EFFECTS OF EARLY SOCIAL ISOLATION AND ADOLESCENT SINGLE PROLONGED STRESS ON ANXIETY-LIKE BEHAVIORS AND ETHANOL SELF-ADMINISTRATION IN FEMALE RATS

BY

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A Thesis Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

Neuroscience

May 2022

Winston-Salem, NC

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ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Jeffrey Weiner, who has been a knowledgeable, enthusiastic, and supportive mentor. I am deeply thankful for his guidance and encouragement throughout the course of this project. I would also like to thank my committee members, Dr. Sara Jones and Dr. Brian McCool. I sincerely appreciate your time, expertise, and perspective on my scientific work thus far.

I would like to express my gratitude for Hannah Carlson and Dr. Eva Bach for their mentorship and immeasurable friendship throughout my past two years in the Weiner lab. I would also like to thank Deepthi Thumuluri and Gray Kinnier for their unwavering support, encouragement, and kindness throughout the most rewarding and most difficult aspects of graduate school. I want to extend many thanks to Olivia Ortelli for her assistance with neurobiological assays and histology, as well as her thoughtful suggestions and comments as this project came to a close. Further, I would like to thank Christina Dyson, Ann Chappell, and Caitlin Clarke for their knowledge and assistance with behavioral paradigms.

I want to extend my appreciation and love for my parents, Gary and Carolyn Pitcairn, and my siblings, Glenn Pitcairn, Claire Pitcairn, and Paige Pitcairn for always supporting my academic and personal endeavors. Lastly, I want to express my profound gratitude to my grandfather, Floyd Lacy Riddle Jr., M.Sc., who has been both a constant source of encouragement and my greatest scientific inspiration.
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<tr>
<td>AUD</td>
<td>alcohol use disorder</td>
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<td>BLA</td>
<td>basolateral amygdala</td>
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<td>CORT</td>
<td>corticosterone</td>
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<td>CRH</td>
<td>corticotropin releasing hormone</td>
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<tr>
<td>DSM-5</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, 5th edition</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ELS</td>
<td>early life stress</td>
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<td>EPM</td>
<td>elevated plus-maze</td>
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<td>EtOH</td>
<td>ethanol</td>
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<td>GH</td>
<td>group housing/group housed</td>
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<td>HPA axis</td>
<td>hypothalamic-pituitary-adrenal axis</td>
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<td>NSF</td>
<td>novelty-suppressed feeding</td>
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<tr>
<td>OFT</td>
<td>open field test</td>
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<tr>
<td>PND</td>
<td>postnatal day</td>
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<td>PTSD</td>
<td>post-traumatic stress disorder</td>
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<td>SAT</td>
<td>successive alleys test</td>
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<tr>
<td>SI</td>
<td>social isolation/socially isolated</td>
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<td>SPS</td>
<td>single prolonged stress</td>
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<tr>
<td>SSRIs</td>
<td>selective serotonin reuptake inhibitors</td>
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<td>vHC</td>
<td>ventral hippocampus</td>
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ABSTRACT

Early life stress is a major risk factor for alcohol use disorder (AUD) and post-traumatic stress disorder (PTSD). Women are twice as likely to be diagnosed with PTSD, but preclinical studies largely focus on males. Our lab has established a rodent social isolation (SI) model that engenders behaviors associated with heightened vulnerability to AUD and PTSD. However, these phenotypes only develop in male rats. Given that neural development is accelerated in females, we tested the hypothesis that there are sex differences in the developmental vulnerability window to SI. Female Long-Evans rats (n=16/gp) were either single-housed or group-housed for 1 week followed by behavioral testing. We next examined whether SI increased vulnerability to adolescent single prolonged stress (SPS), a rodent model of PTSD. Animals were then tested on a novelty-suppressed feeding (NSF) assay followed by fear conditioning and ethanol (EtOH) self-administration. Prior to SPS, SI engendered increased anxiety-like behavior across assays. There was a strong interaction between SI and SPS exposure during NSF with some group differences observed in fear conditioning. No group differences were seen with EtOH self-administration. These findings demonstrate that there may be significant differences in the vulnerability to early life stress, and multiple stressors may interact to exacerbate anxiety-like behaviors. These advances will be leveraged in future studies to better understand the neurobiological substrates underlying behaviors that develop following early life stress.
INTRODUCTION

1. Comorbidity between post-traumatic stress disorder and alcohol use disorder

Alcohol use disorder (AUD) and post-traumatic stress disorder (PTSD) are chronic psychiatric conditions that are highly comorbid in the human population (Debell et al. 2014, Carlson & Weiner 2021). The frequent comorbidity of AUD/PTSD complicates treatment approaches, often resulting in less favorable outcomes than either disorder in isolation; therefore, the comorbidity represents a uniquely debilitating condition (Flanagan et al. 2018, Gilpin & Weiner 2018). In the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), AUD is classified as a single disorder that can be described as mild, moderate, or severe based on the number of criteria that are met (5th ed.; DSM–5; American Psychiatric Association, 2013). Alternatively, PTSD symptoms are categorized in four main clusters: re-experiencing, avoidant symptoms, negative alterations in mood or cognition, and alterations in arousal and reactivity (DSM-5). Both disorders are associated with negative affective states, a decrease in social/recreational activities, and sleep disruptions, suggesting possible overlapping mechanisms or neural circuitry that may underlie behavioral and physiological changes observed in these two pathologies (Carlson & Weiner 2021).

Given the close association of both disorders, one important consideration is the temporal progression of comorbid PTSD/AUD. One theory postulates that individuals with PTSD use alcohol to “self-medicate” or relieve negative feelings associated with stress, causing them to later develop AUD (Khantzian 1997). An alternative theory
proposed by Smith and colleagues suggests that individuals with AUD or other substance use disorders (SUD) may be exposed to environmental circumstances that may make them more likely to experience traumatic events (Smith and Cottler 2018, Carlson & Weiner 2021). Recent studies of the human population have suggested that PTSD typically precedes the onset of AUD, however the temporal progression of PTSD/AUD comorbidity is still debated (Gilpin & Weiner 2017, Smith & Cottler 2018). Additionally, this progression may be affected by factors such as age of drinking onset, biological sex, and nature and duration of the stressor (Lee et al. 2018).

2. Sex differences in PTSD/AUD comorbidity

One critical consideration of PTSD/AUD comorbidity is sex differences. Epidemiological research repeatedly shows that women are about twice as likely as men to meet diagnostic criteria for PTSD according to the DSM-5 classification, even after controlling for differences in trauma type (Breslau et al. 1997, McLean et al. 2011, Christensen 2013, Kilpatrick et al. 2013). Additionally, women with PTSD are at a greater risk for developing AUD compared to women without PTSD. One study found that the prevalence of alcohol abuse/dependence in women with PTSD was 27.9% compared to 13.5% in women without PTSD, although it should be noted that this was analyzed using DSM-3 criteria for both PTSD and AUD (Kessler et al. 1995). Even without the consideration of PTSD, recent evidence has shown higher binge and heavy drinking and increased AUD prevalence in women compared to men (Guinle & Sinha 2020). Though robust in human epidemiological studies, this pattern of sex differences
has not been reflected in preclinical literature, possibly due to the predominant use of male rodents.

