Neuropharmacology 160 (2019) 107780

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Social instability is an effective chronic stress paradigm for both male and female mice

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HIGHLIGHTS

- Chronic CORT administration is not an effective chronic stress paradigm for female mice.
- Social instability is an effective chronic stress paradigm in males and females.
- Social instability was effective at altering behavior regardless of estrous cycle phase.
- Social instability induces anxiety-related behaviors and HPA axis activation.

ARTICLE INFO

Keywords:
Chronic stress
Sex differences
Mood disorders
Social instability

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.

1. 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

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https://doi.org/10.1016/j.neuropharm.2019.107780
Received 31 May 2019; Received in revised form 11 September 2019; Accepted 14 September 2019
Available online 16 September 2019
0028-3908/

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.

2. Materials and methods

2.1. 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.

2.2. 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., 2007, 2010; 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 to 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 2 h of the lights coming on (between 6 and 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).

2.3. 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 μg/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 Fig. 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).

2.4. 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 (40, 41). Estrous phases were identified by the presence or absence of nucleated epithelial cells, cornified epithelial cells, and leukocytes (41, 42 Fig. 3B).

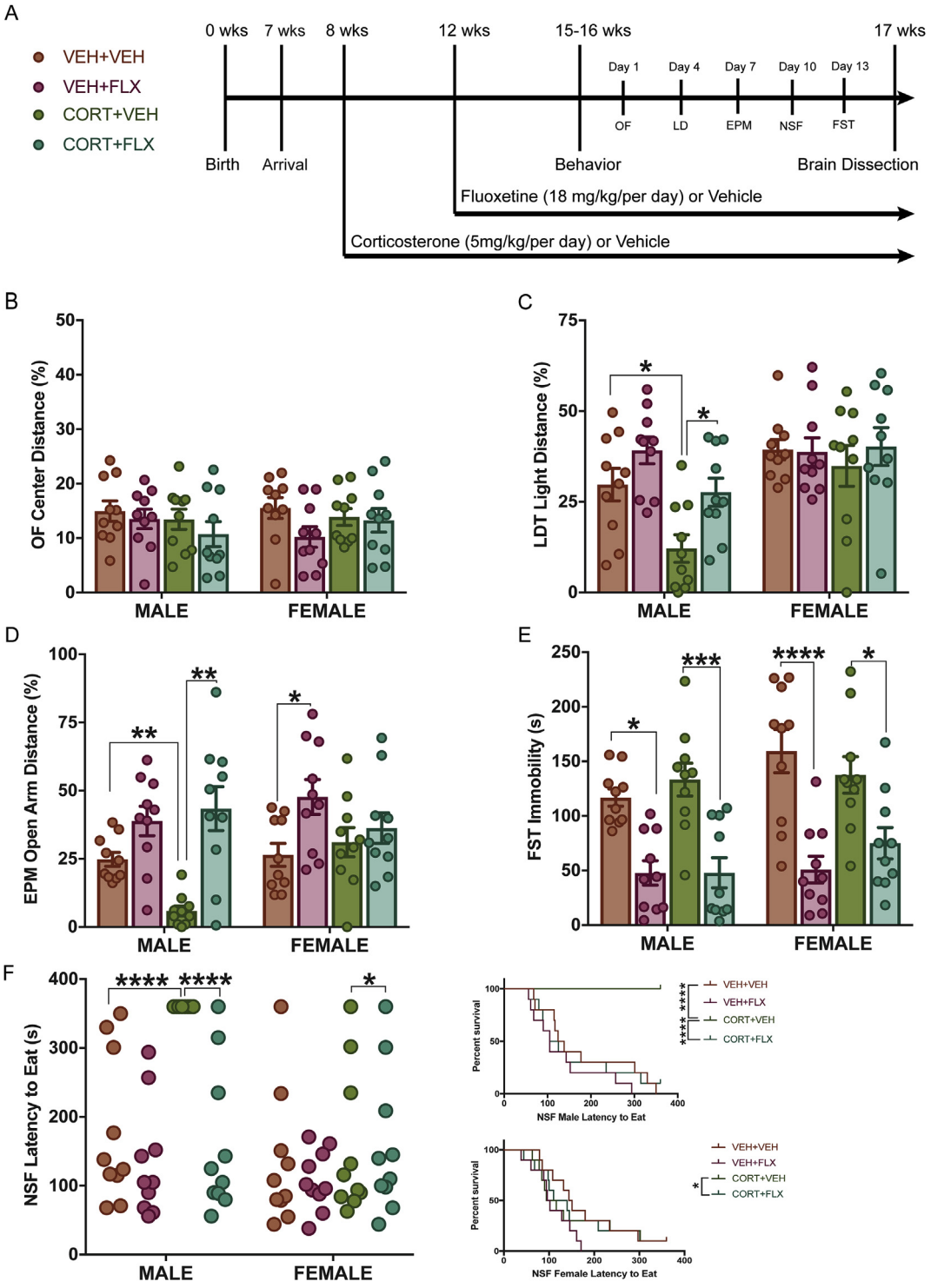

Fig. 1. Chronic Corticosterone has Behavioral Effects in Males but not in Females. (A) Timeline of chronic CORT paradigm and keys for graphs. Panels (B–E) represent separate 2 × 2 × 2 ANOVAs with Bonferroni posthocs exploring effects of CORT and FLX within sex. (B) No effects of CORT or FLX were observed 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.
* $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$.

2.5. Behavioral testing

2.5.1. Open field (OF)

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

2.5.2. 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-min 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.

