

Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice



Jennifer J. Tuscher^a, Julia S. Szinte^a, Joseph R. Starrett^b, Amanda A. Krentzel^b, Ashley M. Fortress^a, Luke Remage-Healey^b, Karyn M. Frick^{a,*}

^a Department of Psychology, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, United States

^b Department of Psychological and Brain Sciences and Neuroscience and Behavior Program, Center for Neuroendocrine Studies, University of Massachusetts Amherst, Amherst, MA, United States

ARTICLE INFO

Article history:

Received 9 March 2016

Accepted 2 May 2016

Available online 10 May 2016

Keywords:

Aromatase

Letrozole

Estradiol

Object recognition

ABSTRACT

The potent estrogen 17 β -Estradiol (E_2) plays a critical role in mediating hippocampal function, yet the precise mechanisms through which E_2 enhances hippocampal memory remain unclear. In young adult female rodents, the beneficial effects of E_2 on memory are generally attributed to ovarian-synthesized E_2 . However, E_2 is also synthesized in the adult brain in numerous species, where it regulates synaptic plasticity and is synthesized in response to experiences such as exposure to females or conspecific song. Although *de novo* E_2 synthesis has been demonstrated in rodent hippocampal cultures, little is known about the functional role of local E_2 synthesis in mediating hippocampal memory function. Therefore, the present study examined the role of hippocampal E_2 synthesis in hippocampal memory consolidation. Using bilateral dorsal hippocampal infusions of the aromatase inhibitor letrozole, we first found that blockade of dorsal hippocampal E_2 synthesis impaired hippocampal memory consolidation. We next found that elevated levels of E_2 in the dorsal hippocampus observed 30 min after object training were blocked by dorsal hippocampal infusion of letrozole, suggesting that behavioral experience increases acute and local E_2 synthesis. Finally, aromatase inhibition did not prevent exogenous E_2 from enhancing hippocampal memory consolidation, indicating that hippocampal E_2 synthesis is not necessary for exogenous E_2 to enhance hippocampal memory. Combined, these data are consistent with the hypothesis that hippocampally-synthesized E_2 is necessary for hippocampus-dependent memory consolidation in rodents.

© 2016 Elsevier Inc. All rights reserved.

Introduction

In recent years, the sex steroid hormone 17 β -estradiol (E_2) has been shown to play a vital role in mediating hippocampal function. Although much has been learned in recent years about how E_2 regulates hippocampal memory (Daniel, 2006; Fortress and Frick, 2014; Frick, 2012; Luine and Frankfurt, 2012; Srivastava and Evans, 2013; Woolley, 2007), many important questions remain. Historically, the beneficial effects of E_2 on memory and hippocampal function have been attributed to ovarian estrogens acting on the brain *via* a traditional endocrine mechanism. However, E_2 can also be synthesized *de novo* in cognitive regions of the brain from cholesterol or androgen precursors, challenging the long-held dogma that these brain regions are merely targets for peripheral endocrine glands. For example, *in vitro* data suggest that rodents are able to synthesize E_2 *de novo* in hippocampal slice cultures (Hojo et al., 2004; Kretz et al., 2004; Prange-Kiel et al., 2003). Additionally, data from

zebra finches demonstrate that E_2 can be rapidly synthesized in the cortex during behavioral experiences including exposure to female conspecifics or conspecific song (Remage-Healey et al., 2008, 2010, 2012). This capacity for local steroidogenesis in the brain suggests that neurosteroids like E_2 may acutely and precisely modulate neural circuitry by acting on a rapid timescale similar to traditional neurotransmitters (Balthazart and Ball, 2006; Saldanha et al., 2011). Despite these findings, little is known about the behavioral significance of *de novo* E_2 synthesis in the rodent brain, particularly in regions critical for regulating learning and memory, such as the hippocampus.

In vitro work conducted over the past few decades has been instrumental to expanding our understanding of neurosteroidogenesis in the adult vertebrate brain. This progress is due to the discovery that aromatase and all other necessary precursors for E_2 synthesis (Abdelgadir et al., 1994; Sanghera et al., 1991; Wehrenberg et al., 2001) are expressed in several regions of the adult brain in a multitude of species, including songbirds, rodents, non-human primates, and humans (Azcoitia et al., 2011; Ivanova and Beyer, 2000; Roselli et al., 1985; Roselli and Resko, 1989; Vockel et al., 1990). At the cellular level, aromatase, the enzyme responsible for converting androgen precursors to estrogens, can be found throughout the hippocampal formation, including

* Corresponding author at: Department of Psychology, University of Wisconsin-Milwaukee, 2441 E. Hartford Ave., Milwaukee, WI 53211, United States.
E-mail address: frick@uwm.edu (K.M. Frick).

granular cells of dentate gyrus and pyramidal cells in CA1 and CA3 in humans and rodents (Azcoitia et al., 2011; Hojo et al., 2011; Yague et al., 2010). Further, intracellular localization studies have documented aromatase throughout the soma and dendrites of hippocampal pyramidal cells, as well as at pre- and postsynaptic terminals (Hojo et al., 2004, 2011; Lephart, 1996). The presence of aromatase in hippocampal synaptic terminals suggests the potential for local E₂ synthesis to rapidly mediate hippocampal plasticity and memory consolidation.

De novo E₂ synthesis was first demonstrated in adult rodent hippocampal neurons *in vitro* (Prange-Kiel et al., 2003), and subsequent work has shown that levels of E₂ are substantially higher in the hippocampus than in circulating plasma in both male and female rats (Hojo et al., 2004). *In vitro*, hippocampal E₂ synthesis is markedly reduced by the aromatase inhibitor letrozole (Fester et al., 2012; Hojo et al., 2004; Kretz et al., 2004; Prange-Kiel et al., 2003). The letrozole-mediated reduction in hippocampal E₂ levels is accompanied by decreased spine synapse density and presynaptic bouton number in rats and mice, suggesting that the loss of local E₂ may reduce synaptic connectivity (Kretz et al., 2004; Zhou et al., 2010; Fester et al., 2012). This notion is supported by other *in vitro* studies demonstrating that aromatase inhibition decreases the expression of the presynaptic membrane protein synaptophysin and the postsynaptic protein spinophilin, key components of spine formation in rat hippocampal neurons (Kretz et al., 2004; Fester et al., 2012). Consistent with these findings, systemic injections of letrozole decrease hippocampal synaptic protein levels in both intact and ovariectomized female mice (Zhou et al., 2010), and impair hippocampal long-term potentiation (LTP) in gonadally-intact male and female mice, as well as ovariectomized female mice (Vierk et al., 2012).

