ROLE OF DENTATE GYRUS IN STRESS AND THE RESPONSE TO ANTIDEPRESSANT TREATMENT

By

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ABSTRACT OF THE DISSERTATION

Role of the Dentate Gyrus in Stress and the Response to Antidepressant Treatment

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Despite stress-associated disorders having a higher incidence rate in females, historically preclinical research in rodents mainly utilizes males. Rodent chronic stress paradigms, such as chronic social defeat and chronic corticosterone administration, were mainly designed and validated in males and subsequent attempts to use these paradigms in females has demonstrated sex differences in the behavioral and HPA axis response to these stressors. We evaluated the behavioral and neuroendocrine response to two novel social stress paradigms, social instability stress (SIS) and chronic non-discriminatory social defeat stress (CNSDS). SIS exposes adult mice to unstable same-sex social hierarchies for 7 weeks. The CNSDS model, a modified social defeat protocol, simultaneously introduces male and female C57BL/6J mice into the home cage of resident CD-1 aggressors for 10 daily 5-minute sessions, with CD-1 aggressors attacking males and females indiscriminately. In the CNSDS paradigm, stress resilient (RES) and susceptible (SUS) subpopulations emerge in both sexes, with SUS mice displaying increased negative valence behaviors relative to RES and control mice in both sexes. SUS male and female mice also displayed hypothalamus-pituitary-adrenal (HPA) axis activation following CNSDS exposure. SIS effectively induces negative valence behaviors and HPA axis activation in both

males and females. Additionally, the effects of SIS on negative valence behaviors are reversed by chronic antidepressant treatment with fluoxetine (FLX) in both males and females.

Chronic stress paradigms in rodents also permit the study of antidepressant treatment resistance. Inhibition of mature Dentate Gyrus (DG) granule cells, through both cell autonomous Gi-coupled receptors and the local microcircuitry, may be critical for mounting a behavioral response to antidepressants. The novelty-suppressed feeding (NSF) task allows us to behaviorally assess response status because a bimodal distribution emerges within the stress+FLX groups. Responders to FLX have a shorter latency to eat the pellet within the brightly light center of the NSF arena than non-responders. We observe that non-responders to FLX have more DG activation and less hippocampal neurogenesis. Additionally, DREADD mediated inhibition of the ventral DG results in a decrease in negative valence behaviors. Interestingly, non-responders to FLX are converted into responders following DREADD mediated inhibition of the ventral DG. Results from these projects can further our understanding of the involvement of the DG in response to stress and antidepressant response.

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TABLE OF CONTENTS

Abstract ii-iii

Acknowledgements iv-v

Introduction 1-7

Figure 0.1. Hippocampal 5HT1A receptors expression 8

General Methods 9-14

Chapter 1: ***Social Instability is an Effective Chronic Stress Paradigm for both Male and Female Mice*** 15-38

Chapter 1 Figures 39-46

Figure 1.1: Chronic Corticosterone has Behavioral Effects in Males but not in Females 39-40

Figure 1.2: Behavioral Effects of Social Instability Stress (SIS) in Male and Female Mice 41-42

Figure 1.3:Behavioral Effects of Social Instability Stress (SIS) in Male and Female Mice 43-44

Figure 1.4: SIS Stress Increases Endogenous Corticosterone Levels in Males and Females 45

Figure 1.5: SIS Stress and FLX Effects on Adult Hippocampal Neurogenesis 46

Chapter 1 Supplementary Figures 47-49

Supplemental Figure 1.1 47

Supplemental Figure 1.2 48

Supplemental Figure 1.3 49

Chapter 2: ***Chronic Non-Discriminatory Social Defeat is an Effective Chronic Stress Paradigm for both Male and Female Mice*** 50-71

Chapter 2 Figures 72-79

Figure 2.1. CNSDS paradigm induces attacks to both male and female C57BL/6J mice 72-73

Figure 2.2. CNSDS SUS male and female mice have an increase in negative valence behaviors 74-76

Figure 2.3. Female negative valence behavior is not impacted by estrous cycle stage during behavioral testing 77-78

Figure 2.4. CNSDS increases plasma corticosterone levels 79

Chapter 2 Supplementary Figures 80-82

Supplemental Figure 2.1. SUS males are attacked quicker and more frequently than SUS females. 80

Supplemental Figure 2.2. No differences between CNTRL groups 81

Supplemental Figure 2.3. CNSDS does not affect estrous cycle 82

Chapter 3: ***The effects of fluoxetine on behavior and adult hippocampal neurogenesis in female C57BL/6J mice across the estrous cycle*** 83-100

Chapter 3 Figures 101-105

Figure 3.1. Behavioral differences between FLX- and vehicle-treated female mice are also mediated by estrous phase 101-102

Figure 3.2. FLX increases all stages of neurogenesis with differences most pronounced in the estrus and diestrus phases 103

Figure 3.3. Estrous cycle impacts adult hippocampal neurogenesis in intact, cycling vehicle-treated female mice 104-105

Chapter 3 Supplementary Figures 106-108

Supplemental Figure 3.1. Null finding of FLX and estrous cycle 106

Supplemental Figure 3.2. Distribution of adult hippocampal neurogenesis across each section of the DG 107-108

Chapter 4: ***Inhibition of Dentate Gyrus Underlies Behavioral Response to Fluoxetine in both Female and Male Mice*** 109-138

Chapter 4 Figures 139-144

Figure 4.1. Male and female behavioral responders and non-responders to chronic fluoxetine treatment 139

Figure 4.2. Differences in DG cFos expression between responders and non-responders to fluoxetine treatment 140

Figure 4.3. DREADD mediated inhibition results in a decrease in negative valence behaviors in males and females 141-142

Figure 4.4. Male and female non-responders to fluoxetine are converted into responders via Gi-DREADD inhibition of DG GCs 143

Chapter 4 Supplementary Figures 144-145

Supplemental Figure 4.1. NSF home cage latency to feed 144

Supplemental Figure 4.2. DREADD mediated vDG inhibition or stimulation impacts vDG activity and does not impact control behavioral measures 145

General Discussion 146-149

Figure 5.1. Adult hippocampal neurogenesis data across males and females 150-151

References 152-162

INTRODUCTION

Mood disorders, such as depression, are prevalent forms of mental illness in today's society, with major depressive disorder (MDD) affecting more than 300 million people worldwide (Levinstein & Samuels, 2014; Murray & Lopez, 1996; Smith, 2014). MDD is a ubiquitous illness characterized by diverse etiologies and a partially overlapping symptomology with highly comorbid disorders, such as anxiety, which has a co-occurrence rate with MDD of up to 60% (Gorman, 1996; B. A. Samuels et al., 2015; Yohn, Gergues, & Samuels, 2017). Although the individual risk for depression is 40-50% genetic, no specific gene polymorphisms with high penetrance have been identified as causative factors for developing MDD (Adachi, Barrot, Autry, Theobald, & Monteggia, 2008). Thus, preclinical research investigating neuropsychiatric disorders typically involve stress paradigms, since the occurrence of stressful life events is associated with onset of depression and anxiety in the clinical population (Kendler et al., 1998). Despite women being more likely to receive a diagnosis of depression and/or anxiety than men (Kessler et al., 1994), majority of preclinical and clinical research have been done in men (Beery & Zucker, 2011). Historically, female rodents were excluded from experiments because researchers assume the hormonal variation of estrous cycle leads to variability. However, while hormonal fluctuations may contribute to behavioral differences in females, individual differences in males can also be attributed to fluctuations in male gonadal hormones (Becker et al., 2016; Prendergast et al., 2014; Shansky 2019). In addition, the daily cort cycle may cause behavioral variations in both sexes. Fortunately, the National Institute of Health now mandates the inclusion of both sexes in experiments, which further pushes efforts to extend scientific findings to females regarding the etiology of neuropsychiatric disorders and response to pharmacotherapies.

However, immediately incorporating females into preclinical research involving chronic stress manipulations is challenging since the vast majority of preclinical chronic stress paradigms were designed and developed using male rodents. When females are simply integrated into the well-documented, valid, and reliable male chronic stress paradigms, the stressor does not always produce a measurable stress effect in females. Either modifications must be made to the existing chronic stress protocols or entirely new paradigms need to be developed. For instance, exposure to exogenous corticosterone (CORT) in the drinking water for four weeks produces a robust behavioral stress effect in male mice (David et al., 2009). However, applying the same protocols to females results in inconsistent findings across several behavioral tasks (Mekiri et al., 2017). Similar to CORT, chronic social defeat stress (CSDS) mimics some pathological dimensions of depression such as decreases in sucrose preference (a possible measure of anhedonia), increases in avoidance behavior when exposed to a potentially anxiogenic situation, and increased social avoidance (Tsankova et al., 2006). Several labs have made successful modifications to the CSDS protocol that allow for the incorporation of female mice (Takahashi et al., 2017; Harris et al., 2018), however, these alterations hinder the ability for direct comparisons between the sexes to be made. Measures need to be taken that not only allow for the usage of both sexes in a stress paradigm, but the ability to make between sex assessments. Two chapters in this thesis focus on development of new chronic stress paradigms that are effective in both sexes.

Overview of the Dentate Gyrus and Antidepressants

The dentate gyrus (DG), a subfield of the hippocampus, is an important mediator in regulating affective state as well as the antidepressant response (Kaidanovich-Beilin, et al., 1995; Moser et al., 1995). The hippocampus is susceptible to various stress induced hormones, such as glucocorticoids, and rat adrenalectomy results in the eventual death of DG granule cells (GCs)

(Sloviter et al., 1989; Gould et al., 1990). Human postmortem data reveals that untreated major depressive disorder (MDD) patients have fewer DG GCs than controls and as the number of depressive episodes increase the DG volume decreases in MDD patients (Boldrini et al., 2013; Treadway et al., 2015). Chronic but not acute, treatment with the antidepressant class selective serotonin reuptake inhibitors (SSRIs) stimulates multiple stages of adult hippocampal neurogenesis within the DG (Wang et al., 2008). In the DG, chronic SSRI treatment in males and females results in increased proliferation of dividing neural precursor cells, as well as faster maturation and integration of young adult born granule cells (abGCs) into the DG (Ming et al., 2005; Wang et al., 2008). Although species and strain dependent, researchers found that adult hippocampal neurogenesis is impacted by varying levels of female gonadal hormones across the estrous cycle (Barha et al. 2009; Tanapat et al. 2005; Lagace et al. 2007). However, no studies have investigated whether SSRIs modulate the effect of the estrous cycle on adult hippocampal neurogenesis. Thus, investigation into the relationship between SSRI treatment and estrous cycle phase on adult hippocampal neurogenesis levels is imperative to understand the impact fluctuating hormones have on response to antidepressant treatment.

Today, antidepressants such as SSRIs are amongst the most frequently prescribed drugs in the world and usage continues to increase. From 1999 to 2012 the percentage of Americans on antidepressants increased from 6.8% to 13% (Kantor et al., 2015). However, these commonly prescribed drugs fail to have any effect for many patients and have incomplete effects for many others. Upon initial monotherapy treatment with SSRIs only a subset of depressed patients (33%) attain remission (Trivedi et al., 2006). Adult hippocampal neurogenesis may be essential for antidepressant effects since ablation of the adult hippocampal neurogenic niche via focal radiological strategies in mice results in loss of antidepressant-mediated behaviors (Santarelli et

al., 2003; David et al., 2009; Airan et al., 2007; Yohn et al., 2017). The mature DG GCs are also critical components of the neural circuit mediating the antidepressant response. Recently, Samuels and colleagues (2015) observed that deletion of Gi-coupled Serotonin 1A receptors (5-HT1ARs) on the mature population of DG GCs abolished the behavioral response to SSRIs, while attenuating the effects of SSRIs on adult hippocampal neurogenesis. By contrast, when 5-HT1ARs were deleted from abGCs in the DG, the SSRI effects on neurogenesis and behavior remained intact. Thus, inhibition of DG GCs via Gi-coupled receptors is a critical feature of the antidepressant response. Recent work suggests that mature DG GCs activity may be inhibited by young abGCs acting on the local microcircuitry or through feedback inhibition mechanisms (Samuels et al., 2015; Ikar et al., 2013; Drew et al., 2016; Lacefield et al., 2012). Specifically, young abGCs can evoke strong inhibitory input to mature DG GCs via activation of GABAergic interneurons (Ikar et al., 2013). An improved understanding of how the DG contributes to behavior and of the relationship between young abGCs and mature DG GCs is essential to resolve differences between responders and non-responders to antidepressant treatment.

While the microcircuitry of the DG may be one facet underlying antidepressant response, the ventral or dorsal pole of the DG is also a main contributor. The dorsal DG mainly contributes to learning and memory, while the ventral DG contributes to mood-related behaviors (Fanselow & Dong, 2010). Differences in expression of 5-HT_{1A} receptors exist in the DG, with them densely packed in the ventral DG (Fanselow & Dong, 2010; Kheirbek et al., 2013; Yohn et al., 2017). Seeing as DG 5-HT_{1A} receptors are necessary and sufficient in underlying the behavioral effects of SSRIs, their location in the ventral pole positions them to have a direct influence on limbic circuitry that regulates mood-related behaviors (Yohn et al., 2017) (Figure 0.1). For instance, acute optogenetic inhibition of the ventral DG, but not dorsal, alters anxiety-related

behaviors in mice (Kheirbek et al., 2013). Moreover, Anacker and colleagues (2018) illustrated that increased neurogenesis results in a decrease in ventral DG GC activity, with resilience to CSDS mediated by decreases in ventral DG activity. Therefore, direct inhibition of ventral DG GCs may be important for SSRI-mediated behavioral effects.

Overview of dissertation experiments

As mentioned above, depression places a major burden on today's society since not all depressed individuals experience remission following response to pharmacotherapy. Additionally, preclinical literature encompassing the effects of stress and antidepressant treatment on brain and behavior have mainly been conducted in males. This poses a huge gap in the literature and in our understanding of how females not only behave, but respond to antidepressant treatment.

Therefore, this dissertation will first evaluate effective stress paradigms that allow direct comparisons to be made between sexes. Social instability stress appears to be an effective social stressor in both sexes, however literature has predominantly focused on effects in juveniles as well as no direct comparisons made between the sexes. Adaptation of this protocol to adult male and female C57BL/6J mice were made, permitting sex comparisons (Chapter 1). Modifications to CSDS protocols have been successful in garnering attacks from the resident mouse (CD-1) to the female intruder mouse. However, these adjustments to the protocol can be time consuming for the researcher as they may entail surgical manipulation of the resident mouse (Takahashi et al., 2017) or collection of male urine and application (Harris et al., 2018) to the female mouse to elicit resident-intruder attacks. Given that pheromones can mask the female identity, alterations to the traditional CSDS protocol were conducted that allowed for simultaneous exposure of a male and a female C57BL/6J mouse. This adaption not only permits direct analyses to be made

between sexes, but the ability to collect experimental data from both sexes simultaneously (Chapter 2).

Next, this dissertation will assess the impact of the SSRI, fluoxetine (FLX), on brain and behavior in both sexes. Before assessing response to FLX in female mice, we first needed to evaluate the potential attenuating effect of FLX on changes in adult hippocampal neurogenesis across the estrous cycle (Chapter 3). Behavioral response to antidepressants can be measured using the Novelty Suppressed Feeding (NSF) task as it is only sensitive to chronic antidepressant effects not acute. Additionally, in mice that are exposed to a stressor and then given FLX (STRESS+FLX) a bimodal distribution occurs in the group, such that non-responders to FLX have a longer latency to eat than the behavioral responders to FLX. Inhibitory 5HT1A receptors on DG GCs are necessary and sufficient for a behavioral response to FLX (Samuels et al., 2015). In conjunction research shows that inhibition of the DG, specifically the ventral pole, mediates resilience to stress (Anacker et al., 2018) and anxiolytic behaviors (Kheirbek et al., 2013). Assesment of the antidepressant behaviroal response and underlying DG GC activation s is pertenent in delinating functional distinctions of the DG in both responders and non-responders to FLX during the NSF (Chapter 4). Additionally, in the final chapter of the dissertation further evaluation of the DG contribution to anxiolytic behaviors were made by funcitionally manipulating the ventral DG (vDG) by Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Specifically, investigation into whether DREADD mediated vDG inhibition produces an antidepressant-like behavioral response, while DREADD mediated vDG activation facilates anxiogenic behavioral responses was made (Chapter 4). Lastly, assessing whether behavioral non-responders could be converted into responders via DREADD mediated vDG

inhibition can shed further light on the importance of vDG inhibtion in underlying antidepressant response.

Three of the four chapters in this dissertation have already been published (Chapters 1-3), while the one chapter was recently submitted for publication (Chapter 4). The citation for these publications are listed below and noted at the beginning of each chapter.

Publication history

Chapter 1: Yohn, C. N., Ashamalla, S. A., Bokka, L., Gergues, M. M., Garino, A., & Samuels, B. A. (2019). Social Instability is an Effective Chronic Stress Paradigm for both Male and Female Mice. *Neuropharmacology*, 160, PUID: 107780

Chapter 2: Yohn, C. N.**, Dieterich, A., Bazer, A. S., Maita, I., Giedraitis, M., & Samuels, B. A. (2019). Chronic non-discriminatory social defeat is an effective chronic stress paradigm for both male and female mice. *Neuropsychopharmacology*, *44*(13), 2220-2229. **co-corresponding author

Chapter 3: Yohn, C.N.**, Shiftman, S., Garino, A., Diethorn, E., Leshya Bokka, Sandra A. Ashamalla, Samuels, B.A**. (2020). The effects of fluoxetine on behavior and adult hippocampal neurogenesis in female C57BL/6J mice across the estrous cycle. *Psychopharmacology*, 1-10 (uploaded on *bioRxiv* (2018), 368449). **co-corresponding author

Chapter 4: Yohn, C.N., Dieterich, A., Maita, I., Bazer, A.S., Emma Diethorn, Sandra Ashamalla, Mark M. Gergues, Benjamin A. Samuels (in preparation). Role of the Dentate Gyrus in facilitating antidepressant response in male and female mice.

Introduction Figure

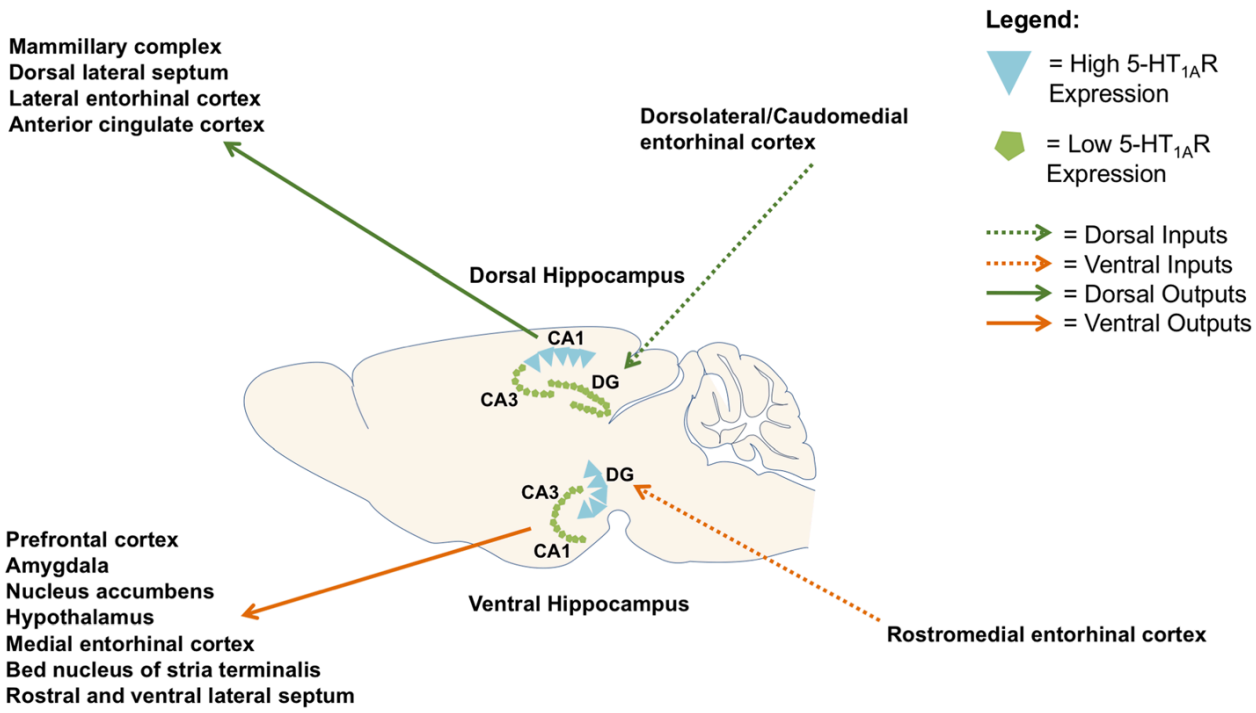


Figure 0.1. Hippocampal 5HT1A receptors expression. The expression of 5-HT1A receptors along the dorsoventral axis of the hippocampus in a rodent brain. 5-HT1AR expression is highest in dorsal CA1 and ventral dentate gyrus. The dorsal and ventral hippocampus participate in distinct circuitry, with the ventral hippocampus projecting to limbic structures. Therefore, 5-HT1ARs on dentate gyrus granule cells are well positioned to exert an influence on mood related behaviors (Yohn et al., 2017)

GENERAL METHODS ACROSS EXPERIMENTS

Mice

All experimental mice were of the C57BL/6J strain, 8 weeks old at the onset of experimentation, and consisted of both sexes. Specific age and sex details are noted for each experiment within the chapters below. All animals were group-housed prior to the beginning of the experiment, with specific housing arrangements specified in each experiment within the chapters. The subjects were housed in a colony room that was maintained on a 12:12-hr light/dark cycle with food and water provided *ad libitum*. All experiments were performed in accordance with protocols approved by the Rutgers University Institutional Animal Care and Use Committee.

FLX Administration

FLX(18 mg/kg/day in deionized water) or vehicle (VEH) (deionized water) were delivered by oral gavage following exposure to the stress paradigm for 3 weeks prior to behavioral testing. The oral gavage of FLX or VEH were continued throughout the behavioral testing and to experimental endpoints. On behavioral testing days, FLX or VEH administrations were conducted after the mice completed the testing in order to avoid any acute effects.

Stress Paradigms

Social Instability Stress (SIS) (Chapter 1&4)

Adult male and female C57BL/6J mice were randomly assigned to either SIS or control (CNTRL). SIS mice experienced unstable social hierarchies where social dynamics were changed every 3 days for 7 weeks [adapted from (Bartolomucci et al., 2004; Schmidt et al., 2010; Schmidt et al., 2007; Sterlemann et al., 2008)]. Social dynamic changes consisted of an individual mouse being introduced to 2-4 novel experimental mice of the same sex from different cages, with total mice per cage ranging from 3-5 mice. All SIS experimental mice experienced a cage change at the same time, with hierarchies in all cages shifting. To prevent stable social

hierarchies from developing during these cage changes, we ensured our rotation schedule was randomized, and prevented SIS experimental mice from encountering a cagemate they were exposed to in the 4 previous cage changes. Thus, no mice were consecutively exposed to recent cage mates. At the end of the 7 weeks, SIS mice remained housed with the mice from the last cage composition. Male and female CNTRL mice had the same cagemates throughout the entire experiment and had cages changed every 3 days. For the duration of SIS conventional mouse cages on open racks were used with corncob bedding (1/4 inch, Anderson lab), with cage changes occurring within the first two hours of the lights coming on (between 6-8am). Clean cages and bedding were provided with each cage change.

Chronic Corticosterone (Chapters 1 & 4)

Adult male and female C57BL/6J mice were randomly assigned to either VEH or corticosterone (CORT) treatment, with weights measured once per week during treatments. CORT (35 ug/mL, equivalent to 5 mg/kg/day) was dissolved in 0.45% beta-cyclodextrin (Sigma) water and delivered ad libitum in opaque drinking bottles (David et al., 2009). VEH treatment consisted of in 0.45% beta-cyclodextrin (Sigma) water and delivered ad libitum in clear drinking bottles.

Chronic Non-Discriminatory Social Defeat Stress (CNSDS) (Chapter 2)

To determine if female mice experience a similar stress outcome as the standard CSDS paradigm in males (Tsankova et al., 2006), both female and male mice were exposed to a novel, dual-sex stress protocol. Adult male and female C57BL/6J mice were randomly assigned to either chronic stress or no stress control groups. Retired CD-1 breeder adult males were purchased from Charles River Laboratory (Wilmington, MA). To screen for aggressive behavior, screener female and male C57BL/6J mice were simultaneously placed in the home cage of a CD-

1 mouse. CD-1 mice that reliably attacked both female and male screener mice within 60 seconds on consecutive days were selected as aggressors (Golden, Covington, Berton, & Russo, 2011). 2 CD-1 mice were selected for each pair of experimental mice (one male and one female C57BL/6J mouse). During the experimental phase, experimental male C57BL/6J mice were matched with the same experimental female C57BL/6J mice throughout the duration of the social defeat. The male and female C57BL/6J pairs were placed into the home cage of novel CD-1 aggressors for 10 consecutive daily 5-minute sessions. Since our cages only permitted one cage divider, we did not cohouse the aggressor CD-1 with both the experimental male and female C57BL/6J mice. Instead, on alternating days, either the male or female C57BL/6J mice were housed with either the CD-1 aggressor mouse with which they had interacted or a novel control aggressor CD-1 (previously screened for aggressive behavior). Co-housed subjects were separated by clear, perforated plexiglass that permitted sensory but not physical interaction. In total, both C57BL/6J male and female mice were exposed to 5 days of sensory exposure to the aggressor CD-1 and 5 days exposure to a novel aggressor CD-1 (previously screened for aggressive behavior). A separate group of female and male C57BL/6J mice were simultaneously placed into a standard cage and allowed to interact for 5 minutes in the absence of an aggressor CD-1 male over 10 days. These opposite sex control mice were housed on either side of a divider and placed on a separate rack from stress mice for 24 hours until the next interaction session.

Behavioral Testing

Within each of the experiments assessment of stress and antidepressant effects were conducted using negative valence behavioral tests. These tests allow for the evaluation of anxiety-like behaviors, which have translation aspect to the clinical assessment of depression.

The main behavioral tests used in the chapters below are the open field (OF), light dark test (LDT), elevated plus maze (EPM), novelty suppressed feeding (NSF), and forced swim test (FST). A general overview of these tests are detailed below.

Open Field (OF)

Motor activity was quantified via infrared photobeams in Plexiglass open field boxes 43x43 cm^2 (Kinder Scientific) in room lighting (300 lux). As previously described (13), activity chambers were computer interfaced for data sampling at 100ms resolution. The computer software predefines grid lines that divide each OF chamber into center and periphery regions, with the center being a square 11cm from the wall. For our analyses we calculated percent distance traveled in the center ((center distance/total distance traveled)* 100).

Light Dark Test (LDT)

LDT was conducted in OF chambers with, a dark plastic box (opaque to visible light, but transparent to infrared light) covering 1/3 of the arena, inserted to separate the OF into light and dark compartments. The dark box contained an opening that allowed passage between the light and dark (13), with the light compartment brightly illuminated (1000 lux; Kinder Scientific). At the beginning of each 5-minute test, mice were placed in the dark compartment, with distance traveled in the light ((distance traveled in the light/total distance) *100) used for analyses.

Elevated Plus Maze (EPM)

The EPM test consisted of a plus-shaped apparatus with two open and two closed arms (side walls), elevated 2 feet from the floor in room lighting (300 lux). During the five-minute test, the mice were recorded from a video camera mounted on the ceiling above each EPM arena. EthoVision (Noldus) software was used to quantify the data, with distance traveled in open arms ((total open arm distance/total distance traveled)*100) used for analyses.

Forced Swim Test (FST)

A modified FST procedure suitable for mice was used (13), with individual cylinders (46x32x30 cm) filled with room-temperature water (25-26°C) conducted in room lighting (250 lux). Two sets of photobeams were mounted on opposite sides of the cylinder (Kinder Scientific) to record swimming behavior during the 6-minute test. Immobility was assessed during only the last 4-minutes of the test since mice habituate to the task during the initial 2-minutes.

Novelty Suppressed Feeding (NSF)

Mice were food deprived for 18 hours within their home cage, prior to being placed in the corner of a testing apparatus (50x50x20 cm) filled with 2 cm of corncob bedding, with a single food pellet attached to a white platform in the brightly illuminated center (1500 lux). The NSF test lasted 6 minutes with latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) recorded in seconds. Mice that timed out were assigned a latency of 360secs. Immediately after the test, mice were transferred to their home cages and given ad libitum access to food for 5 minutes. Latency to eat and amount of food consumed in home cage was measured as a control for feeding behavior observed in the NSF.

Vaginal Lavage

To assess estrous cycle state, daily vaginal lavages were performed two weeks prior to stress exposure to ensure mice were cycling throughout all four stages regularly. Specific collection time points are specified in the chapters below. In general, vaginal smears were collected via a pipette filled with ddH_2O, placed at the opening of the mouse's vaginal canal (without penetration) with ddH_2O gently expelled and suctioned back into the pipette tip (Byers, Wiles, Dunn, & Taft, 2012; McLean, Valenzuela, Fai, & Bennett, 2012). Samples were imaged under an EVOS FL Auto 2.0 microscope (Thermo Fisher Scientific, Waltham, MA) at 10x

magnification. Estrous phases were identified by the presence or absence of nucleated epithelial cells, cornified epithelial cells, and leukocytes (Byers et al., 2012; Felicio, Nelson, & Finch, 1984). Proestrus mice had mostly nucleated and some cornified cells, estrus predominantly displayed cornified epithelial cells, metestrus had cornified epithelial cells and polymorphonuclear leukocytes (Byers et al., 2012), while diestrus contained polymorphonuclear leukocytes. Investigation of whether estrous cycle impacted behavioral performance or response to stress were conducted in the experiments that encompass this dissertation.

CHAPTER 1

Social Instability is an Effective Chronic Stress Paradigm for both Male and Female Mice

The results from this chapter are reported in Yohn, C. N., Ashamalla, S. A., Bokka, L., Gergues, M. M., Garino, A., & Samuels, B. A. (2019). Social Instability is an Effective Chronic Stress Paradigm for both Male and Female Mice. Neuropharmacology, 160, PUID: 107780

Abstract

Despite stress-associated disorders having a higher incidence rate in females, preclinical research mainly focuses on males. Chronic stress paradigms, such as chronic social defeat and chronic corticosterone (CORT) administration, were mainly designed and validated in males and subsequent attempts to use these paradigms in females has demonstrated sex differences in the behavioral and HPA axis response to stress. Here, we assessed the behavioral response to chronic CORT exposure and developed a social stress paradigm, social instability stress (SIS), which exposes adult mice to unstable social hierarchies every 3 days for 7 weeks. Sex differences in response to chronic CORT emerged, with negative valence behaviors induced in CORT treated males, not females. SIS effectively induces negative valence behaviors in the open field, light dark, and novelty suppressed feeding tests, increases immobility in the forced swim test, and activates the hypothalamus-pituitary-adrenal (HPA) axis in both males and females. Importantly, while there were effects of estrous cycle on behavior, this variability did not impact the overall effects of SIS on behavior, suggesting estrous does not need to be tracked while utilizing SIS. Furthermore, the effects of SIS on negative valence behaviors were also reversed following chronic antidepressant treatment with fluoxetine (FLX) in both males and females. SIS also reduced adult hippocampal neurogenesis in female mice, while chronic FLX treatment increased adult hippocampal neurogenesis in both males and females. Overall, these data demonstrate that the SIS paradigm is an ethologically valid approach that effectively induces chronic stress in both adult male and adult female mice.

Introduction

Several mood disorders are precipitated and/or exacerbated by chronic exposure to stressful experiences. These stress-associated disorders occur at higher rates in women than men. However, historically preclinical studies utilize chronic stress paradigms that were designed for and validated with male rodents (Autry et al., 2009; Beery and Zucker, 2011; Shansky, 2015; Trainor, 2011). One reason is that the estrous cycle has effects on neural functions and behavior (Becker et al., 2016; Palanza et al., 2001; Shansky and Woolley, 2016)**.** However, meta-analyses suggest that there is not more variability in female than male rodents (Becker et al., 2016; Prendergast et al., 2014). This realization, coupled with a mandate from National Institutes of Health to include both sexes in grant applications, has increased the number of behavioral neuroscience studies that include both sexes (Shansky, 2018). However, preclinical chronic stress studies continue to lag behind because the experimental paradigms are optimized mainly for male rodents (Beery and Zucker, 2011; Blanchard et al., 1995; David et al., 2009; Golden et al., 2011; Hodes, 2018; Lezak et al., 2017; Russo and Nestler, 2013).

One commonly used chronic stress paradigm, unpredictable chronic mild stress (UCMS), differentially affects negative valence behaviors and hypothalamic-pituitary-adrenal (HPA) axis activation in male and female mice (Guilloux et al., 2011; Mineur et al., 2006; Piantadosi et al., 2016; Zhao et al., 2017). Furthermore, UCMS has historically been plagued with reproducibility and validity issues (Belzung and Lemoine, 2011; Farooq et al., 2012; Willner, 1997). Another widely used paradigm is chronic social defeat stress, where rodents are subjected to larger more aggressive conspecifics (Golden et al., 2011). In males, chronic social defeat stress (CSDS) results in HPA axis activation and increased negative valence behaviors (Keeney et al., 2006; Krishnan et al., 2007). However, standard CSDS protocols are not effective for stressing female

rodents (Greenberg et al., 2013) unless variations are used that require surgeries to activate ventromedial hypothalamus of aggressors or apply male urine to females (Harris et al., 2018; Takahashi et al., 2017).

In males and females, the HPA axis is activated by stress (Herman et al., 2016; Kitay, 1963), resulting in release of cortisol (human) or corticosterone (rodents), and HPA axis dysregulation is observed in many mood disorders (Wardenaar et al., 2011). Therefore, a distinct paradigm that mimics chronic stress in male rodents is chronic corticosterone (CORT) administration (David et al., 2009; Gourley et al., 2008). In females, postpartum chronic CORT increases forced swim test (FST) immobility and decreases maternal behavior in female rats (Brummelte and Galea, 2010; Brummelte et al., 2006). However, one study suggests chronic CORT does not effectively increase negative valence behaviors in females (Mekiri et al., 2017).

Newer paradigms, such as vicarious social defeat (Iniguez et al., 2018) and chronic variable stress (Labonte et al., 2017) were developed to be effective in both sexes. However, there remains a need for new chronic stress paradigms that are effective in both adult male and female rodents because it is important to use more than one type of stress paradigm to understand the neurobiology of stress in both sexes. Furthermore, many preclinical labs currently utilize distinct paradigms for males and females or still focus exclusively on males. Development of a new chronic stress paradigm that is effective in both sexes and permits direct comparisons will help advance our understanding of how chronic stress impacts neural function and behavior. First, we assessed whether chronic CORT administration, which is commonly used in males, is effective in female C57BL/6J mice. We found significant sex differences in negative valence behaviors between CORT treated males and females, suggesting that CORT administration is less effective in females. Next, we describe development of a social instability stress (SIS)

paradigm that is effective in both adult male and female mice. SIS involves exposure to unstable social hierarchical dynamics for several weeks and induces negative valence behaviors and HPA axis activation in adult males and females of the widely used C57BL/6J strain. Negative valence behaviors induced by social instability in both adult males and females can be reversed by subsequent administration with the antidepressant fluoxetine, lending pharmacological validity to SIS. These results demonstrate that social instability is an ethologically valid chronic stress paradigm that is effective for both adult male and female rodents.

Methods

Mice

Adult 8-week-old C57BL/6J mice were purchased from Jackson laboratories and maintained on a 12L:12D schedule with *ad libitum* food and water. All testing was conducted in compliance with the NIH laboratory animal care guidelines and approved by Rutgers University Institutional Animal Care and Use Committee.

Social Instability Stress (SIS)

Adult male and female C57BL/6J mice were randomly assigned to either SIS or control (CNTRL). SIS mice experienced unstable social hierarchies where social dynamics were changed every 3 days for 7 weeks [adapted from (Bartolomucci et al., 2004; Schmidt et al., 2010; Schmidt et al., 2007; Sterlemann et al., 2008)]. Social dynamic changes consisted of an individual mouse being introduced to 2-4 novel experimental mice of the same sex from different cages, with total mice per cage ranging from 3-5 mice. All SIS experimental mice experienced a cage change at the same time, with hierarchies in all cages shifting. To prevent stable social hierarchies from developing during these cage changes, we ensured our rotation schedule was randomized, and prevented SIS experimental mice from encountering a cagemate they were

exposed to in the 4 previous cage changes. Thus, no mice were consecutively exposed to recent cage mates. At the end of the 7 weeks, SIS mice remained housed with the mice from the last cage composition. Male and female CNTRL mice had the same cagemates throughout the entire experiment and had cages changed every 3 days. For the duration of SIS conventional mouse cages on open racks were used with corncob bedding (1/4 inch, Anderson lab), with cage changes occurring within the first two hours of the lights coming on (between 6-8am). Clean cages and bedding were provided with each cage change. The following groups emerged: CNTRL+VEH (male=20; female=20), CNTRL+FLX (male=10; female=10), SIS+VEH (male=25; female=28); SIS+FLX (male=33; female=35).

Chronic Corticosterone

Adult male and female C57BL/6J mice were randomly assigned to either vehicle (VEH) or corticosterone (CORT) treatment, with weights measured once per week during treatments. Corticosterone (35 ug/mL, equivalent to 5 mg/kg/day) was dissolved in 0.45% beta-cyclodextrin (Sigma) water and delivered ad libitum in opaque drinking bottles (David et al., 2009). VEH mice received 0.45% beta-cyclodextrin water ad libitum. Following 4 weeks of CORT or VEH treatment, mice received 3 weeks of fluoxetine (FLX) (18 mg/kg/day) or VEH (water) via oral gavage with CORT or VEH remaining in the drinking water throughout antidepressant treatment (timeline in Figure 1A). On behavioral testing days FLX or VEH was administrated after mice completed the behavioral tests to avoid acute effects. The following groups emerged: VEH+VEH (male=10; female=10), VEH+FLX (male=10; female=10), CORT+VEH (male=10; female=10), and CORT+FLX (male=10; female=9).