2.5.3. 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 5-min 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.

2.5.4. Forced swim test (FST)

A modified FST procedure suitable for mice was used (13), with individual cylinders (46 × 32 × 30 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-min test. Immobility was assessed during only the last 4-min of the test since mice habituate to the task during the initial 2-min.

2.5.5. Novelty suppressed feeding (NSF)

Mice were food deprived for 18 h within their home cage, prior to being placed in the corner of a testing apparatus (50 × 50 × 20 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 min 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 min. Latency to eat and amount of food consumed in home cage was measured as a control for feeding behavior observed in the NSF.

2.6. Brain collection, sectioning, and immunohistochemistry

2.6.1. Brain collection and sectioning

Following behavioral testing (3 days after FST), brains were collected from all experimental mice. Mice were anesthetized with ketamine (80 mg/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.

2.6.2. 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 (40 μm). Slides were washed in 1% TritonX-100-PBS and then 3 PBS washes. Next, slides were incubated in citrate buffer for 30 min followed by 3 PBS washes. Slides were blocked for 1 h 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 h of incubation, 3 PBS washes were performed and then 2 h 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 min, 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 $Ki67^+$ (Fig. 5A) or DCX^+ cells (Fig. 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 (Fig. 5E). The maturation index was defined as the ratio of DCX^+ cells possessing tertiary dendrites over the total DCX^+ cells.

2.7. 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 min after cage change. CNTRL mice had blood collected from the right retro-orbital sinus 40–45 min after a cage change, to control for impact of exposure to a novel cage on plasma corticosterone levels. Blood was also collected 40–45 min 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 and 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).

2.8. Statistical analyses

To investigate SEX, STRESS, and TREATMENT effects we ran 2 × 2 × 2 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 3 × 2 × 2 repeated measures ANOVA. These analyses were then followed up with separate 2 × 3 repeated measures ANOVAs within each sex. GraphPad Prism 7 and SPSS (version 23) was used for analyses.