Despite evidence that *de novo* E₂ regulates hippocampal synaptic plasticity, little is known about the role of hippocampal E₂ synthesis in mediating memory formation. One recent study showed that systemic treatment with the aromatase inhibitor fadrozole prior to fear extinction training impaired the consolidation of extinction memories in male rats (Graham and Milad, 2014). Interestingly, male rats receiving fadrozole four hours after extinction training exhibited no deficits in later fear extinction recall, suggesting that *de novo* synthesis is particularly important for fear memory consolidation within the first four hours. However, the systemic injections used in this study did not permit conclusions to be drawn about the role of hippocampal E₂ synthesis in memory consolidation. Therefore, the present study sought to address the questions of whether hippocampally-synthesized E₂ is essential for either hippocampus-dependent memory consolidation or the memory enhancing effects of exogenous E₂. Using bilateral dorsal hippocampal infusions of letrozole, we found that hippocampal E₂ synthesis is necessary for both object recognition and spatial memory consolidation in ovariectomized female mice. We further found that letrozole blocked what appeared to be a transient increase in hippocampal E₂ synthesis after object training. Finally, our data suggest that hippocampal E₂ synthesis is not necessary for exogenous E₂ to enhance hippocampal memory consolidation, at least at the doses used here. To our knowledge, this is the first demonstration that hippocampal E₂ synthesis is essential for memory consolidation in rodents.

Methods

Subjects

All experiments used young (8–12 week old) female C57BL/6 mice (Taconic, Cambridge City, IN) as subjects. Mice were housed in groups of up to five until surgery, after which they were singly housed. Mice were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and are in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

General experimental design

Approximately four days after arrival in the lab, mice were implanted with guide cannulae and ovariectomized within a single surgical session. At least one week after surgery, mice underwent behavioral training and testing in an object recognition (OR) task to measure object recognition memory and/or an object placement (OP) task to measure spatial memory. Each task used unique sets of objects to maintain novelty and prevent interference from one task to the other. When, as stipulated below, mice participated in both OR and OP, the order of testing varied within each experiment and testing was separated by two weeks to allow the hippocampus to fully recover from each infusion. For a subset of mice (see below), dorsal hippocampi were collected bilaterally two weeks after completing object recognition testing for measurement of E₂ levels.

Surgery

All surgeries were conducted at least one week prior to behavioral testing as described previously (Boulware et al., 2013; Fortress et al., 2013). Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance) in 100% oxygen and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Female mice underwent ovariectomy and cannula implantation in the same surgical session as described previously (Boulware et al., 2013; Fortress et al., 2013). After completion of ovariectomy, mice were implanted with stainless steel bilateral guide cannulae (Plastics One, Roanoke, VA) aimed at the dorsal hippocampus (DH; C232GC, 22 gauge; –1.7 mm AP, ±1.5 mm ML, and –2.3 mm DV (injection site)) or a triple cannula that consisted of a single cannula aimed at the dorsal third ventricle (intracerebroventricular; ICV; C232GC, 22 gauge; –0.9 mm AP, ±0.0 mm ML, and –2.8 mm DV (injection site)) in addition to the bilateral dorsal hippocampal cannulae. This triple infusion protocol is routinely used by our lab to prevent potential damage to the DH from two infusions into the DH in rapid succession (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010, 2012; Boulware et al., 2013; Fortress et al., 2013). Here, we used triple cannulae to administer E₂ adjacent to the DH via the dorsal third ventricle while infusing letrozole bilaterally into the DH. This protocol avoids potential damage to the DH from infusions of both compounds, and precludes possible interactions between the drugs within the DH and reduced drug efficacy that could result from the dilution of each drug in the larger ICV infusion volume. Cannulae were fixed to the skull with dental cement (Darby Dental Supply, New York, NY) that served to close the wound. Dummy cannulae (C232DC; Plastics One) were inserted into each cannula to prevent clogging of the cannula tracts. Mice received 10% ibuprofen in their drinking water for 5 days after surgery, and were allowed a minimum of one week to recover before behavioral testing.

Drugs and infusions

The aromatase inhibitor letrozole (Selleckchem, Houston, TX) was dissolved in sterile 0.9% saline and 2% dimethyl sulfoxide (DMSO) to concentrations of 0.01, 0.05, and 0.1 µg/µl. A volume of 0.5 µl was infused bilaterally into each side of the DH immediately after training. Vehicle-infused controls received infusions of sterile 0.9% saline and 2% DMSO at the same rate and total volume. Hippocampal infusions were conducted at a rate of 0.5 µl/min for 1 min per hemisphere as described previously (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010, 2012), resulting in letrozole doses of 0.005, 0.025, and 0.05 µg/hemisphere. For experiments also involving ICV infusion of E₂, cyclodextrin-encapsulated E₂ (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline to a concentration of 10 µg/µl. The vehicle consisted of 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO) dissolved in saline to the same concentration of cyclodextrin present in the cyclodextrin-encapsulated E₂ solution. ICV infusions were conducted at

the same rate as DH infusions (0.5 $\mu\text{l}/\text{min}$) for 2 min total, to allow for infusion of the same total volume at the same rate as DH infusions.

