore, there were also baseline sex differences in total entry as observed by a significantly greater total number of entries of male mice compared to female mice in the acute CNO administration control experiment; This suggests there may be a baseline effect of sex which may mean sexes should not be combined for this test in experimental conditions. Furthermore, metanalysis of rodent studies found that the estrus cycle can influence anxiety-like behavior, which we did not assess in our study and may have influenced the results (Pestana & Graham, 2024).

As some studies have proposed that CNO may retroconvert into clozapine, an antipsychotic, to have off-target effects, we designed experiments to directly assess this (Lin et al., 1996; MacLaren et al., 2016; Manvich et al., 2018). For the experiment using chemogenetic

inhibition during stress exposure, we included a non-DREADD expressing group that was exposed to stress and injected with CNO. These mice behaved identically to the Gi DREADD mice exposed to SDS and treated with saline, indicating there were no off-target effects of virus or CNO. Additionally, our control experiment which showed a lack of effect of acute CNO injection in non-DREADD expressing mice strengthens this interpretation.

Although our findings suggest a role of SP inputs to the NAC in the mediation stress-induced alcohol consumption, and our Fos/tracing experiments suggest that these projections originate from the PVT, our chemogenetic interventions affected all SP inputs to the NAC. Additional experiments examining specific circuits will further outline the role of SP output from the PVT in the expression of specific post-stress sequelae. Additionally, a shortcoming of the SDS model is that it is most effectively used in male mice due to the sex specific expression of territorial aggression under most conditions, although some groups have utilized approaches that allow for the use of female subjects (Harris et al., 2018; Newman et al., 2019; Takahashi et al., 2017). Additionally, SDS is a physical defeat which theoretically induces some pain and inflammatory response. Utilizing alternative models of stress such as vicarious social defeat stress (Decker Ramirez et al., 2024; Iniguez et al., 2018; Warren et al., 2020), which can effectively incorporate female mice and utilizes a non-physical stress exposure, may further discern if these findings generalize to other stressful responses.

Altogether, our recent findings have implicated the SP/NK1R system involvement in stress-induced alcohol consumption. Our current study found that inhibition of the SP neuronal inputs to the NAC can either prevent or rescue stress-induced alcohol consumption, demonstrating that targeting this system may have potential therapeutic properties. To show bidirectional control, we activated these neuronal inputs, which resulted in increased alcohol

consumption. Future experiments will focus on circuit specific effects, non-physical stressors, and the effect of these projections on stress response in female mice.

Figures

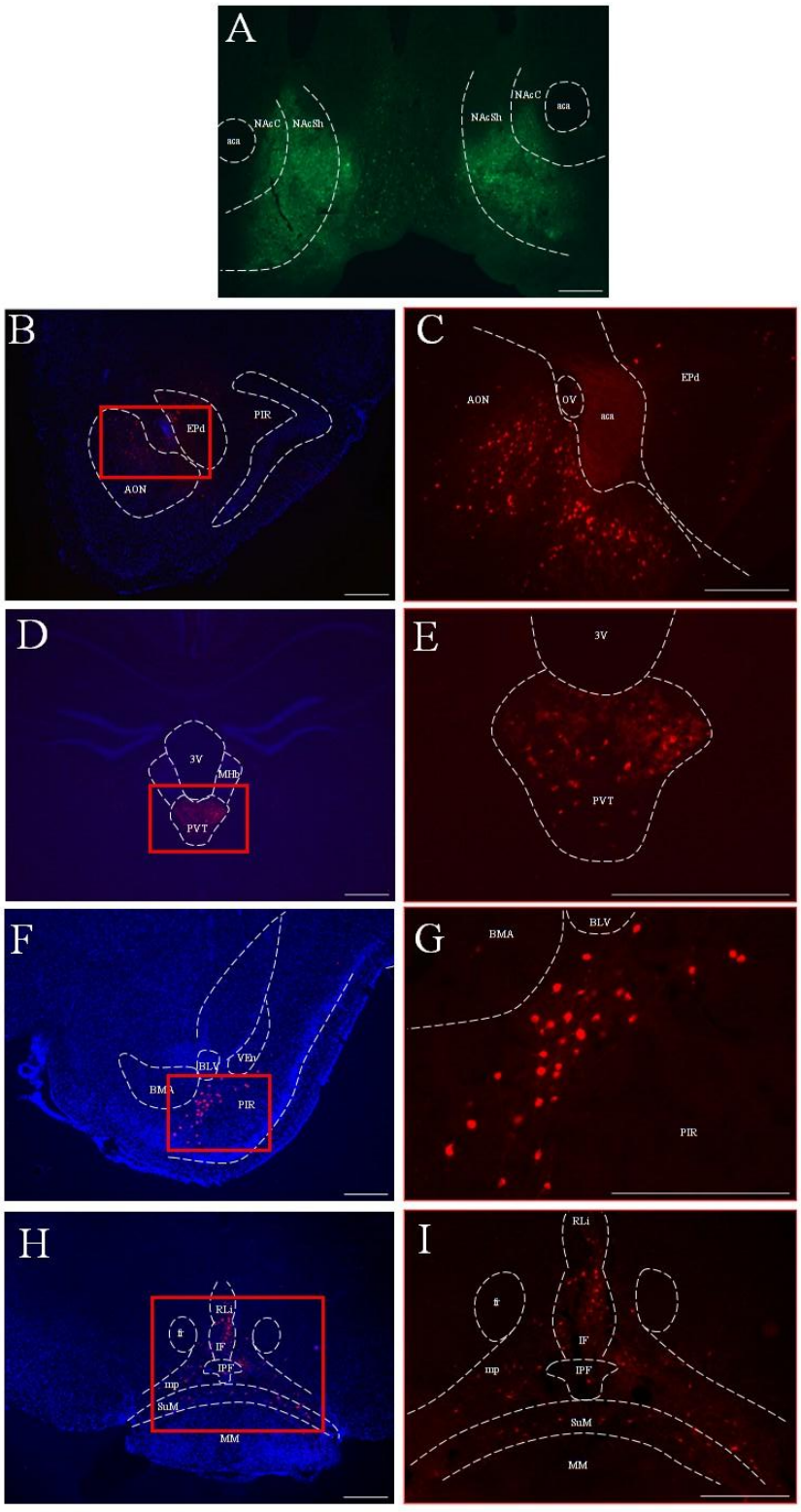

A
NAcC
NAcSh
aca
NAcC
NAcSh
aca
B
EPd
PIR
AON
C
AON
OV
aca
EPd
D
3V
MHb
PVT
E
3V
PVT
F
BLV
VEn
BMA
PIR
G
BMA
BLV
PIR
H
RLi
fi
IF
IPF
mp
SuM
MM
I
RLi
fi
IF
mp
IPF
SuM
MM

Figure 4.1. Tract tracing of substance P neurons that project to the NAC. (A) Representative images of viral placement within the NAC. Representative images of OLF (B-C), PVT (D-E), and PIR (F-G). Representative images containing SuM, RLi, and IF (H-I). Scale bars represent 200 μm.