Vaginal Lavage

Vaginal lavages were performed daily during the stress paradigms and two weeks prior to behavioral testing to ensure mice were cycling throughout all four stages of the estrous cycle regularly. After completing each behavioral test, vaginal smears were collected to assess the estrous state mice were in during the behavior test. Samples were collected via a pipette filled with ddH_2O gently expelled and placed at the vaginal canal opening (without penetration). Samples were suctioned back into the pipette, placed on a microscope slide, and dried on a slide warmer before imaged with an EVOS FL Auto 2.0 microscope (Thermofisher Scientific) at 10x magnification. Estrous phases were identified by the presence or absence of nucleated epithelial cells, cornified epithelial cells, and leukocytes (Figure 1.3B).

Behavioral Testing

Open Field (OF)

Motor activity was quantified via infrared photobeams in Plexiglass open field boxes 43x43 cm^2 (Kinder Scientific) in room lighting (300 lux). As previously described (13), activity chambers were computer interfaced for data sampling at 100ms resolution. The computer software predefines grid lines that divide each OF chamber into center and periphery regions, with the center being a square 11cm from the wall. For our analyses we calculated percent distance traveled in the center ((center distance/total distance traveled)* 100).

Light Dark Test (LDT)

LDT was conducted in OF chambers with, a dark plastic box (opaque to visible light, but transparent to infrared light) covering 1/3 of the arena, inserted to separate the OF into light and dark compartments. The dark box contained an opening that allowed passage between the light and dark (13), with the light compartment brightly illuminated (1000 lux; Kinder Scientific). At

the beginning of each 5-minute test, mice were placed in the dark compartment, with distance traveled in the light ((distance traveled in the light/total distance) *100) used for analyses.

Elevated Plus Maze (EPM)

The EPM test consisted of a plus-shaped apparatus with two open and two closed arms (side walls), elevated 2 feet from the floor in room lighting (300 lux). During the five-minute test, the mice were recorded from a video camera mounted on the ceiling above each EPM arena. EthoVision (Noldus) software was used to quantify the data, with distance traveled in open arms ((total open arm distance/total distance traveled)*100) used for analyses.

Forced Swim Test (FST)

A modified FST procedure suitable for mice was used (13), with individual cylinders (46x32x30 cm) filled with room-temperature water (25-26°C) conducted in room lighting (250 lux). Two sets of photobeams were mounted on opposite sides of the cylinder (Kinder Scientific) to record swimming behavior during the 6-minute test. Immobility was assessed during only the last 4-minutes of the test since mice habituate to the task during the initial 2-minutes.

Novelty Suppressed Feeding (NSF)

Mice were food deprived for 18 hours within their home cage, prior to being placed in the corner of a testing apparatus (50x50x20 cm) filled with 2 cm of corncob bedding, with a single food pellet attached to a white platform in the brightly illuminated center (1500 lux). The NSF test lasted 6 minutes with latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) recorded in seconds. Mice that timed out were assigned a latency of 360secs. Immediately after the test, mice were transferred to their home cages and given ad libitum access to food for 5 minutes. Latency to eat and amount of food consumed in home cage was measured as a control for feeding behavior observed in the NSF.

Brain Collection, Sectioning, and Immunohistochemistry

Brain Collection and Sectioning

Following behavioral testing (3 days after FST), brains were collected from all experimental mice. Mice were anesthetized with ketamine (80mg/kg) and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were collected, stored in 4% paraformaldehyde overnight at 4°C, and then switched to 30% sucrose 0.1% sodium azide (NaN_3) in PBS solution until sectioned. Using a cryostat, the hippocampus was collected (43; Bregma -1.22 to -3.88) and mounted on SuperfrostPlus slides (Thermofisher Scientific) and stored at -20°C until immunostaining.

Immunohistochemistry

The effects of SIS and FLX on adult hippocampal neurogenesis were assessed across the entire hippocampus by counting 1 out of every 6 hippocampal sections (40μm). Slides were washed in 1% TritonX-100-PBS and then 3 PBS washes. Next, slides were incubated in citrate buffer for 30 minutes followed by 3 PBS washes. Slides were blocked for 1 hour in 10% normal goat serum (NGS) diluted in PBS before being incubated overnight at 4°C in either anti-rabbit Ki67 (1:500; Abcam, ab16667) or doublecortin anti-rabbit (1:500; Life technologies; 481200) diluted in 2%NGS-PBS. Following 18 hours of incubation, 3 PBS washes were performed and then 2 hours of incubation in CY-5 goat anti-rabbit (1:1000, Invitrogen, A10523) diluted in 2%NGS-PBS. Following 3 more PBS washes, slides were counterstained with DAPI (1:15000; ThermoFisher Scientific) for 15 minutes, with a final PBS wash before cover slipping using prolong diamond (ThermoFisher Scientific). High-resolution fluorescent images were taken using an EVOS FL Auto 2.0 microscope (Thermofisher Scientific) at 10x magnification for quantification of $Ki67^+$ (Figure 1.5A) or DCX^+ cells (Figure 1.5C) and counted across a total of

12 sections of hippocampus. $Ki67^+$ cells were overlaid with DAPI for quantification purposes. Images were also taken at 40x magnification to subcategorize DCX^+ cells according to their dendritic morphology: DCX^+ cells with no tertiary dendritic processes and DCX^+ cells with complex, tertiary dendrites (Figure 1.5E). The maturation index was defined as the ratio of DCX^+ cells possessing tertiary dendrites over the total DCX^+ cells.

Blood Collection and Corticosterone ELISA

Mice were weighed to ensure that non-terminal blood collection was no more than 1% of the mouse's body weight and were anesthetized with isoflurane for each retro-orbital sinus blood collection. Prior to SIS, all mice had baseline blood samples collected from the left retro-orbital sinus in accordance with IACUC guidelines. To measure corticosterone levels in response to a change in social dynamics (i.e. cage composition change), male and female SIS mice had blood collected from the right retro-orbital sinus approximately 40-45 minutes after cage change. CNTRL mice had blood collected from the right retro-orbital sinus 40-45 minutes after a cage change, to control for impact of exposure to a novel cage on plasma corticosterone levels. Blood was also collected 40-45 minutes following the EPM from the left retro-orbital sinus of SIS and CNTRL mice to measure plasma corticosterone levels in response to a negative valence behavior. For each of the three blood collections, blood was collected in micro centrifuge tubes coated with EDTA. Plasma was isolated from whole blood by centrifugation at 14000 rpm for 10 min at 4 °C, with supernatant collected and stored at - 80°C until assayed. Total yield of plasma per blood collection was between 25 to 40 μL. To assess differences in plasma corticosterone in response to SIS stress and EPM behavior, blood was analyzed from each time point (prior to SIS, SIS cage change, EPM) across 5 mice per sex per stress condition (male: CNTRL=5, SIS=5; female: CNTRL=5, SIS=5). Each sample (total of n=60 across all time points) was diluted 1:100

and assayed in triplicate according to the manufacturer's protocol (Arbor Assays Corticosterone ELISA Kit; sensitivity 18.6 pg/ml).

Statistical Analyses

To investigate SEX, STRESS, and TREATMENT effects we ran 2x2x2 analysis of variance (ANOVA) with Bonferroni post-hoc comparisons. Since NSF data fails to meet basic assumptions of normality, Kaplan-Meier survival analysis (nonparametric test) was used. Lastly, to analyze differences in endogenous corticosterone levels, time point of blood collection, sex, and groups were used to conduct a 3x2x2 repeated measures ANOVA. These analyses were then followed up with separate 2x3 repeated measures ANOVAs within each sex. GraphPad Prism 7 and SPSS (version 23) was used for analyses.

RESULTS

Behavioral Sex Differences following Chronic CORT Administration

Chronic CORT administration mimics chronic stress in male rodents (David et al., 2009; Gourley et al., 2008). However, reports are inconsistent whether CORT administration is effective in female rodents (Brummelte and Galea, 2010; Brummelte et al., 2006; Mekiri et al., 2017). We chronically administered exogenous CORT (5mg/kg/day) or VEH to male and female C57BL/6J mice (timeline in Figure 1.1A). Following 4 weeks of CORT, mice received either FLX (18mg/kg/day) or VEH, resulting in four groups: VEH+VEH (male=10; female=10), VEH+FLX (male=10; female=10), CORT+VEH (male=10; female=10), and CORT+FLX (male=10; female=9). We examined CORT and FLX effects in negative valence behaviors affected by chronic stress paradigms: open field (OF), light/dark test (LDT), elevated plus maze (EPM), novelty suppressed feeding (NSF), and forced swim test (FST).

We ran 2x2x2 three-way ANOVAs investigate SEX, CORT, and FLX effects on negative valence behaviors. We found no significant effects in OF percent center distance traveled in either sex (p>0.05; Figure 1B, Supplemental Figure 1.1A). In LDT we found a significant SEX*x*CORT interaction ($F_{(1,72)}$=4.731, p=0.032; Supplemental Figure 1.1B) on percent light distance traveled, with CORT+VEH males traveling less than CORT+VEH females (p=0.0319) (Supplemental Figure 1B). Post hoc analysis with a Bonferroni correction indicated within sex effects (Figure 1C). CORT+VEH males traveled a smaller distance in the light than VEH+VEH (p=0.032) and CORT+FLX (p=0.048) males. In EPM, a significant interaction between SEX*x*CORT ($F_{(1,72)}$=54.061, p=0.047; Supplemental Figure 1.1C) was observed in open arm percent distance traveled. Males treated with CORT+VEH traveled less distance on open arms than CORT+VEH females (p=0.019; Figure 1.1D; Supplemental Figure 1.1C). Bonferroni post hoc comparisons revealed within sex effects, such that CORT+VEH males travel less percent distance in the open arms than VEH +VEH (p=0.0092) and CORT+FLX (p=0.0016) males. Additionally, VEH+VEH females travel less on the open arms than VEH+FLX females (p=0.016; Figure 1.1D). Additionally, FLX exerted significant main effects ($F_{(1,72)}$=64.71, p<0.0001) on FST immobility, with planned Bonferroni post hoc comparisons revealing within sex effects. Males treated with CORT+FLX were less immobile than CORT+VEH males (p=0.0008; Figure 1.1E) and VEH+FLX were less immobile than VEH+VEH males (p=0.013; Figure 1.1E). Similarly, in females FLX reduced immobility time with CORT+FLX females less immobile than CORT+VEH females (p=0.036; Figure 1.1E). VEH+FLX females were also less immobile (p<0.0001; Figure 1.1E) than VEH+VEH females. Lastly, Kaplan-Meier analysis revealed a significant sex difference in NSF within the CORT+VEH group ($\chi^2_{(1)}$= 16.39, p<0.001; Figure 1F; Supplemental Figure 1.1E), with CORT+VEH males having a longer

latency than CORT+VEH females. Separate analyses within males showed significant group differences ($\chi^2_{(3)}=37$, $p<0.0001$), with CORT+VEH males having a longer latency to eat than VEH+VEH ($\chi^2_{(1)}=37.58$, $p<0.0001$) and CORT+FLX ($\chi^2_{(1)}=15.03$, $p<0.0001$; Figure 1.1F) males. Kaplan-Meier analyses in females revealed a significant difference in NSF between female groups ($\chi^2_{(3)}=9.28$, $p=0.026$), with VEH+FLX females having a shorter latency to eat than VEH+VEH females ($\chi^2_{(1)}=4.54$, $p=0.033$; Figure 1.1F). These data demonstrate that while FLX has behavioral effects in both males and females, CORT only mimics chronic stress effects in males. Taken together, these data support the findings of Mekiri and colleagues (Mekiri et al., 2017) and demonstrate that CORT administration more effectively induces negative valence behaviors in males than in females.

SIS is Effective in both Males and Females

We next developed a social instability paradigm where C57BL/6J mice were exposed to unstable same sex hierarchical conditions within their housing environment (Figure 1.2A). SIS mice were exposed to new cagemates every 3 days for 7 weeks, while CNTRL mice were housed with the same cagemates throughout the experiment. Adult male and female C57BL/6J mice were assigned to SIS or CNTRL housing conditions for 7 weeks, and then received 3 weeks of VEH or FLX (18mg/kg/day) resulting in the following groups: CNTRL+VEH (male=20; female=20), CNTRL+FLX (male=10; female=10), SIS+VEH (male=25; female=28); SIS+FLX (male=33; female=35).

We ran 2x2x2 three-way ANOVAs investigating SEX, SIS, and FLX effects on negative valence behaviors. Importantly, no main effects of SEX were observed in any of the behaviors (Supplemental Figure 1.2A-E). We found a significant SIS*x*FLX interaction ($F_{(1,174)}=20.11$, $p<0.0001$; Figure 1.2B) in OF percent center distance traveled. Within sex Bonferroni post hoc

comparisons revealed that SIS+VEH males traveled less distance than CNTRL+VEH males (p=0.0076; Figure 1.2B). Similarly, SIS+VEH females traveled less than CNTRL+VEH females (p=0.0002). In the LD, we observed a significant SIS*x*FLX interaction ($F_{(1,174)}$= 31.2, p<0.0001; Figure 1.2C) in percent distance traveled in the light. Within sex Bonferroni post-hoc comparisons demonstrated that SIS+VEH males traveled less percent distance in the light than CNTRL+VEH (p=0.013) and SIS+FLX (p<0.0001) males. Within females, SIS+VEH females traveled less than CNTRL+VEH (p=0.021) and SIS+FLX (p<0.0001) females. In the EPM, a significant main effect of FLX ($F_{(1,174)}$= 22.34, p<0.0001; Figure 1.2D) was observed. Within sex Bonferroni post-hoc comparisons showed that FLX treated males traveled more than non-FLX treated males. Specifically, SIS+FLX males traveled more on the open arms than SIS+VEH males (p=0.019) and CNTRL+FLX males also traveled more than CNTRL+VEH males (p=0.016). Similarly, SIS+FLX females traveled more than SIS+VEH females (p=0.005), with CNTRL+FLX females also traveling more than CNTRL+VEH females (p=0.016). Additionally, significant SIS ($F_{(1,92)}$= 6.2, p=0.015) and FLX ($F_{(1,92)}$= 65.7, p<0.0001) main effects were observed in FST immobility. CNTRL+VEH males were more immobile than CNTRL+FLX males (p=0.0006), and SIS+VEH males were more immobile than SIS+FLX males (p=0.0031; Figure 1.2E). Similarly, CNTRL+VEH females were more immobile than CNTRL+FLX females (p=0.014), and SIS+VEH females were more immobile than SIS+FLX females (p=0.0002; Figure 1.2E). Lastly, Kaplan-Meier analysis revealed no significant sex differences in NSF within the SIS+VEH group ($\chi^2_{(1)}$= 47.5, p<0.001). Within males, Kaplan-Meier analysis showed a significant difference across groups ($\chi^2_{(3)}$= 34.06, p<0.0001), and Bonferroni multiple comparisons demonstrated that SIS+VEH males had a longer latency to eat than CNTRL+VEH ($\chi^2_{(1)}$= 33.7, p<0.0001) and SIS+FLX ($\chi^2_{(1)}$= 25.1,

p<0.0001) males (Figure 1.2F). In females, Kaplan-Meier analysis revealed a significant difference across groups ($\chi^2_{(3)}$= 16.41, p=0.0009), with SIS+VEH females having a longer latency to eat than CNTRL+VEH ($\chi^2_{(1)}$= 29.5, p<0.0001) and SIS+FLX females ($\chi^2_{(1)}$= 4.09, p=0.040; Figure 1.2F). Therefore, SIS impacted OF, LDT, FST, and NSF behaviors in both sexes similarly, and FLX treatment reversed the effects of SIS stress in LDT, EPM, FST, and NSF in both sexes. Taken together, these data demonstrate that SIS effectively induces negative valence behaviors in both adult male and female mice.

To further analyze whether stress or estrous contributes to behavioral performance in these negative valence behavioral tasks we also ran separate multiple regressions (Supplemental Figure 1.3A-E). While both stress and estrous phase statistically predicted behavioral outcome in the OF ($F_{(2, 46)}$)=5.99, p=0.005), LD ($F_{2, 46}$)=3.57, p=0.037), EPM ($_{(2, 46)}$)=4.45, p=0.017), and NSF ($F_{(2, 46)}$)=16.99, p<0.0001), stress alone significantly contributed in each of the behavioral measures (OF p=0.008, LD p=0.024, EPM p=0.022, FST p=0.029, NSF p<0.0001). Estrous phase did not independently significantly predict behavioral outcome in these negative valence behavioral tasks (OF p=0.055, LD p=0.19, EPM p=0.078, FST p=0.484, NSF p=0.34; Supplemental Figures 1.3A-E).

Estrous Cycle Effects on Behavior

Since SIS affected female behavior, we next examined the 4 stages of the estrous cycle (Proestrus, Estrus, Metestrus, Diestrus; Figure 1.3B) in CNTRL and SIS females following each behavioral test (Figure 3A). For this experiment FLX was not used. To determine SIS and estrous cycle effects on behavior, separate 2x4 ANOVAs (SIS*x*ESTROUS) were run for each behavior. In OF, significant estrous cycle ($F_{(3,39)}$=45.16, p<0.001) and SIS ($F_{(1,39)}$=19.13, p<0.0001) effects were observed (Figure 1.3C). Specifically, SIS estrus females traveled less

center distance than proestrus (p=0.0003) and metestrus females (p=0.013), and SIS diestrus females traveled less center distance than proestrus (p=0.0016) and metestrus females (p=0.0094) (Figure 1.3C). In CNTRL mice, estrus females traveled less center distance than proestrus (p=0.045) or metestrus females (0.0130), and diestrus females also traveled less center distance than metestrus (p=0.023) and proestrus females (p=0.022; Figure 1.3C). Similarly, in LDT, significant estrous cycle ($F_{(3, 39)}$=10.43, p<0.0001) and SIS ($F_{(1, 39)}$=7.68, p=0.0085) effects were observed, with SIS estrus females traveling less in the light than proestrus (p=0.021) and metestrus females (p=0.003). SIS diestrus females also traveled less light distance than metestrus females (p=0.011; Figure 1.3D). Although significant SIS effects were observed in EPM ($F_{(1, 39)}$=5.87, p=0.0196; Figure 3E) and FST ($F_{(1, 17)}$=4.47, p=0.04; Figure 1.3F), no estrous cycle effects were found (EPM: p=0.849; FST: p=0.97). Within OF, LDT, EPM, and FST, Bonferroni corrected post-hoc comparisons did not reveal differences within estrous states. Lastly, Kaplan-Meier analysis revealed a significant difference in NSF across estrous stages within the SIS group ($\chi^2_{(3)}$= 11.71, p=0.0084), with SIS estrus females having a higher latency to eat than SIS metestrus ($\chi^2_{(1)}$= 15.7, p<0.0001; Figure 1.3G). Thus, although the estrous cycle increases negative valence behaviors during specific stages (mainly estrus and diestrus), the effects of SIS are observed throughout the estrous cycle. Therefore, variability across estrous in freely cycling C57BL/6J females does not impact the overall effects of SIS on negative valence behaviors.

SIS Stress Increases Endogenous Corticosterone Levels in Males and Females

We next sought to determine whether SIS leads to HPA axis activation in males and females. To this end, blood was collected from SIS and CNTRL mice at three different time points: prior to SIS exposure, 40 minutes following a SIS cage change (during the last week of

SIS), and 40 minutes following EPM (Figure 1.4A). Plasma was isolated assayed for corticosterone levels. We performed a 2x2x3 repeated measures three-way ANOVA (SEX*x*SIS*x*TIME POINT) and found no main effect of sex (p=0.48). This suggests that SIS is impacting HPA axis activation similarly in males and females. Next, 2x3 repeated measures ANOVAs (SIS*x*TIME POINT) within each sex were performed. In males, we found a significant interaction (SIS*x*TIME POINT) ($F_{(2, 16)}$=8.55, p=0.003). Specifically, SIS males had higher plasma corticosterone levels following a cage change (p=0.0054) and EPM exposure (p<0.0001) than CNTRL (Figure 1.4B). Significant increases in SIS male plasma corticosterone levels after cage change (p=0.008) and EPM (p=0.0001) were also observed relative to levels before SIS exposure (Figure 1.4B). Therefore, chronic SIS exposure increased HPA axis activation in response to acute stressors in males.

Separate 2x3 repeated measures ANOVAs in females revealed a significant interaction (SIS*x*TIME POINT) ($F_{(2, 16)}$=5.52, p=0.015). SIS females had significantly higher plasma corticosterone levels in response to a cage change than CNTRL females (p=0.0007; Figure 1.4C). Plasma corticosterone levels in SIS females were also significantly higher following a cage change (p=0.0037) and EPM exposure (p=0.0105) relative to corticosterone levels before SIS exposure (Figure 1.4C). Taken together, these results demonstrate that SIS leads to HPA axis activation in response to acute stressors in both males and females.

SIS Stress and FLX Impact Adult Hippocampal Neurogenesis

In addition to behavioral and HPA axis effects, chronic stress can also affect adult hippocampal neurogenesis (Czeh et al., 2002; Pham et al., 2003; Van Bokhoven et al., 2011) and antidepressants increase all stages of adult hippocampal neurogenesis (David et al., 2009; Malberg et al., 2000; Santarelli et al., 2003). Therefore, following behavior, mice were perfused,

and sectioned brains were immunostained for Ki67 (a proliferation marker; Figure 1.5A) and DCX (a newborn/immature neuron marker; Figure 1.5C, E).

First, we investigated sex differences between groups (CNTRL+VEH, CNTRL+FLX, SIS+VEH, and SIS+FLX) with 2x2x2 three-way ANOVAs for each neurogenesis marker. No main effects of sex were found for number of $Ki67^+$ cells (p=0.27), DCX^+ cells (p=0.26), and DCX^+ cells with tertiary dendrites (p=0.29), or maturation index (DCX^+ cells with tertiary dendrites/total DCX^+ cells; p=0.79; Figure 5B, D, F-G smaller panels).

However, the 2x2x2 three-way ANOVAs revealed a significant FLX effect ($F_{(1,32)}$= 48.16, p<0.0001) on proliferation, with SIS+FLX males having more Ki67+ cells in dentate gyrus (DG) than SIS+VEH males ($p=0$.015; Figure 1.5A-B). Similarly, SIS+FLX females had more DG Ki67+ cells than SIS+VEH females ($p=0$.0001). Significant FLX effects were also observed in number of DCX^+ cells ($F_{(1,32)}$= 94.91, $p<0$.0001); DCX^+ cells with tertiary dendrites ($F_{(1,32)}$= 41.97, $p<0$.0001); and maturation index ($F_{(1,32)}$= 11.9, $p=0$.0016), with FLX treated males having more DCX+ cells [CNTRL+FLX*vs*CNTRL+VEH ($p=0$.016) and SIS+FLX*vs*SIS+VEH ($p<0$.0001); Figure 1.5D], more DCX^+ cells with tertiary dendrites [CNTRL+FLX*vs*CNTRL+VEH ($p=0$.038) and SIS+FLX*vs*SIS+VEH ($p=0$.024); Figure 1.5F], and a higher maturation index (SIS+FLX*vs*SIS+VEH ($p=0$.041; Figure 1.5G) than VEH males. Similarly, FLX treated females have more DCX+ cells [CNTRL+FLX*vs*CNTRL+VEH ($p=0$.045) and SIS+FLX*vs*SIS+VEH ($p<0$.0001); Figure 1.5D], more DCX^+ cells with tertiary dendrites (SIS+FLX*vs*SIS+VEH ($p=0$.004); Figure 1.5F), and a higher maturation index (SIS+FLX*vs*SIS+VEH ($p=0$.018; Figure 1.5G) than VEH females. We also observed a significant SIS*x*FLX interaction in number of DCX+ cells ($F_{(1,32)}$= 11.9, $p=0$.0016) and maturation index ($F_{(1,32)}$= 4.87, $p=0$.034) with SIS+VEH females having less DCX+ cells

($p=0.007$) and lower maturation index ($p=0.031$) than CNTRL+VEH females. Thus, while FLX increased adult hippocampal neurogenesis in both sexes, SIS had a larger impact on females.

DISCUSSION

The SIS paradigm effectively induces negative valence behaviors and HPA axis activation in both adult males and females of the widely used C57BL6/J strain. Importantly, there were no sex differences across all behavior tests following SIS exposure, suggesting that SIS can be used to assess the effects of chronic stress in both sexes. Importantly, the effects of SIS were observed throughout the estrous cycle, and therefore, SIS can be performed in adult males and freely cycling adult females without concern of estrous cycle confounds. By contrast, there were behavioral sex differences following CORT administration. Specifically, CORT mimicked chronic stress in males but was less effective in females.

Our study exposes adult male and female C57BL/6J mice to SIS. The most similar approaches in mice were performed in the outbred CD-1 strain, where adolescent mice exposed to unstable housing conditions displayed increased negative valence behaviors in EPM and NSF in males (Sterlemann et al., 2008) and females (Schmidt et al., 2010). In these two studies, SIS began at postnatal day 24, which is right after weaning. Since anxiety can be heavily influenced by development exposure to stress (Leonardo and Hen, 2008), exposure of adolescent CD-1 mice to SIS may be more similar to an early life stress paradigm. In rats, repeated resident-intruder stressful exposure of adolescent females to lactating adult females produces different patterns of effects in negative valence behaviors than exposure of adult females (Ver Hoeve et al., 2013). We utilized adult males and adult females from the widely used inbred C57BL/6J strain, and our data demonstrates that adult exposure to chronic stress impacts negative valence behaviors and HPA axis activation. We also simultaneously ran the male and female cohorts, allowing direct

comparison of potential sex differences. Furthermore, we demonstrate that subsequent FLX treatment reverses the negative valence behaviors in both males and females, lending pharmacological validity to the SIS paradigm.

Subchronic social stress (~15 days) involving periods of social isolation and crowding are also used in rats. Exposure of adolescent male rats, but not female rats, to subchronic social stress induces negative valence behaviors in EPM and HPA axis activation (Roeckner et al., 2017). Another study found effects of subchronic social stress in adult female rats in inducing negative valence behaviors in EPM (Haller et al., 2003). Other studies found that subchronic social stress is effective in inducing HPA axis activation in female rats, but defeat is more effective for male rats (Haller et al., 1999; Nowacka et al., 2015). Chronic social stress (4 weeks) combining periods of social isolation and crowding in adult female rats leads to HPA axis activation and a decrease in sucrose preference (Herzog et al., 2009). Similar chronic social stress leads to activation of the HPA axis but has no effects in EPM or center measures of OF in adult female CD-1 mice (Jarcho et al., 2016).

Repeated social defeat (RSD) stress is similar to CSDS in which a resident C57BL/6J mouse is subjected to daily aggression by a CD1 intruder. The resident C57BL/6J undergoes social disruption for two hours at a time for 6 days where they are subjected to attacks by the aggressive CD1 intruder (Kinsey et al., 2007; McKim et al., 2016). This protocol was recently amended for female mice in which, similar to (Takahashi et al., 2017), male to female aggression was facilitated via DREADD mediated activation of the ventral medial hypothalamus (VMH) in CD1 intruders (Yin et al., 2019). Female C57BL/6J mice were attacked in their home cage for 30 minutes by the male CD1s. In both male and female C57 mice, RSD results in social avoidant behaviors and decreased time in the center of the OF (McKim et al., 2016; Sawicki et al., 2018;

Yin et al., 2019). The SIS paradigm developed here is different from RSD since mice are exposed to social stress by experiencing unstable social hierarchies through repeated changes in cagemates. Unlike RSD, SIS experimental mice are not subjected to attacks by aggressive mice from a different strain, and SIS does not require surgeries in order to effectively induce stress in females.

Given that ovarian hormones can impact stress responses and behavior (Palanza et al., 2001; Sisk and Zehr, 2005; Wood et al., 2001), we tracked the estrous cycle throughout behavior. SIS estrus and diestrus females travel less distance in the center of OF and in the light compartment of LDT than SIS proestrus and metestrus females. These data are in line with findings that socially isolated female mice in the estrus and diestrus phases spend less time in the center of the open field arena than proestrus mice (Palanza et al., 2001). Studies investigating the impact of the estrous cycle on behavior in rats have observed decreases in negative valence behaviors in EPM and OF during the metestrus and proestrus phases relative to the diestrus phase (Frye et al., 2000; Mora et al., 1996). However, even though behavioral differences across estrous phases were observed in both SIS and CNTRL mice, these differences did not impact our results since stress effects remained when we collapsed female mice from all four stages into one group. The notion that tracking females across all stages of the estrous cycle is necessary when analyzing results is unwarranted because males are just as variable as freely cycling females (Becker et al., 2016; Prendergast et al., 2014; Shansky, 2019). Thus, future studies employing the SIS paradigm do not need to track estrous cycle during behavioral experiments. One exception may be in the case of using investigational drugs in combination with SIS, in which case potential interactions between the drugs and cycling gonadal hormones in both females and males may be warranted.

Sex differences in HPA activation between males and females have been found in response to acute stressors (Kirschbaum et al., 1999; Kirschbaum et al., 1992; Kudielka and Kirschbaum, 2005). However, following SIS exposure, we found no sex differences in plasma corticosterone levels in response to either cage changes or EPM exposure. Both males and females exposed to SIS show increased plasma corticosterone levels following these acute stressors. Furthermore, prior to SIS exposure, there were no plasma corticosterone differences between males and females. Future studies may want to assess the long-term neuroendocrine effects of SIS by analyzing corticosterone levels several weeks after the final exposure to unstable social environments.

Chronic stress can affect adult hippocampal neurogenesis in male mice (David et al., 2009). Furthermore, chronic treatment with antidepressants, such as FLX, increases all stages of adult hippocampal neurogenesis in male mice in several strains of mice (David et al., 2009; Santarelli et al., 2003). In rats, both acute and chronic stress can decrease both proliferation and survival of DG granule cells (Czeh et al., 2002; Heine et al., 2004; Malberg and Duman, 2003; Pham et al., 2003; Van Bokhoven et al., 2011). Furthermore, similar to studies in mice, chronic FLX treatment can increase all stages of adult hippocampal neurogenesis and reverse effects of stress in rats (Malberg and Duman, 2003; Malberg et al., 2000; Marcussen et al., 2008). To our knowledge, this is the first study to assess the effects of chronic stress and subsequent antidepressant treatment in both male and female mice simultaneously. Within females, our data shows that SIS impacts multiple stages of adult hippocampal neurogenesis, including proliferation and differentiation. Chronic FLX treatment had effects on all stages of adult neurogenesis in females. By contrast, in males, we did not see effects of SIS on adult hippocampal neurogenesis but found effects of FLX treatment on all stages. In other chronic

stress paradigms, male mice administered CORT only displayed an effect on proliferation (David et al., 2009), while male rats exposed to CSDS displayed transient effects on proliferation and a reduction in total DCX^+ cells (Lagace et al., 2010; Van Bokhoven et al., 2011). However, complete ablation of the hippocampal adult neurogenic niche in mice using focal irradiation does not impact negative valence behaviors, suggesting that decreases in adult hippocampal neurogenesis are not sufficient or necessary to impact behavior (David et al., 2009; Santarelli et al., 2003). Rather, adult neurogenesis is required for the behavioral effects of antidepressant treatment in some, but not all, mouse strains (David et al., 2009; Holick et al., 2008; Santarelli et al., 2003). Importantly, effects of chronic FLX on behavior are also strain-dependent and require an interaction with stress in some strains. For example, in C57BL/6J mice, which are used here, effects of FLX in several negative valence behavioral tasks are only apparent in stressed mice (David et al., 2009; Dulawa et al., 2004). We found that FLX increased all stages of adult hippocampal neurogenesis following SIS in both males and females.

Another important result here is that chronic CORT administration is ineffective in inducing negative valence behaviors in females. These results support the findings of Mekiri and colleagues (Mekiri et al., 2017). In line with these results, a recent review by Kokras and colleagues that details HPA axis sex differences in animal and human studies suggests that these sex differences contribute to the failure of novel HPA axis-based drugs in clinical trials (Kokras et al., 2019). The authors argue this is in part due to, until recently, male-dominated preclinical studies in rodents and increased inclusion of women in clinical trials over the last 25 years. Importantly, when female rats are adrenalectomized and then administered CORT, their behavioral response to stressful experiences is not altered (Kokras et al., 2012; Kokras et al.,

2019). These results indicate that HPA axis activation is not critical for the female behavioral response to stress.

Our data suggest that SIS is an ethologically valid approach that induces chronic stress effects in both adult males and females. In contrast to chronic CORT administration, we found no sex differences in the effects of SIS on negative valence behaviors and HPA axis activation. Future work is necessary to determine how long-lasting the effects of SIS are and whether SIS can be leveraged to study stress resilience and susceptibility in both males and females.

Acknowledgements

The authors would like to thank Emma Diethorn and Sophie Shifman for assistance. This work was supported by NIMH (R01MH112861, BAS).

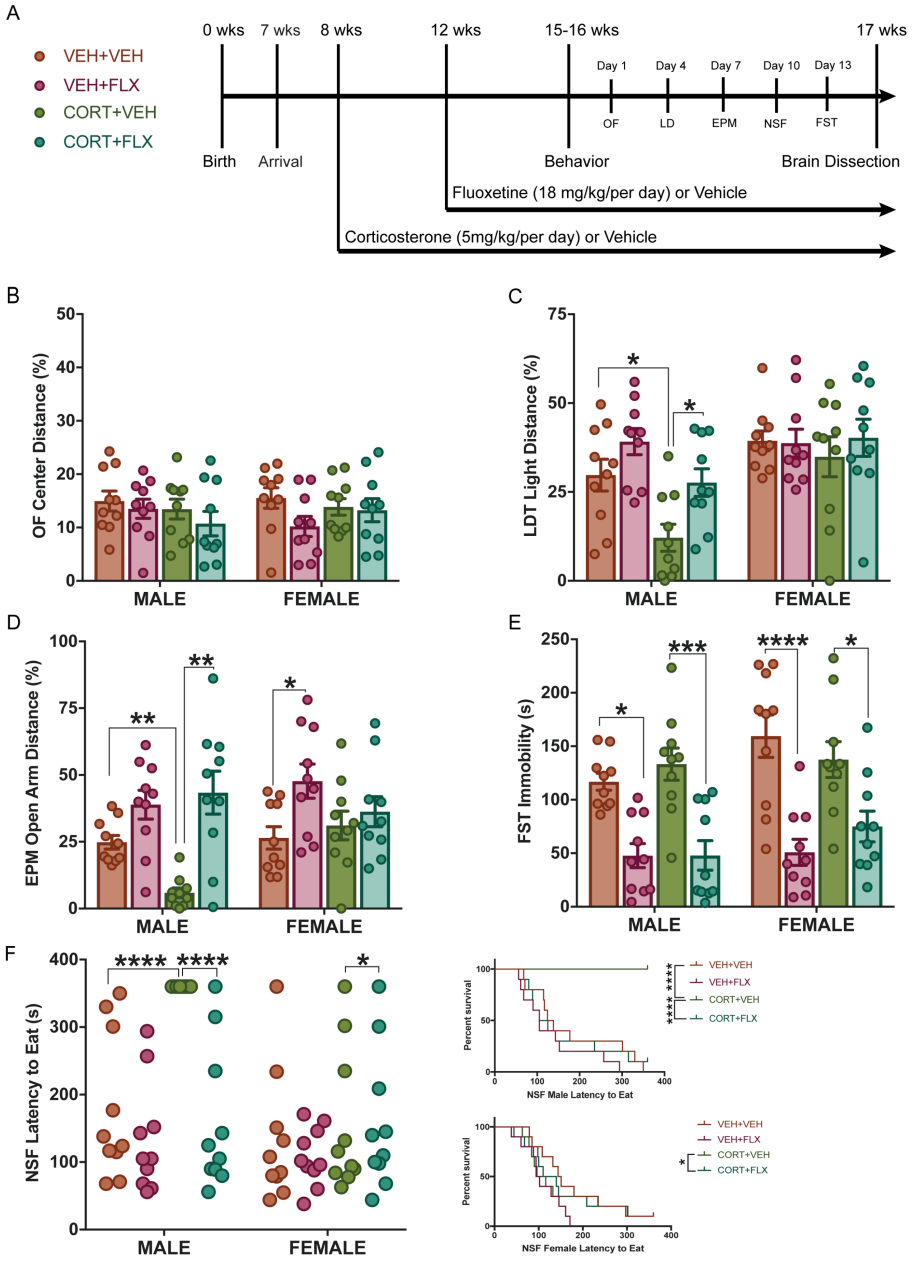


Figure 1.1. Chronic Corticosterone has Behavioral Effects in Males but not in Females. (A) Timeline of chronic CORT paradigm and keys for graphs. Panels (B-E) represent separate 2x2x2 ANOVAs with Bonferroni posthocs exploring effects of CORT and FLX within sex. (B) No effects of CORT or FLX were observed in percent of time in the center of the OF in either sex. (C) CORT affected the percentage of distance traveled in the light in males, but not in females. Males treated with CORT+VEH traveled less distance on the light side than any other group. (D)

In the EPM, CORT impacted distance traveled on the open arms in males, but not in females. Males administered COT+VEH had less distance traveled on the open arms than any other groups. In females, mice treated with VEH+FLX traveled more distance than any other female group. (E) FLX affected immobility time in the FST within both sexes. Males treated with FLX in presence of CORT or absence (VEH) were less immobile than groups without FLX. Similarly, FLX groups in females were less immobile than groups without FLX. Scatterplot and survival curves (F) of NSF data showing individual latency to eat values with Kaplan-Meier survival analysis in males. Males treated with CORT+VEH had a longer latency to eat than any other group. Females treated with FLX without CORT had a short latency to teat than any other group. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$

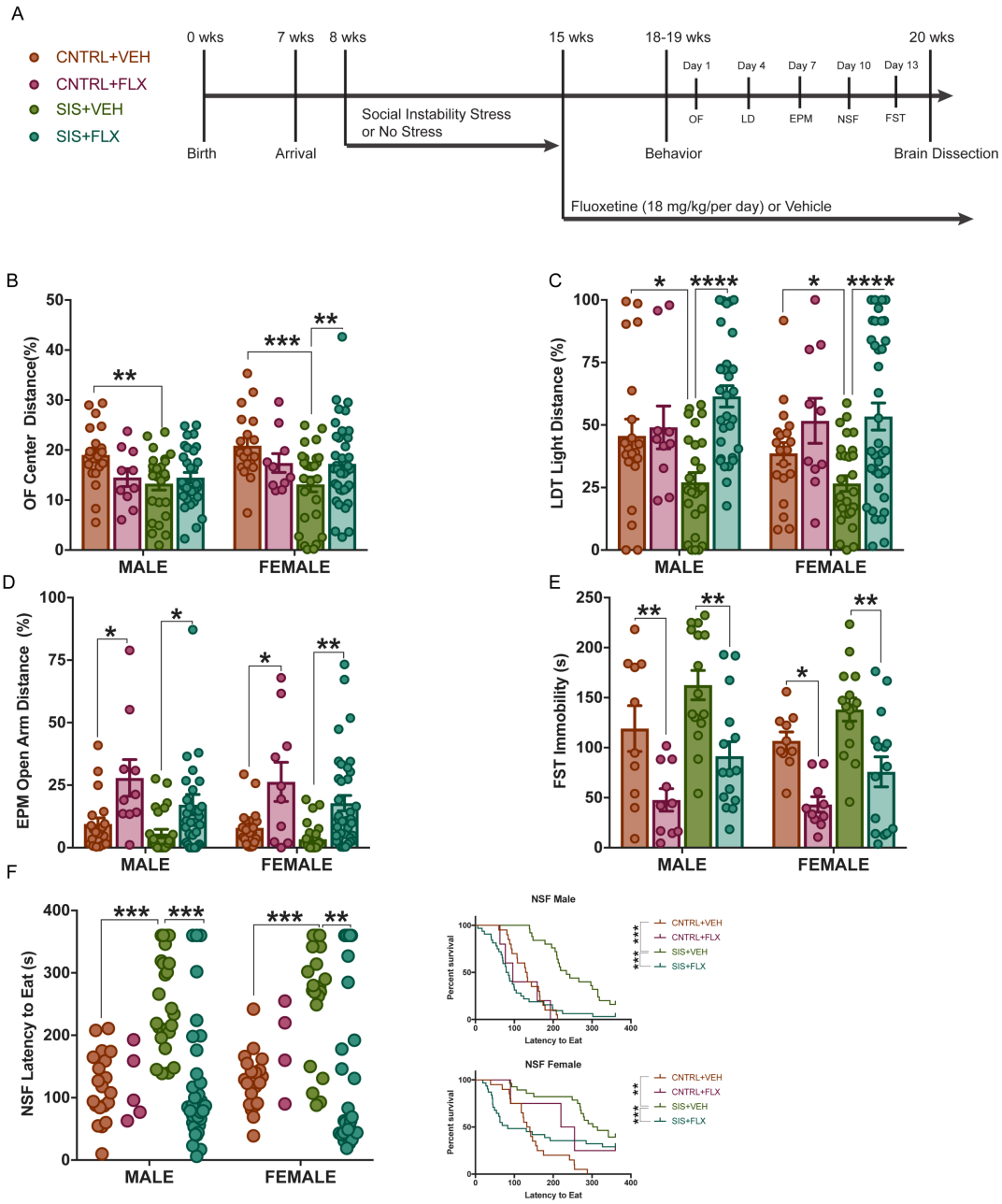


Figure 1.2. Behavioral Effects of Social Instability Stress (SIS) in Male and Female Mice.