Behavioral testing

Object recognition (OR) and object placement (OP) were used to measure object recognition and spatial memory as we have previously described (Boulware et al., 2013; Fortress et al., 2013). Both tasks involve the DH (Baker and Kim, 2002; Cohen et al., 2013; Fernandez et al., 2008). In ovariectomized mice, immediate post-training bilateral infusion of 5 μl E_2 into the dorsal hippocampus enhances OR tested 48 h after infusion (Boulware et al., 2013; Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013, 2014; Pereira et al., 2014; Zhao et al., 2010, 2012) and OP tested 24 h after infusion (Boulware et al., 2013; Fortress et al., 2014). Mice were first handled for one min/day for three days prior to habituation. After the first day of handling, a Lego was placed in each home cage to habituate the mice to objects during the remaining handling days and habituation period. After three days of handling, mice were habituated to the apparatus for two consecutive days by allowing them to explore the empty white arena (60 cm \times 60 cm \times 47 cm) for 5 min/day. For the OR task, mice were then returned to the arena and allowed to accumulate 30 s exploring two identical objects placed about 5 cm from the upper left and right corners of the arena, or until 20 min had elapsed. Immediately after training, mice received DH or DH + ICV drug infusions. Such post-training infusions allowed us to pinpoint effects of aromatase inhibition specifically to the memory consolidation period, while also minimizing the confounding effects of hormones on performance factors (e.g., motivation, anxiety) during training or retention testing (Frick and Gresack, 2003; McGaugh, 1989). Object recognition memory was then tested 24 or 48 h later by measuring the amount of time spent with the novel and familiar object. A 24-h delay was used to measure the potential memory-impairing effects of letrozole because vehicle-infused ovariectomized females show intact OR memory consolidation at this time point (Fortress et al., 2013; Boulware et al., 2013). A 48-h delay was used to measure whether letrozole blocked the memory-enhancing effects of E_2 because vehicle-infused females show impaired OR memory consolidation, whereas E_2 -infused females show intact OR memory consolidation, at this time point (Fortress et al., 2013; Boulware et al., 2013). Moreover, E_2 -infused females spend significantly more time with the novel object than vehicle-infused females 48 h after infusion (Kim et al., 2016). Because most mice inherently prefer novelty, those that remember the training objects spend more time than chance (15 s) exploring the novel object during testing. Chance was set at 15 s because this is the value at which mice spend exactly the same amount of time with each object. Therefore, chance levels of exploration indicate that mice do not remember the training objects.

Training and testing for OP was identical to OR, except that testing was conducted four hours after training, and involved moving one of the identical training objects to a new location in the arena (lower right or lower left corner). Spatial memory consolidation was demonstrated if the mice spent more time than chance with the moved object. At this four-hour delay, vehicle-infused ovariectomized females show intact OP memory (Boulware et al., 2013), thereby permitting observation of letrozole-induced memory impairments. A 24-h delay was used to determine whether letrozole could block the memory-enhancing effects of E_2 on OP memory consolidation. At this time point, E_2 -infused, but not vehicle-infused, mice spend more time than chance with the moved object and E_2 -infused mice spend significantly more time with the moved object than vehicle-infused mice (Boulware et al., 2013; Kim et al., 2016).

E_2 measurement

E_2 levels were measured in the DH using an EIA assay following dual liquid-solid phase extraction (Chao et al., 2011). Briefly, frozen DH tissue was first homogenized in 0.1 M phosphate buffer, followed by

three rounds of ether extraction. The final organic phase was dried under air in a 50 °C water bath, followed by re-suspension in 250 μl of 0.1 M phosphate buffer prior to solid phase extraction. Re-suspended samples were then eluted through C18 columns under vacuum pressure and washed with ddH₂O to elute hydrophilic polar compounds. Hydrophobic compounds, including steroids, were then eluted with a series of washes with 100% methanol, followed by an evaporation step under air in a 50 °C water bath. After drying, samples were suspended in EIA buffer. E_2 concentrations were then measured from EIA plates per the manufacturer's instructions using an Epoch Microplate Spectrophotometer plate reader (Biotek) with a 450 nm filter and Gen5 Software (Chao et al., 2011). The EIA assay used to measure brain estrogen levels in this experiment is highly specific for E_2 (cross-reactivity: 14% for E_2 -3-glucuronide; 12% for estrone; 1% for E_2 -17-glucuronide; < 0.10% for other major steroids including testosterone; Cayman chemical). Data are expressed in pg E_2 per mg wet weight of DH tissue, after correcting for recovery (recovery was 60.6% and intrassay CV was 2.17%).

Data analysis

All statistical analyses were conducted using GraphPad Prism 6 (La Jolla, CA). To determine whether each group demonstrated intact memory for each task, OR and OP data were first analyzed using one sample *t*-tests to determine if the time spent with the novel or moved object differed significantly from chance (15 s; Fortress et al., 2013; Boulware et al., 2013). This analysis is used because time spent with the objects is not independent; time spent with one object reduces time spent with the other object (Frick and Gresack, 2003). To then measure between-group treatment differences for each task, one-way ANOVAs with treatment as the independent variable were conducted for each behavioral experiment followed by Tukey's post hoc tests. EIA data were analyzed using a two-way ANOVA, followed by Tukey's post hocs when appropriate, to determine effects of post-training drug treatments over time. Statistical significance for all analyses was determined as $p \leq 0.05$. Finally, effect sizes were calculated using eta-squared for ANOVAs ($\eta^2 = \text{sum of squares between}/\text{sum of squares total}$) and Cohen's *d* ($d = \text{mean of differences}/\text{standard deviation of differences}$) for pair-wise comparisons.