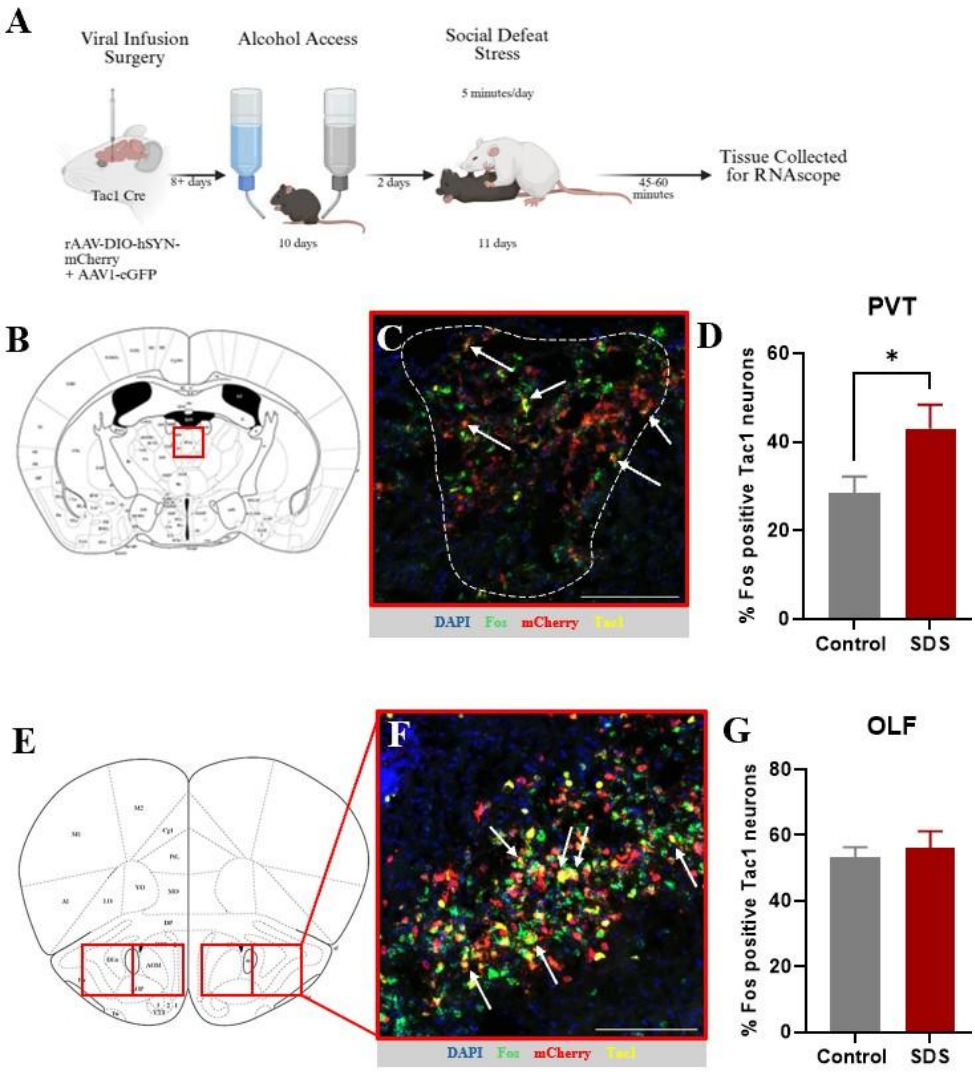
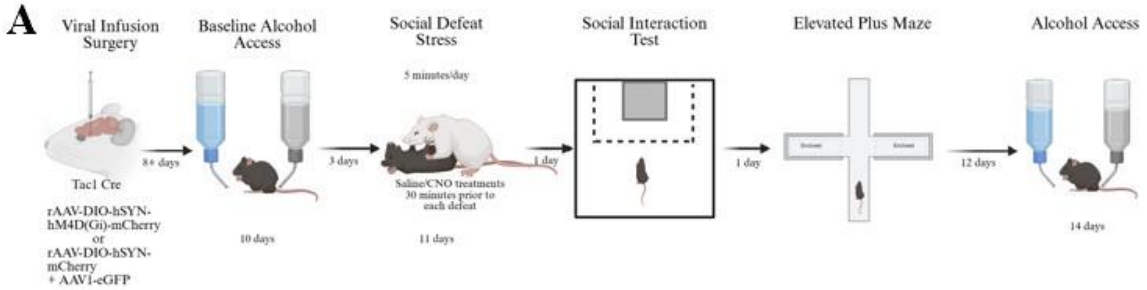

A
Viral Infusion Surgery
Alcohol Access
Social Defeat Stress
5 minutes/day
Tac1 Cre
8+ days
2 days
45-60 minutes
Tissue Collected for RNAscope
rAAV-DIO-hSYN-mCherry + AAV1-eGFP
10 days
11 days
B
C
DAPI Fos mCherry Tac1
D
PVT
% Fos positive Tac1 neurons
60
40
20
0
*
Control
SDS
E
F
DAPI Fos mCherry Tac1
G
OLF
% Fos positive Tac1 neurons
80
60
40
20
0
Control
SDS

Figure 4.2. SDS-induced activation of SP inputs to NAC. (A) Experimental timeline created with BioRender.com. (B) Reference image from Paxinos and Franklin (2004) to indicate site of RNAscope image. (C) Representative image of RNAscope staining of the PVT. (D) SDS increased activation of SP neurons of the PVT which project to the NAC (t_{14}=2.2, p=0.04). (E) Reference image from Paxinos and Franklin (2004) to indicate location of RNAscope image. (F) Representative image of RNAscope staining of the OLF. (G) SDS did not alter activation of SP neurons of the OLF which project to the NAC (t(14)=0.52, p=0.61). Scale bars represent 200 um. *p<0.05

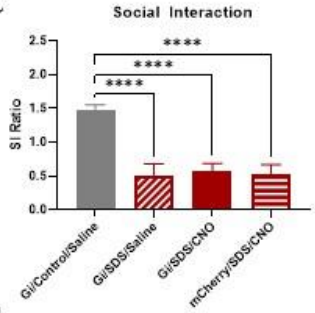
A



B

Virus	Stress	Treatment	Abbreviation	Bar
Gi DREADD	No	Saline	Gi/Control/Saline	
Gi DREADD	Yes	Saline	Gi/SDS/Saline	
Gi DREADD	Yes	CNO	Gi/SDS/CNO	
mCherry	Yes	CNO	mCherry/SDS/CNO	

C



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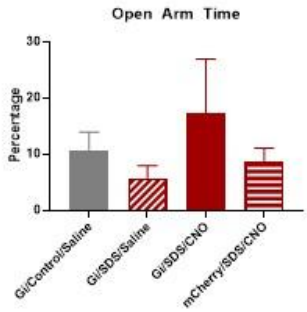

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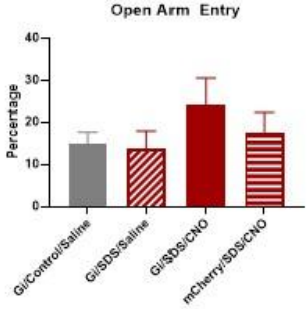