3. Early life stress as a risk factor

The stress response includes physiological, behavioral, and hormonal changes that occur in response to a perceived threat (McEwen 2007). Even acute stressors can alter cognition, mood, behavior, and physiology in both adaptive and maladaptive ways. In the adaptive sense, the stress response acts a powerful signal of avoidance, indicating via hormonal regulation that a threat is imminent and promoting immediate action (Daviu et al. 2019). However, when a threat is no longer present, the response should be terminated. If it does not, lingering changes could lead to chronic dysregulation of the stress response, potentially resulting in long term psychiatric conditions (McEwen et al. 2015, Smith & Pollack 2020). Additionally, physiological and neurological changes can occur through neuroendocrine, autonomic, immune and metabolic mechanisms which can interact and complicate understanding of the maladaptive stress responses and related psychiatric conditions (McEwen et al. 2015).

Importantly, exposure to stress during childhood and adolescence is a major risk factor for comorbid AUD/PTSD and other neuropsychiatric conditions (Dube et al. 2002, Copeland et al. 2007, Müller et al. 2015, Schneider et al. 2013, Baker et al. 2014, Gilpin & Weiner 2017, Lee et al. 2018, Smith & Pollock 2020, Carlson & Weiner 2021). In the human population, early life stress can involve both acute and chronic stressors, including physical or sexual abuse, neglect, violence, witnessing violence and/or death, war,
terrorism, and natural disasters, among others (Copeland et al. 2007, Lee et al. 2018, Smith & Pollack 2020). During critical developmental periods of the nervous system, these experiences can lead to altered stress responding that has been proposed to play a role in stress- and trauma-related disorders, such as PTSD (Lee et al. 2018).

From a prognostic standpoint, these events are additive, such that exposure to multiple traumatic events seems to be strongly associated with the likelihood of developing PTSD and comorbid affective disorders (Khoury et al. 2010, Copeland et al. 2007). A recent study looking at sex differences and the effect of multiple traumatic experiences in civilian post-conflict samples from Northern Uganda, Rwanda, Syria, and Sri Lanka found that the cumulative effect of multiple traumatic experiences was a stronger predictor of PTSD risk than sex (Wilker et al. 2021). In childhood, multiple traumatic events may be even more detrimental. One longitudinal study by Copeland and colleagues followed a representative sample of children ages 9-13 and conducted regular interviews until age 16 in order to conduct a prospective examination of potential trauma and childhood PTSD diagnoses. Interestingly, they found that exposure to potentially traumatic events is fairly common in children and rarely results in DSM-5 childhood PTSD symptoms, except after multiple traumatic experiences or a history of anxiety (Copeland et al. 2007). Childhood PTSD, although distinct from adult PTSD, includes similar symptoms related to re-experiencing, avoidance, and cognitive and behavioral alterations (DSM-5). Despite similarity in symptom clusters, some symptoms may manifest differently due to developmental differences between children and adults; for example, children may express re-experiencing trauma through play behavior (DSM-5).
Additionally, hypervigilance and exaggerated startle response are characteristic of PTSD in both children and adults (DSM-5).

Given that development is a period marked by constant neurobiological changes, many researchers and clinicians have proposed that the developing brain may be more sensitive to stress-induced disruptions (Schneider et al. 2013, see Lee et al. 2018 for review). Children who have experienced stress during early life show lower amygdala gray matter volumes that are associated with both magnitude of cumulative stress as well as frequency of behavioral problems (Hanson et al. 2015, see Gilpin & Weiner 2017 for review). Complicating this further, sex differences are also present throughout development, notably during puberty. Specifically, stress exposure during puberty has been shown to have stronger proximal effects on females than males, including increased risk of developing mood and stress related disorders such as depression, anxiety and PTSD (Hodes & Epperson 2019). Neuroimaging studies of human adolescents have indicated that many brain regions show sex differences throughout development, including the hippocampus, neocortex, amygdala, and many others (for detailed review, see Hodes & Epperson 2019). Given the complex, dynamic factors that can affect the development of psychiatric conditions, there is a tremendous need to elucidate the interaction of variables including biological sex, type and duration of stressor, cumulative amount of stressful experiences, as well as potential biological and genetic predispositions for stress response dysfunction.
4. Preclinical Rodent Studies

Preclinical studies using rodents are particularly useful for investigating how the nervous system responds to acute and chronic stress. Although animal studies cannot account for psychosocial or cultural aspects of sex and gender, or how these aspects may affect responses to stressful experiences, they are nonetheless well-suited to probe the biological and physiological response to stress. Rodent stress paradigms have been extensively reviewed for their biological, behavioral, and peripheral alterations and will not be reviewed in-depth here (see Ganella & Kim 2014, Bali et al. 2015, Deslauriers et al. 2018 for review). Instead, this section will emphasize stress paradigms used in the present experiments as well as common behavioral assays that are used to examine affective behavior in rodents.

a. Social isolation (SI) stress

In preclinical studies, many different paradigms have been used in order to probe the behavioral and neuronal changes associated with early life stress (ELS). One common model involves socially isolating rats during early life, given that rats are naturally social and live in large groups (Gilpin & Weiner 2017). Our lab, among others, has demonstrated various behavioral alterations following ELS. In previous studies, we have employed an SI model where a subset of rats is separated on PND 28 and remain socially isolated for 6 weeks, while the other rats remain group housed (GH). In male rats, this model leads to the emergence of a wide range of behavioral risk factors for AUD,
including increases in anxiety-like behaviors, locomotor activity, and alcohol self-administration (Chappell et al. 2013). Many other studies have supported these findings, showing that SI in rats can result in behavioral and physiological disturbances including hyperlocomotion, anxiety-like behavior, aggression, cognitive alterations, neuroendocrine changes and increased alcohol consumption (Hall 1998, McCool and Chappell 2009, Butler et al. 2014, Robbins 2016, Burke et al. 2017). However, it is important to note that none of these behaviors developed in female rats undergoing same early social isolation paradigm.

b. Single prolonged stress (SPS)

Single prolonged stress (SPS) is a commonly used rodent model of PTSD first described by Liberzon, Krstov and Young in 1997. SPS is a prolonged stressor that occurs in a single day where animals are exposed to three multimodal stressors: 2-hour restraint, followed by a 15-minute forced swim and 10-minute rest period, and concluding with exposure to diethyl ether vapor until loss of consciousness (Liberzon et al. 1997). Following a 7-day period where animals are left undisturbed, SPS elicits behavioral alterations that are reminiscent of PTSD pathology, such as increased anxiety and arousal, increased fear context discrimination, increased fear learning, as well as impaired extinction of conditioned fear, decreased spatial memory, decreased social interaction, and decreased recognition memory, (Souza et al. 2017). SPS also recapitulates neural changes associated with clinical PTSD, such as increased apoptosis in the prefrontal cortex, hippocampus and amygdala (Souza et al. 2017). Additionally,
SPS can induce dysregulation of the HPA axis, downregulate glucocorticoid receptors in the amygdala and hippocampus and increase glucocorticoid receptors in the prefrontal cortex, all of which are seen with clinical PTSD (Souza et al. 2017). Although these neurobiological and behavioral alterations associated with PTSD have been predominantly characterized and validated in male rodents, female rats have been shown to exhibit robust but different neuronal and behavioral changes following SPS, justifying its use as a stress paradigm in females (Pooley et al. 2018).

c. Adolescence in rats

Puberty and adolescence are critical developmental phases that are particularly sensitive to stress in both humans and rodents. However, to study these mechanisms throughout adolescence in humans, it is important to acknowledge the physiology and characteristics of adolescence in animal subjects. As with many other measures in animal studies, “human” adolescence cannot be fully modeled in rodents; instead, studies must carefully consider which aspects of adolescence they want to capture, whether the emphasis is neurobiological, hormonal, behavioral, genetic, or a combination of these approaches.