Results

Blocking dorsal hippocampal E_2 synthesis immediately after training impairs object recognition and object placement memory consolidation

To determine if hippocampal E_2 synthesis is necessary for the consolidation of object memories, young female mice were ovariectomized and then bilaterally implanted with cannulae aimed at the DH one week prior to the start of behavioral training. Immediately after OR training, mice received bilateral DH infusion of vehicle or one of three doses of the aromatase inhibitor letrozole (0.005, 0.025, or 0.05 $\mu\text{g}/\text{hemisphere}$; $n = 9$ –13/group). Object recognition memory was tested 24 h later, a time point at which young ovariectomized vehicle-treated mice remember the familiar object (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010). Vehicle-infused mice spent significantly more time than chance with the novel object ($t_{(12)} = 3.35$, $p = 0.006$; Fig. 1A), demonstrating intact memory for the familiar training object. However, mice infused with 0.005, 0.025, or 0.05 $\mu\text{g}/\text{hemisphere}$ letrozole spent similar amounts of time with the familiar and novel objects (0.005 μg : $t_{(8)} = 1.64$, $p = 0.14$; 0.025 μg : $t_{(9)} = 0.67$, $p = 0.52$; 0.05 μg : $t_{(11)} = 0.26$, $p = 0.80$; Fig. 1A), indicating impaired OR memory. These effects were supported by a one-way ANOVA, which demonstrated a significant main effect of treatment ($F_{(3,40)} = 3.81$, $p = 0.017$, $\eta^2 = 0.22$), and Tukey's post-hoc tests which revealed that mice infused with vehicle spent significantly more time with the novel object than mice infused with 0.025 or 0.05 μg letrozole ($ps < 0.05$; Fig. 1A). Elapsed time to accumulate 30 s of exploration did not differ among the groups during

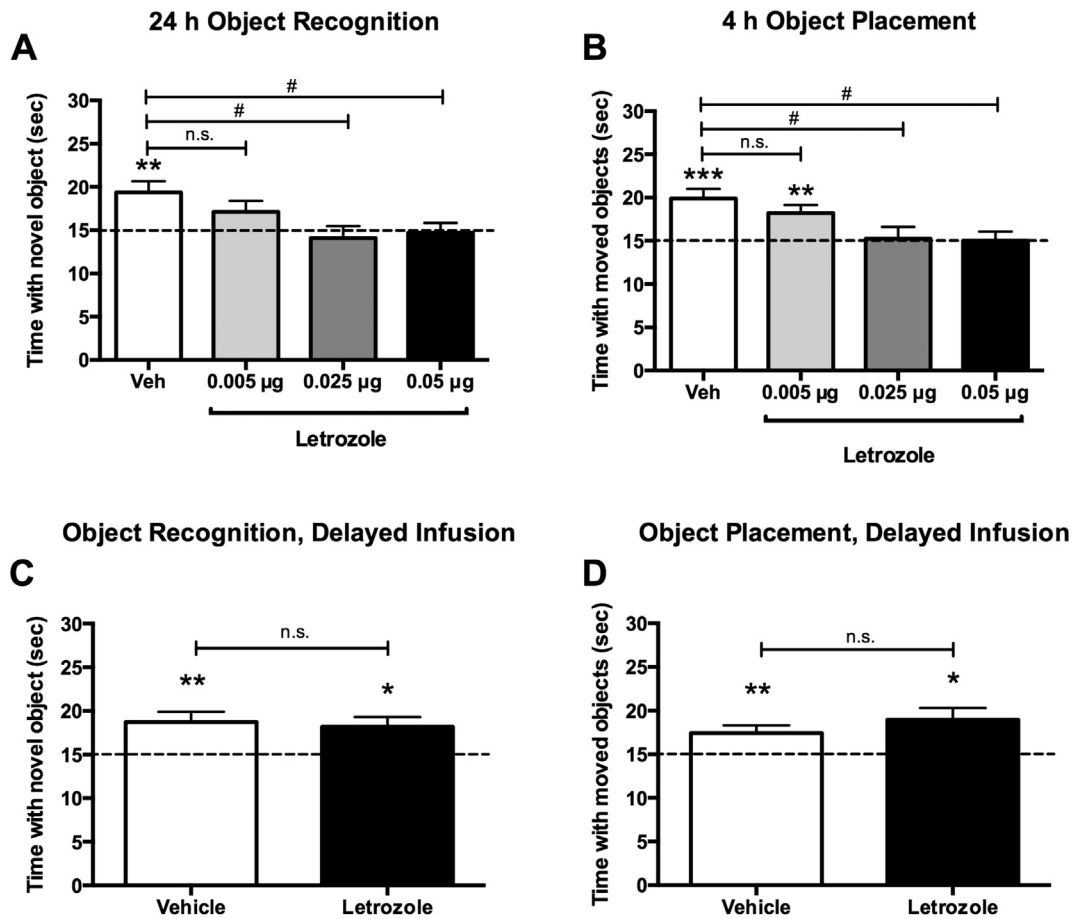


Fig. 1. Letrozole impairs object recognition and object placement memory consolidation. (A) Mice receiving bilateral dorsal hippocampal infusion of vehicle ($n = 13$), but not 0.005 μg ($n = 9$), 0.025 μg ($n = 10$), or 0.05 μg ($n = 12$) letrozole, immediately after training spent significantly more time with the novel object 24 h after training relative to chance (dashed line at 15 s). Vehicle-infused mice also spent significantly more time with the novel object than mice infused with 0.025 or 0.05 μg letrozole. These data suggest that the 0.025 and 0.05 μg doses of letrozole blocked object recognition memory consolidation. (B) Mice receiving bilateral dorsal hippocampal infusions of vehicle ($n = 12$) or 0.005 μg ($n = 9$) letrozole, but not 0.025 μg ($n = 9$) or 0.05 μg ($n = 9$) letrozole, immediately after training spent significantly more time than chance ($*p < 0.05$) with the moved object 4 h after training. Vehicle-infused mice also spent significantly more time with the moved object than mice infused with 0.025 or 0.05 μg letrozole, suggesting that the 0.025 and 0.05 μg doses of letrozole also blocked spatial memory consolidation. This effect was limited to within the first 2–3 h after training, as mice receiving a bilateral infusion of vehicle ($n = 11$) or 0.025 μg letrozole ($n = 10$) 3 h after OR training (C), or 2 h after OP training (vehicle: $n = 13$; letrozole: $n = 9$) (D), spent significantly more time than chance with the novel object ($*p < 0.05$), suggesting that the memory-impairing effects of letrozole are specific to the consolidation period immediately after training. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ relative to chance; $\#p < 0.05$ for between-group differences measured by Tukey's post hoc tests.

testing ($F_{(3,40)} = 1.02$, $p = 0.39$; Vehicle = 649.7 ± 84.61 ; 0.005 $\mu\text{g} = 821.4 \pm 87.07$; 0.025 $\mu\text{g} = 669.7 \pm 46.51$; 0.05 $\mu\text{g} = 714.4 \pm 57.07$).