(A) Timeline of SIS paradigm and keys for larger graphs. (B-E) panels represent Bonferroni posthoc comparisons from the 2x2x2 ANOVAs (SIS*x*SEX*x*FLX) examining effects of SIS and FLX within each sex. (B) SIS affected behavior in the OF with SIS+VEH mice of both sex traveling less distance in the center of the OF than CNTRL+VEH mice of the same sex. (C) An

interaction between SIS*x*FLX was observed in LDT behavior. Males treated with SIS+VEH traveled less in the light than any other group. Similarly, SIS+VEH females traveled less distance in the light than any other female group. (D) In the EPM, an interaction between SIS*x*FLX was also observed. Within males, SIS+VEH males traveled less on the open arms than any other male group. SIS+VEH females also explored the EPM open arms less than any other group. (E) Exposure to FLX affected immobility time in the FST in both sexes. FLX treated males had less immobility time than non-FLX treated males. Similarly, FLX treated females spent less time immobile than non-FLX treated females. Scatterplot and survival curves (F) of NSF data showing individual latency to eat values with Kaplan-Meier survival analysis with SIS+VEH mice having a longer latency to eat in both sexes. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$

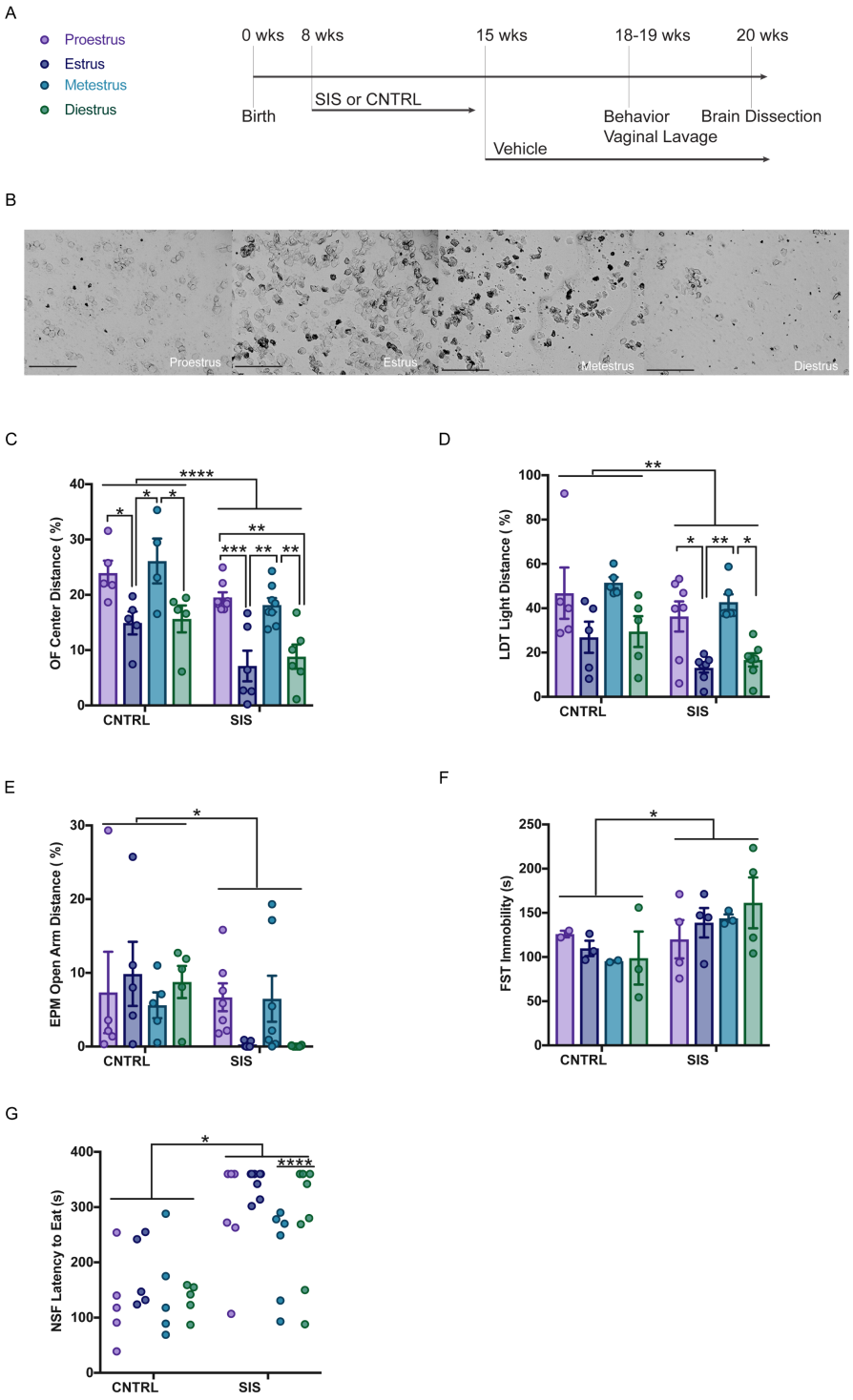


Figure 1.3. Impact of Estrous Cycle in Females on Behavior. Time line of experiment (A) and representative 10x images of vaginal smears (B). (C) Represent 2x4 ANOVAs (SIS*x*ESTROUS) in the OF with differences emerging between estrous phases within both SIS+VEH and CNTRL+VEH females, with estrous and diestrus mice traveling less than their metestrus and

proestrus counterparts. (D) Within SIS+VEH differences between the stages were still present in the LDT with estrous and diestrus females traveling less than proestrus and metestrus females (E-F) No differences existed across the phases within the EPM and FST. Scatterplot (G) form Kaplan-Meier survival analysis of latency to eat across estrous stages within the SIS group with estrous females taking longer to eat than any other group. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p<0.001$

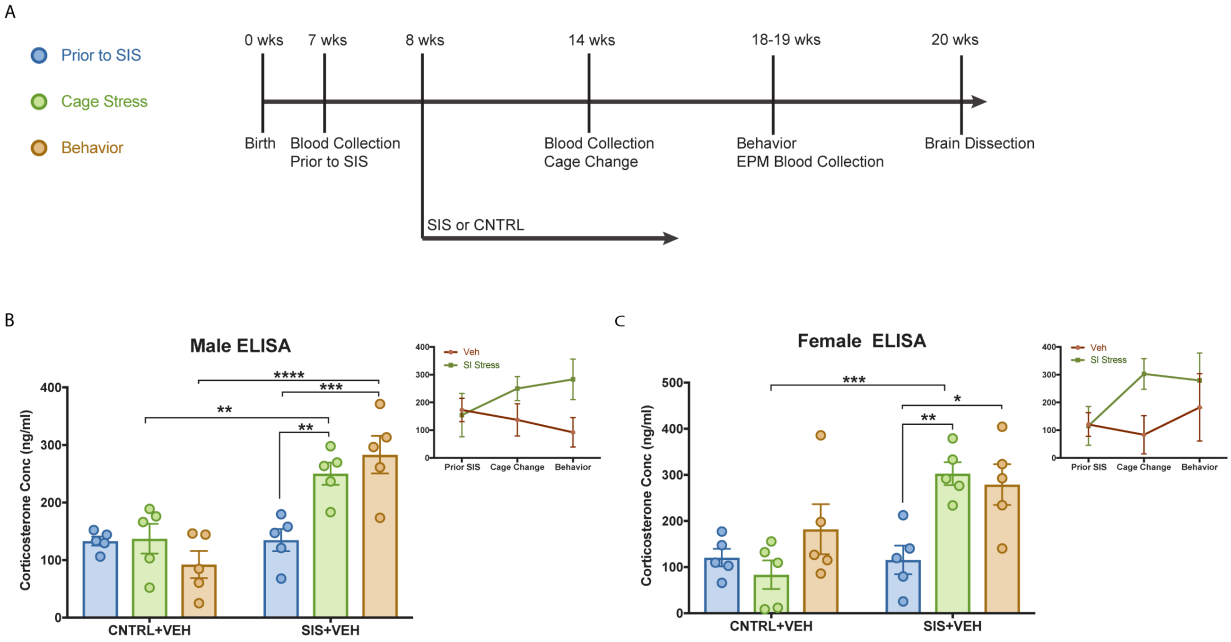


Figure 1.4. SIS Stress Increases Endogenous Corticosterone Levels in Males and Females. Timeline of experiment and legend for figures (A). Smaller panels (B-C) illustrate the changes in plasma corticosterone levels across blood collection time points. Larger panel (B) displays 2x3 ANOVAs (SIS*x*TIME) within males with plasma corticosterone levels increasing in SIS males in response to a cage change and exposure to EPM. Overall, SIS+VEH males have higher corticosterone levels than CNTRL+VEH males in response to both a cage change and EPM exposure. Similarly, in females, larger panel (C), SIS+VEH females experienced an increase in plasma corticosterone levels relative to baseline in response to both a cage change and EPM exposure. SIS+VEH females also had higher corticosterone levels than CNTRL+VEH females in response to a cage change. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$

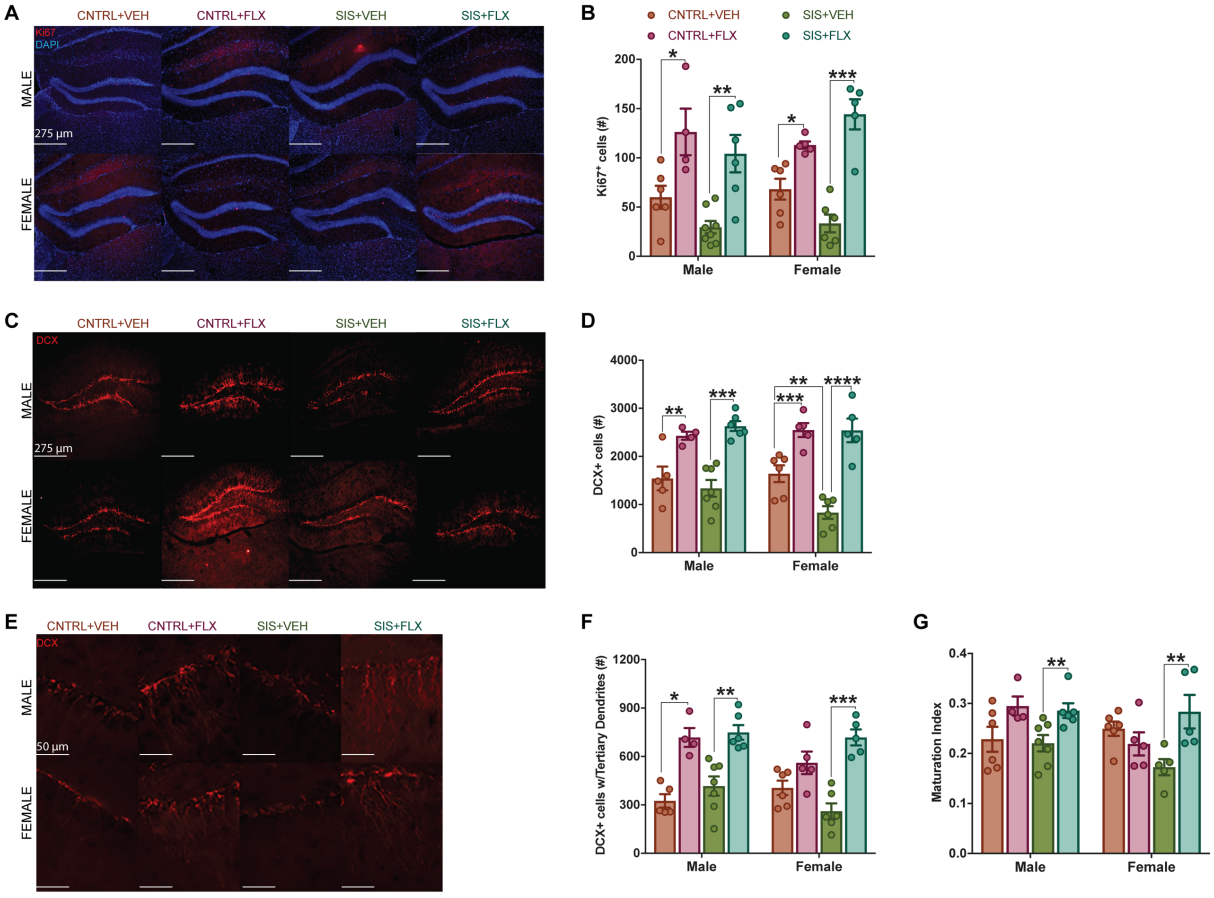
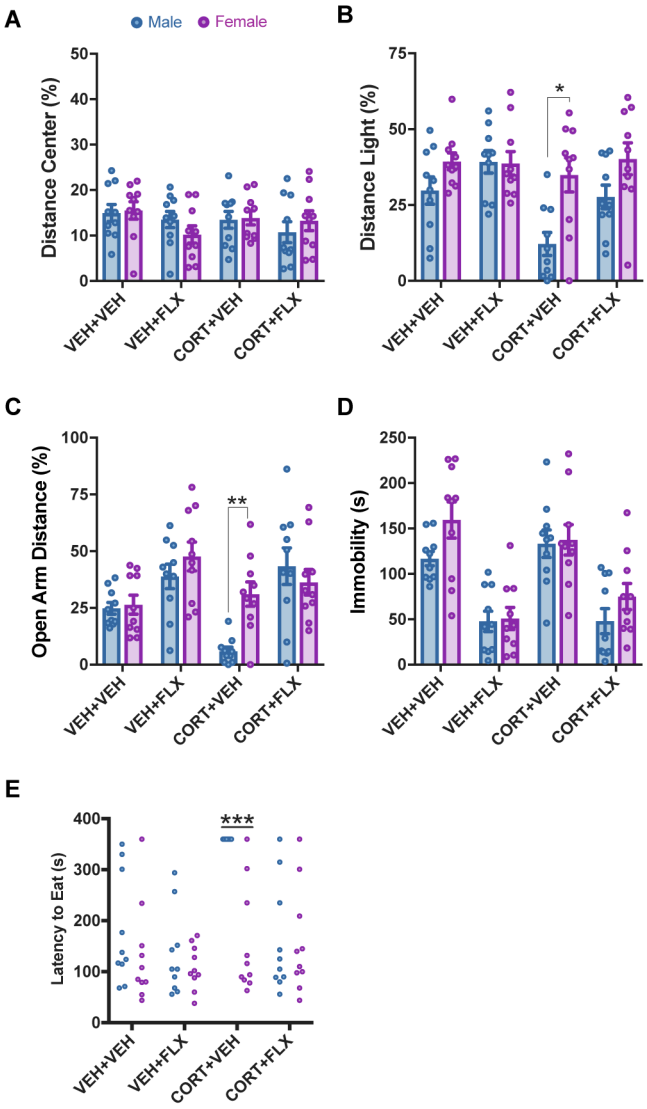
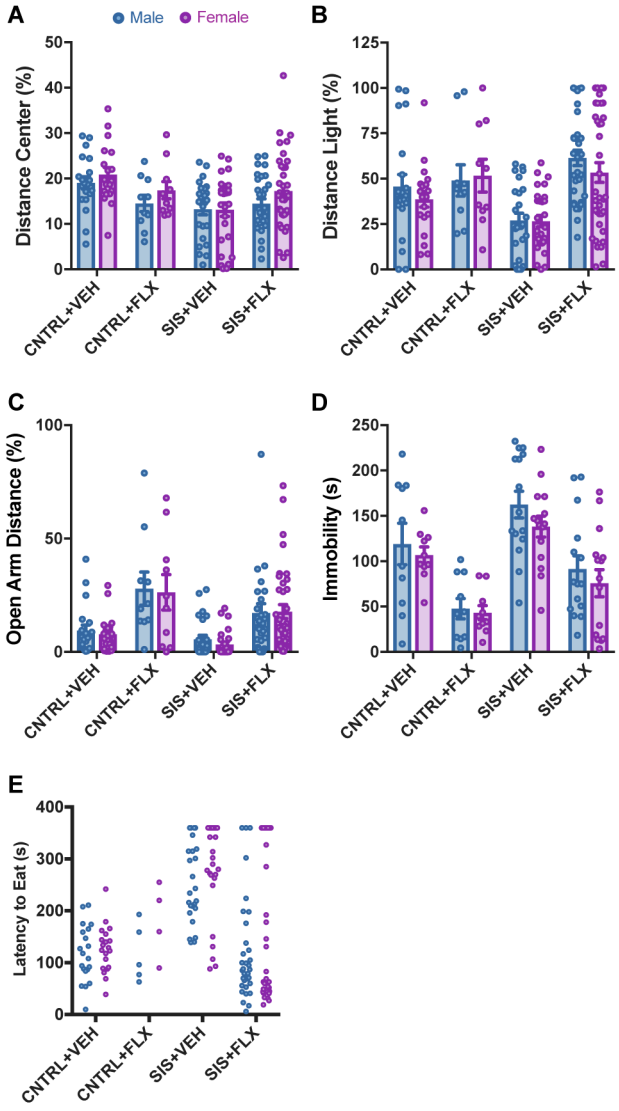


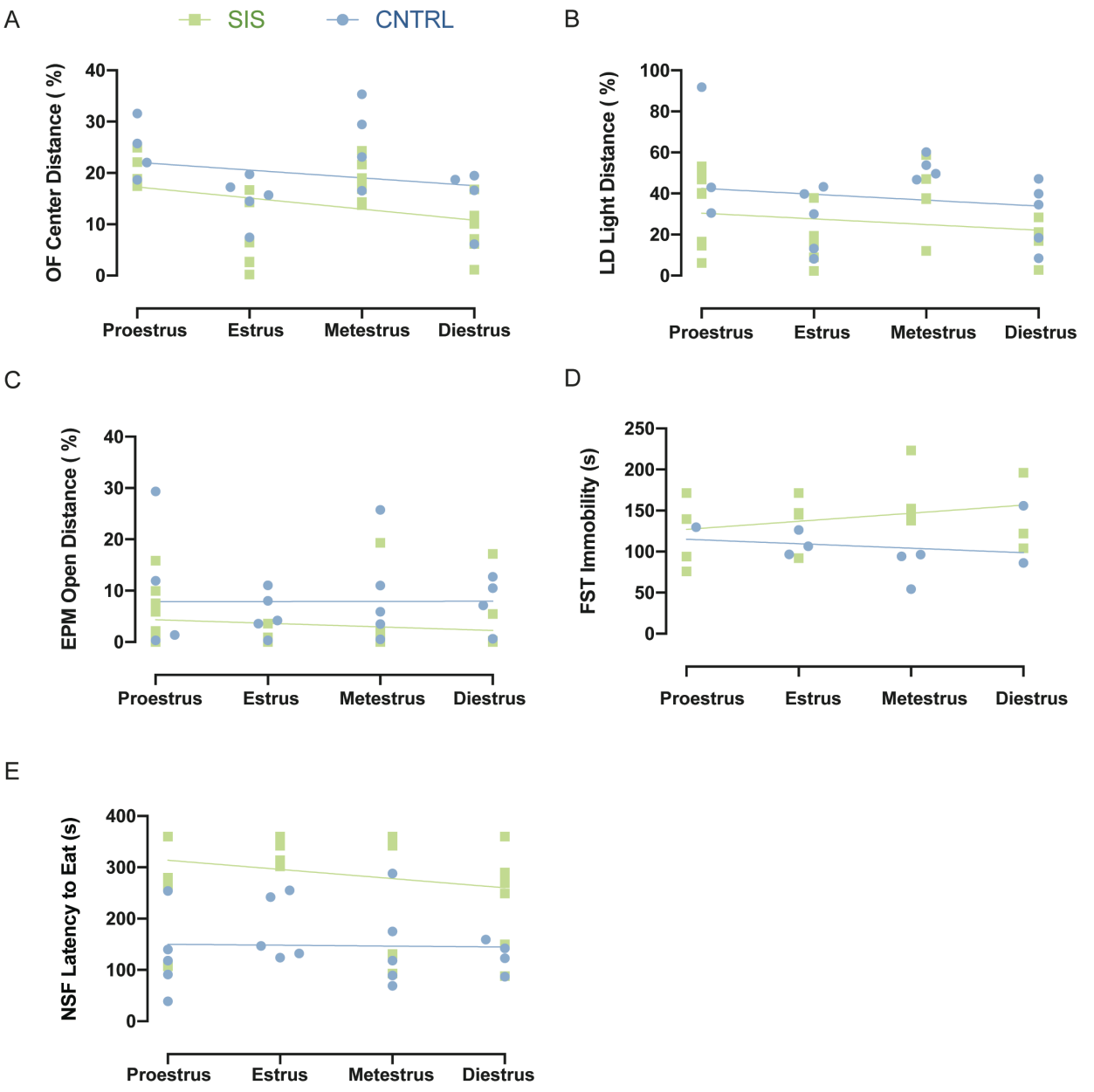
Figure 1.5. SIS Stress and FLX Effects on Adult Hippocampal Neurogenesis. Representative 10x images of Ki67 (A) and DCX (C), with 40x images used to quantify DCX^+ cells with tertiary dendrites (E). Graphs represent Bonferroni posthoc comparisons from the 2x2x2 ANOVAs (SIS*x*SEX*x*FLX), with no sex differences in adult hippocampal neurogenesis observed. In both sexes effects of FLX were seen with FLX treated mice having higher counts of $Ki67^+$, DCX^+, DCX^+ cells with tertiary dendrites, and maturation index. Within females SIS reduced expression of DCX and maturation index. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$



Supplemental Figure 1.1. (A-D) Represent sex differences within the CORT+VEH group observed from 2x2x2 ANOVAs (CORT*x*SEX*x*FLX). Specifically, CORT+VEH males traveled less in the light and less on the open arms than CORT+VEH females. Scatterplot (E) represents sex differences observed by Kaplan-Meier survival analysis in NSF latency to eat with CORT+VEH males having a longer latency to eat than CORT+VEH females. * 0.05> *p* >0.01; ** 0.01> *p* >0.001; ***p<0.001; **** p<0.0001



Supplemental Figure 1.2. (A-D) No sex differences in negative valence behaviors were observed within the SIS paradigm (2x2x2 ANOVAs SIS*x*SEX*x*FLX). Scatterplot (E) shows no sex differences in NSF latency to eat.



Supplemental Figure 1.3. Multiple regressions to assess if estrous cycle phase and SIS stress independently predict performance in negative valence tasks. (A-E) While both stress and estrous phase together predicted behavioral performance, only stress independently significantly contributed to this relationship.

CHAPTER 2

Chronic Non-Discriminatory Social Defeat is an Effective Chronic Stress Paradigm for both Male and Female Mice

The results from this study are published in Yohn, C. N.**, Dieterich, A., Bazer, A. S., Maita, I., Giedraitis, M., & Samuels, B. A. (2019). Chronic non-discriminatory social defeat is an effective chronic stress paradigm for both male and female mice. *Neuropsychopharmacology*, *44*(13), 2220-2229. **co-corresponding author

Abstract

Stress-related mood disorders are more prevalent in females than males, yet preclinical chronic stress paradigms were developed in male rodents and are less effective in female rodents. Here we characterize a novel chronic non-discriminatory social defeat stress (CNSDS) paradigm that results in comparable stress effects in both sexes. Male and female C57BL/6J mice were simultaneously introduced into the home cage of resident CD-1 aggressors for 10 daily 5-minute sessions. CD-1 aggressors attacked males and females indiscriminately, resulting in stress resilient and susceptible subpopulations in both sexes. CD-1 aggressors attacked C57BL/6J male intruders faster and more frequently than female intruders. However, CNSDS similarly induced negative valence behaviors in SUS mice of both sexes relative to RES and CNTRL mice. Furthermore, SUS male and female mice displayed similar increases in plasma corticosterone levels following CNSDS exposure relative to pre-stress exposure levels. The estrous cycle did not impact CD-1 attack behavior or negative valence behaviors. Thus, CNSDS induces chronic stress behavioral and neuroendocrine effects in both male and female C57BL/6J mice and allows direct comparisons between sexes. Adoption of this modified social defeat paradigm will help advance the initiative to include female rodents in preclinical chronic stress research.

Introduction

Stress-associated psychiatric disorders, such as anxiety and depression, occur twice as frequently in women than men (Kessler et al., 1994). However, pre-clinical research investigating stress effects often excludes female subjects (Beery & Zucker, 2011), possibly because female brain and behavior is impacted by fluctuations in gonadal hormones across the estrous cycle (Becker et al., 2005; Shansky, 2015; Yohn et al., 2018). However, the estrous cycle does not result in more variability in female behavioral, molecular, and physiological traits than what is observed in male rodents (Becker, Prendergast, & Liang, 2016; Prendergast, Onishi, & Zucker, 2014; Shansky, 2019). Sex differences in stress response are prevalent at all levels of biological organization, yet the majority of chronic stress paradigms used in rodents were designed for and are only effective in males (Berton, Aguerre, Sarrieau, Mormede, & Chaouloff, 1998; Calvo, Cecchi, Kabbaj, Watson, & Akil, 2011; David et al., 2009; Haller, Fuchs, Halasz, & Makara, 1999; Hodes et al., 2015; Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997; Meerlo, Overkamp, Daan, Van Den Hoofdakker, & Koolhaas, 1996; Trainor et al., 2011). Investigating the validity and reliability of chronic stress paradigms to produce stress susceptibility phenotypes in both sexes is necessary for understanding the etiology of stress-associated mood disorders.

One such chronic stress paradigm, chronic social defeat stress (CSDS), exposes experimental male mice to 10 daily resident-intruder bouts with an aggressor CD-1 male and results in both susceptible (SUS) mice, which display behavioral, neural, and hormonal alterations consistent with chronic stress, and resilient (RES) mice, which do not show these alterations (Berton et al., 1998; Calvo et al., 2011; Chaudhury et al., 2013; Koolhaas et al., 1997; Krishnan et al., 2007; Larrieu et al., 2017; Meerlo et al., 1996). However, when female mice are

exposed to CSDS, CD-1 male aggressors display mounting behaviors rather than aggression toward female intruders (DeBold & Miczek, 1984). Female resident aggressors do not produce the necessary bouts of attack characterizing CSDS, as female-female aggression is limited to territorial species such as Peromyscus californicus (Trainor et al., 2011) and prairie voles (Smith, Lieberwirth, & Wang, 2013), maternal aggression (Bosch & Neumann, 2010; Bourke & Neigh, 2012), or following ovariectomy (DeBold & Miczek, 1984). To circumvent this limitation, Takahashi and colleagues (Takahashi et al., 2017) demonstrated that chemogenetic activation of the ventral medial hypothalamus in CD-1 mice induces aggression toward female intruders and subsequent social avoidance behaviors in the females. Furthermore, Harris and colleagues (Harris et al., 2018) showed that application of male CD-1 urine to C57BL/6J female intruders induces attacks by resident male aggressors, resulting in social avoidance and decreased sucrose preference behaviors in the females. These methods, while clever and useful, require either difficult and time-consuming surgeries that target a deep structure or tedious urine collection.

Here we developed a chronic non-discriminatory social defeat stress (CNSDS) paradigm that is effective in both male and female C57BL/6J mice. We demonstrate that simultaneous exposure of both male and female sexually inexperienced intruder C57BL/6J mice to an aggressive resident CD-1 mouse yields aggression towards both C57BL/6J mice. Over the course of the 10-day CNSDS paradigm, the CD-1 repeatedly attacked both the male and female mice. These attacks resulted in behavioral and neuroendocrine responses in both sexes consistent with chronic stress exposure. This streamlined novel non-discriminatory social defeat paradigm will greatly facilitate the inclusion of female rodents in chronic stress research and permit direct comparisons between males and females.

METHODS

Subjects

8-week-old female and male C57BL/6J strain mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained on a 12L:12D schedule with lights coming on at 6 am and going off at 6pm, with food and water provided *ad libitum*. All behavioral testing took place in the morning, between the hours of 8 am and 11 am. All experiments were conducted in compliance with NIH laboratory animal care guidelines and approved by Rutgers University Institutional Animal Care and Use Committee.

Chronic Non-Discriminatory Social Defeat Stress (CNSDS)

To determine if female mice experience a similar stress outcome as the standard CSDS paradigm in males (Tsankova et al., 2006), both female and male mice were exposed to a novel, dual-sex stress protocol. Adult male and female C57BL/6J mice were randomly assigned to either chronic stress or no stress control groups. Retired CD-1 breeder adult males were purchased from Charles River Laboratory (Wilmington, MA). To screen for aggressive behavior, screener female and male C57BL/6J mice were simultaneously placed in the home cage of a CD-1 mouse. CD-1 mice that reliably attacked both female and male screener mice within 60 seconds on consecutive days were selected as aggressors (Golden, Covington, Berton, & Russo, 2011). 2 CD-1 mice were selected for each pair of experimental mice (one male and one female C57BL/6J mouse). During the experimental phase, experimental male C57BL/6J mice were matched with the same experimental female C57BL/6J mice throughout the duration of the social defeat. The male and female C57BL/6J pairs were placed into the home cage of novel CD-1 aggressors for 10 consecutive daily 5-minute sessions. Since our cages only permitted one cage divider, we did not cohouse the aggressor CD-1 with both the experimental male and

female C57BL/6J mice. Instead, on alternating days, either the male or female C57BL/6J mice were housed with either the CD-1 aggressor mouse with which they had interacted or a novel control aggressor CD-1 (previously screened for aggressive behavior). Co-housed subjects were separated by clear, perforated plexiglass that permitted sensory but not physical interaction. In total, both C57BL/6J male and female mice were exposed to 5 days of sensory exposure to the aggressor CD-1 and 5 days exposure to a novel aggressor CD-1 (previously screened for aggressive behavior). A separate group of female and male C57BL/6J mice were simultaneously placed into a standard cage and allowed to interact for 5 minutes in the absence of an aggressor CD-1 male over 10 days. These opposite sex control mice were housed on either side of a divider and placed on a separate rack from stress mice for 24 hours until the next interaction session.

To determine if the number of mice interacting affected control group behavior, we ran an additional cohort of controls that had three mice interacting (two males and one female; 2-male CNTRL) and compared the results to the original control group that had two mice interacting (one male and one female; 1-male CNTRL). The 2-male CNTRL group consisted of three mice interacting for 5 minutes per day for 10 consecutive days. Following the daily 5-minute interaction, male experimental mice were housed, separated by a divider, with the C57 male they just interacted with. The female experimental mice were then housed with an unfamiliar C57BL/6J male, separated by clear, perforated plexiglass.

Videos were recorded (Sony HDR-CX260) to measure attack latency and frequency, and mounting behavior. CD-1 mounting behavior toward female mice occurred in 8% of the interactions, with an average attack latency of 121 sec for RES and 150 sec for SUS female mice. In males, the CD-1 mice displayed mounting behaviors in 3% of the interactions, with an

average attack latency of 87 sec for RES and 76 sec for SUS male mice. No mounting by the CD-1 led to pregnancy in any of the C57BL/6J females throughout the entire course of the experiment. In total 30 male and 30 female C57BL/6J mice were exposed to the CNSDS paradigm, which resulted in RES (male = 13; female = 11) and SUS (male = 17; female = 19) phenotypes. Additionally, 15 male and 15 female C57BL/6J mice were assigned to the CNTRL group.

Vaginal Lavage

To assess estrous cycle state, daily vaginal lavages were performed two weeks prior to stress exposure to ensure mice were cycling throughout all four stages regularly. Subsequent to daily 5-minute aggressive bouts and after completing each behavioral test or blood collection, vaginal smears were collected via a pipette filled with ddH_2O, placed at the opening of the mouse's vaginal canal (without penetration) with ddH_2O gently expelled and suctioned back into the pipette tip (Byers, Wiles, Dunn, & Taft, 2012; McLean, Valenzuela, Fai, & Bennett, 2012). Samples were imaged under an EVOS FL Auto 2.0 microscope (Thermo Fisher Scientific, Waltham, MA) at 10x magnification. Estrous phases were identified by the presence or absence of nucleated epithelial cells, cornified epithelial cells, and leukocytes (Byers et al., 2012; Felicio, Nelson, & Finch, 1984). Proestrus mice had mostly nucleated and some cornified cells, estrus predominantly displayed cornified epithelial cells, metestrus had cornified epithelial cells and polymorphonuclear leukocytes (Byers et al., 2012), while diestrus contained polymorphonuclear leukocytes. No differences in length of estrous cycle was observed 10 days before stress or the 10 days during stress between the CNTRL and CNSDS mice (Supplemental Figure 2.3A). Additionally, RES and SUS mice had similar cycle lengths both during the 10 days before stress and 10 days after stress (Supplemental Figure 2.3B).

Behavioral Testing

Social Interaction Test (SIT)

To assess responsiveness to social defeat, we ran the SIT to analyze social interaction and avoidance behavior in the presence of a novel CD-1 mouse. Mice were placed in an open field (OF) arena for two consecutive 2.5-minute trials. The first trial had the CD-1 absent, with the CD-1 present in a perforated Plexiglas container within the social interaction zone (14cm*x*24cm) during the second trial (Golden et al., 2011; Tsankova et al., 2006). Overhead cameras recorded behavior, and EthoVision software (Noldus, Wageningen, Netherlands) measured time spent in the interaction zone. Social avoidant and interaction behavior were measured by time spent in the interaction zone during the first (CD-1 absent) and second (CD-1 present) trials, with an interaction ratio calculated: ((interaction time, CD-1 absent) / (interaction time, CD-1 present)).

Light Dark (LDT)

Within OF arenas, dark plastic rectangular boxes (opaque to visible light, but transparent to infrared light) covering 1/3 of the arena, were inserted to separate the arena into light and dark compartments. The dark box contained an opening that allowed passage between the light and dark (David et al., 2009), with the light compartment brightly illuminated (1000 lux; Kinder Scientific). At the beginning of each 5-minute test, mice were placed in the dark compartment, with distance traveled in the light compartment ((distance traveled in the light compartment/total distance) *100) and time in the light compartment used for analyses.

Elevated Plus Maze (EPM)

The EPM test consisted of a plus-shaped apparatus with two open and two closed arms (side walls), elevated 2 feet above the floor. During the 5-minute test, the mice were recorded

from a video camera mounted on the ceiling above each EPM arena. EthoVision software (Noldus, Wageningen, Netherlands) was used to quantify distance traveled in the open arms ((total open arm distance/total distance traveled) *100), and time on open arms used for analyses. EthoVision detected when 2 paws crossed a transition.

Novelty Suppressed Feeding (NSF)

Mice were food deprived for 18 hours in their home cage prior to being placed in the corner of a novel, brightly-lit (1500 lux) testing apparatus (50x50x20 cm) filled with 2 cm of corncob bedding, and a single food pellet attached to a white platform in the center. The NSF lasted 6 minutes with latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) recorded. Mice that timed-out were assigned a latency of 360 secs. Immediately after, mice were transferred to their home cages and given ad libitum access to a food pellet for 5 minutes. Latency to eat and amount of food consumed was measured as a control for feeding behavior observed in the NSF.

Sucrose Preference Test (SPT)

Mice were habituated to a 1% sucrose solution in their home cage for 2 days. Next, a bottle containing water and a bottle containing 1% sucrose were placed side-by-side and switched every 12 hours for 3 days. Consumption of both water and sucrose bottles were measured and compared to determine preference for a 1% sucrose solution. Preference was calculated as 1% sucrose consumed divided by total consumption.

Blood Collection and Corticosterone ELISA

Mice were weighed to ensure that non-terminal blood collection was no more than 1% of the mouse's body weight. Prior to stress, baseline blood samples were collected from the left retro-orbital sinus of all experimental mice, in accordance with IACUC guidelines. To measure

corticosterone levels in response to CNSDS, blood was collected from the right retro-orbital sinus 40-45 minutes after the 10th day of CNSDS. CNTRL mice had blood collected from the right retro-orbital sinus 40-45 minutes after the 10th day of opposite sex exposure. For each blood collections, blood was collected in microcentrifuge tubes coated with EDTA. Plasma was isolated from whole blood by centrifugation at 14000 rpm for 10 min at 4 °C, with supernatant collected and stored at - 80°C until assayed. Total yield of plasma per blood collection was between 25 to 40 μL. To assess differences in plasma corticosterone in response to CNSDS, baseline levels were compared to samples after CNSDS) across 6 mice per sex per stress susceptibility phenotype (male: CNTRL=6, SUS=6, RES=6; female: CNTRL=6, SUS=6, RES=6). Each sample (total n=72 across all time-points) was diluted 1:100 and assayed in triplicate according to the manufacturer's protocol (Arbor Assays Corticosterone ELISA Kit).

