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Neural Mechanisms of Estrogen and Inflammation
in Female Stress Susceptibility

By

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Bachelor of Arts
Clemson University, 2014

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DEDICATION

This dissertation is dedicated to four phenomenal humans who have inspired and supported me in the most magnificent ways, and whom I miss very dearly. First, to my late grandparents, Gramma Terri and Grampa Ken: thank you for giving me the most genuine love I could ever imagine and doing everything for me that you possibly could to facilitate my dreams. To my late aunt, Aunt Margie: no one has been prouder of my accomplishments than you and I will forever appreciate your intensely loving presence in my life. To my late grandmother-in-law, Grandma Luca: I am so grateful for your immeasurable joy and encouragement in me becoming “the docta of the family”. I strive to honor each of your memories every day in showing integrity, humbleness, and thoughtfulness in my roles as a woman, mother, wife, and, especially, scientist.

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ABSTRACT

Posttraumatic stress disorder (PTSD) and major depressive disorder (MDD) are highly debilitating psychiatric disorders that notably share stress as an etiological factor. Stress is a common occurrence experienced daily by virtually everyone and, while most people are resilient, a substantial portion of the population exhibit risk factors that yield them susceptible to developing stress-related psychiatric disorders. One prominent factor that has long been known to increase this risk is the female biological sex with women presenting at least twice as often with disorders like PTSD and MDD compared to men. Interestingly, this bias exists distinctly during the reproductive years and coincides with natural fluctuations in ovarian hormones across the menstrual cycle. Clinical studies support a role for ovarian hormones, namely estrogen, in this susceptibility, although these findings are not always replicated in preclinical studies, particularly in those that do not evaluate behavior in the context of stress. Thus, aim 1 of the current studies tested the hypothesis that females previously exposed to stress exhibit greater anxiety-like behavior when in the presence of heightened estrogen. In support of this, estrogen is known to directly amplify production of the regulatory stress hormone, corticotropin releasing factor (CRF), within the central amygdala (CeA). Studies have shown that this occurs through activation of estrogen receptor beta (ERβ) which binds to an estrogen response element on the promoter region of the *Crf*

gene. It is thought that estrogen signaling through ERβ in the CeA drives the increase in anxiety-like behavior and stress hormone production in females. However, the functionality of this mechanism on behavioral and physiological responses to stress has not been fully evaluated. Therefore, aim 2 of the current studies tested the hypothesis that blocking ERβ during stress exposure will mitigate expression of anxiety-related behavior and the preceding rise in CeA-CRF as a consequence of stress. In addition to its genomic actions on CRF, estrogen also enhances promoter activity of the inflammatory cytokine interleukin-1 beta (IL-1β). Aside from its main functions in fighting injurious and pathogenic insults, IL-1β acts within the central nervous system to increase neuronal firing through the IL-1 receptor (IL-1r). Excess levels of IL-1β that are common in many pathological conditions can cause this signaling to become dysregulated, resulting in overactive neuronal firing or sensitization. This specific action has been shown to occur in the locus coeruleus (LC) which is responsible for arousal state. Moreover, this region is highly sexually dimorphic with the female LC containing a larger population of neurons, exhibiting longer and more complex dendritic trees, and being more densely innervated by other stress-sensitive regions, especially the CeA. Thus, it is hypothesized that stress-induced increases in inflammation can induce LC sensitization that leads to hyperarousal. Therefore, aim 3 of the current studies tested the hypothesis that inhibition of IL-1r signaling will prevent stress-induced increases in anxiety-like behavior. Taken together, these experiments identify a specific role for estrogen in female stress susceptibility and elucidate IL-1r as a novel target for therapeutic endeavors.

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CHAPTER ONE

INTRODUCTION

1.1 Social Stress as an Etiological Factor in Psychiatric Disorders

Organismal survival relies on the ability to maintain bodily homeostasis, a term coined by Walter Cannon to define the concept of internal physiological normalcy (Cannon & Pettit, 1926), when exposed to a challenging event or stimulus known as “stress”. The idea of an innate response designed to combat potentially harmful effects elicited by stress was first described by Hans Selye as the “general adaptation syndrome” (Selye, 1950). Modernly referred to as the “stress response”, Selye’s trailblazing works highlight how the nervous and endocrine systems attempt to maintain homeostatic conditions by mobilizing a discrete set of physiological defenses that yield a broad range of results that are dependent on unique features of the affected organism and initial stressor (Selye, 1950). Pointedly, Selye asserts that factors exclusive to the individual, such as genetics, metabolic reserves, and even prior exposure to stress, direct the overall course and phenotypic outcomes of the response with respect to influential aspects possessed by the stressor itself (Selye, 1950, 1956). Creatively, Selye compares the wide range in which this adaptive response can be expressed to the quizzical array of diseases caused by *tubercle bacillus*:

> *"Before the* tubercle bacillus *had been isolated it would have been considered most improbable that such dissimilar conditions as Pott's disease, phthisis of the lungs, military tuberculosis, and tuberculosis lupus of the skin were all caused by the same pathogen ; yet this is the case".*

Thus, these pivotal findings emphasize how systemic stress can manifest in numerous and seemingly unrelated ways to the benefit of the individual organism.

Though an integral defense mechanism, Selye notes that adaptability is a "finite quantity", explaining that excessive provocation of and/or abnormalities in the stress response can cause detrimental effects (Selye, 1950). For example, activation of the stress response induces release of endogenous glucocorticoids that exert a robust anti-inflammatory effect, which is especially beneficial in individuals with transient insults to (e.g., infection, tissue injury) or chronic diseases of the immune system, like asthma or Multiple Sclerosis (Hodgens & Sharman, 2023; Selye, 1950). However, continuous stress can lead to chronic overexpression of glucocorticoids that, instead, cause harmful elevations in inflammatory load via glucocorticoid resistance (Amasi-Hartoonian et al., 2022; Perrin et al., 2019) or immune system priming (Amasi-Hartoonian et al., 2022; Horowitz et al., 2020). Furthermore, substantial disruptions in the stress response can cause damage to areas like the cardiovascular and renal systems, as well as significantly impair an individual's psychological well-being.

In fact, the most common type of stress that is experienced as part of daily life is psychosocial in nature (Almeida, 2005; Dickerson & Kemeny, 2004; Patel et al., 2019). This type of stress, referred to as social stress, involves direct or indirect

(i.e., witnessing or threatened) exposure to emotionally charged events that lack a physical component. Common instances of social stress include isolation, grief, loss, discrimination, unexpected events or deadlines, and psychological or emotional abuse (Almeida, 2005; Dickerson & Kemeny, 2004). These types of stressors may be individually considered minor disturbances, but, collectively, are considerably more detrimental to overall health than singular, major life events (Almeida, 2005). In addition to being more prevalent, social stress elicits a synonymous internal response to that which is evoked by physical stress (Dickerson & Kemeny, 2004; Selye, 1950, 1956). Moreover, persistent exposure to social stress is known to be a major contributing factor in the development of many debilitating psychiatric conditions, like anxiety and mood disorders. Stress-related anxiety and mood disorders are highly common across the globe, demonstrating a lifetime prevalence of approximately 20% (Faravelli et al., 2013; Gutiérrez-Rojas et al., 2020; Kessler et al., 2005; McLean et al., 2011), though current research indicates that these rates will soar in the wake of the Covid-19 pandemic (Pavlidi et al., 2023; Santomauro et al., 2021).

Posttraumatic stress disorder (PTSD) and major depressive disorder (MDD) are the most common anxiety and mood disorder, respectively (Kessler et al., 2012; Santomauro et al., 2021; Tiller, 2013) and have remained leading causes of disability worldwide for well over three decades (Ferrari et al., 2013; Ferrari et al., 2022; Gutiérrez-Rojas et al., 2020; Pavlidi et al., 2023; Santomauro et al., 2021). PTSD is unique in that it is the only major psychiatric disorder in which the precipitating cause—exposure to a psychologically traumatic event—is known

(Pitman et al., 2012; Sherin & Nemeroff, 2011). In addition to the obvious form of being directly involved in a traumatic event, exposure can also include witnessing a traumatic event, learning that a loved one experienced a traumatic event, or being associated with factors related to a traumatic event (e.g., via employment as crime scene investigators, first responders, or social workers) (Benjet et al., 2016; Mann & Marwaha, 2023). While virtually everyone will experience trauma to some degree during their lifetime (Benjet et al., 2016; Koenen et al., 2017), the leap from initial exposure to meeting diagnostic criteria for a psychiatric disorder relies heavily on the culmination of an individual's risk factors and specific components of the traumatic experience. Those who do go on to develop PTSD often present with characteristic behaviors of the disorder, including re-experiencing the traumatic event (e.g., flashbacks, nightmares), withdrawal from or avoidance of stimuli or settings that serve as reminders of the trauma, and hyperarousal of the nervous system (Grant et al., 2008; Lehner et al., 2021; Lukaschek et al., 2016; Mann & Marwaha, 2023; Pitman et al., 2012; Sherin & Nemeroff, 2011). Moreover, detrimental perturbations in physical and psychological health can arise, including co-morbid MDD (Grant et al., 2008; Hasin et al., 2018; Post et al., 2011; Price & van Stolk-Cooke, 2015). A distinct disorder on its own with widespread etiology, MDD can also result directly from exposure to serious trauma and in conjunction with or as a consequence of other psychiatric disorders like PTSD (Price & van Stolk-Cooke, 2015; Radell et al., 2020). MDD is associated with the expression of prominent behavioral "numbing" symptoms, such as anhedonia, despair, and lack of emotion (Duek et al., 2023; Tiller, 2013) and is also known to elicit a sustained

state of hyperarousal similar to that of PTSD (Hegerl et al., 2012; Horesh et al., 2017; Xie et al., 2024). The high rates of comorbidity and overlapping phenotypic traits of PTSD and MDD can make proper diagnosis and, thus, treatment, difficult to attain (Afzali et al., 2017; Horesh et al., 2017; Price & van Stolk-Cooke, 2015; Tiller, 2013). Compared to those with either disorder alone, individuals suffering from the combined effects of PTSD and MDD are said to have greater functional impairments, more severe symptomology, and poorer responses to treatment (Green et al., 2006; Post et al., 2011; Tiller, 2013; Zhu et al., 2017). Moreover, not only are currently available treatment options critically lacking (Davidson, 2015; Hodes et al., 2015; Tiller, 2013), but only a small percentage of the affected population seeks and/or receives adequate professional treatment (Mansour et al., 2023; Sartori & Singewald, 2019; Tiller, 2013; Williams et al., 2022).

Antidepressant medications like selective serotonin reuptake inhibitors, familiarly known as SSRIs, are first-line treatments for both PTSD (Mann & Marwaha, 2023; Sagarwala & Nasrallah, 2019; Williams et al., 2022) and MDD (Baig-Ward et al., 2023; Holtzheimer & Mayberg, 2011) but pose only modest benefits due to the high rates of insufficient symptom relief and disorder recurrence (Davidson, 2015; Holtzheimer & Mayberg, 2011; Williams et al., 2022). Numerous methods of psychotherapy have also been developed over the years, like cognitive behavioral therapy, as standalone treatments and to enhance the effectiveness of pharmacological treatments (Hetrick et al., 2010; Holtzheimer & Mayberg, 2011; Mansour et al., 2023). Though meaningful decreases in symptom severity have been observed with the use of adjunct psychotherapies, the depth of their impact

remains uncertain due to high participant dropout rates and a lack of participant motivation to continue treatment (Hetrick et al., 2010; Mansour et al., 2023). Adding insult to injury, a considerable portion of individuals with MDD are said to have treatment-resistant depression in which depressive symptoms persist despite having attempted multiple treatment strategies (Baig-Ward et al., 2023; Holtzheimer & Mayberg, 2011). Although there is an immense and continuously growing amount of research surrounding the epidemiology and treatment of PTSD and MDD, available therapeutic options are not significantly more effective today than when they were originally developed almost a century ago (Baig-Ward et al., 2023; Hodes et al., 2015; Holtzheimer & Mayberg, 2011). Therefore, more meticulous investigation is required to elucidate credible therapeutic targets for the prevention and mitigation of these disorders.

1.2 Ethological Models of Social Stress in Preclinical Research

There are numerous limitations in the clinical research setting that make in-depth analyses of physiology near impossible to obtain. One of the greatest hinderances in the field of stress neurobiology is the massive irregularity in clinical subjects in terms of their physiological makeup, social history and experiences, thought processes and intellect, etc. that make direct comparisons within and across study cohorts subpar—not to mention the inability to conduct experiments that require manipulation or collection of brain tissue among other invasive procedures. However, many of these obstacles can be overcome with the use of preclinical animal models. Of course, preclinical research is not without its own limitations and pitfalls, especially since animals cannot dictate their own thoughts and feelings

and different species share different similarities with the clinical population. Nevertheless, recapitulating human pathologies via animal models does provide an enormous advantage by allowing researchers to study important components of neurobiology in a controlled manor, from an entire repertoire of phenotypic traits commonly seen in clinical pathologies to the specific functions of individual proteins. The use of rodent models in particular offers highly informative and translationally relevant methods for studying the mammalian stress response. Mice and rats are the most frequently used species in preclinical research due to their anatomical, physiological, genetic, and behavioral similarities to humans (Bryda, 2013). Compared to other common research models like non-human primates and swine, rodents require less resources since they are much smaller in size, have shorter gestational periods, and progress faster to adulthood (Bryda, 2013). Rats are not simply larger versions of mice, however—both species exhibit characteristics that better model certain aspects of the clinical population. In particular, rats are more often used in behavioral studies because they are more social creatures and better mimic the behavioral repertoire of humans compared to mice (Bryda, 2013). Though not completely identical to the clinical population, rats have been indispensable in the discovery and understanding of pharmacological, mechanistic, and behavioral outcomes that are associated with clinical (patho)physiology. Similarly, there are numerous stress paradigms used throughout the scientific community, each offering unique aspects that simulate clinically relevant stressors and evoked outcomes. Three widely used models of social stress that have generated significant contributions to the understanding of

stress-induced psychopathy include the visible burrow system, chronic restraint stress, and social defeat stress. While each paradigm contains an unavoidable physical component, the social aspect remains the crux of the stressful experience.

The visible burrow system (VBS), originally developed by Robert and D. Caroline Blanchard (Blanchard & Blanchard, 1989) with further use and validation by collaborators Bruce McEwen and Randal Sakai (Blanchard et al., 1993; Herman & Tamashiro, 2017; McEwen et al., 2015), is a semi-naturalistic environment containing a system of underground tunnels and burrows that houses a standard ratio of four male to two female laboratory rats (Nguyen et al., 2007). Strategic placement of infrared cameras and clear Plexiglass at various spots throughout the VBS allows researchers to observe the inhabitants with minimal disruption (McEwen et al., 2015; Nguyen et al., 2007). This paradigm provides critical insight into the dynamics of social groups wherein members form a social hierarchy and experience stress to variable extents depending on their roles as either dominant or subordinate members. Social hierarchies are created in the VBS the same as they are in the wild where male rats fight for social dominance. In this instance, the dominant male can be identified by exhibiting offensive aggression, whereas subordinate males exhibit defensive aggression (Blanchard et al., 1993; McEwen et al., 2015; Melhorn et al., 2017). The dominant male will then (attempt to) prevent subordinate males from accessing food and water resources and mating with females (McEwen et al., 2015). As a result, subordinate males develop numerous physiological deficits, including substantial body weight loss (Blanchard et al., 1993; McEwen et al., 2015; Melhorn et al., 2017; Nguyen et al., 2007; Tamashiro

et al., 2004), reduced thymus gland weights (Blanchard et al., 1993; Tamashiro et al., 2004), adrenal hypertrophy (Blanchard et al., 1993; Nguyen et al., 2007; Tamashiro et al., 2004), greater ratios of visceral to subcutaneous fat (McEwen et al., 2015; Nguyen et al., 2007), heightened basal levels of stress hormones (Blanchard et al., 1993; Melhorn et al., 2017), reduced testosterone and reproductive activity (Blanchard et al., 1993; Nguyen et al., 2007; Tamashiro et al., 2004), and decreased dendritic arborization in the hippocampus (McKittrick et al., 2000). In addition, subordinate males exhibit traits of anxiety- and depressive-like behavior including social withdrawal (Blanchard & Blanchard, 1989; Melhorn et al., 2017). Furthermore, VBS paradigms are typically run for a maximum duration of 14 days because it is at this time that the mortality rate of subordinate males greatly increases (Blanchard et al., 1993; McEwen et al., 2015). Interestingly, this fatal outcome has been shown to be a consequence associated more with the repercussions of social subordination than of physical aggression as the rates and severity of attacks become subdued as time progresses (Blanchard et al., 1993; McEwen et al., 2015). Though muted in comparison to subordinates, dominant males also experience negative effects of stress in the VBS paradigm. In rodent models, dominant males exhibit experience reductions in thymus gland weights (Blanchard et al., 1993; Tamashiro et al., 2004), adrenal gland hypertrophy (Blanchard et al., 1993; Nguyen et al., 2007; Tamashiro et al., 2004), reductions in overall body fat (Nguyen et al., 2007; Tamashiro et al., 2004), elevated basal levels of stress hormones (Blanchard et al., 1993; Nguyen et al., 2007), heightened levels of testosterone (Nguyen et al., 2007; Tamashiro et al., 2004), and dendritic

shrinkage in the hippocampus (McKittrick et al., 2000). Subordination under these circumstances is associated with greater repercussions of stress due to the despotic nature of social hierarchy formation that is inherent to many commonly studied species like rats, mice, rhesus monkeys, and savanna baboons (Sapolsky, 2005). In this type of social structure, dominance is first achieved through brute force and resource guarding and is maintained through psychological intimidation tactics (Sapolsky, 2005). It is suggested that subordinate males in these environments experience greater physiological deficits and eventual mortality due to their consistent exposure to stress and resource inequity, and that dominant males are less affected due to the lack of physical aggression required to maintain their status (Sapolsky, 2005). Conversely, other species employ hierarchy formations in which the dominant male experiences greater physical and psychological distress than subordinates and, thus, is more susceptible to stress-related pathologies. For example, African wild dogs and chimpanzees exhibit a despotic social hierarchy in which social dominance is maintained via continued physical aggression, even in the absence of overt challenge (Sapolsky, 2005). Additionally, dominant males bear the burden of stress under circumstances of unstable or shifting social rankings, periodic or seasonal mating, and misinterpreting ambiguous social cues to be threatening (Sapolsky, 2005).

In some species like the ring-tailed lemur, females also form social hierarchies that involve continued physical aggression to maintain high-ranking social status that renders dominant females more susceptible to the negative effects of stress (Sapolsky, 2005). However, like women, female non-human

primates most often display non-physical forms of aggression that entail threatened harassment or psychological distress (Michopoulos et al., 2012). Moreover, female rodents are not typically studied using this particular method of stress since they do not develop social hierarchies or exhibit reliable aggression against same-sex conspecifics (Kuske & Trainor, 2022; Michopoulos et al., 2012). According to the limited data that do exist in regard to the rodent VBS paradigm, dominant males exhibit consistently more biting attacks on females than on other males; however, this has been found to be in relation to mating attempts rather than acts of overt aggression. So, while the data may broadly signify a greater number of dominant male-on-female attacks than dominant male-on-subordinate male attacks, the severity of aggression and wounding are notably less severe for females. Indeed, sexually receptive females have been found to engage in agonistic sexual interactions and spend increasingly more time with the dominant males as a progression of time (Blanchard et al., 2001). Overall, findings from studies conducted in rodents using the VBS model highlight the critical impact that subordination can have on metabolic, endocrinological, and neurobiological responses to stress that occur exclusively in males.

In addition to the VBS paradigm, preclinical studies often employ chronic restraint stress (CRS) as a model psychosocial stress that is typically conducted over a duration of approximately six hours per day over a period of seven consecutive days (Burzynski et al., 2022; Chiba et al., 2012; Pansarim et al., 2023). Though there is no overt social component like in the VBS, CRS is known to confer a high degree of social isolation that functions as a form of social stress in its own

right and also elicits notable impairments in social behavior. Moreover, CRS is especially advantageous in studies on learning and memory because it has been shown to reliably reduce hippocampal volume and alter neuronal morphology to an extent that impairs critical aspects of cognition (Burzynski et al., 2022; McLaughlin et al., 2007). The CRS paradigm involves the restriction of movement to an extent that does not hinder necessary physiological processes like breathing which is most commonly achieved in rats with the use of an adjustable acrylic tube (Chiba et al., 2012; Pansarim et al., 2023) or a mesh cylinder that can be clamped on both ends (Burzynski et al., 2022). Physiological outcomes of CRS are similar to those observed in subordinate rats exposed to the VBS, including blunted weight gain (Burzynski et al., 2022; Chiba et al., 2012; Doremus-Fitzwater et al., 2009; Pansarim et al., 2023), elevated stress hormone levels (Chiba et al., 2012; Doremus-Fitzwater et al., 2009; McLaughlin et al., 2007; Pansarim et al., 2023), and reductions in hippocampal volume and dendritic branching (Burzynski et al., 2022; McLaughlin et al., 2007). Behavioral outcomes following CRS include elevations in anxiety-like behaviors and anhedonia (Chiba et al., 2012; Doremus-Fitzwater et al., 2009; Peay et al., 2020) that resemble behavioral features of anxious and depressive phenotypes seen in clinical cases of PTSD and MDD. Exposure to CRS has also been shown to evoke aspects related to social dysfunction, like reductions in social motivation and social preference (Doremus-Fitzwater et al., 2009). Furthermore, unlike the VBS, male and female rodents can be equally stressed under the CRS model which allows for important intra- and inter-sex comparisons on stress-related outcomes to be conducted. However,

studies that include both sexes remain to be the exception rather than the rule, and, in those that do, CRS females are more resilient to the effects of stress in that they exhibit negligible differences compared to non-stressed females and males (Pansarim et al., 2023; Peay et al., 2020). The CRS paradigm is incredibly useful for studies that explore cognitive and hormonal implications of stress, but there remains a significant need for further research to support or refute its worth in studies that are heavily focused on social behavior and females/sex differences.

The social defeat stress (SDS) paradigm is arguably the most widely used model of social stress in recent years due to its predictable and robust evocation of pathophysiological and behavioral deficits that align with clinical presentations of PTSD and MDD. Originally developed by Klaus Miczek (Miczek, 1979), SDS, otherwise known as the resident-intruder paradigm, involves the placement of a sexually naïve, smaller male rat ("intruder") into the home cage of an older, territorial, retired breeder male rat ("resident") in which fighting for dominance ensues, similar to the social hierarchy that is established in VBS colonies (Carnevali et al., 2020; Hollis & Kabbaj, 2014). A typical SDS encounter involves offensive aggression on the part of the resident ranging from lateral threats to biting attacks (Carnevali et al., 2020) that are inflicted on the intruder shortly after entry into the resident's cage (Wood et al., 2015). In turn, the intruder exhibits defensive behaviors and is eventually forced into submission by the resident, which most often involves the intruder lying motionless in a supine position (Wood et al., 2015). Though details of study design can be minorly modified depending on the research question (Hollis & Kabbaj, 2014), SDS typically entails exposure of the intruder to

physical and psychological stress by the resident for approximately 15 to 30 minutes that is followed by either separation of the males via a partition in the resident's home cage or return of the intruder to its home cage (Finnell et al., 2017; Finnell et al., 2019; Hollis & Kabbaj, 2014; Wood et al., 2010). Though the physical component of stress in the SDS paradigm is notable, research has shown that the psychological effects elicited by SDS exposure are highly impactful. For example, studies that employ this stress paradigm have found substantial impairments in cardiovascular function that persist long after the final SDS exposure is experienced (Finnell et al., 2017; Tidey & Miczek, 1997; Wood et al., 2017; Wood, 2014; Wood et al., 2012). This is especially important in terms of clinical relevance because development of stress-induced psychiatric disorders serves as a prominent risk factor for cardiovascular dysfunction and disease later in life (Larsen & Christenfeld, 2009). Other physiological consequences of SDS include reduced weight gain (Berton et al., 1998; Meerlo et al., 1996; Pulliam et al., 2010), decreased thymus weights (Berton et al., 1998; Calvo et al., 2011), adrenal gland hypertrophy (Berton et al., 1998; Finnell et al., 2017; Wood et al., 2012), elevated stress hormone levels (Finnell et al., 2017; Razzoli et al., 2006; Wood et al., 2012), and reduced testosterone levels (Razzoli et al., 2006). Likewise, there are considerable changes in the brain that occur following exposure to the SDS paradigm, including reductions in volume and perturbations in neurogenesis in the prefrontal cortex and hippocampus (Czéh et al., 2001; Czéh et al., 2007; Van Bokhoven et al., 2011), though there is evidence to show that these changes are only transient (Lagace et al., 2010). SDS exposure also elicits long-term increases

in objective anxiety- and depressive-like behaviors, including social avoidance (Berton et al., 1998; Hollis et al., 2010; Laman-Maharg & Trainor, 2017; Meerlo et al., 1996), reduced locomotor activity and exploration (Meerlo et al., 1996; Tidey & Miczek, 1997), reduced sucrose preference (Finnell et al., 2017), and increased immobility in the forced swim test (Berton et al., 1998; Hollis et al., 2010). Moreover, SDS is particularly useful because, unlike most all other forms of social stress, rats do not habituate even when repeatedly exposed to the SDS paradigm (Hollis & Kabbaj, 2014; Tidey & Miczek, 1997), and profound physiological and behavioral changes can be seen acutely after a single exposure, long-term that extends days to weeks following the last exposure, and everything in between. Overall, SDS provides a sustainable model of stress that reliably evokes physiological and behavioral changes in rats that greatly resemble the changes seen in clinical presentations of PTSD and MDD. However, there is one prominent drawback to using this model: it is not as effective or ethologically valid in females.

Historically, rather than aggression from a same sex conspecific, females confer buffering effects through social interaction that ameliorate the negative impact of stress and promote resilience (Ishii et al., 2016; Smith & Wang, 2014), reflecting the “tend and befriend”, support seeking reaction that women adopt in response to stress (Taylor et al., 2000; Youssef et al., 2018). Moreover, studies conducted on victims of aggression often use the term “intimate partner violence”, or IPV, since the vast majority of perpetrators of sexual, physical, and psychological acts of aggression have been documented as being a current or former intimate partner of the victim at the time of the abuse (Black et al., 2011;

Dokkedahl et al., 2019; Lagdon et al., 2014). For women in the United States as of 2010, approximately 15% have experienced some form of sexual IPV, 25% have experienced some form of severe physical IPV, and 50% have experienced some form of psychological IPV (Black et al., 2011). Hence, in female subjects, scientists have begun to shift the focus from physical stress to psychological stress considering its high prevalence and association with development of PTSD and MDD (Black et al., 2011; Dokkedahl et al., 2019; Lagdon et al., 2014). This is not surprising considering psychosocial stress is the most common type experienced by the general public, as previously mentioned. However, in preclinical studies, females are rarely, if ever, antagonized into submission and are typically allowed free access to resources, unlike subordinate male rats. It can be challenging, then, to model social stress in females because behavioral intent cannot be discerned nor can covert behavioral expression be personified. In other words, male rats do not normally attack female rats, instead, exhibiting copulatory behavior, so standard paradigms of SDS are ineffective when studying females. Additionally, simply observing rats and making subjective assumptions regarding their psychological states is invalid. Furthermore, across a wide range of species, females are not commonly found to engage in physical acts of aggression against one another, like fighting for social dominance. In humans, women more often exhibit indirect forms of aggression that lack any physical components, such as gossiping about, intentionally excluding, and criticizing others (Archer & Coyne, 2005; Denson et al., 2018; Österman et al., 1998), whereas men primarily engage in direct and verbal forms of aggression (Bjorkqvist et al., 1992; Österman et al.,

1998). Thus, a modified form of the SDS paradigm was developed to better mimic this type of social stress in humans and, importantly, provide an effective model for use in females.

Witness stress (WS) is a form of vicarious social defeat stress in which a rat ("witness") is exposed to the SDS encounter between a resident and intruder from the safety of an adjacent compartment. In general, the typical SDS setup remains the same with an intruder being placed into the home cage of a resident for social dominance to be established. However, for WS, the witness is also inserted into the cage where it is sequestered to one end away from the fighting males via a perforated partition (Carnevali et al., 2020; Kuske & Trainor, 2022; Warren et al., 2020). This design prevents the witness from receiving physical acts of any sort while also providing auditory, visual, and olfactory information of the SDS (Carnevali et al., 2020; Warren et al., 2020). Like SDS, WS can be performed under acute or chronic conditions and has shown to elicit robust changes akin to clinical manifestations of PTSD and MDD (Bouknight et al., 2023; Finnell et al., 2017; Finnell et al., 2018; Pate et al., 2023). Frequent physiological outcomes of WS include cardiovascular dysfunction (Finnell et al., 2017; Pate et al., 2023), inhibited body weight gain (Patki et al., 2014), reduced thymus gland weights (Patki et al., 2014), adrenal hypertrophy (Finnell et al., 2017), and elevated stress hormone levels (Bouknight et al., 2023; Carnevali et al., 2017; Finnell et al., 2017). Behavioral changes induced by WS include social avoidance (Carnevali et al., 2017), anxiety-like defensive burying (Bouknight et al., 2023; Finnell et al., 2018; Pate et al., 2023), reduced sucrose preference (Bouknight et al., 2023; Finnell et

al., 2017; Patki et al., 2015), and increased immobility in the forced swim test (Finnell et al., 2018; Patki et al., 2015). The WS paradigm has allowed numerous doors to open in the scientific community with its ability to adequately model psychological stress in rodents of essentially any species, sex, or age. This critical milestone enables more translational and rigorous studies to be conducted in sorely underrepresented populations like females.

1.3 Sex Differences in Stress-related Psychiatric Disorders

Since its inception, scientists have made enormous advancements in the field of behavioral neurobiology to identify specific factors and mechanisms that give rise to the development of stress-induced psychiatric disorders. Despite the breadth of integral discoveries that have been made, the bulk of preclinical research has been conducted solely in male species (Bolea-Alamanac et al., 2018; Kuske & Trainor, 2022; Shansky & Woolley, 2016), which fills in only a portion of the puzzle. Like rats are not simply larger mice, females are not simply smaller males—yet, even if that were the case, neither sex deserves to have to fall under that assumption without due diligence from the research community at large. Especially now with the utility of the WS paradigm, there are no justifiable reasons to exclude females from preclinical studies of social stress. Rather, females need to be prioritized in these types of studies due to women's overall higher rates of stress-related psychiatric disorders (Kessler et al., 1993; Kessler et al., 2012).

As previously mentioned, PTSD and MDD are some of the most burdensome and common disorders present in today's society and occur twice as often in women than men (Altemus et al., 2014; Kessler et al., 2012; Kundakovic

& Rocks, 2022(Lehner et al., 2021)). In fact, women are more likely to meet diagnostic criteria for most all clinical anxiety and mood disorders (Altemus et al., 2014) in addition to presenting with more subclinical symptoms of these disorders compared to men (Hankin, 2009). Though many important aspects of physiology and behavior contribute to this bias, those related to its establishment are of particular interest. Notable factors include females' heightened responsivity to stress that coincides with a predisposition for internalized coping tactics, in addition to the sharp rise in production of essential glucocorticoids as part of the physiological stress response.

The increased prevalence of stress-related psychiatric disorders in women begins during the transition into adolescence due to marked changes that arise from puberty and the accompanying social challenges (Oldehinkel & Bouma, 2011). During this time period, clinical studies have shown that girls display a propensity to view changes like body shape and weight in a negative light (Sandanger et al., 2004; Stroud et al., 2002), and also point to a rise in dependence on interpersonal relationships which sets the stage for social instability (Hamilton et al., 2015; Oldehinkel & Bouma, 2011). This overt negative perception of self (e.g., rumination and neuroticism (Brown et al., 2020; Michl et al., 2013; Roley et al., 2015)) that appears during early adolescence has been linked to women's heightened responsiveness to negative social stressors (Kelly et al., 2008; Stroud et al., 2002; Villada et al., 2017). For example, in response to a laboratory administered psychosocial stress paradigm, women exhibited greater subjective stress as described by feelings of "fear, irritability, confusion, and less happiness"

immediately following the stressful experience compared to men (Kelly et al., 2008). Similarly, in another study of sex differences, women experienced more distress from events surrounding close relationships, illness, and network, and that exposure to an event in one of these categories was more strongly related to anxiety and depression, especially when illness and network stress overlapped (Sandanger et al., 2004). Thus, it is the enhanced recognition of and response to negative valence situations by women that is said to drive the detrimental reciprocity between internal coping and symptoms of anxiety and depression (Brown et al., 2020; Hamilton et al., 2015; Hankin, 2009; Villada et al., 2017). In support of this, research has shown that women's increased risk for developing PTSD and MDD is, in part, due to their tendency to exhibit elevated rumination and neuroticism at baseline (Brown et al., 2020; Michl et al., 2013; Villada et al., 2017), and that continued expression of these behaviors maintains and exacerbates PTSD and MDD symptomology (Mellick et al., 2019; Roley et al., 2015).