Depending on the experimental aims, it can also be crucial to distinguish puberty from adolescence. Adolescence overlaps with puberty, but the developmental periods are not equivalent and puberty is much more rigidly defined by the onset of sexual maturity (Spear 2000, Schneider et al. 2013). In rats and humans, female puberty is characterized by cyclic ovarian hormone production, which includes surges of estradiol and
progesterone, while puberty in males is typically associated with fluctuating testosterone production which leads to spermatogenesis (Koss & Frick 2017). Therefore, puberty is typically considered to signal the beginning of adolescence, but adolescence refers to gradual changes that occur in the brain and behavior until adulthood is reached and does not fall into precise time points (Spear 2000, Schneider et al. 2013).

For rats, a common classification has been early adolescence/prepubescence from PND21-34, mid-adolescence from PND34-46, late adolescence from 46-59, and adulthood from PND60+ (McCormick and Green 2013). In this classification, early adolescence/prepubescence begins when animals are typically weaned from their mothers and mid-adolescence encapsulates the pubertal window (McCormick and Green 2013). However, the developmental window between the onset of puberty in female and male rodents are notably distinct. In male rats, puberty is thought to be indicated by balano-preputial separation, which typically occurs around PND38-45 (Schneider et al 2013). For female rats, there is a juvenile period from PND22 to PND 30-32 (Schneider et al 2013). Following this juvenile period is a peri-pubertal period that culminates with the first ovulation, when animals begin freely cycling (Schneider et al 2013). Additionally, vaginal opening occurs between PND32 and PND40 and is another external marker of puberty in female rats (Schneider et al. 2013).

In rodents, adolescence is associated with various neuronal changes, including changes to long range connectivity, extinction-induced plasticity, spine density and synaptic plasticity, as well as inhibitory systems in regions such as the basolateral amygdala and prefrontal cortex (see Gerhard et al. 2021 for review). However, considerably less is known about how stress may impact these neural maturation
processes and relatively few studies have addressed these interactions in females (Gerhard et al. 2021).

d. Validity of rodent behavioral assays

i. Open field test (OFT)

The open field test (OFT) is a behavioral test first used to examine affective behavior in rodents by Calvin S. Hall in 1934. In recent years, it has been commonly used to assess anxiety-like behavior, exploratory behavior, and general locomotion (Bao et al. 2021). The open field is a brightly lit arena enclosed in walls, where the center of the arena is more brightly lit than the margins. It has been observed ethologically that rodents show thigmotaxis, a tendency to remain in the perimeter of a novel environment (Treit & Fundytus 1988, Prut & Belzung 2003). Therefore, a common measure of anxiety-like behavior in rodents is examining the time spent in the center versus time spent in the margins, where animals exhibiting high amounts of anxiety-like behavior would be expected to spend more time in the margins compared to the center. The OFT has been pharmacologically validated as an assay of anxiety-like behavior using classical anxiolytic drugs such as benzodiazepines and serotonin receptor full or partial agonists. However, its validity for other anxiolytic drugs such as selective serotonin reuptake inhibitors (SSRIs) has not been as well-validated and this assay has shown some poor predictive validity for novel anxiolytic drugs (Treit & Fundytus 1988, Prut & Belzung 2002, Haller et al. 2013). The OFT can also be used to assess general locomotor activity
in a novel environment by examining the total distance the rodent travels during the session. Although locomotion in a novel environment is not commonly used as a measure of anxiety-like behavior, hyperactivity in the OFT has been seen with rats undergoing social isolation (Zhang et al. 2011, Chappell et al. 2013, Butler 2014) and may itself be considered a risk factor for addictive disorders (Flagal et al. 2014).

ii. Elevated plus-maze (EPM)

The elevated plus-maze (EPM) is a rodent assay that has been used for decades to assess brain regions and mechanisms underlying anxiety-related behavior and to test anxiolytic properties of novel drugs (Walf & Frye 2007). The EPM was developed based on the ethological observation that rodents prefer dark, enclosed spaces (approach) and tend to avoid bright, open areas (avoidance) (Montgomery & Monkman 1955). By placing the rat in the junction of a bright open arm and a dark enclosed arm, there is a conflict of approach versus avoidance (Garner 2014, Walf & Frye 2007, Montgomery & Monkman 1955). This approach versus avoidance conflict has been labeled “anxiety,” but is different from the pathological anxiety that characterizes disorders like generalized anxiety disorder (Garner 2014). In humans diagnosed with anxiety disorders, a critical aspect of the disorder is that the anxiety is experienced when there is no conflict, making the EPM limited in its translational capability to understand human psychopathologies (Garner et al. 2014). However, the EPM has been validated pharmacologically for many years and has remained the most common assay to examine states of unconditioned anxiety in a rodent and to assess anxiolytic properties of novel drugs.
iii. Successive alleys test (SAT)

The successive alleys test (SAT) is a more recently developed assay of unconditioned anxiety that expands upon the premise of the EPM. The SAT was developed to create a gradient of anxiogenic conditions by including decline in wall height, decline in alley width and increasing brightness (Fig. 1), in contrast to the EPM where changes in wall height, alley width, and brightness are more abrupt (Deacon 2013). The design of the SAT also eliminates the junction that is present in the EPM and therefore eliminates the difficult task of interpreting time spent in this zone (Deacon 2013). An additional consideration of behavior on this assay is the phenomenon of one-trial tolerance. One-trial tolerance relates to the interaction of learning and unconditioned anxiety-like behavior on the EPM (File 1993). When an animal is first placed on the EPM, typically an animal exhibiting anxiety-like behavior will avoid open and bright areas due to the innate avoidance of open areas that may indicate risk of predation. When given an anxiolytic drug, such as a benzodiazepine, the animal will show anxiolytic behavioral phenotypes in the EPM. However, when animals are re-run on the EPM, the benzodiazepine does not elicit this anxiolytic phenotype (File 1993). For this reason, behavioral studies do not typically use the EPM multiple times in the same animal. Due to the inability to re-run rats on the EPM, using both the EPM and SAT in the same experiments allows similar measures of unconditioned anxiety to be assessed in two distinct novel environments. Although more studies are required to determine validity and sensitivity of this modified assay, it provides another measure of unconditioned
anxiety-like behavior and allows animals to be tested even if they have already been run on the EPM.