Next, to determine if local E_2 is also essential for spatial memory consolidation, a different set of mice received bilateral DH infusion of vehicle or one of the same three doses of letrozole ($n = 9$ –12/group) immediately after OP training. Spatial memory was tested four hours later, a time point at which young ovariectomized vehicle-treated mice remember the unmoved object (Boulware et al., 2013). Mice infused with either vehicle ($t_{(11)} = 4.40$, $p = 0.001$) or 0.005 μg /hemisphere letrozole ($t_{(8)} = 3.53$, $p = 0.008$) spent significantly more time than chance with the moved object (Fig. 1B), demonstrating intact memory, whereas those infused with 0.025 or 0.05 μg /hemisphere letrozole exhibited impaired OP memory (0.025 μg : $t_{(8)} = 0.18$, $p = 0.86$; 0.05 μg : $t_{(8)} = 0.02$, $p = 0.98$; Fig. 1B). As with OR, one-way ANOVA demonstrated a significant main effect of treatment ($F_{(3,35)} = 4.60$, $p = 0.008$, $\eta^2 = 0.28$) and Tukey's post-hoc tests revealed that mice infused with vehicle spent significantly more time with the moved object than mice infused with 0.025 or 0.05 μg letrozole ($ps < 0.05$; Fig. 1B). Elapsed time to accumulate 30 s of exploration did not differ among the groups during testing ($F_{(3,35)} = 2.12$, $p = 0.12$; Vehicle = 569.8 ± 61.30 ; 0.005 $\mu\text{g} = 408.8 \pm 43.65$; 0.025 $\mu\text{g} =$

442.3 ± 50.43 ; 0.05 $\mu\text{g} = 564.0 \pm 63.16$). Together, these data provide the first evidence that local E_2 synthesis in the DH is necessary for the consolidation of hippocampus-dependent object recognition and spatial memories in young female mice.

To demonstrate that the post-training effects of letrozole on memory are specific to the memory consolidation period immediately after training, we next infused new mice with vehicle or 0.025 μg letrozole three hours after OR training or two hours after OP training. These delays were chosen because the memory-enhancing effects of post-training systemic or intrahippocampal E_2 treatment are restricted to between 1 and 3 h after training (Fernandez et al., 2008; Frye et al., 2007; Packard and Teather, 1997; Walf et al., 2006). In our own hands, post-training intrahippocampal infusion of E_2 delayed 3 h after training did not enhance OR memory consolidation in ovariectomized mice (Fernandez et al., 2008). We used this same delay for OR in the present study, and used a 2-h delay for OP based on our experience that OP memory decays faster than OR memory (Boulware et al., 2013). In the present study, memory was tested 24 h later in OR, or 4 h later in OP (as in Fig. 1A and B). We found that mice infused with either vehicle ($t_{(10)} = 3.16$, $p = 0.01$) or 0.025 μg /hemisphere letrozole ($t_{(9)} = 2.83$, $p = 0.02$) three hours after OR training spent significantly more time

than chance with the novel object (Fig. 1C). Moreover, mice infused with vehicle or letrozole did not differ in the time spent with the novel object ($t_{(19)} = 0.33$, $p = 0.74$, $d = 0.15$). Together, these results demonstrate that letrozole infusion did not block OR memory consolidation when delayed three hours after training. Similarly, we found that mice infused with either vehicle ($t_{(12)} = 2.86$, $p = 0.01$) or $0.025 \mu\text{g}/\text{hemisphere}$ letrozole ($t_{(8)} = 2.91$, $p = 0.02$) two hours after OP training spent significantly more time than chance with the moved object, and the groups again did not differ from each other ($t_{(20)} = 0.99$, $p = 0.33$, $d = 0.15$), demonstrating that a delayed infusion of letrozole also failed to block OP memory consolidation. Together, these findings support the notion that hippocampal *de novo* synthesis is particularly critical for memory formation during the consolidation period that occurs immediately after training.

Letrozole reduces dorsal hippocampal E_2 levels 30 min after object training

We next measured levels of E_2 in the DH at various points after object training to determine: 1) the extent to which letrozole reduced E_2 levels within the DH, and 2) if letrozole could block a learning-induced increase in DH E_2 levels. Two weeks after the completion of OR testing, 28 mice included in Fig. 1A were trained with two new identical objects and then immediately received a DH infusion of vehicle or the lowest dose of letrozole shown to impair memory in both OR and OP ($0.025 \mu\text{g}/\text{hemisphere}$). The DH was then dissected 30, 60, and 120 min later (30 min: $n = 3$ Veh, $n = 6$ Let; 60 min: $n = 3$ Veh, $n = 7$ Let; 120 min: $n = 3$ Veh, $n = 5$ Let) on wet ice and stored at -80°C prior to dual liquid and solid phase extraction for quantification of DH E_2 content by EIA. A two-way ANOVA revealed a significant interaction between treatment and time ($F_{(2, 21)} = 8.55$, $p = 0.002$, $\eta^2 = 0.303$; Fig. 2) and a main effect of treatment ($F_{(2, 21)} = 8.58$, $p = 0.002$, $\eta^2 = 0.025$). Post hoc analyses revealed that DH E_2 levels were significantly higher 30 min after training in mice infused with vehicle relative to those infused with letrozole ($t_{(7)} = 4.06$, $p = 0.005$, $d = 2.87$; Fig. 2). DH E_2 levels in vehicle-treated mice dropped substantially by 60 min after training, and were similar to those of letrozole-treated mice at the 60 min and 120 min time points (60 min: $t_{(8)} = 1.11$, $p = 0.31$, $d = 0.75$; 120 min: $t_{(6)} = 0.82$, $p = 0.44$, $d = 0.60$; Fig. 2). DH E_2 levels were similar in absolute quantity to those reported in other studies of

hippocampal and brain DH E_2 content in mice (Overk et al., 2013) and songbirds (Fokidis et al., 2015; Heimovics et al., 2016), although most studies, including the current study, focus on relative quantity of brain E_2 content between regions or time periods. These data suggest that letrozole suppresses an increase in DH E_2 levels 30 min after infusion at a dose shown to impair OR and OP memory consolidation *in vivo*.