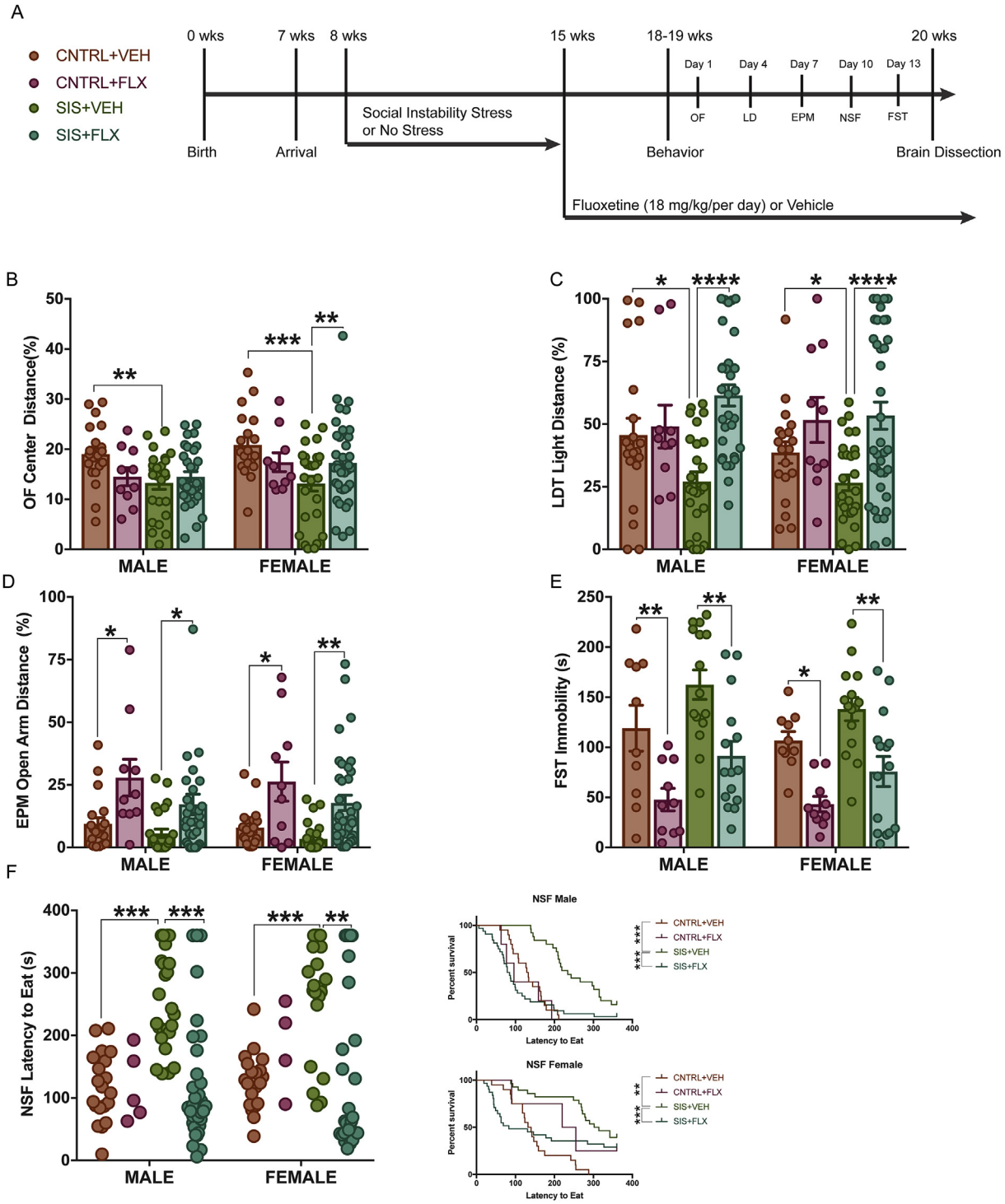

Fig. 2. Behavioral Effects of Social Instability Stress (SIS) in Male and Female Mice. (A) Timeline of SIS paradigm and keys for larger graphs. (B–E) panels represent Bonferroni posthoc comparisons from the 2 × 2 × 2 ANOVAs (SIS*x*SEX*x*FLX) examining effects of SIS and FLX within each sex. (B) SIS affected 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. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$.