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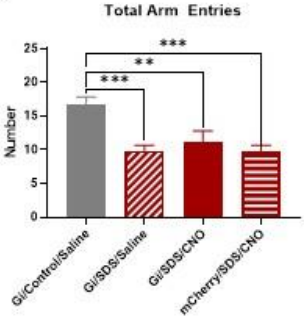

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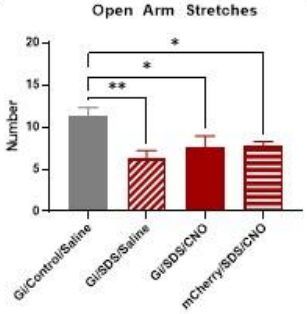

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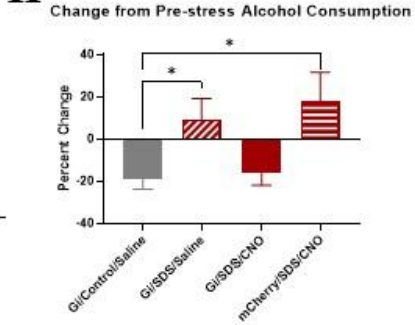


Figure 4.3. Inhibition of SP innervation of the NAC during SDS. (A) Experimental timeline created with BioRender.com. (B) Legend of treatment groups. (C) Social interaction following SDS ($F_{3,40}$=15.9, p<0.0001), where each defeated group had significantly reduced SI ratio compared to the Gi/Control/Saline group (p<0.0001). (D) Percent open arm time(Bartlett's test <0.0001, Kruskal-Wallis test p=0.52). (E) Percent open arm entries ($F_{3,40}$=1.021, p=0.39). (F) Significant effect in total arm entries ($F_{3,40}$=8.86, p=0.0001), where each defeated group had significantly reduced arm entries compared to the control (p<0.05 for all). (G) Significant effect on stretches into the open arm ($F_{3,40}$=5.36, p=0.003), where each defeated group had significantly reduced number of stretches compared to the Gi/Control/Saline group (p<0.05 for all). (H) Percent change from baseline alcohol consumption. mCherry/SDS/CNO (p=0.03) and Gi/SDS/Saline (p=0.01) groups significantly differed from the Gi/Control/Saline group. *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001

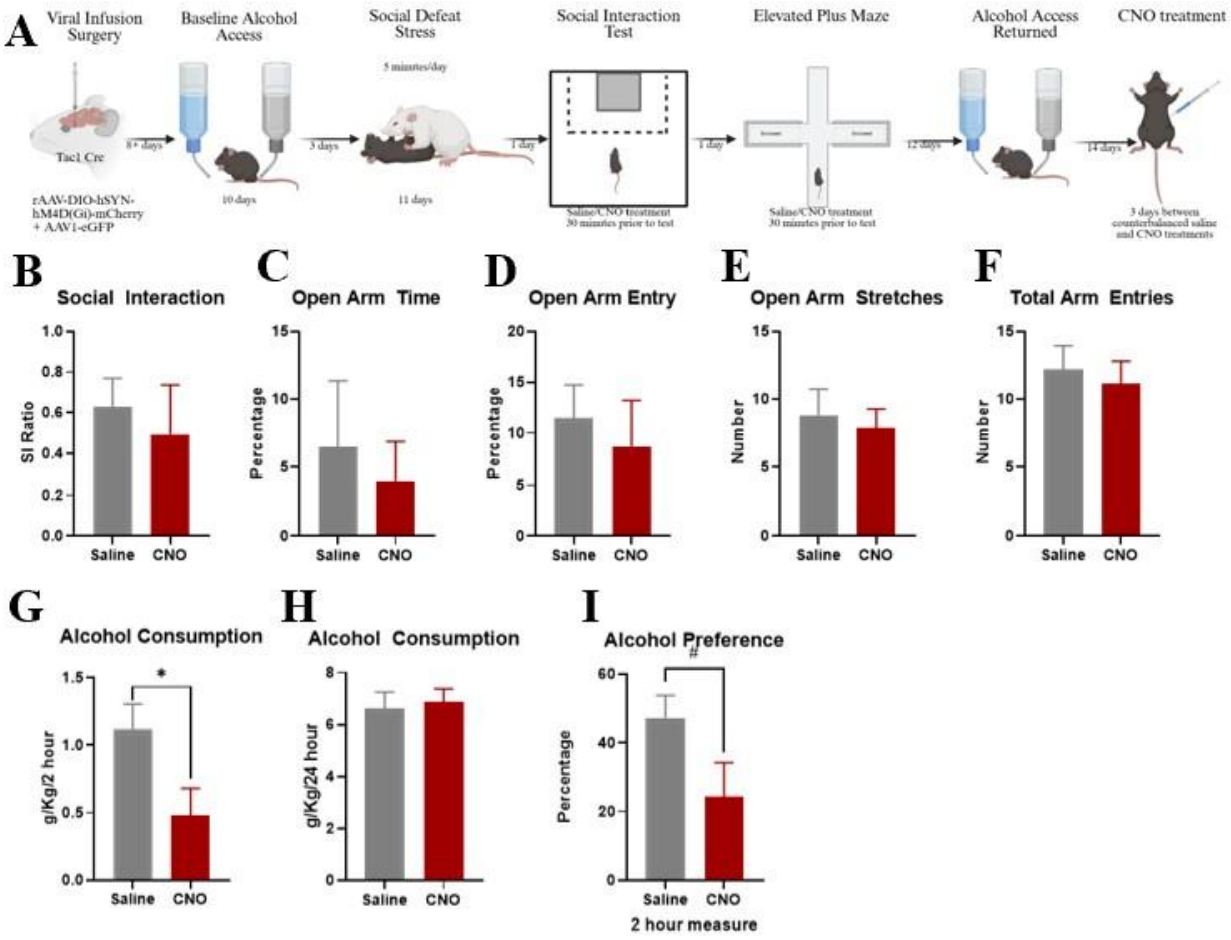

A
Viral Infusion Surgery
Baseline Alcohol Access
Social Defeat Stress
Social Interaction Test
Elevated Plus Maze
Alcohol Access Returned
CNO treatment
Tac1 Cre
rAAV-DIO-hSYN-hM4D(Gi)-mCherry + AAV1-eGFP
8+ days
10 days
3 days
5 minutes/day
11 days
1 day
Saline/CNO treatment 30 minutes prior to test
1 day
Saline/CNO treatment 30 minutes prior to test
12 days
14 days
3 days between counterbalanced saline and CNO treatments
B
Social Interaction
SI Ratio
Saline
CNO
C
Open Arm Time
Percentage
Saline
CNO
D
Open Arm Entry
Percentage
Saline
CNO
E
Open Arm Stretches
Number
Saline
CNO
F
Total Arm Entries
Number
Saline
CNO
G
Alcohol Consumption
g/Kg/2 hour
*
Saline
CNO
H
Alcohol Consumption
g/Kg/24 hour
Saline
CNO
I
Alcohol Preference
#
Percentage
Saline
CNO
2 hour measure

Figure 4.4. Post-SDS acute inhibition of SP innervation of the NAC. (A) Experimental timeline for inhibition of SP innervation of the NAC during behavior created with BioRender.com. (B) CNO treatment had no effect on the SI ratio (t_{26}=1.4, p=0.18). (C) Percent open arm time (t_{10}=0.46, p=0.66). (D) Percent open arm entry (t_{10}=0.47, p=0.65). (E) Number of open arm stretches (t_{10}=0.41, p=0.69). (F) Total arm entries (t_{10}=0.43, p=0.68). (G) CNO significantly reduced alcohol consumption at the two hour measure (t_{11}=2.7, p=0.02). (H) There was no difference between CNO and saline treatment on the 24 hour measure of alcohol consumption (t_{11}=0.45, p=0.66). (I) There was a trending effect of CNO on alcohol preference at the 2 hour measure (t_{11}=1.8, p=0.10).