Dorsal hippocampal E_2 synthesis is not necessary for exogenous E_2 to enhance hippocampal memory consolidation

Previous work from our laboratory has demonstrated that $5 \mu\text{g}$ E_2 infused bilaterally into the DH or $10 \mu\text{g}$ infused into the dorsal third ventricle (ICV) enhances the consolidation of OR and OP memories in young ovariectomized female mice in an ERK-dependent manner (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010). However, these studies did not take into account the potential contribution of local E_2 synthesis to the observed memory enhancements. In the hypothalamus of ovariectomized monkeys, exogenous estradiol benzoate infusion increases the release of local E_2 (Kenealy et al., 2013), suggesting potentially important interactions between exogenous and local E_2 . To determine if local and exogenous E_2 may synergistically facilitate memory consolidation, we next investigated the role of local E_2 synthesis in the memory-enhancing effects produced by exogenous E_2 . Mice were ovariectomized and then implanted with bilateral DH cannulae and a unilateral ICV cannula as in our previous work (e.g., Boulware et al., 2013; Fortress et al., 2013). Immediately after object training, mice received a DH infusion of 2% DMSO in sterile saline vehicle or letrozole ($0.025 \mu\text{g}/\text{hemisphere}$), followed immediately by an ICV infusion of HBC vehicle or $10 \mu\text{g}$ E_2 . OR and OP memory were then tested 48 or 24 h later, respectively, because exogenous E_2 enhances memory in these tasks at these time points (e.g., Boulware et al., 2013; Fortress et al., 2013). The same mice were used in both tasks, with the order of presentation of OR and OP varied within each group. Mice receiving vehicle infusion into the DH, followed by ICV infusion of vehicle or letrozole, did not spend more time than chance with the novel object in the OR task (Veh/Veh: $t_{(9)} = 0.51$, $p = 0.62$; Let/Veh: $t_{(10)} = 0.27$, $p = 0.79$; Fig. 3A), or the moved object in the OP task (Veh/Veh: $t_{(9)} = 1.91$, $p = 0.09$; Let/Veh: $t_{(10)} = 0.10$, $p = 0.93$; Fig. 3B). In contrast, mice who were bilaterally infused with vehicle or letrozole into the DH, followed by ICV infusion of E_2 , spent more time than chance with the novel object 48 h after OR training (Veh/ E_2 : $t_{(11)} = 4.75$, $p = 0.0006$; Let/ E_2 : $t_{(13)} = 3.40$, $p = 0.005$; Fig. 3A), and with the moved object 24 h after OP training (Veh/ E_2 : $t_{(11)} = 3.20$, $p = 0.01$; Let/ E_2 : $t_{(11)} = 3.73$, $p = 0.003$; Fig. 3B). For OR, one-way ANOVA demonstrated a significant main effect of treatment ($F_{(3,43)} = 5.32$, $p = 0.003$, $\eta^2 = 0.27$), and Tukey's post-hoc tests revealed that Veh/ E_2 mice spent significantly more time with the novel object than Veh/Veh or Let/Veh mice ($ps < 0.05$; Fig. 3A). Let/ E_2 mice spent significantly more time with the novel object than Let/Veh mice ($p < 0.05$; Fig. 3A). Similarly, for the OP task, one-way ANOVA demonstrated a significant main effect of treatment ($F_{(3,41)} = 6.28$, $p = 0.001$, $\eta^2 = 0.32$), and Tukey's post-hoc tests revealed that Let/ E_2 mice spent significantly more time with the moved object than Veh/Veh mice ($ps < 0.05$; Fig. 3B). Veh/ E_2 mice also spent significantly more time with the moved object relative to Veh/Veh or Let/Veh mice ($p < 0.05$; Fig. 3B). Elapsed time to accumulate 30 s of exploration did not differ among the groups during OP testing ($F_{(3,41)} = 0.36$, $p = 0.783$; Veh/Veh = 746.1 ± 67.01 ; Let/Veh = 748.8 ± 69.77 ; Let/ E_2 = 785.0 ± 78.24 ; Veh/ E_2 = 837.2 ± 67.40). Elapsed time to accumulate 30 s of exploration in OR did vary among the groups, seemingly due to the increased time taken by Veh/ E_2 mice ($F_{(3,43)} = 4.33$, $p = 0.01$; Veh/Veh = 649.4 ± 65.93 ; Let/Veh = 588.0 ± 62.02 ; Let/ E_2 = 767.5 ± 72.10 ; Veh/ E_2 = 901.1 ± 55.86). However, this increase is unlikely to have significantly contributed to the time spent with the novel object because time spent with the objects and elapsed time were not correlated in any behavioral experiment reported here or in previous publications (e.g., Fernandez et al., 2008). Together, these data suggest

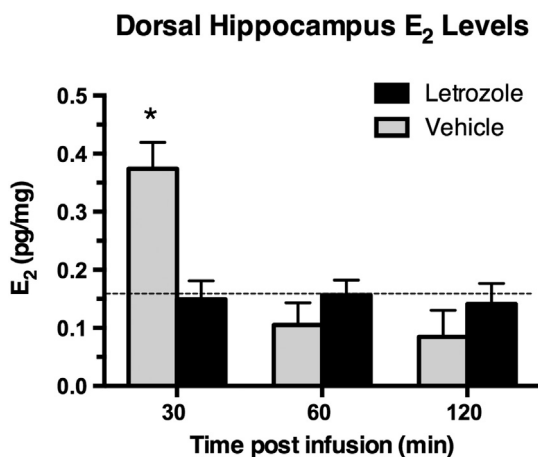


Fig. 2. Letrozole reduces hippocampal E_2 levels at a dose that impairs spatial and object recognition memory consolidation. Mice receiving bilateral dorsal hippocampal infusion of $0.025 \mu\text{g}$ letrozole had significantly lower dorsal hippocampal E_2 levels than vehicle-treated mice 30 min after infusion ($*p < 0.05$; vehicle $n = 3$; letrozole $n = 6$). Dorsal hippocampal E_2 levels in vehicle-treated mice dropped to the level of those in letrozole-treated mice by 60 min after training, and were similar to that of letrozole-treated mice at the 60 min (vehicle $n = 3$; letrozole $n = 7$) and 120 min (vehicle $n = 3$; letrozole $n = 5$) time points. Dashed horizontal line indicates the average background E_2 concentration as reported by EIA for control wells; all E_2 levels above this concentration are validated as detectable levels on the EIA.