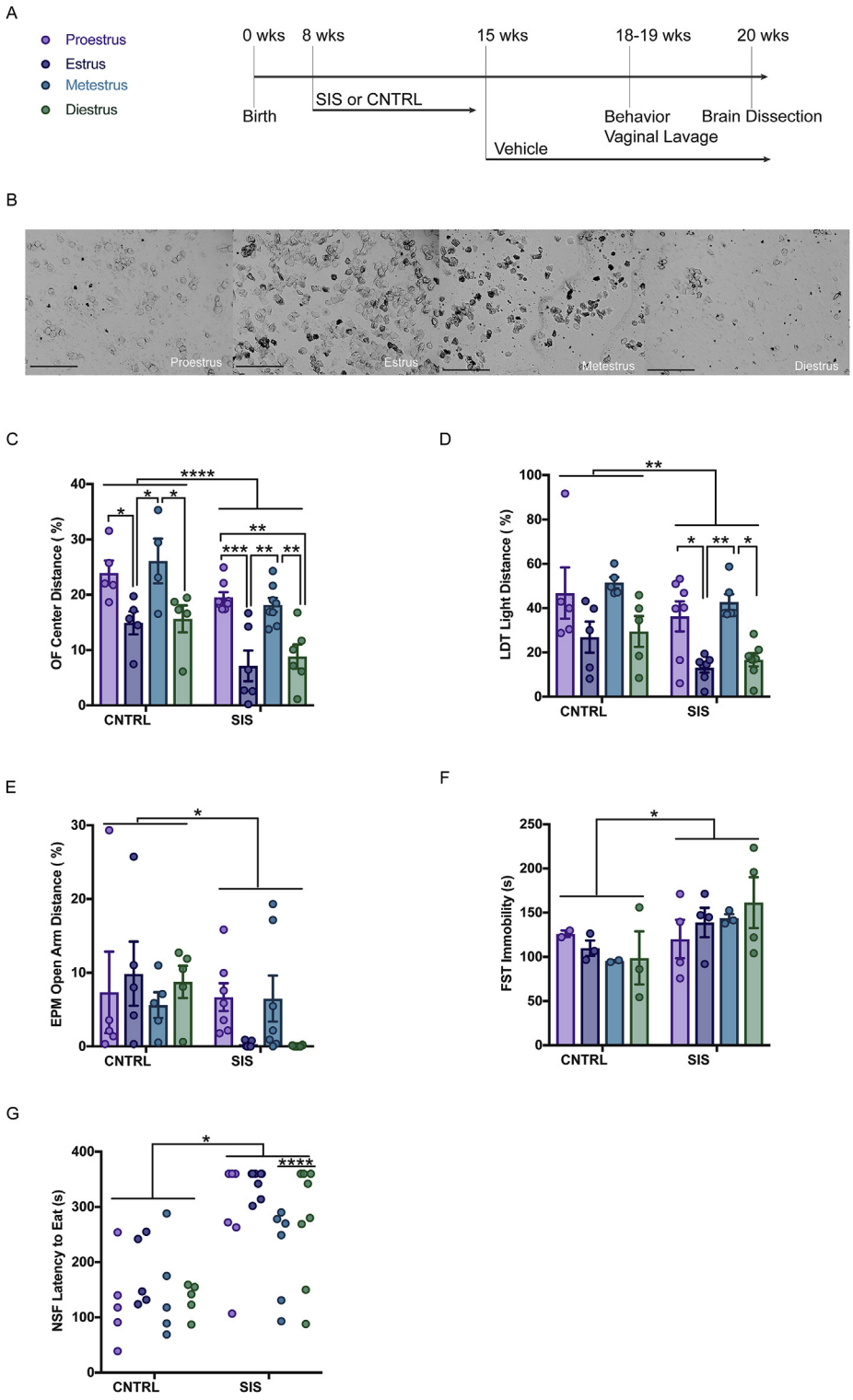

Fig. 3. Impact of Estrous Cycle in Females on Behavior. Time line of experiment (A) and representative 10x images of vaginal smears (B). (C) Represent 2 × 4 ANOVAs (SIS*x*ESTROUS) in the OF with differences emerging between estrous phases within both SIS + VEH and CNTRL + VEH females, with estrous and 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.