The adolescent transition is also marked by elevations in the production of glucocorticoids, namely cortisol (CORT), as part of the physiological stress response. The final product of the hypothalamic-pituitary-adrenal (HPA) axis, CORT is generated by the adrenal glands and serves to mobilize bodily resources in preparation of warding off impending threats (Bangasser & Valentino, 2014; Stroud et al., 2011). Though critical to survival, dysregulation of the HPA axis can incur pathophysiological changes that ultimately lead to the development of stress-related psychiatric disorders. Indeed, PTSD and MDD have been heavily linked to disruptions in HPA axis function (Bangasser & Wiersielis, 2018; Kudielka &

Kirschbaum, 2005), such as elevated basal levels of CORT and a slower return to baseline following a challenge. Studies conducted during the peri-adolescent stage support this by showing positive associations between baseline CORT levels and depressive symptoms (Chafkin et al., 2022; Charbonneau et al., 2009; Stroud et al., 2011), as well as impediments in the speed of recovery after exposure to a stressful experience (Stroud et al., 2011). Importantly, these relationships are exclusive to females, arising distinctly during the onset of puberty and remaining upregulated until the completion of menopause (Barth et al., 2015; Kundakovic & Rocks, 2022). In line with this evidence, further connections have been discovered regarding the stage of pubertal maturation, such that elevations in basal CORT levels and the appearance of anxiety and depressive symptoms are also positively correlated with increases in pubertal stage of girls (Chafkin et al., 2022; Stroud et al., 2011). Taken together, these data point to female gonadal hormones as factors responsible for the increased development of stress-related psychiatric disorders that are biased in women.

1.4 The Role of Ovarian Hormones in Promoting Stress Susceptibility

Pubertal maturation marks the development of secondary sex organs that facilitate species continuation. In females, the ovaries produce the vast majority of sex hormones, aptly referred to as ovarian hormones, that belong to either the estrogen or progestogen family of steroid hormones. The predominant form of estrogen in females of reproductive age is estradiol, or E2, which exists in greater concentrations, is more potent, and has significantly greater binding affinity for the canonical receptors, estrogen receptor-alpha (ERα) and -beta (ERβ), compared

to the other estrogen subtypes (Baker, 2013). Likewise, the predominant progestogen is natural progesterone, or P4, and is considered the “mother molecule” due to its distinct effects throughout the body that differ from synthetic progestogens via binding to the P4 receptor (Mesiano, 2022; Piette, 2020). Both classes of hormones exhibit genomic and non-genomic, or “rapid”, mechanisms of action that allow for their well-known involvement in controlling menstruation and driving reproductive processes (García-Sáenz et al., 2023; Mesiano, 2022). However, E2 and P4 are also known to exert significant influence over other bodily functions, including cognition, memory, and non-reproductive behavior, the latter of which being central to the studies described in later chapters (Albert & Newhouse, 2019; Newhouse et al., 2010; Ogawa et al., 2020; Sartin-Tarm et al., 2020). Specifically, research has shown that E2 and P4 both critically alter components of the stress response and related social behaviors (García-Sáenz et al., 2023; Morgan et al., 2004; Ogawa et al., 2020), however, the contribution of E2 appears to be especially crucial. As discussed above, numerous clinical studies have reported a robust connection between the onset of anxiety and depressive symptoms and the rise in ovarian hormones, namely E2, during pubertal maturation (Chafkin et al., 2022; Stroud et al., 2011). Other studies have expanded on these data, revealing a stark correlation between development of stress-related psychiatric disorders and cycling ovarian hormones. Specifically, women’s increased prevalence of developing stress-related psychiatric disorders compared to men is present exclusively during the reproductive years, from puberty onset

through completion of menopause, when naturally cycling estrogen is at its highest (Barth et al., 2015; Kundakovic & Rocks, 2022).

Support for this association is described in an array of research conducted across the lifespan. In a study on a group of young adults of both sexes (Rattel et al., 2019), participants were shown highly aversive movie clips depicting severe forms of IPV that were preceded by a neutral sound serving as a conditioned stimulus. Results from this study revealed that women exhibited greater and more distressing intrusive thoughts, a characteristic behavior of PTSD and MDD, in response to hearing the conditioned stimulus on subsequent days compared to men. In addition, the authors reported that women experienced greater magnitudes of distress that coincided with the intrusions. A similar study by a different research lab was conducted using a series of neutral and negative valence images in addition to the presence of absence of cold-water stress (Cheung et al., 2013). Results indicated that women exhibited greater unwanted intrusions than men, and, also, that elevated levels of salivary estrogen correlated with increased intrusions of negative images in women. These findings are also in line with studies that were conducted exclusively in healthy, postmenopausal women with respect to differences in circulating levels of exogenous estrogen. In two distinct studies conducted in the same lab, separate groups of postmenopausal women, half of which received three months of oral E2 treatment prior, in response to a common laboratory-administered psychosocial stressor called the Trier Social Stress Test (TSST). Results showed that women treated with E2 displayed greater negative affect in response to the TSST, independent of

age, compared to those treated with placebo (Dumas et al., 2012; Newhouse et al., 2008). Collectively, these studies highlight elevated levels of E2 as a notable risk factor for stress-related psychiatric disorders in women.

Surprisingly, preclinical studies in females do not always exhibit the same results that are apparent in the clinical setting and, instead, often support E2 as being behaviorally "protective" in response to stress. These findings are present in relation to endogenous elevations in E2 based on a rodent's estrous cycle phase, the murine equivalent of the human menstrual phase, and to exogenously elevated levels of E2. In two studies conducted by separate research groups, female rats in the putatively High E22 phase of the estrous cycle were compared to those in the Low E22 phase for specific behaviors exhibited during an anxiogenic task (Fabris et al., 2022; Sayin et al., 2014). Both studies reported that rats in the High E22 phase displayed less anxiety-like behavior than rats in the Low E22 phase, thereby assuming an anxiolytic effect of E2. Similarly, in two studies conducted independently by different research groups (Ghazvini et al., 2021; Liu et al., 2022), following surgical remove of the ovaries, rats were treated with E2 or placebo, and then tested in the same anxiogenic task wherein rats that received E2 treatment exhibited reduced anxiety-like behavior than those treated with placebo. One critical aspect of these studies stands out, however: no stress paradigm is implemented prior to undergoing behavioral testing. Without the use of a stressor prior to assessing behavior, "stress-induced" assessments cannot be concluded, and, thus, these studies provide information about baseline behavior at best. Indeed, when a stressor is utilized prior to behavioral assessments, results often

mirror those that are seen in clinical studies (Bouknight et al., 2023; Finnell et al., 2018; Hokenson et al., 2021; Jasnow et al., 2006; Lynch et al., 2014; Pate et al., 2023). Preclinical studies are not routinely designed in this manner, though, and, so, are unable to be directly compared to much of the current literature, therefore chapter two explores the underpinnings of these dichotomous findings and bridges the gap that remains in preclinical research regarding the female response to stress.

1.5 Estrogenic Regulation of the Stress Response in the Amygdala

Though the hippocampus receives considerable attention in stress neurobiology research due to its vital role in learning and memory, many studies that investigate other behavioral outcomes of stress often focus on another important region that is responsible for emotional salience: the amygdala. At their core, stress-related neuropsychiatric disorders like PTSD and MDD involve impairments in emotional regulation and, thus, concern the amygdala. Indeed, many studies, preclinical and clinical alike, that examine the amygdala in relation to stress have noted distinct changes in various parameters, from volume and morphology to neurochemical alterations and induced hyperactivity (Barry et al., 2017; McEwen et al., 2016; Zhang et al., 2018). Thus, it is no surprise that the stress-sensitive amygdala has also been found to be closely intertwined with HPA axis functionality. Activation of the HPA axis in response to stress begins with production of the trophic hormone, corticotropin releasing factor (CRF), in the paraventricular nucleus (PVN) of the hypothalamus. CRF production in this region then elicits production of adrenocorticotropic hormone from the pituitary, which then acts on the adrenal

glands to stimulate CORT production (Kudielka & Kirschbaum, 2005). This pathway directed by hypothalamic CRF makes up the endocrine branch of the response that is largely responsible for peripheral and physiological effects of stress (Van Bockstaele et al., 1998; Wiersielis et al., 2016). However, the amygdala serves as an additional, extrahypothalamic site of CRF production that dictates the behavioral response to stress and is directly implicated in the manifestation of stress-related psychiatric disorders like PTSD and MDD (Arborelius et al., 1999; Barry et al., 2017; Van Bockstaele et al., 1998). In support of these claims, clinical studies conducted on the brains of depressed patients that died by suicide show an accumulation of CRF in extrahypothalamic regions that are highly interconnected with the amygdala (Austin et al., 2003; Bissette et al., 2003), as well as in the cerebrospinal fluid of military veterans diagnosed with PTSD (Bremner et al., 1997). Furthermore, preclinical studies have shown that local administration of CRF into the amygdala induces expression of PTSD- and MDD-related behaviors (Keen-Rhinehart et al., 2009) while CRF blockade prevents their expression (Howard et al., 2008). Thus, these findings reveal a critical role for intra-amygdalar CRF in the dysregulation of the stress response and development of stress-related psychiatric disorders.

CRF also plays a noteworthy role in the sexual dimorphism of these disorders due to its notable enhancement via estrogen signaling. The estrogens are steroid hormones that can elicit neurochemical changes through a classical genomic pathway or a rapid, non-genomic pathway (Handa et al., 2012; Ogawa et al., 2020), the former being the method by which estrogen is known to promote

CRF production. In brief, the genomic mechanism involved in amplifying CRF expression in the amygdala begins with E2 binding to either nuclear estrogen receptor, ERα or ERβ, thus forming a complex which then acts as a transcription factor and binds to the estrogen response element (ERE) located within the promoter region of the CRF gene (Chakraborti et al., 2007; Hwang et al., 2020; Vamvakopoulos & Chrousos, 1993). While findings are mixed, the general consensus among the scientific community is that ERα regulates more reproductive functions while ERβ regulates more of the non-reproductive functions (e.g., stress-related function and behaviors) (Acevedo-Rodriguez et al., 2015; Bodo & Rissman, 2006; Lynch et al., 2014). However, more research is needed to determine the accuracy in which this describes both the preclinical and clinical populations, in addition to each receptor's discrete functions with respect to stress (Hwang et al., 2020). Furthermore, many pharmacological studies utilize select agonists to study the effects of receptor activation on the stress response, however there is an extreme paucity of research that employs the use of equally informative receptor antagonists. In an elegant study that did include an estrogen receptor antagonist that specifically targeted ERβ (Isgor et al., 2003), the authors found an overall reduction in stress-related behavioral outcomes, indicating an overall positive effect. However, it is not guaranteed that effects of an antagonist will directly and precisely oppose those elicited by agonist activity. For these reasons, chapter three examines how stress affects estrogen receptor expression, and, further, how blockade of intra-amygdalar ERβ during exposure to repeated social stress affects subsequent physiological and psychological outcomes.

1.6 The Sexually Dimorphic Locus Coeruleus: Beyond Estrogen

While E2 and estrogen receptors play considerable roles in directing behavioral expression, they are also well known for their function in maintaining adequate bone density (Gamsjaeger et al., 2021). Bone loss due to estrogen deprivation during menopause can confer detrimental effects on women's overall health, thus hormone replacement therapy (HRT) has become a popular remedy (Caufriez, 2007; Gamsjaeger et al., 2021). However, uncertainty regarding the weight of benefits versus costs of HRT have given many scientists and clinicians pause based on the increasing amount of studies documenting immense complexities and adversities as a result of this treatment method (Artero et al., 2012; Cucciniello et al., 2023; Mills et al., 2023), not to mention the profound evidence highlighting a causal role for estrogen treatment in stress-related negative affective behavior in postmenopausal women. Conversely, treating symptoms of stress-related psychiatric disorders in premenopausal with estrogen blockers is unheard of due to estrogen's necessary functions in reproduction and other normative behaviors. Therefore, while estrogen is not directly a sufficient target for the treatment of these disorders, adjacent, upstream factors should be highly considered. In addition to having a strong link with estrogen, an ideal therapeutic candidate will also exhibit strong interactions with amygdalar CRF. The locus coeruleus (LC)-norepinephrine (NE) system is one such component that meets these requirements.

The LC is a primitive brain region located in the pons that contains a homogenous population of NE-producing cells and functions as a key regulator of the stress response in two prominent ways (Bangasser et al., 2016). First, the LC

has been shown to stimulate the HPA axis in response to perceived threat via neuronal innervation of the PVN, in which NE release from the LC exerts excitatory effects through activation of presynaptic α1-adrenergic receptors (Milanick et al., 2019). Second, the LC exhibits prominent reciprocal connections with the amygdala, specifically its central nucleus (CeA), which involve LC-directed increases in CeA-CRF production through β-adrenergic receptor activation (Kravets et al., 2015) in addition to amygdalar CRF-directed increases in LC-NE production that are elicited through CRF receptor-1 (Curtis et al., 1997). These relationships serve to potentiate main biological actions of the LC that involve directing attention and arousal (Bangasser et al., 2018; Bangasser et al., 2016). As such, the LC-NE system is a notable contributor to the development of stress-induced psychiatric disorders particularly when tonic activation becomes awry (Borsody & Weiss, 2002, 2004, 2014). Behavioral hyperarousal, as mentioned previously, is a hallmark of common stress-related psychiatric disorders, especially PTSD (Pitman et al., 2012; Sherin & Nemeroff, 2011) and MDD (Hegerl et al., 2012; Xie et al., 2024). Support for this is derived from post-mortem analyses that show significant elevations in CRF levels within the LC regions of depressed subjects that died by suicide (Austin et al., 2003; Bissette et al., 2003).

The severity of these effects may also shed light on the elevated rates of PTSD and MDD that are seen in women due to distinct sex differences that are displayed within this region. In terms of morphology, the LC of female rats has been shown to be larger in size, contain a larger population of NE-producing cells, and exhibit longer and more extensive dendritic processes (Bangasser et al.,

2016). Although no study to date has directly assessed these anatomical differences in the clinical population, there is information in the literature to support some degree of similarity in humans (Busch et al., 1997; Ohm et al., 1997). Regarding functionality, estrogen has been shown to enhance neuronal activation of the LC by upregulating the major enzymes involved in the synthesis of NE, including tyrosine hydroxylase (TH) (Chakraborti et al., 2007; Serova et al., 2002). Additionally, neuroinflammatory factors (i.e., proinflammatory cytokines) have been identified as another critical modulator of the LC-NE system that are also known to exhibit stark sex differences in regard to disorder development and presentation. Critically, estrogen is known to interact with proinflammatory cytokines in the central nervous system (Ruh et al., 1998) to enhance the stress response which perpetuates females' risk of developing psychiatric disorders.

1.7 Sensitization of the Locus Coeruleus via Inflammatory Cytokines

There is a mountain of information in both preclinical and clinical literature that show a strong reciprocal connection between stress and inflammation, which was briefly touched upon in earlier sections. In general, exposure to stress evokes proliferation of glucocorticoids that exert anti-inflammatory effects (Hodgens & Sharman, 2023), though these specific anti-inflammatory actions have been shown to be greater in males compared to females (Duma et al., 2010). This evolutionary trait prevents the body from exerting its limited resources on superfluous functions that do not immediately or largely enhance the rate of survival in the presence of a threat. However, when stress becomes dysregulated and persists, the body can experience unnecessary increases in cytokine load as

a result of glucocorticoid resistance among other things, thereby exacerbating the stress response in addition to systemic inflammation (Amasi-Hartoonian et al., 2022; Bauer & Teixeira, 2019; Horowitz et al., 2020; Perrin et al., 2019). This concept is particularly relevant to females considering basal levels of both, glucocorticoids and inflammation, are inherently greater in females compared to males and, as a result, women are at greater risk for developing autoimmune diseases (Belem da Silva et al., 2017; Derry et al., 2015; Duma et al., 2010; Martinez-Muniz & Wood, 2020). This may also explain the increased rates and severity of MDD in women because heightened inflammatory profiles have been shown to promote susceptibility to depression and disrupt therapeutic actions of common antidepressant medications (Beurel et al., 2020; Derry et al., 2015). For example, a study conducted in healthy adults explored the physiological and psychological response following a subthreshold dose of intravenous lipopolysaccharide (LPS), a commonly used endotoxin in preclinical and clinical research, that was administered based on weight (Engler et al., 2016). Immediately prior to and six hours after LPS administration, participants were assessed for plasma concentrations of cytokines and stress hormones. Results revealed a significant increase in post-LPS plasma concentrations of proinflammatory cytokines and CORT in women. In contrast, men displayed significant elevations in anti-inflammatory cytokines, though no differences were observed in emotional state. Another study that was conducted in a large cohort of healthy subjects, also compared effects of LPS to placebo, and did report a significant elevation in feelings of depression that were exhibited only by women in the LPS group (Moieni

et al., 2015). Together, these findings support an inherent sensitivity of the inflammatory system as a basis for women's susceptibility to stress-induced psychiatric disorders.

The interleukin (IL)-1 superfamily of proinflammatory cytokines is of keen interest due to substantial research implicating these molecules in the direct pathogenesis of psychiatric disorders. IL-1β in particular is a potent proinflammatory cytokine within this family that functions through the IL-1 receptor (IL-1r) and is highly associated with numerous disease states, especially stress-related psychiatric disorders (Konsman et al., 2008; Martinez-Muniz & Wood, 2020). In a study comparing adolescents diagnosed with depression to healthy controls, serum levels of IL-1β were positively correlated with anxiety scores exclusively in the female subpopulation of depressed adolescents (Pallavi et al., 2015). This is unsurprising considering IL-1β expression can be upregulated via estrogen-mediated mechanisms (Ruh et al., 1998). More importantly, IL-1β has been shown to directly exponentiate LC-NE activity (Borsody & Weiss, 2002, 2004, 2014). On the other hand, the endogenous anti-inflammatory cytokine, IL-1r antagonist (IL-1ra), has been shown to significantly blunt LC-NE neuronal firing (Borsody & Weiss, 2004). Taken together, these data suggest a prominent circuit by which E2 and IL-1β synergistically enhance LC-NE activation which leads to elevated CRF production within the PVN and CeA that promotes HPA axis activation and stress-induced behavioral outcomes, respectively. Though much is known in this regard, there remains a lack of vital research on distinct effects of pro- and anti-inflammatory cytokines within the female LC, especially in relation to

stress and subsequent behavioral outcomes. Thus, chapter four will investigate differences in stress-related behavioral and physiological endpoints that result from intra-LC LPS and, especially, IL-1ra administration.

1.8 Summary of Information and Overarching Hypotheses

Stress-related psychiatric disorders pose a significant burden on the global population with PTSD and MDD serving as leading causes of worldwide disability (Ferrari et al., 2013; Santomauro et al., 2021). Though incredible advancements have been made in research over the last century, it remains a critical problem that therapeutic treatment options have improved only minorly (Hetrick et al., 2010; Holtzheimer & Mayberg, 2011; Mansour et al., 2023; Sartori & Singewald, 2019). Women are at a particular disadvantage in this regard because they are twice as likely to develop stress-related psychiatric disorders like PTSD and MDD compared to men (Altemus et al., 2014; Kessler et al., 1993; Kessler et al., 2012; McLean et al., 2011). This bias, however, exists distinctly during the reproductive years which alludes to a causal role of ovarian hormones, particularly E2, in this enhanced susceptibility (Barth et al., 2015; Kundakovic & Rocks, 2022). However, studies conducted in preclinical rodent models often oppose these findings, which is hypothesized to be driven by an overt lack of prior exposure to stress. Notably, E2 has been shown to direct estrogen-mediated non-reproductive behavioral responses via upregulation of CRF in the CeA through activation of the nuclear receptor ERβ; yet, exactly how this occurs in the context of stress remains unknown. In addition, women exhibit inherently greater levels of proinflammatory cytokines and, thus, are at greater risk of developing autoimmune disorders, as

well (Derry et al., 2015; Duma et al., 2010). It is well known that stress and inflammation reciprocally enhance the other's effects, thereby accelerating the impending development and subsequent severity of stress-related psychiatric disorders in this susceptible population (Bauer & Teixeira, 2019). A prominent way by which this occurs is through direct activation of the LC-NE system via the major proinflammatory cytokine, IL-1β, and its receptor, IL-1r (Borsody & Weiss, 2002, 2004, 2014). The female rat (and likely human) LC exhibits robust features of sexual dimorphism that render it more sensitive to stimuli that increase rates of neuronal firing (Bangasser et al., 2016). It is in this way that IL-1β is thought to sensitize the female LC to promote stress-induced hypervigilance, a characteristic behavior of PTSD and MDD (Lehner et al., 2021; Xie et al., 2024). Alternatively, the anti-inflammatory actions of IL-1ra have been shown to dose-dependently decrease LC neuronal activation (Borsody & Weiss, 2004), which alludes to a critical circuit level mechanism of stress regulation that has not been fully elucidated. Thus, the current overarching hypothesis is that the naturally elevated levels of E2 and IL-1β that exist in females work independently and together to promote a highly sensitive stress response that, when dysregulated, induces debilitating symptoms of disorders like PTSD and MDD. The following specific aims were generated to explore this hypothesis and answer lingering questions:

Specific Aim 1

A: Determine if repeated social stress evokes subsequent hypervigilance during behavioral challenges, and if these effects are exacerbated in rats with a stress history when assessed explicitly during the High E22 phase of the estrous cycle.

Furthermore, assess if these changes are reflected by increases in neuronal activation in the LC and CeA.

B: Determine if depletion of the ovarian hormones via surgical removal prevents manifestation of stress-induced hypervigilance in subsequent behavioral challenges in addition to increased neuronal activation in the LC and CeA.

Specific Aim 2

A: Determine if exposure to repeated social stress increases subsequent anxiety-like behavior and upregulates protein expression of ERβ and CRF within the CeA.

B: Determine if intra-CeA ERβ antagonism during repeated social stress prevents subsequent stress-induced elevations in anxiety- and depressive-like behaviors and CeA-CRF protein expression.

Specific Aim 3

A: Determine if intra-LC LPS promotes hypervigilance and elevations in plasma CORT and IL-1β in response to an acute social stressor.

B: Determine if intra-LC IL-1ra prevents hypervigilance and the preceding increases in LC neuronal activation in response to an acute social stressor.

C: Determine if intra-LC IL-1ra during repeated social stress prevents subsequent stress-induced increases in hypervigilance and social avoidance.

CHAPTER TWO

STREStrogen: History of Stress and Cycling Ovarian Hormones Intersect to Enhance Neuronal Sensitization and Hypervigilance in Female Rats

2.1 Background and Significance

Stress is one of life's few certainties to which the current population is undeniably accustomed, especially in the wake of the COVID-19 pandemic. Exposure to stress elicits an array of physiological and behavioral adaptations ingrained in the primitive brain for the purpose of survival that typically dissipate upon cessation of the stressor (Chrousos & Gold, 1992; Kudielka & Kirschbaum, 2005). However, dysregulation or persistence of this response can occur, thereby inducing pathophysiological changes in the brain that ultimately give rise to debilitating psychiatric disorders (Kudielka & Kirschbaum, 2005). Prior to and especially following the pandemic, stress-related psychiatric disorders have remained a leading cause of disability worldwide despite the numerous advancements in therapeutic research that have been made over the last several decades (Ferrari et al., 2013; Santomauro et al., 2021). Women are at a particular disadvantage in this regard because one factor known to majorly contribute to the development of these disorders is female biological sex (Bangasser & Valentino, 2014; Kessler et al., 2005; Kessler et al., 1993).

Research has shown that women are more susceptible to and suffer greater effects of stress compared to men (McLean et al., 2011; Oldehinkel & Bouma, 2011; Sandanger et al., 2004; Stroud et al., 2002) and, as such, are more than twice as likely to develop stress-related psychiatric disorders that share hyperarousal as a key symptom, including post-traumatic stress disorder (PTSD) and major depressive disorder (MDD) (Altemus et al., 2014; Barth et al., 2015; Lehner et al., 2021; Xie et al., 2024). Interestingly, this bias exists distinctly during the reproductive years and coincides with the natural fluctuations in ovarian hormones across the menstrual cycle (Barth et al., 2015; Kundakovic & Rocks, 2022). Clinical studies support this by evidencing positive associations between pubertal maturation, stress hormone levels, and depressive symptoms in adolescent girls but not boys (Chafkin et al., 2022; Charbonneau et al., 2009; Oldehinkel & Bouma, 2011; Stroud et al., 2011). Likewise in adults, premenopausal women have reported experiencing more distressing and greater numbers of intrusive images, a common symptom of PTSD, compared to age-matched men (Cheung et al., 2013; Rattel et al., 2019). Upon completion of menopause, however, the risk of developing these disorders is reduced and comparable to that of men (Barth et al., 2015; Dennerstein et al., 2004; Kundakovic & Rocks, 2022), thus indicating a notable role of ovarian hormones in this enhanced susceptibility.

Indeed, further support is derived from clinical studies that show the relative amount of circulating estrogen differentially affects stress-induced behavioral outcomes among women. Elevated levels of estrogen in premenopausal women exposed to stress have been shown to predict greater intrusion of negative images

(Cheung et al., 2013; Rattel et al., 2019) and enhance re-experiencing one's trauma (i.e., flashbacks) (Garcia et al., 2018). Similarly, postmenopausal women receiving estrogen treatment have reported greater negative affect following psychosocial stress than their placebo-treated counterparts (Dumas et al., 2012; Newhouse et al., 2008). Together, these findings emphasize a major role of ovarian hormones, namely estrogen, in women's heightened risk of developing stress-induced psychiatric disorders and displaying robust hypervigilant phenotypes.

Studies conducted in rodent models, however, often oppose these clinical findings. When directly comparing the sexes, many behavioral studies show that males exhibit greater anxiety-related and hypervigilant behaviors than females (Börchers et al., 2022; Scholl et al., 2019). This is especially true when implementing locomotor-dependent behavioral assays such as the elevated plus maze and open field test. These types of behavioral assessments were largely developed and validated in males without further investigation for applicability to female animal models, or for evaluating effects of sex hormones (Börchers et al., 2022). In fact, many preclinical studies support an influence of estrogen in reducing rather than elevating behaviors indicative of negative affect, whether occurring naturally in circulation (Fabris et al., 2022; Marcondes et al., 2001; Milad et al., 2009; Sayin et al., 2014) or by exogenous treatment (Ghazvini et al., 2021; Liu et al., 2022; Marcondes et al., 2001; Milad et al., 2009). Importantly, though, preclinical studies yielding these outcomes frequently omit the use of an initial stressor prior to conducting behavioral assessments, which likely drives this divergence between study populations. While these findings are important for understanding innate,

baseline levels of behavior, they fail to address the explicit effects of stress. As such, there is a substantial lack of information regarding critical components of stress-related neurocircuitry that drive behavior and uniquely position cycling females at greater risk of these unfavorable outcomes.

Two interconnected brain regions that are essential mediators of the stress response are the locus coeruleus (LC) and central amygdala (CeA) (Kravets et al., 2015). As the primary source of central norepinephrine (NE), the LC is responsible for regulating arousal and awareness (Bangasser et al., 2016; Benarroch, 2017; Chrousos & Gold, 1992). Excessive firing or sensitization of LC-NE neurons as a result of stress can lead to maladaptive states of hyperarousal that are common to stress-related psychiatric disorders, including hypervigilance, aversiveness, and avoidance (Chaijale et al., 2013; Howard et al., 2008; Naegeli et al., 2018; Pate et al., 2023; Seo et al., 2021). In fact, studies using rodents have shown that increased tonic LC activity is required for manifestation of these stress-induced behaviors (Howard et al., 2008; McCall et al., 2015). One prominent way by which LC neurons increase firing in response to stress occurs from direct innervation of corticotropin releasing factor (CRF)-producing neurons that originate within the CeA (Curtis et al., 2002; McCall et al., 2015; Van Bockstaele et al., 1998). CeA-CRF projections to the LC maintain a key role in directing the behavioral response to stress (Keen-Rhinehart et al., 2009; Van Bockstaele et al., 1998; Wiersielis et al., 2016). Furthermore, the structure of the LC is sexually dimorphic and both NE and CRF systems are greatly impacted by estrogen (Bangasser et al., 2016). Thus, heightened activation of LC-NE and CeA-CRF neuronal populations is more likely

to occur in females and is capable of eliciting behavioral perturbations that are highly characteristic of disorders like PTSD and MDD.

Taken together, it is evident that the LC and CeA play integral roles in enhancing the female stress response, which may be driven, at least in part, by heightened levels of circulating estrogens within these regions. However, there remains an overt inconsistency between preclinical and clinical literature regarding the impact of estrogen on stress-induced behavioral outcomes. Specifically, studies conducted in the clinical population typically report an anxiogenic effect of estrogen while those conducted in preclinical rodent models often report an anxiolytic effect. Therefore, it is hypothesized that prior exposure to stress will lead to exacerbated anxiety-like behavior and neuronal activation in naturally cycling female rats with the greatest effects occurring in those experiencing high estrogen phases of the rodent estrous cycle at the time of behavioral testing. Specifically, the following two experiments were conducted to identify how, 1) putative estrogen levels, assessed indirectly via estrous cycle stage, and 2) complete elimination of circulating ovarian hormones via surgical removal of the ovaries, may influence LC-NE and CeA-CRF neuronal activity and subsequent anxiety-like and hypervigilant behavior in rats following repeated exposure to social stress.

2.2 Materials and Methods

2.2.1 Rats and housing

A total of 71 sexually naïve Sprague-Dawley female rats ("controls" and "witnesses"; intact cohort, 9-10 weeks upon arrival; Sham/OVX cohort, 8-9 weeks upon arrival) were obtained from Charles River (Raleigh, North Carolina, USA).

Additionally, sexually naïve Sprague-Dawley male rats (“intruders”; 250-275 g upon arrival; Charles River) and retired breeder Long Evans male rats (“residents”; Charles River and Envigo; Dublin, Virginia, USA) were used for the social stress aspect. From the time of arrival, rats were single housed in standard polycarbonate cages with Teklad Sani-Chip® bedding (Envigo) and ad lib access to pelleted chow (Teklad Rodent Diet® 8904, Envigo) and filtered water, except during testing. All rats were kept in the same colony room but were separated on different racks by sex and, further, on different shelves by stress condition (i.e., control vs. witness) or role (i.e., intruder vs. resident). The room was kept on a 12-hour light/dark cycle (lights on at 0700 hours) and maintained at 25°C and 40% humidity. Rats were habituated for at least five days prior to beginning behavioral testing (intact cohort, all males), which occurred during the light cycle between 0800 and 1400 hours, or before receiving surgery (Sham/OVX cohort). All components of the study were conducted with prior approval from the University of South Carolina IACUC and in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals*.