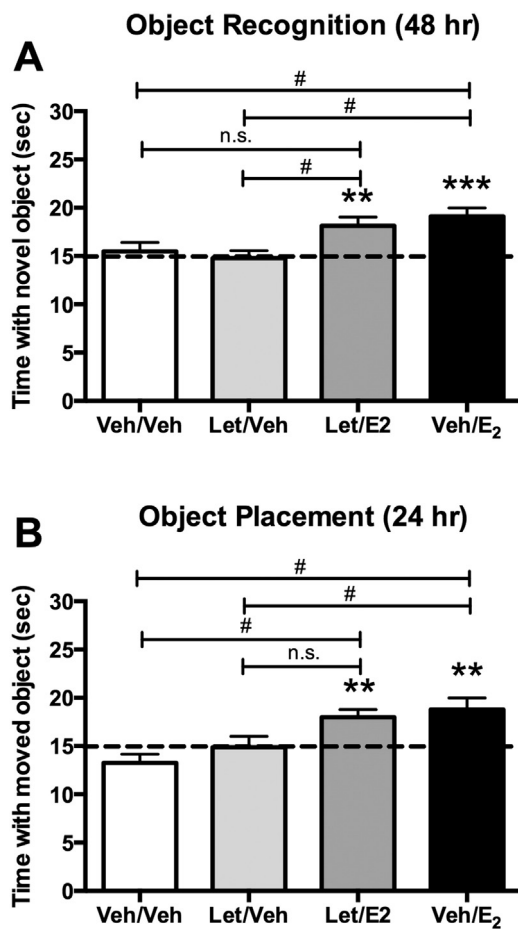


Fig. 3. Dorsal hippocampal E_2 synthesis is not necessary for exogenous dorsal hippocampal infusion of E_2 to enhance object recognition or spatial memory consolidation. Mice receiving infusions of vehicle or 0.025 μ g letrozole into the dorsal hippocampus, followed by ICV infusion of vehicle (Veh/Veh, Let/Veh) did not spend significantly more time than chance with the novel object (A) or moved object (B). In contrast, mice receiving bilateral infusion of vehicle or 0.025 μ g letrozole into the dorsal hippocampus, followed by ICV infusion of E_2 (Veh/ E_2 , Let/ E_2) spent significantly more time than chance (dashed line at 15 s, $^*p < 0.05$) with the novel object 48 h after training (A) and the moved object 24 h after training (B). For the OR task, the Veh/ E_2 group ($n = 12$) spent significantly more time with the novel object than both the Let/Veh ($n = 11$) and the Veh/Veh group ($n = 10$), and the Let/ E_2 group ($n = 14$) spent more time with the novel object relative to the Let/Veh control group. For the OP task, the Veh/ E_2 group ($n = 12$) spent significantly more time with the novel object than both the Let/Veh ($n = 11$) and the Veh/Veh groups ($n = 10$), and the Let/ E_2 group ($n = 12$) spent more time with the novel object relative to the Veh/Veh control group. These data suggest that inhibition of local E_2 synthesis in the dorsal hippocampus is not sufficient to prevent exogenous E_2 from enhancing object recognition or spatial memory consolidation. $^{**}p < 0.01$, $^{***}p < 0.001$ relative to chance; $\#p < 0.05$ for between-group differences measured Tukey's post hoc tests.

that inhibition of local E_2 synthesis in the DH does not prevent exogenous E_2 from enhancing object recognition or spatial memory consolidation, and suggest that hippocampal *de novo* E_2 synthesis is not necessary for exogenous E_2 to enhance memory, at least at the 10 μ g dose.

Discussion

Exogenous estrogens have long been known to regulate many types of learning and memory mediated by the hippocampus and other brain regions (see Bean et al., 2015; Daniel et al., 2015; Duarte-Guterman et al., 2015; Ervin et al., 2015; Foster, 2012; Frick, 2015; Frick et al., 2015; Korol and Pisani, 2015; Luine, 2014 for recent reviews). The current findings provide novel insight into the functional role of brain-derived estrogens on learning and memory in rodents. First, we demonstrated that hippocampal E_2 synthesis immediately after training is

necessary for object recognition and spatial memory consolidation in young ovariectomized mice. Next, we found that DH E_2 levels are elevated 30 min after novel object training relative to later time points, and that this elevation is blocked by DH infusion of an aromatase inhibitor *in vivo*. Together, these data suggest E_2 synthesis may increase acutely in an experience-dependent manner, and that this increase is necessary for the consolidation of recognition and spatial memories. We also found in a subsequent experiment that local E_2 synthesis is not necessary for exogenous E_2 to enhance object recognition and spatial memory consolidation, at least at the 10 μ g dose of E_2 we used. Collectively, these findings are the first to demonstrate that *de novo* E_2 synthesis in the DH is necessary for the consolidation of hippocampus-dependent memories in female rodents. Further, these experiments provide novel insights into the role of DH E_2 synthesis in the memory-enhancing effects of exogenous E_2 in rodents.