Figure 1: Schematic of successive alleys test. The successive alleys test creates a gradient of anxiogenic conditions. The first zone is enclosed and dark in color. Each successive alley becomes brighter in color, narrower, and has decreased wall height.

iv. Novelty-suppressed feeding (NSF)

Novelty-suppressed feeding (NSF) is a test that is used to examine negative affect in rodents, although it is used as both an assay of anxiety-like (De Gregorio et al. 2019, Kauffman et al. 2020), and depressive-like (Brachman et al. 2016, Ramaker 2017) behavior in preclinical rodent studies. The test is a modified version of the OFT and consists of a similar brightly lit arena enclosed within walls. However, for NSF, food is placed in the center of the arena. The placement of food in the center of the arena creates a more motivating reason for the animal to approach the typically-avoided region of the center. Additionally, animals are often food deprived prior to NSF, creating increased drive to seek food at the cost of being in an anxiogenic environment (De Oliveira Sergio et al. 2021). Studies typically use measures such as latency to approach food, latency to consume the food, as well as amount of food consumed to assess negative affect. Additionally, this assay may show sex-specific effects. One recent study showed that female Wistar rats showed increased anxiety-like behavior compared to males and that
administration of a benzodiazepine altered these behaviors in females but not males (De Oliveira Sergio et al. 2021).

v. Fear conditioning

Fear conditioning is a reliable paradigm that has been prominently used in the study of behavioral, neurobiological and neuroendocrine responsivity to stress, particularly related to learning and memory of stressful experiences. This paradigm involves Pavlovian conditioning, creating an association between either a context or cue and a stress response. In contextual fear conditioning, the noxious stimulus (e.g. mild footshock) is paired with a context on the first day of conditioning, and this context often includes distinct odors, wall color, floor texture, and/or lighting conditions (Ferland-Beckham 2021). On day 2, the animal is re-exposed to the context without the shock to assess extinction of the conditioned fear. Day 3 is typically identical to day 2, but the goal of the third day is to examine if extinction memory or fear memory persists even if another day has passed (Ferland-Beckham 2021).

For cued fear conditioning, the first day includes pairing a neural stimulus, such as a tone cue or a light cue (conditioned stimulus), to a noxious stimulus such as a mild footshock (unconditioned stimulus) by presenting both stimuli together repeatedly. Day 2 and day 3 are similar to what was described in contextual fear conditioning, where the tone is played without the shock in order to assess extinction. Animals are typically assessed for behavior associated with fear or startle, such as freezing or darting. However, one critical consideration for analyzing behavior during this assay is reliance
on locomotor measures, given that males and females can exhibit different coping strategies (e.g. freezing vs. darting) in response to the stressor (Colom-Lapetina et al. 2019). The ability to assess fear extinction is the primary translational strength of this assay, as extinction deficits in rodents parallels the extinction deficits seen with clinical PTSD (VanElzakker et al. 2014).

5. Neurobiology and potential mechanisms

Human functional imaging studies have observed differences in connectivity between a number of brain regions involved in the stress response of both healthy individuals and PTSD patients, including the ventromedial prefrontal cortex, locus coeruleus, hypothalamus, hippocampus, amygdala, and others (Fenster et al. 2018, Daviu et al. 2019). Many of these brain regions have also been shown to be sensitive to alcohol (Vilpouxi et al. 2009, Squeglia et al. 2014). However, less is known about the development of these regions in rodent stress models throughout development and even less is known about potential differences in females. Despite the limited studies in female rodents, and even more limited understanding of dynamic changes throughout female development, brain regions sensitive to stress and alcohol will be briefly discussed.

a. Hippocampus

The hippocampus is part of the limbic circuitry and plays a role in learning and memory as well as spatial navigation (Jarrard et al. 1993). In addition to these well-
established functions, the hippocampus has been shown to be sensitive to stress, particularly the ventral hippocampus (vHC) (Kim et al. 2015, Premachandran et al. 2020). The vHC receives strong excitatory input from the basolateral amygdala (BLA) and the BLA-vHC projection has been shown to modulate anxiety-like behaviors in a bidirectional manner (Felix-Ortiz 2013). A recent study from our lab examined the effect of alcohol on the BLA-vHC circuit using ex vivo electrophysiology and found that withdrawal from chronic alcohol, which increases anxiety-like behaviors, significantly increases excitability in this pathway (Bach et al. 2021). In addition to changes in neuronal circuitry, the hippocampus shows morphological changes throughout development in rats. One study examining young female rats found that PND21 female rats have a greater number of dendritic segments on dentate gyrus granule cells than male counterparts, a difference that disappeared by PND60 (Juraska 1990, see Premachandran et al. 2020 for detailed review). Additionally, there are sex differences in GABAergic signaling in the hippocampus. During early development, a switch from GABA$_A$-mediated excitation to inhibition occurs in a wide array of brain structures, including the hippocampus (Premachandran et al. 2020). In female rats, the switch from GABA$_A$-mediated excitation to inhibition in CA1 and CA3 pyramidal neurons occurs earlier in females than males, which suggests that males may have a longer window of GABA$_A$-mediated excitation compared to females (Premachandran et al. 2020). Although this switch occurs earlier in development than the developmental window used in the present study, it may suggest that slight shifts in experimental protocol may differently affect neural circuitry in males and females that may be the same chronological age but are at different time points in neural development.
b. Basolateral amygdala (BLA)

The basolateral amygdala (BLA) is a group of amygdala nuclei that contains the lateral nucleus, the basal nucleus and basomedial nucleus and has been implicated in emotional valence encoding (Sah et al. 2003, for review see Daviu et al. 2019). Emotional valence refers to the subjective value assigned to a sensory stimulus, typically either positive (leading to approach or consummatory behaviors) or negative (leading to avoidance behaviors) (Daviu et al. 2019). Although assigning emotional valence to a stimulus can be adaptive to an individual in its environment, emotional valence encoding has been implicated in anxiety and stress disorders (Daviu et al. 2019). Regarding ethanol (EtOH) exposure, the BLA has also been implicated in human and animal studies. In one human neuroimaging study, males (but not females) with alcohol dependence had dose dependent smaller gray matter volumes of the BLA nucleus (Grace et al. 2021).

c. Hypothalamic-pituitary-adrenal (HPA) axis and corticosterone (CORT)

The hypothalamic-pituitary-adrenal axis (HPA axis) is a critical aspect of the brain stress response. During exposure to stress, the HPA axis becomes active and begins to release several hormones, including corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). ACTH stimulates the synthesis and secretion of glucocorticoids by the adrenal glands, which go on to target brain regions such as the hypothalamus, hippocampus, and PFC (Baker et al. 2014). The HPA axis terminates the stress response through negative feedback, which reduces the production and release of
CRH and ACTH (Baker et al. 2014). This mechanism of stress responding is well-understood in adult humans; However, the manner in which the HPA axis is involved in PTSD/AUD or its potentially sex-dependent changes throughout development are not as well-characterized. In humans, the role of HPA axis functioning in the development and maintenance of PTSD has been extensively studied, yet results remain inconsistent (Dunlop & Wong 2019).