Statistical Analyses

To investigate sex differences in CD-1 attack behavior (attack frequency and attack latency) independent samples t-tests were used. Separate chi-square analyses were used to assess if frequency of attack behavior was different between sex and estrous cycle phases. To assess sex and stress susceptibility effects 2x3 analysis of variance (ANOVA) with Bonferroni post-hoc comparisons were used to analyze behavioral results from SIT, LD, EPM, Sucrose preference, and Emotionality Index. Prior to conducting each ANOVA, we ran a Sharpio-Wilk normality test. Each ANOVA reported in the manuscript passed the Sharpio-Wilk (p-values > 0.05), which justified usage of parametric analyses. When there was no main effect of sex in the ANOVAs, the mice were collapsed and Bonferroni post-hoc comparisons were used to compare stress susceptibility phenotype groups. Planned Bonferroni post-hoc comparisons (based on the collapsed mice) were then used to investigate effects with each sex. To investigate differences in

stress susceptibility across the estrous cycle 3x4 ANOVAs were used for all behaviors except NSF. Since NSF data fails to meet basic assumptions of normality, Kaplan-Meier survival analysis (nonparametric test) was used. Lastly, to analyze differences in endogeneous corticosterone levels, time point of blood collection, sex, and stress susceptibility were used to conduct a 3x2x2 repeated measures ANOVA. Bonferroni post-hocs in collapsed mice and planned comparisons with each sex were then performed as was done for behavior analyses. GraphPad Prism 7 was used for all analyses.

RESULTS

CNSDS induces Susceptible and Resilient Stress Susceptibility Phenotypes and Negative Valence Behaviors in both Males and Females

To determine whether simultaneous exposure of C57BL/6J male and female intruder mice to resident CD-1 aggressors results in attacks of both sexes, we modified a standard CSDS protocol. To this end, C57BL/6J male (n=30) and female (n=30) mice were exposed simultaneously to male CD-1 aggressor mice for 10 daily 5-minute aggressive bouts. Non-stressed control (CNTRL) C57BL/6J male (n=15) and female (n=15) mice experienced 5-minute daily interactions with each other and were then co-housed (Figure 2.1A). Independent samples t-tests revealed sex differences in CD-1 attack latency ($t(28)=3.85$, $p=0.006$) and number of attacks ($t(28)=3.7$, $p=0.009$; Figure 2.1B), with males on average being attacked more quickly and frequently across the 10 days than females. We next analyzed attack and mounting behaviors from resident CD-1 males on both male (n=30) and female (n=30) C57BL/6J intruder mice (n=60). Male C57BL/6J mice were attacked in a total of 83.77% and were mounted in a total of 2.6% of their interactions with CD-1 aggressors (Figure 2.1C). 14.94% of resident-intruder interactions resulted in neither aggression nor mounting of the male C57BL/6J mice (Figure

2.1C). Female C57BL/6J mice were attacked in a total of 64.33%, mounted in 7.64%, and neither in 31.21% of these interactions (Figure 2.1C). A chi-square test revealed a significant sex difference (X^2=15.75, p<0.0001), with males being attacked more frequently than females. Estrous cycle stage in females did not impact resident-intruder aggression by the CD-1 aggressor (X^2=9.03, p=0.434; Figure 2.1C). In addition, 71.34% of the CD-1 aggressors attacked both males and females (Figure 2.1D), while just 16.56% and 9.55% of CD-1 aggressors attacked males or females only, respectively. Taken together, these data demonstrate that in most CNSDS interactions CD-1 aggressors will attack both the male and female C57BL/6J mice.

Similar to classic CSDS paradigms, we next used a social interaction test (SIT) to assess stress susceptibility in a new cohort of CNSDS and CNTRL male and female C57BL/6J mice (Figure 2.2A-B). An interaction ratio ((interaction time with aggressor present) / (interaction time with aggressor absent)) score of less than 1 resulted in a susceptible (SUS) classification, whereas resilient (RES) mice had an interaction ratio greater than 1 (Krishnan et al., 2007; Razzoli, Carboni, Andreoli, Ballottari, & Arban, 2011). A 2x3 ANOVA (sex x stress susceptibility) was used to analyze stress susceptibility phenotype and sex differences in SIT behavior between SUS, RES, and CNTRL mice. This 2x3 ANOVA revealed no main effect of sex within SIT (p=0.38). By contrast, a main effect of stress ($F_{(2, 83)}$=23.21, p<0.001) was observed. Therefore, given that no sex differences were observed, we first collapsed all mice and ran Bonferroni post-hoc comparisons (Figure 2.2B left), which showed that SUS mice had a lower interaction ratio than RES (p<0.0001) and CNTRL (p=0.0003; Figure 2.2B left) mice. We next ran planned Bonferroni post-hoc comparisons (based on the collapsed comparisons) in both males and females. These separate planned Bonferroni post-hoc comparisons within sex showed that SUS males had a lower interaction ratio than RES (p<0.001) and CNTRL (p=0.036) males

(Figure 2.2B right) and that SUS females had a lower interaction ratio than RES (p<0.001) and CNTRL (p=0.005) females (Figure 2.2B right).

Similar to our observations in Figure 2.1, 2x2 ANOVAs (sex x stress susceptibility) revealed an effect of sex in average attack latency ($F_{(1, 26)}$=16.99, p=0.0003; Supplemental Figure 2.1A small panel) and total number of attacks ($F_{(1, 26)}$=14.65, p=0.0007; Supplemental Figure 2.1B small panel) by CD-1 aggressors across the 10 days, with SUS males on average attacked more quickly (p=0.0017; Supplemental Figure 2.1A large panel) and more frequently (p=0.0053; Supplemental Figure 2.1B large panel) than SUS females. Nevertheless, similar to other findings in classic CSDS paradigms (Krishnan et al., 2007), no main effect of stress susceptibility phenotype on attack latency (p=0.51) or number of attacks (p=0.15) across the 10 days existed between SUS and RES mice within males and within females (Supplemental Figure 2.1A-B large panel).

We next subjected the CNSDS and CNTRL mice to behavioral tests that are affected by classic CSDS, including LDT, EPM, SPT, and NSF (Figure 2.2A). A 2x3 ANOVA (sex x stress susceptibility) revealed no main effect of sex within LDT (light time p=0.51; light distance p=0.37), EPM (open arm time p=0.61; percent distance in open arms p=0.91), or sucrose preference (p=0.91). Within the LDT a significant main effect of stress was observed in time spent ($F_{(2, 84)}$=16.64, p<0.001) and distance traveled ($F_{(2, 84)}$=14.79, p<0.001) in the light, with SUS mice spending less time (RES: p<0.0001; CNTRL: p=0.0006; Figure 2.2C left) and traveling less distance (RES: p<0.0001; CNTRL: p<0.0001; Figure 2.2D left) than RES and CNTRL mice. Planned Bonferroni post-hoc comparisons revealed SUS males spent less time (p=0.002; Figure 2.2C right) and traveled less in the light (p=0.003; Figure 2.2D right) than RES males, while SUS females spent less time (RES: p<0.001; CNTRL: p<0.001; Figure 2.2C right)

and traveled less (RES: $p<0.001$; CNTRL: $p=0.01$; Figure 2.2D right) in the light than RES and CNTRL females. Significant effects of stress were also found in time spent ($F_{(2, 83)}=15.33$, $p<0.001$) and percent distance traveled ($F_{(2, 83)}=15.7$, $p<0.001$) in EPM open arms, with SUS mice spending less time (RES: $p<0.0001$; CNTRL: $p=0.0018$; Figure 2.2E left) and traveling less on the EPM open arms (RES: $p<0.0001$; CNTRL: $p=0.0002$; Figure 2.2F left) than RES and CNTRL mice. Planned Bonferroni post-hoc comparisons indicated that SUS males spent less time (RES: $p=0.003$; CNTRL $p=0.047$; Figure 2.2E) and traveled less (RES: $p=0.004$; CNTRL $p=0.015$; Figure 2.2F) in EPM open arms than RES and CNTRL males and that SUS females also spent less time (RES: $p<0.001$; CNTRL $p=0.035$; Figure 2E right) and traveled less (RES: $p<0.001$; CNTRL $p=0.045$; Figure 2.2F right) than RES and CNTRL females in the EPM open arms. There was also a significant effect of stress ($F_{(2, 80)}=4.79$, $p=0.011$; Figure 2.2G) in sucrose consumption, with SUS mice having a lower preference for 1% sucrose solution than RES mice ($p=0.0125$; Figure 2.2G left). Planned Bonferroni post-hoc comparisons revealed that this effect was observed in females, with SUS females showing less preference for a 1% sucrose solution than RES females ($p=0.044$; Figure 2.2G right). Lastly, in the NSF, Kaplan-Meier survival analyses between the stress susceptibility phenotypes showed a significant effect of stress susceptibility phenotype (X^2 (2)=20.53, $p<0.0001$), with SUS mice having a longer latency to eat than RES (X^2 (1)=17.75, $p=0.0035$) and CNTRL (X^2 (1)=14.94, $p=0.0065$) mice (Figure 2.2H left) with both sexes collapsed. We also examined the effects of CNSDS on NSF within each sex. SUS males had a longer latency to eat than RES ($X^2(1)=8.51$, $p=0.0035$; Bonferroni corrected) and CNTRL ($X^2(1)=7.4$, $p=0.0065$; Bonferroni corrected) males (Figure 2.2H right) and SUS females had a longer latency to eat than RES ($X^2(1)=6.81$, $p=0.0009$; Bonferroni

corrected) and CNTRL ($X^2(1)=6.4$, $p=0.01$; Bonferroni corrected) females (Figure 2.2H right). No differences in home cage feeding behavior were observed (Supplemental Figure 2.3C).

Next, we used an emotionality index to assess CNSDS-induced behavioral differences across LD, EPM, Sucrose Preference, and NSF. As previously described (Guilloux, Seney, Edgar, & Sibille, 2011), this emotionality index calculates z-scores for each behavioral test (LD, EPM, Sucrose, NSF) by normalizing individual mice against CNTRL averages and the standard deviation for that specific behavior. This allows a comprehensive analysis of multiple behavioral modalities. A score above zero represents a mouse with less time and distance in the light of the LD, less time and distance on EPM open arms, low preference for 1% sucrose solution, and longer latency to feed in the NSF task relative to CNTRL. A 2x3 ANOVA showed that there was a significant effect of stress susceptibility phenotype ($F_{(2,84)}=43.34$, $p<0.0001$), but no main effect of sex (p=0.085). Bonferroni post-hoc comparisons showed that SUS mice have a higher emotionality index than RES ($p<0.0001$) and CNTRL ($p<0.0001$) with all mice collapsed (Figure 2.2I left). Planned Bonferroni post-hoc comparisons within sex showed that SUS males have a higher emotionality index score than CNTRL ($p=0.0001$) and RES ($p<0.0001$) males (Figure 2.2I right). Similarly, SUS females had a higher emotionality index score than CNTRL ($p<0.0001$) and RES ($p<0.0001$) females (Figure 2.2I right).

To further assess the relationship between stress susceptibility phenotype and the other behaviors, separate correlations revealed that SIT ratio is significantly related to LD light time (r=0.387, $p=0.0022$; Figure 2.2J), EPM open arm time (r=0.519, $p<0.0001$; Figure 2.2J), and NSF latency to eat (r=-0.393, $p=0.0019$; Figure 2.2J) when both sexes were collapsed. There was not a significant relationship between SIT ratio and sucrose preference (r=0.216, $p=0.096$). We also examined the effects of CNSDS on these behavior correlations within each sex. SIT ratio in

males was related to EPM open arm time (r=0.625, p=0.0002; Figure 2.2K), and NSF latency to eat (r=-0.496, p=0.0089; Figure 2.2K). In females, SIT ratio was significantly related to LD light time (r=0.482, p=0.007; Figure 2.2L) and EPM open arm time (r=0.418, p=0.021; Figure 2.2L).

We also ran an additional control group in which three mice interacted (two males and one female; 2-male CNTRL) and compared the effects on behavior to what was observed when two mice interacted (one male and one female; 1-male CNTRL) (Supplemental Figure 2.2). We found no differences in any of the behavioral tasks (Supplemental Figure 2.2B-I).

Taken together, these results demonstrate that the CNSDS paradigm effectively induces susceptible and resilient behavioral phenotypes in both males and females. Both SUS male and SUS female mice displayed increases in negative valence behaviors relative to RES and CNTRL mice and there were no sex differences in negative valence behaviors or sucrose preference. Thus, the CNSDS paradigm yields comparable stress effects on behavior in both sexes.

Estrous Cycle does not Impact Stress Susceptibility Phenotype

Within females, the behavioral results were not impacted by the estrous cycle since 3x4 ANOVAs (stress susceptibility x estrous cycle phase) revealed no main effect of estrous cycle phase in SIT (p=0.159), LDT (p=0.472), or EPM (p=0.105) (Figures 2.3A-D). Kaplan Meier analysis also showed no difference in latency to eat in NSF across estrous (p=0.768) (Figure 2.3E). Furthermore, when analyzing potential effects of the four stages of estrous on behavior, the main effect of stress susceptibility persisted in SIT ratio ($F_{(3, 33)}$=6.44, p=0.004; SUS vs. RES p<0.001; SUS vs CNTRL p=0.018 Bonferroni corrected), LDT time in light ($F_{(3, 33)}$=7.62, p=0.002; SUS vs. RES p<0.001; SUS vs CNTRL p=0.007 Bonferroni corrected), and EPM time in open arms ($F_{(3, 33)}$=6.65, p=0.004; SUS vs. RES p<0.001; SUS vs CNTRL p=0.041 Bonferroni corrected) (Figures 2.3B-D). Kaplan-Meier analysis also revealed stress susceptibility

phenotype differences in NSF latency to eat ($X^2(11)=57.69$, $p<0.001$; SUS vs. RES $p=0.01$; SUS vs CNTRL $p=0.01$ Bonferroni corrected) (Figure 2.3E). No significant differences were observed in home cage feeding behavior between estrous phases and stress susceptibility phenotype (Supplemental Figure 2.3D). These data indicate that the estrous cycle does not impact stress susceptibility or behavior in stressed and non-stressed freely cycling female C57BL/6J mice. Furthermore, CNSDS did not affect the estrous cycle. CNSDS-exposed and CNTRL females had similar cycle lengths prior to and during either CNSDS or CNTRL exposure (Supplemental Figure 2.3A). RES and SUS females also had similar cycle lengths both prior to and during CNSDS exposure (Supplemental Figure 2.3B).

CNSDS Increases Plasma Corticosterone Levels

Increases in HPA axis activation are found in response to CSDS stress (Hammels et al., 2015; A. Keeney et al., 2006; A. J. Keeney, Hogg, & Marsden, 2001). Therefore, we next evaluated whether the CNSDS paradigm elicited increases in plasma corticosterone levels, with blood collected before CNSDS (baseline) and again on the final day of CNSDS exposure, following the last 5-minute interaction (defeat; Figure 2.4A). To analyze differences between sex and stress susceptibility phenotype (RES, SUS, CNTRL) across the two blood collection time points (baseline, defeat) a repeated measures 2x2x3 ANOVA revealed significant main effects of stress ($F_{(2, 60)}=15.6$, $p<0.0001$) and time point $F_{(1, 60)}=17.17$, $p=0.0001$), with a significant interaction between stress susceptibility phenotype and blood collection time point ($F_{(2, 60)}=10.86$, $p<0.0001$). There were no significant effects of sex ($p=0.215$) on plasma corticosterone levels. Bonferroni post-hoc comparisons demonstrated that CNTRL mice had lower corticosterone levels than RES ($p=0.0001$) and SUS ($p<0.0001$) following exposure to CNSDS (Figure 2.4B) when all mice were collapsed. Additionally, corticosterone levels

following CNSDS interaction were significantly different relative to baseline in both SUS (p<0.0001) and RES (p=0.0059; Figure 2.4B). We next ran planned Bonferroni post-hoc comparisons within each sex. In males, CNTRL mice had lower corticosterone levels than SUS (p<0.0001) and RES (p<0.0001) males following the final CNSDS interaction (Figure 2.4C), and CNSDS increased corticosterone levels relative to baseline in both SUS (p=0.0018) and RES (p=0.0098) males (Figure 2.4C). In females, SUS mice had higher corticosterone plasma levels than RES (p=0.0043) and CNTRL (p=0.0002) females following the final CNSDS interaction (Figure 2.4D), and CNSDS significantly increased corticosterone levels between baseline and defeat only in SUS females (p=0.0018; Figure 4D). RES females subjected to CNSDS did not show an increase in corticosterone levels relative to their baseline (p=0.99). Taken together, these data indicate that CNSDS results in a significant increase in HPA axis activation in both SUS males and SUS females as well as in RES males.

DISCUSSION

Several chronic stress paradigms that are used in rodents are ineffective in females. One such example is chronic social defeat stress. Recently described modifications that involve chemogenetics or application of male urine to females have permitted usage of social defeat in females. However, these clever modifications require difficult surgeries or tedious urine collection (Harris et al., 2018; Takahashi et al., 2017). Here we develop a streamlined novel non-discriminatory social defeat paradigm (CNSDS), that, while similar to CSDS, induces chronic stress behavioral and neuroendocrine phenotypes in both males and females of the widely-used C57BL6/J strain. CNSDS has face validity as it permits stratification of stressed male and female mice into SUS and RES groups. This stratification is similar to the stress response observed in males in classic CSDS (Golden et al., 2011; Razzoli et al., 2011) and in females in modified

CSDS paradigms (Harris et al., 2018; Takahashi et al., 2017). Therefore, the CNSDS paradigm successfully induces chronic stress behavioral phenotypes in both males and females.

To the best of our knowledge, the only other chronic stressor that simultaneously exposes males and females to stress is the vicarious social defeat paradigm (Iniguez et al., 2018; Sial, Warren, Alcantara, Parise, & Bolanos-Guzman, 2016). Within this paradigm, male C57BL/6J mice undergo physical defeat stress, while female mice experience emotional stress through exposure (separated by a divider) to the aggressive bouts displayed from CD-1 aggressors to C57BL/6J mice. However, unlike CNSDS, in which the same physical stressor is applied to both sexes, comparison between the sexes in vicarious social defeat is difficult because the males experience physical stress and females experience emotional stress. Although female-to-female aggression occurs infrequently, a recent study showed that housing intact swiss webster (CFW) female mice with a castrated male resulted in CFW females displaying aggressive behavior to intruding C57BL/6J female mice (Newman et al., 2019). These defeated females displayed reduced social interaction, which was then reversed by ketamine treatment. This paradigm is very interesting, but one advantage that CNSDS offers is that it permits some direct comparisons of chronic stress responses between sexes, as both males and females are being exposed to the same aggressor and experience simultaneously.

Male-to-female aggression is rare in classic CSDS, as resident CD-1 males are more likely to display mounting behaviors against female intruders (DeBold & Miczek, 1984). However, CD-1 males attack male intruders (Golden et al., 2011; Miczek, Maxson, Fish, & Faccidomo, 2001; Van Loo, Van Zutphen, & Baumans, 2003). In CNSDS, CD-1 aggressors attack both males and females possibly because of a generalized aggressive response to the male intruders. Although the CD-1 aggressors attacked males more frequently and with a shorter

latency than females, the attacks of the females were sufficient to induce RES and SUS behavioral and neuroendocrine phenotypes similar to what was observed in males. Thus, similar to what is observed when comparing SUS and RES mice in traditional CSDS (Krishnan et al., 2007), the number of attacks and latencies to attack do not impact stress susceptibility phenotypes when comparing males and females.

Overall the CNSDS paradigm affected both sexes similarly in the LDT, EPM, NSF, and SPT. SUS male and female mice displayed increases in negative valence behaviors associated with anxiety relative to RES and CNTRL same sex counterparts. However, CNSDS also resulted in a significantly increased sucrose preference in RES females relative to SUS females, a finding that was not observed in males. While classic CSDS is often reported to result in differences in sucrose preference between SUS and RES males, some studies report more mixed results (Covington, Maze, Vialou, & Nestler, 2015; Croft, Brooks, Cole, & Little, 2005). Furthermore, we found no effect of C57BL/6J estrous stage on CD-1 attack behavior of females in the CNSDS paradigm, which could be due to the presence of C57BL/6J male mice during all defeat interactions (Campbell, Ryan, & Schwartz, 1976; Whitten, 1958). Similarly, Harris and colleagues (Harris et al., 2018) also found that estrous did not impact CD-1 attack behavior in their modified version of CSDS that applied urine to C57BL/6J females. Thus, CNSDS permits direct comparison of chronic stress effects on males and females without the need for estrous cycle tracking.

Previous studies have demonstrated that female mice spend less time in the open arms than male equivalents during diestrus, but spend more time in the open arms during proestrus (Frye, Petralia, & Rhodes, 2000; Walf & Frye, 2007). Studies that collapse all rodents regardless of estrous phase have found that female rats spend more time in the open arms than males

(Johnston & File, 1991). Previous studies also found baseline sex differences in sucrose preference, with female rats and mice consuming more sucrose than males (Dalla et al., 2008; Hong et al., 2012). The lack of baseline sex differences in this study may be due to strain, as other studies have also observed no sex differences in C57BL6 mice at baseline (Adamec, Head, Blundell, Burton, & Berton, 2006; O'Leary, Gunn, & Brown, 2013).

To assess the effects of CNSDS on the HPA axis, blood samples were collected prior to CNSDS and again on the last day of CNSDS. Overall, both SUS male and female mice showed an increase in corticosterone levels after 10 days of defeat relative to baseline levels before stress exposure and compared to CNTRL. Similarly, classic CSDS increases plasma corticosterone levels in male experimental mice (A. Keeney et al., 2006), and modified CSDS increases plasma corticosterone levels in female experimental mice (Harris et al., 2018). However, within males we found that CNSDS increased corticosterone levels in both RES and SUS mice, which is similar to the findings of Krishnan and colleagues (Krishnan et al., 2007) using the traditional CSDS paradigm. By contrast, SUS females displayed higher corticosterone levels than RES females following CNSDS. These data suggest that CNSDS induces a robust stress response specifically in SUS females.

While clever modifications of the classic CSDS protocol exist for inducing stress in female mice, CNSDS is a streamlined paradigm that does not require difficult, time-consuming surgeries or urine collection (Harris et al., 2018; Takahashi et al., 2017) and doubles experimental output by allowing the simultaneous defeat of two mice (one male and one female). However, one potential drawback to CNSDS is that experimental C57BL6/J males and females receive different levels of attack from CD-1 aggressors. While CNSDS does induce similar behavioral effects and stress susceptibility phenotypes in males and females, the attack

differences may be a confound. One advantage of the Takahashi and colleagues paradigm (Takahashi et al., 2017) is that different levels of CD-1 aggression can be achieved through chemogenetic approaches.

Overall, the CNSDS paradigm leads to similar behavioral and neuroendocrine effects in both male and female C57BL/6J mice. Adoption of this streamlined CNSDS paradigm will help advance the initiative to include female rodents in preclinical chronic stress research. Thus, we present a CNSDS paradigm to simultaneously investigate the mechanisms underlying stress susceptibility and resilience in both sexes as well as the potential to study the neural circuitry underlying therapeutic interventions in both sexes.

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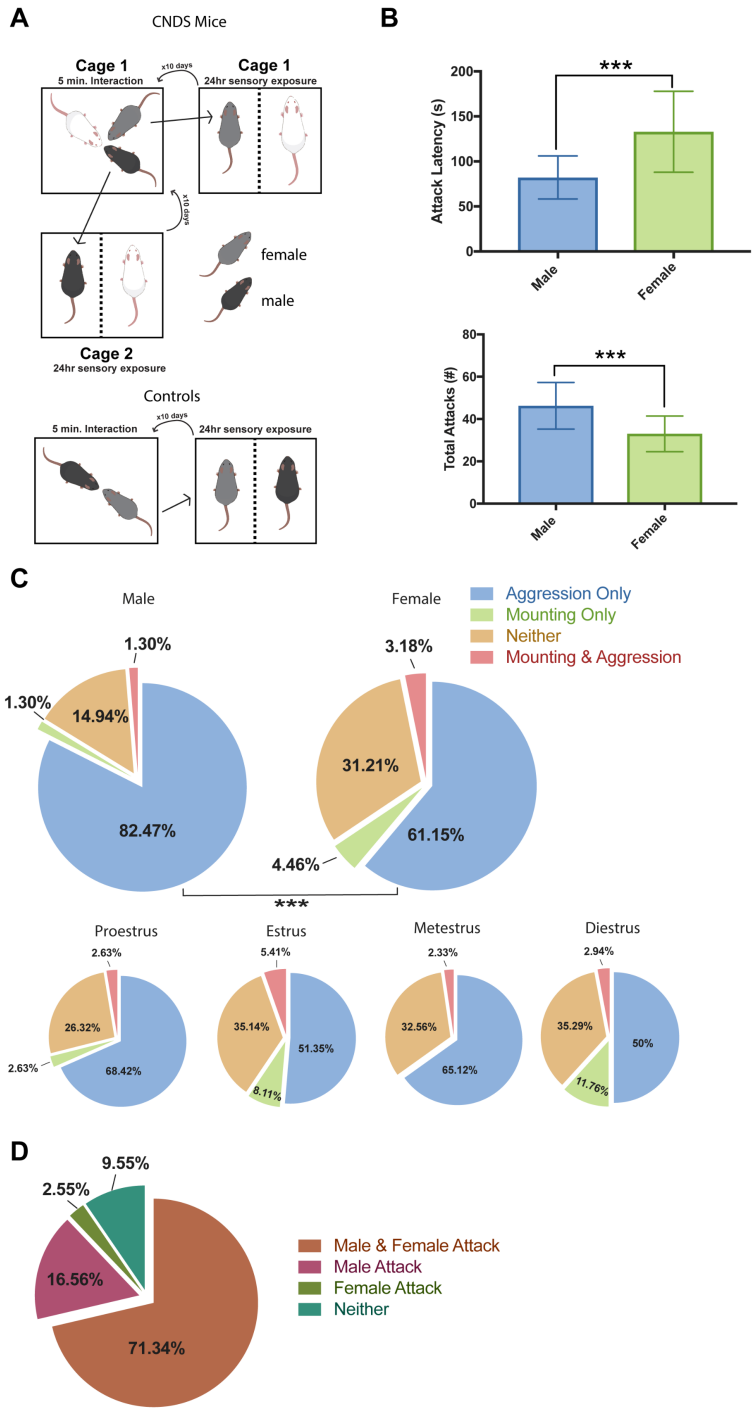


Figure 2.1. CNSDS paradigm induces attacks to both male and female C57BL/6J mice. (A) Schematic representation of CNSDS paradigm. (B) CD1 aggressors attack male quicker (***t(28) = 3.85, p = 0.006) and more (***t(28) = 3.7, p = 0.009) than female C57BL/6J mice.

(C) The percentage of CD1 behaviors directed towards male and female mice over the 10-day CNSDS protocol. Behaviors directed towards females were further investigated over the course of the estrous cycle. (D) Percentage of CD1s that attacked males or females over the 10-day CNSDS protocol.

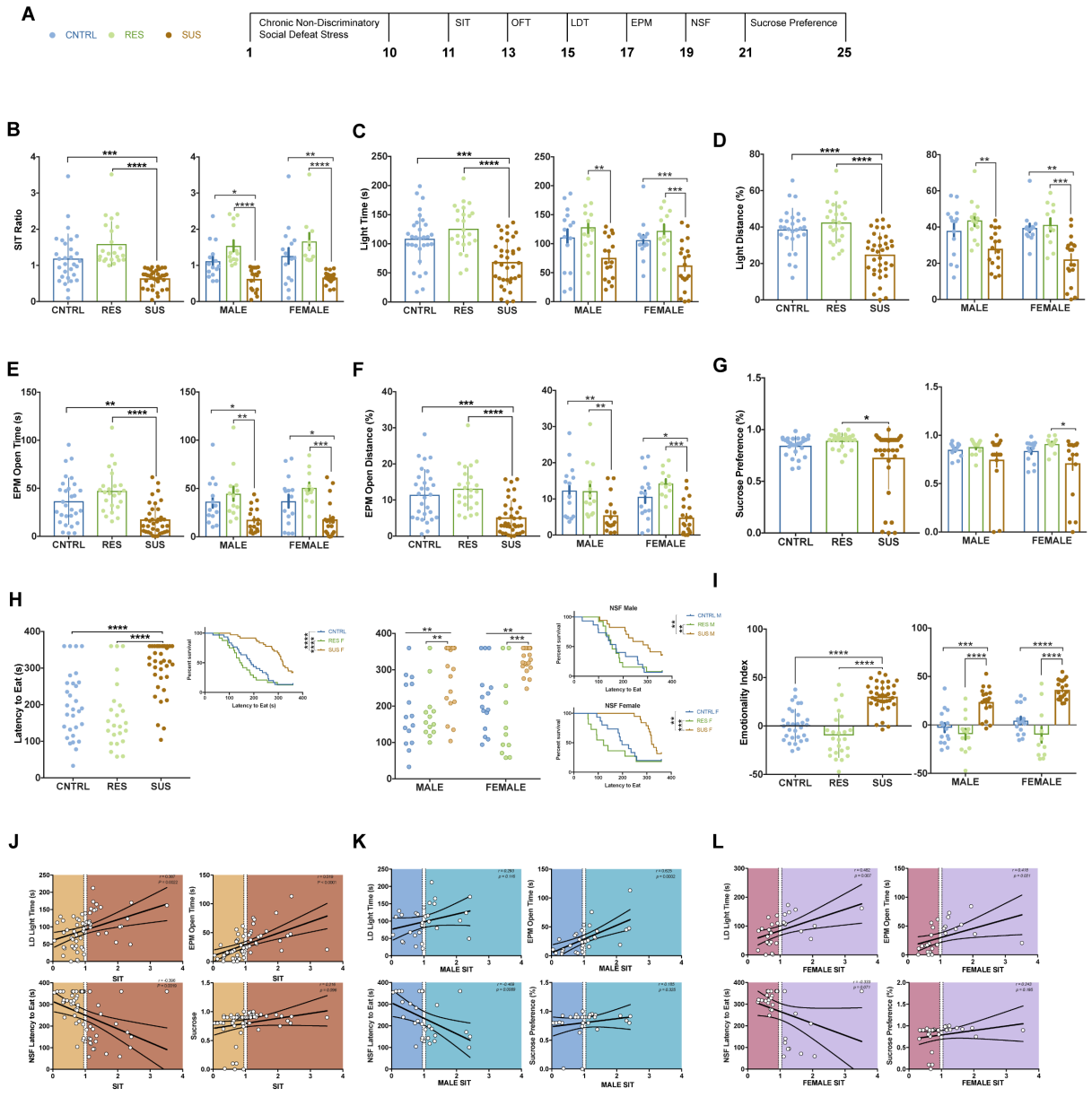


Figure 2.2. CNSDS SUS male and female mice have an increase in negative valence behaviors. (A) Timeline of CNSDS paradigm and behavior. (B-G) represent separate 2x3 ANOVAs with the left graph representing collapsed groups to explore the main effect of stress susceptibility phenotype, while the right displays planned Bonferroni post-hoc comparisons (SEXxSTRESS). Main effect of stress was observed in SIT ($F_{(2, 83)}$=23.21, p<0.001; ****SUSvsRES p<0.0001; ***SUSvsCNTRL p=0.0003; male ****SUSvsRES p<0.0001;

*SUSvsCNTRL p=0.036; female ****SUSvsRES p<0.0001; **SUSvsCNTRL p=0.005), LDT light time ($F_{(2, 84)}$ = 16.64, p < 0.001; ****SUSvsRES p<0.0001; ***SUSvsCNTRL p=0.0006; male **SUSvsRES p=0.002; female ***SUSvsRES p<0.001; ***SUSvsCNTRL p<0.001), LDT light distance ($F_{(2, 84)}$ = 14.79, p < 0.001; ****SUSvsRES p<0.0001; ****SUSvsCNTRL p<0.0001; male **SUSvsRES p=0.003; female ***SUSvsRES p<0.001; **SUSvsCNTRL p=0.01), EPM open arm time ($F_{(2, 83)}$ = 15.33, p<0.001; ****SUSvsRES p<0.0001; **SUSvsCNTRL p=0.0018; male **SUSvsRES p=0.003; *SUSvsCNTRL p=0.047; female ***SUSvsRES p<0.001; *SUSvsCNTRL p=0.035), EPM open arm distance ($F_{(2, 83)}$ = 15.7, p<0.001; ****SUSvsRES p<0.0001; ***SUSvsCNTRL p=0.0002; male **SUSvsRES p=0.004; **SUSvsCNTRL p=0.015; female ***SUSvsRES p<0.001; *SUSvsCNTRL p=0.045), in sucrose preference ($F_{(2, 80)}$ = 4.79, p=0.011; *SUSvsRES p=0.0125; females *SUSvsRES p=0.044). Scatterplots (H) represents feeding behavior differences from Bonferroni Kaplan Meier survival analysis between the stress susceptibility phenotypes ****SUSvsRES (X^2 (1) 17.75, p=0.0035) ***SUSvsCNTRL (X^2 (1)=14.94, p=0.0065). Separate Bonferroni Kaplan Meier survival analysis were also conducted between sexes with male **SUSvsRES (X^2 (1) 8.51, p=0.0035), **SUSvsCNTRL (X^2 (1)=7.4, p=0.0065) and female ***SUSvsRES (X^2 (1) 6.81, p=0.0009), **SUSvsCNTRL (X^2 (1)=6.4, p=0.01). (I) represents separate 2x3 ANOVAs with the left graph representing collapsed groups to explore the main effect of stress susceptibility phenotype, while the right displays planned Bonferroni post-hoc comparisons (SEXxSTRESS) for emotionality index ($F_{(2,84)}$=43.34, p<0.0001, ****SUSvsRES p<0.0001; ****SUSvsCNTRL p<0.0001; male ****SUSvsRES p<0.0001; ***SUSvsCNTRL p=0.0001; female ****SUSvsRES p<0.0001; ****SUSvsCNTRL p<0.0001. (J) Correlations collapsed by sex showed significant relationships between SIT ratios and time spent in the light, in EPM open

arms, and NSF latency to eat. (K) Correlations within males revealed significant relationships between SIT ratios and time spent on the EPM open arms as well as NSF latency to eat. (L) In females, significant correlations emerged between SIT ratios and time spent light, and on the EPM open arms.

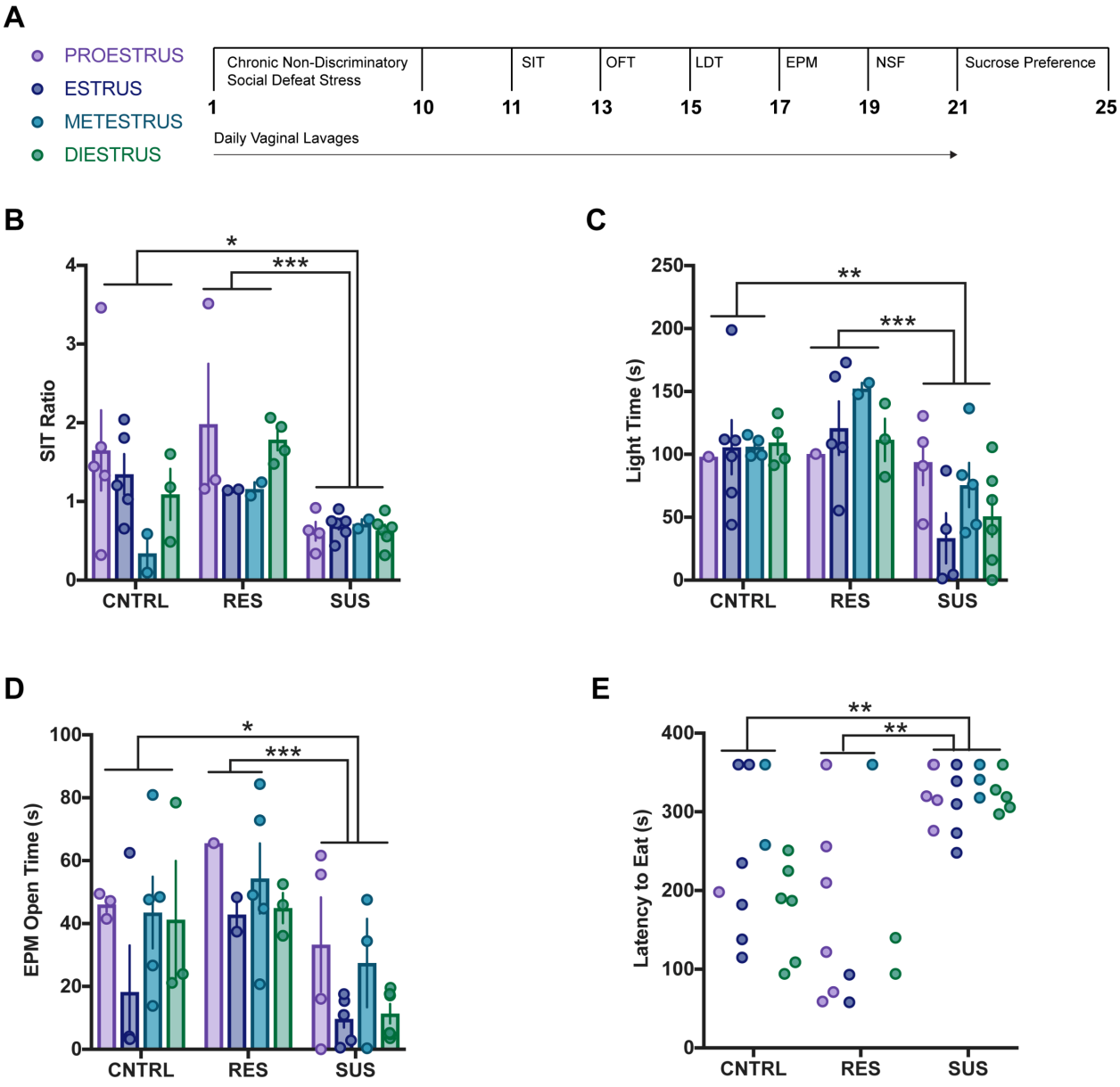


Figure 2.3. Female negative valence behavior is not impacted by estrous cycle stage during behavioral testing. (A) Time line of experiment. (B-D) 3x4 ANOVAs revealed that stress susceptibility phenotype effects persisted in the SIT ($F_{(3, 33)} = 6.44$, p=0.004; ***SUS vs. RES p<0.001; *SUS vs CNTRL p=0.018), LDT ($F_{(3, 33)} = 7.62$, p=0.002; ***SUS vs. RES p<0.001; **SUS vs CNTRL p=0.007 Bonferroni corrected), and EPM ($F_{(3, 33)} = 6.65$, p=0.004; ***SUS vs. RES p<0.001; *SUS vs CNTRL p=0.041 Bonferroni corrected). (E) Kaplan-Meier analysis

also revealed differences in NSF latency to eat (X^2 (11)=57.69, p<0.001; **SUS vs. RES p=0.01; **SUS vs CNTRL p=0.01 Bonferroni corrected).