3. Results

3.1. 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 (5 mg/kg/day) or VEH to male and female C57BL/6J mice (timeline in Fig. 1A). Following 4 weeks of CORT, mice received either FLX (18 mg/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 2 × 2 × 2 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 ($p > 0.05$; Fig. 1B, Supplemental Fig. 1A). In LDT we found a significant SEX-*x*CORT interaction ($F_{(1,72)} = 4.731$, $p = 0.032$; Supplemental Fig. 1B) on percent light distance traveled, with CORT + VEH males traveling less than CORT + VEH females ($p = 0.0319$) (Supplemental Fig. 1B). Post hoc analysis with a Bonferroni correction indicated within sex effects (Fig. 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 Fig. 1C) was observed in open arm percent distance traveled. Males treated with CORT + VEH traveled less distance on open arms than CORT + VEH females ($p = 0.019$; Fig. 1D; Supplemental Fig. 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 ($p = 0.016$; Fig. 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 ($p = 0.0008$; Fig. 1E) and VEH + FLX were less immobile than VEH + VEH males ($p = 0.013$; Fig. 1E). Similarly, in females FLX reduced immobility time with CORT + FLX females less immobile than CORT + VEH females ($p = 0.036$; Fig. 1E). VEH + FLX females were also less immobile ($p < 0.0001$; Fig. 1E) than VEH + VEH females. Lastly, Kaplan-Meier analysis revealed a significant sex difference in NSF within the CORT + VEH group ($\chi^2_{(1)} = 16.39$, $p < 0.001$; Fig. 1F; Supplemental Fig. 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 ($\chi^2_{(1)} = 15.03$, $p < 0.0001$; Fig. 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 ($\chi^2_{(1)} = 4.54$, $p = 0.033$; Fig. 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.

3.2. 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 (Fig. 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 (18 mg/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 2 × 2 × 2 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 Fig. 2 A-E). We found a significant SIS*x*FLX interaction ($F_{(1,174)} = 20.11$, $p < 0.0001$; Fig. 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 ($p = 0.0076$; Fig. 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 ($F_{(1,174)} = 31.2$, $p < 0.0001$; Fig. 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 ($F_{(1,174)} = 22.34$, $p < 0.0001$; Fig. 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 ($p = 0.0031$; Fig. 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 ($p = 0.0002$; Fig. 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 (Fig. 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 ($\chi^2_{(1)} = 4.09$, $p = 0.040$; Fig. 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 Figs. 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 Figs. 3A–E).

3.3. Estrous cycle effects on behavior

Since SIS affected female behavior, we next examined the 4 stages of the estrous cycle (Proestrus, Estrus, Metestrus, Diestrus; Fig. 3B) in CNTRL and SIS females following each behavioral test (Fig. 3A). For this experiment FLX was not used. To determine SIS and estrous cycle effects on behavior, separate 2 × 4 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 (Fig. 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 ($p = 0.0094$) (Fig. 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 ($p = 0.022$; Fig. 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 ($p = 0.011$; Fig. 3D). Although significant SIS effects were observed in EPM ($F_{(1,\ 39)} = 5.87$, $p = 0.0196$; Fig. 3E) and FST ($F_{(1,\ 17)} = 4.47$, $p = 0.04$; Fig. 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 ($\chi^2_{(1)} = 15.7$, $p < 0.0001$; Fig. 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.

3.4. 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 min following a SIS cage change (during the last week of SIS), and 40 min following EPM (Fig. 4A). Plasma was isolated assayed for corticosterone levels. We performed a 2 × 2 × 3 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, 2 × 3 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 (Fig. 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 (Fig. 4B). Therefore, chronic SIS exposure increased HPA axis activation in response to acute stressors in males.

Separate 2 × 3 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 ($p = 0.0007$; Fig. 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 (Fig. 4C). Taken together, these results demonstrate that SIS leads to HPA axis activation in response to acute stressors in both males and females.

3.5. 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; Fig. 5A) and DCX (a newborn/immature neuron marker; Fig. 5C, E).

First, we investigated sex differences between groups (CNTRL + VEH, CNTRL + FLX, SIS + VEH, and SIS + FLX) with 2 × 2 × 2 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; $p = 0.79$; Fig. 5B, D, F-G smaller panels).