In rats, studies have shown that basal levels of ACTH and corticosterone (CORT), a major glucocorticoid in rats, are comparable between adolescent and adult rats when they are not exposed to stress (for review, see Baker et al. 2014). However, when rats are acutely stressed, released ACTH and CORT is higher in adolescent rats compared to adults. Additionally, adolescent rats show a much slower return to baseline levels of stress hormones (Baker et al. 2014). When animals are chronically exposed to the same stressor, the HPA-axis response in adult rats habituates, whereas adolescent rats continue to exhibit robust release of ACTH and corticosterone (Baker et al. 2014). However, conflicting findings have been observed in the literature. One study found that male and female rats exposed to a juvenile stress paradigm, and then challenged in either juvenility or adulthood, showed comparable corticosterone levels, emphasizing the notion that CORT is merely one aspect of the stress response and may not be directly proportional to stress levels (Jacobson-Pick & Richter-Levin 2010). Although HPA axis functioning and CORT levels may change across development or be affected by levels of other circulating hormones (e.g. sex hormones), it provides one peripheral measure that can be assessed repeatedly in vivo and allow researcher to further characterize sex differences in anxiety-like behavior across development.
6. Novel early social isolation paradigm and current study

Rodents have been useful in providing information about the neural substrates linking early life stress and behavioral alterations related to anxiety. However, much of this work has focused exclusively on male subjects. As a result, much less is known how early life stress impacts neural circuitry related to anxiety-like behaviors in females. To begin to address this discrepancy, the current study was designed to examine how stress during development may drive anxiety-like behaviors and ethanol consumption in female rats. This study used two of the previously discussed paradigms, chronic social isolation stress and single prolonged stress, at different developmental time points. Prior studies from our lab used a social isolation paradigm where rats arrive on PND21 and are separated into SI/GH housing one week after arrival for a six week housing manipulation. In male rats, this model leads to the emergence of a wide range of behavioral risk factors for AUD, including increases in anxiety-like behaviors, locomotor activity, and alcohol self-administration. Interestingly, none of these behaviors developed in females rats following this social isolation paradigm. Because many neurodevelopmental changes occur earlier in females (Premachandran et al. 2020), the present study used a novel early social isolation paradigm where female rats arrived on PND21 and were immediately separated into group-housed or single housed conditions rather than including a week of acclimation, such that animals were isolated one week earlier than our previous social isolation paradigm. This study sought to test the hypothesis that females have an earlier developmental vulnerability window to social isolation and that social isolation may
interact with single prolonged stress to modulate various anxiety-like behaviors and ethanol drinking.
METHODS

Animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Wake Forest University Institutional Animal Care and Use Committee. 32 female Long-Evans rats (Envigo, Indianapolis IN) arrived for two separate experiments with 16 animals in each experiment. The design of the experiments was identical except for the drinking procedures. All animals arrived on PND21 and were randomly separated into group housed (n=8, 4 rats/cage) or socially isolated conditions (n=8) on the day of arrival. Subjects were housed in standard polypropylene cages and given ad libitum access to standard rodent chow and water. Rats were maintained on a 12-hour reverse light/dark cycle with lights off at 9:00 AM. Animals were handled once weekly for weighing and marking. When drinking procedures began, animals were weighed daily to ensure accurate measures of alcohol consumption (g/kg).

Behavioral assays

All behavioral procedures began at 10:00AM, one hour into the active cycle. Animals were brought into the testing room 20 minutes prior to each behavioral assay to allow for acclimation. Housing condition groups were counterbalanced during each assay to account for potential order effects.

Open field test (OFT)

Subjects were run in the open field test (OFT) on PND30, nine days after assignment to housing conditions. Rats were placed in a brightly lit standard locomotor
activity chamber (42 cm × 42 cm × 30 cm) for a duration of 30 minutes to assess locomotor activity (Digiscan animal activity monitors, Omnitech, Columbus, OH, USA). General locomotor activity was measured by the total distance traveled. Time spent in the center of the arena was examined, but no group differences were seen. The reason for this lack of an observed effect is possibly due to the size of the Omnitech chambers in our lab, which may not be large enough to capture center versus margin time with rats.

_Elevated plus maze (EPM)_

On PND32, rats were placed in a standard EPM (Med Associates, St. Albans, VT) consisting of two closed arms and two open arms (each arm 10.2 cm wide by 50.8 cm long). Rats were placed in the junction facing the open arm and recorded in the assay for 5 minutes. In this assay, the time spent in open arms and number of entries into open arms were used as measures of anxiety-like behavior. Number of entries into closed arms was used as a measure of general locomotor activity.

_Successive alleys test (SAT)_

On PND36, rats underwent the successive alleys test (SAT) as an additional assay of anxiety-like behavior. The SAT consists of four segments, each of which are 45 cm in length. The first segment is enclosed and black in color (9.0 cm width, walls 29.0 cm height), the second segment is dark gray (9.0 cm in width, walls 2.5 cm height), the third segment is light gray (6.7 cm width, walls 0.5 cm height), and the final segment is white (3.5 cm width, contains walls 0.3 cm in height). In this assay, locomotor activity was measured by recording the total distance traveled. Anxiety-like behavior was assessed using time spent in each zone, number of entries into the lighter zones (dark gray, light
gray, and white), and latency to enter the lighter zones. Rats were recorded in this assay for 5 minutes and were tracked using Ethovision software (Noldus, Leesburg, VA).

*Single prolonged stress (SPS)*

Following initial behavioral testing, animals were randomly assigned from prior SI/GH groups and subdivided into SI, SI + SPS, GH, or GH + SPS groups (n=4/group). On PND46, SPS was conducted in a novel testing room with overhead lights off and a red lit lamp. Control animals were placed in a smaller room behind the testing room throughout the duration of SPS with identical environmental conditions. Rats in the +SPS groups were first placed in hard plastic cylindrical restraints for a duration of 2 hours. Following restraint, rats (n = 4 at a time) were placed into a 15 minute forced group swim in a plastic tub (21 in width x 21 in length x 17 in height) filled ⅔ full with 24°C water. Following the forced swim, rats underwent a 10 minute rest period on dry cloth towels. Ether exposure occurred under a fume hood to four animals at a time in a standard cage with a ventilated lid. Cotton balls with diethyl ether (Sigma-Aldrich, St. Louis, MO) were placed in the cage at a rate of 3 mL/min in a plastic barrier that prevented physical contact with the rats. Animals were removed upon loss of consciousness and lack of righting response, placed in a clean housing cage, and monitored for recovery. Animals remained in their initial housing conditions following ether exposure and were returned to their housing room upon return of consciousness for a 1 week undisturbed period.
Novelty-suppressed feeding (NSF)

Novelty-suppressed feeding is an assay that is commonly used to examine negative affect in rodents. In this assay, rodents were placed in the corner of a clear acrylic arena (26.6 in x 34 in). Bright light sources were placed at each of the four corners of the arena and a shortbread cookie (Nabisco, East Hanover NJ) was placed in the center. Animals were recorded in the assay for 10 minutes and video recorded and analyzed using Ethovision software (Noldus, Leesburg, VA). Total distance traveled was used as a measure of general locomotor activity and latency to approach the food was used as a measure of anxiety-like behavior. On a separate day, a shortbread cookie was placed in the home cage and latency to approach was measured. Latency to approach food in the home cage is used to control for differences in general motivation to eat (i.e. hunger) in a non-anxiogenic environment.

Fear conditioning

On PND59, all animals underwent a 3-day fear conditioning paradigm. The first day was an acquisition day. Animals were placed in fear conditioning chambers (42 cm × 42 cm × 30 cm) with an electric grid floor and tone speakers (Omnitech, Columbus, OH, USA). Following a 5-minute habituation period in the chamber, the tone (8000 Hz) was presented for 29.5 s followed by a shock (0.25 mA) for 0.5 s. The acquisition day consisted of 5 episodes of the tone/shock pairing. The second and third days were extinction days, where the tone was presented alone with no shock. Animals were presented with 14 episodes of the tone alone on each day to assess extinction of
conditioned fear. Animals were assessed for time spent freezing and percent of the episode spent freezing to assess acquisition and extinction of conditioned fear.