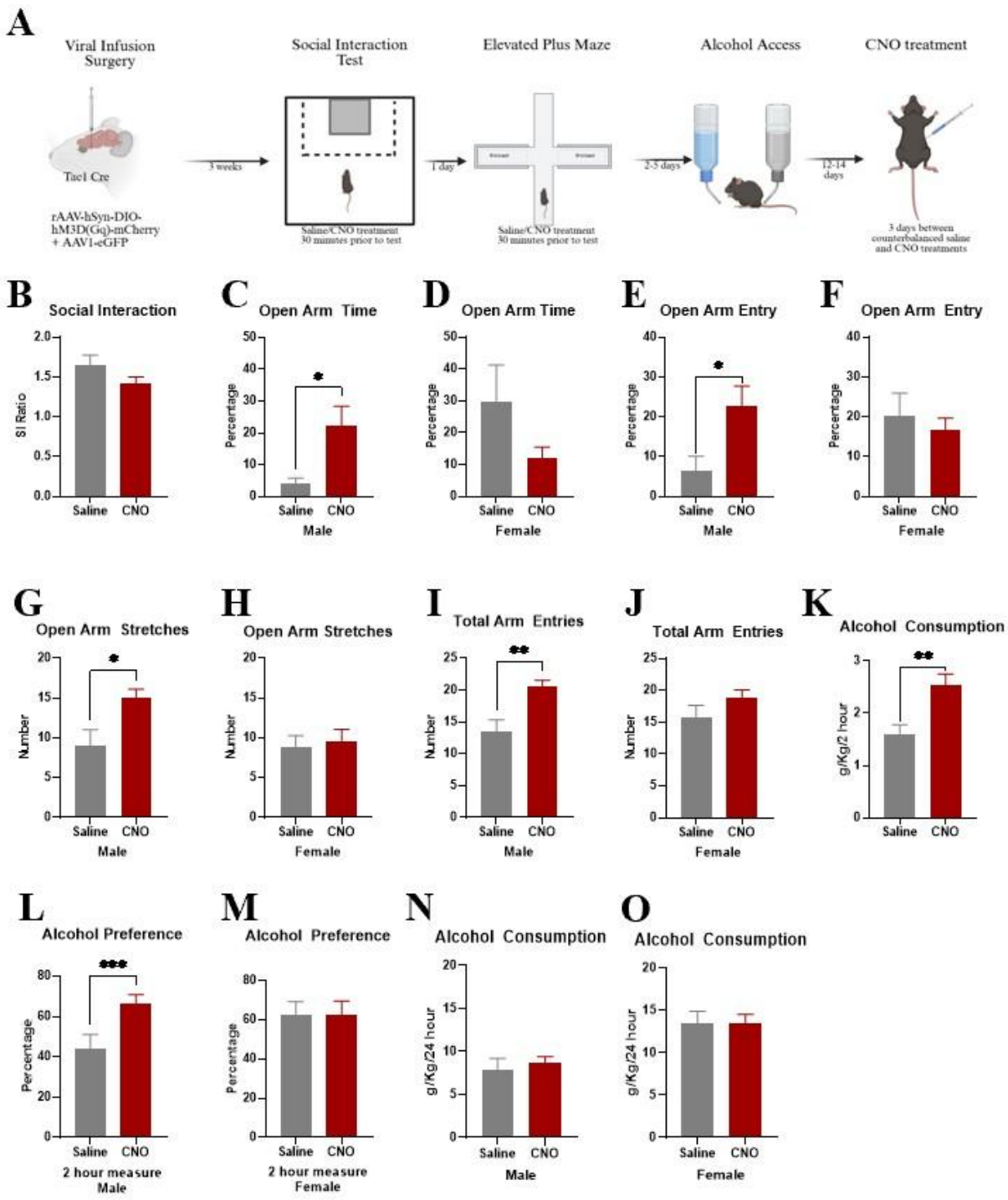

A
Viral Infusion Surgery
Social Interaction Test
Elevated Plus Maze
Alcohol Access
CNO treatment
Tac1 Cre
rAAV-hSyn-DIO-hM3D(Gq)-mCherry + AAV1-eGFP
3 weeks
Saline/CNO treatment 30 minutes prior to test
1 day
Saline/CNO treatment 30 minutes prior to test
2-5 days
12-14 days
3 days between counterbalanced saline and CNO treatments
B
Social Interaction
SI Ratio
Saline CNO
C
Open Arm Time
Percentage
Saline CNO
Male
D
Open Arm Time
Percentage
Saline CNO
Female
E
Open Arm Entry
Percentage
Saline CNO
Male
F
Open Arm Entry
Percentage
Saline CNO
Female
G
Open Arm Stretches
Number
Saline CNO
Male
H
Open Arm Stretches
Number
Saline CNO
Female
I
Total Arm Entries
Number
Saline CNO
Male
J
Total Arm Entries
Number
Saline CNO
Female
K
Alcohol Consumption
g/Kg/2 hour
Saline CNO
L
Alcohol Preference
Percentage
Saline CNO
2 hour measure
Male
M
Alcohol Preference
Percentage
Saline CNO
2 hour measure
Female
N
Alcohol Consumption
g/Kg/24 hour
Saline CNO
Male
O
Alcohol Consumption
g/Kg/24 hour
Saline CNO
Female

Figure 4.5. Acute chemogenetic activation of SP innervation of the NAC.

(A)Timeline of experiment created in Biorender.com. (B) CNO treatment had no affect on SI Ratio (t_{26}=1.4, p=0.18). (C) On the elevated plus mace, CNO increased percent open arm time in male mice(F-statistic difference in variation $F_{7,4}$=18.56, p=0.013Mann-Whitney test p=0.04). (D)There was no effect of CNO on percent open arm time in female mice (F-statistic analysis of variation $F_{7,6}$=0.006, Mann-whitney test p=0.61). (E) Percent open arm entry was increased in male mice treated with CNO (t_{11}=2.36, p=0.03). (F) CNO treatment did not alter percent open arm entry in female mice (t_{13}=0.34, p=0.74). (G) CNO treatment increased stretches onto the open arm in male mice (t_{11}=2.85, p=0.02). (H)Stretches onto the open arm in female mice was not impacted by treatment (t_{13}=0.34, p=0.74). (I)CNO increased total arm entries in male mice(t_{11}=3.579, p=0.0043), but (J) did not significantly affect total arm entry in female mice (t_{13}=1.347, p=0.2011). (K) CNO treatment increased alcohol consumption at the two-hour measure (t_{27}=3.4, p=0.002). (L) CNO increased alcohol preference in male mice at this 2 hour measure(t_{12}=4.45, p=0.0008). (M) CNO did not alter female alcohol preference at the 2 hour measure (t_{14}=0.014, p=0.99). (N) CNO did not impact 24 hour alcohol consumption in male mice (t_{12}=0.32, p=0.75) or (O) in female mice (t_{14}=0.018, p=0.88).

