



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

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Abstract

Rationale 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.

Objective Very few studies have looked at the effects of antidepressants on behavior and neurogenesis across the estrous cycle in naturally cycling female rodents.

Methods 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.

Results 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.

Conclusions 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

Abbreviations

DG Dentate gyrus

FLX Fluoxetine

OVX Ovariectomized

EPM Elevated plus maze

NSF Novelty-suppressed feeding

FST Forced swim test

LD Light dark

OF Open field

SSRI Selective serotonin reuptake inhibitor

DCX Doublecortin

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

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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 FST (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 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 OVX 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 12-L:12-D (6 am:6 pm) 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 h of the light phase via oral gavage with volume administered contingent on bodyweight. Specifically, mice were individually weighed with oral gavage volume calculated via $((18 \text{ mg/kg (dose of FLX)} \times \text{mouse weight kg}) / \text{concentration mg/ml})$. The final volume delivered by gavage each day ranged from 0.15 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 2 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 the 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 ddH₂O, placed at the opening of the mouse's vaginal canal (without penetration) with ddH₂O 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 min and imaged under a EVOS FL Auto 2.0 microscope (ThermoFisher Scientific) at $\times 10$ 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 (Fig. 1(B)). Estrus was recorded as the presence of mostly cornified epithelial cells, with the presence of a few nucleated cells in early estrus (Fig. 1(B)). 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 (Fig. 1(B)).