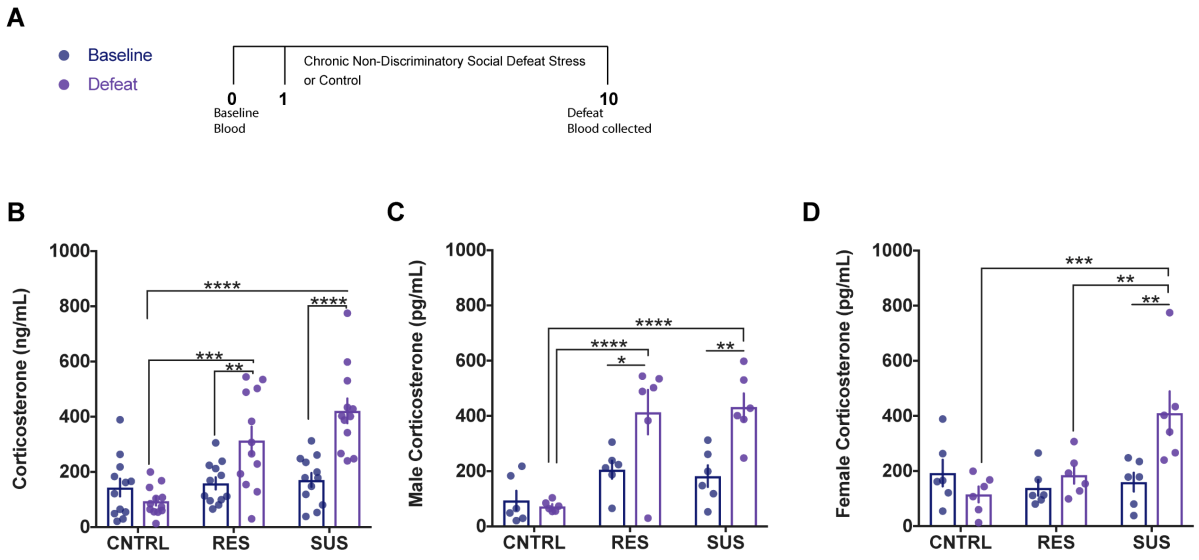
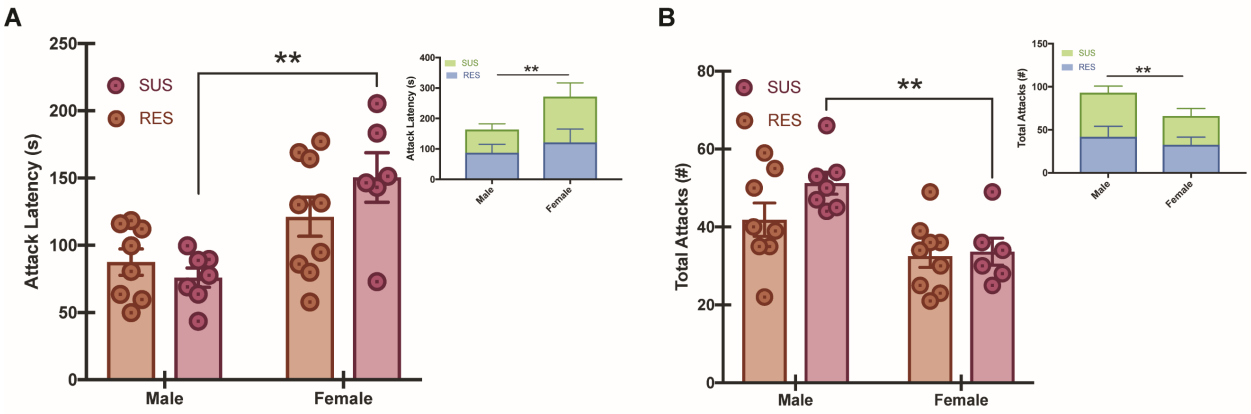
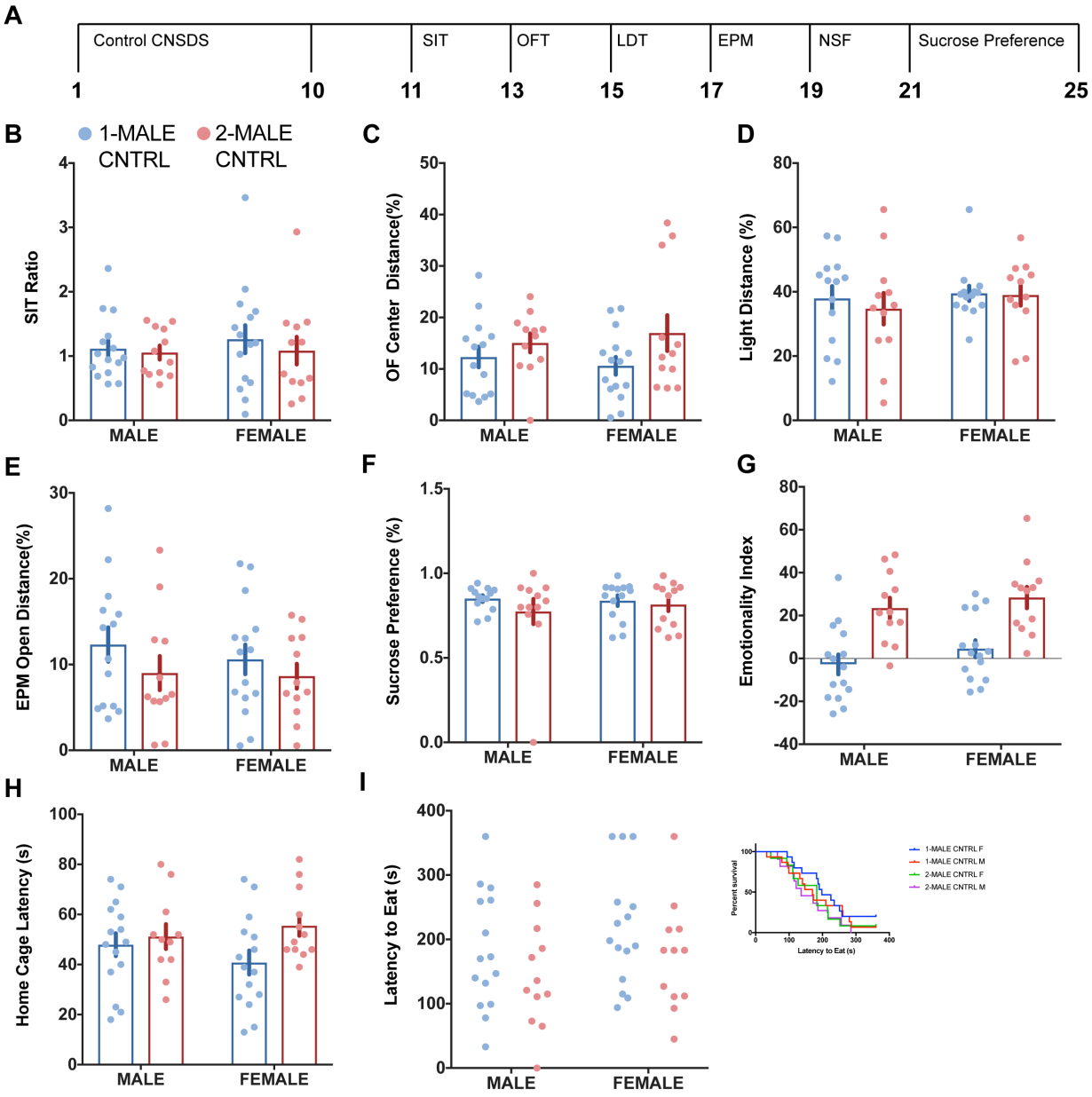


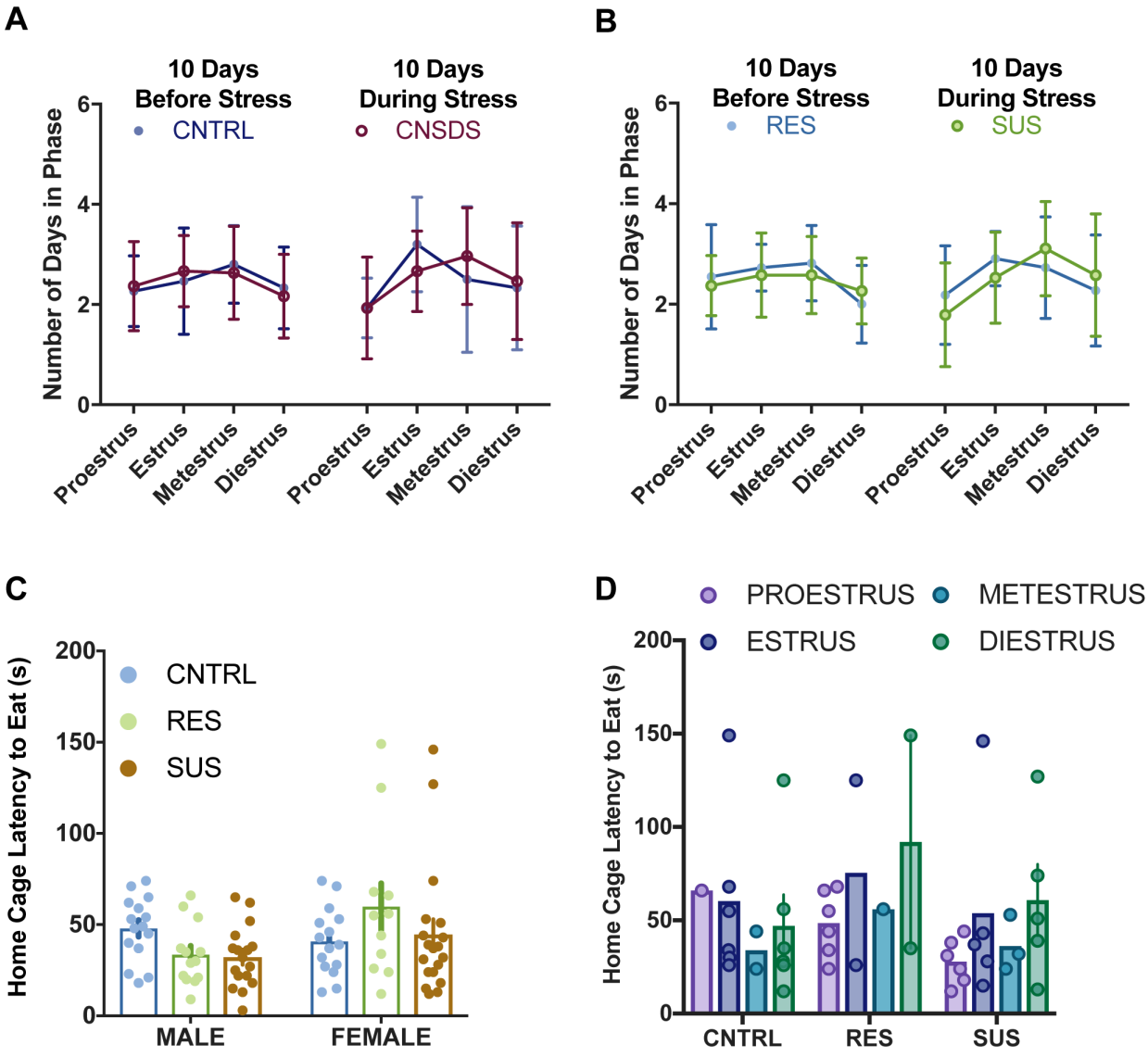
Figure 2.4. CNSDS increases plasma corticosterone levels. (A) Timeline of retro-orbital eye bleeds. (B-D). 2x2x3 repeated measures ANOVA revealed significant main effects of stress susceptibility phenotype ($F_{(2, 60)}$=15.6, p<0.0001), time point $F_{(1, 60)}$=17.17, p=0.0001), and interaction between stress susceptibility phenotype and blood collection time point ($F_{(2, 60)}$=10.86, p<0.0001). Bonferroni post-hoc comparisons collapsed by sex (B) showed significant differences in corticosterone response following CNSDS exposure (****SUS vs CNTRL p<0.0001; ***RES vs CNTRL p=0.0001). CNSDS also increased corticosterone levels relative to baseline in both RES (**p=0.0059) and SUS (****p<0.0001). In males (C) Bonferroni post-hoc comparisons showed differences in corticosterone in response to CNSDS exposure (****SUS vs CNTRL p<0.0001 and ****RES vs CNTRL p<0.0001). Additionally, CNSDS increased corticosterone levels relative to baseline in both SUS (**p=0.0018) and RES (*p=0.0098) males. (D) Bonferroni post-hoc comparisons in females showed differences in corticosterone in response to CNSDS exposure (***SUS vs CNTRL p=0.0002 and **SUS vs RES p=0.0043). Lastly, CNSDS significantly increased corticosterone levels between baseline and defeat only in SUS females (**p=0.0018).



Supplemental Figure 2.1. SUS males are attacked quicker and more frequently than SUS females. (A) A 2x2 ANOVA (sex x stress susceptibility) indicates an effect of sex on average attack latency ($F_{(1, 26)}$=16.99, p=0.0003) (inset). Bonferroni post-hoc comparisons showed the CD1 aggressor attacked the C57BL/6J SUS males with a shorter latency than for SUS females (**SUS males vs SUS females p=0.0017). (B) A 2x2 ANOVA (sex x stress susceptibility) indicate an effect of sex on total number of attacks ($F_{(1, 26)}$=14.65, p=0.0007) (inset). Bonferroni post-hoc comparisons showed the CD1 aggressor attacked the SUS males more frequently than the SUS females (**SUS males vs SUS females p=0.0053).



Supplemental Figure 2.2. No differences between CNTRL groups. We compared behavioral differences between two different CNTRL groups, where females were exposed to either 1 or 2 C57BL/6J males for 5 minutes daily for 10 consecutive days (A). (B-H) 2x2 ANOVAs (sex x CNTRL group) revealed no significant differences in SIT, OF, LDT, EPM, Sucrose Preference, Emotionality Index, or Home Cage feeding behavior. (I) Kaplan-Meier analysis also revealed no differences in latency to eat in the NSF between the CNTRL groups.



Supplemental Figure 2.3. CNSDS does not affect estrous cycle. Line graphs represent the average amount of days a mouse was observed in each estrous phase during the 10 days before stress and 10 days after stress. We compared these averages between CNTRL and CNSDS females (A) and between RES and SUS female mice (B). No differences in feeding behavior was observed in latency to eat in the home cage between the different stress phenotypes (C) and across the estrous cycle (D).

CHAPTER 3

The effects of fluoxetine on behavior and adult hippocampal neurogenesis in female C57BL/6J mice across the estrous cycle

Results from this chapter were published in Yohn, C.N.**, Shiftman, S., Garino, A., Diethorn, E., Leshya Bokka, Sandra A. Ashamalla, Samuels, B.A**. (2020). The effects of fluoxetine on behavior and adult hippocampal neurogenesis in female C57BL/6J mice across the estrous cycle. *Psychopharmacology*, 1-10 (uploaded on *bioRxiv* (2018), 368449). **co-corresponding author

ABSTRACT

Some mood disorders, such as major depressive disorder, are more prevalent in women than in men. However, historically preclinical studies in rodents have a lower inclusion rate of females than males, possibly due to the fact that behavior can be affected by the estrous cycle. Several studies have demonstrated that chronic antidepressant treatment can decrease anxiety-associated behaviors and increase adult hippocampal neurogenesis in male rodents. Very few studies have looked at the effects of antidepressants on behavior and neurogenesis across the estrous cycle in naturally cycling female rodents. Here we analyze the effects of chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac) on behavior and adult hippocampal neurogenesis in naturally cycling C57BL/6J females across all four phases of the estrous cycle. In naturally cycling C57BL/6J females, fluoxetine decreases negative valence behaviors associated with anxiety in the elevated plus maze and novelty suppressed feeding task, reduces immobility time in forced swim test, and increases adult hippocampal neurogenesis. Interestingly, the effects of fluoxetine on several negative valence behavior and adult hippocampal neurogenesis measures were mainly found within the estrus and diestrus phases of the estrous cycle. Taken together these data are the first to illustrate the effects of fluoxetine on behavior and adult hippocampal neurogenesis across all four phases of the murine estrous cycle.

Keywords: Estrous Cycle, Adult Neurogenesis, Anxiety, Depression, Antidepressants, Females

INTRODUCTION

Although several mood disorders, including major depressive disorder, are more prevalent in women than men (Kessler 2003; Sloan and Kornstein 2003), historically females are excluded from rodent preclinical studies because fluctuations in ovarian steroid hormones (Arakawa et al. 2014; Lovick 2012) across the estrous cycle may confound experimental results. Many preclinical studies have used rodents to demonstrate behavioral effects of treatment with various antidepressants, including selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (FLX), on negative valence behaviors associated with anxiety such as elevated plus maze and novelty suppressed feeding and on forced swim test (Bodnoff et al. 1988; Cryan et al. 2002; David et al. 2009; Dulawa et al. 2004; Lucki et al. 2001; Samuels et al. 2015; Santarelli et al. 2003). However, nearly all of these studies only studied the effects of antidepressants in male rodents. Similarly, although treatment with antidepressants such as FLX was shown nearly two decades ago to increase adult hippocampal neurogenesis in male rodents (Malberg et al. 2000), and follow-up work suggested this effect on neurogenesis is required for the behavioral effects of antidepressants (Santarelli et al. 2003), surprisingly little is known about how FLX affects adult hippocampal neurogenesis in naturally cycling females.

In humans, women can experience depression and anxiety due to premenstrual syndrome, where variations in mood states are correlated with different secretion patterns of estrogen and progesterone across the menstrual cycle (Shors and Leuner 2003). Rodents display similar fluctuations in behavior, with diestrus female rodents showing increased stress responses, immobility time in the forced swim test (FST), and anxiety-associated behaviors within the elevated plus maze (EPM) relative to proestrus females (D'Souza and Sadananda 2017; Lovick 2012; Marcondes et al. 2001; Sayin et al. 2014). Administration of estradiol to ovariectomized

(OVX) rats reduces immobility time in the forced swim test (Bekku and Yoshimura 2005), an effect that is similar to antidepressant treatment (Green and Galea 2008; Mahmoud et al. 2016). Finally, in comparing two different mouse strains, Meziane and colleagues observed that unlike BALB/cByJ females, C57BL/6J female mice have less behavioral variation across the estrous cycle in the open field (OF), tail flick, and tail suspension tests (Meziane et al. 2007).

In addition to effects on behavior, variations in gonadal steroid hormone secretion patterns, as seen in cycling females, contribute to alterations in adult hippocampal neurogenesis (Barha et al. 2009; Tanapat et al. 2005) within the subgranular zone of the dentate gyrus (DG) (Tanapat et al. 1999). For instance, proestrus rats have higher DG cell proliferation than estrus or diestrus rats possibly due to higher estradiol levels during the proestrus phase (Pawluski et al. 2009; Sadrollahi et al. 2014; Tanapat et al. 1999). Whether the estrous cycle impacts other phases of adult hippocampal neurogenesis, such as differentiation into and maturation of young neurons (Plumpe et al. 2006) remains unknown. Furthermore, while many studies document that estrogen administration to ovariectomized (OVX) rats affects DG cell proliferation, these effects may be species specific since Lagace and colleagues observed no significant differences in adult hippocampal cell proliferation in ovariectomized C57BL/6J mice (Lagace et al. 2007). While Sayin and colleagues illustrate that citalopram, a selective serotonin reuptake inhibitor (SSRI), alleviates differences in anxiety-associated behaviors between proestrus and non-proestrus rats (Sayin et al. 2014), no studies have examined the impact of SSRIs on behavior and neurogenesis in intact, cycling females across all four phases of estrous.

Therefore, given the gaps in knowledge in how FLX affects behavior and adult hippocampal neurogenesis in naturally cycling female rodents, coupled with the fact that the

estrous cycle has clear effects, this study aims to resolve the behavioral and adult neurogenesis effects of FLX across all four phases of the estrous cycle.

METHODS

Subjects

Adult 8-week-old female C57BL/6J strain mice were purchased from Jackson laboratories. All mice were grouped housed (up to 5 per cage) in standard cages with corncob bedding, maintained on a 12L:12D (6am:6pm) schedule with food (Purina 5002 LabDiet) and autoclaved water provided *ad libitum*. For the duration of the project FLX (n= 25; 18 mg/kg/day; BioTrend, BG0197) or vehicle (n = 20; deionized water) was administered within the first 3 hours of the light phase via oral gavage with volume administered contingent on bodyweight. Specifically, mice were individually weighed with oral gavage volume calculated via ((18mg/kg (dose of FLX)*mouse weight kg)/ concentration mg/ml). The final volume delivered by gavage each day ranged from 0.15ml to 0.23 ml. After 3 weeks of FLX treatment behavioral testing began and was run during the morning hours (8:00-12:00), with two days separating each behavioral test. On behavioral testing days FLX or vehicle was administered after mice completed the behavioral test to avoid acute effects. All testing was conducted in compliance with the NIH laboratory animal care guidelines and approved by Rutgers University Institutional Animal Care and Use Committee.

Vaginal Lavage

To examine estrous cycle state vaginal lavages were performed throughout FLX/vehicle treatment, after completing each behavioral test, and prior euthanasia. In order to collect the samples, a pipet was filled with ddH_2O, placed at the opening of the mouse's vaginal canal (without penetration) with ddH_2O gently expelled and suctioned back (Byers et al. 2012;

McLean et al. 2012). Samples were then placed on a slide warmer to dry for approximately 5 minutes and imaged under a EVOS FL Auto 2.0 microscope (Thermofisher Scientific) at 10x magnification. Estrous phase was identified by the presence or absence of nucleated epithelial cells, cornified epithelial cells, and leukocytes (Byers et al. 2012; Felicio et al. 1984). Mice in proestrus displayed mostly nucleated and some cornified cells (Figure 3.1B). Estrus was recorded as the presence of mostly cornified epithelial cells, with the presence of a few nucleated cells in early estrus (Figure 3.1B). Metestrus was determined by the presence of cornified epithelial cells and polymorphonuclear leukocytes (Byers et al. 2012), while mice in diestrus contained mainly polymorphonuclear leukocytes with few epithelial cells being present (Figure 3.1B).

Behavioral Testing

Open Field (OF)

Motor activity was quantified in five Plexiglass open field boxes 43 x 43 cm (Kinder Scientific). The recording of x-y ambulatory movements was recorded by two sets of 16 pulse-modulated infrared photobeams placed on opposite walls 2.5 cm apart to. As previously described, activity chambers were computer interfaced for data sampling at 100ms resolution (David et al. 2009). The computer software predefines grid lines that divide each open field chamber into center and periphery regions, with the center being a square 11cm from the wall. The number of entries, distance traveled, and total time spent in the center were recorded, as well as percent of distance traveled in the center defined as center distance divided by total distance traveled (Supplemental Figure 3.1A). To measure overall motor activity total distance (cm) was quantified.

Light Dark (LD)

The light/dark test was conducted in an open field chamber measuring 43 x 43 cm (Kinder Scientific, USA), with a clear floor and walls. To divide the open field into separate light and dark compartments, a dark plastic box that covered one third of the chamber was inserted. The dark box was opaque to visible light, but transparent to infrared light, and contained an opening that allowed passage between the light and dark compartments (David et al. 2009). The light compartment was brightly illuminated at 1000 lux. At the beginning of each 5-minute test, mice were placed in the dark compartment. An observer blind to treatment groups recorded the latency to emerge into the light (Supplemental Figure 3.1C). Using Activity Monitor (Kinder Scientific, USA) software, total time in the light and ambulatory distance in both compartments was analyzed. To calculate percent distance traveled in the light, distance traveled in the light was divided by total distance traveled (Supplemental Figure 3.1B).

Elevated Plus Maze (EPM)

The EPM test consisted of a plus-shaped apparatus with two open and two closed arms (side walls), elevated 2 feet from the floor. During the five-minute test, the mouse's behavior was recorded from a video camera mounted above each EPM arena. EthoVision (Noldus) software was then used to score time spent in the open arms, entries, and distance traveled in both open and closed arms. By dividing total open arm distance traveled by total distance traveled, we were able to analyze percent distance traveled in open arms.

Novelty Suppressed Feeding (NSF)

After undergoing 18 hours of food deprivation within their home cage, mice were placed in the corner of a testing apparatus (50x50x20 cm) filled with approximately 2 cm of corncob bedding and a single pellet of food attached via a rubberband to a white platform in the center of the box

(Samuels and Hen 2011). The center of the box was illuminated at 1500 lux. The NSF test lasted 6 minutes, where the latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) was timed/recorded. If a mouse did not consume food during the NSF a latency of 360secs was recorded. Immediately afterwards, the mice were transferred to their home cage to assess home cage feeding behavior for 5 minutes (Supplemental Figure 3.1D-F). During this task latency to eat and amount of food consumed was measured as a control for feeding behavior observed in the NSF task. Each mouse was weighed before food deprivation and after home cage feeding to assess the percentage of body weight loss. Following home cage feeding, mice were placed in a new home cage with cage mates and returned to the colony room.

Forced Swim Test (FST)

A modified FST procedure suitable for mice was used (David et al. 2009). Individual cylinders (46 cm tall x 32 cm in diameter x 30 cm deep) were filled with room-temperature water (25-26°C) before placing each mouse into the cylinder. Two sets of photobeams were mounted on opposite sides of the cylinder (Kinder Scientific, USA) to allow for the recording of swimming behavior during the 6-minute test. Immobility times (measured by beam breaks over 5-second intervals) were assessed during the last 4-minutes of the test since mice are habituating to the task during the initial 2-minutes of the test.

Brain Collection, Sectioning and Immunohistochemistry

Brain Collection and Sectioning

48 hours after completion of behavioral testing and 24 hours after the last FLX dosage, brains were collected from all experimental mice. To ensure mice were euthanized in each estrous cycle phase, vaginal lavages were collected prior to anesthesia to ensure that enough mice were sacrificed during different phases. Mice were anesthetized with ketamine (80mg/kg)

and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were collected and stored in 4% paraformaldehyde overnight at 4°C. Next, brains were switched to a 30% sucrose 0.1% sodium azide (NaN_3) in PBS solution and stored at 4°C until they were sectioned. Using a cryostat, every sixth section (40μm) of the hippocampus (Bregma -1.22 to -3.88) was collected onto Superfrost Plus slides (Thermofisher Scientific) and stored at -20°C until staining and further analysis.

Ki67 labeling

The effects of FLX treatment on cell proliferation were assessed across a total of 12 sections (every sixth section) of the hippocampus. Slides were washed in 1% Triton X-100 PBS for 5 minutes before undergoing three PBS washes. Next, slides were incubated in warm citrate buffer for 30 minutes and then washed three times in PBS. Slides were then transferred to an opaque moisture chamber (TedPella) for the blocking and overnight incubation step. Slides were blocked for 1 hour in 10% normal goat serum (NGS) diluted in PBS before being incubated overnight at 4°C in anti-rabbit Ki67 (1:250; polyclonal abCam, ab15580) diluted in 2% NGS PBS. Following 18 hours of incubation slides were washed 3 times in PBS before being incubated at room temperature for 2 hours in CY-5 goat anti-rabbit (1:1000, Invitrogen, Thermo Fisher Scientific, A10523) diluted in 2% NGS PBS. Next slides were washed with PBS then counterstained with DAPI (1:15000) for 15 minutes. Finally, slides were washed with PBS and cover slipped using the mounting medium Prolong Diamond (Thermofisher Scientific). Fluorescent images were taken using a EVOS FL Auto 2.0 microscope (Thermofisher Scientific) at 10x or 40x magnification, where $Ki67^+$ cells overlayed with DAPI (Thermofisher Scientific) across the 12 sections of hippocampus were collected and counted via an observer blind to treatment (Supplemental Figure 3.2A-E).

Doublecortin (DCX) labeling

12 hippocampal sections (every sixth section) for doublecortin (DCX) were stained using the primary antibody doublecortin anti-goat (1:500; Life technologies; 481200) and secondary antibody CY-5 goat anti-rabbit (1:1000, Invitrogen, Thermo Fisher Scientific, A10523). Fluorescent images were taken using using a EVOS FL Auto 2.0 microscope (Thermofisher Scientific) at 10x or 40x magnification, where DCX^+ cells across the 12 sections of hippocampus were collected and counted by an observer blind to treatment (Supplemental Figure 3.2F-O). Following imaging, DCX^+ cells were counted and subcategorized according to their dendritic morphology: DCX^+ cells with no tertiary dendritic processes and DCX^+ cells with complex, tertiary dendrites. The maturation index was defined as the ratio of DCX^+ cells possessing tertiary dendrites over the total DCX^+ cells.

Statistical Analyses

To analyze both behavioral and molecular differences between treatment groups and estrous cycle phases separate 2x4 analyses of variance (ANOVA) were conducted. Each ANOVA was followed by subsequent Bonferroni post-hoc analyses to further assess between and within group differences across the estrous cycle. Since we imposed a cutoff time during the NSF, we ran a Kaplan Meier survival analysis (nonparametric test) that permits censoring of these data points to analyze differences in feeding latencies (Samuels and Hen 2011). GraphPad Prism 7 was used for all statistical analyses.

RESULTS

FLX and vehicle behavioral differences across estrous cycle

We treated adult C57BL/6J female mice (n= 45) with 18mg/kg FLX for three weeks (Figure 3.1A) and then exposed these mice to the Open Field (OF), Light Dark Test (LD),

Elevated Plus Maze (EPM), Novelty Suppressed Feeding (NSF), and Forced Swim Test (FST). To assess estrous cycle phase (Figure 3.1B), vaginal lavages were performed daily beginning two weeks prior to behavior until the end of the experiment. On behavioral days lavages were performed upon completion of testing. We found significant treatment effects in the EPM and FST, such that FLX mice spent more time in the open arms (Two-Way ANOVA cycle x treatment $F(1,35)=22.8$, $p<0.001$, $\eta^2 = 0.373$; Figure 3.1C) and less time immobile (Two-Way ANOVA cycle x treatment $F(1,35) = 25.77$, $p < 0.001$, $\eta^2 = 0.356$; Figure 3.1D) than vehicle mice. In the EPM, planned Bonferroni post-hoc comparisons revealed treatment differences in open arm time within the estrus phase, such that estrus FLX-treated mice spent more time in the open arms than estrus vehicle-treated mice, $p = 0.003$ (Figure 3.1C). Additionally, planned Bonferroni post-hoc comparisons revealed immobility time in the FST was significantly different between treatment groups within the estrus and diestrus phases, such that vehicle mice spent significantly more time immobile than FLX mice within both the estrus (Bonferroni post-hoc comparison, $p = 0.025$) and diestrus (Bonferroni post-hoc comparison, $p = 0.006$) phases (Figure 3.1D). We found no significant effects of treatment or estrous phase nor interaction effect on behaviors within the OF and LD tests (Supplemental Figure 3.1A-C).

In the NSF, a Kaplan Meier survival analysis log rank test revealed that FLX significantly reduces latency to feed, $p = 0.0038$ (Figure 3.1E, F), as compared to vehicle mice. Additionally, we observed an effect of FLX treatment in the estrus phase of the estrous cycle, with FLX females in estrus having lower latencies to feed than estrus vehicle females, $p = 0.008$. There was no treatment effect nor estrous cycle effect on home cage latency to eat, amount of food consumed in home cage, or percent weight change (Supplemental Figure 3.1D-F).

Taken together, these data suggest that behavior across the estrous cycle is consistent across estrous phases, and that FLX treatment reduces anxiety-associated behaviors in the estrus phase and decreases FST immobility in both the estrus and diestrus phases.

Fluoxetine treatment and estrous cycle state impact adult neurogenesis

We next performed immunostaining to determine the effects of FLX on the distinct stages of adult hippocampal neurogenesis across the different phases of the estrous cycle. We first stained for the cell proliferation marker Ki67 and found that FLX-treated mice had more $Ki67^+$ cells than vehicle-treated mice (Two-Way ANOVA cycle x treatment, $F(1, 32) = 11.79$, $p = 0.002$, $\eta^2 = 0.234$) (Figure 3.2A-B, Supplemental Figure 3.2A-E). We observed no estrous cycle effects nor interaction on number of $Ki67^+$ cells ($p > 0.05$ for all phases). However, planned Bonferroni post-hoc comparisons showed that FLX-treated mice had more Ki67+ cells than vehicle mice within the estrus ($p = 0.007$) and there was a trend in diestrus ($p = 0.053$). Additionally, within the vehicle group we found no differences in the number of $Ki67^+$ cells within the DG subgranular zone (SGZ) across all phases of the estrous cycle (Figure 3.3A).

We next performed immunostaining with the young neuron marker DCX, and observed that FLX-treated mice had more DCX^+ cells within the DG than vehicle-treated mice (Two-Way ANOVA cycle x treatment, $F(1, 37) = 13.44$, $p < 0.001$, $\eta^2 = 0.280$) (Figure 3.2C-D, Supplemental Figure 2F-J). We found a significant interaction effect between treatment and estrous phase (Two-Way ANOVA cycle x treatment, $F(3, 37) = 4.57$, $p = 0.008$, $\eta^2 = 0.275$). Bonferroni post-hoc comparisons revealed that FLX-treated mice had more DCX^+ cells than vehicle mice within the estrus ($p < 0.001$) and diestrus ($p = 0.009$; Figure 3.2D) phases. Within the vehicle group, Bonferroni post-hoc comparisons revealed that proestrus females had significantly more DCX^+ cells than both estrus ($p = 0.001$) and diestrus ($p = 0.01$) female mice

(Figure 3.3B). Additionally, metestrus vehicle-treated mice had significantly more DCX^+ cells than both estrus ($p < 0.001$) and diestrus ($p = 0.008$) vehicle-treated mice (Figure 3.3B). Within the FLX group no differences in DCX^+ cell expression was observed across the estrous cycle.

To assess maturation of the young neurons, we counted the DCX+ neurons that displayed tertiary dendrites. As expected, FLX-treated mice had more mature neurons than vehicle mice as indicated by the number of DCX^+ cells with tertiary dendrites (Two-Way ANOVA cycle x treatment, $F(1, 37) = 15.85$, $p < 0.001$, $\eta^2 = 0.401$; Figure 3.2E-F, Supplemental Figure 2K-O). Planned Bonferroni post-hoc comparisons revealed that FLX-treated mice had more DCX^+ cells with tertiary dendrites than vehicle mice within the estrus ($p < 0.001$) and diestrus ($p = 0.001$; Figure 3.2F) phases. We also observed a significant interaction effect between treatment group and estrous cycle phase (Two-Way ANOVA cycle x treatment, $F(3, 37) = 3.26$, $p = 0.032$, $\eta^2 = 0.126$). Within the vehicle group, planned post-hoc comparisons revealed that proestrus females have more DCX^+ cells with tertiary dendrites than estrus ($p = 0.007$) and diestrus ($p = 0.012$) females (Figure 3.3C). Planned comparisons also revealed that metestrus vehicle-treated females had more mature neurons than estrus ($p = 0.004$) and diestrus ($p = 0.006$) vehicle-treated females (Figure 3.3C). Within the FLX group, we observed no differences in expression of DCX^+ cell with tertiary dendrites across the estrous cycle.

Lastly, we observed that FLX mice have a higher maturation index (Two-Way ANOVA cycle x treatment, $F(1, 37) = 21.71$, $p < 0.001$; Figure 3.2G) than vehicle mice, with FLX females having a significantly higher maturation index than vehicle females in both the estrus (Bonferroni post-hoc comparison, $p = 0.003$) and diestrus ($p = 0.013$) phases (Figure 3.2G). Within both the vehicle (Figure 3.3D) and FLX group we observed no differences in the maturation index across the estrous cycle. Taken together, these data suggest that the effects of

fluoxetine on adult hippocampal neurogenesis are most pronounced during both the estrus and diestrus phases.

DISCUSSION

FLX behavioral effects across estrous cycle

We used negative valence behavioral tests to evaluate the impact of antidepressant treatment on behavior across the estrous cycle. Prior to behavioral testing we tracked the females estrous cycle for two weeks to assess whether females were cycling together within the same housing room. Although Meziane and colleagues observed that females within the same room cycle together (Meziane et al. 2007), we found that females in our vivarium had out of sync cycles, which allowed us to investigate the different estrous cycle phases during each behavioral test. Similar to previous studies in males (David et al. 2009), our data illustrate that FLX treatment in females reduces anxiety-associated behaviors within the EPM and NSF, and decreases immobility in the FST, with no effects seen on behavior within the OF and LD. However, our data demonstrate that estrous cycle significantly impacts the effects of FLX on behavior. FLX-treated females in the estrus phase display a reduction in anxiety-associated behavior in the EPM and NSF, and reduced immobility in the FST relative to vehicle-treated estrus females. Furthermore, diestrus FLX-treated females had lower immobility times in the FST than diestrus vehicle females, but FLX did not affect the anxiety-associated EPM and NSF tasks in this phase. The behavioral effects of FLX were not significant in any of these tasks during metestrus and proestrus. Sayin and colleagues observed that proestrus rats display decreased anxiety-associated behaviors relative to non-proestrus rats in the EPM, with estrous cycle differences attenuated following citalopram administration (Sayin et al. 2014). Compared to our data, these findings suggest that species differences may exist in anxiety-associated behaviors across the estrous

cycle. Although we did not observe an effect of the estrous cycle on behavior within treatment, a previous study observed that C57BL/6J females have less variation in anxiety-associated behaviors across the estrous cycle than BALB/cByJ females (Meziane et al. 2007). Despite this, our data illustrate that FLX treatment has significant effects on behavior. However, our detailed analyses suggest that the behavioral effects of FLX in the EPM and NSF are mainly driven by changes during the estrus phase, while the effects of FLX in the FST are driven by changes during the estrus and diestrus phases.