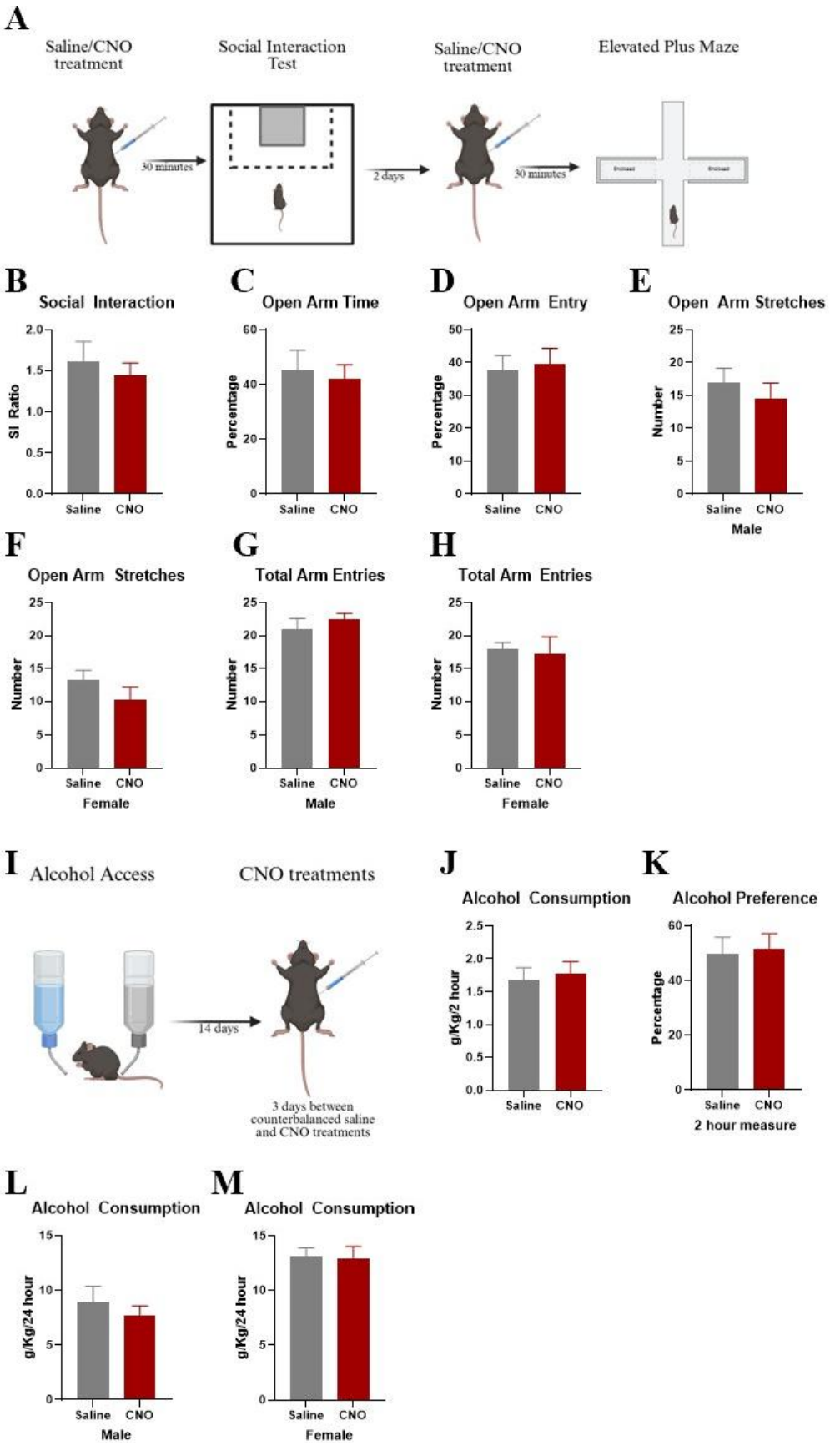

A
Saline/CNO treatment
Social Interaction Test
Saline/CNO treatment
Elevated Plus Maze
30 minutes
2 days
30 minutes
B
Social Interaction
SI Ratio
Saline
CNO
C
Open Arm Time
Percentage
Saline
CNO
D
Open Arm Entry
Percentage
Saline
CNO
E
Open Arm Stretches
Number
Saline
CNO
Male
F
Open Arm Stretches
Number
Saline
CNO
Female
G
Total Arm Entries
Number
Saline
CNO
Male
H
Total Arm Entries
Number
Saline
CNO
Female
I
Alcohol Access
CNO treatments
14 days
3 days between counterbalanced saline and CNO treatments
J
Alcohol Consumption
g/Kg/2 hour
Saline
CNO
K
Alcohol Preference
Percentage
Saline
CNO
2 hour measure
L
Alcohol Consumption
g/Kg/24 hour
Saline
CNO
Male
M
Alcohol Consumption
g/Kg/24 hour
Saline
CNO
Female

Figure 4.6. Acute CNO administration control experiment. (A)Experimental timeline of acute administration of counterbalanced saline/CNO treatments prior to behavioral measures created in Biorender.com. (B) CNO treatment did not affect SI Ratio (t_{24}=0.63, p=0.54). CNO administration did not impact percent of open arm time (C) (t_{23}=0.35, p=0.72) or percent of open arm entry (D)(t_{23}=0.26, p=0.80) CNO did not impact number of stretches into the open arms in male (E) (t_{8}=0.76, p=0.47) or female mice (F) (t_{13}=1.25, p=0.23). CNO did not impact total arm entries in male mice (G)(t_{8}=0.72, p=0.49) or female mice (H)(F-test to compare variances $F_{6,7}$=6.69, p=0.02; Mann-Whitney test p>0.9999). (I)Experimental timeline of acute administration of counterbalanced saline/CNO treatments during alcohol consumption created in Biorender.com. CNO treatment did not impact alcohol consumption (J)(t_{25}=0.34, p=0.74) or alcohol preference (K)(t_{24}=0.29, p=0.78) at the 2 hour timepoint. At the 24 hour timepoint, CNO treatment did not impact alcohol consumption in male mice (L)(t_{9}=0.70, p=0.50) or female mice (M) (t_{15}=0.13, p=0.90).