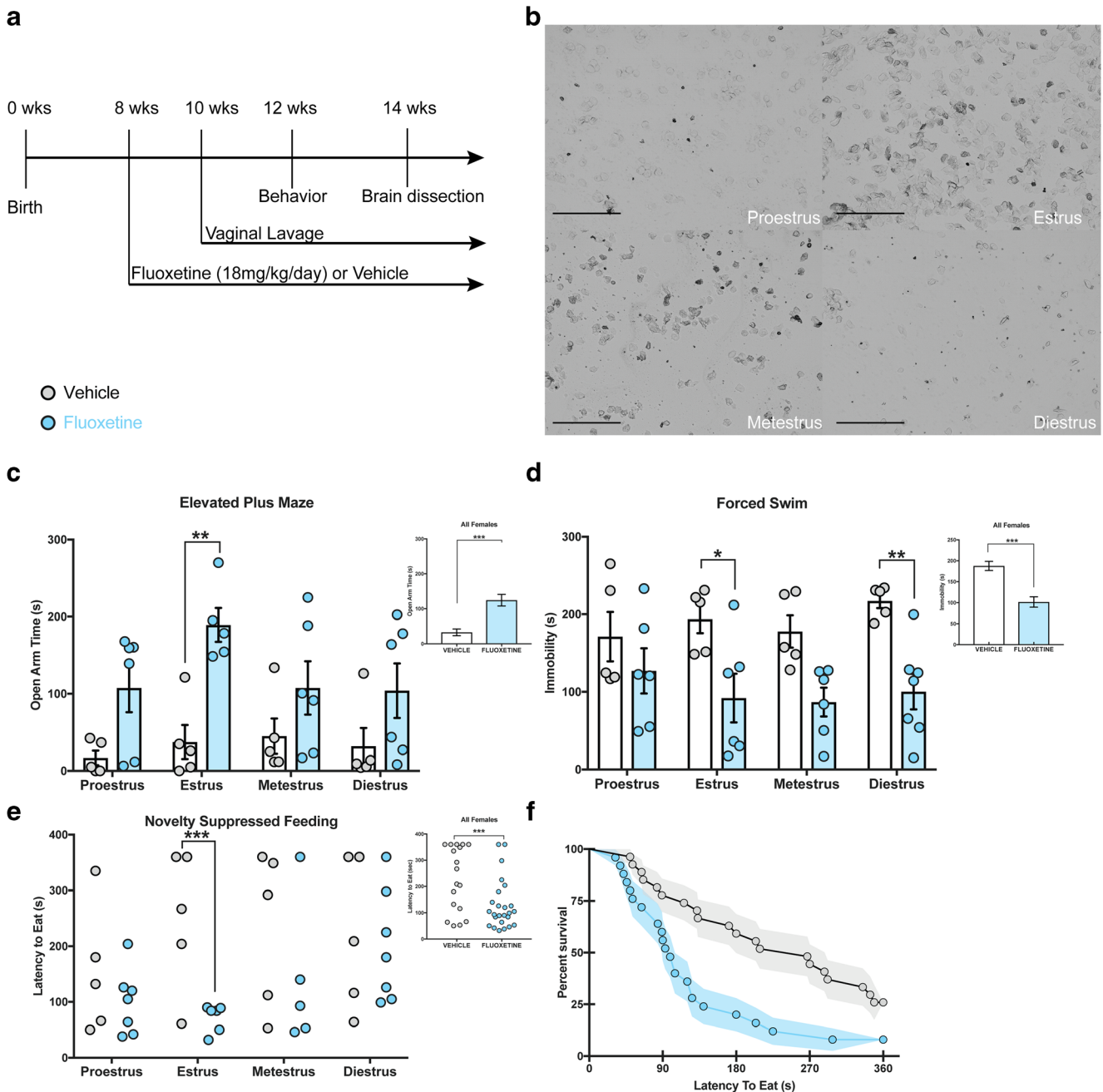


Fig. 1 Behavioral differences between FLX- and vehicle-treated female mice are also mediated by estrous phase. A timeline depicting the experimental study is depicted (A) with representative images of the four phases of the estrous cycle ($\times 10$ magnification; scale = 500 μm ; B). (C) Separate 2×4 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 ($\chi^2(1) = 8.95, p = 0.0028$) task than vehicle-treated females. Larger graph (E) represents Kaplan–Meier survival analysis within group differences being evident within the estrus phase ($***\chi^2(1) = 10.5, p = 0.008$)

Behavioral testing

Open field

Motor activity was quantified in five Plexiglass OF boxes 43 × 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 a 100-ms resolution (David et al. 2009). The computer software predefines grid lines that divide each OF chamber into the center and periphery regions, with the center being a square 11 cm 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 1A). To measure overall motor activity, total distance (cm) was quantified.

Light dark

The light/dark test was conducted in an OF chamber measuring 43 × 43 cm (Kinder Scientific, USA), with clear floor and walls. To divide the OF 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 lx. At the beginning of each 5-min test, mice were placed in the dark compartment. An observer blind to treatment groups recorded the latency to emerge into the light (Supplemental Figure 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 1B).

Elevated plus maze

The EPM test consisted of a plus-shaped apparatus with two open and two closed arms (side walls), elevated 2 ft from the floor. During the 5-min 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

After undergoing 18 h of food deprivation within their home cage, mice were placed in the corner of a testing apparatus (50 × 50 × 20 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 lx. The NSF test lasted 6 min, 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 360 s was recorded. Immediately afterwards, the mice were transferred to their home cage to assess home cage feeding behavior for 5 min (Supplemental Figure 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

A modified FST procedure suitable for mice was used (David et al. 2009). Individual cylinders (46-cm tall × 32 cm in diameter × 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-min test. Immobility times (measured by beam breaks over 5-s intervals) were assessed during the last 4 min of the test since mice are habituating to the task during the initial 2 min of the test.

Brain collection, sectioning, and immunohistochemistry

Brain collection and sectioning

Forty-eight hours after completion of behavioral testing and 24 h 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 (80 mg/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 (NaN₃) in PBS solution and stored at 4 °C until they were sectioned. Using a cryostat, every sixth section (40 μm) of the hippocampus (Bregma –1.22 to –3.88) was collected onto Superfrost

Plus slides (ThermoFisher Scientific) and stored at $-20\text{ }^{\circ}\text{C}$ 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 min before undergoing three PBS washes. Next, slides were incubated in warm citrate buffer for 30 min 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 h in 10% normal goat serum (NGS) diluted in PBS before being incubated overnight at $4\text{ }^{\circ}\text{C}$ in anti-rabbit Ki67 (1:250; polyclonal abCam, ab15580) diluted in 2% NGS PBS. Following 18 h of incubation, slides were washed 3 times in PBS before being incubated at room temperature for 2 h 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 min. 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 $\times 10$ or $\times 40$ magnification, where Ki67⁺ cells overlaid with DAPI (ThermoFisher Scientific) across the 12 sections of hippocampus were collected and counted via an observer blind to treatment (Supplemental Figure 2A–E).