**Tail blood collection and CORT analysis**

On the first day of fear conditioning, blood was collected from a tail snip prior to fear conditioning and 20 minutes after completion of fear conditioning in order to have a within-subject pre/post comparison. Blood was collected in serum separator tubes and centrifuged for 2 minutes at 10,000 rpm. Serum was pipetted into 1-mL Eppendorf tubes and frozen at -20°C until analysis. Analysis was completed with an enzyme-linked immunosorbent assay (ELISA) corticosterone kit (Arbor Assays, Ann Arbor MI) according to the manufacturer instructions. All blood samples were analyzed in triplicates. Samples were balanced so each group was equally represented in each run. Assay sensitivity was 17.5 pg/mL in the 100 μL format. Intra-assay variability was less than 20%.

**Ethanol self-administration: Experiment 1**

![Figure 2A. Experiment 1 Timeline of Behavioral Tests. GH animals separated on PND61 following completion of fear conditioning.](image)

In the first experiment, drinking began on PND64 (Fig. 2A). Rats were separated into single housing three days prior to the start of drinking procedures to allow formerly
group housed animals to acclimate to housing conditions. EtOH self-administration was assessed in all animals in both experiments using a two-bottle choice paradigm. For the first 8 days, animals had continuous access to EtOH and water in their home cage that was available for 24 hours. Water and ethanol bottles were alternated daily to account for side preference and weighed daily in order to obtain measures of alcohol consumption (g/kg/day). Ethanol concentration was increased over the 8-day period. Rats were given two days 3% EtOH, two days of 5% EtOH, two days of 7% EtOH, and concluding with two days of 10% (v/v). This gradual increase of ethanol concentration was used because a prior experiment with female Long-Evans rats showed an escalation of EtOH consumption with this regimen (Dyson and Weiner 2021, unpublished data). All animals were single housed throughout drinking procedures. Following 1-week of continuous access, animals had intermittent access (T-Th-Sat) with 2 weeks of 10% EtOH and approximately 1 week of 15% EtOH.

*Ethanol self-administration: Experiment 2*

![Figure 2B. Experiment 2 Timeline of Behavioral Tests. Note that all rats in this experiment were separated on PND37 and exposed to 8-day continuous access paradigm from PND37 to PND45.](image)

Following the first experiment, we hypothesized that exposure to alcohol in early life may be important to the development of drinking phenotypes in later life (Spear
2015, Anderson et al. 2016, Becker 2017). In the second experiment, animals underwent the same 8-day continuous access two bottle choice procedure, but animals had EtOH access during the period following the SAT and prior to SPS, from PND37-PND45 (Fig. 2B). Because animals must be single-housed for drinking procedures, animals remained in single housed conditions from PND36 through the completion of the experiment. NSF, fear conditioning, and later drinking procedures occurred as described for experiment 1. Experiment 2 intermittent drinking procedures are ongoing.

Statistical analysis

All statistical analyses were performed in Sigmaplot 14.0 or R. Two-tailed unpaired Student’s t-tests were used to evaluate effects of SI or GH housing condition on behavior in the OFT and EPM and total distance in the SAT. If Shapiro-Wilk normality test failed (P<0.050), Mann-Whitney Rank Sum Test was used. Following SPS, two-way ANOVAs with housing condition (SI/GH) as one factor and SPS exposure (+/-) as the second factor. For fear conditioning, a two-way mixed model repeated measures ANOVA was used to analyze the effect of housing condition and SPS exposure across each episode. For ethanol self-administration, a two-way mixed model repeated measures ANOVA was used to analyze the effect of housing condition and SPS exposure on ethanol consumption across drinking sessions. The minimal level of significance was p<0.05 for all analyses. All data expressed as mean ± SEM.
RESULTS

Early SI leads to increased anxiety-like behavior across different assays

There was a main effect of housing condition on distance traveled in the OFT (Fig. 3A; \(t(30)=3.809, p=0.001\) with SI rats showing increased locomotion compared to GH. In the EPM, there was a strong trend of SI animals to show decreased time in the open arm (Fig. 3C; \(t(30)=-1.828, p=0.07\)). No significant difference was observed between SI and GH animals in the number of entries into the open arm (Fig. 3D; Mann-Whitney U=88.000, \(p=0.130\)). There was no significant difference in the number of entries into the closed arm, which is used as a measure of general locomotor activity (Fig. 3B; \(t(30)=0.462, p=0.648\)). In the SAT, SI animals spent less time in the white zone (Fig. 4A; (Mann-Whitney, \(U=75.000, p<0.03\)) and decreased frequency to white zone (Fig. 4B; Mann-Whitney, \(U=75.000, p<0.03\)). SI animals also showed significantly increased latency to enter the white zone (Fig. 4C; Mann-Whitney \(U=43.00, p<0.001\)). There was no difference between groups on total distance traveled on the SAT (Fig. 4D; \(t(30)=-1.597, p=0.121\)).
Figure 3. Effects of SI on anxiety-like behaviors in the OFT and EPM. (A) SI rats had increased locomotor activity in the OFT compared to GH rats. (B) There was no difference in locomotor activity in the EPM between the groups, measured by number of entries into the closed arm. (C and D) SI rats increased anxiety-like behavior, indicated by less time spent in the open arms and fewer entries into the open arm. ***=p<0.01.
Figure 4. Effect of SI on behavior in the SAT. (A) SI rats spent significantly less time in the white zone of the SAT. (B) SI rats made significantly fewer visits to the white zone. (C) SI rats had increased latency to enter the white zone compared to GH rats. (D) There were no differences between SI and GH rats on total distance traveled during the SAT session. * = p<0.05, ***=p<0.01
SI and SPS show strong synergistic effects on anxiety-like behavior in the NSF test

To examine the effect of stress exposure on latency to approach the food in the NSF, a two-way ANOVA was conducted with housing condition as factor 1 and SPS exposure as factor 2. There was a significant effect of housing condition (Fig. 5A; F(1,26)=10.852, p= 0.003) and a significant effect of SPS (Fig. 5A; (F(1,26)=12.597, p=0.001. There was a strong housing x SPS interaction (Fig. 5A; (F(1,26)=12.597, p=0.002). Two outliers were excluded from the NSF analysis based on an outlier test, but were not excluded in any other analyses. Latency to approach food in the home cage was analyzed as a control. No effect of housing condition (Fig. 5B; (1,27)=2.405, p= 0.133), SPS exposure (Fig. 5B; (F(1,27)=0.274, p=0.605), or interaction effect was observed (Fig. 5B; (F1,27)=1.362, p=0.253) on latency to approach food in the home cage.
Figure 5: Effect of SI and SPS on anxiety-like behavior in the NSF test. (A) Social isolation rats showed significantly higher latency to approach food in the arena than all other groups. (B) No group differences were seen in latency to approach food in the home cage.***=p<0.01.