CHAPTER 5

SUMMARIES AND CONCLUSIONS

5.1 Overview

AUD affects millions of people in the U.S. each year. As current treatments are either not well utilized or are ineffective for many people, better understanding of the mechanisms which drive AUD and discovery of potential therapeutic targets are extremely important. SP-NK1R is involved in various mechanisms, but it has been particularly studied for its role in stress and inflammation. SP-NK1R may act as a bridge between stress and inflammation and may serve as an interesting target for conditions such as MDD and AUD which are both influenced by stress and inflammation. Many studies support that women may have greater physiological responses to stress and stronger inflammatory reactions, so better studying this system in female models is particularly important.

The aim of this dissertation was to better characterize SP and the NK1R in various rodent models of alcohol use. Furthermore, models that included female mice were also utilized to characterize the ability of NK1R antagonists to work in the female sex. The described studies furthered the understanding of SP-NK1R as a potential therapeutic target for AUD.

5.2 Summaries of Conducted studies

The effects of Lipopolysaccharide on social interaction, cytokine expression, and alcohol consumption in male and female mice

Chapter 2 utilized a model of inflammation to induce depressive-like phenotypes and alcohol consumption. Only female social interaction was found to be reduced following LPS-

administration which aligns with females having a more prolonged inflammatory response to LPS. LPS induced increased inflammation as measured by IL-6 and TNFα upregulation in the HPC, PFC, and VS 24 hours following administration. Trending or significant effects were observed in HPC TNFα expression and VS IL-6 expression, where LPS-treated female mice had significantly greater expression in response to LPS compared to the LPS-treated male mice. This aligns with other research that demonstrates female mice can have greater or more prolonged inflammatory responses to LPS (Dockman et al., 2022; Klein & Flanagan, 2016; Sharma et al., 2018).

In another experiment, LPS induced increased alcohol consumption in both male and female mice. The NK1R antagonist was administered, but only significantly reduced alcohol consumption in both saline and LPS-treated female mice. This suggests that NK1R antagonism may particularly be effective in females, which are known to have more upregulated inflammatory response, and suggests that female mice may be more sensitive to this dose of the NK1R antagonist. The main findings of this chapter are illustrated in Figure 5.1.

Vicarious Defeat Stress induces increased alcohol consumption in female mice: Role of neurokinin-1 receptor and interleukin-6

Chapter 3 utilized a model of vicarious social defeat stress (VDS) to study stress in female mice. As traditional SDS does not work for female mice as male mice will not be territorially aggressive towards them, this model allows the female mice to witness the defeat of another male mouse while not in any physical danger themselves. This model may be a more translationally as it relates more to a psychological stressor which people are more likely to experience. As discussed previously, inflammation can occur in response to stress models and can be associated with stress-induced depressive-like phenotypes. This experiment found

increased IL-6 expression in the PFC and HPC of the VDS-treated female mice. These experiments additionally found that VDS induced decreased social interaction time and increased alcohol consumption and alcohol preference. The change in alcohol consumption negatively correlated with their social interaction time. Figure 5.2 highlights these results.

Substance P innervation of the nucleus accumbens mediates alcohol consumption following chronic social stress

The aim of chapter 4 was to better characterize and understand the role of the SP projections to the nucleus accumbens. This builds on past research indicating that the NK1R within the NAC is particularly important for stress susceptibility as well as stress-induced alcohol consumption. This study determined regions which project SP to the NAC and looked at activation of 2 circuits of regions which are known to be involved in stress: PVT and OLF →NAC. We found that PVT→NAC projections were activated by SDS. Chemogenetic inhibition during SDS prevented SDS-induced alcohol consumption. Chemogenetic inhibition during alcohol consumption following SDS also reduced drinking. This indicates that inhibition of these SP inputs can prevent or rescue behavior. Meanwhile, acute chemogenetic activation of these inputs resulted in increased alcohol consumption for both sexes although preference was only increased in male mice. There was no impact on social interaction. There were mixed effects observed on the elevated plus maze which limited conclusions. The conclusions of this chapter are further illustrated in figure 5.3.

5.3Role of inflammatory markers in VDS and LPS models

Inflammation has been associated with both MDD and AUD. The VDS and LPS experiments demonstrated that LPS or VDS induced increased inflammatory markers within certain regions. LPS-induced elevated TNF alpha and IL6 gene expression within each region tested, indicating that it may induce brain-wide vs. regional inflammation as seen in VDS-treated

mice compared to controls. IL6 was increased by LPS and VDS in the hippocampus (HPC) and prefrontal cortex (PFC). This is particularly interesting as inflammation within the HPC reduces neurogenesis and long-term potentiation and may contribute to decreased neurogenesis as observed in patients with MDD (Boldrini et al., 2019; Boldrini et al., 2013). Meanwhile, these parameters within the PFC may indicate dysfunctional excitatory/inhibitory balance. As these were the only regions measured that were impacted by VDS at the same time-point as the post-VDS SI test, this indicates that IL6 expression in these regions may particularly influence this depressive-like measure in female mice. Meanwhile, as no effects were observed in LPS-treated male mice in the SI test, this could suggest that IL6 or TNF alpha in these regions do not impact this behavior in male rodents. This is further supported by studies indicating that only women's depressive symptoms correlate with inflammatory markers (Elgellaie et al., 2023). Alternatively, this behavior may be impacted at earlier timepoints in the male mice, closer to their peak LPS response.

5.4 Role of NK1R antagonists in reducing alcohol consumption in LPS and VDS models

As a mechanism involved both in stress and inflammatory mechanisms, the NK1R is a particularly interesting target for conditions like MDD and AUD which are influenced by both stress and inflammation. Interestingly, NK1R antagonism reduced alcohol consumption in LPS and saline-treated female rodents. However, no effects were observed in LPS-treated male mice. As many studies have found that non-escalated drinking in male mice is not necessarily impacted by NK1R antagonism, the lack of the effects in the saline-treated males is unsurprising. However, this suggests that the anti-inflammatory action of antagonizing the NK1R is only effective in female mice. Similarly, both control and VDS-treated mice had significantly reduced alcohol consumption. This suggests that in both an inflammatory model and in a stress model, an NK1R antagonist is effective in reducing alcohol consumption in female mice. However, as the

saline-treated or control female mice also have a reduction in alcohol consumption, there may be a sex difference which contributes to increased sensitivity to NK1R antagonists in female mice. More research into the sex differences of this system will better elucidate cause of these findings.

5.5 Current understanding of SP circuitry involvement in stress and alcohol consumption

Substance P has been studied as a stress-related neuropeptide within the central nervous system that may contribute to alcohol use. Past research from our lab has greatly implicated the NK1R in the nucleus accumbens as particularly important in stress susceptibility and alcohol consumption, and a previous study found that forced swimming stress can significantly increase SP release into the NAC (Berton et al., 2007; Solomon et al., 2024). The current study advanced our knowledge of which regions project substance P to the nucleus accumbens and assessed SDS-induced activation in some brain regions. Importantly, this study demonstrated a bidirectional influence on alcohol consumption by SP in the NAC. By demonstrating the prevention of the SDS-induced increase in alcohol consumption, this study demonstrates a causal relationship of SDS-induced SP signaling in the NAC and subsequent alcohol consumption.