2.2.2 Surgical sham and bilateral ovariectomy (OVX) procedures

Female rats in the Sham/OVX cohort received abdominal surgery under sterile conditions to manipulate the ovaries in one of two ways. First, rats were anesthetized via isofluorane vapor then, after proving unreactive to a toe pinch, a portion of the lower abdomen was shaved and cleaned. A vertical incision of approximately two cm was made into the skin and the tissue around the edges was gently separated from the underlying muscle, and then a similar incision was

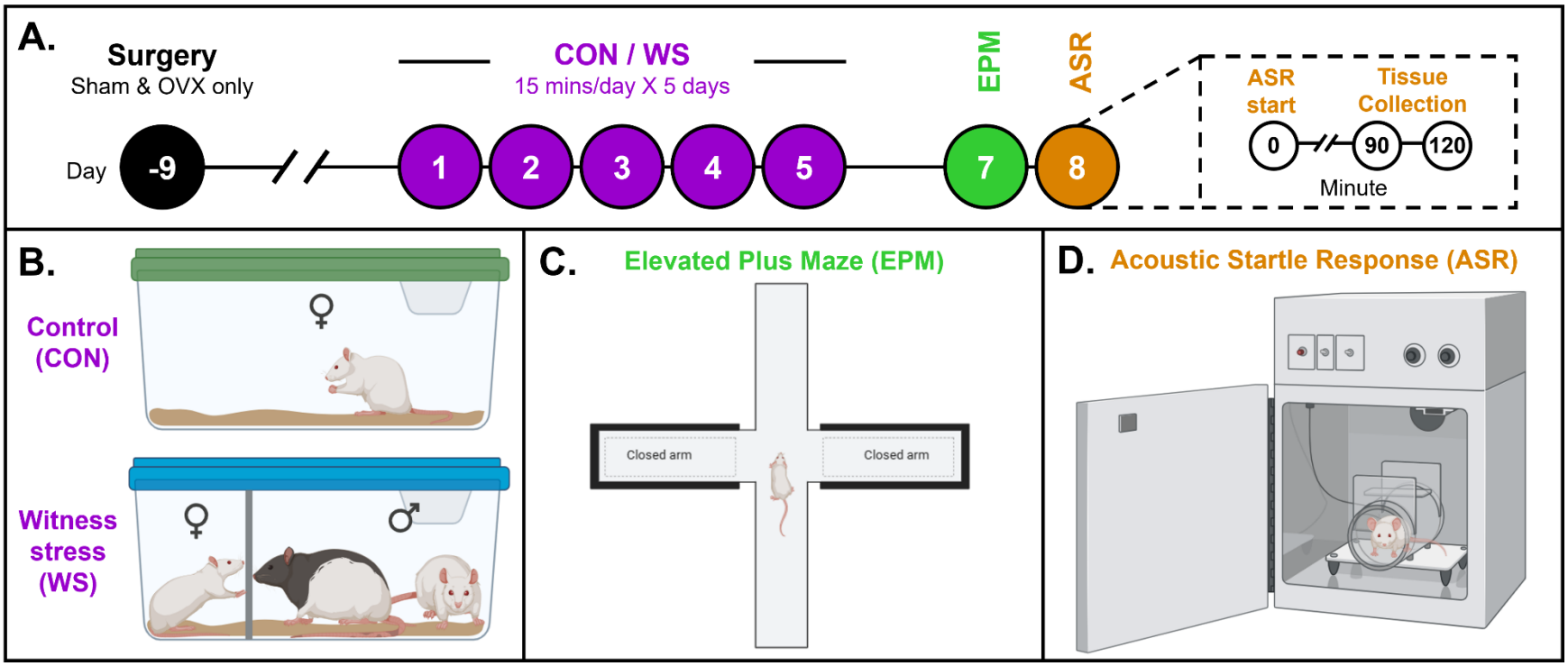

Figure 2.1 Experimental timeline and behavioral task descriptions. A) Two cohorts of rats were assessed using the same experimental design to assess behavioral and neuronal consequences of repeated witness stress with respect to the presence or absence of naturally cycling ovarian hormones. **B)** Rats in the CON group were briefly held then returned to their home cage whereas WS rats were exposed to the auditory, olfactory, and visual components of social defeat stress between two males from within a protected compartment of the same cage. **C)** Following repeated CON/WS exposure, female rats were assessed in the EPM for anxiety-like behaviors **D)** and then in ASR for hypervigilance. CON=control, WS=witness stress, OVX=ovariectomized, EPM=elevated plus maze, ASR=acoustic startle response

made into the muscle. Each ovary was then identified and returned to the abdominal cavity (Sham) or occluded from the attached uterine horn and fully excised (OVX). Lastly, each incision was sutured closed. Post-operative analgesia (Flunazine® Injectable Solution, 0.25 mg/kg, s.c.) was administered at the start of and roughly 18 hours after surgery, along with nutritional supplementation (Bacon Softies, Bio-Serv; Flemington, New Jersey, USA). Rats were given at least five days of recovery prior to beginning testing.

2.2.3 Repeated witness stress (WS) and non-stressed control handling (CON)

Witness stress (WS) is an ethologically relevant form of psychosocial stress modified from the widely used resident-intruder social defeat paradigm. For this study, WS was conducted for 15 minutes per day for five consecutive days (**Figure 2.1A,B**). Briefly, the female witness is confined to one side of a resident's home cage to observe the auditory, olfactory, and visual stimuli of a social defeat encounter between the resident and intruder as described previously (**Figure 2.1B**) (Bouknight et al., 2023; Finnell et al., 2018; Pate et al., 2023). Importantly, prior to testing, all residents were screened during at least two subsequent sessions of social defeat with a novel Sprague-Dawley male, and only those exhibiting adequate aggression without causing injury were used. Additionally, while there was never physical contact between the witnesses and intruders, they were paired such that the same witness and intruder were exposed in their respective manners to a novel resident each day to prevent establishment of social dominance and provide a consistent level of social defeat over the five-day period.

For comparison, females in the non-stressed control (CON) group were briefly handled then returned to their home cages (**Figure 2.1B**) to mimic the transfer of the witnesses to the residents' cages while remaining non-stressed. Our lab has previously shown that this version of a control condition produces equivalent outcomes to when a female rat is placed into a novel cage behind a partition in the presence of a non-stressed intruder male (Bouknight et al., 2023).

2.2.4 Elevated Plus Maze testing (EPM) testing

Elevated plus maze (EPM) testing occurred on day 7 (**Figure 2.1A**) for evaluation of hypervigilant behaviors including general risk assessment and avoidance. At least 30 minutes prior to beginning testing, rats were transported to a quiet area adjacent to the EPM room for environmental habituation. Rats were then individually assessed in a standard EPM apparatus (**Figure 2.1C**) (previously described in detail (Bouknight et al., 2023)) beginning with placement of the rat in the center of the maze facing an open arm. An overhead video camera captured each rat's five-minute session for retrospective analysis of three conventional measures including total distance traveled, duration in the open arms, and duration in the closed arms, as well as four ethological behaviors—stretching, grooming, head dipping, and rearing. An experienced researcher blinded to treatment groups manually scored the ethological behaviors while distance traveled and arm durations were automatically scored using ANY-maze software (Stoelting Co.; Wood Dale, Illinois, USA). Total durations of each behavior were then converted to percents of total time.

2.2.5 Acoustic Startle Response (ASR) testing

Acoustic startle response (ASR) testing occurred on day 8 (**Figure 2.1A**) for assessment of hypervigilance via the SR-LAB Startle Response System (San Diego Instruments; San Diego, California, USA). Rats were individually placed in an acrylic enclosure secured over an accelerometer within one of three adjacent sound-attenuating isolation cabinets (**Figure 2.1D**). Rats were tested two or three at a time in a counterbalanced fashion based on stress condition and, when applicable, surgical procedure. To prevent disturbances from outside sounds, 65 decibels (dB) was played continuously throughout the session to serve as background noise. All rats were exposed to the same protocol that began with a five-minute habituation period in which only background noise was played, followed by a series of ten different tones (65, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB) that were each played a total of five times in a pseudorandom order. All tones were played in 40-millisecond bursts and the following 100 milliseconds were recorded for the maximum startle amplitude exhibited by each rat for that specific trial. To ensure equivalence across test sessions, calibration of each cabinet's accelerometer reading and dB level emission was conducted according to the manufacturer's directions in the associated manual.

2.2.6 Estrous cycle phase determination

Immediately following their respective EPM and ASR sessions, vaginal lavage samples from each rat were collected and evaluated by an experienced researcher based on previously published criteria (Cora et al., 2015; Long & Evans, 1922). Samples were obtained by inserting a sterile transfer pipette containing ~200 µL of saline into the vaginal opening and aspirating the liquid. Each sample was then

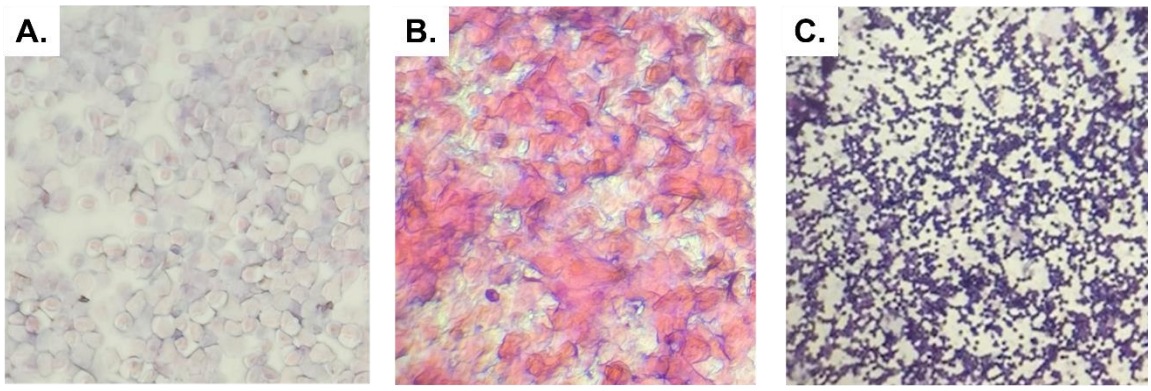

Figure 2.2 Estrous cycle phase determination based on cell types present in vaginal lavage samples. A) Clusters of small, round cells containing large, stained nuclei are predominantly found in the proestrus phase when estrogen levels peak and progesterone is minimal. **B)** Numerous jagged, keratinized cells that often lack nuclei and appear in thick sheets of pink are found in the mid-to-late estrous phase when estrogen and progesterone levels are both low. **C)** Tiny, round, darkly stained leukocytic cells are in abundance during the metestrus and diestrus phases when circulating estrogen is low and progesterone is high.

plated on a microscope slide, fully dried, and then stained with hematoxylin and eosin Y. Under 20X magnification, cells were identified as nucleated epithelial (**Figure 2.2A**), anucleated epithelial (**Figure 2.2B**), or leukocytes (**Figure 2.2C**) and the abundance and proportion of each were considered for classification into either the “Low E2” or “High E2” phase group. Low levels of estradiol occur during the late estrus through mid-diestrus phases (Lovick & Zangrossi, 2021; Maeng & Milad, 2015) which are characterized by a lack of nucleated cells and a majority of leukocytes (**Figure 2.2C**) (Cora et al., 2015; Long & Evans, 1922). Peak estradiol occurs during proestrus, but high levels are present throughout the late diestrus to early estrus phases (Lovick & Zangrossi, 2021; Maeng & Milad, 2015) which are characterized predominantly by nucleated cells with sparse anucleated cells and an overall absence of leukocytes (**Figure 2.2A**) (Cora et al., 2015; Long & Evans, 1922).

2.2.7 Tissue collection and preparation

Tissue was collected via transcardial perfusion occurring 90-120 minutes following the start of ASR testing on day 8 (**Figure 2.1A**). This timeframe was chosen for assessment of peak cFos expression, a widely used marker of neuronal activation (Hoffman et al., 1993). Briefly, rats were heavily sedated under isofluorane anesthesia then perfused with ~200 mL of cold 0.1M phosphate-buffered saline (PBS) followed by ~250 mL of cold 4% paraformaldehyde. Brains were then removed and post-fixed at 4°C for at least 24 hours followed by complete saturation in a sucrose-azide solution (20% sucrose + 1% sodium azide in 0.1M phosphate buffer (PB)). Brains were then flash frozen in isopentane and stored at -80°C until coronally sliced at 30-µm thickness for serial collection of region-specific tissue. Slices were stored in an anti-freezing solution (30% sucrose + 30% ethylene glycol in 0.1M PB) at -20°C until analyses were ready to be conducted.

2.2.8 Immunohistochemical staining of cFos and tyrosine hydroxylase (TH) in free-floating LC slices of the intact cohort

Slices containing core LC were selected from a subset of rats in the intact cohort (Bregma -9.96 to -9.60 mm, corresponding to the Rat Brain Atlas (Paxinos & Watson, 2006)). Tissue was first washed in fresh 0.1M PBS three consecutive times for ten minutes each followed by a 30-minute incubation in blocking buffer (0.9% H2O2 + 4% Triton X-100 in PBS). After another round of washes, tissue was incubated in an antibody solution (2% normal donkey serum + 0.25% Triton X-100 in 0.1M PBS) containing rabbit anti-cFos (1:500, No. ABE457; Millipore Sigma; Burlington, Massachusetts, USA) for two days. On the morning of the third day,

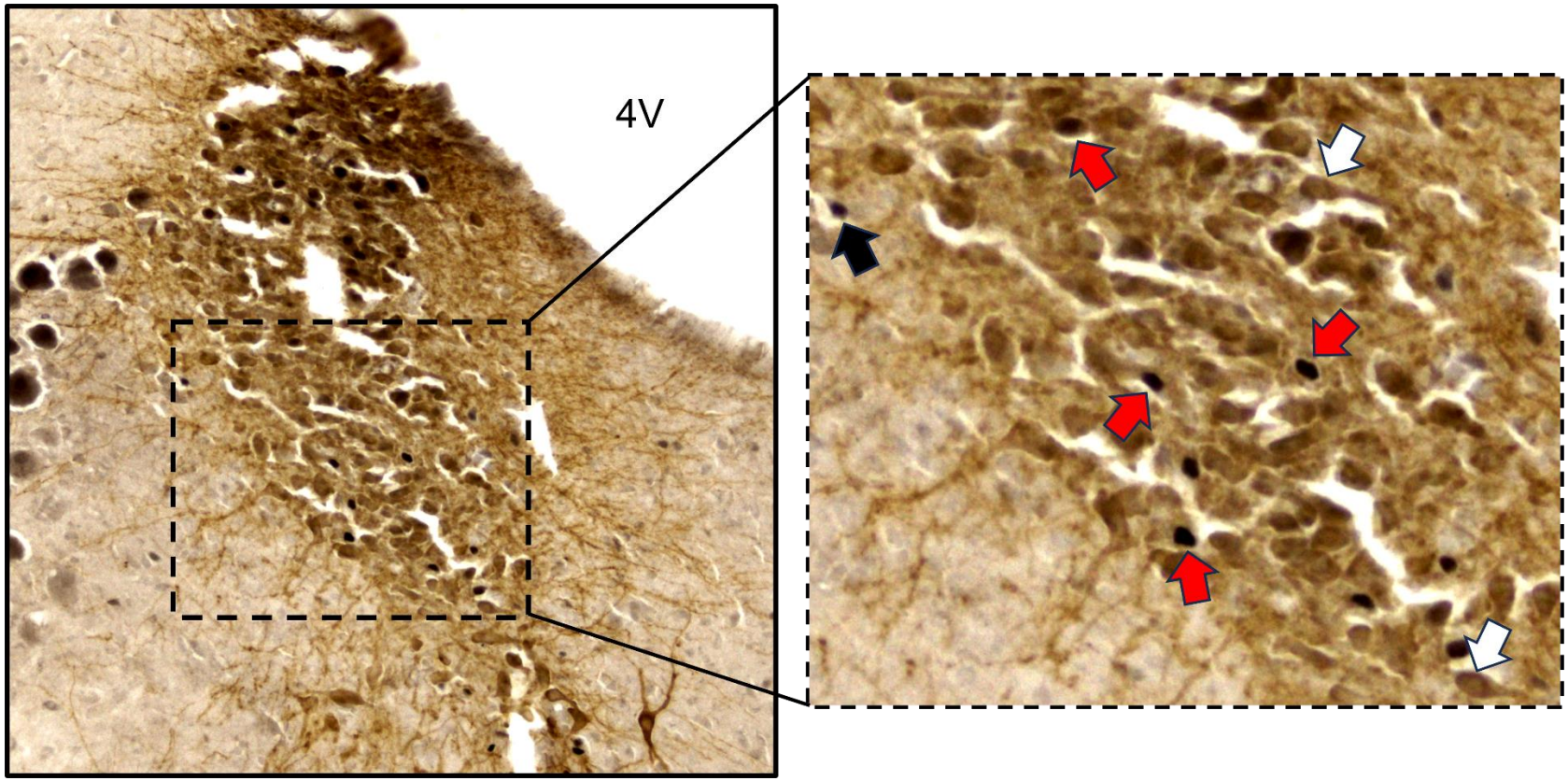

Figure 2.3 Photomicrograph of the locus coeruleus stained via immunohistochemistry for identification of tyrosine hydroxylase- and cFos-expressing neurons. Noradrenergic cells of the LC are indicated by copper-brown staining that resulted from TH immunoreactivity while putative neuronal activation is indicated by the dark blue-black nuclei that resulted from cFos immunoreactivity. The zoomed-in image on the right denotes “non-activated” noradrenergic cells solely expressing TH (white arrows), “activated” non-noradrenergic cells solely expressing cFos (black arrow), and “activated” noradrenergic cells co-expressing TH and cFos (red arrows). LC=locus coeruleus, TH=tyrosine hydroxylase, 4V = 4th ventricle

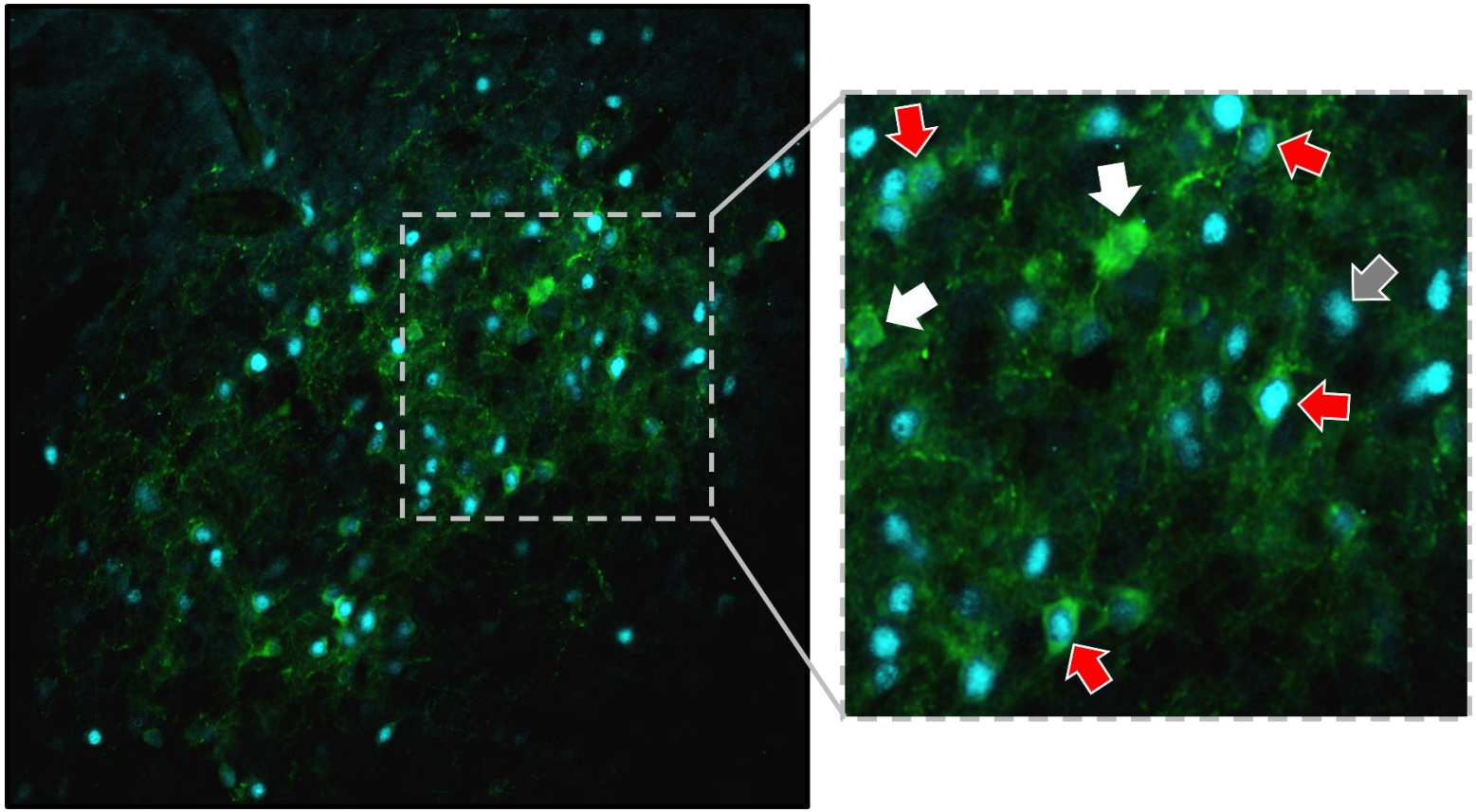

Figure 2.4 Photomicrograph of the central amygdala stained via immunofluorescence for identification of corticotropin releasing factor- and cFos-expressing neurons. CRF-producing cells in the CeA are indicated by green fluorescence that resulted from CRF immunoreactivity while putative neuronal activation is indicated by the cyan fluorescence that resulted from cFos immunoreactivity. The zoomed-in image on the right denotes “non-activated” cells solely expressing CRF (white arrows), “activated” cells solely expressing cFos (gray arrow), and “activated” cells co-expressing CRF and cFos (red arrows). CeA=central amygdala, CRF=corticotropin releasing factor

tissue underwent another round of washes before being incubated in biotinylated goat anti-rabbit (1:200, No. BA-1000; Vector Laboratories; Newark, California, USA) antibody solution for two hours. Tissue was then incubated in an avidin/biotin complex (ABC) (VECTASTAIN®, No. PK-6100; Vector Laboratories) solution for one hour prior to undergoing another round of washes. Next, tissue was incubated in nickel-enhanced diaminobenzidine (DAB) (SIGMAFAST™, No. D2493; Millipore Sigma) solution for two minutes and thirty seconds which was immediately followed by another round of washes. For TH staining, this process was repeated beginning at the second round of washes with the following changes: tissue was incubated overnight at in mouse anti-TH (1:2500, No. MAB5280; Millipore Sigma) antibody solution, for two hours in biotinylated donkey anti-mouse (1:1000, No. ab208001; Abcam; Boston, Massachusetts, USA) antibody solution, and for 15 seconds in DAB (Millipore Sigma) solution. After the final round of washes, tissue was mounted onto microscope slides (Fisherbrand™ Superfrost™ Plus; No. 12-550-15, Thermo Fisher Scientific; Waltham, Massachusetts, USA) which were then dehydrated then cover slipped. Images of the slices were then taken at the level of the LC corresponding to plates 30 and 31 of the Rat Brain Atlas (Paxinos & Watson, 2006) for quantification of cells co-expressing cFos and TH (**Figure 2.3**).

2.2.9 Immunohistochemical staining of cFos and TH in slide-mounted LC slices of the Sham/OVX cohort

To further preserve the integrity of the tissue, the above staining process was adjusted using a more conservative approach for the Sham/OVX cohort. Slices containing core LC were first mounted onto microscope slides (Fisherbrand™

ProbeOn Plus™, No. 22-230-900; Thermo Fisher Scientific) and then briefly dried. Slides then underwent three consecutive ten-minute washes in fresh wash buffer (0.5% Tween-20 in 0.1M PBS) followed by a 30-minute incubation in blocking buffer (2% normal donkey serum + 0.05% Tween-20 + 0.1% Triton X-100 + 0.01% bovine serum albumin (BSA) + 50mM glycine in 0.1M PBS). Slides were then incubated in primary antibody solution (0.1% Triton X-100 + 0.05% Tween-20 + 0.03% H_2O_2 + 1% sodium azide + 10mM glycine in 0.1M PBS) containing rabbit anti-cFos (1:350; Millipore Sigma) at 4°C for roughly 48 hours. Following a second round of washes in wash buffer, slides were incubated for two hours in a secondary antibody solution (0.1% Tween-20 in 0.1M PBS) containing biotinylated goat anti-rabbit (1:400; Vector Laboratories). Next, slides were incubated in ABC (Vector Laboratories) solution for 30 minutes followed by three consecutive ten-minute washes in 0.1M PBS. Slides were then incubated in nickel-enhanced DAB (Vector Laboratories) solution for five minutes prior to undergoing another round of PBS washes. This process was repeated beginning at the second round of washes in wash buffer for TH staining with the following changes: slides were incubated overnight at 4°C in mouse anti-TH primary antibody (1:5000; Millipore Sigma) solution, for two hours in biotinylated donkey anti-mouse secondary antibody (1:1000; Abcam) solution, and for 90 seconds in DAB (No. SK-4100; Vector Laboratories) solution. After a final round of PBS washes, slides were dried, dehydrated, and then cover slipped. Identically to the intact cohort, images of LC slices from Sham/OVX rats were then taken at the level of the LC corresponding

to plates 30 and 31 of the Rat Brain Atlas (Paxinos & Watson, 2006) for quantification of cells co-expressing cFos and TH (**Figure 2.3**).

2.2.10 Immunofluorescent staining of cFos and CRF in free-floating CeA slices

Slices containing the CRF-rich region of the CeA were selected from a subset of rats in each cohort (Bregma -3.14 to -2.56 mm, corresponding to the Rat Brain Atlas (Paxinos & Watson, 2006)). To begin, tissue underwent three consecutive ten-minute washes in fresh 0.1M PBS, followed by a 90-minute incubation in blocking buffer (10% normal donkey serum + 0.3% Triton X-100 in 0.1M PBS). Tissue was then incubated for roughly 48 hours at 4°C in antibody solution (5% normal donkey serum + 0.3% Triton X-100 in 0.1M PBS) containing, for the intact cohort, mouse anti-cFos (1:500; No. NPB2-50037, Novus Biologicals; Centennial, Colorado, USA), and, for the Sham/OVX cohort, rabbit anti-cFos (1:500; Millipore Sigma). On the third day, tissue underwent a round of PBS washes, and then was incubated for two hours in antibody solution containing donkey anti-mouse Alexa Fluor 647 (1:500; No. ab150107, Abcam; Boston, Massachusetts, USA) or donkey anti-rabbit Alexa Fluor 647 (1:500; No. ab150075, Abcam), respectively. Next, after another round of PBS washes, tissue was incubated in antibody solution containing guinea pig anti-CRF (1:1000; No. TA364372, Origene; Rockville, Maryland, USA) for roughly 36 hours at 4°C. The next day, following another round of PBS washes, tissue was incubated for two hours in donkey anti-guinea pig Alexa Fluor 488 (1:500; No. 706-545-148, Jackson ImmunoResearch Laboratories; West Grove, Pennsylvania, USA). After a final round of PBS washes, tissue was mounted onto microscope slides (Fisherbrand™ Superfrost™ Plus; Thermo Fisher

Scientific), dried, and then cover slipped. Images were taken of the CeA corresponding to plates 59 and 60 in the Rat Brain Atlas (Paxinos & Watson, 2006) for quantification of cells co-expressing CRF and cFos (**Figure 2.4**).

2.2.11 Statistical analyses

Statistical outliers were identified and removed from each data set if they were greater than two standard deviations outside of the mean. EPM data from the intact cohort were first analyzed using two-tailed unpaired t-tests to compare groups based on prior stress condition (i.e., CON vs WS). These assessments were then expanded to compare rats based on stress X estrous cycle phase using two-way analysis of variance (ANOVA) followed by Tukey's post-hoc analyses. ASR data were analyzed via two-way ANOVAs to compare stress X startle amplitude followed by planned comparisons using Fisher's least significant difference (LSD) post-hoc analyses. Neuronal data from the LC and CeA were analyzed via unpaired t-tests to compare groups based on stress. For the Sham/OVX cohort, EPM data were analyzed via two-way ANOVAs, first, to compare stress X ovarian status, then again to compare stress X estrous or OVX, all of which were followed by Tukey's post-hoc analyses. ASR data were analyzed via three-way ANOVAs with planned comparisons using Fisher's LSD post-hoc analyses to compare stress X ovarian status X startle amplitude. Cell counts from the LC and CeA were analyzed via two-way ANOVAs followed by Tukey's post-hoc analyses to compare stress X ovarian status. Main effects resulting from these analyses are reported in the main text of the results section, while significant post-hoc and t-test findings are denoted by symbols on figures and reported in corresponding figure legends.

Statistical significance was defined as $\alpha = 0.05$, and data are presented as mean ± standard error of the mean (SEM).

2.3 Results

2.3.1 Anxiety-like behavior in the EPM is not discretely influenced by history of WS in the intact cohort

Prior exposure to repeated WS alone did not alter any of the behavioral outcomes for which rats were evaluated in their respective five-minute EPM sessions. Unpaired t-tests for distance traveled (**Figure 2.5A**; $t_{26}=0.0070$, $p=0.9944$), duration of time spent in the open arms (**Figure 2.5B**; $t_{27}=0.5462$, $p=0.5894$), in the closed arms (**Figure 2.5C**; $t_{27}=0.1086$, $p=0.9143$), grooming (**Figure 2.5D**; $t_{27}=0.9911$, $p=0.3305$), head dipping (**Figure 2.5E**; $t_{27}=0.4164$, $p=0.6804$), rearing (**Figure 2.5F**; $t_{27}=0.9810$, $p=0.3353$), and stretching (**Figure 2.5G**; $t_{27}=0.7082$, $p=0.4849$) revealed no significant differences between control and WS groups.

2.3.2 Hypervigilance in the EPM is influenced by estrous cycle phase in a dichotomous manner in the intact cohort

When compared by stress in conjunction with the estrous cycle phase at the time of testing, multiple differences in behavioral outcomes were revealed. In general, rats with a history of WS that were tested during the High E2strogen phases of the estrous cycle (WS / High E2) exhibited increased hypervigilance and reduced exploration, whereas the opposite was true for rats without a history of WS (CON / High E2). Two-way ANOVAs revealed significant interactions of stress X estrous for distance traveled (**Figure 2.6A**; $F_{1,24}=18.13$, $p=0.0003$), duration in the open arms (**Figure 2.6B**; $F_{1,25}=55.43$, $p<0.0001$), closed arms (**Figure 2.6C**;

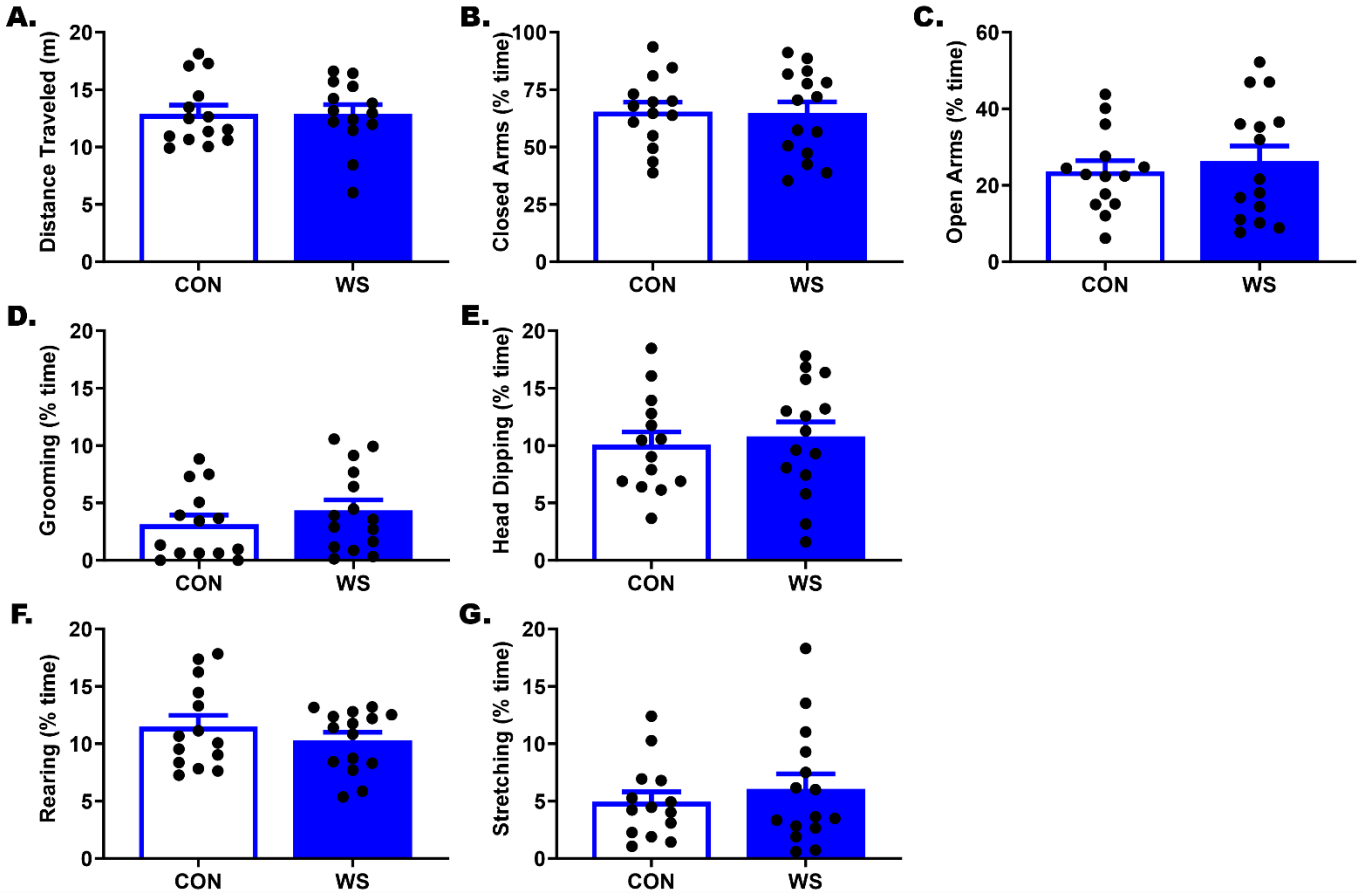

Figure 2.5 Elevated plus maze testing revealed no differences in behavioral outcomes based on history of witness stress alone in the intact cohort. A) Total distance traveled, **B)** duration in the closed arms, **C)** duration in the open arms, **D)** duration of grooming, **E)** duration of head dipping, **F)** duration of rearing, and **G)** duration of stretching remained unaffected by prior exposure to WS throughout the 5-minute test duration. CON=control, WS=witness stress
No statistical significance.