Differences in behavior between treatment groups within estrus and diestrus may be related to fluctuations in estradiol and progesterone levels (Lovick 2012; Pawluski et al. 2009). Specifically, estradiol levels are lowest during estrus and diestrus (Pawluski et al. 2009; Wood et al. 2007). Exogenous estradiol treatment to mimic diestrus in OVX rats results in decreases in anxiety-associated behavior in the EPM compared to non-estrogen treated freely cycling diestrus rats (Marcondes et al. 2001). In mice, females in the estrus and diestrus phases are more susceptible to individual housing stress and spend less time in the center of the open field arena than proestrus mice (Palanza et al. 2001). The impact of FLX administration on ovarian steroid hormones across the estrous cycle is understudied in rodents, and future studies will need to assess the relationship between antidepressant and endogenous ovarian hormone levels.

One potential caveat of this study is that behavioral testing is performed during the first few hours of the light phase. Some studies report that testing during the light vs dark phase can influence anxiety-associated behaviors in rodents. For example, wild-type males show more rears and time in the light side of the light/dark test than males with a dysfunctional androgen receptor during the dark phase but not during the light phase (Chen et al. 2014). Therefore, it will be informative for future studies to also assess behavioral testing during the dark phase.

Fluoxetine treatment and estrous cycle state impact adult neurogenesis

Similar to several other studies (Lagace et al. 2007; Pawluski et al. 2014), we observed that chronic FLX administration increased adult hippocampal neurogenesis levels relative to vehicle-treated females. Within the DG, females treated with FLX had increased numbers of proliferating cells and young neurons, and increased maturation of young neurons relative to vehicle-treated females. However, similar to the effects on behavior, our study is the first to illustrate that the effects of FLX on adult hippocampal neurogenesis are most pronounced during the estrus and diestrus phases. This is in part due to the relatively lower levels of neurogenesis in the vehicle-treated mice during these phases, which may be attributable to natural low levels of estradiol in these phases. Estrogens, such as estradiol, impact both cell proliferation and cell survival in the DG (Ormerod et al. 2003) and more proliferating cells are found in the proestrus phase than the non-proestrus phases (Pawluski et al. 2009; Sadrollahi et al. 2014; Tanapat et al. 1999). Discrepancies in proliferating cell numbers across the estrous cycle can be attributed to endogenous estrogen levels naturally peaking during the proestrus phase and decreasing during the estrus and diestrus phase (Butcher et al. 1974; Pawluski et al. 2009; Tanapat et al. 1999). However, Lagace and colleagues show that endogenous levels of estradiol do not appear to impact adult hippocampal cell proliferation in C57BL/6J mice, since OVX female mice have similar number of proliferating cells ($BrdU^+$) and immature neurons (DCX^+) in the hippocampus as intact female mice (Lagace et al. 2007). Furthermore, in assessing cell proliferation across three phases of the estrous cycle (proestrus, estrus, and diestrus), Lagace and colleagues observed no differences in cell proliferation in the different phases. Similar to Lagace and colleagues, we show that estrous cycle phase does not significantly impact DG cell proliferation levels within vehicle-treated mice (Lagace et al. 2007).

We found that proestrus and metestrus vehicle-treated female mice have higher numbers of young neurons (DCX^+) within the DG than estrus and diestrus vehicle-treated female mice. Additionally, we show that estrus and diestrus vehicle treated female mice have less maturation of young neurons in the DG than proestrus and metestrus vehicle-treated female mice. Interestingly, FLX specifically increased the number of young neurons and maturation of the young neurons in the DG during estrus and diestrus relative to vehicle-treated mice. This finding is surprising, since individual phases of the estrous cycle only last about twenty-four hours and the processes of fate specification and maturation can take several days (Ming and Song 2005). However, chronic FLX accelerates maturation of immature neurons (Wang et al. 2008) and our data indicate that these effects are primarily observed during estrus and diestrus. One possible explanation is that the effects of FLX on DCX expression are in part due to dematuration of mature granule cells within the DG (Kobayashi et al. 2010), which could happen on a fast timescale. A second possible explanation is that DCX as a neurogenesis marker has been widely described in male rodents, where it is only transiently expressed during an intermediate phase in which the cells are young neurons. However, DCX expression in these cells may be more dynamic than previously appreciated in females. Ultimately, our data suggest that the effects of FLX on adult hippocampal neurogenesis are mainly driven by changes in the estrus and diestrus phases of the estrous cycle. Interestingly, estrogen treatment of OVX or castrated rats has antidepressant-like effects (Martinez-Mota et al. 2008). We found that adult hippocampal neurogenesis levels were relatively lower (although not significantly different from other phases) in the estrus phase when estrogen levels are lowest. It is therefore possible that FLX acts as a substitute for estrogen during estrus but that the naturally higher levels of estrogen during proestrus mask or prevent any effects of FLX on adult hippocampal neurogenesis.

Interestingly, the effects of FLX on DCX expression appear to be more pronounced in the ventral dentate gyrus during estrus and diestrus (Supplemental Figure 3.2). A few lines of evidence suggest that dorsal (posterior in primates) and ventral (anterior in primates) hippocampus play different roles in mediating behavior (Fanselow and Dong 2010). First, dorsal and ventral hippocampus have distinct anatomical connections (Fanselow and Dong 2010). Second, relatively specific manipulations suggest that dorsal performs primarily cognitive functions while ventral hippocampus performs functions related to stress and affective state (Kheirbek et al. 2013). Therefore, more profound effects of FLX on DCX in the ventral DG may permit a larger effect on affect-related behaviors.

Overall our study illustrates that different estrous phases show significant differences in behavior and adult hippocampal neurogenesis. Furthermore, the effects of FLX treatment on behavior and adult hippocampal neurogenesis are mainly driven by changes during the estrus and diestrus phases. Future studies should assess whether antidepressants influence endogenous levels of ovarian hormones, such as estrogen and progesterone, since fluctuations in these hormones across the estrous cycle would also affect behavior and adult hippocampal neurogenesis. Given that sex differences in the etiologies of mood disorders and symptomologies exist, preclinical studies that determine differences across the estrous cycle are critical for developing a better understanding of how these disorders develop and should be treated in females.

ACKNOWLEDGMENTS

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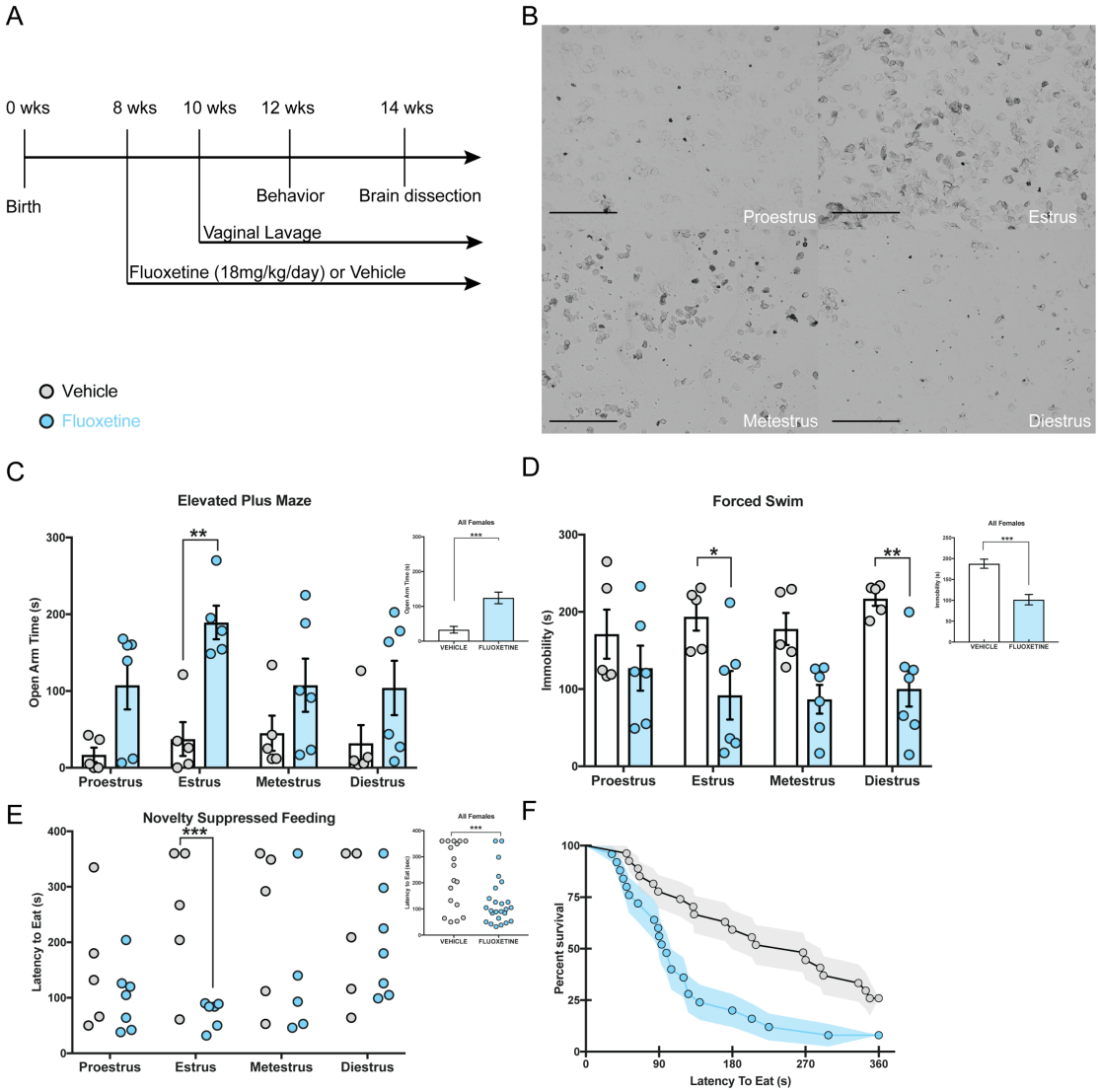


Figure 3.1. Behavioral differences between FLX- and vehicle-treated female mice are also mediated by estrous phase. A timeline depicting the experimental study is depicted (A) with representative images of the four phases of the estrous cycle (10x magnification; scale = 500 µm; B). (C) Separate 2x4 ANOVAs were run and revealed that overall females treated with FLX (smaller inset panel) spend less time in the open arms than vehicle mice ($F(1,35)=22.8$, $p<0.001$, $\eta^2 = 0.373$), with this group difference being evident within the estrus phase of the estrous cycle ($p = 0.003$, Bonferroni corrected). (D) In the FST, FLX appeared to reduce overall immobility time (treatment $F(1,35) = 25.77$, $p < 0.001$, $\eta^2 = 0.356$), with FLX-treated females in the estrus (p

= 0.025) and diestrus (p = 0.006) phases having significantly lower immobility scores than vehicle-treated females. (E) Small scatter plot represents Kaplan Meier survival analysis (F) with FLX-treated females have shorter latencies to eat in the NSF ($x^2(1)=8.95$, p=0.0028) task than vehicle-treated females. Larger graph (E) represents Kaplan Meier survival analysis within group differences being evident within the estrus phase (*** $x^2(1)=10.5$, p = 0.008).

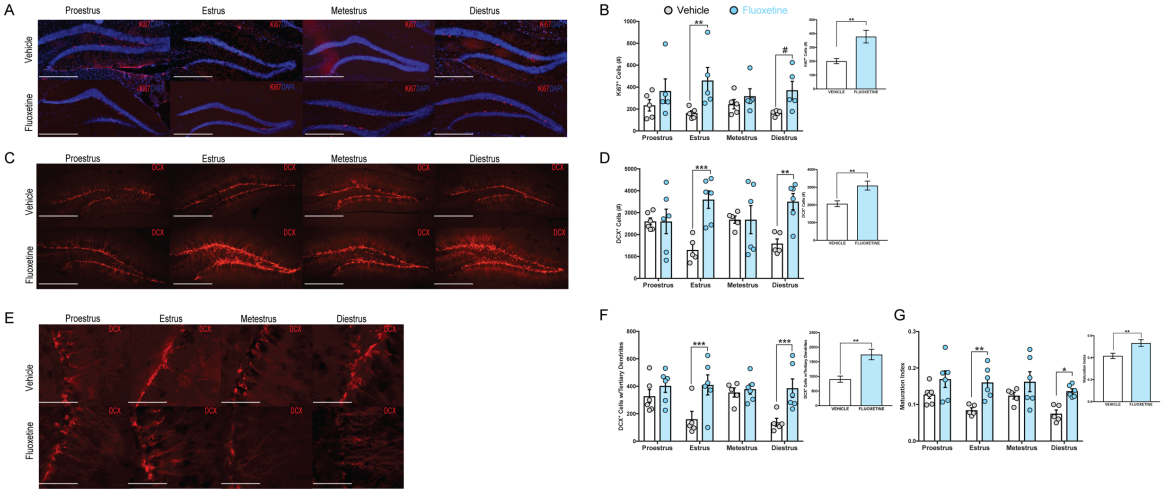


Figure 3.2. FLX increases all stages of neurogenesis with differences most pronounced in the estrus and diestrus phases. Visual representation of Ki67 and DCX immunostaining is depicted above for each treatment group across the estrous cycle phases. Ki67 (A) and DCX (C) images were taken at 10x magnification (scale = 500 µm) with mature neurons (E) taken at 40x magnification (scale = 125 µm). Smaller inset panels (B, D, F, G) represent 2x4 ANOVAs (cycle x treatment FLX-treated females had higher adult cell proliferation ($Ki67^+$, B, ** $F(1, 32) = 11.79$, $p = 0.002$, $\eta^2 = 0.234$) more immature (D, *** $F(1, 37) = 13.44$, $p < 0.001$, $\eta^2 = 0.280$) and mature (F, ***$F(1, 37) = 15.85$, $p < 0.001$, $\eta^2 = 0.401$) neurons as well as a higher maturation index (G, ***$F(1, 37) = 21.71$, $p < 0.001$) compared to vehicle animals. Larger panels represent the estrous effect from 2x4 ANOVAs (cycle x treatment) with FLX-treated females having more cell proliferation (B **estrus p = 0.002 and #diestrus p = 0.053), more immature neurons (D $F(3, 37) = 4.57$, $p = 0.008$, $\eta^2 = 0.275$, estrus ***$p < 0.001$, diestrus ** $p=0.009$) and mature (F, $F(3, 37) = 3.26$, $p = 0.032$, $\eta^2 = 0.126$; **estrus p = 0.007 and *diestrus p = 0.012) neurons as well as a higher maturation index (G, **estrus p = 0.002 and *diestrus p = 0.013) compared to vehicle-treated females.

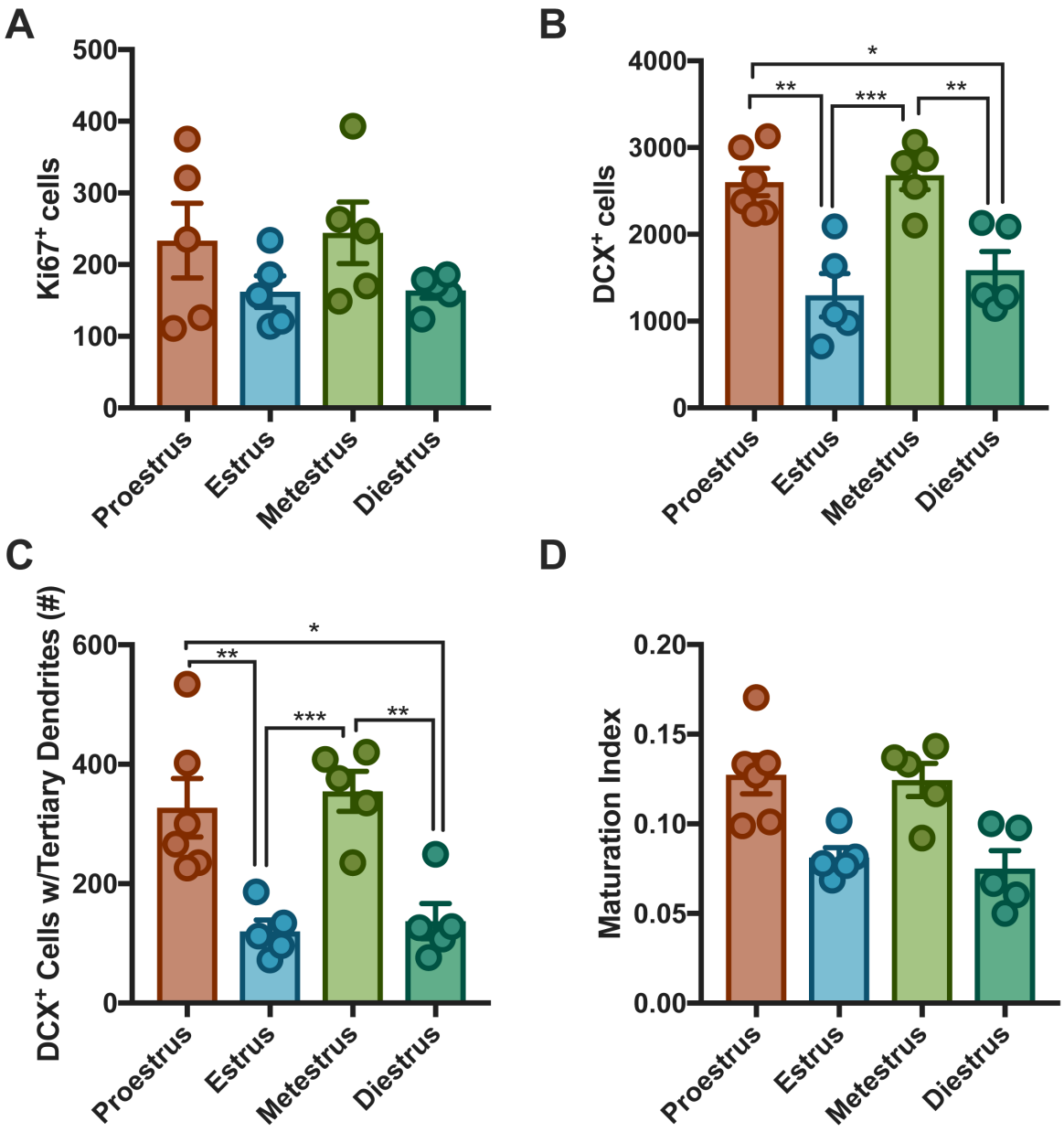
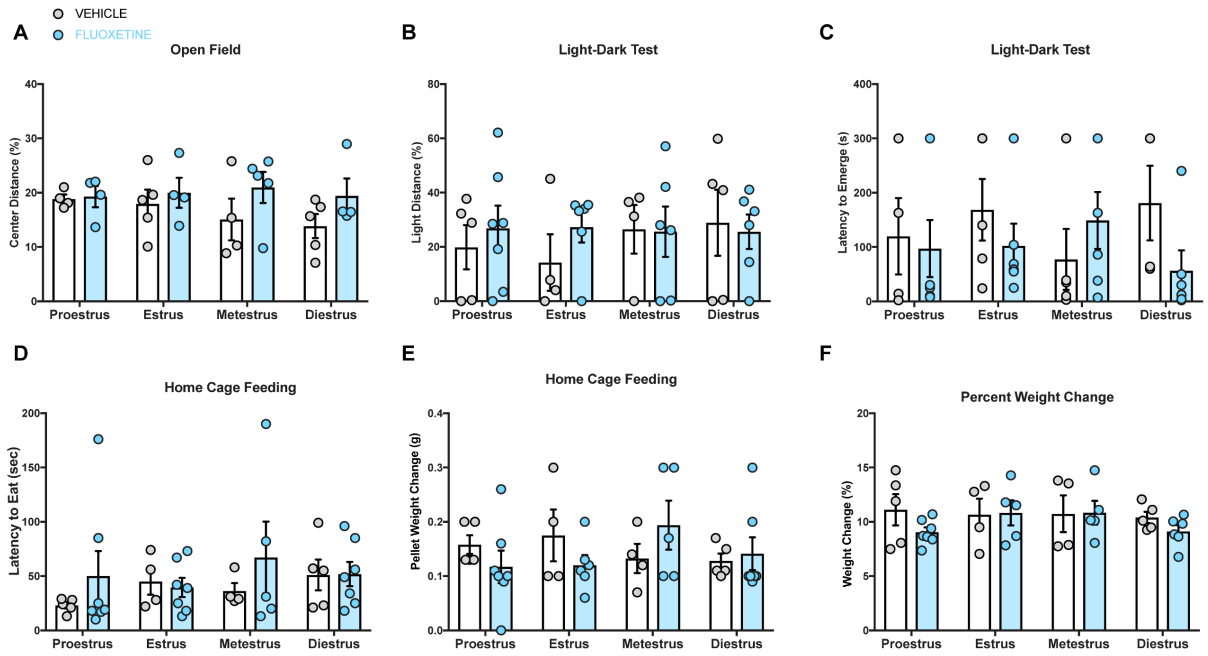
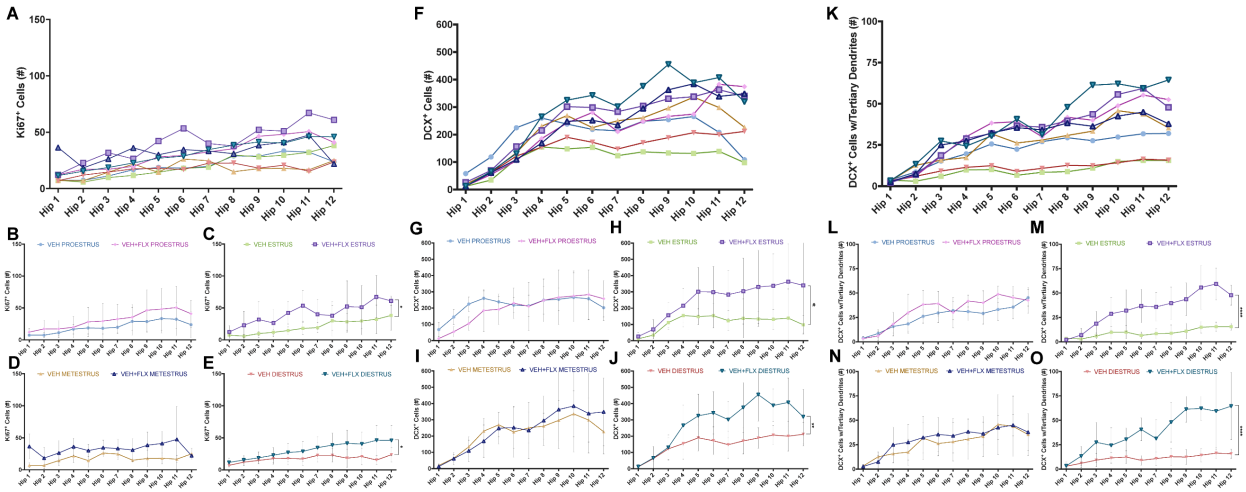


Figure 3.3. Estrous cycle impacts adult hippocampal neurogenesis in intact, cycling vehicle-treated female mice. Planned post-hoc analyses within the vehicle group was conducted to assess impact of estrous cycle on adult hippocampal neurogenesis. No differences in adult hippocampal cell proliferation was noted across the estrous cycle in vehicle females (A). Proestrus females had higher expression of immature (B **estrus p=0.001, *diestrus p=0.01) and mature (C, **estrus p=0.007; *diestrus p=0.012) neurons than estrus and diestrus

females. Metestrus females had more immature (***estrus $p < 0.001$; **diestrus $p = 0.008$) and (C) mature (***estrus $p=0.004$; **diestrus $p=0.006$) neurons estrus and diestrus. There was no impact of estrous cycle on maturation index (D).



Supplemental Figure 3.1. Null finding of FLX and estrous cycle. FLX treatment and estrous cycle do not impact percent center distance in the open field (A), distance traveled in the light compartment (B) or latency to emerge into the light compartment in the light dark test (C) or latency to eat in the home cage following NSF (D), amount of food eaten in the first five minutes in the home cage following NSF (E), or percentage of body weight lost in the food deprivation period prior to NSF (F).



Supplemental Figure 3.2. Distribution of adult hippocampal neurogenesis across each section of the DG. Line graphs showing the distribution of Ki67+(A), DCX+ (F), and DCX+ cells with tertiary dendrites (K) across the treatment groups of each estrous phase. To further investigate differences within each estrous phase, repeated measures ANOVAs were used across the 12 sections of the DG to analyze differences in adult hippocampal neurogenesis between treatment groups. (B-E) Interaction effects between VEH and FLX within each phase to illustrate the effects of FLX treatment on Ki67^{+}cell counts in each DG section along the dorsoventral axis: proestrus ($F_{(11,88)}$=9.89, p=0.345), estrus ($F_{(11,88)}$=4.55, p=0.045), metestrus ($F_{(11,88)}$=8.76, p=0.124), diestrus ($F_{(11,88)}$=10.07, p=0.048). (G-J) Interaction effects between VEH and FLX within each phase to illustrate the effects of FLX treatment on Dcx^{+}cell counts in each DG section along the dorsoventral axis: proestrus ($F_{(11,99)}$=1.25, p=0.264), estrus (#$F_{(11,99)}$=1.83, p=0.058), metestrus ($F_{(11,99)}$=0.73, p=0.705), diestrus (**$F_{(11,99)}$=2.69, p=0.0045). (L-O) Interaction effects between VEH and FLX within each phase to illustrate the effects of FLX treatment on Dcx^{+} with tertiary dendrites cell counts in each DG section along the dorsoventral axis: proestrus ($F_{(11,99)}$=1.47, p=0.144), estrus (****$F_{(11,99)}$=6.43, p<0.0001), metestrus ($F_{(11,99)}$=0.45, p=0.927), diestrus (***$F_{(11,99)}$=3.76, p=0.002). n=5 mice per stage per treatment.

Error bars indicate SEM. Error bars are not shown for panels A, F, and K in order to permit visualization of the line graphs.

CHAPTER 4

Inhibition of Dentate Gyrus Underlies Behavioral Response to Fluoxetine in both Female and Male Mice

This paper is in the process of being submitted to Neuropsychopharmacology: Yohn, C.N., Dieterich, A., Maita, I., Bazer, A.S., Emma Diethorn, Sandra Ashamalla, Mark M. Gergues, Benjamin A. Samuels

Abstract

Depression is a complex psychiatric disorder that is a major burden on society, with 33% of depressed patients attaining remission upon initial monotherapy with a selective serotonin reuptake inhibitor (SSRI). In preclinical studies using mice, chronic stress paradigms, such as chronic corticosterone and social instability stress, are used to induce negative valence behaviors. Chronic fluoxetine (FLX; an SSRI) treatment reverses these stress-induced behavioral changes in some, but not all mice, permitting stratification of mice into behavioral responders and non-responders to FLX. In using the novelty-suppressed feeding (NSF) task, we can behaviorally assess response status in both sexes, with a bimodal distribution emerging within the stress+FLX groups. Responders to FLX have a shorter latency to eat the pellet within the brightly light center of the NSF arena than non-responders. Research indicates that inhibition of mature Dentate Gyrus (DG) granule cells, through both cell autonomous Gi-coupled receptors and the local microcircuitry, may be critical for mounting a behavioral response to antidepressants. We observe that FLX-responders, have less DG cFos expression, indicative of less DG activation compared to same sex stress controls and FLX-non-responders. Additionally, chronic DREADD mediated inhibition of the ventral DG results in a decrease in negative valence behaviors in the presence or absence of stress. Furthermore, non-responders to FLX are converted into responders following chronic DREADD mediated inhibition of the ventral DG. Interestingly, in using two distinct chronic stress paradigms we illustrate that DG function in mediating antidepressant-like behaviors is independent of applied stressor.

INTRODUCTION

Mood disorders, such as depression, are increasingly prevalent in today's society. Nearly 16% of Americans experience an episode of major depression in their lifetime and over 300 million people are affected worldwide (Kessler et al., 2003; World Health Organization, 2017). Stressful life events are risk factors for the onset of mood disorders as patients show decreased levels of neuroplasticity, structural changes due to increased glucocorticoid levels, and decreased expression of neurotrophic factors. Antidepressant treatment enhances neuroplasticity and counteracts the impact of chronic stress (Calabrese et al., 2009; Joëls, 2011; Pittenger & Duman, 2008). The most commonly prescribed class of antidepressants are selective serotonin reuptake inhibitors (SSRIs), which ultimately lead to an increase in available serotonin (5-HT) levels by blocking reuptake into serotonergic neurons. Despite the popularity of SSRIs, 2 out of 3 patients with major depressive disorder do not remit after initial antidepressant treatment (Rush et al., 2006). Therefore, understanding the physiology of the antidepressant response and treatment resistance will lead to more effective treatments and therapies.

The dentate gyrus (DG), a subregion of the hippocampus, is strongly implicated in mediating the antidepressant response. Chronic antidepressant treatment increases all stages of adult neurogenesis in the DG. Specifically, chronic antidepressant treatment increases proliferation of neural precursor cells, differentiation into young adult-born granule cells (abGCs), and the rate of maturation of young abGCs into DG granule cells (GCs) and integration into the existing neural circuitry (REF). Ablation of adult neurogenesis in the hippocampus via x-irradiation blocks the behavioral response to antidepressants, suggesting that adult hippocampal neurogenesis is necessary for the antidepressant response (Malberg, Eisch, Nestler, & Duman, 2000; Yohn, Gergues, & Samuels, 2017; David et al., 2009; Santarelli et al., 2003;

Wang et al., 2008). Fourteen distinct 5-HT receptors exist, but 5-HT_{1A} receptors, which are Gi-coupled heteroreceptors, on DG GCs are implicated in the antidepressant response (Samuels et al., 2015; Yohn, Gergues, & Samuels, 2017). Specifically, Samuels and colleagues (2015) observed that 5-HT_{1A}-receptor-deficient mice engineered to express 5-HT_{1A} receptors on DG GCs respond to SSRIs, while mice lacking mature DG GC 5-HT_{1A} receptors show no behavioral response to SSRIs (Samuels et al., 2015). The ventral DG regulates avoidance behaviors and facilitates the response to antidepressants via 5-HT receptors. Optogenetic inhibition of the ventral DG, but not the dorsal DG, suppresses innate avoidance behaviors in tasks such as the open field (Bagot et. al., 2015; Kheirbek et. al., 2013), suggesting that inhibition of the ventral DG mimics an antidepressant response. Furthermore, chemogenetic inhibition of the ventral DG also promotes resilience to chronic stress, whereas excitation promotes susceptibility (Anacker). More specifically, inhibition of mature DG GCs in the ventral DG increases susceptibility to chronic stress, while increased neurogenesis promotes resilience to chronic stress and decreased activity in mature DG GCs, suggesting that young adult-born neurons inhibit mature DG GCs to decrease susceptibility to chronic stress (Anacker et al., 2018; Luna et al., 2019).

Novelty suppressed feeding (NSF), a behavioral paradigm, can be used to assess responder/non-responder patterns to antidepressant treatment (Gergues, Yohn et al, bioRxiv). In NSF, latency to approach and eat a food pellet in the center of a brightly lit arena after 18 hours of food deprivation is observed. A longer latency to eat suggests increased maladaptive avoidance behavior. Chronic SSRI treatment tends to significantly reduce latency to eat. However, data from NSF often indicates a bimodal distribution in which a subset of antidepressant-treated mice retain a long latency to eat (Samuels & Hen, 2011; Gergues, Yohn et al, bioRxiv). This bimodal distribution permits stratification of mice into presumptive responders

(shorter latency to eat) and non-responders (longer latency to eat) to SSRI treatment. This study aims to assess the role of the DG in the underlying antidepressant response. Inhibition of mature DG GCs, via activation of Gi-coupled 5-HT_{1A} receptors, is important for the antidepressant response. Therefore, by utilizing chemogenetic DREADD-mediated inhibition and excitation, we seek to clarify the role of the ventral DG in the antidepressant response. We predict that Gi-DREADD-mediated inhibition of ventral DG GCs will mimic an antidepressant response, while Gq-DREADD-mediated activation of ventral DG GCs will increase maladaptive avoidance behaviors. We also aim to understand if Gi-DREADD-mediated silencing of DG GCs can convert FLX non-responders into responders.

Subjects

Adult male and female C57BL/6J mice (age 8 weeks) were purchased from Jackson Labs (Bar Harbor, ME). Mice were maintained on a 12L:12D schedule with lights turned on at 6 am and lights turned off at 6pm. Food and water were provided ad libitum. All behavioral testing occurred in the light phase between the hours of 8 a.m. and 11 a.m. All testing was conducted in compliance with the NIH lab animal care guidelines and was approved by the Rutgers University Institution of Animal Care and Use Committee (Protocol # 17-015; 15-070).

Stress Paradigms

Chronic Corticosterone

Adult male C57BL/6J mice were randomly divided into Corticosterone (CORT) and VEH treatments, with weights measured on a weekly basis during treatment. VEH treated mice were administered 0.45% beta-cyclodextrin dissolved in their drinking water, whereas CORT treated mice received a CORT (35 μg/mL) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.45% beta-cyclodextrin (4.5 mg/mL) (Sigma-Aldrich, St. Louis, MO). CORT was administered in opaque

bottles due to the light sensitivity of the drug (David et al., 2009). CORT treatment lasted the duration of all experiments it was involved in.

Social Instability Stress (SIS)

Female C57BL/6J mice were randomly assigned into SIS or control (CNTRL) groups. SIS mice were subject to fluctuating and unstable social environments in which social dynamics are changed twice a week for 7 weeks (Yohn et al., 2019). During the 7 weeks, the cage composition changed twice a week in which the mice became housed with novel mice of the same sex. The total number of mice per cage at any given time ranged from 3-5 mice. The rotation schedule was randomized to prevent mice from being housed with a recent house mate. Following the initial 7 week exposure to SIS, we continued SIS for the duration of the experiments with changing of cage composition not occurring on behavioral testing days. Female mice in the CNTRL group were housed with the same mice during the entire duration of the SIS paradigm and cages were changed twice per week.

Viral Injections

To assess antidepressant-like effects of inactivation or activation of ventral DG GC one of three DREADD viruses AAV8-CamKIIa-hm4D(Gi)-mCherry, AAV8-CamKIIa-hm3D(Gq)-mCherry, AAV8-CamKIIa-EGFP-mCherry were injected into the ventral DG of 6 week old mice: 3.5mm, +/-2.8mm relative to the bregma line and midline respectively at a depth of 3.6 mm from the skull at the bregma.

Drug Treatment

Fluoxetine (FLX)

Subsequent CORT treatment or SIS, fluoxetine (18 mg/kg/day in deionized water) or VEH (deionized water) was administered via oral gavage for three weeks prior to behavioral testing.

Oral gavaging of FLX or VEH solution continued into the behavioral testing phase. During behavioral testing days, oral gavage was delivered following the test to avoid any acute effects.

CNO

Water bottles were filled on alternating days with 0.05 mg/ml CNO. CNO was dissolved in DMSO, for a final concentration of 0.25% DMSO, and added to 0.5% saccharine water. Based off of mice weights ranging between 30–35 g and water consumption approximately 3 ml/day, each mouse received an approximate dose of 5 mg/kg CNO daily.

Behavioral Testing

Open Field (OF)

To assess motor activity, mice were placed in the corner of a Plexiglas open field chamber measuring 43cm X 43 cm and monitored using Motor Monitor software (Kinder Scientific, Poway, CA). Through infrared photobeams on the wall, the software measured the distance traveled (cm) and both time and entry into the center of the OF. The center was defined as a sequare 11 cm from each wall of the OF. In our analysis, we ...

Novelty Suppressed Feeding (NSF)

After an 18-hour food deprevation period, mice were placed in the corner of a novel, brightly lit VSF chamber which contained a food pellet in the center. The mice were observed and the latency to eat the food pellet was recorded. NSF was used the conflict between the need to forage for food in a food deprived state and the uncertainty of a new, brightly lit environment.

Forced Swim test (FST)

Mice were placed in a Forced Swim Test chamber filled with room temperature water for 6 minutes. Motor Monitor software (Kinder Scientific, Loway, CA) was used to measure

immobility, defined as 6 or less beam breaks over the span of 5 seconds, in the last 4 minutes of the test.

Light Dark (LD)

Within the OF arena, a dark opaque rectangular box is placed inside to cover ⅓ of the arena area and to separate the area into a light compartment from a dark compartment. The box contains a small opening for the mice assess the light part of the arena, which is brightly illuminated (1000 lux; KinderScientific). Mice are placed in the dark compartment of the arena and monitored for 5 minutes. Percent distance traveled in the light compartment (distance traveled in the light compartment/ total distance * 100) is measured for analysis.

Elevated Plus Maze (EPM)

The elevated plus maze is a plus shaped apparatus raised 2 feet above floor level and consists of two dark arms that are dimly lit and two light arms that are brightly lit. At the start of the 5 minute test, mice are placed in the center of the plus maze and video recorded by a camera mounted on the ceiling above the EPM. Using EthoVision software (Noldus, Wageningen, Netherlands) time spent in the open arms and distance traveled in both open and closed arms were assessed. In our analysis, the percent distance traveled was derived (total open arm distance traveled/ total distance traveled).

IHC

cFos staining

The effects of stress (CORT or SIS) in the presence or absence of fluoxetine treatment on DG GC activity was assessed (timelines Figure 4.2a & e). We also confirmed DG GC activity to confirm inhibition or excitation of the vDG in mice undergoing the DREADD experiments (Figure 3a & i). Forty minutes after the NSF task, when cFos peaks in expression, animals were

anesthetized with ketamine and xylazine (100 mg/ml ketamine; 20 mg/ml xylazine), and perfused transcardially (cold saline for 2 min, followed by 4% cold paraformaldehyde at 4°C). The brains were removed and stored in 4% paraformaldehyde overnight at 4°C. Next brains were cryoprotected in 30% sucrose, 1% sodium azide and stored at 4°C. Serial sections (40 μM) were cut on a cryostat and collected through the entire hippocampus (Franklin and Paxinos, 1997). Sections were collected in wells and wet mounted prior to staining. Sections were washed in 1% Triton X- 100 PBS for 5 minutes before undergoing three PBS washes. Slides were incubated in warm citrate buffer for 30 minutes. After washing with PBS, slides were blocked for 1 hour in 10% normal goat serum (NGS) before being incubated overnight at 4°C in anti-rabbit cFos (1:750; Cell Signal). Next, slides were washed with PBS then incubated at room temperature for two hours in the secondary antibody (CY-5 goat anti-rabbit; 1:1000). Following the incubation slides were washed with PBS then counterstained with DAPI (1:15000) for 15 minutes. Finally, slides were washed with PBS and cover slipped using the mounting medium prolong diamond. Fluorescent images were taken using an inverted microscope (ThermoFisher), where cFos positive cells overlayed with DAPI across the 12 sections of hippocampus will be counted.