Housing condition and SPS exposure affect percent freezing during fear conditioning

For fear conditioning, a two-way mixed model repeated measures ANOVA was used to analyze the effect of housing condition and SPS exposure across each episode. On day 1, a significant effect of SPS exposure was observed (Fig. 6A; (F(1,28)=6.125, p=0.02). A significant effect of episode was also observed (Fig. 6A; (F(4,112)=31.11, p<0.001), suggesting that subjects in all groups acquired fear conditioning. On day 2, there was a significant effect of housing condition (Fig. 6C; F(1,28)=5.630, p=0.025) and episode on percent freezing (Fig. 6C; F(7.01, 196.19)=7.233, p<0.001). Additionally, a housing x SPS exposure interaction was observed on percent freezing on day 2 (Fig. 6C; F(1,28)=9.914, p=0.004). On day 3, only a significant effect of housing condition was observed (Fig. 6D; F(1,28)=10.007, p=0.004). CORT results were underpowered and therefore were not formally analyzed.
SI and SPS have no effect on EtOH consumption, regardless of prior EtOH exposure

For ethanol self-administration, a two-way mixed model repeated measures ANOVA was used to analyze the effect of housing condition and SPS exposure on ethanol consumption across drinking sessions. For drinking procedures, experiment 1 animals had no prior exposure to alcohol. No group differences were observed in continuous or intermittent access to EtOH in either experiment. In experiment 2, animals received 8-day continuous access to EtOH prior to SPS. In contrast to findings from three recent experiments in which early SI significantly increased ethanol drinking in females, no group differences were observed in EtOH consumption (or preference, not shown) prior to SPS. Following SPS and the completion of behavioral testing, rats underwent the same 8-day continuous access and intermittent drinking paradigms. As in experiment 1, no group differences were observed.
Figure 6. Housing condition and SPS exposure affect percent freezing during fear conditioning. (A) Group differences were seen in the percent freezing during the tone/shock episodes on day 1 (acquisition day). There was a significant effect of housing condition, SPS exposure, and episode on percent freezing during day 1. (B) Results of serum CORT analysis. Sample size is too small for formal analysis (n=2/group). (C) On day 2, there was a significant effect of housing condition, episode, and a housing x stress interaction on percent freezing. (D) On day 3, only a significant effect of housing condition was observed. * = p<0.05.
Figure 7. Results of experiment 1 ethanol self-administration. Rats in experiment 1 had no prior EtOH exposure and all began continuous access two-bottle choice following the completion of behavioral testing. (A) Housing condition and SPS exposure had no effect on alcohol consumption in an 8-day continuous access paradigm. (B) Housing condition and SPS exposure had no effect on alcohol consumption in an intermittent access paradigm that occurred immediately following the continuous access paradigm. ABS=abstinence.

Figure 8. Results of experiment 2 ethanol self-administration. Rats in experiment 2 had EtOH exposure prior to SPS. Animals underwent 8-day continuous access paradigm from PND37-45 with SPS exposure (+/-) on PND46. Rats underwent the same 8-day continuous drinking paradigm from PND37-PND45, following completion of behavioral testing. (A) Housing condition and SPS exposure had no effect on early alcohol consumption that occurred prior to SPS. (B) Housing condition and SPS exposure had no effect on later alcohol consumption that occurred following SPS.
Early life stress is a major risk factor for both AUD and PTSD, both of which are debilitating psychiatric conditions that are often comorbid and notably prevalent in the human female population. However, most preclinical studies examining the effect of early life stress on alcohol drinking have predominantly used male rodents. Thus, many of the neurobiological and behavioral alterations resulting from early life stress have been predominantly characterized in male animals, leading to a lack of understanding of how early life stress may affect circuitry and behavior in females. Over the past decade, our lab has established a rodent social isolation model that engenders many behaviors associated with heightened vulnerability to AUD and PTSD, including increases in anxiety-like behaviors, deficits in fear extinction, and increased proclivity to consume alcohol in the two-bottle choice paradigm (Skelly et al. 2015) and in an operant paradigm (McCool & Chappell 2009). However, for unknown reasons, these phenotypes only develop in male rats undergoing this paradigm (Butler et al. 2014). Given that females progress more rapidly through neuronal development, the present study sought to determine if there are possible sex differences in the vulnerability window to early SI. Additionally, we sought to examine the effect of combined SI and SPS to examine the potential synergistic effect of multiple stressors on anxiety-like behavior.

The SI model employed in this study is a variation on the model commonly used by our lab. In the present study, female rats were isolated one week earlier than in the previous model. The female rats exposed to this modified early SI paradigm starting on PND21 displayed a variety of behavioral differences from their GH counterparts,
including hyperlocomotion in the OFT and increased anxiety-like behavior in the EPM and the SAT. Females displaying hyperlocomotion in an open field is consistent with previous observations from our lab and others and has been reproduced in multiple cohorts run by different experimenters (Dyson & Weiner 2021 unpublished data, Chappell & Weiner 2021 unpublished data, Gildawie et al. 2021). The results from the EPM are consistent with what has been seen in males that are socially isolated starting on PND28 (Chappell et al. 2013). Taken together, these results support the notion that initiating SI a week earlier in female rodents leads to the emergence of reproducible anxiety-like behavioral phenotypes.

Later in adolescence, a subset of each group was exposed to SPS. There was a strong interaction between SI and SPS exposure in the NSF test. Rats exposed to combined SI/SPS showed strikingly higher latency to approach the food in the NSF arena but not in their home cage, supporting the hypothesis that multiple stressors have a synergistic effect that impacts behavioral responses to stress. This has been observed in other studies that implement multiple stressors on rodents throughout development (Gildawie et al. 2021).

Finally, all animals were run on a 3-day fear conditioning paradigm, during which both cohorts acquired fear conditioning and extinguished conditioned fear. However, the effects of housing condition and SPS exposure on percent freezing are complex on the different days. On day 1, there was a significant effect of housing condition such that GH animals froze more than SI animals. A significant effect of SPS exposure was also observed on day 1 such that GH animals exposed to SPS froze less than GH alone, with no differences seen between SI groups. On day 2, there was a significant effect of
housing condition, episode, and an interaction of housing and SPS exposure on percent freezing. On day 3, only a significant effect of housing condition was observed. The lack of an observed effect in some of the groups may be related to the sensitivity of the assay. Like other rodent assays, fear conditioning was established and characterized using male rodents and therefore does not necessarily fully encapsulate sex differences in “normal” behavior. Rodents are expected to display freezing behavior, but many researchers have argued that darting may be a more accurate measure of the fear response in female rats (Gruene et al. 2015, Shansky et al. 2015). Future directions of this study include using an operant measure of fear responding rather than relying on locomotor measures that often differ in females (Sengupta et al. 2016).

The results from the EtOH self-administration sessions are the least consistent with previous literature and other findings from our lab. The results from this experiment showed no effect of housing condition or SPS exposure on EtOH consumption, regardless of prior EtOH exposure. In contrast, several recent studies from our lab saw a robust escalation of EtOH consumption in female rodents following the same modified SI protocol and using the same continuous and intermittent access EtOH paradigm described in this experiment (Dyson & Weiner 2021 & 2022, unpublished data). Although every effort was made in this study to replicate the experimental conditions used in the prior experiments, rats from the previous experiments began drinking on PND40, while those in the present study began drinking on PND37 in the early exposure experiment (experiment 2). While this is a seemingly minor difference, the rapid and constantly changing nature of rodent development could potentially result in a highly sensitive vulnerability window. Indeed, starting our isolation protocol one week earlier produced
vastly different effects in female rodents. Early in development, animals seem far more sensitive to even slight temporal differences.