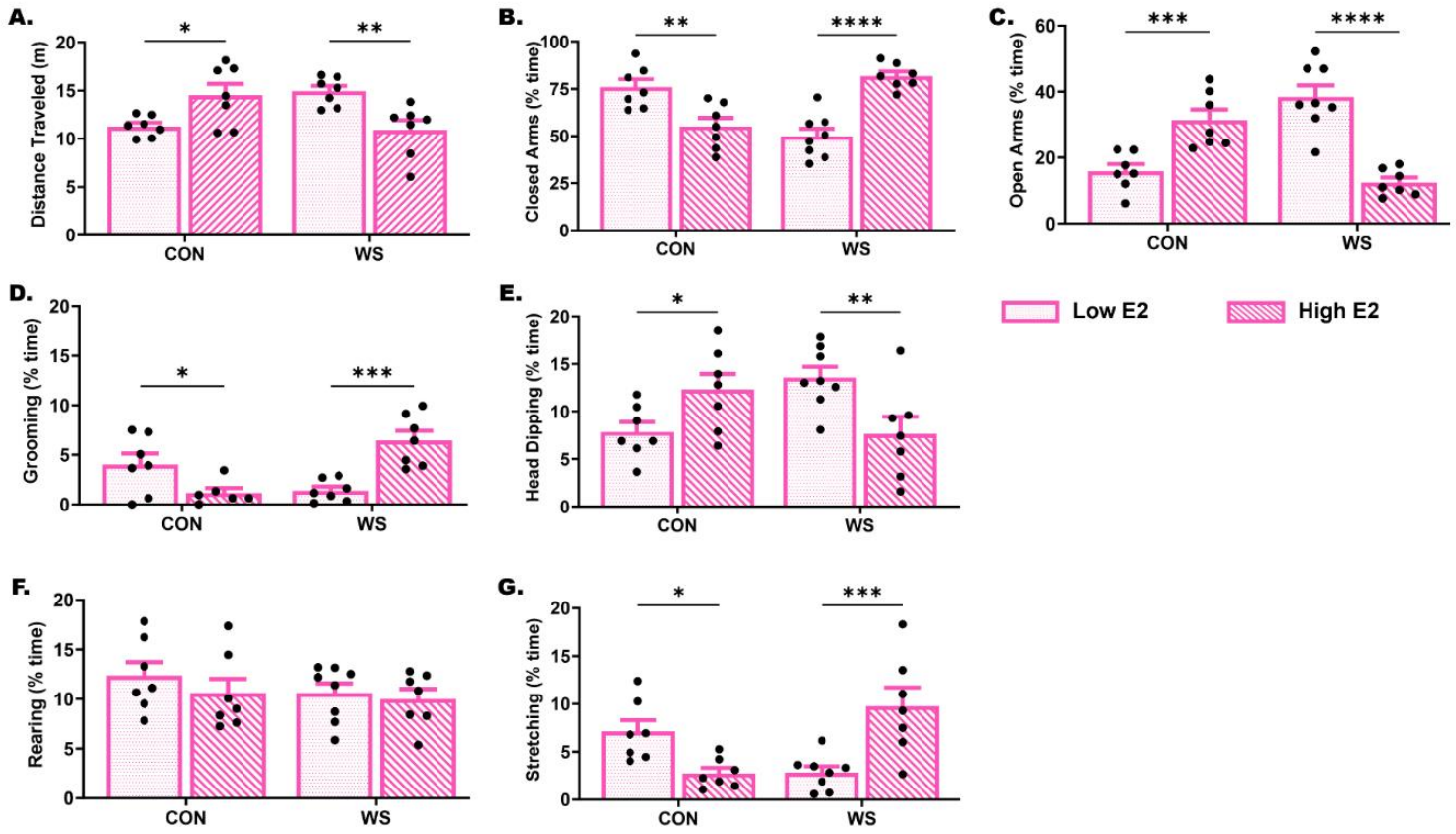

Figure 2.6 Behavioral assessments during elevated plus maze testing revealed a dichotomous role of estrous phase in a stress-dependent manner for rats in the intact cohort. Rats with a history of WS displayed greater anxiety-like and avoidant behaviors (e.g., greater duration **B)** in the closed arms, **D)** grooming, and **G)** stretching) and less risk-taking, exploratory behaviors (e.g., reduced **A)** distance traveled and lesser duration **C)** in the open arms and **E)** head dipping) when tested during High E2 versus Low E2 phases, though **F)** rearing was not affected. These behaviors were completely reversed for CON rats such that those in High E2 phases exhibited less anxiety-like and more risk-taking behaviors those in Low E2 phases. CON=control, WS=witness stress, E2=estrogen

** p < 0.05, ** p < 0.01, *** p < 0.001,**** p < 0.0001 versus stress-matched counterparts; Tukey's post-hoc*

$F_{1,25}$=45.12, p<0.0001), grooming (**Figure 2.6D**; $F_{1,23}$=22.86, p<0.0001), head dipping (**Figure 2.6E**; $F_{1,25}$=13.21, p=0.0013), and stretching (**Figure 2.6G**; $F_{1,25}$=22.60, p<0.0001). No main effects were observed for duration of rearing (**Figure 2.6F**; effect of stress X estrous: $F_{1,25}$=0.2219, p=0.6417).

2.3.3 Rats with a history of WS in the intact cohort exhibited heightened startle amplitude as well as greater neuronal activation in the LC and CeA

All rats were exposed to the same range of tones in the same pseudorandom order, and all responded with increasingly heightened startle amplitude as the tones grew louder (**Figure 2.7A**; effect of tone: $F_{9,254}$=69.78, p<0.0001). Overall, rats with a history of WS exhibited greater startle compared to CON rats independent of estrous cycle phase (**Figure 2.7A**; effect of stress: $F_{1,254}$=9.851, p=0.0019). Notably, rats with a history of WS had significant elevations in startle amplitude at the 105 dB, 110 dB, and 120 dB tone bursts.

Brains were collected to reflect peak levels of cFos protein, a prominent marker of neuronal activation, following ASR testing. Rats with a history of WS displayed significantly greater numbers of cFos-positive cells in the LC (**Figure 2.7B**; t_{14}=7.841, p<0.0001), though there were no differences in the total area of the LC that was analyzed (t_{20}=0.2449, p=0.8090, data not shown. Rats with a history of WS also showed a greater percent of total CRF-positive cells co-expressing cFos in the CeA (**Figure 2.7C**; t_{10}=5.175, p=0.0004) which were mirrored by differences in total number of cells co-expressing CRF and cFos (t_{11}=2.304, p=0.0418, data not shown), though no differences were observed in the overall number of CRF-positive cells regardless of cFos co-expression (t_{11}=0.1294,

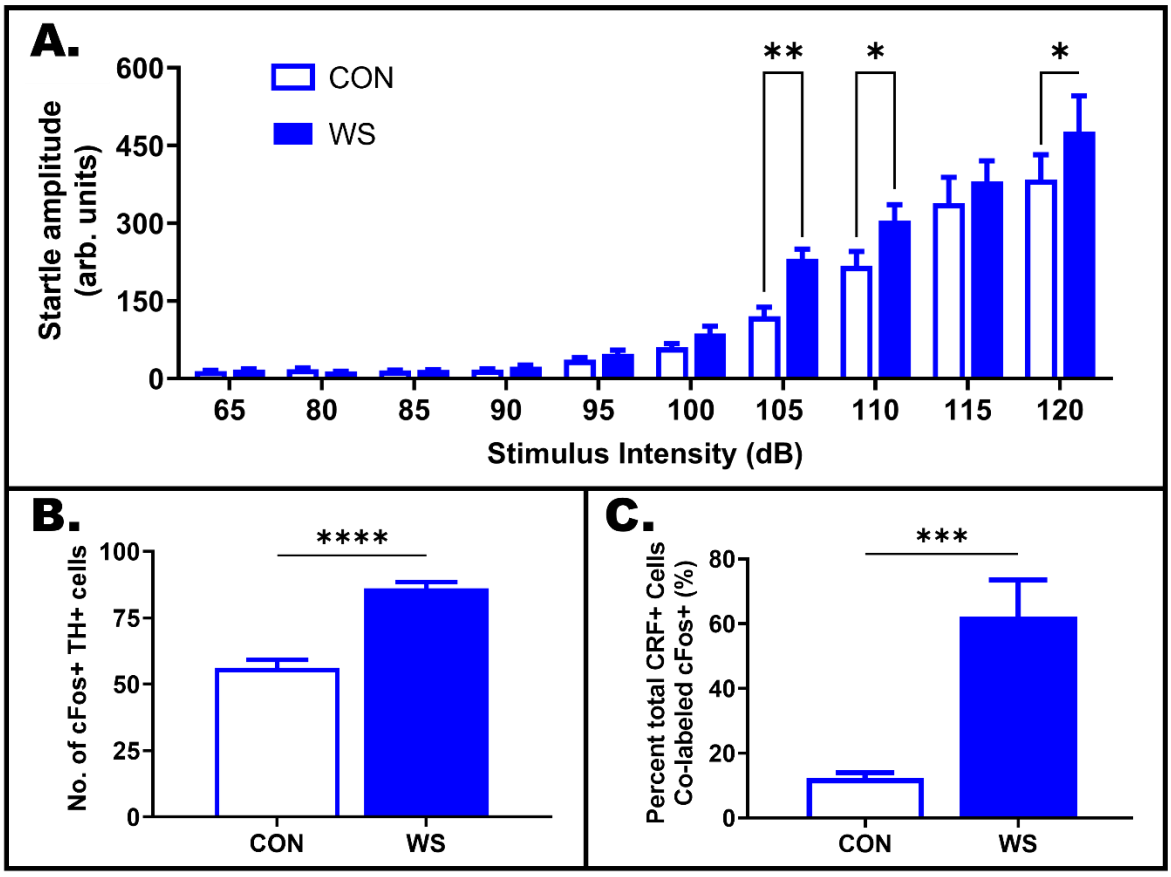

Figure 2.7 History of witness stress elicited greater startle amplitude during acoustic startle response testing as a result of neuronal sensitization in the locus coeruleus and central amygdala of rats in the intact cohort. A) All rats exhibited increased startle amplitude as a function of stimulus intensity, though WS induced an enhanced response compared to CON. History of WS also evoked greater neuronal activation as evidenced by **B)** increased numbers of TH-positive cells co-expressing cFos in the LC, and **C)** an increased percentage of total CRF-positive cells co-expressing cFos in the CeA. CON=control, WS=witness stress, dB=decibel, LC=locus coeruleus, CeA=central amygdala, TH=tyrosine hydroxylase, CRF=corticotropin releasing factor
** p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, Tukey's post-hoc*

p=0.8994, data not shown). Together, these results indicate greater activation of LC-NE and CeA-CRF cells during acoustic startle response testing as a result of prior WS.

2.3.4 Anxiety-like behavior in the EPM was not altered discretely by differences in stress history or ovarian status in the Sham/OVX cohort

Neither history of WS nor absence of ovaries directly influenced any of the behaviors that were measured in the EPM. Similar to the intact cohort, all rats in the Sham/OVX cohort exhibited approximately the same distance traveled (**Figure 2.8A**; effect of stress X ovarian status: $F_{1,37}$=1.341, p=0.2543), duration in the open arms (**Figure 2.8B**; effect of stress X ovarian status: $F_{1,36}$=0.2423, p=0.6255), and the closed arms (**Figure 2.8C**; effect of stress X ovarian status: $F_{1,37}$=0.0110, p=0.9170), duration of stretching (**Figure 2.8D**; effect of stress X ovarian status: $F_{1,37}$=0.7427, p=0.3944), grooming (**Figure 2.8E**; effect of stress X ovarian status: $F_{1,35}$=0.04356, p=0.8359), head dipping (**Figure 2.8F**; effect of stress X ovarian status: $F_{1,37}$=0.2044, p=0.6538), and rearing (**Figure 2.8G**; effect of stress X ovarian status: $F_{1,37}$=0.1747, p=0.6784).

2.3.5 Estrous cycle phase dichotomously influenced anxiety-like behavior in the EPM with respect to prior WS in Sham but not OVX rats

Similar yet muted differences in behavior were observed for rats in the Sham group of the Sham/OVX cohort as were seen for rats in the intact cohort during EPM testing when comparing estrous-related differences. Behaviors exhibited by rats in the OVX group remained indifferent when compared by history of WS. In addition, data obtained from the OVX group generally aligned with the data acquired from the Sham / Low E2 group with respect to stress. The one exception to this finding included a main effect of ovarian status that occurred for distance traveled (**Figure 2.9A**; $F_{2,35}$=5.990, p=0.0058) with OVX rats exhibiting greater locomotor activity than Sham rats as a whole. Significant interactions of stress X estrous or OVX were present for duration in the open arms (**Figure 2.9B**; $F_{2,34}$=4.186, p=0.0237),

which was exhibited more by rats in the WS / Low E2 group compared to those in the WS / High E2 group, and duration in the closed arms (**Figure 2.9C**; $F_{2,35}$=5.993, p=0.0058), which was exhibited more by rats in the WS / High E2 group compared to those in the WS / Low E2 group. No main effects were observed for duration of stretching (**Figure 2.9D**; effect of stress X estrous or OVX: $F_{2,35}$=0.9496, p=0.3966), duration of grooming (**Figure 2.9E**; effect of stress X estrous or OVX: $F_{2,33}$=0.08627, p=0.9176), duration of head dipping (**Figure 2.9F**; effect of stress X estrous or OVX: $F_{2,35}$=0.5491, p=0.5824), or duration of rearing (**Figure 2.9G**; effect of stress X estrous or OVX: $F_{2,35}$=0.1069, p=0.8989) among groups in the Sham/OVX cohort.

2.3.6 History of WS and cycling ovarian hormones elicited the greatest startle response and corresponding neuronal activity in the LC and CeA

Rats in the Sham/OVX cohort were exposed to the same acoustic startle protocol as the intact cohort, and all Sham and OVX rats responded with the expected increases in startle amplitude as the dB level of each tone increased (**Figure 2.10A**; effect of tone: $F_{9,268}$=36.46, p<0.0001). Three-way ANOVA revealed a trend of stress, such that all rats with a history of WS, regardless of ovarian status, exhibited elevated startle amplitude compared to CON rats across the entire range of tones (**Figure 2.10A**; effect of stress: $F_{1,268}$=3.234, p=0.0733). A significant interaction of stress X ovarian status was observed in which WS / Sham rats exhibited greater startle amplitude than CON / Sham and WS / OVX rats, but not CON / OVX rats (**Figure 2.10A**; effect of stress X ovarian status: $F_{1,268}$=7.795, p=0.0056). OVX abolished the stress-related sensitization in acoustic startle in

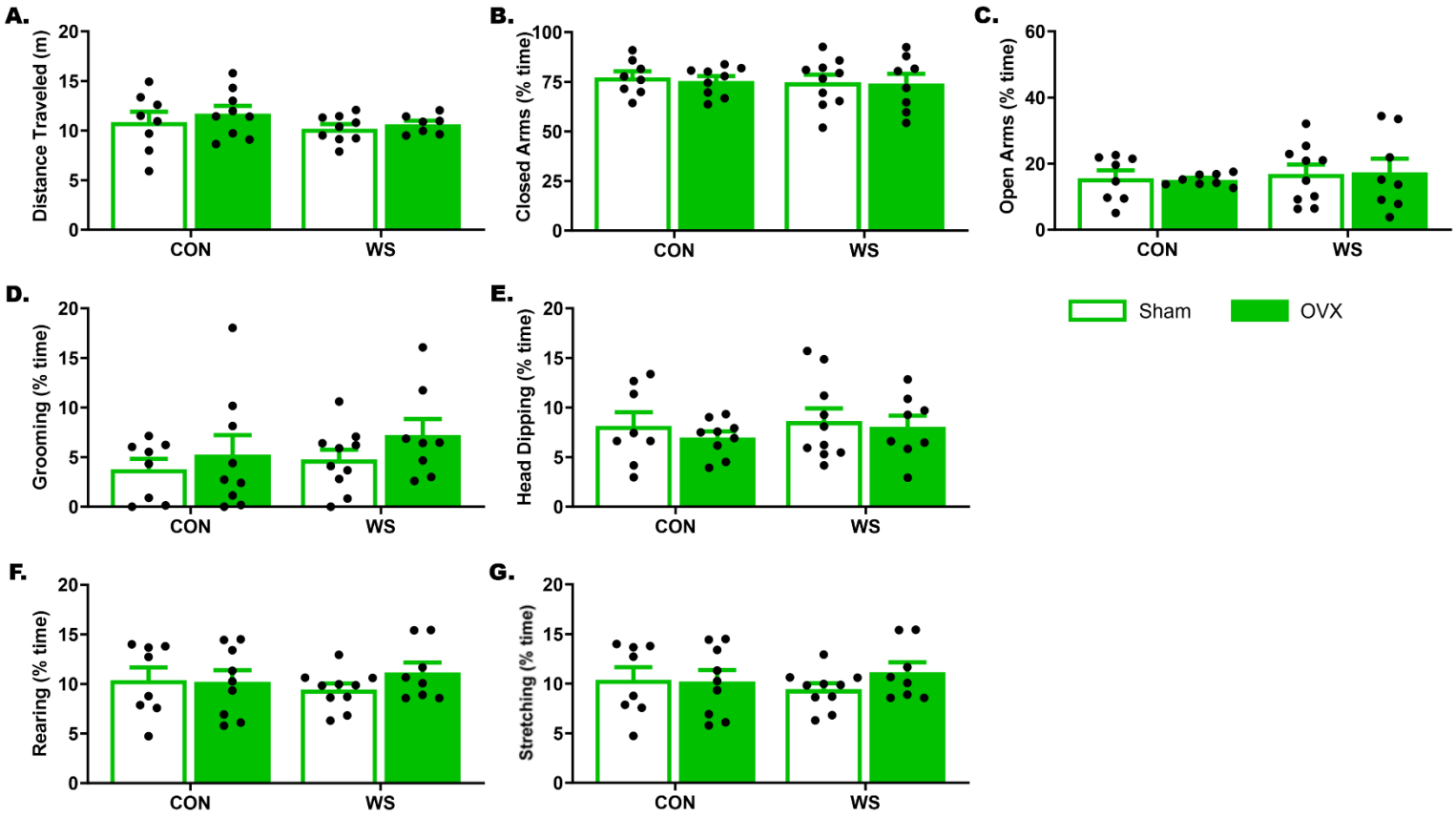

Fig 2.8 Neither history of witness stress nor retention of cycling ovarian hormones discretely altered behavior during elevated plus maze testing for rats in the Sham/OVX cohort. A) Total distance traveled, **B)** duration in the closed arms, **C)** duration in the open arms, **D)** duration of grooming, **E)** duration of head dipping, **F)** duration of rearing, and **G)** duration of stretching were not overtly affected by prior exposure to WS or retention of cycling ovarian hormones. CON=control, WS=witness stress, OVX=ovariectomized
No statistical significance.

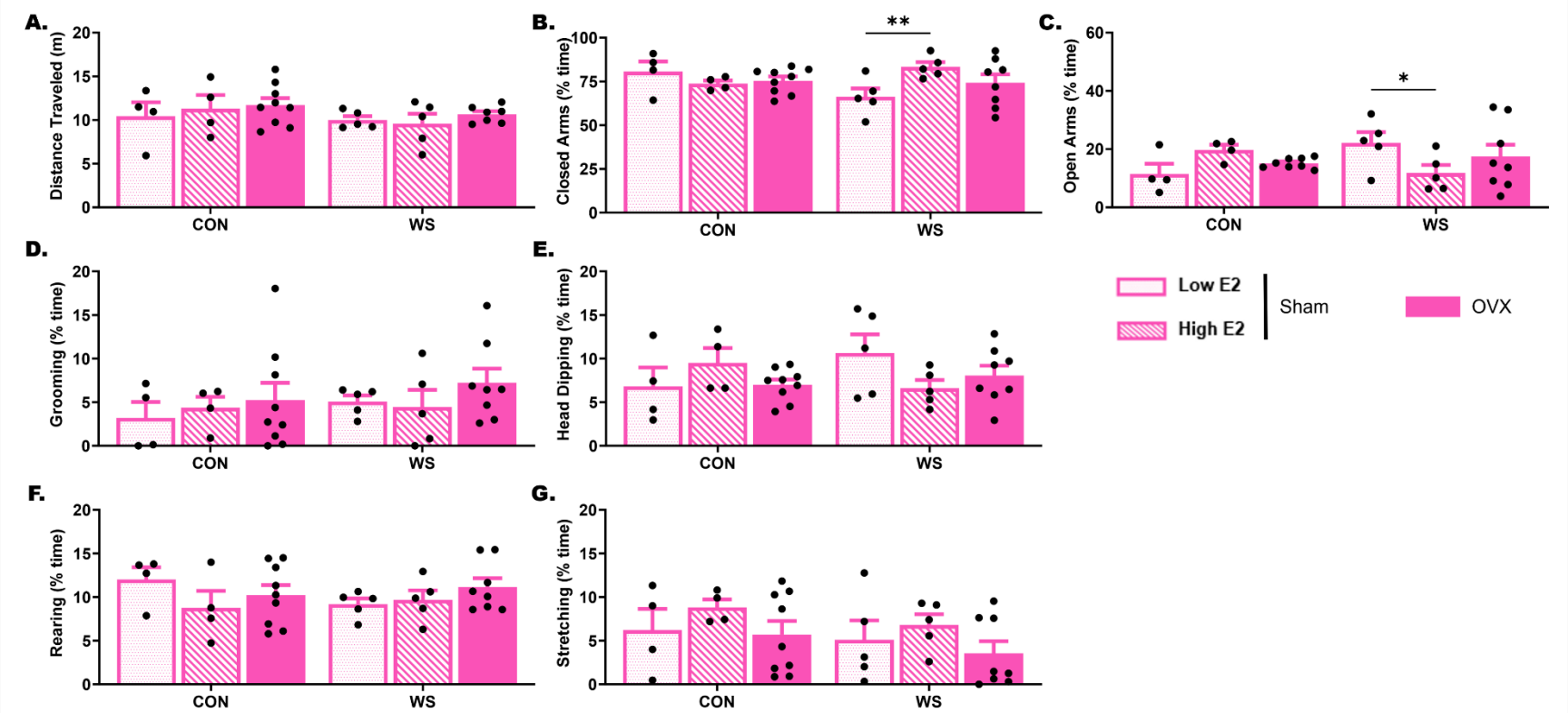

Figure 2.9 Anxiety-like behavior in the elevated plus maze was affected by estrous cycle phase in a dichotomous manner based on prior history of witness stress only in Sham rats of the Sham/OVX cohort. Sham rats with a history of WS that were tested during the High E2 phases of the estrous cycle exhibited greater anxiety-like and avoidant behavior than those in the Low E2 phases as indicated by **B)** more time spent in the closed arms and **C)** less time spent in the open arms. However, no differences were observed by estrous cycle within or between groups for the remainder of the behaviors, including **A)** distance traveled, **D)** grooming duration, **E)** head dipping duration, **F)** rearing duration, and **G)** stretching duration. CON=control, WS=witness stress, OVX=ovariectomized, E2=estrogen

** p < 0.05, ** p < 0.01 versus stress-matched counterparts, Tukey's post-hoc*

WS-exposed rats across the range of tones.

In line with above data, two-way ANOVAs revealed stark differences in neuronal activation levels among all four groups in the Sham/OVX cohort. In the LC, main effects of stress (**Figure 2.10B**; $F_{1,19}$=40.51, p<0.0001) and ovarian status (**Figure 2.10B**; $F_{1,19}$=23.38, p=0.0001) were observed, indicating a greater number of cFos-positive cells in WS compared to CON rats, and, further, in Sham compared to OVX rats. Overall, WS / Sham rats displayed the greatest number of cFos-positive cells in the LC, while CON / OVX rats displayed the lowest. In the CeA, no significant differences occurred, however there was a trend such that rats with a history of WS had a greater percentage of CRF-positive cells co-expressing cFos than CON rats (**Figure 2.10C**; effect of stress: $F_{1,8}$=4.095, p=0.0776) as well as a greater total number of cells co-expressing CRF and cFos ($F_{1,11}$=4.577, p=0.0557, data not shown). There was also a trend of ovarian status on total number of CRF-positive cells (effect of ovarian status: $F_{1,10}$=4.953, p=0.0502) indicating that Sham rats had greater numbers of CRF-immunoreactive cells in the CeA than OVX rats as a whole (data not shown).

2.4 Discussion

The present data indicate that behavioral responses during EPM testing, reflecting measures related to anxiety-like behaviors in rodents (i.e., arms' durations), exploration (i.e., distance, head dips, and rearing), and vigilance (i.e., grooming and stretch attend postures), are exquisitely sensitive to the current state of estrous. The rodent estrous cycle is categorized into four stages and, like the menstrual phase in women, can be further condensed into two based on general

reproductive properties. Specifically, proestrus and estrus are defined by greater concentrations of estradiol than progesterone which promotes sexual receptivity, whereas metestrus and diestrus are essentially anestrus phases characterized by relatively low levels of both hormones similar to OVX rats (Marcondes et al., 2002).

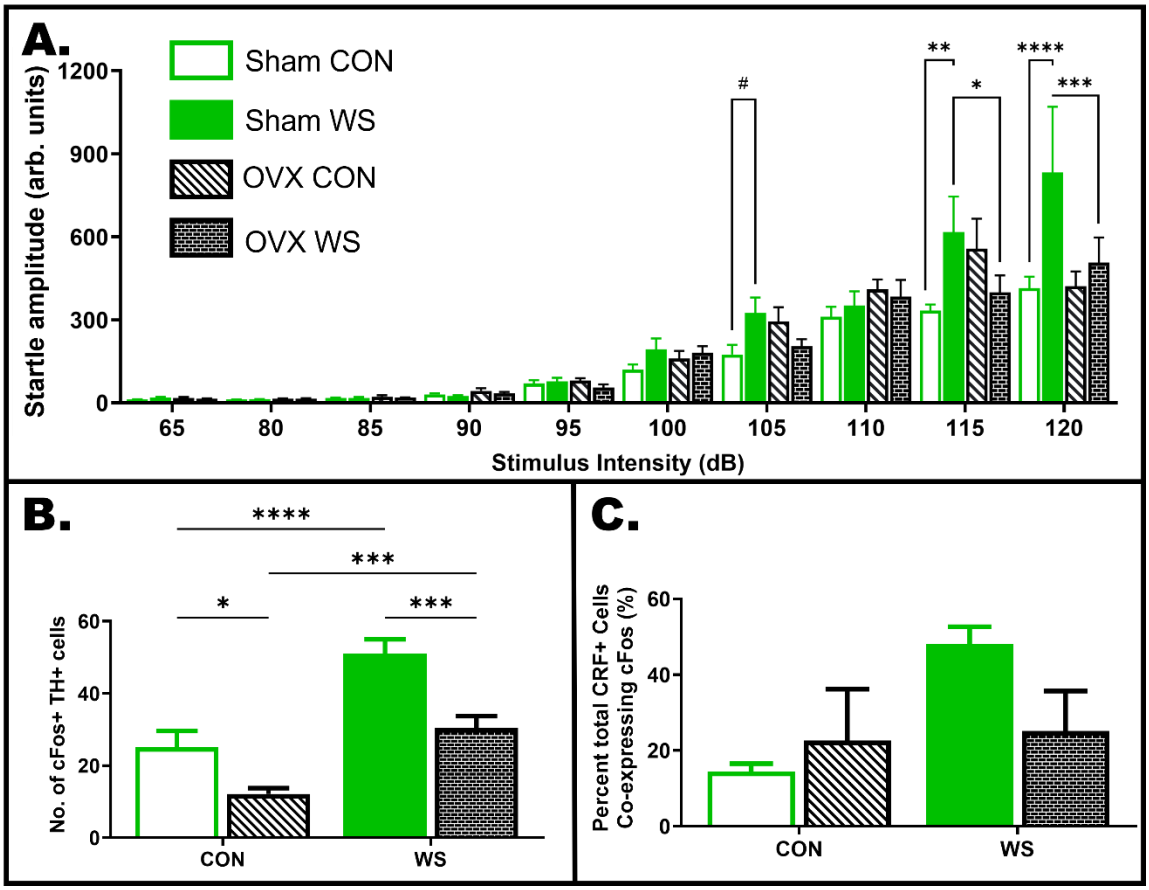

Figure 2.10 Heightened startle amplitude as a result of elevated neuronal activation was evident in all Sham/OVX rats with a history of witness stress, though the effects were greater in the Sham group. A) All rats exhibited increased startle that positively correlated with increasing stimulus intensity, though a history of WS significantly enhanced this reaction, especially in Sham rats. This effect is supported by coinciding elevations in neuronal activitation which is represented by **B)** significantly increased cFos-positive cells within the LC of WS rats compared to CON, and, further in Sham rats compared to OVX. **C)** In addition, there is a trend indicating elevated percentages of cFos-expressing CRF-positive cells in the CeA of WS versus CON rats. CON=control, WS=witness stress, OVX=ovariectomized, dB=decibel, LC=locus coeruleus, CeA=central amygdala, TH=tyrosine hydroxylase, CRF=corticotropin releasing factor
*# $p \leq 0.09$ * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Tukey's post-hoc*

No differences in EPM were found between the OVX groups, though their collective results aligned with those exhibited by their respective Sham / Low E2 counterparts, which is no surprise considering the expectation of similar hormonal profiles.

Traditionally, the duration of time spent in the open arms is described as being a reflection of anxiety (Pellow et al., 1985) such that increased duration represents reduced anxiety-like behavior. In the current study, CON / High E2 rats spent more time in this region of the maze, indicating an association between putatively high levels of estrogen and reduced anxiety-like behavior in unstressed females. This makes sense from an evolutionary standpoint being a high risk-high reward situation where, for example, entering the brightly lit, open arms equates to venturing into the open wild where the risk of predation is high but so are opportunities for prime foraging and mating. However, for Low E2 rats, reproduction is not an immediate consideration, so foraging and mating opportunities are not perceived as highly rewarding nor outweigh the risk of predation, thus potentially explaining the cause of reduced time spent in the open arms exhibited by CON / Low E2 rats. More strikingly, the data show a dichotomous effect of estrous phase that is dependent on stress history. Contrary to non-stressed CON group findings, rats with a history of WS rats spent less time in the open arms if they were in a High E2 phase during EPM, compared to WS / Low E2 rats. Again, in the perspective of evolution and reproductive success, the risk of predation may be perceived to an even greater extent when the brain has been

primed to be on “high alert” by prior stress, thus the reward in this scenario is, instead, preservation of reproductive capabilities and maintaining survival.

Rats were also evaluated for measures of locomotor activity and exploration including total distance travelled and head dipping (Pellow et al., 1985; Pellow & File, 1986) which paralleled the results gleaned from open arms duration. These outcomes were not surprising yet not necessarily expected, considering time spent in the open arms has been described as being a function of overall locomotor and exploratory activity that cannot be explained fully by these measures alone (Pellow et al., 1985). These findings are further supported by time spent in the closed arms of the maze that directly opposes open arms duration. Stretch attend postures and grooming, both indicators of vigilance, followed this same pattern. Interestingly, no differences were observed in rearing durations for any of the groups when directly compared or in relation to any distinguishing factor. While many locomotor-based tasks use total distance travelled as a basis for equalizing other parameters, EPM is not such a task. Though, if needed, rearing may be better suited to identify meaningful differences in locomotor activity. Overall, when measured via behavioral responses to the EPM, CON / High E2 and WS / Low E2 rats maintained a “non-anxious” phenotype across all behavioral parameters while CON / Low E2 and WS / High E2 rats exhibited anxiety-like and hypervigilant behaviors across the board. While an overt effect of WS was not evident in EPM behavior, a remarkable interaction of stress history X estrous cycle phase is revealed that supports the hypothesis that the absence of a prior stressor is the missing link between preclinical and clinical translational relevance.

When assessed in ASR, however, robust effects of WS were revealed. In both study cohorts, significant elevations in startle amplitude began to diverge around the 105 dB tone, but, strikingly, no distinct effects of WS were observed between the OVX groups. It is important to note, however, that all rats exhibited significantly increased startle amplitude that matched the logarithmic rise in stimulus intensity, thus emphasizing the validity of, both, the ASR test and WS paradigm. It is important to note that the acoustic startle reflex is a response that is conserved in rodents and humans (Börchers et al., 2022). The present data show increased startle responses of rats that were previously exposed to WS that mirror outcomes reported in clinical studies that utilize acoustic startle testing in clinical populations, highlighting the clinical relevance of these findings. Collectively, these findings support ASR as a superior test of stress-induced behavioral consequences in female rats that are not impacted by current state of estrous, demonstrating reliability, replicability, and translational relevance.