RESULTS

Behavioral Response Phenotypes to Fluoxetine Emerge in both Male and Female mice

To better understand the potential treatment resistance phenotype following chronic stress and antidepressant treatment, we exposed a cohort of group housed 8-week-old male C57BL/6J mice to chronic vehicle (VEH) or corticosterone (CORT, 5mg/kg/day via drinking water) administration. Chronic CORT administration at this dosage induces several maladaptive behaviors, including increased latency to feed in NSF and decreased open arm entries and duration in the elevated plus maze (EPM). We administered vehicle or CORT for 4 weeks and

then co-administered either VEH or the SSRI fluoxetine (FLX, Prozac, 18mg/kg/day) for an additional 3 weeks (timeline Figure 1a). As expected, we found significant group differences in latency to feed in the NSF ($x(3)=36.75$, $p<0.000001$, logrank Mantel-Cox test; Figure 4.1b) with coadministration of CORT+FLX resulting in significantly reduced latency to feed relative to CORT+VEH treated mice ($p=0.00045$, logrank Mantel-Cox with Bonferroni correction) and VEH+VEH mice($p<0.000001$, logrank Mantel-Cox with Bonferroni correction) (Figure 1b). Similar to our previous findings (Gergues, Yohn et al bioRxiv), closer inspection of the individual latencies of CORT+FLX mice demonstrated a bimodal distribution, providing a potential basis for dividing mice into responders and non-responders to FLX treatment groups. Furthermore, food consumption in the home cage was similar among all mice (Supplemental Figure 4.1a).

We next exposed the same cohort of C57BL/6J mice to elevated plus maze (EPM) and the forced swim test (FST), which is a commonly used test of antidepressant efficacy. In the EPM, separate two-way ANOVAs revealed effects of CORT administration and FLX treatment in distance traveled on the open arms (CORT: $F(1,67)=16.12$, $p=0.0002$, FLX: $F(1,67)=8.83$, $p=0.0041$; Figure 1c left) and open arm duration (CORT: $F(1,67)= 40.3$, $p<0.0001$, FLX: $F(1,67)=15.81$, $p=0.0002$; ; Figure 4.1d left). To investigate behavioral differences between CORT-only treated mice, NSF-defined CORT+FLX responders, and non-responders in the EPM, we used one-way ANOVAs and found significant differences in distance traveled on open arms ($F(2,43)=30.03$, $p<0.001$; Figure 4.1c right) and duration ($F(2,43)=73.71$, $p<0.001$; Figure 1d right), with Bonferroni-corrected post hoc tests demonstrating that responders had significantly increased open arm exploration (Figure 4.1c right)and duration relative (Figure 1d right) to VEH treated mice and non-responders (entries and duration: CORT+VEH vs CORT+FLX-R and

CORT+FLX-R vs CORT+FLX-NR, all $p<0.001$). These data suggest that FLX response status is conserved across the NSF and EPM. Similarly, in the FST, a two-way ANOVA revealed effects of both CORT administration ($F(1,67)= 6.44$, $p=0.013$) and FLX treatment ($F(1,67)=26.68$, $p<0.0001$) on immobility (last four minutes of the six minute test) (Figure 1e left). A separate one-way ANOVA demonstrated significant differences in immobility ($F(2,43)=72.13$, $p<0.001$), with Bonferroni-corrected post hoc tests demonstrating that responders had significantly decreased immobility relative to VEH treated mice and non-responders (CORT+VEH vs CORT+FLX-R and CORT+FLX-R vs CORT+FLX-NR, both $p<0.001$) (Figure 4.1e right). Taken together, these data suggest that FLX response status across NSF, EPM, and FST is conserved in CORT-treated male mice.

We next wanted to examine whether antidepressant response status is conserved across behaviors using a distinct stressor in female mice, as the effects of CORT on behavior are more prominent in male mice (Yohn et al, 2019; Guilloux et al., 2017). We recently developed a chronic social stress paradigm, social instability stress (SIS), that induces maladaptive behaviors in female mice (Yohn et al., 2019) through exposure to unstable social hierarchies. To this end, we exposed a cohort of group housed 8-week-old female C57BL/6J mice to chronic SIS or control (CNTRL). The SIS paradigm is a social stressor where mice are exposed to different same-sex cage-mates every 3 days over 7 weeks (Yohn et al 2019). Following the initial 7 weeks of SIS, we administered VEH or FLX for an additional 3 weeks, while continuing to expose mice to SIS (Figure 4.1f). As expected, we found significant group differences in latency to feed in the NSF ($x(3)=22.62$, $p=0.000048$, logrank Mantel-Cox test; Figure 4.1g) with SIS+FLX treated mice having a significantly reduced latency to feed relative to SIS+VEH mice ($p=0.018$, logrank Mantel-Cox with Bonferroni correction), indicative of an antidepressant response. CNTRL+VEH

mice showed a significant reduced latency to feed relative to SIS+VEH mice ($p<0.000001$, logrank Mantel-Cox with Bonferroni correction). Similar to what we observed with CORT+FLX males, the individual latencies of SIS+FLX females demonstrated a bimodal distribution. Furthermore, food consumption in the home cage was similar among all mice (Supplemental Figure 4.1c).

We next exposed the same cohort of C57BL/6J female mice to EPM and FST. In the EPM, separate two-way ANOVAs revealed effects of SIS and FLX treatment in distance traveled on the open arms (SIS: $F(1,49)=8.94$, $p=0.0043$, FLX: $F(1,49)=14.73$, $p=0.0004$) (Figure 4.1h left) and open arm duration (SIS: $F(1,49)=34.9$, $p<0.0001$, FLX: $F(1,49)=23.39$, $p<0.0001$) (Figure 4.1i left). To investigate behavioral differences between SIS-only treated mice, NSF-defined SIS+FLX responders, and non-responders in the EPM, we used one-way ANOVAs and found significant differences in distance traveled on open arms ($F(2,30)=18.99$, $p<0.001$) (Figure 4.1h right) and duration ($F(2,30)=74.2$, $p<0.001$) (Figure 4.1i right), with Bonferroni-corrected post hoc tests demonstrating that responders had significantly increased open arm exploration and duration relative to vehicle treated mice and non-responders (distance and duration: SIS+VEH vs SIS+FLX-R and SIS+FLX-R vs SIS+FLX-NR, $p\leq0.002$ for all). These data suggest that FLX response status is conserved across the NSF and EPM. Similarly, in the FST, a two-way ANOVA revealed effects of both SIS ($F(1,49)=12.53$, $p=0.0009$) and FLX treatment ($F(1,49)=40.15$, $p<0.0001$) on immobility (Figure 4.1j left). A separate one-way ANOVA demonstrated significant differences in immobility ($F(2,30)=55.8$, $p<0.001$) (Figure 1j right), with Bonferroni-corrected post hoc tests demonstrating that responders had significantly decreased immobility relative to vehicle treated mice and non-responders (SIS+VEH vs SIS+FLX-R and SIS+FLX-R vs SIS+FLX-NR, $p\leq0.002$ for both). Taken together, these data

suggest that FLX response status across NSF, EPM, and FST is conserved in SIS treated female mice. Moreover, these data demonstrate that using two different stressors (CORT and SIS) and different sexes, FLX response status is conserved across behaviors.

DG GC activity during NSF task

To assess the role of the DG in facilitating the behavioral response to antidepressants, we treated a separate cohort of male mice with 4 weeks of CORT or VEH administration, then administered either VEH or FLX for 3 additional weeks (timeline Figure 4.2a). Following chronic FLX or VEH treatment, mice underwent the NSF task to assess behavioral response status and were sacrificed 40 minutes post-NSF in order to investigate expression of the immediate early gene cFos. We collected and stained 1 out of every 6 sections of the DG (total of 12 sections counted) to assess cFos expression across the full DG granule cell (GC) layer (Figure 4.2d). As expected, we found significant group differences in latency to feed in the NSF ($x(3)=22.85$, $p=0.000043$, logrank Mantel-Cox test) (Figure 4.2b left) with coadministration of CORT and FLX resulting in significantly reduced latency to feed relative to CORT+VEH treated mice ($p=0.018$, logrank Mantel-Cox with Bonferroni correction) and VEH+VEH treated mice ($p=0.000018$, logrank Mantel-Cox with Bonferroni correction), indicative of an antidepressant response (Figure 4.2b right). No differences in home cage latency to feed were observed (Supplemental figure 4.1b). A two-way ANOVA revealed FLX treatment ($F(1,36)=33$, $p<0.0001$), but not CORT administration ($F(1,36)= 0.96$, $p=0.332$) significantly reduces cFos expression in the DG (Figure 2c left). Bonferroni post-hoc comparisons revealed that both VEH-only and CORT+VEH mice have more cFos expression than mice treated with FLX (VEH: $p=0.0027$; CORT: $p<0.0001$) (Figure 4.2c right). To investigate differences of cFos expression between CORT-only treated mice, NSF-defined CORT+FLX responders, and non-responders,

we used a one-way ANOVA that showed CORT+FLX-R mice have fewer DG cFos+ cells than CORT+VEH ($p<0.001$) and CORT+FLX-NR mice ($p=0.016$) (Figure 4.2c right).

We next exposed a cohort of female mice to 7 weeks of SIS, then administered either VEH or FLX for 3 additional weeks. Following chronic FLX or VEH treatment, mice underwent the NSF task to assess behavioral response status and were sacrificed 40 minutes post-NSF (timeline Figure 4.2e). We collected and stained 1 out of every 6 sections of the DG to assess cFos expression (Figure 4.2h). As expected, we found significant group differences in latency to feed in the NSF ($x(3)=21.16$, $p=0.000097$, logrank Mantel-Cox test) (Figure 4.2f left). Bonferroni post-hoc comparisons revealed that SIS+VEH mice displayed a significantly increased latency to eat compared to SIS+FLX ($p=0.0099$, logrank Mantel-Cox with Bonferroni correction) and CNTRL+VEH mice ($p=0.000018$, logrank Mantel-Cox with Bonferroni correction) (Figure 4.2f right). No differences in home cage latency to feed were observed (Supplemental figure 4.1d). A two-way ANOVA revealed FLX treatment ($F(1,36)=40.07$, $p<0.0001$), but not SIS ($F(1,36)=2.57$, $p=0.117$) significantly reduces cFos expression in the DG (Figure 4.2g left). To investigate differences of cFos expression between SIS-only treated mice, NSF-defined SIS+FLX responders, and non-responders, we used a one-way ANOVA that showed SIS+FLX-R mice have fewer DG cFos+ cells than SIS+VEH ($p<0.001$) and SIS+FLX-NR mice ($p<0.001$) (Figure 4.2g right). Taken together, these data demonstrate across two distinct chronic stressors and both sexes that non-responders to FLX treatment show an increased number of activated DG GCs in response to NSF exposure.

Ventral DG DREADD mediated regulation of behavior

Males

Based on the cFos data, and the fact that inhibition of DG GCs is important for mediating the antidepressant response and stress resilience (Samuels et al 2015, Anacker et al), we next wanted to investigate the effects of Gi- and Gq-DREADD expression and activation in DG GCs on behavior. We hypothesized that Gi-DREADD-mediated inhibition of DG GCs in male mice exposed to CORT would mimic an antidepressant response and that Gq-DREADD-mediated activation of DG GCs in unstressed mice would mimic the effects of chronic stress. To this end, we bilaterally injected either AAV8-CamKIIa-hm4D(Gi)-mCherry, AAV8-CamKIIahm3D(Gq)-mCherry, AAV8-CamKIIa-EGFP-mCherry into the ventral DG of 6 week old mice: 3.5mm, +/-2.8mm relative to the bregma line and midline respectively at a depth of 3.6 mm from the skull at the bregma (Figure 4.3a). We then treated these mice with either VEH or CORT beginning at 8 weeks of age. After 4 weeks of CORT treatment, we then co-administered either VEH or CNO (0.05 mg/ml for average consumption of 5mg/ml CNO daily) in the drinking water (Figure 4.3a). To confirm that CNO activated the DREADD viruses we measured activity of DG GCs following exposure to the NSF by cFos expression. A two-way ANOVA between treatment (CNO or VEH) and viral groups (Gi-DREADD, Gq-DREADD, GFP), showed significant effects of treatment ($F(2, 30)=9.78$, $p=0.004$) and virus ($F(2, 30)=13.71$, $p<0.001$), with a significant interaction also present ($F(2,30)=21.2$, $p<0.001$) (Supplemental figure 4.2c &d). Bonferroni corrected post hoc comparisons revealed that Gi-DREADD+CNO mice had lower vDG cFos expression compared to GFP+CNO ($p<0.001$) and Gq-DREADD+CNO ($p<0.001$) mice. Additionally, Gi-DREADD+CNO mice had less vDG cFos expression than Gi-DREADD+VEH ($p<0.001$), while Gq-DREADD+CNO mice had more vDG cFos expression than Gq-DREADD+VEH ($p=0.046$). Gq-DREADD+CNO mice also had more vDG cFos expression than GFP+CNO mice ($p=0.040$) (Supplemental figure 4.2c &d). These results suggest that the

different DREADD viruses resulted in modification of vDG GC activity, with application of CNO in Gi-DREADD mice leading to vDG inhibition, while CNO administration in the Gq-DREADD mice activated the vDG. Lastly, we investigated differences in latency to eat within the NSF with a Mantle Cox log-rank test revealing significant differences between the VEH groups ($x^2(5)=17.99$, $p=0.003$). Bonferroni post-hoc comparisons showed that Gq-DREADD+CNO mice have a longer latency to eat than Gi-DREADD+CNO ($p<0.0001$) and Gq-DREADD+VEH ($p=0.0015$) male VEH mice (Figure 4.3b). No differences were observed in home cage latecy to eat (Supplemental Figure 4.2h).

We next ran separate two-way ANOVAs within VEH and CORT groups to investigate differences between viral groups and treatment (CNO or VEH). Within the VEH group we observed a significant interaction between virus and treatment on both OF center distance traveled ($F(2, 24)=3.791$, $p=0.0371$) and time ($F(2, 24)=6.85$, $p=0.0044$; Figure 4.3). Significant effects of virus (distance: $F(2, 24) = 4.628$,$p=0.02$; time: $F(1, 24)=7.658$, $p=0.0107$) and treatment (distance: $F(1, 24)=16.81$, $p=0.0004$; time: $F(1, 24) = 7.658$, $p=0.0107$) were also found on OF center distance traveled (Figure 4.3c)and time (Figure 4.3d). Bonferroni post-hoc comparisons revealed that within the VEH group Gq-DREADD+CNO mice traveled significantly less distance ($p=0.0004$) in the center than Gq-DREADD+VEH male mice (Figure 4.3c). Additionally, Gi-DREADD+CNO mice spent more time in the OF center than Gi-DREADD+VEH ($p=0.0022$), Gq-DREADD+CNO ($p=0.0003$), and GFP+CNO ($p=0.0014$) male mice (Figure 3d). In the LD task we investigated group differences in distance traveled and time spent in the light and found significant viral (distance: $F(2, 24)=6.72$, $p=0.0048$; time: $F(2, 24)=9.86$, $p=0.0007$) and treatment (time: $F(1, 24)=6.24$, $p=0.0197$) effects as well as an interaction (distance: $F(2, 24)=4.883$, $p=0.016$; time: $F(2, 24)= 11.44$, $p=0.0003$) (Figure 3e & f).

We observed no significant treatment effect in distance traveled in the LD light (p=0.2948). Bonferroni post-hoc comparisons revealed that Gq-DREADD+CNO traveled and spent less time in the light than GFP+CNO (distance: p=0.0019, time: p=0.0037) and Gi-DREADD+CNO (distance: p=0.0011; time: p<0.0001) VEH male mice (Figure 4.3e & f). VEH Gi-DREADD+CNO male mice also spent significantly more time in the light than Gi-DREADD+VEH mice (p<0.0001) (Figure 3f). We next investigated differences in the EPM with significant viral (distance: $F(2, 24)=9.197$, $p =0.0011$; time: $F(2, 24)=12.7$, $p=0.0002$) and treatment (time: $F(1, 24)=6.656$, $p=0.0164$) effects as well as a significant interaction (distance: $F(2, 24)=8.534$, $p=0.0016$; time: $F(2, 24)=12.7$, $p=0.0002$) (Figure 4.3g & h). We observed no significant treatment effect in distance traveled on EPM open arms (p=0.9565) within VEH males. Bonferroni post-hoc comparisons showed that within the VEH group Gi-DREADD+CNO mice traveled more distance (Figure 4.3g) and spent more time (Figure 4.3h) on the open arms than Gi-DREADD+VEH (distance: p=0.0078; time: p<0.0001), Gq-DREADD+CNO (distance: p<0.0001; time: p<0.0001), and GFP+CNO (distance: p=0.0004; time: p<0.0001) male mice. Across the behavioral task OF, LD, and EPM we found no differences in total distance traveled (p's=0.05) (Supplemental figure 4.2 e-g). Taken together, these data indicate that cell autonomous inhibition of the vDG results in anxiolytic behaviors, while vDG stimulation exerts anxiogenic effects on a non-stress background.

Next, we analyzed differences between viral and treatment groups within the CORT treated male mice using separate two-way ANOVAs for each behavioral task. We found significant group differences in NSF latency to feed ($x^2(5)=23.58$, $p=0.0003$) with Bonferroni post-hoc comparisons revealing Gi-DREADD+CNO mice eat significantly faster than Gi-DREADD+VEH (p=0.0035), Gq-DREADD+CNO (p<0.0001), and GFP+CNO (p=0.0025) male

CORT mice (Figure 4.3b). No differences were observed in home cage latecy to eat (Supplemental Figure 4.2h). In the OF, we observed distance traveled and time spent in the center was significantly affected by viral (distance: $F(2, 24) = 4.519$, $p = 0.0216$; time: $F(2, 24) = 4.375$, $p = 0.024$) and treatment (distance: $F(1, 24) = 4.927$, $p = 0.0379$; time: $F(1, 24) = 4.401$, $p = 0.046$) condition (Figure 4.3c & d). In both OF center measurements we found significant interactions (distance: $F(2, 24) = 3.791$, $p = 0.0241$; time: $F(2, 24) = 2.97$, $p = 0.07$). Bonferroni post-hoc comparisons showed Gi-DREADD+CNO mice travel (Figure 4.3c) and spend more time (Figure 3d) in the center than Gi-DREADD+VEH (distance: $p = 0.0064$; time: $p = 0.036$) and Gq-DREADD+CNO (distance: $p = 0.001$; time: $p = 0.0027$). Analyzing group differences in distance traveled (Figure 4.3e) and time (Figure 3f) spent in the light we found significant viral (distance: $F(2, 24) = 6.72$, $p = 0.0002$; time: $F(2, 24) = 22.89$, $p < 0.0001$) and treatment (distance: $F(1, 24) = 1.147$, $p < 0.0001$; time: $F(1, 24) = 24.84$, $p < 0.0001$) effects as well as a significant interaction (distance: $F(2, 24) = 13.04$, $p = 0.0001$; time: $F(2, 24) = 26.57$, $p < 0.0001$). Bonferroni post-hoc comparisons revealed that Gi-DREADD+CNO mice significantly travel (Figure 3e) and spend more time (Figure 4.3f) in the light than Gi-DREADD+VEH (distance: $p < 0.0001$; time: $p < 0.0001$), Gq-DREADD+CNO (distance: $p < 0.0001$; time: $p < 0.0001$), and GFP+CNO (distance: $p = 0.0004$; time: $p < 0.0001$) mice. We also found that Gq-DREADD+CNO mice spend less time in the light than GFP+CNO ($p = 0.0037$) mice (Figure 4.3f). Investigating group differences in distance and time spent on the EPM open arms we found significant viral (distance: $F(2, 24) = 8.021$, $p = 0.0021$; time: $F(2, 24) = 16$, $p < 0.0001$) and treatment (distance: $F(1, 24) = 8.383$, $p = 0.0079$; time: $F(1, 24) = 4.83$, $p = 0.0377$) effects together with a significant interaction (distance: $F(2, 24) = 8.446$, $p = 0.0017$; time: $F(2, 24) = 17.03$, $p < 0.0001$) (Figure 4.3g & h). Bonferroni post-hoc comparisons showed that Gi DREADD+CNO mice travel more (Figure

4.3g) and spend more time (Figure 4.3h) on EPM open arms than Gi-DREADD+VEH (distance: $p=0.0001$; time: $p<0.0001$), Gq-DREADD+CNO (distance: $p<0.0001$; time: $p<0.0001$), and GFP+CNO (distance: $p=0.0003$; time: $p<0.0001$) male mice (Figure 4.3g & h). Within the OF, LD, and EPM we observed no significant group differences in overall distance traveled (all p's>0.05) (Supplemental figure 4.2 e-g). Taken together, these data indicate that inhibition of the vDG on a stress background decreases negative valence behaviors, while stimulation of vDG does not further exacerbate the stress effects. We also observe no implications of CNO administration on behavior.

Females

In order to examine the impact of DREADD-mediated modulation of ventral DG GCs on female behavior, we ran separate two-way ANOVAs within CNTRL and SIS groups to investigate differences between viral groups and treatment (Figure 4.3i). Similar to the males, we confirmed CNO activation of the DREADD by measuring cFos expression in the vDG following exposure to the NSF (Supplemental figure 4.2 a, b, i. j). A two-way ANOVA between treatment (CNO or VEH) and viral groups (Gi-DREADD, Gq-DREADD, GFP), showed significant effects of treatment ($F(2, 30)=11.2$, $p=0.002$) and virus ($F(2, 30)=17.07$, $p<0.001$), with a significant interaction also present ($F(2,30)=27.87$, $p<0.001$) (Supplemental figure 4.2i & j). Bonferroni corrected post hoc comparisons revealed that Gi-DREADD+CNO mice had lower vDG cFos expression compared to GFP+CNO ($p<0.001$) and Gq-DREADD+CNO ($p<0.001$) mice. Additionally, Gi-DREADD+CNO mice had less vDG cFos expression than Gi-DREADD+VEH ($p<0.001$), while Gq-DREADD+CNO mice had more vDG cFos expression than Gq-DREADD+VEH ($p=0.026$) (Supplemental figure 4.2i & j). Furhtermore, Gq-DREADD+CNO mice had more cFos expression than GFP+CNO mice ($p=0.039$). These results suggest that the

different DREADD viruses resulted in modification of vDG GC activity, with application of CNO in Gi-DREADD mice leading to vDG inhibition, while CNO administration in the Gq-DREADD mice activated the vDG. We investigated differences in latency to eat within the NSF with a Mantle Cox log-rank test, which revealed significant differences between the CNTRL groups ($x^2(5)=20.89$, p=0.0008) (Figure 4.3j). Bonferroni post-hoc comparisons showed that Gq DREADD+CNO mice have a longer latency to eat than Gi DREADD+CNO mice (p=0.0005), GFP+CNO mice (p=0.0007), and Gq DREADD+VEH mice (p=0.0007). No differences in home cage latency to feed were observed (Supplemental figure 4.2n).

Within the CNTRL group, we observed no effect of virus, treatment, nor interaction on distance traveled or time spent in the center of the OF (all p's>0.05; Figure 4.3k & l). Next, looking at distance and time traveled in the light portion of the LD chamber, we found a significant viral effect (distance: F(2, 30)=3.71,p=0.036; time: F(2, 30)=9.86, p<0.0001) with no significant effect of treatment (distance: F(1, 30)=2.062,p=0.1614; time: F(1, 30)=6.24,p=0.213). We also found significant interactions (distance: F(2, 30)=6.955,p=0.0033 ; time: F(2,30)=9.5, p=0.0006) (Figure 4.3m & n). Bonferroni post-hoc comparison showed that Gq DREADD+CNO traveled and spent less time in the light than Gi DREADD+CNO (distance: p=0.0006; time: p<0.0001), GFP+CNO (distance: p=0.0036; time: p=0.008), and Gq DREADD+VEH (distance: p=0.0018) (Figure 4.3m & n). Also, Gi DREADD+CNO mice spent more time in the light than Gi DREADD+VEH (p<0.0001). Lastly, looking at distance and time traveled on EPM open arms, we found a significant viral effect (distance: F(2, 30)=12.33, p =0.0001; time: F(2,30)=3.803,p=0.0337) with no significant effect of treatment (distance: F(1, 30)=1.857, p=0.1831; time: F(1, 30)=0.63, p=0.43) (Figure 4.3o & p). We also found significant interactions (distance F(2, 30)=6.817, p=0.0036; time: F(2, 30)=5.34, p=0.0103). Bonferroni post-hoc

comparisons showed that Gq DREADD+CNO mice traveled and spent less time on the open arms than Gi DREADD+CNO (distance: $p=0.001$; time: $p<0.0001$) and GFP+CNO (time: $p=0.0041$). Also, Gi DREADD+CNO mice spent more time on the EPM open arms than Gi DREADD+VEH ($p=0.0053$) (Figure 4.3o & p). We found no significant difference in total difference traveled across the behavioral tasks OF, EPM, and LD (all p's>0.05) (Supplemental figures 4.2 k-m). Taken together, these data indicate that on a non-stress background inhibition of the vDG results in anxiolytic-like behavioral effects, while vDG stimulation has anxiogenic-like effects on female mice behavior.

Next, we analyzed differences between viral and treatment groups within the SIS treated female mice using separate two-way ANOVAs for each behavioral task. we investigated differences in latency to eat within the NSF with a Mantle Cox log-rank test, which revealed significant differences between the SIS groups ($x^2(5)=34.01$, $p<0.0001$). Bonferroni post-hoc comparisons showed that Gi DREADD+CNO have a shorter latency to eat than Gi DREADD+VEH ($p=0.0007$), Gq DREADD+CNO ($p=0.0007$), and GFP+CNO mice ($p=0.0007$) (Figure 4.3j). No differences in home cage latency to feed were observed (Supplemental figure 4.2n). Looking at distance traveled and time spent in the center of the OF, we found a significant viral (distance: $F(2,30)=10.18$, $p=0.0004$; time: $F(2, 30)=15.66$,$p<0.0001$) and treatment (distance: $F(1, 30)=30.18$,$p<0.0001$; time: $F(1, 30)=18.04$,$p=0.0002$) effect (Figure 4.3k & l). We also found significant interactions (distance: $F(2, 30) =12.32$,$p=0.0001$; time: $F(2, 30)=18.79$,$p<0.0001$). Bonferroni post-hoc comparisons showed that Gi DREADD+CNO mice traveled and spent more time in the center of the OF than GFP+CNO (distance: $p=0.0007$; time: $p<0.0001$), Gq DREADD+CNO (distance: $p<0.0001$; time: $p<0.0001$), and Gi DREADD+VEH (distance: $p<0.0001$; time: $p<0.0001$) (Figure 4.3k & l). Next, looking at distance and time

traveled in the light portion of the LD chamber, we found a significant viral (distance: $F(2,30)=6.72, p=0.003$; time: $F(2,30)=40.92, p<0.0001$) and treatment effect (distance: $F(1,30)=1.147, p=0.0049$; time: $F(1,30)=42.24, p<0.0001$) (Figure 4.3m & n). We also found significant interactions (distance: $F(2,30)=13.04, p=0.0099$; time: $F(2,30)=43.69, p<0.0001$). Bonferroni post-hoc comparisons showed that Gq DREADD+CNO mice traveled and spent less time in the light than Gi DREADD+CNO (distance: $p<0.0001$; time: $p<0.0001$) and spent less time, but did not travel more, in the light than GFP+CNO (time: $p=0.0025$) (Figure 4.3m & n). Also, Gi DREADD+CNO mice spent traveled and spent more time in the light than GFP+CNO (distance: $p=0.0222$; time: $p<0.0001$) and Gi DREADD+VEH (distance: $p<0.0001$; time: $p<0.0001$) (Figure 4.3m & n). Finally, looking at distance and time traveled on the EPM open arms, we found a significant viral (distance: $F(2,30) =15.58, p<0.0021$; time: $F(2, 30)=15.58, p<0.0021$) and treatment effect (distance: $F(1,30)=10.65$, $p=0.0028$; time: $F(1, 30)=10.65, p=0.0028$) (Figure 4.3o & p).. We also found significant interactions (distance: $F(2,30)=9.631, p=0.0017$; time: $F(2,30)=9.631, p=0.0017$). Bonferroni post-hoc comparisons showed that Gq DREADD+CNO mice traveled and spent less time in the open arms of the EPM than GFP+CNO (distance: $p=0.0123$; time: $p=0.0002$) and Gi DREADD+CNO (distance: $p=0.0002$; time: $p<0.0001$). Also, Gi DREADD+CNO mice traveled and spent more time in the open arms of the EPM than Gi DREADD+CNO (distance: $p=0.0014$; time: $p=0.0002$) (Figure 4.3o & p).. We found no significant difference in total difference traveled across the behavioral tasks OF, EPM, and LD (all p's>0.05) (Supplemental figures 4.2 k-m). Taken together, these data indicate that Gi-DREADD inhibition of the vDG via CNO on a stress background results in a reduction of negative valence behaviors, while vDG Gq-DREADD+CNO stimulation has mild effects in the presence of stress.

DREADD-mediated modulation of DG GCs reverses effects of FLX treatment on behavior

To assess whether inhibition of vDG is sufficient to convert a behavioral non-responder to FLX treatment into a responder, we expressed a Gi-DREADD virus into the vDG of all mice. Next, male mice were exposed to 4 weeks of chronic CORT before 3 weeks of CORT+FLX treatment (Figure 4.4a). Mice underwent the NSF to assess behavioral responses status with NR (n=10) and R (n=12) emerging. We then randomly assigned half the NR and R to either CORT+FLX+VEH or CORT+FLX+CNO for 3 weeks and then exposed them to second NSF trial (Figure 4.4a). In the second NSF we found significant differences between the 4 groups (NR-CORT+FLX+VEH, NR-CORT+FLX+CNO, R-CORT+FLX+VEH, R-CORT+FLX+CNO) $x(3)=14.86$, $p=0.002$ (Figure 4.4c). Bonferroni post-hoc comparisons within each response group showed significant differences within NR, such that NRs that remained on CORT+FLX+VEH had higher latencies than NR-CORT+FLX+CNO ($x(1)=10.26$, $p=0.001$) (Figure 4.4c). No differences in NSF latency to eat were noted within the R group ($x(1)=0.816$, $p=0.366$). This indicates that inhibition of the vDG is sufficient in converting FLX NR male's into R, since NSF latency to eat was decreased following three weeks of vDG inhibtion.

Similarly, in female mice before undergoing SIS stress all females were injected with a Gi-DREADD virus (n=30). Following the initial 7-weeks of SIS exposure females were given FLX for 3 weeks and then exposed to the NSF to assess behavioral response status to FLX, with both NR (n=12) and R (n=18) emerging (Figure 4.4b). Next, mice within both response categories were given either SIS+FLX+VEH (NR= 6, R=9) or SIS+FLX+CNO (NR=6, R=8) for 3 weeks and then exposed to the second NSF trial (Figure 4.4b). During the second NSF we found significant differences between the 4 groups (NR-SIS+FLX+VEH, NR-SIS+FLX+CNO, R-SIS+FLX+VEH, R-SIS+FLX+CNO) $x(3)=35.7$, $p<0.0001$ (Figure 4.4d). Bonferroni post-hoc

comparisons within each response group revealed that NR-SIS+FLX+CNO mice had shorter latencies than NR-SIS+FLX+VEH female mice $x(1)=12.09$, $p=0.0005$ (Figure 4.4d). No differences in NSF latency to eat were noted within the R group ($x(1)=0.478$, $p=0.489$). As evident in male mice, vDG inhibition converted female FLX NR into R by reducing NSF latency.

Ventral DG DREADD-mediated stimulation converts responders in non-responders

Following a similar design to the experiments converting non-responders into responders via Gi-DREADD mediated mechanisms, we wanted to explore if stimulation of the ventral DG would lead to behavioral responders turning into non-responders. All male mice were injected with a Gq-DREADD virus, exposed to CORT for 4 weeks and then 3 weeks of CORT+FLX prior to the first NSF (Figure 4.4a). Both behavioral response phenotypes emerged during the first NSF, with all mice then randomly assigned to either CORT+FLX+VEH or CORT+FLX+CNO for 3 weeks. Mice were then exposed to a second NSF trial with group differences emerging between the 4 Gq-DREADD groups (NR-CORT+FLX+VEH, NR-CORT+FLX+CNO, R-CORT+FLX+VEH, R-CORT+FLX+CNO) $x(3)=23.46$, $p<0.001$ (Figure 4.4e). Bonferroni post-hoc comparisons within each response group showed significant differences within R, such that responders that remained on CORT+FLX+VEH had shorter latencies than R-CORT+FLX+CNO ($x(1)=9.701$, $p=0.002$). No differences in NSF latency to eat were noted within the NR groups ($x(1)=0$, $p>0.99$). Results suggest that stimulation of the vDG for 3 weeks is sufficient in converting FLX R in the NSF to FLX NR by increasing NSF latency to eat during the second exposure.

Lastly, we followed the same design as in the female Gi-DREADD conversion study, and wanted to assess if stimulation of the ventral DG converted R to FLX into NR. All male mice

were injected with a Gq-DREADD virus, exposed to SIS for 7 weeks, then 3 weeks of SIS and FLX prior to the first NSF (Figure 4.4b). Both behavioral response phenotypes emerged during the first NSF, with all mice then randomly assigned to either SIS+FLX+VEH or SIS+FLX+CNO for 3 weeks. Mice were then exposed to a second NSF trial with group differences emerging between the 4 Gq-DREADD groups (NR-SIS+FLX+VEH, NR-SIS+FLX+CNO, R-SIS+FLX+VEH, R-SIS+FLX+CNO) $x(3)=38.6$, $p<0.0001$ (Figure 4.4f). Bonferroni post-hoc comparisons within each response group showed significant differences within R, such that responders that remained on SIS+FLX+VEH had shorter latencies than R-SIS+FLX+CNO ($x(1)=24.79$, $p<0.0001$). No differences in NSF latency to eat were noted within the NR groups ($x(1)=0$, $p>0.99$). Similar to male observations, vDG stimulation converted female FLX R into NR by increasing NSF latency to eat following chronic vDG stimulation.

Discussion

In the current study we illustrate the importance of the DG in mediating the behavioral response to antidepressant treatment in both male and female mice. Across two valid chronic stress experimental systems, we find that behavioral response to FLX results in a decrease in negative valence behaviors. Behavioral response to FLX is associated with a decrease in DG GC activity, with chronic inhibition of the vDG via DREADDs mounting antidepressant-like behavioral responses in both stress and non-stress backgrounds. In both sexes we observe that chronic stimulation of the vDG in the presence or absence of stress has mild effects on negative valence behaviors. Furthermore, we illustrate that behavioral non-responders to FLX can be converted into behavioral responders following chronic inhibition of the vDG via DREADDs. Interestingly, behavioral FLX responders are converted into non-responders following chronic

chemogenic stimulation of the vDG. Together these results suggest that DG GCs inhibition is a critical component of the behavioral response to FLX.

Behavioral response to FLX

Within the United States, 16% of the population will experience an episode of major depression in their lifetime (Smith, 2014). Although commonly used treatments, such as SSRIs, are readily prescribed to reprieve depressed patients of their symptoms, only a subset of patients (~33%) achieve remission with intial treatment (Trivedi et al., 2006). In using separate, but valid chronic stress experimental systems in both sexes, we illustrate that treatment resistant behavioral phenotypes are not impacted by type of stressor (pharmacological or social stressor). In both males and females, we observed a bimodal distribution in both stress+FLX groups (CORT+FLX and SIS+FLX). Across both chronic CORT and SIS paradigms, we illustrate that behavioral response to FLX (CORT+FLX-R and SIS+FLX-R) results in a decrease in negative valence behaviors in the EPM and FST compared to same sex behavioral FLX non-responders (CORT+FLX-NR and SIS+FLX-NR) and stress controls (CORT+VEH and SIS+VEH). Given that females have long been excluded form preclinical research, our results are the first to document this behavioral response status to FLX in female mice.

Our preclinical data highlights a growing dilemma in modern psychiatry, with a subset of the population non-responsive to first line pharmacotherapies. Within the clinical population, studies have tried to address this issue by switching non-remitters to SSRIs to a second antidepressant. Specifically, Rush and colleagues (2006) switched citalopram (SSRI) non-remitters to either bupropion (norepinephrine dopamine reuptake inhibitor), venlafaxine (serotonin norepinephrine reuptake inhibitor), or sertraline (SSRI) and measured their response to these second line antidepressants. Surprisingly, Rush and colleagues (2006) found that similar

remittance profiles still persisted in these patients, despite these drugs acting on different monoaminergic neurotransmitters. These results highlight the need for a better understanding in the molecular and cellular action of antidepressants as well as novel pharmacotherapies that address the heterogeneous nature of depression. Moreover, while we cannot directly compare our results between sexes, utilization of different stressors in each sex denotes that type of stressor does not significantly impact response to antidepressants. Rather the underlying factor leading to individual differences in antidepressant response most likely reside within the DG activity. Specifically, in using the NSF, which is susceptible to chronic antidepressant effects not acute, we observe that responders to FLX (CORT+FLX-R and SIS+FLX-R) have less DG cFos expression than non-responders to FLX (CORT+FLX-NR and SIS+FLX-NR) and same-sex stress controls. Given that cFos is an indicator of neuronal activity within an area (Hoffman et al,, 1993), our results indicate that response to FLX is related to a decrease in activation of the DG GCs.