However, the 2 × 2 × 2 three-way ANOVAs revealed a significant

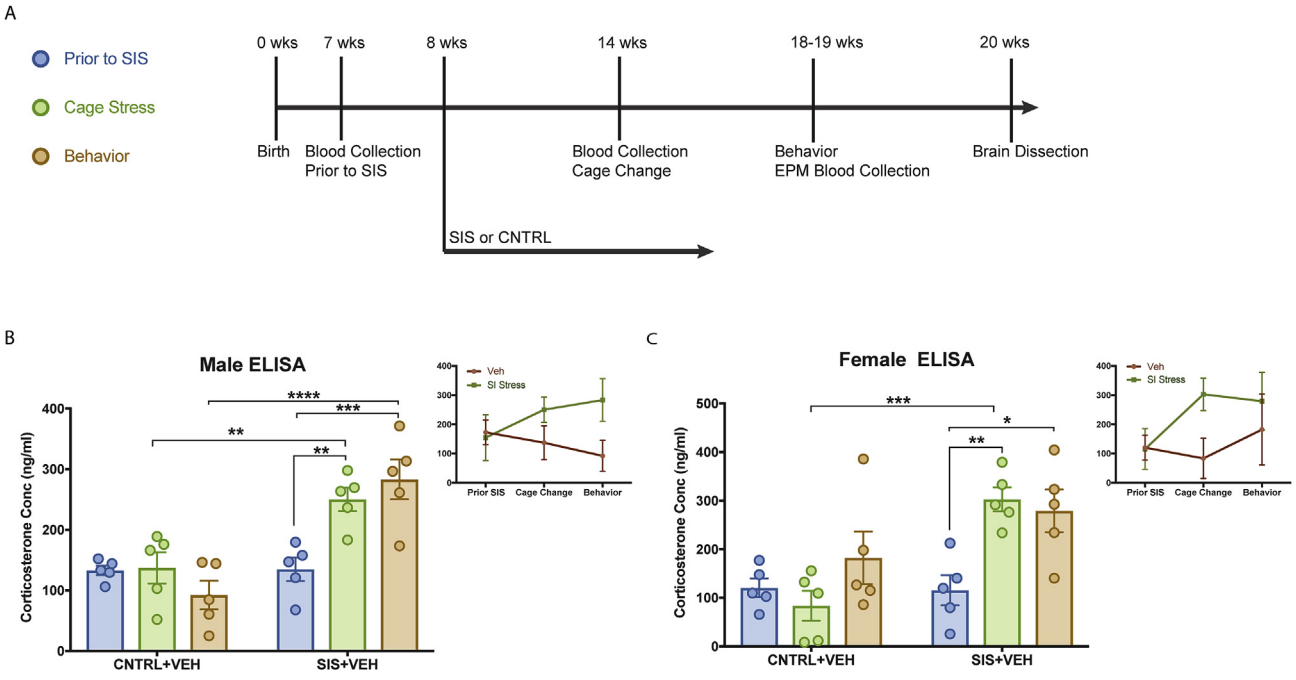

Fig. 4. SIS Stress Increases Endogenous Corticosterone Levels in Males and Females. Timeline of experiment and legend for figures (A). Smaller panels (B–C) illustrate the changes in plasma corticosterone levels across blood collection time points. Larger panel (B) displays 2 × 3 ANOVAs (SIS*x*TIME) within 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. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$.