The present data further demonstrate that cycling ovarian hormones are necessary for elevated neuronal activity levels, as measured by cFos-positive staining, within the LC and CeA that precede the overt hypervigilance expressed during ASR testing. These experiments highlight the critically important fact that exposure to five days of repeated WS is enough to elicit robust neuronal and behavioral outcomes. Additionally, while the general presence of cycling ovarian hormones is required to elicit the observed responses, neither explicit nor relative amounts of circulating ovarian hormones override the strong effects of stress. Specifically in the intact cohort, prior exposure to WS resulted in significantly

greater neuronal activation in the LC and CeA along with higher startle output than those exposed to the CON condition. Similarly in Sham rats of the Sham/OVX cohort, those with a history of WS exhibited greater neuronal activation and subsequent startle amplitude than their CON counterparts. Most importantly, exclusive assessment of ovarian status revealed that WS / Sham rats exhibited greater neuronal activation and startle amplitude in response to ASR compared to WS / OVX rats.

2.5 Conclusion

These experiments are the first to demonstrate that a history of repeated WS exposure can sustain enhanced sensitivity to acoustic startle stimuli that are reflected in the increased neuronal activation of the LC and CeA. We propose that, in turn, a sensitized LC to CeA bidirectional circuit drives the elevations in behavioral hypervigilance. Furthermore, these experiments highlight a critical role of cycling ovarian hormones on females' enhanced susceptibility to the effects of stress. These findings also bridge the gap between clinical and preclinical research regarding the opposing impact of ovarian hormones on behavior by revealing their stress-dependent, dichotomous nature. Finally, the data support the use of non-locomotor-based behavioral tasks like ASR to obtain ethologically relevant and reliable results when using female rodent models, especially in the context stress.

In regard to EPM testing, though results can be impressive at first glance, like in the present study, they warrant considerable caution in their interpretations. The EPM test was originally developed and validated exclusively in adult male, hooded Lister rats for the purpose of testing anxiolytic and anxiogenic effects of

pharmacological compounds (Pellow et al., 1985; Pellow & File, 1986). This is stated in no uncertain terms in the seminal publications by its creators wherein the authors pointedly note that even antidepressants and other non-anxiety-related compounds bear no effects on behavioral outcomes in the EPM (Pellow et al., 1985). While it is true that different outcomes may consistently arise when various species, strains, and sexes, are tested, no study to date has set out to specifically validate the use of this test for these varying purposes, like sex differences or hormonal conditions. Therefore, extreme caution must be used to avoid interpretation pitfalls when comparisons are made across different groups or studies with expanding degrees of separation.

Furthermore, it is important to remember, that, in the “big picture”, females exposed to stress exhibit notable increases in various aspects of the stress response. While elevated levels of estrogen, whether presumed or quantitatively assessed, have been shown to enhance these effects, a response is mounted regardless. Thus, the fact that there were no distinct differences in behavioral outcomes of EPM testing that were precipitated solely by stress is striking. This statement is most certainly not to discredit the EPM or disavow its useful purpose in stress neurobiology research, but, instead, remind the scientific community that behavioral tasks and their associated interpretations are not one-size-fits-all.

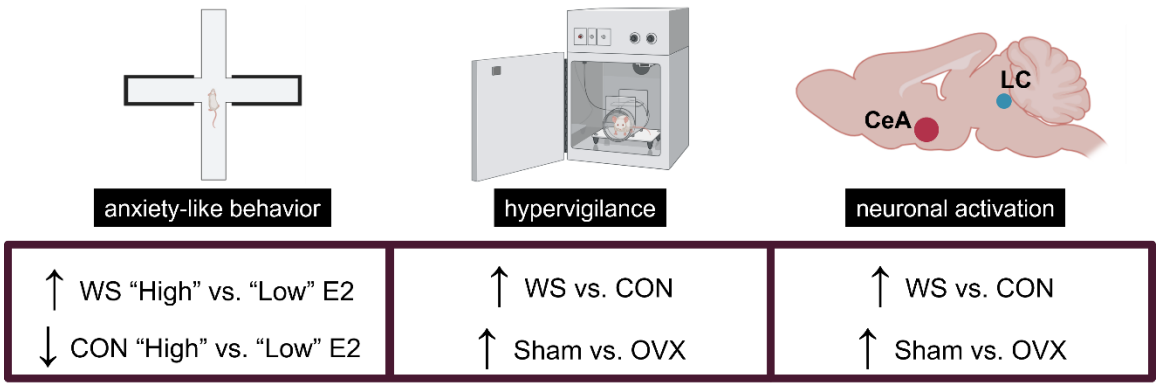

Figure 2.11 Graphical summary. The previous studies revealed a dichotomous effect of estrous cycle as a function of WS history such that rats in the high E2 phases exhibited greater anxiety-like behavior if they had a history of WS versus reduced anxiety-like behavior if they had a history of CON. However, regardless of discrete cycle phase, all naturally cycling rats with a history of WS exhibited heightened startle amplitude, a measure of hypervigilance, which was associated with elevations in cFos immunoreactivity, a marker of neuronal activation, in the stress sensitive CeA and LC regions. CON=control, WS=witness stress, E2=estradiol/estrogen, OVX=ovariectomized, CeA=central amygdala, LC=locus coeruleus

CHAPTER THREE

Estrogen Receptor beta in the Central Amygdala Regulates the Deleterious Behavioral and Neuronal Consequences of Repeated Social Stress in Female Rats

3.1 Background and Significance

Social stressors are one of the most commonly experienced and highly impactful forms of stress in the clinical population. Exposure to these stressors is a risk factor for the development of many debilitating psychiatric disorders, including depression and anxiety (Kessler et al., 1993; Kessler et al., 2012). As a leading cause of disability, such stress-related conditions pose a significant burden on both the patient and society. While the overall incidence of mental health disorders is around 30%, females exhibit over twice the rate of neuropsychiatric conditions, including anxiety, depression, and post-traumatic stress disorder, when compared to males, possibly due to increased exposure and vulnerability to stress (Kendler et al., 2001; Sandanger et al., 2004). Importantly, this incidence is not correlated with differences in demographic factors such as marital status, employment, or number of children (Klose & Jacobi, 2004; Steel et al., 2014). Therefore, neurobiological factors inherent to females may underlie this increased prevalence of mental health disorders following stress exposure.

Despite a large body of literature suggesting that, in healthy patients, estrogen mediates protective effects under non-stressful conditions, recent advances in the field have established estrogen as a factor that may promote susceptibility to the consequences of stress (Finnell et al., 2018; Hokenson et al., 2021). Higher levels of circulating estrogen increase physiological and neuroendocrine responses to stress (Hwang et al., 2020) and, in post-menopausal women without circulating estrogen, treatment with estrogen lead to a heightened response to laboratory administered social stressors (Dumas et al., 2012; Newhouse et al., 2008). Stress sensitization in the presence of estrogen is also replicated in preclinical models. Elevated levels of estrogen in rodent models promotes an exaggerated release of stress hormones including corticotropin releasing factor (CRF), a critical regulator of the stress response, and amplifies neuronal activation in brain regions responsible for the production of these hormones (Babb et al., 2013; Zuloaga et al., 2020).

Due to these known interactions with CRF, estrogen signaling within specific stress-sensitive brain regions may be instrumental in exacerbating stress sensitivity in females. The central amygdala (CeA) is one such region that contains a prominent CRF network that is activated in response to stress (Callahan et al., 2013; Kravets et al., 2015; Ventura-Silva et al., 2013; Wiersielis et al., 2016). This region is also critically involved in regulating behaviors related to hypervigilance, including the acoustic startle response (Keen-Rhinehart et al., 2009) and long-term negative responses to stress cues (Weera et al., 2021). Further, the CeA extends dense CRF projections to the locus coeruleus (LC), a brain region known to

integrate multiple aspects of the stress response and is responsible for the emergence of hypervigilant behaviors (Curtis et al., 2002; Howard et al., 2008; Kravets et al., 2015; Van Bockstaele et al., 1998). Importantly, estrogen is also able to modulate cellular activity of the CeA by signaling through ER beta (ERβ), the predominant ER subtype in this region (Osterlund et al., 1998; Shughrue & Merchenthaler, 2001). While ERβ has traditionally been shown to be anxiolytic (Lund et al., 2005), this effect is only exhibited in behavioral tasks among naïve rats without a history of stress (Le Moëne et al., 2019). The dichotomous role of ERβ activity is revealed in rats that are exposed to stress prior to testing on measures of anxiety-like behaviors, suggesting that ERβ may, instead, have anxiogenic effects in the context of stress (Jasnow et al., 2006; Lynch et al., 2014).

Therefore, these experiments sought to identify a specific role for intra-CeA ERβ in the negative behavioral responses to social stress that have been observed in female rats. Previous experiments in our lab using the witness stress model in females have determined that cycling ovarian hormones are required for behavioral susceptibility to social stress (Finnell et al., 2018), thus, the present studies were designed to explore the neuronal mechanisms responsible for estrogen-mediated behavioral responses to stress. Specifically, these studies determined 1) how expression of ERβ and CRF are altered in the CeA following social stress exposure in females and 2) if pharmacological blockade of ERβ during stress prevents the subsequent manifestation of anxiety-, depressive-, and hypervigilant-like behaviors when later assessed.

3.2 Materials and Methods

3.2.1 Experimental procedures

Two main experiments were completed under the hypothesis that, during witness stress exposure, estrogen signals through ERβ to promote CRF expression, leading to heightened stress responsivity in females. Rats in Experiment #1 (**Figure 3.1A**) were tested on a baseline marble burying (MB) task prior to experiencing five consecutive days of control handling (CON) or witness stress (WS) (15 minutes/day for 5 consecutive days). MB was conducted again following CON/WS to determine the impact on anxiety-like behavior, and then tissue collection occurred following re-exposure to the initial CON/WS context (CTXT). Rats in Experiment #2 (**Figure 3.1B**) were surgically implanted with indwelling bilateral guide cannulas aimed at the CeA in both hemispheres for microinfusion of the selective ERβ antagonist, PHTPP, or vehicle one hour prior to each of the

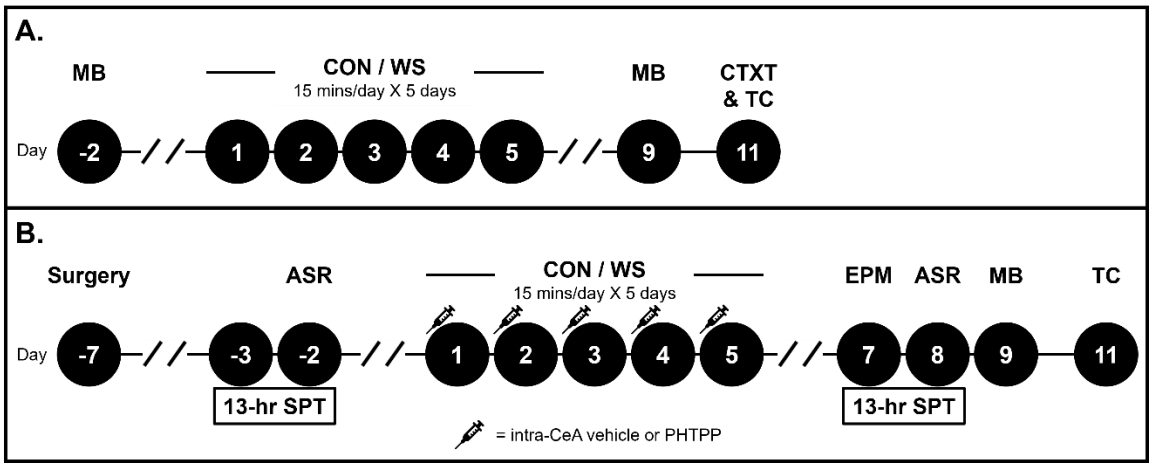

Figure 3.1 Study timelines. A) Experiment #1: a preliminary study was conducted to determine the impact of WS on behavior and protein expression of neuroendocrine factors in the CeA. **B)** Experiment #2: a separate study investigated the effects of intra-CeA ERβ antagonism on behavior during and following repeated CON/WS. CON=control, WS=witness stress, MB=marble burying, CTXT=context, TC=tissue collection, ASR=acoustic startle response, SPT=sucrose preference test, EPM=elevated plus maze, CeA=central amygdala, ERβ=estrogen receptor-β

five daily CON/WS sessions. Rats underwent pre- and/or post-CON/WS behavioral assessments in the sucrose preference, acoustic startle response, marble burying, and elevated plus maze tests, followed by tissue collection at rest.

3.2.2 Rats and housing

Female Sprague-Dawley rats (8-9 weeks and ~150 g on arrival, "controls" and "witnesses"; Charles River; Raleigh, North Carolina, USA), male Sprague-Dawley rats (~250 g on arrival, "intruders"; Charles River), and male Long-Evans retired breeders (600-800 g, "residents"; Envigo; Dublin, Virginia, USA) were all housed individually for the duration of the experiment in standard polycarbonate cages and maintained on a 12-hour light/dark cycle with lights on at 0700 hours and *ad libitum* access to standard rat chow and water, except during behavioral testing. Additionally, female rats that experienced control conditions were housed in an adjacent room from the stressed females and all male rats but were maintained identically. These experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee and maintained adherence to the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

3.2.3 CeA Cannulation Surgery

Rats in Experiment #2 were implanted with indwelling bilateral guide (26 ga., P1 Technologies; Roanoke, Virginia, USA) aimed to terminate directly dorsal to the CeA (in mm from Bregma and the surface of the dura, A/P: -2.3; M/L: ± 4.1, D/V: -6.5). Post-operative analgesic injections (Flunazine® Injectable Solution, 0.25 mg/kg, s.c.) were given on the day of and day following surgery, along with

nutritional supplementation (Bacon Softies, Bio-Serv; Flemington, New Jersey, USA), and rats were given at least 7 days of recovery prior to the start of testing.

3.2.4 Repeated witness stress (WS) or non-stressed control handling (CON)

Females from both experiments were exposed for 15 minutes/day over 5 consecutive days to either WS or CON conditions. Briefly, during each WS session, the female witness was placed behind a perforated, acrylic partition in a protected region (10 x 5 cm) of a territorial resident's home cage. Immediately after placement of the witness, a sexually naïve male intruder was placed into the side of the cage with the resident and social defeat commenced. This paradigm allows the witness to observe auditory, olfactory, and visual cues without experiencing physical aspects of social defeat (Finnell et al., 2018). Matched witness-intruder pairs for all five WS sessions were exposed to a novel resident each day to prevent habituation. Prior to experimentation, residents were screened for appropriate levels of aggression and those that either did not respond to an intruder or caused physical injury to intruders were unused.

3.2.5 Drug information

One hour prior to each CON/WS exposure, females in Experiment #2 received either vehicle (10% dimethyl sulfoxide [DMSO] in saline, Fisher Scientific; Pittsburgh, Pennsylvania, USA) or the potent and selective ERβ antagonist, 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolol[1,5-a]pyrimidin-3-yl]phenol (PHTPP, 10 μM dissolved in 10% DMSO, TOCRIS; Minneapolis, Minnesota, USA). Regardless of treatment, all solutions were delivered at a volume of 0.5 μL per side and infused over a one-minute period followed by a one-minute wait post-infusion before

injectors were removed from the cannula (Compton et al., 2004). This dose was chosen based on a dose response pilot in which 0, 1, 3 and 10 μM of PHTPP were administered prior to WS. The 10 μM dose effectively reduced WS-evoked CRF expression in the CeA compared to vehicle and, thus, was the dose used for all further experiments.

3.2.6 Behavior during CON/WS exposure

CON/WS sessions were video-recorded on the first (D1) and last (D5) day of exposure, and behaviors including burying, freezing, and rearing (Finnell et al., 2018; Pate et al., 2023) were manually quantified retrospectively by an experimenter blinded to treatment groups using ANY-maze (Stoelting Co., Wood Dale, IL). Scores were validated by a second blinded experimenter. Briefly, behaviors were defined by the following criteria: burying was rapid, non-exploratory digging or pushing of the bedding; freezing was complete bodily immobility with the exception of respiratory movements and vocalizations; and rearing was upward extension of the rat's body while simultaneously raising both forepaws from the bedding with or without the hindlimbs extended, in the absence of other behaviors such as grooming, eating, or drinking. Analyses included the latency to the first occurrence of each behavior as well as the total duration of each behavior throughout the 15-minute session. Importantly, the burying exhibited during stress is a spontaneous and rapid shoveling of the cage bedding that is distinct from the behaviors observed during the marble burying paradigm wherein the burying of a discrete object is measured

3.2.7 Sucrose Preference Test (SPT)

To assess the role for intra-CeA ERβ on WS-induced reductions in reward seeking, rats in Experiment #2 underwent a two-bottle choice SPT performed three days pre- as well as three days post-CON/WS exposure. The SPT paradigm utilized in previously published experiments (Finnell et al., 2018) was completed with minor modifications: Phase 1: for the first 24 hours, rats were acclimated to the presence of two bottles in their home cage filled with standard drinking water; Phase 2: rats had free access to two standard bottles filled with 1% sucrose in standard drinking water for the next 24 hours; Phase 3: rats were water deprived for 10 hours prior to the start of the dark cycle (1900 hours), when two bottles, one with standard drinking water and one with 1% sucrose, were placed in the cage overnight for 13 hours. The location of the bottles was switched at 2000 hours to rule out the potential for side bias. Total liquid consumption was measured by subtracting bottle weights at the end of the 13 hours from weights at the start of the test. Percent sucrose preference was calculated as follows:

$$100 * \left(\frac{1\%\ \text{sucrose consumed (g)}}{\text{water consumed (g)} + 1\%\ \text{sucrose consumed (g)}}\right)$$

Rats were excluded if they exhibited sucrose aversion indicated by a sucrose preference below 60% in the pre-stress SPT. Data are presented as the change in percent sucrose preference from pre- to post-stress to determine how WS specifically alters this measure of reward seeking.

3.2.8 Acoustic Startle Response Test (ASR)

ASR testing utilized the automated SR-LAB Startle Response System (San Diego Instruments San Diego, CA). Briefly, this system consists of a sound-attenuating isolation cabinet which contains an acrylic enclosure placed over an

accelerometer. Females in Experiment #2 were tested three days prior to and three days following CON/WS exposure to determine how WS specifically alters the hypervigilant startle response. Although this test occurred the day following SPT, rats maintained access to water for the 16 hours preceding the start of ASR and, therefore, were not tested under water-deprived conditions. Each session lasted for approximately 30 minutes beginning with an initial 5-minute habituation period of exposure to a constant 65-dB (decibel) tone that served as background noise, followed by 30 total trials that included 10 trials each of 95, 105, and 115 dB tones played in 50-millisecond bursts. Tones were presented in a pseudorandom order with inter-trial intervals that varied between 35-45 seconds. This task is known to elicit an innate reflex that has previously been established as a translational method of measuring hypervigilance (Koch, 1999; Koch & Schnitzler, 1997). Since our ongoing experiments indicate that WS impacts startle at the 105 dB level in accordance with the current literature (Börchers et al., 2022), data were analyzed at 105 dB as the change in startle amplitude from pre- to post-CON/WS testing.

3.2.9 Elevated Plus Maze Test (EPM)

Rats in Experiment #2 were tested in the EPM two days following the end of CON/WS exposure. On the day of testing, rats were transported to the testing room at least 30 minutes prior for environmental habituation. The maze was placed 52 cm above the ground and consisted of five total zones: a 10 x 10 cm central square that connected two opposing 50 x 10 cm extensions enclosed by 40 cm-tall side walls (closed arms) and an additional two opposing 50 x 10 cm extensions without side walls (open arms). Each rat was placed into the center zone facing an open

arm, and the session was video recorded to track the location of the animal for the 5-minute duration. Videos were automatically scored using ANY-maze (Stoelting Co.) to determine the total distance traveled and duration of time spent in each pair of arms. This technique provides a measure of general anxiety-like and avoidance behavior based on rodents' inherent aversion to brightly lit and unfamiliar open spaces (i.e., the open arms) and the efficacy of anxiolytics to reduce this aversion (Pellow et al., 1985; Pellow & File, 1986).

3.2.10 Marble Burying Test (MB)

Rats in both experiments were tested in the MB paradigm, a behavioral task used to assess general anxiety-like behavior in rodents based on the ability of clinically efficacious anxiolytic drugs to reduce the burying of marbles (Dixit et al., 2020). The MB testing chamber consisted of a clean, standard rat cage with Teklad Sani-Chip® bedding (Envigo; 5 cm deep, leveled), the same bedding used for all rat's home cages. Fifteen glass marbles (1.5 cm in diameter) were arranged in 3 x 5 rows symmetrically distributed across the bedding (**Figure 3.3A**). Rats were placed into the cage such that the marbles were not disturbed, and each session was video recorded for 15 minutes. Upon completion, rats were carefully removed so as not to disturb the final marble locations, and the number of marbles buried was recorded. A marble was counted as buried if ¾ or more was covered in bedding. To confirm the covering of a buried marble as intentional, videos were reviewed by a researcher blinded to the treatment conditions. Durations of marble burying and marble interaction (defined as any physical manipulation of the marble excluding burying) during the 15-minute testing period were manually quantified

using ANY-maze. Rats in Experiment #1 underwent a pre- and post-CON/WS MB test while rats from Experiment #2 underwent only post-CON/WS testing. Thus, MB data from Experiment #1 are presented as change from baseline and as a repeated measure while MB data from Experiment #2 represent only behavior during the single MB test conducted following CON/WS exposure.

3.2.11 CON/WS context (CTXT)

On the day of tissue collection, rats from Experiment #1 were exposed to the context in which they originally experienced CON/WS to allow for quantification of stress cue-evoked changes in behavior and neural alterations. Females with a history of WS were placed behind a partition in a soiled resident's home cage with their paired male intruder on the opposite side in the absence of the resident. The resident's cage used for context exposure was from the same rat that was used as the resident on the final day of WS for each witness. CON females were briefly handled then returned to their home cages. CTXT sessions were video recorded for 15 minutes and behaviors were scored identically to D1 and D5 of CON/WS as described above.

3.2.12 Tissue collection

Brains were collected 30 minutes after the start of CTXT for rats in Experiment #1. For rats in Experiment #2, brains were collected under home cage resting conditions two days after the final behavioral test (MB). Rats were deeply anesthetized with isoflurane vapor prior to quick decapitation. Brains were dissected into anterior and posterior sections, flash-frozen in isopentane chilled on dry ice, and stored at -80°C. Anterior sections were sliced coronally, and 1 x 1 mm

(diameter x depth) bilateral punches of the CeA were taken using a tissue biopsy punch (Harvard Apparatus; Holliston, Massachusetts, USA) prior to storage at -80°C until processing. Histological assessment verified accuracy of punch placement at 10X magnification. Brain slices from female rats in Experiment #2 were also assessed to confirm cannula placement within the CeA. Additionally, a separate rat was injected with Hoechst dye (Bio-Rad; Hercules, California, USA) into the CeA to ensure drug spread was limited to the CeA. The spread of the dye was measured at a total area of 153,976 pixels^2 while the entire area of the CeA was established as 388,980 pixels^2, further reinforcing that drug spread was limited to approximately half of the total area of the CeA bilaterally.

CeA punches were homogenized and assessed for protein concentration using a Pierce Bicinchoninic Acid (BCA) assay (Thermo Scientific; Rockford, Illinois, USA) per manufacturer's protocol. Briefly, each sample was mixed with 100 µL of zirconium oxide beads (Next Advance, Inc.; Averill Park, New York, USA) and 200 µL of lab-made lysis buffer consisting of 137 mM NaCl, 20 mM Tris, 1% Igepal, 10% glycerol, and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific, No. 87786). Samples then underwent mechanical disruption in a Bullet Blender (Next Advance, Inc.) at speed 8 for 3 minutes at 4°C followed by centrifugation at 1400 rcf for 15 minutes at 4°C. The resulting homogenate for each sample was stored at -80° and a 12 µL aliquot was mixed in a 1:4 dilution with phosphate buffered sodium azide (PB-az) for BCA analysis. A 25-µL aliquot of each sample along with 25 µL of each albumin standard was pipetted in duplicate onto a 96-well plate. Working reagent was added at a volume of 200 µL to each well, and the plate was

incubated at 37°C for 30 minutes before reading at 562 nm on a Synergy plate reader (Agilent Technologies; Santa Clara, California, USA). Using the resulting protein concentration, homogenates containing 20 µg of protein were aliquoted and stored at -80°C for later use.

3.2.14 Western blotting

Western blots were completed using CeA homogenates in the manner previously described (Finnell et al., 2018; Pate et al., 2023). Briefly, each sample was mixed at a 1:6 ratio with a solution of β-mercaptoethanol and 6X sample buffer, heated at 75°C for 5 mins, and then loaded into mini-protean gels (Bio-Rad). Gels were submerged in 1X running buffer solution (Bio-Rad) in electrophoresis chambers for one hour at 135 V. Proteins were then transferred from the gels onto PVDF membranes in 1X transfer buffer (Bio-Rad) in transfer chambers for 90 minutes at 100 V. Membranes were then submerged in blocking buffer at room temperature for one hour (1:1 ratio of 1X PBS: Fluorescent Blocking Buffer, Millipore Sigma; Burlington, Massachusetts, USA). After blocking, membranes were incubated overnight at 4°C in the following primary antibodies diluted in a solution of blocking buffer with 0.2% Tween-20: mouse anti-GapDH (1:1000, No. sc-365062; Santa Cruz Biotechnology, Inc.; Santa Cruz, California, USA), rabbit anti-ERβ (1:250, #PA1-310B; Thermo Fisher Scientific; Waltham, Massachusetts, USA), and rabbit anti-CRF (1:2500, No. ab-184238; Abcam; Boston, Massachusetts, USA). The next day, membranes underwent three 10-minute washes in 1X TBS with 10% Tween-20 and were incubated in LI-Cor fluorescent secondary antibodies (IR-Dye anti-rabbit 800 nm, No. 926-32213; IR-Dye anti-mouse 680 nm, No. 962-68072;

LI-Cor Biosciences; Lincoln, Nebraska, USA) diluted 1:20,000 in a solution of blocking buffer with 0.2% Tween-20 and 0.1% sodium dodecyl sulfate for one hour. Membranes were washed in TBS with 10% Tween-20 (3 x 10 minutes), imaged on a LI-Cor Odyssey scanner (LI-Cor Biosciences), quantified using LI-Cor Image Studios Software, and normalized to GapDH protein levels. Importantly, the GapDH concentrations used did not differ between treatment groups.

3.2.15 Statistical analyses

The data included in Experiment #1 were analyzed using unpaired t-tests to compare CON versus WS. All behavioral and molecular statistical analyses for Experiment #2 were performed using a two-way analysis of variance (ANOVA) with Tukey's post-hoc analyses. Behavioral testing that was completed prior to and following stress exposure were additionally analyzed using a three-way repeated measures ANOVA. Main effects of stress, drug, and stress x drug interactions are provided in the main text while post-hoc analyses are indicated by symbols on figures and denoted in figure legends. Outliers were identified and removed if they were more than two standard deviations from the group mean. Data are presented as mean ± standard error of the mean (SEM) with an $\alpha = 0.05$.

3.3 Results

3.3.1 *WS evoked greater anxiety-like behavior during the initial five-day exposure period for rats in Experiment #1*

For rats in Experiment #1, those that experienced WS exhibited greater durations of anxiety-like burying (**Figure 3.2A**; $t_{10}=2.368$, $p=0.0394$) and a shorter latency to begin burying (**Figure 3.2B**; $t_{10}=4.005$, $p=0.0025$) when compared to CON rats on

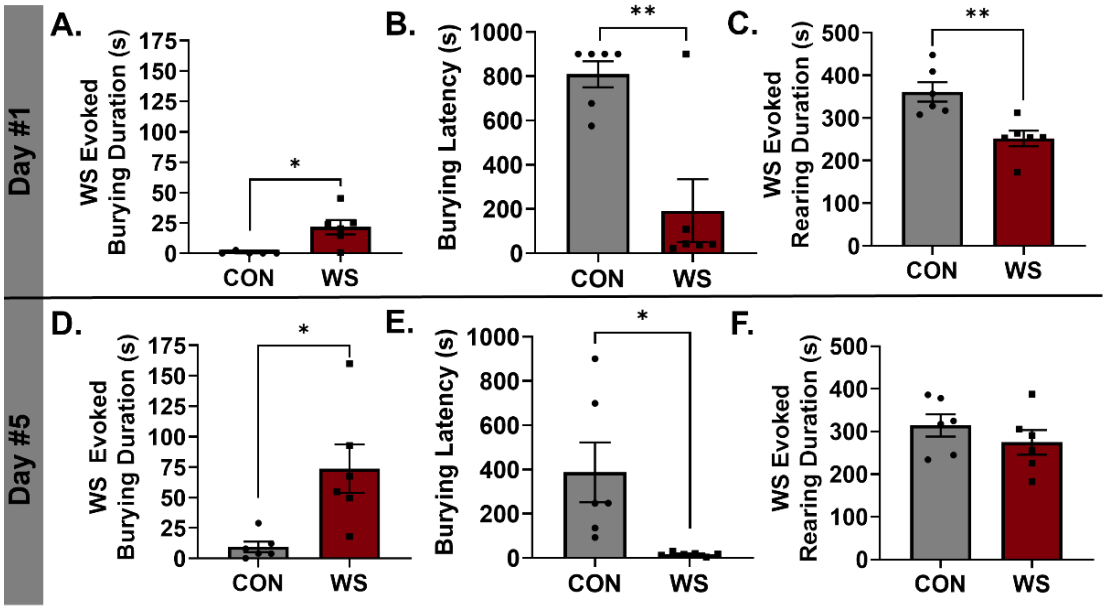

Figure 3.2 Throughout the five-day exposure, rats in Experiment #1 that were exposed to witness stress exhibited a greater duration of and shorter latency to begin burying. On D1 of CON/WS, WS-exposed rats **A)** buried for longer, **B)** were quicker to begin burying, and **C)** reared less than CON rats. On the final day, D5, WS-exposed rats **D)** buried for longer durations and **E)** were quicker to begin burying but **F)** did not rear more than CON rats. CON=control, WS=witness stress, D1=day 1, D5=day 5
** p < 0.05, ** p < 0.01, Tukey's post-hoc*

D1. This behavior was paralleled by a decrease in rearing behavior in WS rats on D1 (**Figure 3.2C**; t_{10}=3.728, p=0.0039) with no effect on freezing behavior (t_9=1.165, p=0.2741, data not shown). Anxiety-like burying remained elevated in WS rats on D5 with increased duration (**Figure 3.2D**; t_{10}=3.185, p=0.0102) and decreased latency (**Figure 3.2E**; t_{10}=2.737, p=0.0210). However, there were no differences observed in rearing (**Figure 3.2F**; t_{10}=1.011, p=0.3357) or freezing duration on D5 (t_{10}=0.2656, p=0.796, data not shown).

3.3.2 Rats with a history of WS in Experiment #1 exhibited greater anxiety-like behavior in the MB test compared to CON rats and when assessed across time

Three days prior to and four days after CON/WS exposure, rats underwent MB testing (**Figure 3.3A**) and were evaluated for durations and latencies of marble burying, freezing, rearing, and marble interaction via individual analyses and repeated measures comparisons. Post-CON/WS analyses revealed that rats with a history of WS exhibited a greater number of total marbles buried (**Figure 3.3B**; t_{10}=4.385, p=0.0014) and of marble burying duration (**Figure 3.3D**; t_{10}=2.363, p=0.0397) compared to CON. Repeated measures comparisons further revealed a significant interaction between stress and time for the number of marbles buried (**Figure 3.3C**; $F_{1,10}$=16.76, p=0.0022) and marble burying duration (**Figure 3.3E**; $F_{1,10}$=5.584, p=0.0397), in which rats with a history of WS buried more marbles and for a longer duration than they did pre-WS, but CON rats did not. Additionally, rats with a history of WS exhibited a trend towards decreased duration of marble interaction over time (**Figure 3.3F**; t_{10}=1.692, p=0.0608), which further indicates an anxiety-like phenotype, though no other differences were observed (**Figure 3.3G**; $F_{1,10}$=2.862, p=0.1216). Additional measures of freezing (t_{10}=1.988, p=0.0749) and rearing (t_{10}=1.34, p=0.2098), were not different between groups (data not shown).

3.3.3 Prior exposure to WS elicited greater anxiety-like behavior during CTXT for rats in Experiment #1

For CTXT assessments, rats were exposed to their previous respective CON/WS conditions (**Figure 3.3H**) for 15 minutes to mimic the context of the prior week's CON or WS setting. When compared to CON rats, which were handled and returned to their home cage, rats previously exposed to WS exhibited significantly

increased anxiety-like burying (**Figure 3.3I**; t_{10}=11.23, p<0.0001) along with decreased latency to begin burying (**Figure 3.3J**; t_{10}=2.996, p=0.0141) in response to the context in which they previously experienced WS. Further, rats with a history of WS exhibited a decrease in rearing (**Figure 3.3K**; t_{10}=3.785, p=0.0036) compared to CON.