As the previous study had implicated the NK1R involvement in inducing stress susceptibility as measured by social interaction and alcohol consumption, this study demonstrated that inhibition of the SP inputs would prevent stress-induced alcohol consumption. Interestingly, our study found no impact of chemogenetic activation or inhibition on the social interaction behavior. One explanation is that our retrograde virus has a hSyn promotor, making it neuron specific. While this is beneficial in determining neuronal circuit involvement, it does not account for local microglia within the NAC that may also be releasing SP. Another contributing factor may be that the current study utilized different coordinates and hit both the core and shell within the NAC whereas the previous study looked at NK1R overexpression specifically within

the NAC shell. Furthermore, the inclusion of additional behavioral tests as described in section 5.6 may better illustrate if SP in the NAC influences other depressive-like phenotypes or if it only consistently impacts alcohol consumption.

5.6 Limitations and Future Directions

Although these findings have greatly improved the validity of NK1R as a target for alcohol use, there are some limitations of these studies. Additional experiments, described below, will aid in drawing a more causal relationship between SP-NK1R involvement in inflammation and stress-driven alcohol consumption.

Our study found that a single administration of LPS can escalate alcohol consumption in both male and female mice. This single administration timeline should allow for better analysis of LPS-induced changes. One limitation is that our study regarding gene expression of inflammatory markers was taken 24 hours following LPS administration whereas alcohol access was returned 3 days following administration. An additional timepoint to observe these inflammatory markers at 3-days post-treatment may draw a better conclusion at ongoing inflammation which may influence the alcohol consumption. It is also important to note that all markers were still significantly elevated at the 24 hour timepoint, but that some of these markers may be resolved 3-days post-LPS. To further determine a role of SP in the LPS-induced alcohol consumption, NK1R antagonist treatment either before the LPS treatment or between the LPS treatment and return to alcohol access may help determine a more causal role of SP in LPS-induced alcohol consumption.

Similarly, NK1R antagonism reduced alcohol consumption in female mice following VDS; however, there is no causal relationship between VDS and the NK1R or SP. Determining an alteration of gene or protein expression of NK1R post-VDS would better illuminate a specific

role of SP-NK1R in the stress vs. a general role in alcohol consumption. Although we have previously shown that SDS increases NK1R in the NAC, this experiment could include other regions that have demonstrated NK1R expression and involvement in alcohol consumption such as the central amygdala. Furthermore, NK1R antagonism during stress may demonstrate if SP is driving the effects of VDS in female mice. Furthermore, as discussed below, analyzing activation of SP innervation of the NAC during VDS may help illuminate a direct role of SP in VDS.

Another limitation is that little is known in regards to sex differences seen in the SP-NK1R system. In our studies, female mice seem to be more sensitive to the NK1R antagonists. Furthermore, female mice had no change in alcohol preference although they significantly increased alcohol consumption when activating SP inputs into the NAC, indicating that there may be a difference in the SP circuitry compared to male mice. Several factors may contribute to this such as difference in NK1R expression or difference in NK1R expression on varying cell types. There could be a different amount of SP released or a different number of neurons which release SP. Importantly, future behavioral studies should include observation of the estrous cycle as the estrous cycle has been shown to influence both serum and regional expression of SP (Duval et al., 1996). Tracking the estrous cycle may determine if there is a hormonal influence involved in NK1R antagonist response in female mice.

To better illustrate the role of specific circuit involvement in SDS, the Fos activation experiment should be repeated with the other regions which project SP to the NAC. Many of the other observed regions may be of particular interest for their involvement in stress and stress-induced behavior. For example, the supramammillary nucleus of the hypothalamus (SuM) is involved in coping behaviors, and the piriform cortex has been shown to be activated by stress in other studies (Escobedo et al., 2024; Matsuda et al., 1996; Okuda et al., 2025). Analyzing these

specific areas may determine additional circuits of interest. Another confounding variable of SDS is that defeats can induce pain and injuries. This is important as certain circuits such as glutamatergic PVT→NAC projections have been shown to be activated by pain (Liu et al., 2025). Utilizing another stressor which induces similar effects on behavior and alcohol consumption, such as VDS, may better illustrate that differences in neuronal activation are stress-specific rather than a pain response.

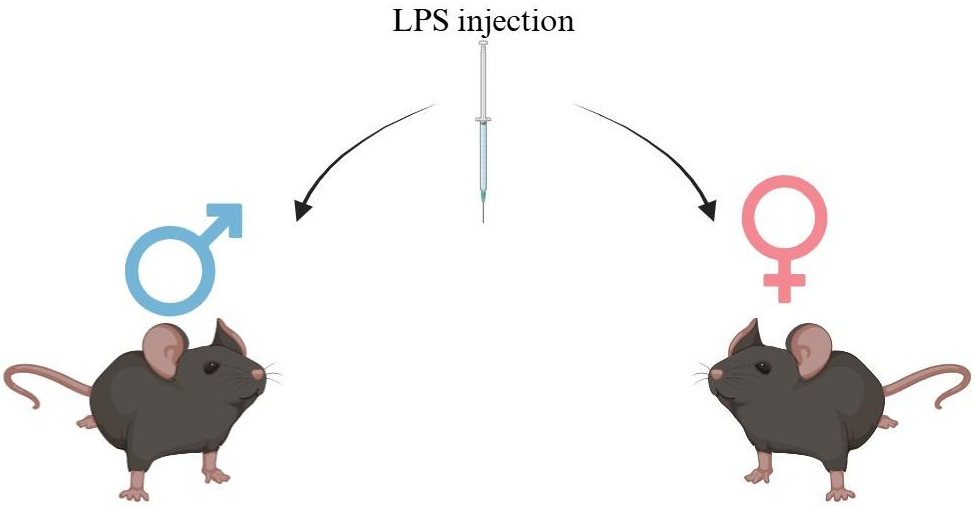
Furthermore, this study determined that inhibition or activation of SP innervation of the NAC bidirectionally impacts alcohol consumption. However, the current study utilized activation or inhibition of all SP inputs. Future experiments should utilize the same viral infusion techniques with the addition of a cannula placement into the region of the specific circuit of interest (e.g. PVT to determine PVT→NAC circuit effects) will tease apart specific circuit involvement and may uncover that only specific regional projections to the NAC play a role. Inclusion of additional behavioral measures may identify circuit-specific influences on different behaviors. For example, utilizing a tail suspension test for passive-coping behavior may be particularly helpful in determining effects of the SuM→NAC SP projections, as this region is involved in active coping behaviors. Additionally, utilizing the same viral technique for chemogenetic activation and implanting a cannula within the NAC to allow administration of the NK1R antagonist following CNO administration would further support that the observed effects are specifically due to SP rather than other co-expressed neurotransmitters such as glutamate.