Additionally, there is an extensive body of literature that suggests that prior exposure to alcohol is a critical risk factor for escalation of EtOH drinking following stress (Anderson et al. 2016, Spear 2015). However, the present study also reports no effect of either stress condition during post-SPS EtOH consumption in rats that had been previously exposed to EtOH. Males have shown increased preference for EtOH following SPS (Yu et al. 2016) and females have shown increased EtOH consumption following acute restraint (Willie-Billie et al. 2017). Additionally, one study that examined ethanol consumption following SPS in female rats found a positive relationship between anxiety-like behaviors and ethanol intake following SPS (Denny et al. 2021). However, it is important to highlight that this study used adult female rats rather than female rats during early life and adolescence. Though many of the behavioral outcomes of early SI were replicated, we observed no difference in drinking between SI and GH females. This may imply a dissociation between these behavioral phenotypes and the escalation in ethanol consumption previously observed in our lab. It is possible that increased drinking may be related to an increase in the positive reinforcing properties of alcohol, rather than an increase in consumption due to negative reinforcement (i.e. to relieve distress). However, studies examining EtOH drinking in adolescent female rodents following SPS have been limited, so there may be changes induced by SPS that have not been fully elucidated that could affect later drinking. Therefore, future experiments will be necessary to determine the effect of SPS on female EtOH consumption and the potential interaction with additional stressors during different periods of development.
One limitation of the current study is that female rats were not monitored for
estrous cycling. This was chosen in order to keep handling to a minimum and to remain
consistent with previous studies from our lab. However, the estrous cycle has been
reported to play a role in fear conditioning such that females that underwent extinction
during the metestrus phase showed significantly higher freezing during the recall test
compared to males (Milad et al. 2009). Literature on EPM is more mixed. Some studies
report no effect of estrous cycle stage on anxiety-like behavior (Scholl et al. 2019), while
others have shown that rats in proestrus-estrus demonstrated reduced anxiety-like
behavior in the EPM (D’Souza 2017). Estrous cycle does not seem to affect locomotion
in the OFT (Miller et al. 2020). Future studies should include the use of large sample
sizes that could capture behavioral differences related to estrous cycle stage.

Studies examining the effect of stress on behavioral alterations and neural
circuitry in females have been extremely limited. The findings from this study support the
hypothesis that females have an earlier developmental vulnerability window to stressors
such as social isolation, and that social isolation can interact with other stressors to effect
anxiety-like behaviors. The findings from this study demonstrate that a modified SI
paradigm results in reproducible and robust anxiogenic phenotypes in females during
development, therefore providing a model that can be used to assess neurobiological and
circuitry changes associated with these behaviors. The further characterization of
vulnerability periods during adolescence and the underlying neural circuitry involved
provides important avenues to understand the interaction between stressful experiences,
alcohol exposure, biological sex, and other potential risk factors for psychiatric
conditions.
REFERENCES


Guinle, M. I. B., & Sinha, R. (2020). The Role of Stress, Trauma, and Negative Affect in the Development of Alcohol Misuse and Alcohol Use Disorders in Women. *Alcohol Research: Current Reviews, 40*(2), arcr.v40.2.05. https://doi.org/10.35946/arcr.v40.2.05


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EDUCATION

May 2022  M.S. Neuroscience, Wake Forest Graduate School of Arts & Sciences, Winston-Salem, NC

May 2020  B.S. Neuroscience, College of William & Mary, Williamsburg, VA

B.A. Hispanic Studies, College of William & Mary, Williamsburg, VA

ABSTRACTS AND PRESENTATIONS

**Pitcairn SR,** Dyson CD, Weiner JL. Examining the effects of early social isolation and adolescent single prolonged stress on anxiety-like behaviors and alcohol self-administration in adult female rats. Expected presentation at 2022 Research Society on Alcoholism Meeting, Orlando, FL.

**Pitcairn SR,** Weiner JL. Sex differences in common rodent assays for anxiety-like behavior following single exposure to predator odor. Accepted for the 2021 Society for Neuroscience Meeting, virtual due to the Covid-19 pandemic.

**Pitcairn SR,** Weiner JL. Sex differences in anxiety-like behavior in rats following single exposure to predator odor. Presented at the 2021 Wake Forest School of Medicine Biomedical Sciences Research Day Poster Session, virtual due to the Covid-19 pandemic.


**Pitcairn SR,** Burk JA. Effects of nicotine on attention: role of orexin-1 receptors. Accepted for the 2020 William & Mary Undergraduate Research Symposium, canceled due to the Covid-19 pandemic.

Blumenthal SA, **Pitcairn SR,** Salas B, Burk JA. Effects of an orexin-2 receptor agonist on attention following loss of cortical cholinergic inputs. Presented at the 2019 Central Virginia Chapter of Society for Neuroscience Conference, Charlottesville, VA.
SKILLS

- Rodent behavioral assays
- Rodent stress paradigms
- Animal care
- Ethovision
- MED-PC
- Data analysis
- Data visualization
- Advanced statistics
- SPSS
- R
- Sigmaplot
- Tissue preparation
- Immunostaining
- Western blotting
- Enzyme-linked immunoassay (ELISA)
- Science writing
- Public speaking
- Spanish language (fluent)

ACCOMPLISHMENTS

2020 Departmental Honors in Neuroscience, College of William & Mary
2020 Dean’s List, College of William & Mary
2018–2020 Sigma Delta Pi Inductee, National Academic Honor Society for Hispanic Studies, College of William & Mary

COMMUNICATION AND OUTREACH

2022– Friends-In-STEM Mentor to K-12 Students
2021– “The Science Of…” Scientific Blog Contributor
2021– Brain Awareness Council Steering Committee Member
2021 W&M Neuroscience Student Organization Alumni Panelist (Virtual)
2020– Wake Up to Science STEM Outreach Volunteer
2019–2020 William & Mary Neuroscience Program Admissions Representative
2019–2020 William & Mary Neuroscience Student Organization President
2019–2020 WCWM 90.9 Radio Broadcaster
2019 William & Mary Neuroscience Student Organization Outreach Chair
2018 Medical Spanish Translation Drive Participant
PUBLIC-FACING TALKS AND LECTURES*
*ordered by audience age

**Pitcairn SR.** Unhappy Hour: Stress, Anxiety, and the Neuroscience of Alcohol Addiction. Presented at CONgregate Science Fiction Convention, July 10th 2021, Winston-Salem NC.

**Pitcairn SR.** Your Brain and Fear. Presented at W&M Neuroscience Student Organization Outreach Event, October 20th 2019, Williamsburg VA.


PROFESSIONAL AFFILIATIONS

2019–
Society for Neuroscience (SfN), Member

MENTORSHIP/ TRAINING

*Undergraduate students*

2022 Mia Santucci, Wake Forest University
2021 – Matt Blumberg, Wake Forest University
2021 Sarah Bussey, Wake Forest University
2019 – 2020 Bella Salas, College of William & Mary
2019 – 2020 Paige Little, College of William & Mary
2019 – 2020 Mia Peri, College of William & Mary
2019 – 2020 Kaitlyn Maniscalco, College of William & Mary
2019 – 2020 Grace Smith, College of William & Mary