Chronic Inhibition of the DG mimics antidepressant behavioral response

Research suggests that inhibition of the DG appears to be a critical component of the behavioral response to SSRIs. Samuels and colleagues (2015) showed that behavioral response to FLX is related to 5-HT_{1A} heteroreceptors inhibition of mature DG GCs within the vDG. Direct optogenetic inhibition of the vDG, also suppresses negative valence behaviors (Bagot et. al., 2015; Kheirbek et. al., 2013), suggesting that inhibition of the vDG mimics a behavioral response to antidepressants. Use of chemogenetics, such as DREADDs, allow for the functional manipulation of a brain nuclei or circuit that permits further exploration in the regions activity level during behavioral tasks (Smith et al., 2016; Sternson & Roth, 2014). To this end we assessed whether chronic inhibition of DG GCs would mimic an antidepressant behavioral

response, since FLX responders have less DG cFos expression, indicative of an underlying reduction in DG GC activity during a behavioral response to FLX. In both male's and female's, we observed that chronic inhibition (Gi DREADD+CNO) of the vDG on a non-stress background resulted in anxiolytic behavioral effects in the LD and EPM (Figure 3d-h; m-p). Specifically, Gi-DREADD+CNO male and female mice traveled and spent more time in the light of the LD and on the EPM open arms than same sex Gi-DREADD+VEH, Gq-DREADD+CNO, and GFP+CNO mice. In males vDG inhibition in the absence of stress resulted in an increase in the time traveled in the center, however, within females no effects were found. Additionally, in the absence of stress we also assessed the impact of chronic vDG stimulation on negative valence behaviors and found that in both sexes vDG stimulation (Gq DREADD+CNO) has mild anxiogenic effects in the LD and EPM (Figure 4.3e, f, m, n, & p). Overall, in the absence of stress our results support our hypothesis that vDG inhibition exerts anxiolytic effects on behavior, while vDG stimulation mounts anxiogenic behavioral responses.

Seeing as preclinical stress paradigms are used to delineate the impact chronic antidepressant treatment has on reversing stress-related behavioral effects, we wanted to separately investigate the impact of a stress background on functional modulation of the vDG. Considering that antidepressants like FLX exert inhibitory mechanisms within the DG, we used chronic inhibition of the vDG to mimic an antidepressant-like effect on behavior. In the presence of stress (CORT or SIS) in both sexes, we show that chronic vDG inhibition (Gi-DREADD+CNO) results in a reduction of stress-related behavioral responses in the OF, LD, and EPM compared to same sex mice that received no inhibition (Gi DREADD+VEH and GFP+CNO) or chronic vDG stimulation (Gq-DREADD+CNO). Interestingly, in the presence of stress chronic stimulation of the vDG only mildly exacerbated stress effects (CORT and SIS),

with Gq-DREADD+CNO mice spending less time in the light of the LD compared to same sex GFP+CNO mice. Within SIS females, we observed that chronic vDG stimulation also resulted in more pronounced behavioral effects in the EPM with Gq-DREADD+CNO females spending less time and distance on EPM open arms than GFP+CNO. These results indicate that a floor effect may be present in the present of stress allowing for no differences to be observed between stimulated and unstimulated Gq-DREADDs. Moreover, we show no behavioral differences between GFP+CNO and GFP+VEH mice controlling for the behavioral effects of CNO being reversed-metabolized to clozapine in vitro (Webster et al., 2018).

Behavioral response to FLX is reversed by chemogentic stimulation or inhibition of vDG

Seeing as chronic inhibition of the vDG in the presence of stress resembled antidepressant-like behavioral effects, we wanted to assess whether functionally manipulating the vDG could alter the behavioral response to FLX. After delineating mice into responders or non-responders following the initial three weeks of stress+FLX treatment, we activated either the inhibitory (Gi-DREADD) or stimulatory (Gq-DREADD) DREADD that was expressed in the vDG of mice prior to exposure to the stressor. We observed that three weeks of chronic vDG inhibition converted non-responders (NR-Gi DREADD+ stress+FLX+CNO) into responders with NSF latencies reduced following second exposure compare to non-responder mice that did not receive vDG inhibition (NR-Gi-DREADD+STRESS+FLX+VEH). Ultimately, inhibition of the vDG in the presence of FLX is sufficient in converting non-respodners into responders. Surprisingly, chronic vDG stimulation for 3 weeks in FLX responder mice (R-Gq-DREADD+STRESS+FLX+CNO) increased NSF latencies to eat during the second exposure compared to non-vDG stimulated FLX responder mice (R-Gq DREADD+STRESS+FLX+VEH).

Thus, it appears that chronic stimulation of the vDG in the presence FLX, which exerts inhibitory mechanisms within the DG (Sameuls et al., 2015), can alter the behavioral response to FLX. While our data supports that the type of stressor used results in comparable effects within both sexes, our results are limited in that direct analyses between sexes cannot be performed.

Most strikingly these results indicate that modulation of the DG is independent of an applied stressor. Using two distinct stress paradigms, chronic CORT and SIS, we illustrate that DG GC inhibition is sufficient in mounting antidepressant-like effects and converting behavioral FLX non-responders into responders on both stress backgrounds. These distinctions are important to note given that in the clinical population depressed populations experience an array of stressful events that may contribute to the etiology of depression. Understanding that treatment resistant depression is independent from stress allows researchers to focus on the individual differences in the circuits that mount responses to antidepressants. Furthermore, these results point to the importance in understanding the DG role in mediating behavioral responses. While there are direct connections between the DG impact on negative valence behavior (Anacker et al., 2018; Luna et al., 2019; Samuels et al., 2015), the exact projections into and out of the DG that are mediating these effects are unknown. Future research is needed to delineate these DG projections.

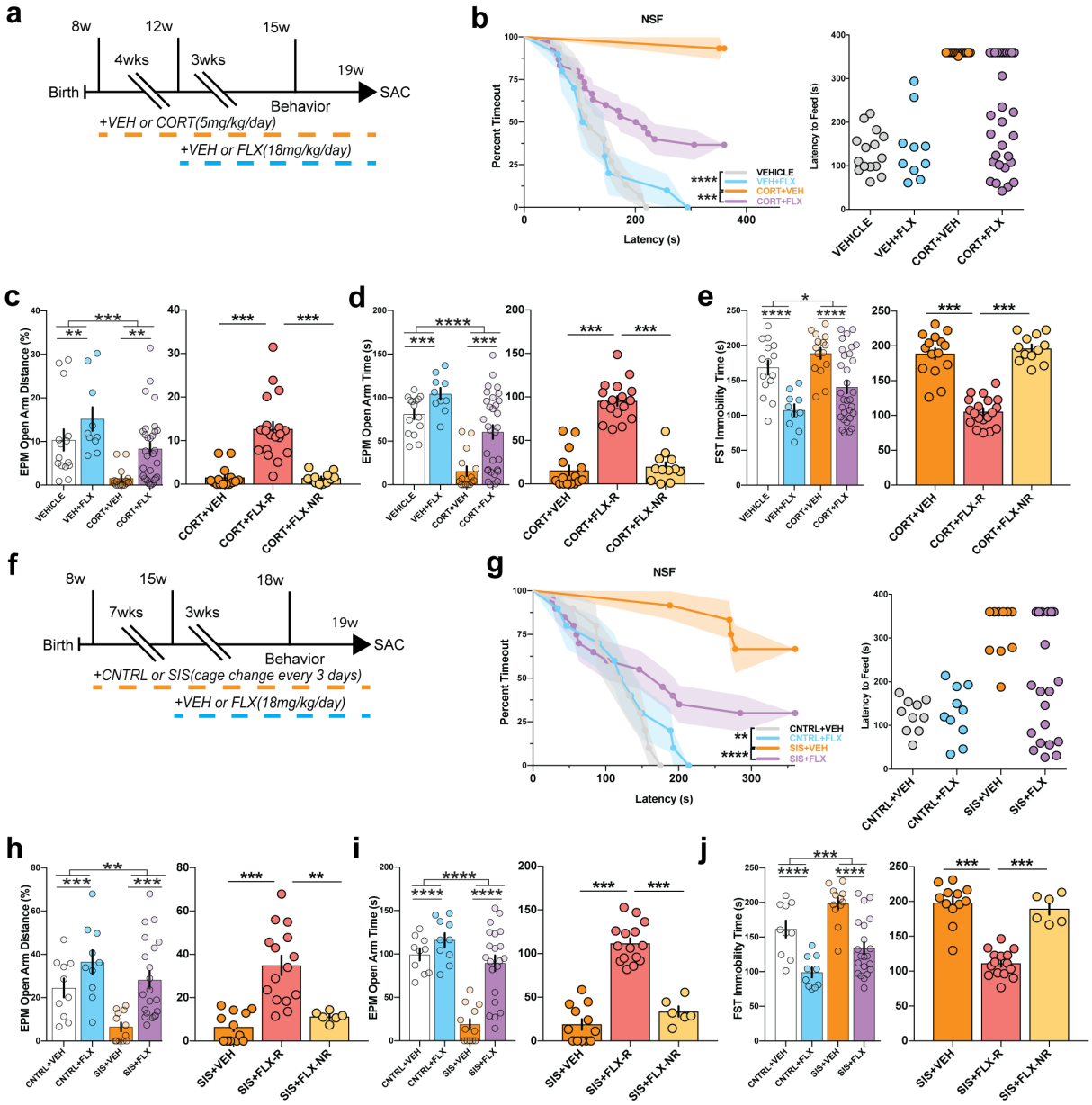


Figure 4.1. Male and female behavioral responders and non-responders to chronic fluoxetine treatment. Timeline of experiment males (a) and females (f). (b & g) NSF is used to distinguish between responders and non-responders to chronic FLX treatment. 2x2 ANOVAs (left graphs c-e; h-j) were conducted to examine stress and treatment effects with separate one-way ANOVAs (right graphs c-e; h-j to investigate differences between stressed, responders and non-responders to FLX. **p<0.01; ***p<0.001, ****p<0.0001.

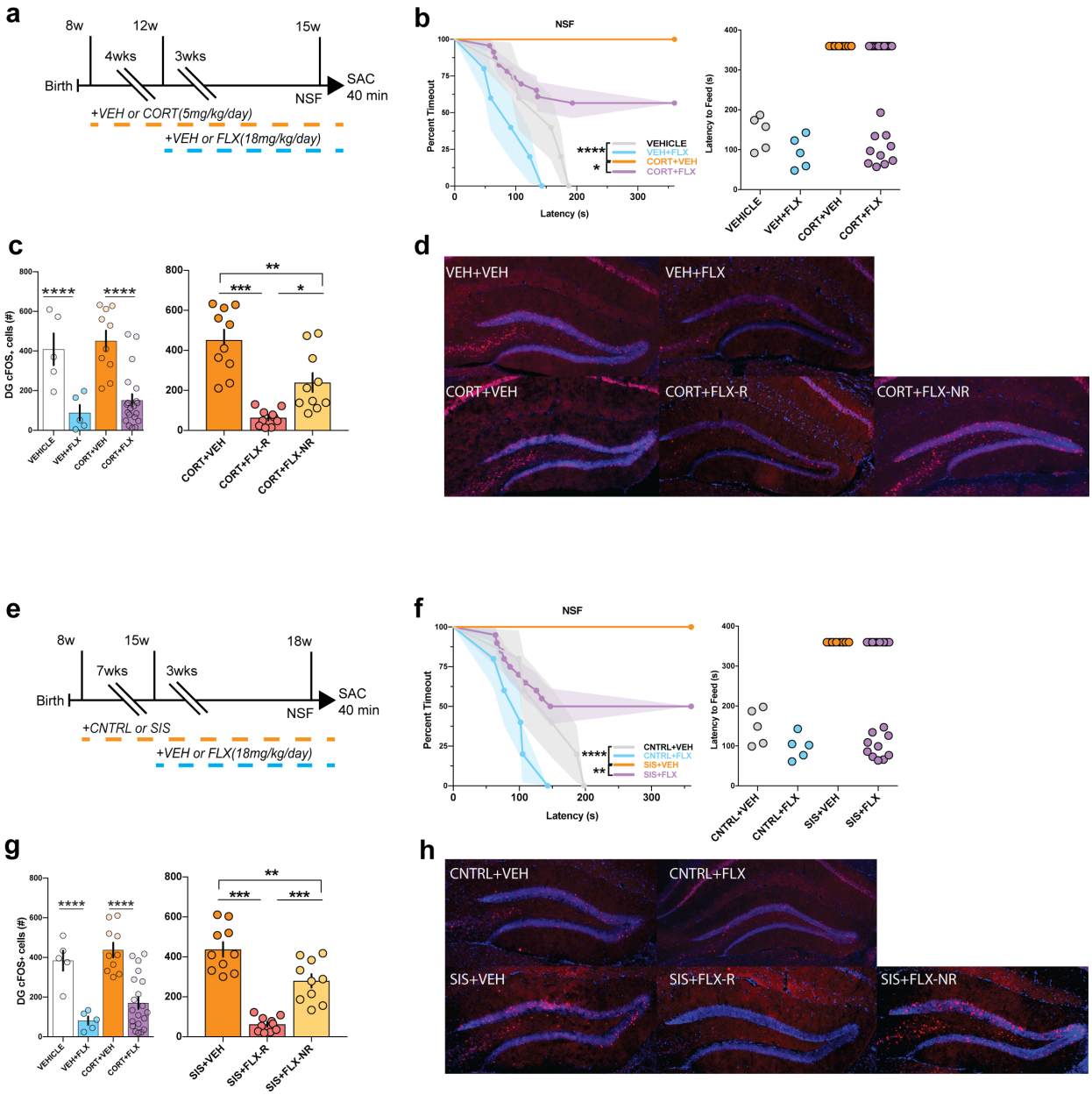


Figure 4.2. Differences in DG cFos expression between responders and non-responders to fluoxetine treatment. (a) Male and (e) female experimental timelines. (b & f) NSF latencies used to assess treatment response. (c & g) Following NSF mice were perfused to assess DG GC activity in response to NSF exposure. Both males (c) and females responders had less DG cFos expression than their stress and non-responder counterparts. (d & h) Representative cFos images (10x).

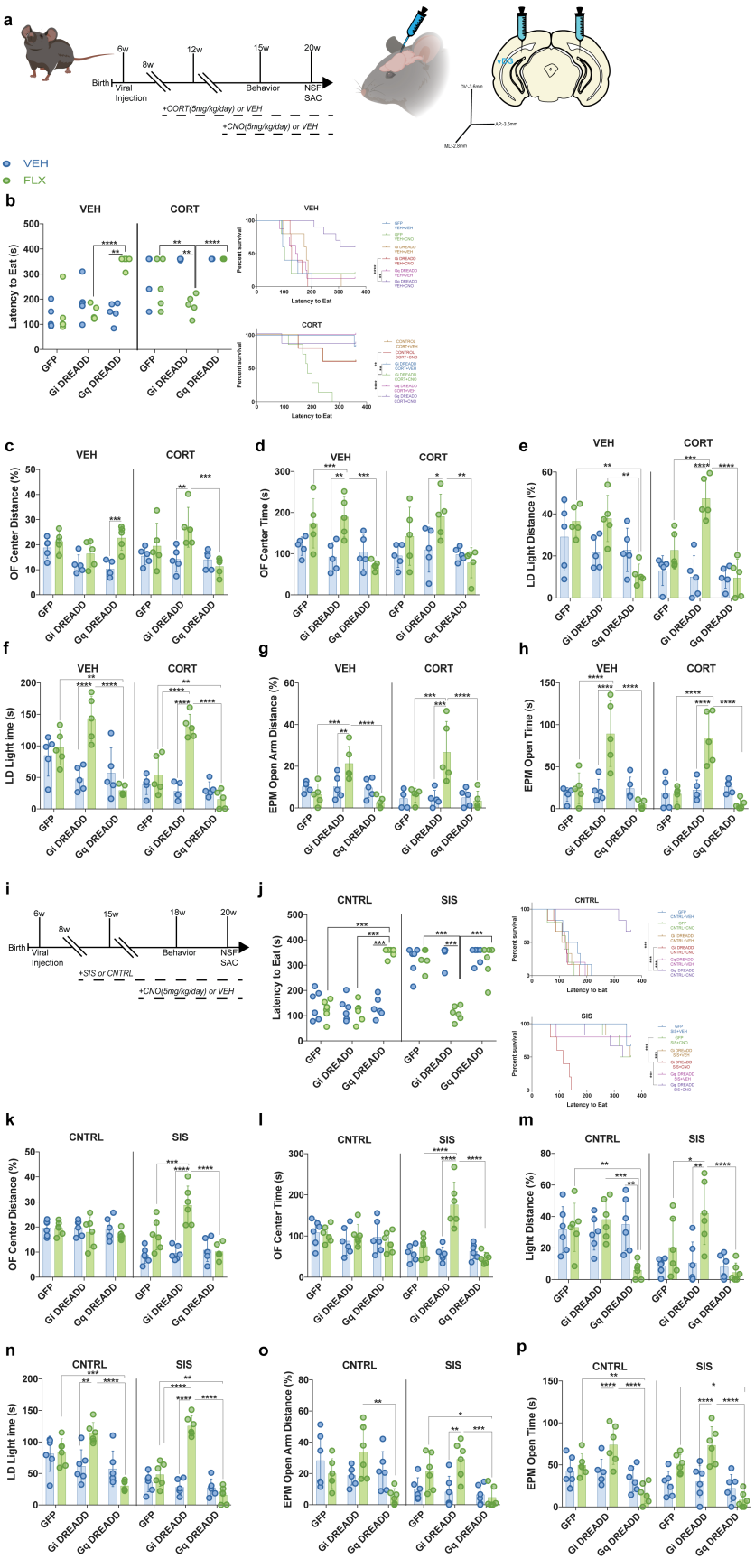

a
6w
8w
12w
15w
20w
Birth
Viral Injection
Behavior
NSF SAC
+CORT(5mg/kg/day) or VEH
+CNO(5mg/kg/day) or VEH
VEH
FLX
b
VEH
CORT
Latency to Eat (s)
GFP
Gi DREADD
Gq DREADD
Percent survival
Latency to Eat
c
OF Center Distance (%)
d
OF Center Time (s)
e
LD Light Distance (%)
f
LD Light ime (s)
g
EPM Open Arm Distance (%)
h
EPM Open Time (s)
i
+SIS or CNTRL
j
CNTRL
SIS
k
l
m
Light Distance (%)
n
o
p

Figure 4.3. DREADD mediated inhibition results in a decrease in negative valence behaviors in males and females. (a) Experimental timeline. (b) Gi DREADD+CNO mice had a quicker latency to eat than Gq DREADD+CNO in the NSF. (c-d) Gi-DREADD+CNO resulted in more distance traveled and time spent in the OF center. (e-f) Gi-DREADD+CNO mice traveled more distance and spent more time in the light than GFP+CNO and Gq DREADD+CNO mice. (g-h) Gi-DREADD+CNO mice traveled more and spent more time on the EPM open arms than GFP+CNO and Gq-DREADD+CNO treated mice. (i) Experimental timeline. (j) Stressed Gi DREADD+CNO mice had a quicker latency to eat than Gq DREADD+CNO in the NSF. (k-l) Stressed Gi-DREADD+CNO mice traveled more and spent more time in the center of the open field than GFP+CNO and GqDREADD+CNO mice (m-n) Gi-DREADD+CNO mice traveled more distance and spent more time in the light than GFP+CNO and Gq DREADD+CNO mice. (o-p) Gi-DREADD+CNO mice traveled more and spent more time on the EPM open arms than GFP+CNO and Gq-DREADD+CNO treated mice.

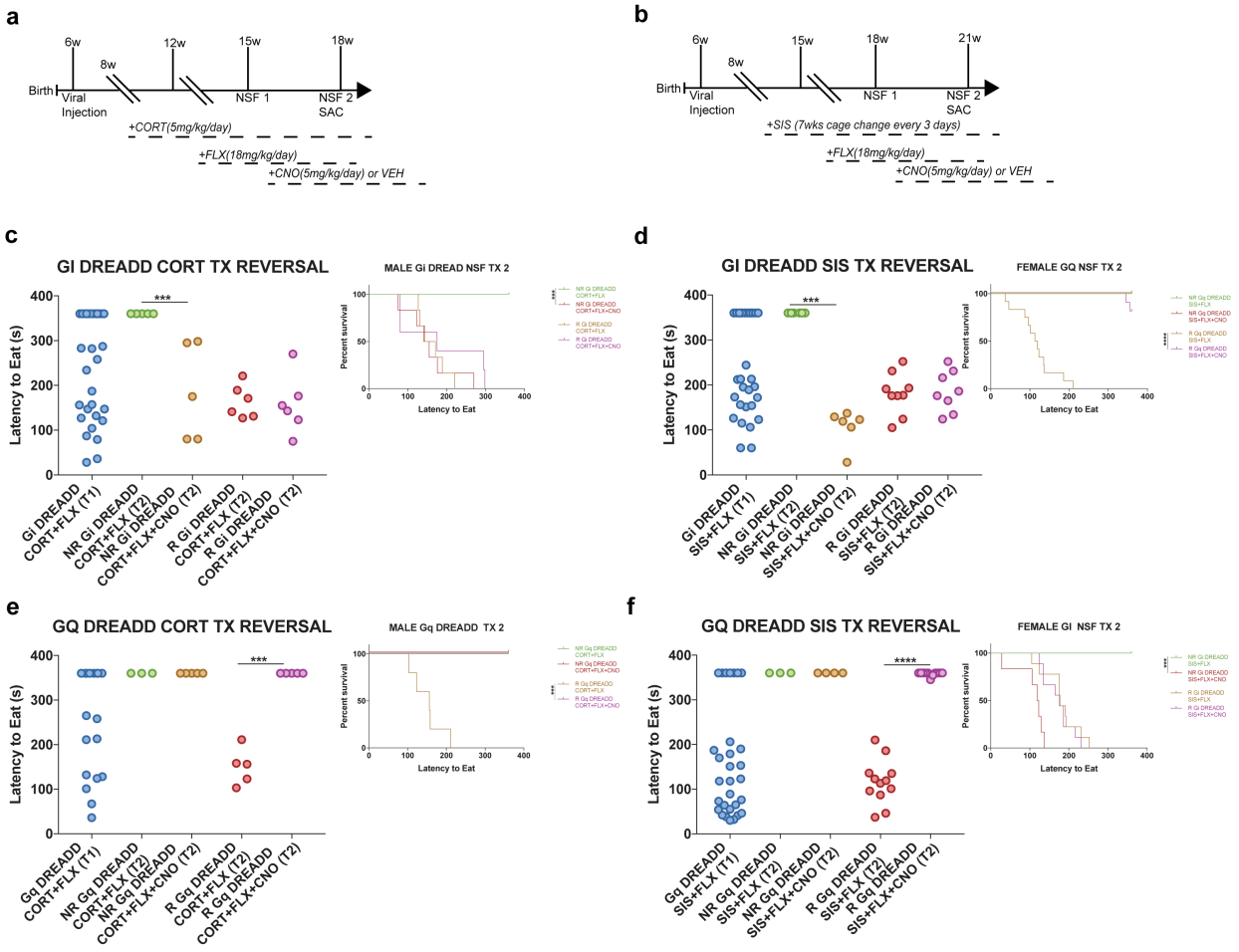
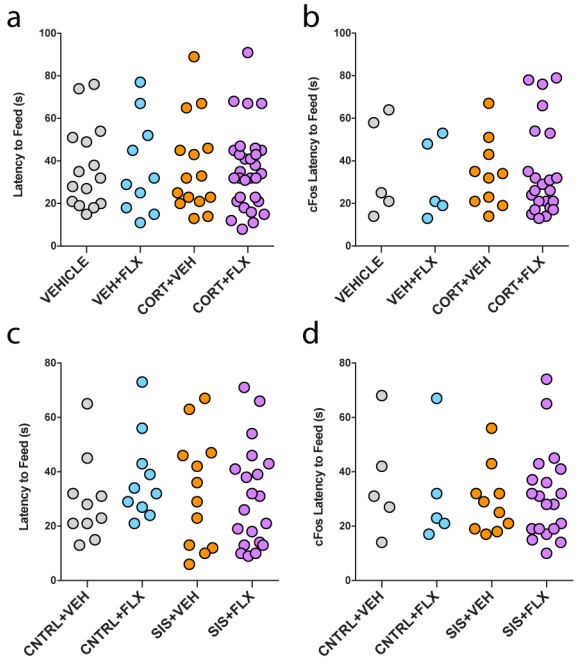
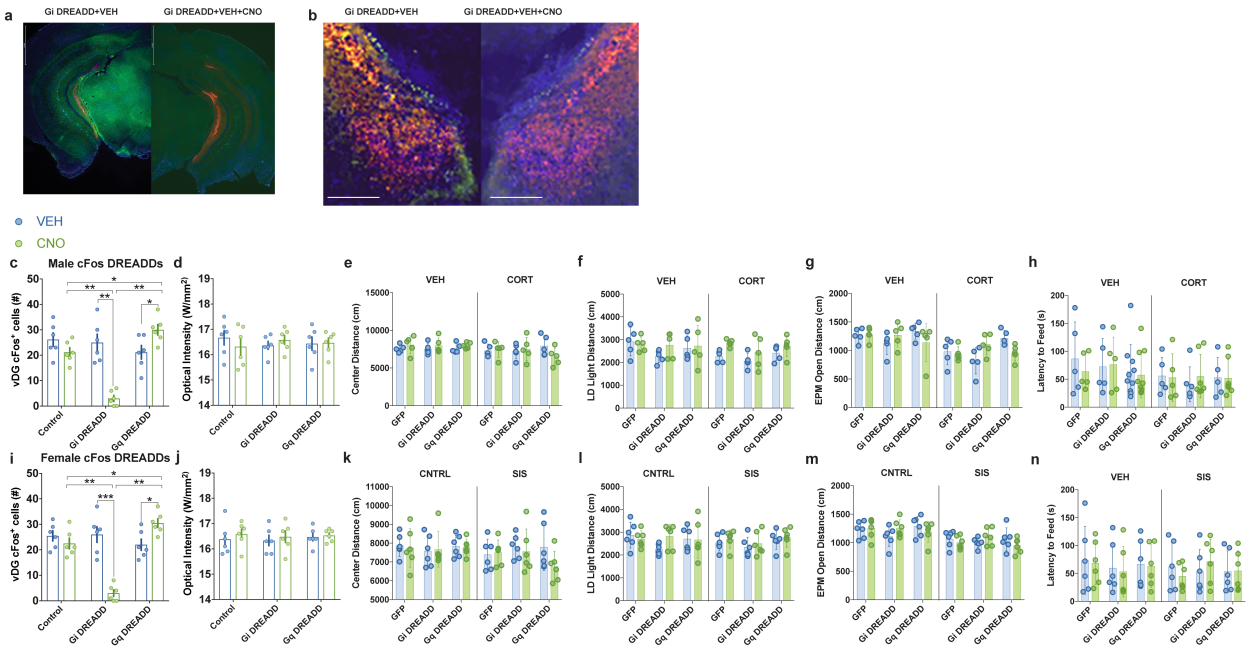


Figure 4.4. **Male and female non-responders to fluoxetine are converted into responders via Gi-DREADD inhibition of DG GCs**. (a & b) Experimental timelines. (c & d) NSF latencies for Gi DREADD, with FLX non-responders (NR) decreasing latency to feed following vDG inhibition. (e & f) Chonic vDG stimulation via Gq-DREADD resulted in increased latency to feed in stimulated FLX responders compared to vDG unstimulated FLX responders.



Supplemental Figure 4.1. NSF home cage latency to feed. (a & c) Home cage latency to feed for NSF related to characterizing responders and non-responders to FLX in males (a) and females (c). (b & d) Home cage latency to feed for cFos experiment males (b) and females (d).



Supplemental Figure 4.2. DREADD mediated vDG inhibition or stimulation impacts vDG activity and does not impact control behavioral measures. (a) Gi-DREADD 4x images virus (mCherry), nuclear stain (DAPI), cFos expression (FITC). (b) Higher magnification of Gi-DREADD images (40x) used to count vDG cFos+ cells. (c & i) Male and female cFos counts to confirm CNO activation of DREADD virus. (d &j) Optical intensity to control for viral expression. (e-h, k-n) No differences were found in total distance traveled in the OF, LD, EPM or in latency to eat within the home cage.

General Discussion

Majority of this dissertation aimed at incorporating females into the scientific literature regarding depression and anxiety, given that females are twice as likely to be diagnosed than males (Kessler et al., 1994). In adapting SIS to adult mice and designing CNSDS, both sexes can now be studied comparatively and without the daunting task of tracking the female estrous cycle (Yohn et al., 2019a, b). Although variations in behavior have been documented across the female estrous cycle (Yohn et al., 2020), these differences are also seen in male behavioral data (Becker et al., 2016; Prendergast et al., 2014; Shansky 2019). Understanding the relationship between FLX and the female estrous cycle without the involvement of stress can help in pharmacotherapy development and understanding underlying factors that may influence behavioral response to antidepressants. Knowledge gained from experimental results found in Chapters 1 thru 3 gave a basis for the experimental design in Chapter 4.

While comparing males and females is an ideal experimental design to assess sex differences in the etiology of neuropsychiatric disorders and disease states. Designing experiments that use two distinct stress paradigms, in this case a pharmacological approach (CORT) or social stressor (SIS), is advantageous in directly assessing whether response to pharmacotherapies is independent of applied stressors. Sex differences were only documented in Chapter 1 in response to chronic CORT application, with a robust effect in males, not female mice (Yohn et al., 2019a). Lack of sex differences in regards to FLX treatment in both negative valence behaviors and adult hippocampal neurogenesis, further permitted us to use separate stress paradigms for Chapter 4. Moreover, while cFos expression in certain nuclei of the brain are impacted by sex (Menedez-Lopez et al., 2009), sex differences in regard to DG cFos expression are not confirmed (Jordan et al., 2019). Stress effects in regard to behavior were

analyzed throughout Chapter 1 and 2, with stress effects confirmed in both stressors and FLX responder and non-responders emerging in both sexes and stressors. Results from Chapter 4 illustrate that the DG role in mounting antidepressant-like behavioral effects is impartial to stress. In other words, what appeared to be an experimental limitation in using distinct stressors in each sex, has translational value since in the clinical population MDD can arise from a multitude of stressors (Kendler et al., 1998). Thus, it is not the type of stressor endured that mounts lack of a behavioral response to SSRIs, but rather potential individual differences within the DG and its circuitry in treatment resistant individual. In confirming that DREADDs functionally stimulated or inhibited the vDG, we counted cFos expression. Overall, results from Chapter 4 could have been strengthened with use of *in vivo* electrophysiology or imaging techniques.

Hypothesis of DG microcircuitry

As shown in Chapter 1 and Chapter 3 application of FLX increases all stages of adult hippocampal neurogenesis. An experiment that was left out of Chapter 4 (Figure 5.1), but will be discussed briefly here, illustrates differences in adult hippocampal neurogenesis between FLX responders and non-responders. In using the brains of mice collected from the cFos experiments from Chapter 4, different markers of hippocampal neurogenesis were stained for. Responders to FLX (CORT+FLX-R, SIS+FLX-R) have more young adult born GCs (abGCs) (proliferating cells ki67), and higher expression of both immature and mature neurons (DCX) than FLX non-responders (CORT+FLX-R, SIS+FLX-R) and stress controls (CORT+VEH, SIS+VEH). These results support studies showing that antidepressant treatment increases adult hippocampal neurogenesis (Wang et al., 2008; David et al., 2009), and adds to the literature that differences exist between behavioral responders and non-responders. In conjunction with the cFos data form

Chapter 4 it appears higher hippocampal neurogenesis in FLX responders is related to lower DG activation, while lower hippocampal neurogenesis is associated with higher DG activation in FLX non-responders.

Mature DG GCs appear critical in mounting a behavioral response to commonly prescribed antidepressants, like FLX. Samuels and colleagues (2015) illustrated that deletion of 5-HT_{1A} heteroreceptors from mature DG GCs, not abGCs, abolished the behavioral response to SSRIs and attenuated the effects of SSRIs on adult hippocampal neurogenesis. Moreover, Anacker and colleagues (2018) showed that increased neurogenesis results in a decrease in vDG mature GC activity, with decreases in vDG activity facilitating resilience to stress. Our data in combination with these studies illustrates that decreases in mature DG GCs activity facilitates a behavioral response to FLX. Mature DG GCs may be inhibited by young abGCs acting on the local microcircuitry or through feedback inhibition mechanisms (Drew et al., 2016; Ikrar et al., 2013; Lacefield, Itskov, Reardon, Hen, & Gordon, 2012; Samuels et al., 2015). Specifically, young abGCs can evoke strong inhibitory input to mature DG GCs via activation of GABAergic interneurons (Ikrar et al., 2013). After ablating abGCs, Burghardt and colleagues (2012) observed an increase in behaviorally induced immediate early gene (IEG) expression in mature DG GCs. Behavioral responses to SSRIs may also be mediated by inhibition of GABA release from DG cholecystokinin (CCK) neurons (Medrihan et al., 2017). Specifically, by inhibiting the inhibitory DG CCK neurons, parvalbumin (PV) interneurons are disinhibited, leading to an inactivation of DG GCs. Actions of abGCs, however, are dependent on afferent inputs into the DG. Specifically, DG abGCs activated via inputs from the lateral entorhinal cortex exert inhibition on DG mature GCs through metabotropic glutamate receptors (Luna et al., 2019). On the other hand, medial entorhinal cortex projections into the DG lead to abGCs exciting mature

GCs through NMDA receptors. Results from Luna and colleagues (2019) elucidate how incoming synaptic information can exert bidirectional effects on abGCs that impact DG neuronal activity, with downstream effects on DG role in cognitive and affective state. Previous research in conjunction with results presented in this dissertation highlight the direct role of the DG in impacting behavior, however, more research is needed to understand the underlying inputs to and outputs from the DG.

Results presented exemplify the importance of including both sexes in preclinical research regarding neuropsychiatric disorders. Moreover, while direct comparisons between males and females are necessary to understand sex differences within disease states, we show that DG exerts direct actions on behavior independent of stressors. Delineating the role of the DG GCs in mediating the behavioral response to antidepressants serves translation relevance and could lead to the development of novel pharmacotherapies.

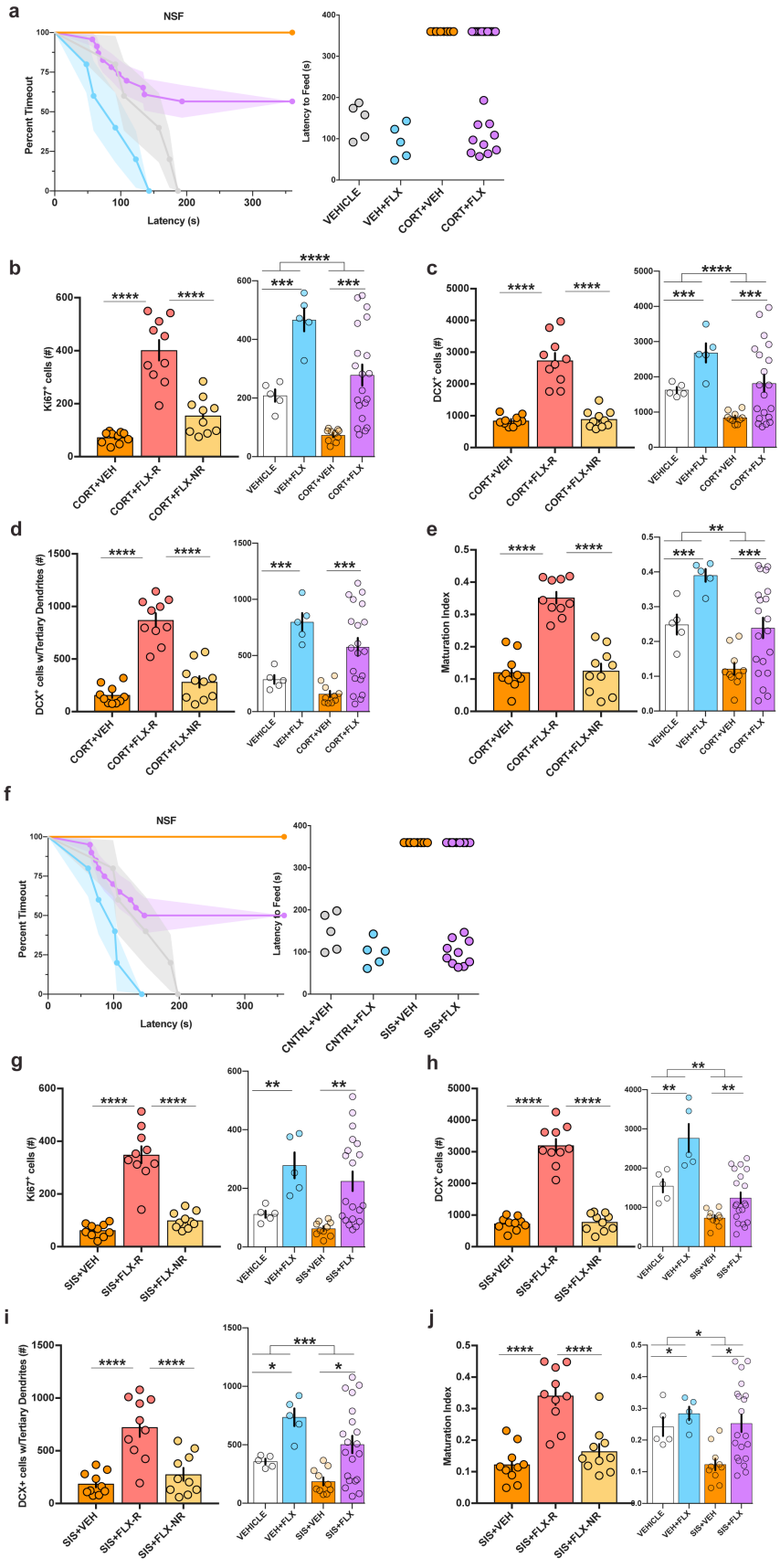

a
NSF
Percent Timeout
Latency (s)
Latency to Feed (s)
VEHICLE
VEH+FLX
CORT+VEH
CORT+FLX
b
Ki67+ cells (#)
CORT+VEH
CORT+FLX-R
CORT+FLX-NR
c
DCX+ cells (#)
d
DCX+ cells w/Tertiary Dendrites (#)
e
Maturation Index
f
NSF
Percent Timeout
Latency (s)
Latency to Feed (s)
CNTRL+VEH
CNTRL+FLX
SIS+VEH
SIS+FLX
g
Ki67+ cells (#)
SIS+VEH
SIS+FLX-R
SIS+FLX-NR
h
DCX+ cells (#)
i
DCX+ cells w/Tertiary Dendrites (#)
j
Maturation Index

Figure 5.1. Adult hippocampal neurogenesis data across males and females. Male data is depicted (a-e) with female data (f-j). (a & f) Mice were used from the cFos experiments in Chapter 4 with the same NSF latency to feed as depicted in Figure 4.2. (b & g left) Differences in expression of ki67+ across 12 sections of the DG, with effects of stress and treatment depicted in (b & g right). (c & h left) FLX responders have more DG DCX+ expression and tertiary dendrites (d & i left) than non-responders and stress controls, with effects of stress and treatment depicted in (c & h left; d & I right). Maturation indexes are higher in FLX responders (e & j right) compared to non-responders and stress controls.

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