3.3.4 Re-exposure to the WS CTXT exhibited elevated protein expression of ERβ and CRF in the CeA of rats in Experiment #1

Brain tissue was collected 15 minutes following the end of CTXT then prepared for Western blot analysis to identify protein expression levels of ERβ and CRF in the CeA (**Figure 3.3L**). The CRF antibody yields a separate band for the CRF precursor, proCRF (45 kD), and the mature protein (25 kD). Rats with a history of WS exhibited a significant increase in expression levels of ERβ (**Figure 3.3M**; t_9=2.928, p=0.0168) as well as proCRF (**Figure 3.3N**; t_{10}=2.113, p=0.0304) with a trend towards increased CRF (**Figure 3.3O**, t_{10}=1.648, p=0.0652) in the CeA.

3.3.5 WS-evoked burying was attenuated by intra-CeA ERβ antagonism during repeated WS exposure for rats in Experiment #2

Similar to previous findings in our lab (Finnell et al., 2018; Pate et al., 2023) and in accordance with Experiment #1, WS was shown to enhance anxiety-like burying duration (**Figure 3.4A**; effect of stress: $F_{1,41}$=37.52, p<0.0001) with a shorter latency to begin burying (**Figure 3.4B**; effect of stress: $F_{1,42}$=41.71, p<0.0001) on D1. There were no significant differences in rearing (**Figure 3.4C**; effect of stress: $F_{1,44}$=0.0022, p=0.9624; effect of drug: $F_{1,44}$=0.3217, p=0.5735) or freezing (effect of stress: $F_{1,46}$=1.957, p=0.1685; effect of drug: $F_{1,46}$=0.07395, p=0.7869, data not

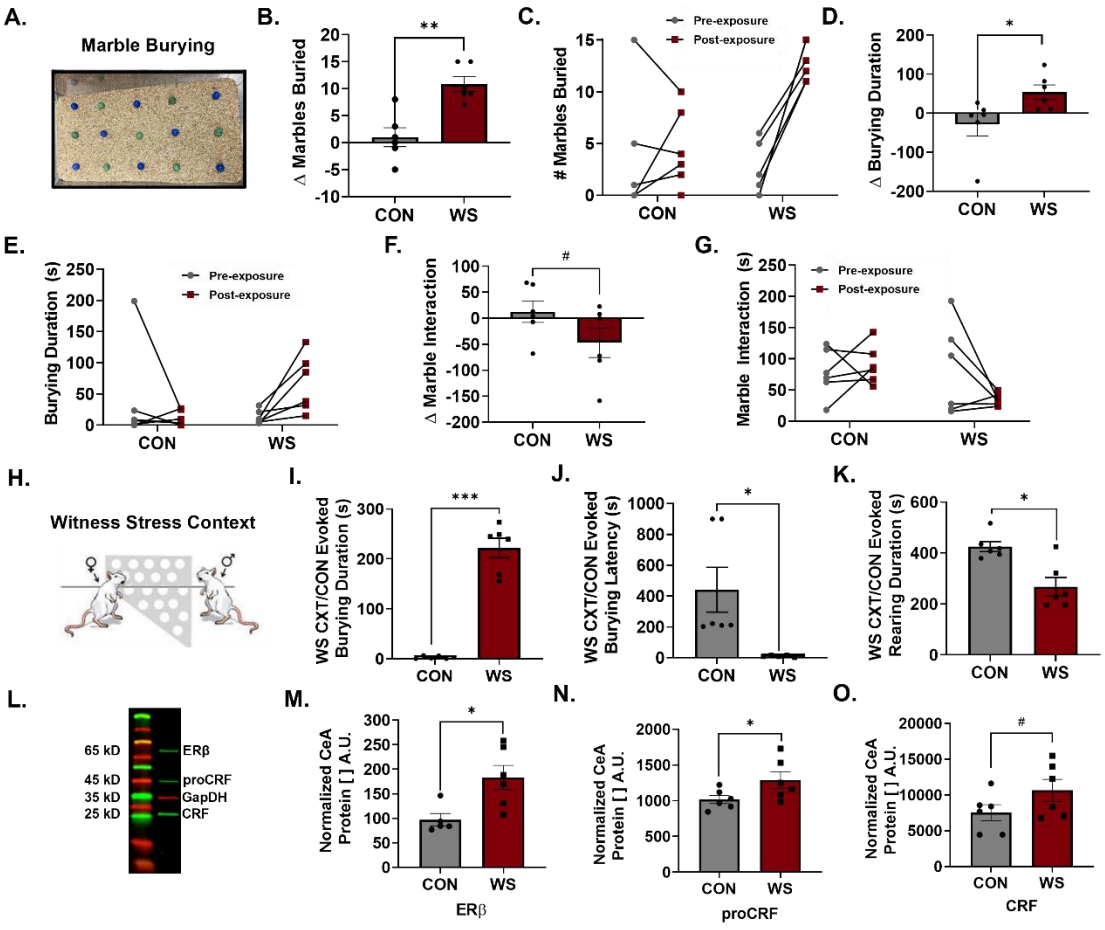

Figure 3.3 Rats with a history of witness stress in Experiment #1 exhibited an overall increase in anxiety-like behavior that was associated with elevations in protein expression of neuroendocrine factors in the central amygdala. A) MB test setup. **B)** Rats with a history of WS buried more marbles compared to CON, **C)** as well as to their respective pre-WS values. **D)** Rats with a history of WS exhibited greater anxiety-like marble burying during the MB test compared to CON, **E)** as well as to their respective pre-WS values. **F)** Rats with a history of WS exhibited reduced exploratory marble interaction compared to CON, **G)** as well as to their respective pre-WS durations. **H)** Representation of the WS CTXT model. During CTXT, rats with a history of WS **I)** buried for longer durations, **J)** were quicker to begin burying, and **K)** reared for shorter durations compared to CON. **L)** Representative Western blot image depicting the appearance of bands associated with the proteins of interest in comparison to a standard protein latter denoted by molecular weights. **M)** Rats with a history of WS displayed a more reactive physiological stress response evidenced by greater intra-CeA protein expression of ERβ, **N)** proCRF, and **O)** CRF compared to CON. CON=control, WS=witness stress, MB=marble burying, CTXT=context, CeA=central amygdala, ERβ=estrogen receptor-β, CRF=corticotropin releasing factor

*# p ≤ 0.09 * p < 0.05, ** p < 0.01, *** p < 0.001, Tukey's post-hoc*

shown) on D1. Similarly, on D5, WS rats exhibited an increase in burying compared to CON (**Figure 3.4D**; effect of stress: $F_{1,41}$=36.71, p<0.0001). Additionally, while there was no effect of drug on anxiety-like burying upon the initial D1 exposure, PHTPP treatment significantly reduced anxiety-like burying measured on D5. Further, WS rats exhibited a shorter latency to begin burying on D5 (**Figure 3.4E**; effect of stress: $F_{1,44}$=48.56, p<0.0001) with no effects on rearing (**Figure 3.4F**; effect of stress: $F_{1,44}$=0.0339, p=0.8548; effect of drug: $F_{1,44}$=1.557, p=0.2187) or freezing (effect of stress: $F_{1,44}$=0.1455, p=0.7052; effect of drug: $F_{1,44}$=0.00281, p=0.958, data not shown).

Behavior data obtained from D1 and D5 were also assessed via a repeated measures ANOVA. When comparing burying behavior between D1 and D5 (**Figure 3.4G**), there was a significant time X stress ($F_{1,38}$=7.969, p=0.0075) and stress X drug interaction ($F_{1,38}$=4.187, p=0.0477). To further analyze the effect of PHTPP exclusively on WS between D1 and D5, burying behavior was compared explicitly between the two WS groups and revealed a significant drug effect (**Figure 3.4H**; $F_{1,76}$=3.281, p=0.0016) such that PHTPP drastically reduced the time spent burying of WS rats compared to VEH.

3.3.6 Prior exposure to WS and treatment with PHTPP differentially affected behavioral outcomes of EPM, ASR, SPT, and MB for rats in Experiment #2

Rats were tested on a variety of behavioral paradigms to determine the impact of prior exposure to WS ± prior intra-CeA PHTPP treatment on anxiety-like, depressive-like, and hypervigilant outcomes. No differences were observed in the EPM for time spent in the open arms (**Figure 3.5A**; effect of stress: $F_{1,36}$=2.523,

p=0.1210; effect of drug: $F_{1,36}$=0.1591, p=0.6923) or distance traveled (**Figure 3.5B**; effect of stress: $F_{1,35}$=0.04819, p=0.8275; effect of drug: $F_{1,35}$=0.2297, p=0.6347). During ASR testing, a history of WS elicited greater startle amplitude at the 105 dB tone which was diminished via prior PHTPP treatment (**Figure 3.5C**; effect of stress X drug: $F_{1,40}$=9.237, p=0.0042). A three-way repeated measures ANOVA determined that there was a trend towards a time X stress X drug interaction ($F_{1,40}$=4.002, p=0.0523, data not shown) indicating a slight increase in post-CON/WS startle in the CON + PHTPP and WS + VEH groups but not in the

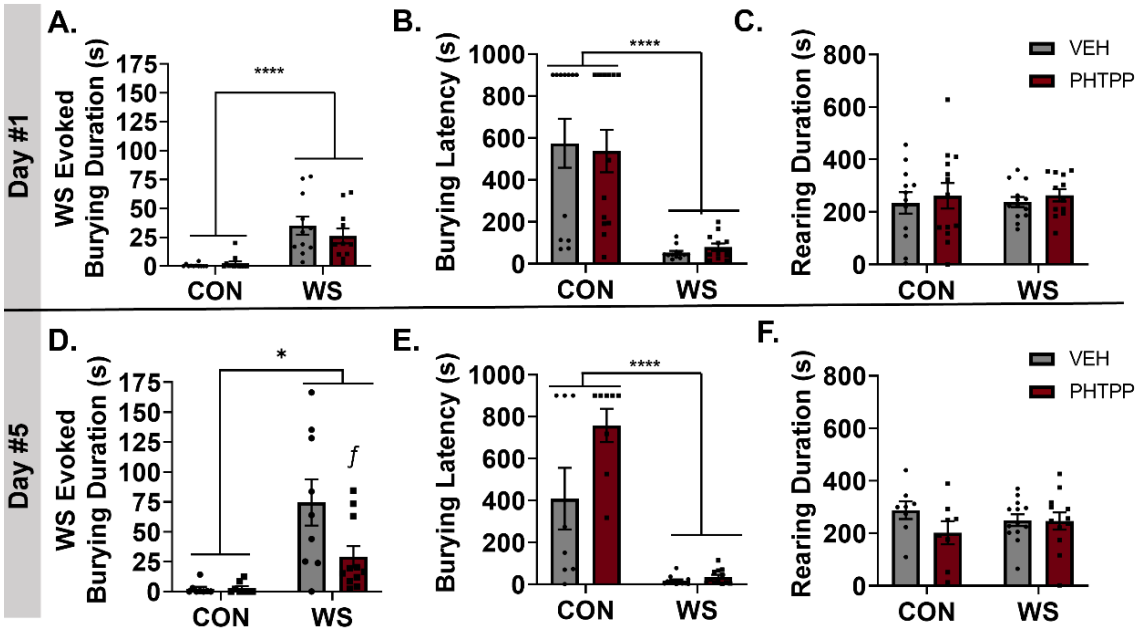

Figure 3.4 PHTPP treatment prevented the exacerbated anxiety-like burying response associated with prior witness stress exposure for rats in Experiment #2. A) On D1 of CON/WS exposure, rats exposed to WS exhibited an increased duration of and **B)** latency to begin burying than CON rats, though no effects of drug were evident. **C)** No differences were observed in rearing duration between groups based on prior stress or drug condition. **D)** On D5 of CON/WS exposure, PHTPP treatment significantly prevented the WS-evoked increase in duration of anxiety-like burying, but **E)** did not alter the latency to begin burying which remained elevated in WS rats compared to CON. **F)** No effects of stress or drug condition were observed for rearing during the final CON/WS exposure on D5. CON=control, WS=witness stress, D1=day 1, D5=day 5, VEH=vehicle
*ƒ < 0.05 vs. WS + VEH, * p < 0.05, ** p < 0.01, *** p < 0.001, Tukey's post-hoc*

CON + VEH and WS + PHTPP groups. In the SPT, sucrose preference was reduced following exposure to repeated WS which was prevented via prior PHTPP treatment given during CON/WS exposure (**Figure 3.5D**). A three-way repeated measures ANOVA of these data revealed significant time X stress ($F_{1,30}$=4.505, p=0.0422), time X drug ($F_{1,30}$=5.863, p=0.0217), and stress X drug ($F_{1,30}$=4.637, p=0.0395) interactions indicating an increase in anhedonia specifically in WS + VEH rats. In the final behavioral task, MB, no effects of stress or drug were evident in relation to the number of marbles buried (**Figure 3.5E**; effect of stress: $F_{1,32}$=0.1475, p=0.7035; effect of drug: $F_{1,32}$=2.448, p=0.1275), though there was a significant drug effect for duration of marble burying (**Figure 3.5F**; $F_{1,31}$=5.149, p=0.0304). No additional effects were observed for other behaviors including marble interaction (effect of stress: $F_{1,31}$=0.0868, p=0.7703; effect of drug: $F_{1,31}$=2.039, p=0.1633, data not shown). It is important to emphasize that these data represent behaviors from a single post-CON/WS test as opposed to an assessment involving within-subjects change from baseline which contributes to the differences between these data and the robust results in Experiment #1.

3.3.7 PHTPP treatment induced long-term alterations in CRF and ERβ protein expression levels in the CeA of rats in Experiment #2

Tissue was collected under resting conditions two days following the final behavioral assay. Samples of the CeA were analyzed using Western blot to identify protein expression levels of ERβ and CRF. There was a significant effect of previous PHTPP treatment on resting levels of ERβ in the CeA (**Figure 3.5G**; effect of drug: $F_{1,39}$=10.11, p=0.0029) such that PHTPP-treated rats exhibited higher

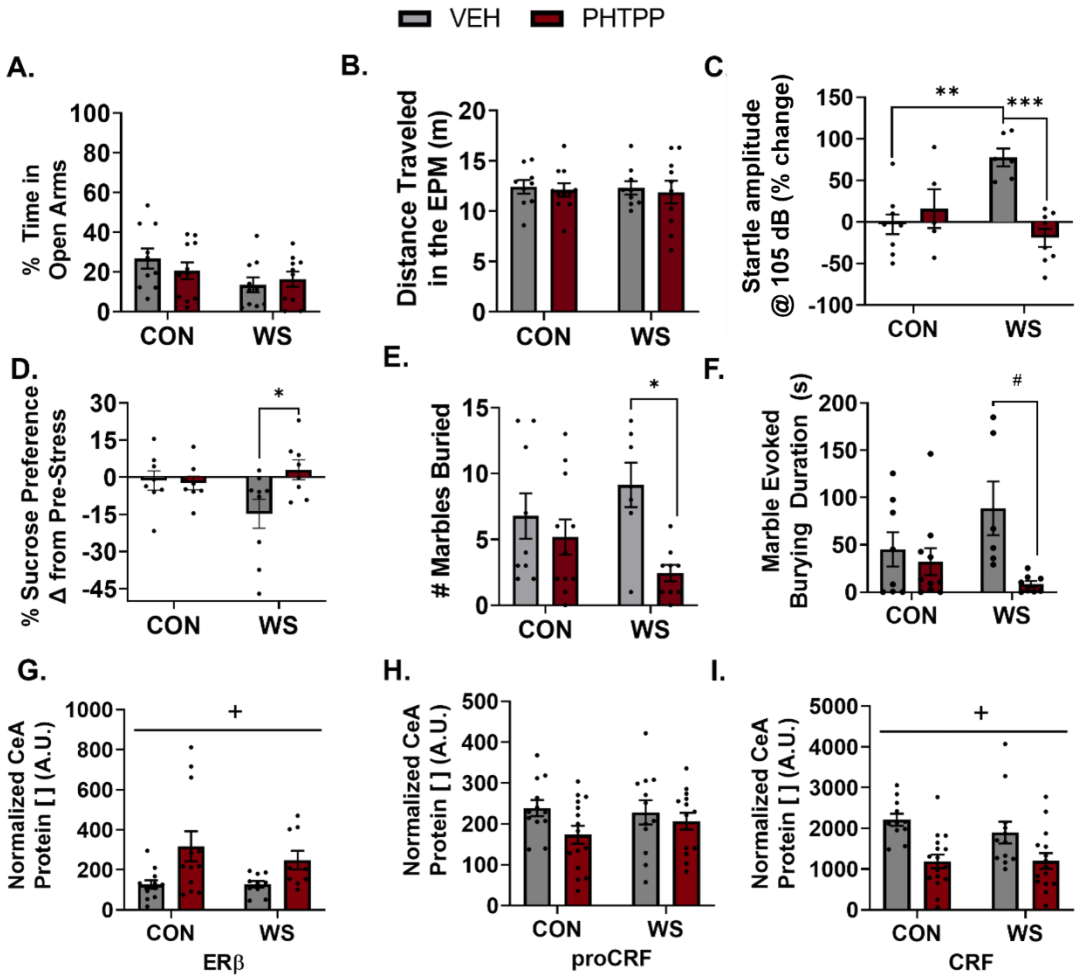

Figure 3.5 Prior treatment with PHTPP attenuated the negative effects of prior witness stress on behavioral outcomes but revealed a compensatory increase in expression of neuroendocrine factors in the central amygdala. No overt effects of prior stress or drug were observed for anxiety-like behavior assessed during the EPM test, indicated by no differences in **A)** open arms duration or **B)** distance traveled. **C)** PHTPP treatment prevented the WS-evoked increase in hypervigilance during ASR testing, represented as an increase in startle amplitude compared to baseline at the 105 dB tone. **D)** PHTPP treatment prevented anhedonia that was elicited by history of WS, indicated by retained sucrose preference in the SPT. **E)** In post-CON/WS MB, PHTPP attenuated the WS-induced increases in number of marbles buried and **F)** duration of marble burying. **G)** Resting levels of intra-CeA ERβ were elevated in PHTPP-treated rats regardless of prior exposure. **H)** No differences were revealed in resting levels of intra-CeA proCRF protein expression, **I)** though PHTPP caused elevations in the mature form of the CRF protein. CON=control, WS=witness stress, EPM=elevated plus maze, ASR=acoustic startle response, MB=marble burying, CeA=central amygdala, ERβ=estrogen receptor-β, CRF=corticotropin releasing factor

*# p ≤ 0.09 * p < 0.05, ** p < 0.01, *** p < 0.001, + = effect of PHTPP, Tukey's post-hoc*

levels of ERβ independent of stress history. Likewise, PHTPP treatment led to a decrease in CRF expression in the CeA regardless of stress history (**Figure 3.5I**; effect of drug: $F_{1,39}$=18.76, p<0.0001) though no significant changes were seen in proCRF (**Figure 3.5H**; effect of stress X drug, $F_{1,49}$=0.8941, p=0.349).

3.4 Discussion

These studies show for the first time that ERβ signaling in the CeA increases sensitivity to social stress in females and further strengthens the hypothesis that estrogen plays a notable role in regulating behavior in the context of stress. Overall, these data demonstrate that WS increases hypervigilant and negative valence behaviors that were accompanied by increases in intra-CeA ERβ and CRF protein expression. Importantly, these studies went on to prove that stress-evoked negative valence was regulated by intra-CeA ERβ, as pharmacological blockade of ERβ in the CeA prevented these effects. These studies build on previous work investigating the role of estrogen signaling on stress-related outcomes in females and highlight a novel target for the treatment of stress-induced neuropsychiatric disorders. The increased prevalence of such stress-related conditions among women in their reproductive years is well documented (Barth et al., 2015; Kessler et al., 2005; Kundakovic & Rocks, 2022) and, while we cannot rule out contributing roles of other ovarian hormones such as progesterone, accumulating evidence from both clinical (Hlavacova et al., 2008; Newhouse et al., 2008) and preclinical (Flores et al., 2020; Hokenson et al., 2021; Jasnow et al., 2006) studies demonstrate that, in the context of stress, estrogen signaling in the brain plays a critical role in this heightened susceptibility.

Estrogen is known to directly increase the expression of CRF mRNA and heighten behavioral responses to stressful stimuli (Jasnow et al., 2006). Importantly, the CRF gene contains an estrogen response element which allows nuclear estrogen receptor activation to modulate CRF transcription (Ni & Nicholson, 2006; Vamvakopoulos & Chrousos, 1993). Using the same WS model as utilized for these studies, we previously reported a stress-evoked increase in CeA-CRF among intact but not ovariectomized female rats (Finnell et al., 2018). Furthermore, this sensitized CRF response was accompanied by increased hypervigilant behavioral responses in natural cycling, but not ovariectomized, females. While this evidence highlights the contribution of estrogen-mediated effects on CRF in the increased stress susceptibility observed in females, there is a pressing need to determine the specific neural mechanisms underlying this phenomenon. It is reasonable, then, to consider estrogen signaling through ERβ within the CeA resulting in increased CRF expression as a promising mechanism by which estrogen facilitates this enhanced stress response among females. Our findings support this hypothesis as the first to identify a regulatory role for intra-CeA ERβ in the development of negative valence and hypervigilant phenotypes that emerge among naturally cycling females as a consequence of repeated social stress exposure.

While Experiment #1 determined that exposure to the WS CXT increased both pro-CRF and CRF in rats with a history of WS, Experiment #2 showed that under resting conditions at this protracted timeline, WS rats did not demonstrate changes in CRF. However, PHTPP treated rats exhibited decreased levels of CRF

regardless of stress history. These data suggest that exposure to stress-related cues induces de-novo production of CRF from its precursor, while, in the long term, a history of ERβ blockade prevents the long-term accumulation of CRF. Together, these results suggest that beyond the expected stress evoked increase in CRF production signaling in the CeA, baseline levels of CRF in the CeA may be maintained by ERβ signaling. Further, given the robust impact that ERβ blockade had on CeA CRF levels, it is important to consider the impact of this pharmacological manipulation on CeA-CRF projection regions that are known to directly regulate these behaviors. Importantly, CRF-projecting neurons from the CeA are a major source of stress-induced activation of the noradrenergic LC (Curtis et al., 2002; Curtis et al., 1997), which is responsible for initiating the hypervigilant burying behavior assessed in our studies (Howard et al., 2008). In fact, stress- or CRF-evoked hypervigilant burying is dependent upon intact LC-norepinephrine (NE) signaling. Therefore, decreased stress-induced CRF expression achieved in the PHTPP-treated rats is likely to reduce LC-NE activity and could explain their blunted WS-evoked burying response (Finnell et al., 2018). Unfortunately, the protracted time point of euthanasia in these experiments did not allow for the investigation of this hypothesis within our current dataset. Future studies exploring this avenue include dynamic methods of analysis, like in vivo electrophysiology, to better understand the impact of CeA ERβ antagonism on LC-NE signaling and behavior.

These studies determined that estrogen signaling through ERβ plays a role in the behavioral sensitization to stress that occurs during repeating exposure to

social stressors. Specifically, PHTPP did not induce a significant effect on the burying response during stress on the initial stress exposure, but significantly decreased this behavior on the final day of stress. It is also important to note that while intra-CeA ERβ appears to mediate many stress-induced behaviors, EPM was unaffected by stress and intra-CeA PHTPP treatment. As such, it is important to highlight the sex differences in anxiety-like behaviors that are established. It has recently been established that adult female rats do not exhibit anxiety-like phenotypes on the EPM when compared to males (Börchers et al., 2022), and this may underlie the lack of stress-induced alterations observed in these studies. Further, there is major involvement of other brain regions in the regulation of EPM-specific behaviors, including the medial amygdala (Troakes & Ingram, 2009) and bed nucleus of the stria terminalis (Butler et al., 2016; Callahan et al., 2013) and, thus, sole modulation of ERβ in the CeA would be insufficient to alter these behaviors. Further, while the focus of these experiments was on the alterations in ERβ in stressed females, there are sex differences in ERβ expression within the amygdala that may lead to sex differences if these experiments were replicated in males. Females exhibit higher levels of ERβ expression in the amygdala compared to males (Weiser et al., 2008; Zuloaga et al., 2020) which may lead to increased CRF release in response to stress. Further experiments directly comparing ERβ and CRF expression followings stress between males and females would be required to determine the presence of sex differences.

An additional unexpected finding was that, in the long-term, treatment with PHTPP for five consecutive days resulted in a compensatory increase in ERβ

expression within the CeA regardless of stress exposure. While PHTPP is established as a potent and selective antagonist of ERβ (Compton et al., 2004), there is a lack of research examining neuronal adaptations that occur in response to repeated pharmacological blockade of ERβ. Importantly, these are the first results to show that five days of intra-CeA PHTPP administration leads to compensatory increases in receptor expression. While the timescale of ERβ alterations following chronic PHTPP treatment are unclear, one major limitation comes from the fact that there may be increased ERβ during the behavioral testing post-stress. Despite this compensatory increase, ERβ antagonism during stress exposure was still effective at preventing the stress-induced behavioral alterations previously observed. Additionally, PHTPP treatment did not alter any behaviors when administered to controls, demonstrating that an upregulation of ERβ within the CeA alone is not sufficient to facilitate changes in these behavioral tasks. Regardless of our data indicating that drug spread should be confined to the CeA, we cannot definitively rule out the possibility that PHTPP may have spread to the other amygdala regions that are in close proximity to the CeA including the lateral and basolateral subregions. However, these findings support the hypothesis that estrogen signaling via ERβ within the CeA during stress exposure is critical for the hypervigilant and anhedonic behavioral responses observed following repeated stress. In parallel, we established that exposure to the WS context in rats with a history of WS increases CRF in the CeA while we show for the first time that ERβ signaling serves to regulate long lasting resting/basal CRF expression levels at 6 days following treatment. Although there is a lack of a stress effect in resting CRF

levels at the protracted timeline in Experiment #2, stress-cue evoked CRF levels were heightened, indicating that prior WS exposure sensitizes stress-evoked CRF.

3.5 Conclusions

Overall, these experiments have established that WS exposure increases intra-CeA CRF expression and promotes the development of negative valence and hypervigilant behaviors across multiple behavioral paradigms. Further, despite the compensatory elevation in CeA ERβ, local PHTPP treatment during WS exposure was effective in attenuating the stress-induced neuronal and behavioral shifts identified in these experiments. These data highlight the critical role for CeA ERβ signaling during stress exposure since, regardless of resting ERβ levels following stress, blocking ERβ with PHTPP during WS prevents the development of hypervigilant behaviors. Future studies examining the downstream neural correlates involved in these behavioral responses and impacted by ERβ signaling, including the LC-NE system, will further establish these pathways as viable targets for the treatment and prevention of stress-induced psychiatric disorders in females.

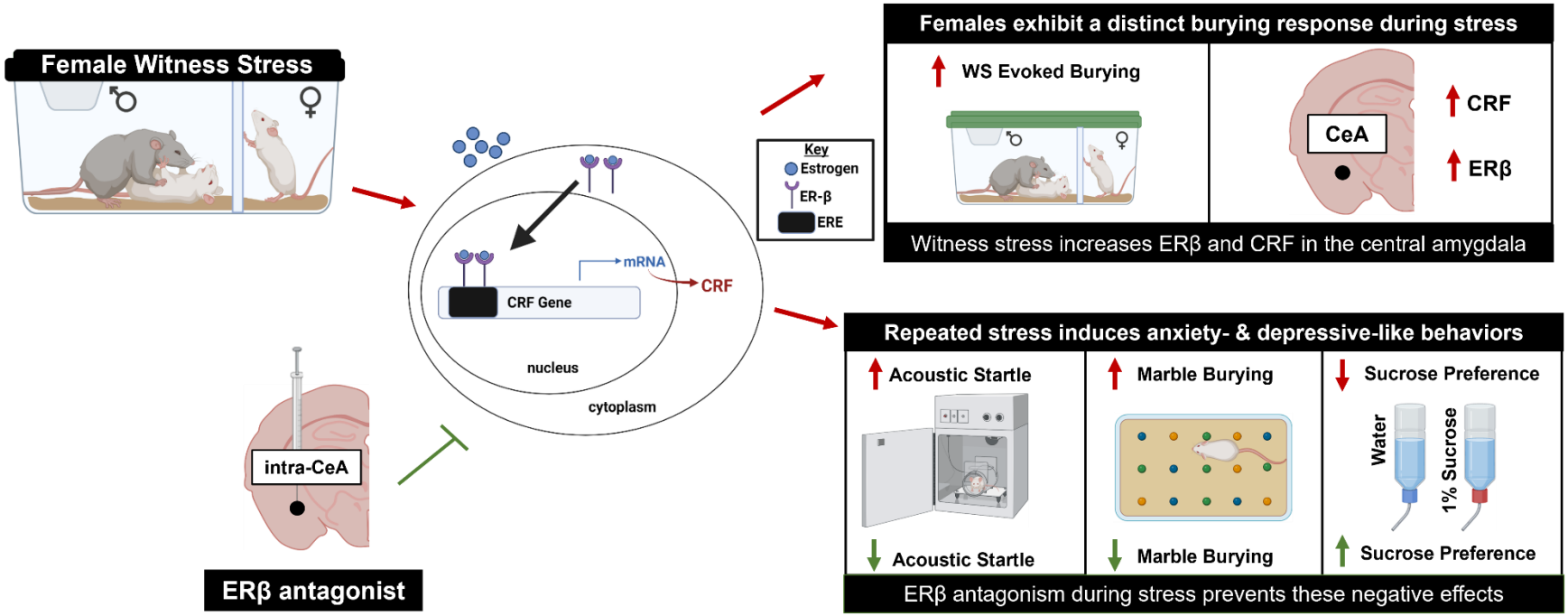

Figure 3.6 Graphical summary. Exposure to repeated WS evokes heightened protein expression levels of CRF and ERβ in the CeA via a proposed mechanism by which ERβ binds to the promoter region on the *Crf* gene in response to stress to increase transcription. These actions then enhance negative effects of stress like hypervigilance, anxiety-like behavior, and anhedonia. However, when ERβ signaling is inhibited, the expression of these behaviors is abolished. ERβ=estrogen receptor-β, CRF=corticotropin releasing factor, ERE=estrogen response element, WS=witness stress

CHAPTER FOUR

INTERLEUKIN-1 RECEPTOR ANTAGONISM IN THE LOCUS COERULEUS OF FEMALE RATS ATTENUATES THE CONSEQUENCES OF SOCIAL STRESS

4.1 Background and Significance

Immune signaling plays a pivotal role in maintaining systemic health and homeostasis by responding to physiological insults with a complex orchestra of proinflammatory cytokines. In this manner, proinflammatory cytokines function to eradicate the present threat, whether it be from tissue damage or pathogenic infiltration (Ishijima & Nakajima, 2021; Saijo & Glass, 2011; Wautier & Wautier, 2023). These widespread actions occur throughout the periphery and central nervous system where they are also reported to engage in mediating normative processes like those related to neurodevelopment (Bollinger & Wohleb, 2019; Sominsky et al., 2018). Microglia are the resident immune cells in the brain that are primarily responsible for the generation and release of cytokines (Saijo & Glass, 2011; Smith et al., 2012) and facilitate processes related to learning and memory (Parkhurst et al., 2013), cognition (McNamara et al., 2023), social behavior (Kana et al., 2019; Zhan et al., 2014), myelination (McNamara et al., 2023), and synaptic pruning (Paolicelli et al., 2011; Parkhurst et al., 2013) to name a few. However, as with many great things in life, too much or too little can cause

negative effects. This is also true for microglia, of which under- and, especially, overactivation can precipitate the onset of myriad neurodevelopmental and psychiatric disorders (Wohleb, 2016; Yirmiya et al., 2015).

As a consequence of microglial dysfunction, aberrant immune signaling and an overabundance of cytokines promote the development of disorders like anxiety and depression (Kritas et al., 2014; Pate et al., 2023; Wang et al., 2022; Yirmiya et al., 2015). Clinical studies have shown that post-traumatic stress disorder (PTSD) (D'Elia et al., 2022; Garrett et al., 2021; Lalonde et al., 2021; Lindqvist et al., 2014; Spivak et al., 1997) and major depressive disorder (MDD) (D'Elia et al., 2022; Garrett et al., 2021; Henje Blom et al., 2012; Zhang et al., 2023) are causally associated with immune perturbations. Preclinical research also supports the relationship between enhanced anxiety- and depressive-like phenotypes and heightened levels of proinflammatory cytokines (Finnell et al., 2019; Jones et al., 2018; Pate et al., 2023; Wood et al., 2015), specifically, that of interleukin (IL)-1 beta (IL-1β). IL-1β is a potent proinflammatory cytokine implicated in the development and expression of anxiety and depressive disorders including PTSD and MDD (Belem da Silva et al., 2017; D'Elia et al., 2022; Hsieh et al., 2020; Konsman et al., 2008; Lan et al., 2023; Zhang et al., 2023) that exerts its central actions through the IL-1 receptor (IL-1r) located directly on neurons.