5.7 Conclusions

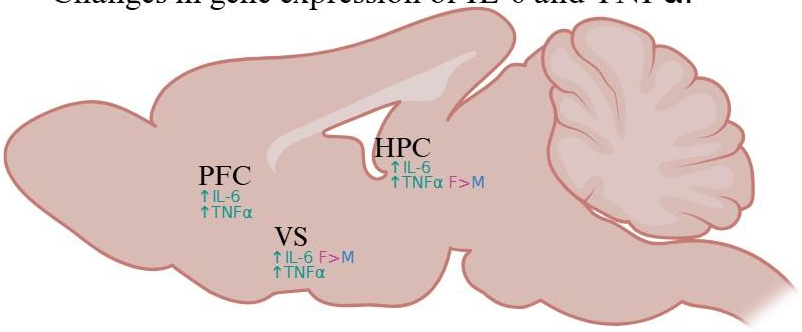
Chapter 2 and 3 have further validated two new models of escalated alcohol consumption. Additionally, these studies demonstrated that the neurokinin-1 receptor antagonist reduced LPS-induced and VDS-induced alcohol consumption in female mice. Furthermore, chapter 4 better

characterized the SP projections to the NAC and determined the SP-NK1R system is involved in SDS-induced alcohol consumption in male mice. Together these studies demonstrate SP and the NK1R may contribute to stress and inflammation-induced alcohol consumption. As NK1R antagonism is effective in reducing alcohol consumption across various models and has now been further studied in female rodents, these findings further support a potential therapeutic target of NK1R for AUD.

LPS: Model of inflammation/immune response



- ↓ SI time in female mice
- Changes in gene expression of IL-6 and TNFα:



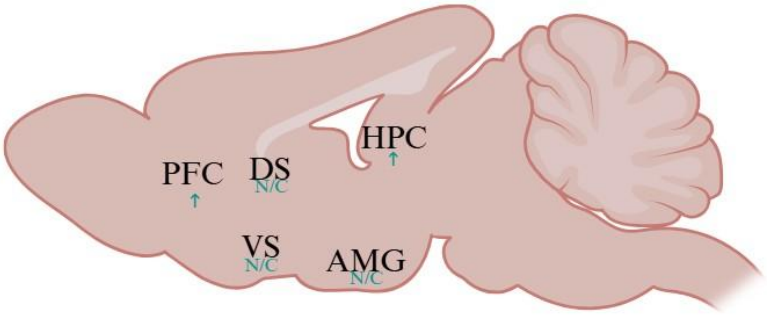
- LPS ↑alcohol consumption in both sexes
- NK1R antagonist treatment ↓ alcohol consumption only in female mice

Unknown: Sex difference in NK1R expression, distribution, or function which contributes to sex difference in response

Figure 5.1 Conclusions of chapter 2. Aligning with past results, female mice appeared more sensitive to LPS as evident in the decreased social interaction (SI) time and the change in proinflammatory gene expression. LPS increased both interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) in each brain region observed: prefrontal cortex (PFC), ventral striatum (VS), and hippocampus (HPC). Female mice had a more profound increase of IL-6 in the VS and TNFα in the HPC in response to LPS compared to LPS-treated male mice. LPS increased alcohol consumption in both sexes, but the neurokinin-1 receptor (NK1R) antagonist only significantly reduced alcohol consumption in female mice. Sex differences in NK1Rs unknown which may contribute to this observed sex difference in response to NK1R antagonist treatment.

VDS: novel stress model for psychosocial stress in female mice

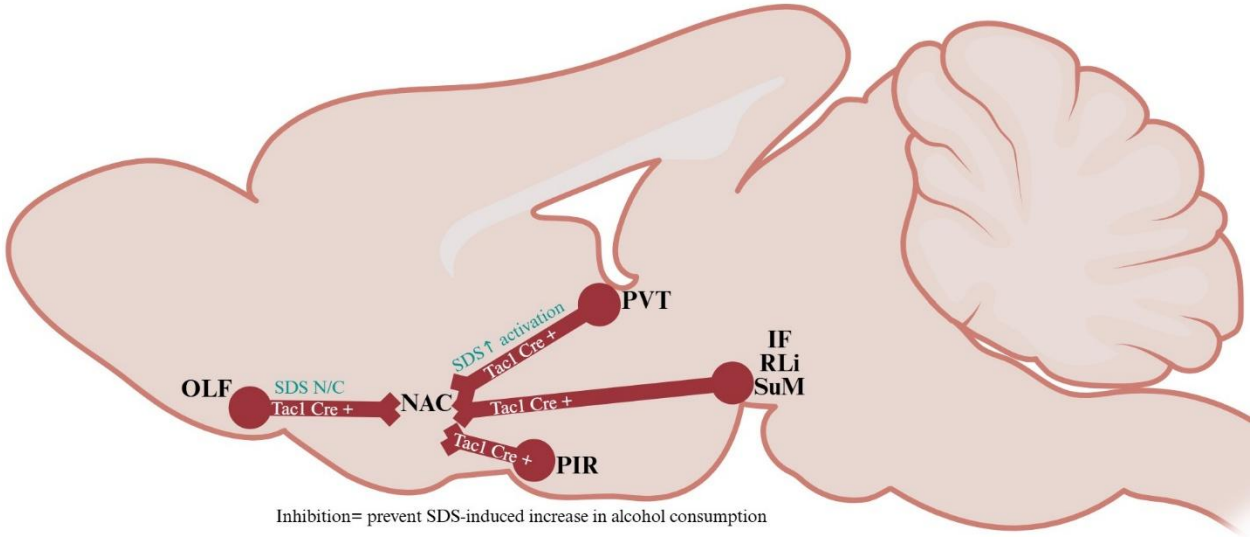
- Changes in IL-6 expression:



- ↓ SI time
- ↑alcohol consumption
- NK1R antagonist treatment ↓ alcohol consumption

Unknown:
- Male response to VDS
- Circuitry involved in VDS

Figure 5.2 Conclusions of chapter 3. Vicarious social defeat stress (VDS) elicited several stress-induced phenotypes in female mice. Following VDS, female mice had increased interleukin 6 (IL-6) expression in the hippocampus (HPC) and prefrontal cortex (PFC) with no change (N/C) observed in the ventral striatum (VS), dorsal striatum (DS), or amygdala (AMG). VDS reduced social interaction (SI) time and increased alcohol consumption. The neurokinin-1 receptor (NK1R) reduced alcohol consumption. It is currently unknown if male mice would exhibit these same results or which circuits are involved in VDS.



Inhibition= prevent SDS-induced increase in alcohol consumption

Activation= increased alcohol consumption

Unknown: circuit specific roles in behavioral phenotypes

Figure 5.3 Conclusions of chapter 4. Utilizing a retrograde, cre-dependent mCherry virus infused into the nucleus accumbens (NAC) of a Tac1 mouse, we found several regions with cell bodies expressing mCherry, indicating they express substance P (SP) and project to the NAC. These regions include the olfactory area (OLF), the piriform cortex (PIR), the interfascicular nucleus (IF), the rostral linear nucleus (RLi), the supramammilary nucleus of the hypothalamus (SuM), and the paraventricular nucleus of the thalamus (PVT). We analyzed fos activation of the PVT→NAC and the OLF→NAC SP projections and found that the PVT→NAC projections were activated by SDS. Through chemogenetic inhibition, SDS-induced alcohol consumption could be prevented or reduced. Through chemogenetic activation, mice increased alcohol consumption. Circuit-specific effects on behavior is currently unknown but will be tested in future experiments.

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