Doublecortin labeling

Twelve hippocampal sections (every sixth section) for doublecortin (DCX) were stained using the primary antibody doublecortin anti-goat (1:500; Life technologies; 481,200) and secondary antibody CY-5 goat anti-rabbit (1:1000, Invitrogen, Thermo Fisher Scientific, A10523). Fluorescent images were taken using a EVOS FL Auto 2.0 microscope (ThermoFisher Scientific) at $\times 10$ or $\times 40$ magnification, where DCX⁺ cells across the 12 sections of hippocampus were collected and counted by an observer blind to treatment (Supplemental Figure 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 2×4 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 18 mg/kg FLX for 3 weeks (Fig. 1(A)) and then exposed these mice to the OF, light dark test (LD), EPM, novelty-suppressed feeding (NSF), and FST. To assess estrous cycle phase (Fig. 1(B)), vaginal lavages were performed daily beginning 2 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 \times treatment $F(1,35) = 22.8, p < 0.001, \eta^2 = 0.373$; Fig. 1(C)) and less time immobile (two-way ANOVA cycle \times treatment $F(1,35) = 25.77, p < 0.001, \eta^2 = 0.356$; Fig. 1(D)) 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, $p = 0.003$ (Fig. 1(C)). 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 (Fig. 1(D)). We found no significant effects of treatment or estrous phase nor interaction effect on behaviors within the OF and LD tests (Supplemental Figure 1A–C).

In the NSF, a Kaplan–Meier survival analysis log-rank test revealed that FLX significantly reduces latency to feed, $p = 0.0038$ (Fig. 1(E, 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 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 × treatment, $F(1, 32) = 11.79$, $p = 0.002$, $\eta^2 = 0.234$) (Fig. 2(A–B), Supplemental Figure 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 (Fig. 3(A)).

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 × treatment, $F(1, 37) = 13.44$, $p < 0.001$, $\eta^2 = 0.280$) (Fig. 2(C–D), Supplemental Figure 2F–J). We found a significant interaction effect between treatment and estrous phase (two-way ANOVA cycle × 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 ($p = 0.009$; Fig. 2(D)) 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 (Fig. 3(B)). Additionally, metestrus vehicle-treated mice had significantly more DCX⁺ cells than both estrus ($p < 0.001$) and diestrus ($p = 0.008$) vehicle-treated mice (Fig. 3(B)). Within the FLX group, no differences in DCX⁺ cell expression were 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 × treatment, $F(1, 37) = 15.85$, $p < 0.001$, $\eta^2 = 0.401$; Fig. 2(E–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 ($p = 0.001$; Fig. 2(F)) phases. We also observed a significant interaction effect between treatment group and estrous cycle phase (two-way ANOVA cycle × 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 (Fig. 3(C)). Planned comparisons also revealed that metestrus vehicle-treated females had more mature neurons than estrus ($p = 0.004$) and diestrus ($p = 0.006$) vehicle-