This is especially relevant when occurring in stress-sensitive brain regions like the locus coeruleus (LC) where IL-1r activation has been shown to induce neuronal sensitization and sustain heightened levels of tonic firing (Borsody & Weiss, 2002, 2004, 2014). The LC is composed of a homogenous population of

norepinephrine (NE)-containing cells that direct behavioral arousal, thus persistent activation of this region can lead to chronic states of hyperarousal, or hypervigilance (Bangasser et al., 2018; Bangasser et al., 2016; Lehner et al., 2021; Xie et al., 2024). This is also known to occur in response to stress which, in combination with elevated levels of IL-1β, exacerbates pathological perturbations and enhances the risk of developing PTSD and MDD. Women are especially disadvantaged in this regard due to their inherently greater levels of proinflammatory cytokines (Derry et al., 2015; Duma et al., 2010; Engler et al., 2016; Pallavi et al., 2015; Powers et al., 2019), heightened susceptibility to stress (Kendler et al., 2001; Sandanger et al., 2004), and more expanded and sensitive LC network (Bangasser et al., 2016; Naegeli et al., 2018; Ohm et al., 1997). Despite decades of pharmacological research, effective treatment options for stress-related psychiatric disorders remain scarce (Davidson, 2015; Hodes et al., 2015; Tiller, 2013) which highlights the need for deeper investigation into the underpinnings of cytokine-mediated neuronal activation in the LC. Therefore, the following studies seek to identify how the LC-NE system is discretely affected by stress, inflammation, and the combined effects of stress and inflammation in terms of behavior, neuronal activity, and neuroendocrine factors. Importantly, this will be assessed in a manner of varying degrees, all directly targeting the LC via local pharmacological manipulations. First, by assessing differences in stimulation and suppression of the local immune response in the context of an acute stressor, and second, by evaluating long-term behavioral outcomes of an attenuated immune response.

This first component of the present studies was conducted using the endotoxin lipopolysaccharide (LPS). LPS was chosen as the inflammatory stimulant because of its reliable and widespread use throughout clinical and preclinical research alike. LPS is known to exert the majority of its stimulatory effects through activation of toll-like receptor (TLR)-4 which is located primarily on microglia but is also present on neurons (Batista et al., 2019). Research shows that LPS promotes the reciprocal actions between activating microglia and inducing a cascade of proinflammatory cytokines that serve as common factors in several pathological conditions (Kritas et al., 2014; Wohleb, 2016; Yirmiya et al., 2015). Importantly, LPS is known to enhance production of the major proinflammatory cytokine IL-1β through microglial activation (Ishijima & Nakajima, 2021), which has been specifically implicated in stress-related disorders like PTSD and MDD (Lan et al., 2023; Spivak et al., 1997). Thus, LPS was used to evaluate how excess inflammation may upregulate hypervigilant behavior and stress-related neuroendocrine factors.

Secondly, IL-1r antagonist (IL-1ra) was used to identify differences in behavioral and neuronal outcomes in the presence of a reduced or attenuated inflammatory load. IL-1ra is an endogenous anti-inflammatory cytokine that functions as a competitive antagonist of and with equal affinity to IL-1β that prevents it from binding to the IL-1r (Borsody & Weiss, 2004; Frank et al., 2012; Greenhalgh et al., 2010). Similar to TLR-4, IL-1r can be found on multiple cell types within the central nervous system including microglia and neurons, the latter of which has been shown to robustly occur in the LC (Borsody & Weiss, 2002, 2004).

Therefore, to determine the therapeutic capacity of IL-1ra in the LC, it was first evaluated in relation to an acute social stressor in terms of behavior and neuronal activation, and then assessed for its effectiveness in preventing long-term stress-induced behavioral deficits. Collectively, these studies sought to investigate the overarching hypothesis that inhibiting inflammation-induced neuronal activation within the LC during exposure to social stress will prevent later expression of behavioral deficits in terms of hypervigilance and social apprehension that are commonly seen in clinical manifestations of stress-related psychiatric disorders.

4.2 Materials and Methods

4.2.1 Rats and housing

Sexually naïve Sprague-Dawley female rats ("controls" and "witnesses", 8-9 weeks upon arrival; "conspecifics" 9-10 weeks upon arrival; Charles River; Raleigh, North Carolina, USA), sexually naïve Sprague-Dawley male rats ("intruders"; 250-275 g upon arrival; Charles River), and retired breeder Long Evans male rats ("residents"; Charles River and Envigo; Dublin, Virginia, USA) were obtained for use in the present studies. From the time of arrival and throughout the duration of the experiments, rats were single housed in standard polycarbonate cages with the exception of female conspecifics that arrived and remained pair housed. All rats had *ad lib* access to pelleted chow (Teklad Rodent Diet® 8904, Envigo) and filtered water, except during testing. All female and male rats were housed in separate colony rooms that were kept on a 12-hour light/dark cycle (lights on at 0700 hours) and maintained at 25°C and 40% humidity. Control, witness, and

conspecific females were kept in the same room but on different racks from each other. This was the case for males, as well, with intruders and residents living in the same room but on different racks from each other. All components of these studies were conducted with prior approval from the University of South Carolina IACUC and in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

4.2.2 Intra-LC bilateral guide cannula surgery

Approximately one week following arrival, control and witness females were implanted with indwelling bilateral guide cannulas (26 ga., P1 Technologies; Roanoke, Virginia, USA) aimed at the LC (caudal: -3.9 mm from Bregma; lateral: ± 1.1 mm; and ventral: -5.1 mm from skull, nose at a 30° angle). For placement accuracy, cannula surgeries were guided by multi-unit electrophysiology in which the precise location of the LC was ascertained via neuronal firing in direct response to a toe pinch. Rats received standard post-operative care (Flunazine® Injectable Solutions, 0.25mg/kg, s.c.) on the day of and the day following surgery, in addition to at least 7 days of recovery prior to experimental procedures.

4.2.2 Drug information

LPS used in Study A was selected as an agent to induce microglial activation. LPS (Sigma-Aldrich; St. Louis, Missouri, USA) was dissolved in 0.9% sterile saline at a concentration of either 0 (vehicle) or 3 μg/μL and delivered as a single 1-μL dose that was microinfused bilaterally into the LC over one minute followed by a one-minute post-infusion pressure normalization period before injectors were removed. Drug administration occurred one hour prior to experimental testing and the dose

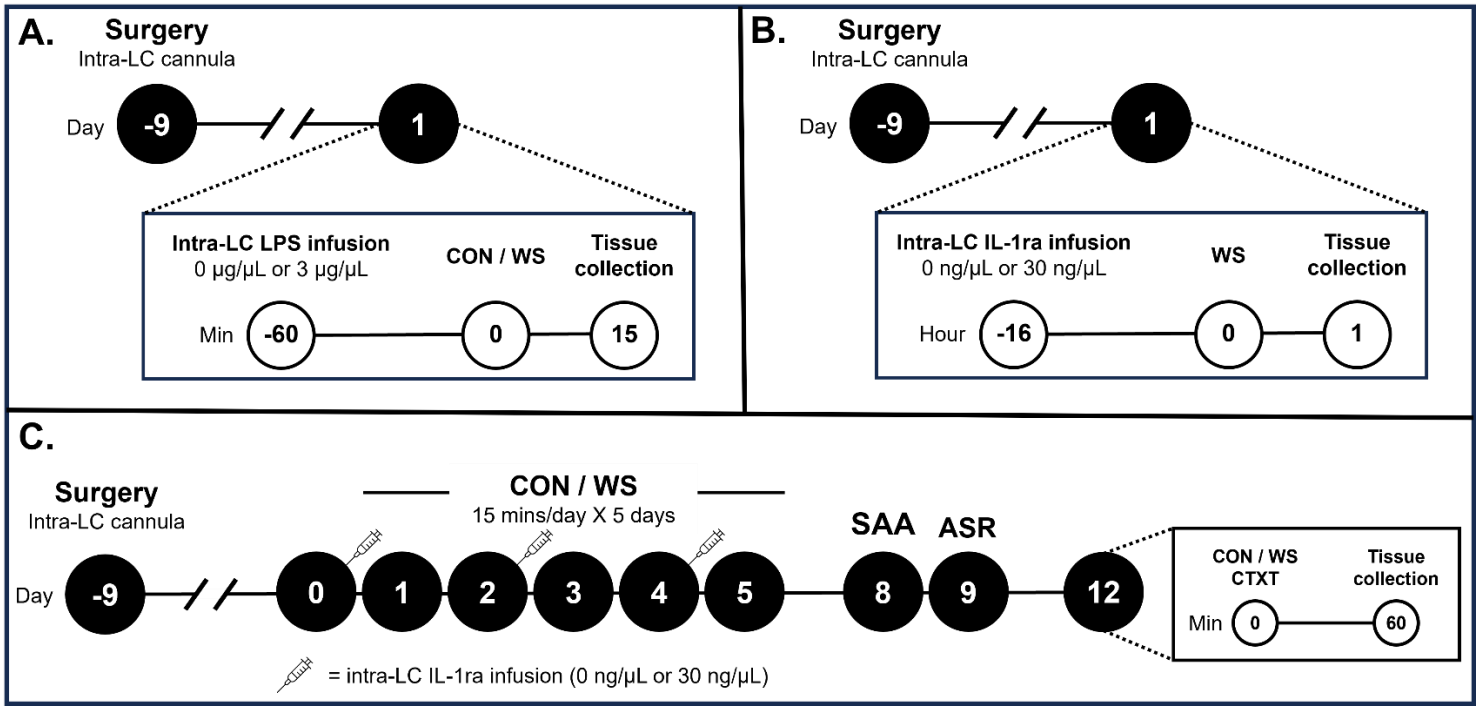

Figure 4.1 Experimental timelines. A) Study A: rats were exposed to a single experimental day in which either vehicle or LPS was administered intra-LC roughly one hour before undergoing CON/WS. Tissue was collected immediately following the 15-minute CON/WS exposure. **B)** Study B: rats received an intra-LC infusion of IL-1ra on the evening prior to undergoing an acute WS exposure that was followed by collection of fixed tissue one hour later. **C)** Study C: rats received the first of three intra-LC infusions of IL-1ra the evening before undergoing the first CON/WS session. The second and third doses were given 48 and 96 hours following the first dose on the evenings prior to the third and fifth CON/WS session, respectively. Two days after the final CON/WS session, rats were tested for sociability in the SAA test followed the next day by ASR testing for hypervigilance. Two days later, rats underwent CTXT in which they re-experienced their initial CON or WS exposure settings, which was followed by tissue collection one hour later. CON=control, WS=witness stress, LC=locus coeruleus, LPS=lipopolysaccharide, IL-1ra=interleukin-1 receptor antagonist, SAA=social approach avoidance, ASR=acoustic startle response, CTXT=context

and timing were chosen based on pilot studies demonstrating the effectiveness of LPS in eliciting an inflammatory response without producing sickness behaviors.

IL-1ra used in Study B and Study C was chosen for its selective effect on the endogenous IL-1r and proven ability to decrease tonic firing of the LC (Borsody & Weiss, 2002, 2004). IL-1ra (R & D Systems; Minneapolis, Minnesota, USA) was dissolved in lab-made artificial cerebrospinal fluid (aCSF) at a concentration of 0 (vehicle) or 30 ng/μL. Rats in Study B received a single 1-μL dose microinfused bilaterally into the LC over one minute followed by a one-minute post-infusion pressure normalization period that occurred approximately 16 hours prior to experimental testing. Rats in Study C received this same dose administered at three separate times roughly 48 hours apart with the first dose given approximately 16 hours prior to beginning experimental testing. This dose was chosen based on prior studies conducted in female Sprague-Dawley rats that effectively suppressed tonic firing of LC cells (Borsody & Weiss, 2002, 2004). The timing of administration was chosen based on other preclinical studies that have shown robust effects beginning within hours (Borsody & Weiss, 2002, 2004; Greenhalgh et al., 2010) and lasting for up to 14 days (Frank et al., 2012) after central infusion. Importantly, were first piloted the 30 ng dose and 48-hour timeframe to ensure viable effects on stress-induced outcomes were attainable.

4.2.4 Acute or repeated witness stress (WS) and control handling (CON)

Witness stress (WS) is an ethologically relevant form of psychosocial stress that has been modified from the widely used resident-intruder social defeat paradigm to impart a robust source of stress on females for preclinical research. Briefly, the

female witness is confined to one side of a resident's home cage to observe the auditory, olfactory, and visual stimuli of a social defeat encounter between the resident and intruder as described previously (Bouknight et al., 2023; Finnell et al., 2018; Pate et al., 2023). For Studies A (**Figure 4.1A**) and B (**Figure 4.1B**), WS was conducted as a single, acute session. For Study C, WS was repeated over a period of five consecutive days for 15 minutes per day (**Figure 4.1C**). Importantly, prior to testing, all residents were screened during at least two subsequent sessions of social defeat with a novel Sprague-Dawley male, and only those exhibiting adequate aggression without causing injury were used. Additionally, while there was never physical contact between the witnesses and intruders, they were paired such that the same witness and intruder were exposed in their respective manners to a novel resident each day to prevent establishment of social dominance and provide a consistent level of social defeat.

For comparison, females in the non-stressed control (CON) group were briefly handled then returned to their home cages to mimic the transfer of the witnesses to the residents' cages while remaining non-stressed. Our lab has previously shown that this version of a control condition produces equivalent outcomes to when a female rat is placed into a novel cage behind a partition in the presence of a non-stressed intruder male (Bouknight et al., 2023).

4.2.5 Quantification of anxiety-like burying during CON/WS

CON/WS sessions were video-recorded and retrospectively assessed for anxiety-like burying behavior by an experimenter blinded to treatment groups using ANY-maze (Stoelting Co.; Wood Dale, Illinois, USA). In brief, burying is defined as a

non-exploratory digging or pushing of the bedding that con be conducted with any of the four paws or head/nose. This behavior was evaluated during the acute CON/WS sessions in Study A, the WS sessions in Study B, the first (D1) and fifth (D5) CON/WS sessions in Study C, and for the CON/WS context (CTXT) sessions in Study C. This behavior is noteworthy because it is known to be highly characteristic of an anxiety-like phenotype that occurs robustly in cycling females (Finnell et al., 2018) and requires the LC for its manifestation (Howard et al., 2008).

4.2.6 Social Approach – Avoidance (SAA) test

In Study C, rats were assessed during the social approach – avoidance (SAA) test on day 8 (**Figure 4.1C**) that occurred in a standard open field arena containing only a perforated, acrylic "stranger cage" in the center of one side of the arena (**Figure 4.5A**). Rats underwent two back-to-back five-minute sessions, first, without the presence of a conspecific, referred to as "target absent", and then with a novel conspecific inhabiting the stranger cage, referred to as "target present". During each session, rats were scored for total distance traveled as well as duration in the close half and far half that were compared as ratios for both the target absent and present conditions. The zones are termed in relation to the stranger cage, such that the close half indicates the half of the arena closest to the stranger cage. Study rats were first placed into the arena facing the far wall in the far half of the arena for both sessions. Between each of their target absent and present sessions, they were briefly placed in a novel, clean cage while the conspecific was positioned in the stranger cage. Sessions were recorded then

automatically scored using Noldus EthoVision XT software (Leesburg, Virginia, USA) which was set to determine the rat's location based on its nose point.

4.2.7 Acoustic Startle Response (ASR) test

In Study C, acoustic startle response (ASR) testing occurred on day 9 (**Figure 4.1C**) for assessment of hypervigilance via the SR-LAB Startle Response System (San Diego Instruments; San Diego, California, USA). Rats were individually placed in an acrylic enclosure secured over an accelerometer within one of three adjacent sound-attenuating isolation cabinets. Rats were tested two or three at a time in a counterbalanced fashion based on stress condition and drug treatment. To prevent disturbances from outside sounds, 65 decibels (dB) was played continuously throughout the session to serve as background noise. All rats were exposed to the same protocol that began with a five-minute habituation period in which only background noise was played, followed by a series of five different tones (95, 100, 105, 110, and 115 dB) that were each played a total of ten times in a pseudorandom order. All tones were played in 50-millisecond bursts with the following 100 milliseconds recorded for the maximum startle amplitude exhibited by each rat for that specific trial. To ensure equivalence across test sessions, calibration of each cabinet's accelerometer reading and dB level emission was conducted according to the manufacturer's directions in the SR-LAB manual.

4.2.8 CON/WS context (CTXT)

To assess long-term effects of either the stress or drug intervention, rats in Study C were re-exposed to their initial CON or WS condition for a single, acute 15-minute session. For CON rats, this entailed brief handling followed by return to

their home cage. For WS rats, this entailed being placed in the home cage of a familiar resident behind a partition with their paired intruder on the other side, as previously done during WS, but without the resident male, thus, in the absence of true social defeat. This paradigm served as a stress cue to evaluate how prior WS and/or IL-1ra treatment may dictate negative behavioral outcomes to reminders of the stressful experience at a later point, which is a major hallmark of clinical PTSD (Mann & Marwaha, 2023).

4.2.9 Tissue collection

Rats in Studies A and C were briefly anesthetized with isoflurane prior to quick decapitation for collection of fresh tissue. This occurred immediately following CON/WS exposure in Study A, and 60 minutes following CTXT in Study C. Trunk blood was collected into EDTA-lined tubes then centrifuged for extraction of plasma. Brains were immediately dissected into posterior and anterior halves, flash-frozen in isopentane, and stored at -80°C until later use.

Rats in Study B were briefly anesthetized with isofluorane prior to being transcardially perfused with fixative, which occurred 60 minutes following the start of CON/WS (**Figure 4.1B**). This timeframe was chosen for assessment of peak cFos expression, a widely used marker of neuronal activation (Hoffman et al., 1993). Briefly, rats were heavily sedated using isofluorane vapor then transcardially perfused with ~200 mL of cold 0.1M phosphate-buffered saline (PBS) followed by ~250 mL of cold 4% paraformaldehyde solution. Brains were subsequently removed then post-fixed for at least 24 hours at 4°C followed by being completely saturated in a sucrose-azide solution (20% sucrose + 1% sodium

azide in 0.1M phosphate buffer (PB)) for approximately 3 days. Brains were then flash frozen in isopentane and stored at -80°C until coronally sliced at 30-µm thickness for serial collection of LC tissue. Slices were stored in an anti-freezing solution (30% sucrose + 30% ethylene glycol in 0.1M PB) at -20°C until ready for immunohistochemistry.

4.2.10 Corticosterone (CORT) and IL-1β ELISAs

Plasma samples acquired from rats in Study A were analyzed for the major peripheral stress hormone corticosterone (CORT) using a commercially available CORT ELISA kit (Enzo Life Sciences; Farmingdale, New York, USA) that was performed per the manufacturer's instructions. Similarly, plasma levels of IL-1β were measured using a commercially available ELISA kit (R&D Systems) performed per the manufacturer's instructions.

4.2.11 Immunohistochemical analysis of cFos and tyrosine hydroxylase (TH) in free-floating LC slices

For Study B, slices containing core LC (Bregma -9.96 to -9.60 mm (Paxinos & Watson, 2006)) were double-labeled with cfos and TH to quantify putative cellular activation that occurred within the LC as a result of WS and, further, to identify if IL-1ra treatment attenuated this effect. Slices were first washed five times for five minutes each in 0.1M phosphate buffered saline (PBS) then incubated in 0.9% H_2O_2 in PBS for 20 minutes for free aldehyde scavenging purposes. Tissue was then washed three times in 0.1M PBS for five minutes each prior to incubating for one hour in blocking buffer (3% normal donkey serum + 0.2% Triton-X 100 in 0.1M PBS). Slices were then transferred into a solution containing rabbit anti-cFos

antibody (1:500, No. ABE457; Millipore Sigma; Boston, Massachusetts, USA) made up in 2% normal donkey serum + 0.25% Triton-X 100 in 0.1M PBS for incubation over two days at 4°C. Following three consecutive ten-minute washes in fresh PBS, tissue was placed into a solution containing biotinylated anti-rabbit antibody (1:200, No. BA-1000; Vector Laboratories; Newark, California, USA) for two hours at room temperature. Slices were then incubated in an avidin/biotin complex (ABC) (VECTASTAIN®, No. PK-6100; Vector Laboratories) solution for one hour then washed in fresh 0.1M PBS three times for five minutes each. Next, slices were incubated in a nickel-enhanced diaminobenzidine (DAB; SIGMAFAST™, No. D2493; Millipore Sigma) solution for approximately two minutes. Following another round of three five-minute washes in PBS, tissue was transferred into mouse anti-tyrosine hydroxylase (TH) antibody solution (1:2500, No. MAB5280; Millipore Sigma) made up in 2% normal donkey serum + 0.25% Triton-X 100 in 0.1M PBS for incubation overnight at room temperature. The next morning, slices were washed for three rounds of five-minutes each in 0.1M PBS, and then were incubated in biotinylated anti-mouse antibody (1:1000, No. ab208001; Abcam; Waltham, Massachusetts, USA). Slices were, again, incubated for an hour in ABC (Vector Laboratories) solution followed by incubation in DAB (Millipore Sigma) solution for roughly 10 seconds prior to undergoing a final round of three five-minute PBS washes. Slices were then mounted onto slides (Fisherbrand™ Superfrost™ Plus; No. 12-550-15, Thermo Fisher Scientific; Waltham, Massachusetts, USA), dehydrated, and cover slipped.

4.2.11 Statistical analyses

Datasets were first evaluated for outliers, which were considered to be outside two standard deviations from the group mean, that were removed before performing statistical analyses. For Study A, CON/WS burying duration, CORT, and IL-1β were each assessed via a two-way analysis of variance (ANOVA) to identify effects of stress and/or LPS treatment. For Study B, WS burying duration and intra-LC cFos-positive cell counts were evaluated using unpaired T-tests for effects of IL-1ra treatment. For Study C, D1 and D5 CON/WS burying durations were assessed using a two-way ANOVA to identify the effects of stress and/or IL-1ra treatment in addition to a three-way repeated measures ANOVA to identify how these factors altered burying over time. SAA parameters and ASR startle amplitude were evaluated using three-way ANOVAs to compare stress X IL-1ra treatment X social target status and stress X IL-1ra treatment X stimulus intensity, respectively. CTXT was assessed using a two-way ANOVA to identify the impact of prior stress and/or IL-1ra treatment on long-term burying behavior in response to stress cues. All statistical significance was followed by Tukey's post-hoc analyses, except for ASR data that utilized Fisher's Least Significant Difference (LSD) for planned comparisons. Data are presented as mean ± standard error of the mean (SEM) with an $\alpha = 0.05$.

4.3 Results

4.3.1 WS elicited robust increases in behavioral and neuroendocrine outcomes reflective of anxiety-like behavior which were augmented by intra-LC LPS treatment in Study A

WS rats treated with vehicle exhibited a significantly greater burying duration compared to CON that was further enhanced by intra-LC LPS selectively in the WS-exposed rats (**Figure 4.2A**; effect of stress: $F_{1,17}$=7.19, p=0.02; effect of drug: $F_{1,17}$=51.53, p<0.0001; effect of stress X drug: $F_{1,17}$=7.20, p=0.02). Intra-LC LPS also increased circulating CORT levels regardless of stress condition (**Figure 4.2B**; effect of drug $F_{1,15}$ = 6.67, p = 0.02). However, treatment with intra-LC LPS did not further enhance the WS-evoked peripheral CORT response (**Figure 4.2B**). In contrast, intra-LC LPS augmented the WS-induced increase in plasma IL-1β (**Figure 4.2C**; effect of stress: $F_{1,18}$=7.68, p=0.013; effect of drug: $F_{1,18}$=18.86, p=0.0004), with the combination of WS + LPS producing the greatest effect.

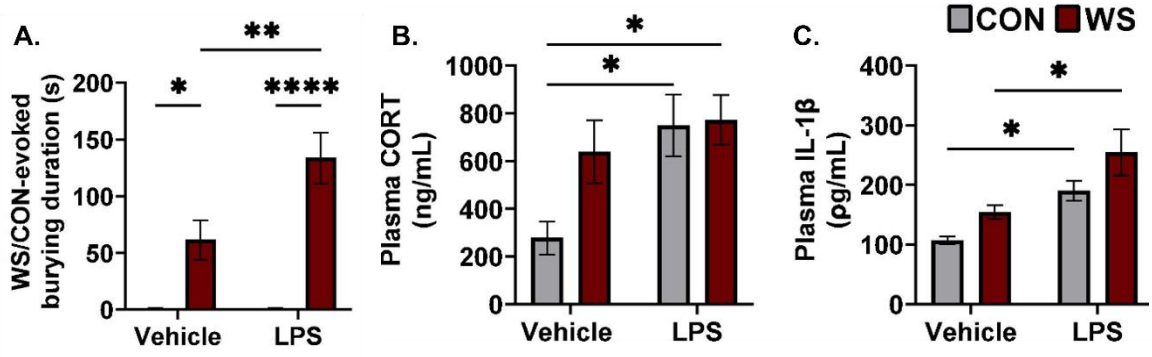

Figure 4.2 Infusion of lipopolysaccharide into the locus coeruleus exacerbated the behavioral and neuroendocrine responses to an acute episode of witness stress. A) WS rats exhibited significantly greater anxiety-like burying than CON rats during their respective exposures, and intra-LC LPS treatment potentiated this effect in WS rats. **B)** WS and intra-LC LPS individually and together induced a robust increase in circulating levels of CORT, though the combined effects were not significantly greater than WS or LPS treatment alone. **C)** Peripheral IL-1β levels were elevated in response to the individual effects of WS and LPS treatment and were synergistically enhanced by their combined effects, such that CON + VEH rats exhibited significantly lower while WS + LPS rats exhibited significantly greater plasma IL-1β among the four groups. CON=control, WS=witness stress, LPS=lipopolysaccharide, CORT=corticosterone, IL-1β=interleukin-1 β, LC=locus coeruleus, VEH=vehicle
** p < 0.05, ** p < 0.01, *** p < 0.001*, *Tukey's post-hoc*

4.3.2 IL-1ra attenuated the increase in anxiety-like burying during acute WS as well as the preceding increase in LC neuronal activation in Study B

A pilot study was designed to determine the dose of IL-1ra that was effective in reducing WS-evoked burying and LC activity. WS + VEH rats tended to display greater anxiety-like burying during the acute WS exposure compared to WS + IL-1ra rats (**Figure 4.3A**; effect of drug: t_6=2.442, p=0.0504) evidenced by an increase in duration of burying in vehicle-treated over IL-1ra-treated rats. A similar attenuation in LC neuronal activation was observed, indicated by a reduced number of neurons co-expressing cFos and tyrosine hydroxylase (TH), a marker of noradrenergic cells (**Figure 4.3B**; effect of drug: t_4=2.415, p=0.0732).

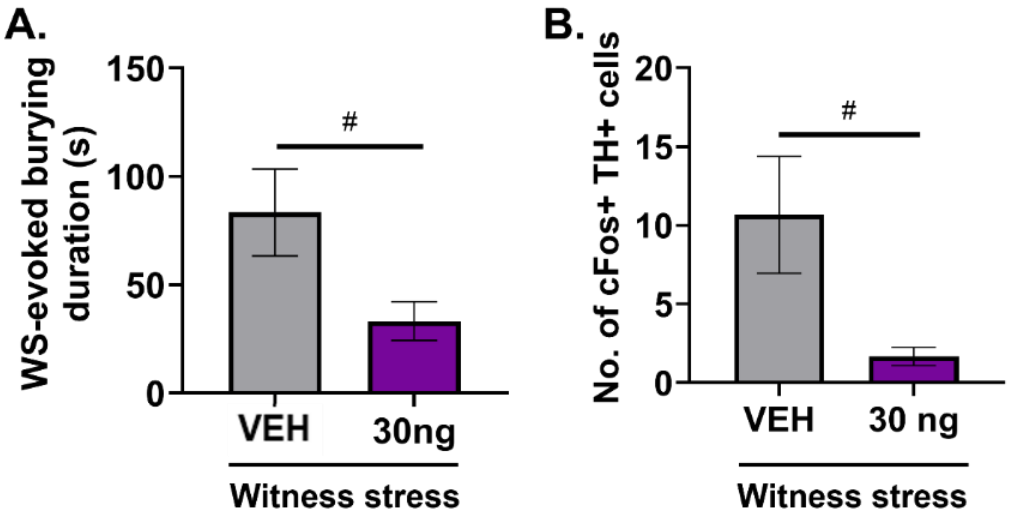

Figure 4.3 Blockade of interleukin-1 receptor in the locus coeruleus prior to witness stress modestly reduced the duration of anxiety-like burying and preceding increase in local expression of cFos. A) VEH-treated rats exhibited greater anxiety-like burying during WS exposure than IL-1ra-treated rats. **B)** Increased cFos immunoreactivity in the LC of WS + VEH rats was attenuated by treatment with IL-1ra, indicating a reduction in LC neuronal activation. WS=witness stress, VEH=vehicle, IL-1ra=interleukin-1 receptor antagonist, LC=locus coeruleus *# p ≤ 0.09, Tukey's post-hoc*

4.3.3 IL-1ra treatment prevented the WS-evoked increased in anxiety-like burying during repeated exposure to repeated WS

On CON/WS D1, rats exhibited greater anxiety-like burying during WS compared to CON, and IL-1ra treatment significantly ameliorated this effect (**Figure 4.4A**; effect of stress: $F_{1,26}$=37.17, p<0.0001; effect of drug: $F_{1,25}$=5.315, p=0.0294). These effects were paralleled on D5 with WS rats exhibiting greater anxiety-like

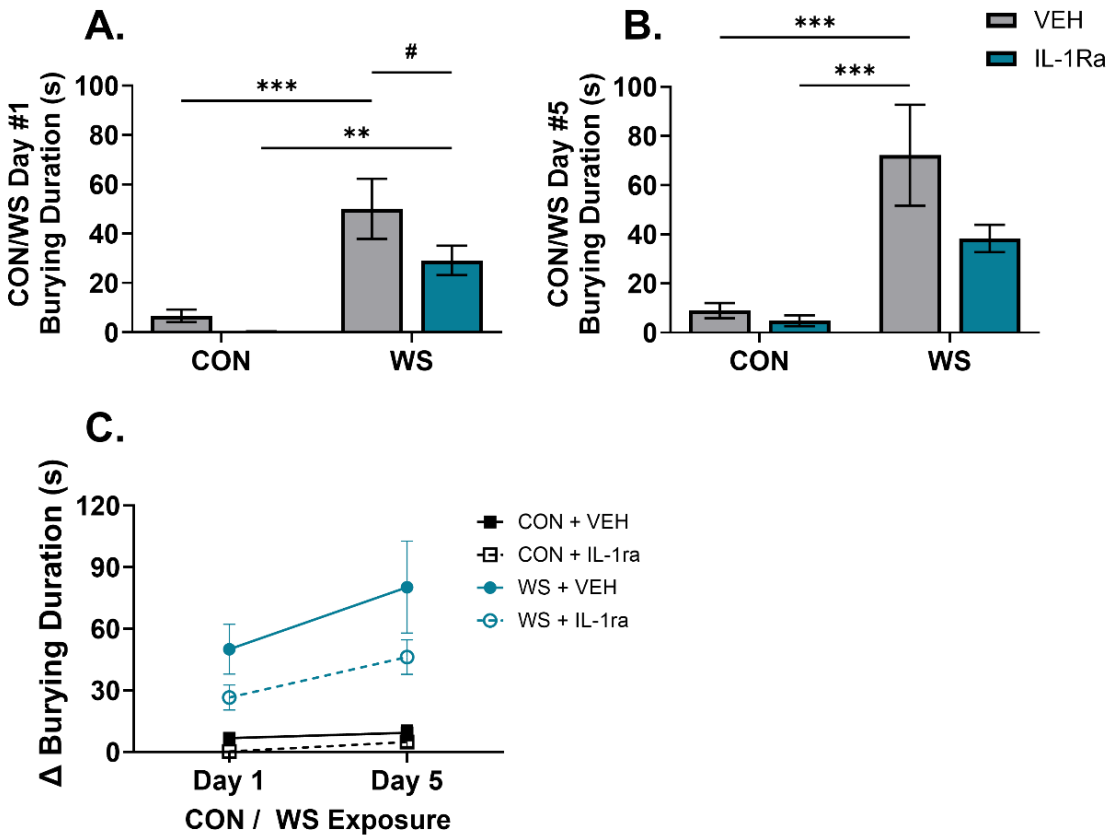

Figure 4.4 Exposure to witness stress induced anxiety-like burying that steadily increased from the first to the fifth episode but was notably reduced with interleukin-1 receptor antagonist treatment in the locus coeruleus. A) On day 1 of CON/WS, rats exposed to WS exhibited significantly greater anxiety-like burying compared to CON, and **B)** this pattern continued through the final day 5. **C)** Comparing day 1 to day 5, WS elicited a gradual increase in anxiety-like burying but this effect was significantly muted by IL-1ra treatment. CON=control, WS=witness stress, VEH=vehicle, IL-1ra=interleukin-1 receptor antagonist
*# p ≤ 0.09, ** p < 0.01, *** p < 0.001, Tukey's post-hoc*

burying behavior than CON, and IL-1ra treatment tending to reduce this behavior (**Figure 4.4B**; effect of stress: $F_{1,26}$=22.83, p<0.0001; effect of drug: $F_{1,26}$=3.520, p=0.0719). Over time, WS rats exhibited greater anxiety-like burying from D1 to D5 versus CON regardless of treatment, though this effect was significantly attenuated with IL-1ra treatment (**Figure 4.4C**; effect of stress: $F_{1,24}$=40.18, p<0.0001; effect of drug: $F_{1,26}$=5.702, p=0.0252).