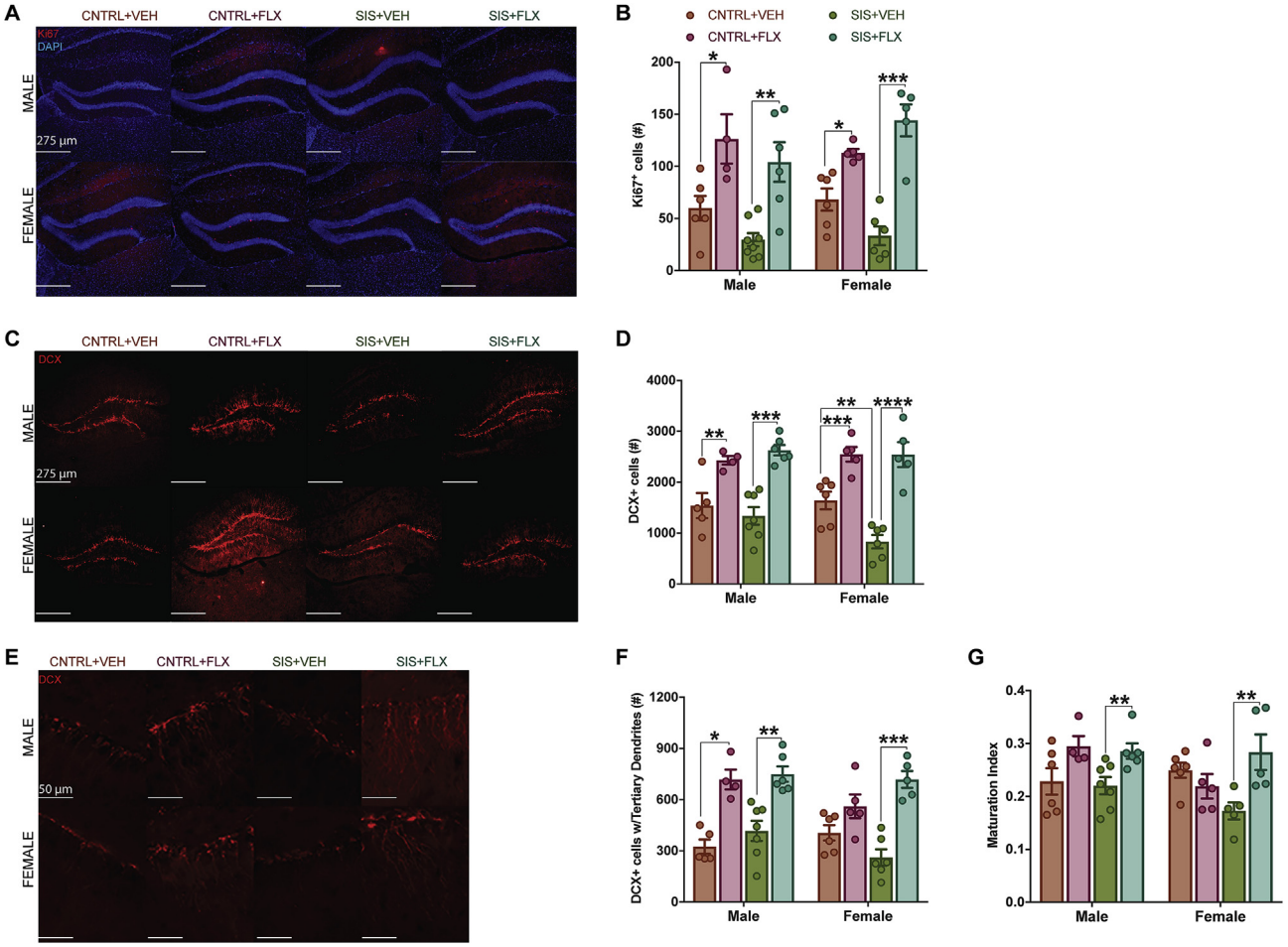

Fig. 5. SIS Stress and FLX Effects on Adult Hippocampal Neurogenesis. Representative 10x images of Ki67 (A) and DCX (C), with 40x images used to quantify DCX^+ cells with tertiary dendrites (E). Graphs represent Bonferroni posthoc comparisons from the 2 × 2 × 2 ANOVAs (SIS*x*SEX*x*FLX), with no sex differences 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. * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; ***$p < 0.001$.

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 ($p = 0.015$; Fig. 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 ($p < 0.0001$); Fig. 5D], more DCX^+ cells with tertiary dendrites [CNTRL + FLX*vs*CNTRL + VEH ($p = 0.038$) and SIS + FLX*vs*SIS + VEH ($p = 0.024$); Fig. 5F], and a higher maturation index (SIS + FLX*vs*SIS + VEH ($p = 0.041$; Fig. 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 ($p < 0.0001$); Fig. 5D], more DCX^+ cells with tertiary dendrites (SIS + FLX*vs*SIS + VEH ($p = 0.004$); Fig. 5F), and a higher maturation index (SIS + FLX*vs*SIS + VEH ($p = 0.018$; Fig. 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.

4. 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 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 2 h 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 min 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 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., 1992, 1999; 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, 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.

Disclosure

The authors declare no conflicts of interest.

Acknowledgements

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2019.107780.

Author contributions

C.N.Y. and B.A.S. conceived of experiments. C.N.Y., S.A.A., L.B., and A.G. performed the experiments. C.N.Y., M.M.G., and B.A.S. analyzed the data and made figures. C.N.Y., M.M.G., and B.A.S. wrote the manuscript.

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