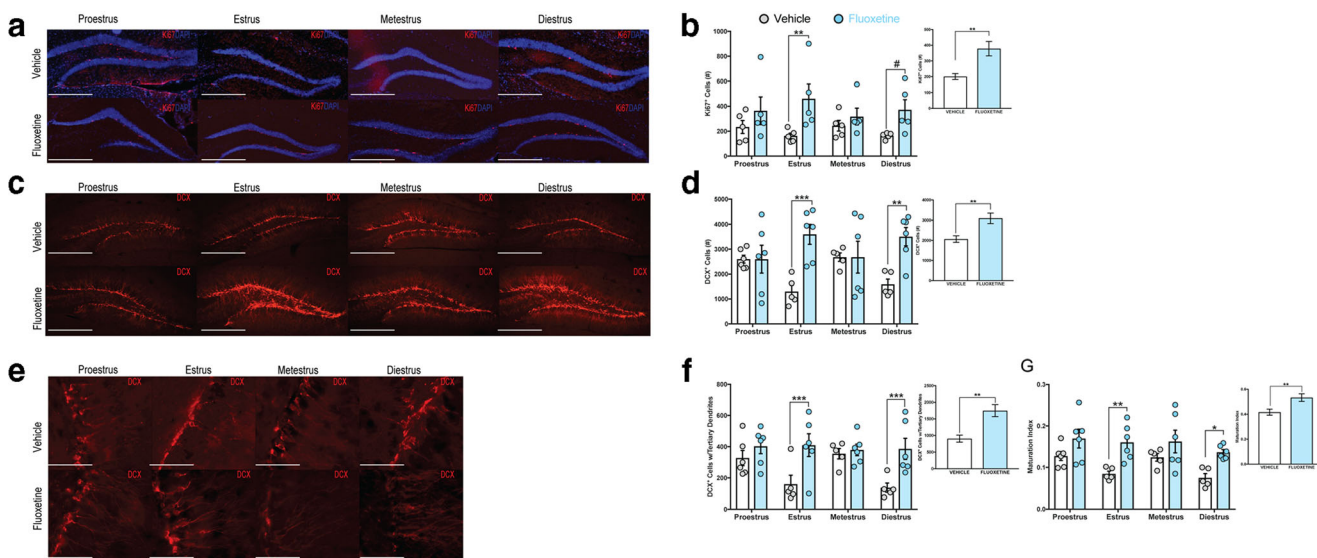
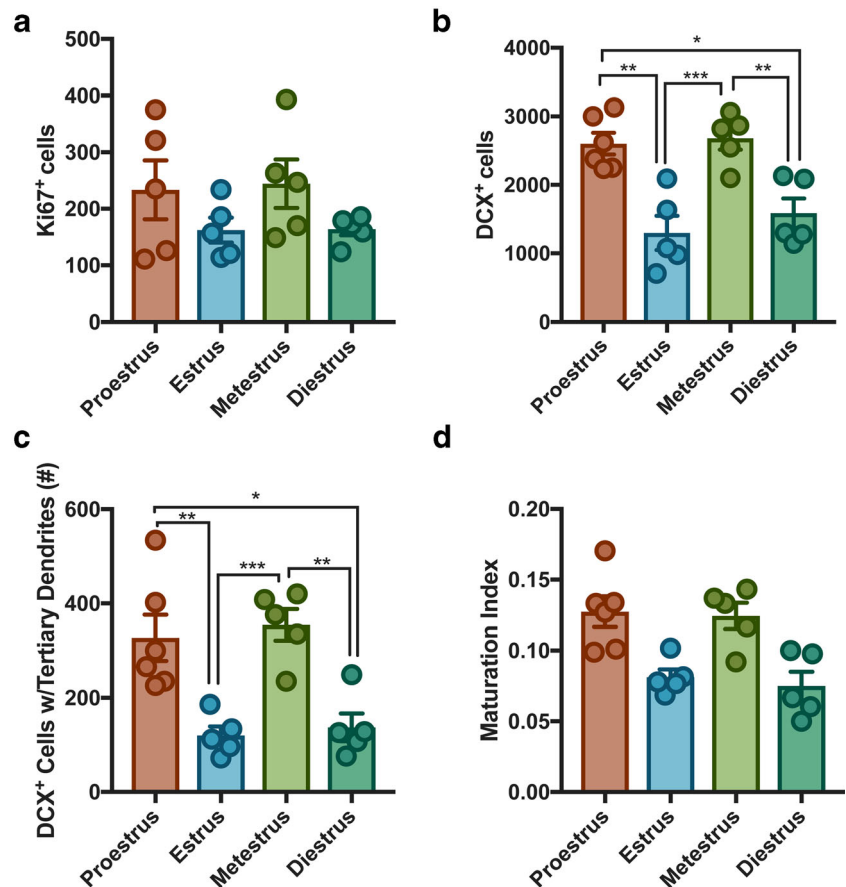


Fig. 2 FLX increases all stages of neurogenesis with differences most pronounced in the estrus and diestrus phases. Visual representation of Ki67 and DCX immunostaining is depicted above for each treatment group across the estrous cycle phases. Ki67 (A) and DCX (C) images were taken at $\times 10$ magnification (scale = 500 μm) with mature neurons (E) taken at $\times 40$ magnification (scale = 125 μm). Smaller inset panels (B, D, F, G) represent 2×4 ANOVAs (cycle \times 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 2×4 ANOVAs (cycle \times 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

Fig. 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)



treated females (Fig. 3(C)). 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 \times treatment, $F(1, 37) = 21.71$, $p < 0.001$; Fig. 2(G)) 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 (Fig. 2(G)). Within both the vehicle (Fig. 3(D)) and FLX group, we observed no differences in the maturation index across the estrous cycle. Taken together, these data suggest that the effects of FLX 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 2 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 24 h 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 DG during estrus and diestrus (Supplemental Figure 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.

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Compliance with ethical standards

Conflict of interest They authors declare that they have no conflict of interest.

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