4.3.4 History of WS induced a socially avoidant phenotype during SAA testing that was not altered by prior IL-1ra treatment

When assessed for sociability in the SAA test (**Figure 4.5A**), there were no differences observed between groups in terms of locomotor activity for distance traveled in either the target absent (effect of stress X drug: $F_{1,31}$=1.692, p=0.2030,

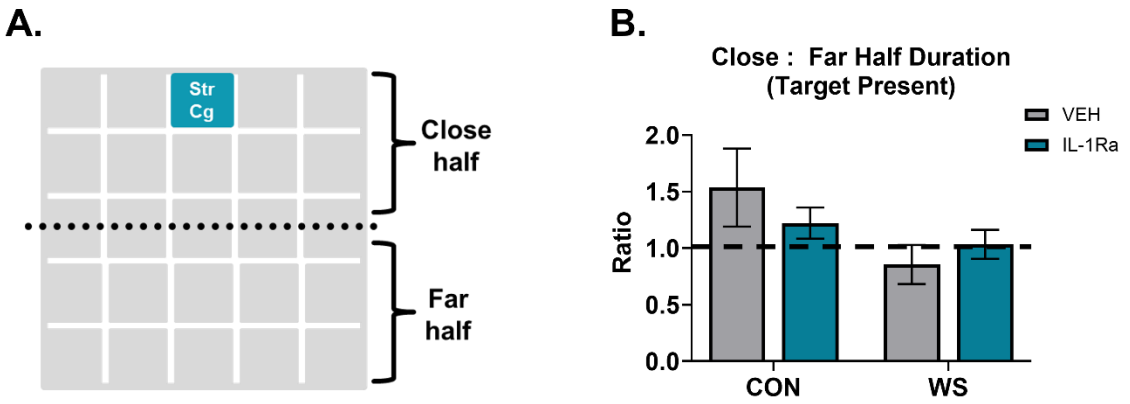

Figure 4.5 Rats previously exposed to witness stress spent less time in the close half of the arena when the conspecific female was present, indicating a socially avoidant phenotype. A) Representation of the SAA apparatus and the imaginary separation line delineating the close and far halves. **B)** History of WS induced a socially avoidant phenotype indicated by a reduced ratio of time spent in the close to far half of the arena under target present conditions. Prior treatment with IL-1ra did not attenuate the effect of WS. CON=control, WS=witness stress, Str Cg=stranger cage, VEH=vehicle, IL-1ra=interleukin-1 receptor antagonist, SAA=social approach-avoidance
No post-hoc significance.

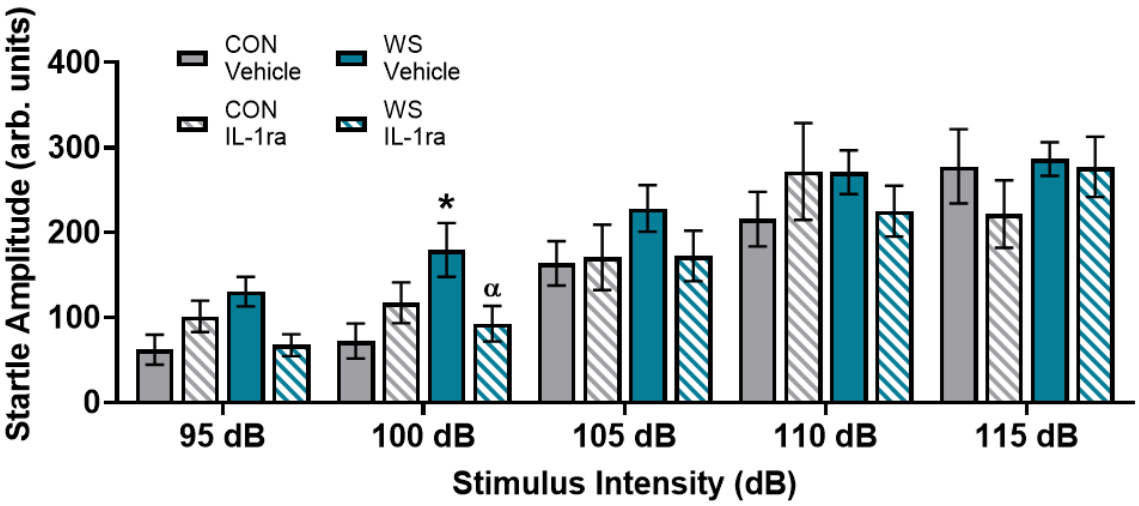

Figure 4.6 Rats with a history of witness stress startled more when played aversive sounds, though this effect was modestly reduced in those previously treated with interleukin-1 receptor antagonist in the locus coeruleus. IL-1ra treatment during the initial repeated WS exposure lessened the WS-evoked increase in startle amplitude that was most apparent at the 100 dB stimulus; however, prior treatment potentiated the effects of startle in CON rats. CON=control, WS=witness stress, IL-1ra=interleukin-1 receptor antagonist
$^{\alpha}$ *p < 0.09, * p < 0.05, Tukey's post-hoc*

data not shown) or target present (effect of stress X drug: $F_{1,29}$=1.292, p=0.2650, data not shown) conditions. Additionally, no effects were found when close and far durations were compared as a ratio of time when the target was absent (effect of stress X drug: $F_{1,29}$=1.133, p=0.7389, data not shown), though robust effects of stress were observed in the target present condition (**Figure 4.5B**; effect of stress: $F_{1,29}$=4.825, p=0.0362). These data show that rats with a history of WS, exhibiting a ratio ≤ 1.0 for time spent in the close versus far half when the target was present, indicate a lack of "social preference" and an overall socially avoidant phenotype. There are no indications, however, that prior intra-LC IL-1ra treatment modified this behavioral outcome.

4.3.6 Prior treatment with IL-1ra ameliorated the WS-induced increase in startle amplitude, but potentiated this effect in CON rats

Subsequent testing of hypervigilant behavior in ASR revealed dual effects of IL-1ra treatment in which CON rats exhibited an increase in startle amplitude while WS rats exhibited a decrease compared to their respective VEH-treated counterparts (**Figure 4.6**; effect of stress X drug $F_{1,100}$=4.946, p=0.0284). Post-hoc analysis revealed the origin of this significance to stem from the 100 dB stimulus. At this specific tone there were no differences observed between CON + VEH and CON + IL-1ra groups; however, the WS + VEH group exhibited a significantly heightened startle amplitude compared to CON + VEH (p=0.0263) and a trend compared to WS + IL-1ra (p=0.0805). Collectively, these findings indicate that a robust impact of LPS on potentiation and IL-1ra on attenuation of the stress response. These results are in line with previous reports from our lab that specify

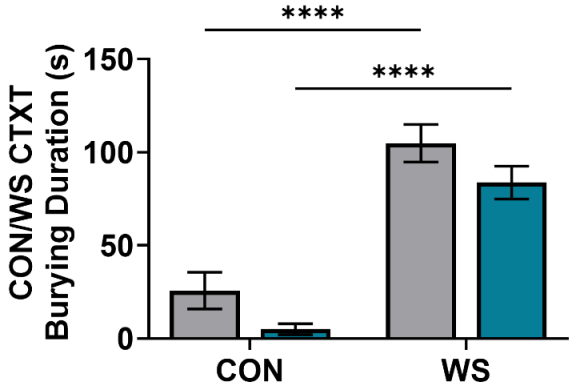

Figure 4.7 Previous treatment with interleukin-1 receptor antagonist in the locus coeruleus attenuated the latent effects of prior witness stress on anxiety-like burying during context re-exposure. Rats with a history of WS exhibited a greater duration of anxiety-like burying compared to CON, though previous treatment with IL-1ra reduced this behavior across the board. CON=control, WS=witness stress, CTXT=context, IL-1ra=interleukin-1 receptor antagonist
***** p < 0.0001, Tukey's post-hoc*

history of WS induced greater reactivity in the ASR test, and that prior IL-1ra treatment limited this response, especially at the 100 dB stimulus.

4.3.7 Prior treatment with IL-1ra attenuated WS-evoked increases in anxiety-like burying during CTXT re-exposure

Finally, when assessed during CTXT, individual effects of stress and drug treatment occurred, such that prior WS elicited a robust increase in anxiety-like burying while IL-1ra attenuated this behavior (**Figure 4.7**; effect of stress: $F_{1,28}$=92.05, p<0.0001; effect of drug: $F_{1,28}$=6.423, p=0.0171).

4.4 Discussion

The present studies are among the first to show how inflammatory factors within the LC can alter the response to social stress in females. Overall findings indicate a role for LC microglia in the exacerbation of stress-related outcomes (Pate et al., 2023). The current study adds another critical layer of knowledge to this information by pinpointing the causative actions of IL-1r in the LC in relation to behavioral, neuronal, and physiological manifestations of stress that serve as a promising therapeutic target for disorders like PTSD and MDD that exhibit reciprocal relationships with inflammation.

The LC-NE system is of particular interest in this area of research because of its sexually dimorphic anatomy that renders females more susceptible to stress and stress-related psychiatric disorders, especially PTSD and MDD which women are at least twice more prone to developing than men (Bangasser et al., 2016; Sandanger et al., 2004). In addition, females have inherently greater levels of inflammation that begin to starkly differ from males around pubertal onset (Derry

et al., 2015) which also coincides with the age of onset of females' enhanced physiological response to stress (e.g., elevated CORT and a delayed recovery time compared to males) and expression of anxiety and depression symptoms (Chafkin et al., 2022; Stroud et al., 2011). Thus, immune regulation within the LC serves as a prime candidate for therapeutic potential in alleviating the burdens of stress in the female population.

The outcomes of these studies support this by evidencing a strong association between intra-LC inflammation and behavioral manifestations of the stress response in naturally cycling, female rats. Specifically, the use of LPS robustly enhanced anxiety-like burying during an acute stressor while IL-1ra reduced this outcome. Moreover, IL-1ra treatment remained effective in the long-term by lowering expression of anxiety-like burying in response to the original WS CTXT. These effects on behavior are of vital importance because LC function is required for anxiety-like burying (Howard et al., 2008), so the ability of these drugs to directly influence this response gives credence to the local immune system as a promising candidate.

In addition, IL-1ra treatment muted the WS-induced hypervigilant response that was observed in the ASR test. Heightened startle is a characteristic behavior of many anxiety disorders, especially PTSD, that is heavily linked to hyperarousal of the nervous system (Bangasser et al., 2018; Nwokafor et al., 2021). ASR is an incredibly useful measure of hypervigilance because of its translational relevance between clinical and preclinical research settings (Börchers et al., 2022). When considering female psychopathology, ASR is one of the few, if not only, major

behavioral tests where results from rodent models accurately represent the sex differences in stress-related outcomes that are found in human studies (Börchers et al., 2022). Expectedly, WS induced a heightened startle response that was most obvious in VEH-treated rats. Importantly though, no differences were observed in startle amplitude between both CON groups and WS + IL-1ra rats. While there is no overt statistical significance between the WS groups to polarize a distinct effect of IL-1ra treatment, the considerable lack of difference that exists only with the WS + IL-1ra group is important to note. The present study is the first to show that IL-1ra in the LC exhibits long-term attenuation of stress-induced hypervigilant behavior in the ASR test. Moreover, these findings replicate previous results acquired from our lab using this paradigm which supports its reliable and critical use in modeling social stress in females. Thus, it is imperative to continue exploring this fruitful avenue for therapeutic possibilities and improving female-based research.

Finally, exposure to stress is known to impair aspects of social functioning that are highly common in PTSD and MDD, including social withdrawal and avoidance (Ottenbreit et al., 2014). The current data support this by showing a reduction in social approach, or social avoidance, in rats with a history of WS compared to CON. This was represented by a preference for being in the close half near the stranger cage versus the far half of the arena when the conspecific target was present. Interestingly, this strong social preference was not observed if rats had a history of WS. While social avoidance of an aggressive conspecific has been routinely observed in mice following social stress (Hollis & Kabbaj, 2014), the

present findings are novel in reporting the induction of avoidance of a same sex conspecific following witness stress in females. Interestingly, despite the robust effect of IL-1ra treatment on other hypervigilant endpoints, there was no effect of IL-1ra treatment on social avoidance. This may be explained by the LC being less involved in directing aspects of social behaviors compared to other regions, like the amygdala, prefrontal cortex, and nucleus accumbens (Gellner et al., 2021). However, the evident effect of WS in hampering prosocial behavior is a pertinent finding that has yet to be shown using this social stress paradigm in female rats which opens many doors to advancing stress neurobiology research.

4.5 Conclusion

Taken together, the data from the current studies mark the inflammatory system, namely IL-1r and its activating proteins, as a notable mediator of the stress response. More specifically, that its functions within the LC critically regulate female susceptibility to the hypervigilant repercussions of social stress, in conjunction with inherently greater levels of inflammation, create a perfect storm of sorts that promotes the development of stress-related psychiatric disorders. With the palpable lack of effective treatment options, novel, druggable targets are in desperate need of investigation: here, the case is made for IL-1 in the LC. However, the direct infusion of IL-1ra into the LC is not a viable option for the clinical population, thus further studies are needed to assess bioavailability via a peripheral method of administration. In addition, further research should be conducted on deeper mechanistic qualities to understand how IL-1ra treatment is efficacious and what, if any, other aspects may provide more precise targeting.

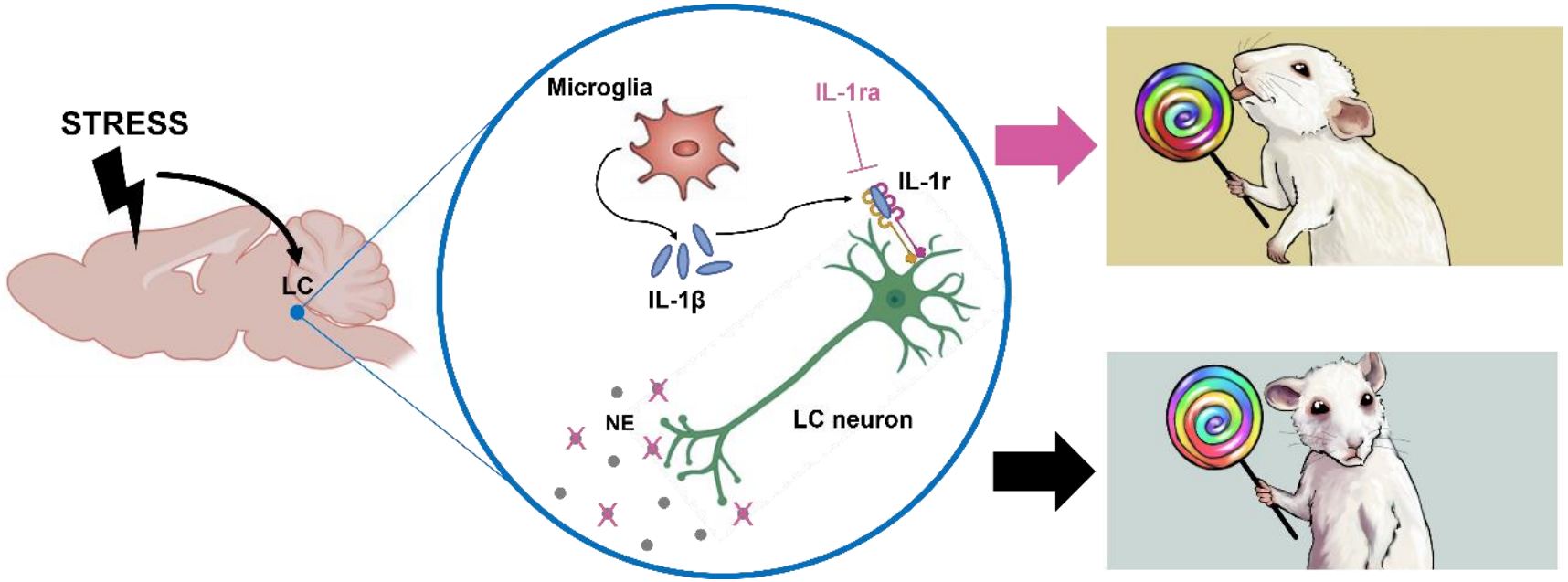

Figure 4.8 Graphical summary. Proposed mechanism by which IL-1β binds to IL-1r located on LC neurons to enhance their activation and subsequent production of NE in response to stress (black arrows) versus to the ability of IL-1ra to prevent IL-1β from binding, thus resulting in an attenuated stress response (pink arrows). IL-1β=interleukin-1 β, IL-1r=interleukin-1 receptor, IL-1ra=interleukin-1 receptor antagonist, LC=locus coeruleus, NE=norepinephrine

CHAPTER FIVE

CONCLUSIONS

5.1 Mechanisms by which Estrogen may Facilitate Greater Stress Reactivity

Although decades of research have supported the female sex as being a risk factor for development of stress-related psychiatric disorders, a majority of the research conducted on stress and subsequent psychopathological changes has been conducted in males. While there are certainly many similarities and considerable findings that can be taken from male-dominated studies and applied to females, there are numerous aspects specific to females that cannot be recapitulated in males. This is not only in reference to prevalence rates of these disorders, but also concerns vast differences in mechanisms of susceptibility, treatment outcomes, and behavioral responses, to name a few. Thus, the present studies offer novel insight into this largely underrepresented population in stress neurobiology research. The associated findings highlight two essential aspects of physiology that put females at this detrimental risk: estrogen and inflammation.

It is common knowledge that women are more likely to develop stress-related disorders like PTSD and MDD than age-matched men, specifically during the reproductive years (Altemus et al., 2014; Barth et al., 2015; Kessler et al., 1993; McLean et al., 2011). Clinical studies support this by evidencing estrogen as the

culprit behind the rise in peripheral markers like cortisol (Elzinga et al., 2003; Hamidovic et al., 2020; Nakajima et al., 2019; Stroud et al., 2011) and central markers like CRF (Bangasser et al., 2010; Bangasser & Wiersielis, 2018; Wiersielis et al., 2016), in addition to behavioral perturbations (Brown et al., 2020; Kelly et al., 2008; Newhouse et al., 2008). However, preclinical studies that investigate the role of estrogens in relation to anxiety-like behaviors frequently oppose these findings, and, instead, report that estrogen serves a behaviorally protective effect (Ghazvini et al., 2021; Marcondes et al., 2001). Most studies that yield this outcome, though, do not employ stress paradigms prior to behavioral testing. In actuality, these studies better measure the effects of estrogen under baseline conditions which is highly valuable in its own right. The studies conducted in chapter two merge these findings by evidencing a dichotomous effect of estrous cycle, such that when rats are tested during the high estrogen phases of the cycle, those with a history of stress exhibit greater anxiety-like behavior, while those that were in the control condition exhibit greater risk-taking and exploratory behaviors. Findings also confirmed that prior stress induces a state of neuronal sensitization in the stress sensitive CeA and LC that led to an increased acoustic startle response, indicative of hypervigilance, and was denoted by elevated cFos expression within these regions. Studies in chapter three built on these findings, highlighting a mechanism by which these effects may be induced via estrogen signaling through its canonical receptor, ERβ. Specifically, these studies revealed that repeated exposure to WS elicits an increase in protein expression of CRF and ERβ in the CeA and that blockade of ERβ in this region prevents latent effects of

WS on promoting anxiety- and depressive-like behaviors. Based on their main functionality as nuclear receptors, it is hypothesized that these behaviors are largely driven by ER-mediated genomic signaling pathways. Under these circumstances, genomic signaling involves E2 binding to ERβ within the cytoplasm, and then ERβ acting as a transcription factor binds to an estrogen response element on the gene of interest, (i.e., *Crf*) (Ni & Nicholson, 2006; Vamvakopoulos & Chrousos, 1993; Wilkenfeld et al., 2018). This enhances transcription and, thus, production of the CRF protein. However, genomic actions are known to occur over a period of multiple hours (Wilkenfeld et al., 2018) which is relatively slow and unlikely to have been totally responsible for the effects observed on D1 burying which occurred one hour following the first local administration of the ERβ antagonist, PHTPP. There is evidence for non-genomic actions of ER, though, that are known to be fast-acting and work in concert with the genomic signaling pathway. One example of this has been shown in the paraventricular nucleus of the hypothalamus in mice via estrogenic regulation of voltage-gated potassium channels on CRF neurons (Hu et al., 2016; Power et al., 2023). ERβ is the main ER subtype in the brain, especially in the hypothalamus where it is known to colocalize with CRF neurons (Power et al., 2023). It is in this region that E2 has been shown to increase CRF neuron excitability by suppressing the M-current which results in an enhanced cellular response to excitatory synaptic input and causes more rapid cell firing (Hu et al., 2016). Overall, in accordance with existing literature, the findings from chapters two and three support a critical role for E2, specifically signaling through ERβ, in facilitating stress-evoked

outcomes that resemble anxiety- and depressive-like phenotypes and contribute to females' heightened rates of stress-related pathologies.

5.2 The Impact of Hormonal Contraceptives on Behavior

With respect to this line of research, it is important to discuss the impact that exogenous administration of sex hormones can have on behavior in the clinical population, especially due to the widespread use of hormonal contraceptives in today's society. As reported in earlier chapters, there is an abundance of clinical literature that reveal a positive association of E2 levels with greater negative affect particularly in women with stress-related psychiatric disorders like PTSD (Pineles et al., 2016). The converse, however, has been found for trauma-exposed women not diagnosed with a clinical disorder, such that low levels of E2 are associated with greater symptoms of PTSD (Wegerer et al., 2014). Furthermore, these findings have been shown to carry over to the population of women that receive hormonal contraceptive treatment. In a study conducted on women reporting to an emergency room for sexual assault, a leading cause of PTSD development especially for women, researchers found that when participants were given emergency contraception higher in synthetic progestogen at the time of the attack, they reported significantly lower rates of PTSD symptoms six months later compared to those who had declined and abstained from contraceptive treatment (Ferree et al., 2012). However, in a study of premenopausal women without a history of psychiatric or medical illness, pharmacologically induced hypogonadism that effectively suppressed naturally cycling E2 elicited impairments akin to

menopause (e.g., hot flashes, loss of sexual interest), though no overt effects on mood were observed (Ben Dor et al., 2013).

These discoveries allude to an effect that is regulated more so by differences in circuitry and neural processing than discrete levels of the hormones in circulation. In support of this, a study conducted on naturally cycling, premenopausal women highlighted differences in regional activation based on endogenous levels of E2 or hormonal contraceptive use during a laboratory-administered stressor involving the viewing of traumatic film clips (Miedl et al., 2018). The authors of this study showed the areas of the brain that were activated in response to the traumatic film clips were different in naturally cycling women versus women taking conventional hormonal contraceptives. Specifically, naturally cycling women during the high E2 phase of the menstrual cycle exhibited greater activation in regions that enhance regulatory prefrontal activity (e.g., ventromedial prefrontal cortex and rostral anterior cingulate cortex), while the regions activated in women taking hormonal contraceptive therapy were those that are commonly hyperactivated in PTSD patients (e.g., dorsal anterior cingulate cortex and insula) (Miedl et al., 2018). Other studies investigating the etiology of and therapeutic options for anxiety and depression that arise from syndromes exclusively associated with ovarian hormones like premenstrual dysphoric disorder and postpartum depression have yielded similar findings that point to a causative role of altered neuronal sensitivity to the fluctuations of these hormones rather than discrete concentration levels (Standeven et al., 2020). Taken together, while the use of progestogen-based hormonal contraceptives may be beneficial in the direct

aftermath of trauma or when dealing with psychiatric disorders centered around drastic changes in the milieu of ovarian hormones, best practices advise personalized strategies that may not always include standard contraceptive use. Overall, more research needs to be done to elucidate critical factors that mediate ovarian hormone-sensitive neurocircuitry in this vulnerable population.

5.3 Targeting Inflammatory Signaling in the Brain as a Therapeutic Avenue

While ovarian hormone signaling certainly plays a pivotal role in women's heightened risk for psychiatric disorders, estrogens, progestogens, and their receptors are not ideal candidates for therapeutic manipulations. However, as mentioned in chapter four, women have inherently elevated immune profiles which further give rise to their heightened risk of developing stress-related psychiatric disorders (Derry et al., 2015) due to the strong interplay between stress and inflammatory cytokines that potentiate each other's effects (Amasi-Hartoonian et al., 2022; Bauer & Teixeira, 2019; Belem da Silva et al., 2017; Beurel et al., 2020; D'Elia et al., 2022; Duma et al., 2010; Fagundes et al., 2013; Kritas et al., 2014; Slavich & Irwin, 2014). Importantly, E2 is known to enhance promoter activity of the regulatory proinflammatory cytokine, IL-1β (Ruh et al., 1998). This may also explain why autoimmune diseases are more common in women due to the pathological nature of excessive levels of IL-1β (Migliorini et al., 2020). Many common autoimmune disorders that are known to occur more frequently in women and that are associated with heightened IL-1β in the periphery and/or central nervous system include multiple sclerosis, rheumatoid arthritis, lupus, and neurodegenerative diseases like Alzheimer's dementia (Derry et al., 2015; Duma

et al., 2010; Migliorini et al., 2020; Rothwell & Luheshi, 2000). However, novel treatments for these and related disorders have recently become available by means of recombinant IL-1ra. IL-1ra is an endogenous antagonist with potent anti-inflammatory effects that prevent and inhibit further damage from heightened inflammatory responses and have been effectively reported to enter the brain in the preclinical (Arakawa et al., 2009; Frank et al., 2012; Greenhalgh et al., 2010; Hsieh et al., 2020; Konsman et al., 2000; Pradillo et al., 2012; Wahab et al., 2015) and clinical (Clark et al., 2008; Galea et al., 2011) settings.

Chapter four of the present studies shows that intra-LC IL-1ra administration attenuates stress-induced neuronal activation as measured by cFos immunoreactivity and also inhibits anxiety-like behavior following repeated WS exposure, thus giving further credence to the volatile relationship between stress and proinflammatory cytokine signaling. Though the exact mechanism by which IL-1r exerts its effects is not known, recent research has identified a neuron-specific isoform of the IL-1r accessory protein that seems to be required for IL-1r signaling in the central nervous system (Huang et al., 2011; Smith et al., 2009). Specifically, the addition of this isoform in IL-1r signaling has been shown to direct IL-1β-mediated calcium influx, thereby eliciting a state of neuronal excitability (Huang et al., 2011). It is possible that the use of IL-1ra in the current studies prevents this action of IL-1β signaling through IL-1r and its accessory protein isoform on LC neurons, thus, attenuating the exacerbated cellular activation induced by prior stress. Further research detailing the unique and beneficial uses

of IL-1ra within the central nervous system is necessary and should be prioritized, especially due to the promising and impactful results obtained in these studies.

5.4 Modulation of Startle by the Central Amygdala and Locus Coeruleus

Arguably, however, the most impactful finding from the present set of studies involves the robust elevations in startle amplitude that resulted as a consequence of repeated WS exposure. This innate reflex and its underlying neural correlates are highly conserved across species, thus, the ASR test provides a translational measure of hypervigilance that is, importantly, capable of being augmented by stress (Albrechet-Souza et al., 2021; Gonzales et al., 2008; Hitchcock et al., 1989; Keen-Rhinehart et al., 2009; Yang et al., 2021). Because of the extremely short latency at which this reflex is enacted following an aversive auditory stimulus (<15 milliseconds) (Ison et al., 1973), the circuitry involved must also be relatively short and contain few synapses. Indeed, scientists have shown that the pathway responsible for directing this reflex is centralized in the brainstem and includes the cochlear root nucleus (CRN), caudal pontine reticular nucleus (PnC), spinal interneurons, and lower motor neurons in the periphery (Davis et al., 1982; Gómez-Nieto et al., 2014; Koch, 1999; Lee et al., 1996; Zheng & Schmid, 2023). While not directly implicated in this pathway, the CeA and LC, as well as other areas, synapse with these regions and can dictate shifts in the startle response as a result.

The CeA exhibits direct efferent projections to the PnC which is suggested to be the principle method by which it affects startle (Davis et al., 1997; Rosen et al., 1991). The majority of studies that investigate the role of the CeA in the ASR conclude that it mediates fear-potentiated startle (i.e., conditioned) versus anxiety-

related startle (i.e., unconditioned) which is said to be governed by the bed nucleus of the stria terminalis (BNST) (Butler et al., 2016; Davis, 1998; Davis et al., 1993; Davis et al., 1997). Additionally, it is suggested that a stressful or threatening stimulus first activates the CeA which elicits the rapid response, and then activates the BNST which is responsible for the sustained response (Walker et al., 2009). The CeA and BNST are both a part of the extended amygdaloid complex and are highly interconnected and similar in terms of cellular morphology and cellular content with both regions containing notable populations of CRF that are involved in the startle response (Davis et al., 1997; Liang et al., 1992; Ventura-Silva et al., 2020). Though there is some discrepancy in the literature regarding these distinct populations of CRF neurons that invariably exhibit sex differences, an elegant study conducted in female rats revealed that overexpression of CRF specifically within the CeA robustly elevated startle amplitude (Keen-Rhinehart et al., 2009). This is in accordance with findings from the present collection of studies indicating an association between CeA-CRF and enhanced WS-induced startle. However, these studies do not explicitly rule out any impact of the BNST on startle and should be further examined with remaining tissue and/or in future studies. In fact, it is likely that the BNST is involved. Studies have shown that the CeA sends efferent CRF projections to the BNST and that CRF-1 receptors (CRF-r1) within this region may serve as the primary source of CRF-mediated behavioral outcomes in response to stress (Jasnow et al., 2004; Walker et al., 2009).

Deeper investigation into the involvement of region-specific CRF signaling, specifically via CRF-1 receptors, is warranted since this aspect of startle has received even less attention (Flandreau et al., 2015; Gresack & Risbrough, 2011). The LC is another critical region that has been shown to affect startle possibly due to its abundance of CRF-r1 (Bangasser et al., 2010), direct projections onto CRNs (Gómez-Nieto et al., 2008; Hormigo et al., 2017), and control over arousal state (Yang et al., 2021). In support of this, a clinical study found that participants with PTSD exhibited greater eye-blink reflexes in response to acoustic startle that was associated with greater LC activity (Naegeli et al., 2018). Thus, results from chapter two that highlight an association between elevated LC neuronal activity and heightened startle amplitude were unsurprising. Other preclinical studies support this effect of the LC on startle outcomes (Gresack & Risbrough, 2011; Sabban et al., 2018; Yang et al., 2021); however, research has also shown that its involvement may be limited because lesioning of the LC does not abolish this response (Davis et al., 1977; Hormigo et al., 2017). Interestingly, though, the LC is involved in regulating another aspect of the startle response, pre-pulse inhibition, in which a weaker, softer stimulus that precedes a louder, aversive stimulus attenuates the rise in startle amplitude (Alsene et al., 2011). This component of the startle response, additionally referred to as sensorimotor gating, is often dysregulated in patients with common psychiatric disorders like schizophrenia and obsessive compulsive disorder, though there is a paucity of research on the prevalence of this symptom in other disorders including PTSD (Kohl et al., 2013). With substantial evidence implicating this region in anxiety disorders like PTSD, it

is imperative to investigate how LC-mediated sensorimotor gating impairments may be involved.

5.5 Overview of Findings and Future Directions

In summary, the present studies highlight signaling pathways of E2 and IL-1r in the CeA and LC as key components that amplify and perpetuate the negative effects of stress. Furthermore, because of the sexual dimorphism within these specific regions, the mechanisms by which E2 and IL-1r confer their effects plausibly contribute to the higher rates of stress-induced pathologies that exist in females. Important future avenues of investigation include delineating specific components of neurocircuitry that confer maladaptive responses via ovarian hormone signaling, investigating the validity of IL-1ra as a therapeutic treatment for stress-related psychiatric disorders, and detailing differences in regulatory capacities of CRF-containing neuron populations within the CeA and BNST on the stress-evoked startle response in addition to the impact of LC-NE on startle and sensorimotor gating in the context of PTSD.

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