Our data showing that letrozole blocks OR and OP memory consolidation are consistent with previous *in vitro* studies suggesting that *de novo* E_2 synthesis regulates the expression of synaptic proteins, synaptic spine density, and LTP (Fester et al., 2012; Kretz et al., 2004; Vierk et al., 2012, 2014). Our present findings are also consistent with recently published *in vivo* studies showing that *de novo* E_2 synthesis in the hippocampus is important for spatial memory in male and female zebra finches (Bailey et al., 2013; Rensel et al., 2013), and a recent study conducted in male rats demonstrating the necessity of *de novo* E_2 synthesis for the extinction of fear memories (Graham and Milad, 2014). The latter study administered the aromatase inhibitor fadrozole systemically, precluding definitive conclusions about the key locus of E_2 synthesis in the brain for extinction. However, given the important role of the basolateral amygdala in fear extinction (Zeidan et al., 2011), *de novo* E_2 synthesis in this brain region may play a role. Other recent data support the importance of local E_2 synthesis by showing that lentiviral delivery of estrogen receptor α ($ER\alpha$) to the hippocampus of ovariectomized $ER\alpha$ knockout mice or middle-aged rats reverses spatial memory deficits and increases ERK activation in the absence of exogenous E_2 (Foster et al., 2008; Witty et al., 2012). These data suggest that endogenously synthesized E_2 may have bound to the virally-introduced $ER\alpha$ to facilitate spatial memory formation. Together with the present data, the findings from zebra finches and rodents suggest that regulation of memory by *de novo* E_2 synthesis may be a general property of both male and female adult vertebrate brains, and that this process is particularly important during the consolidation phase of memory in male and female rodents. Although numerous rodent studies have demonstrated that exogenous E_2 treatment drives p42 ERK activation (Boulware et al., 2013; Fernandez et al., 2008; Srivastava et al., 2008), the mechanisms through which *de novo* E_2 facilitates memory consolidation in rodents are currently unknown. Therefore, future studies should address whether local E_2 synthesis is necessary and/or sufficient to activate the MAPK/ERK pathway and its downstream targets, which would ultimately influence the expression of genes that support learning and memory.

The current study also provides the first evidence that a learning experience may increase local E_2 synthesis in the rodent hippocampus. The fact that the elevated E_2 levels in vehicle-treated mice observed 30 min after training was not found 60 or 120 min later, suggests a transient increase in hippocampal E_2 synthesis driven by object training. Although the high E_2 levels at 30 min cannot be definitively attributed to learning in the absence of samples collected at 0 min, the fact that hippocampal E_2 levels were suppressed 30 min after letrozole infusion into the DH infusion suggests that aromatase inhibition blocked an acute elevation due to learning or methodological parameters (e.g., infusion). The nature of such a putative change must therefore be tested in future experiments, and the role of DH E_2 synthesis, degradation, and conjugation vis-à-vis our quantification of dynamic DH E_2 levels are still unclear. Nevertheless, the 30-min data serve to confirm that DH infusion of letrozole suppresses local E_2 synthesis in the DH at a dose that impairs

recognition and spatial memory consolidation *in vivo*. The putative experience-induced increase in hippocampal E₂ levels observed here is consistent with avian studies reporting experience-induced changes in the male zebra finch forebrain after social interactions with female conspecifics or exposure to various auditory stimuli (Remage-Healey et al., 2008, 2010, 2011). Also, our finding that DH delivery of the aromatase inhibitor letrozole suppresses local E₂ synthesis is consistent with zebra finch studies showing suppression of acute changes in E₂ in the caudomedial nidopallium (NCM) of male zebra finches after retrodialysis of the aromatase inhibitor fadrozole (Remage-Healey et al., 2008, 2010). In the zebra finch forebrain, it has also been established that rapid changes in E₂ synthesis are dependent upon Ca²⁺ influx, much like classical neurotransmitters (Remage-Healey et al., 2011). Evidence from zebra finches also shows that blocking *de novo* E₂ synthesis in the NCM disrupts neuronal response properties, such as spike rate and burst firing activity, during the processing of auditory stimuli (Remage-Healey et al., 2010). Furthermore, suppressing *de novo* E₂ synthesis in the NCM changes firing rate and stimulus selectivity in the higher vocal center (HVC), a structure that receives indirect afferent input from the NCM. Thus, suppressing the ability of one region to synthesize E₂ impacts auditory processing in other downstream target regions that receive input from the NCM (Remage-Healey and Joshi, 2012). Although the present study did not measure the electrophysiological consequences of blocking local E₂ in female mice *in vivo*, the avian data and *ex vivo* rodent data suggest that such investigations may provide valuable additional insight into the mechanisms through which local E₂ synthesis influences cognitive function.

Finally, the present findings also suggest that local E₂ synthesis is not essential for exogenous E₂ to exert its beneficial effects on object recognition and spatial memory consolidation. These findings do not align with previously reported *in vitro* data showing that treatment of hippocampal cells with an aromatase inhibitor prevents exogenous E₂ from increasing mRNA and protein expression of synaptic plasticity markers PSD-95 and Arc (Chamniansawat and Chongthammakun, 2012), or of the presynaptic marker synaptophysin in hippocampal slice cultures (Kretz et al., 2004). There are several possible reasons for this discrepancy. First, the previous studies were conducted *in vitro* and the duration of exposure to the aromatase inhibitor was chronic (4–12 days), instead of the single acute infusion used in the current study. It is possible that chronic delivery in our study would have prevented exogenous E₂ from enhancing hippocampal memory consolidation. Second, the synaptic proteins measured in the *in vitro* studies discussed above may not be necessary for E₂ to enhance object recognition or spatial memory consolidation, as the necessity of these specific proteins for E₂-induced memory enhancement has not been directly evaluated in our behavioral paradigms. Third, the dose of exogenous E₂ used in the present study may have been too high for local inhibition to matter, as much lower doses of E₂ were used in the aforementioned *in vitro* studies (10⁻⁷–10⁻¹² M E₂ compared to 10 μg E₂ in this study). Finally, the timing of aromatase inhibitor and E₂ administration may have played an important role in our findings. That is, local E₂ synthesis may not have been sufficiently suppressed by the time exogenous E₂ was administered. Thus, exogenous E₂ may have activated the cell-signaling cascades (*i.e.*, ERK, PI3K, mTOR) necessary for E₂ to enhance memory (Boulware et al., 2013; Fortress et al., 2013) before letrozole had time to suppress local E₂ levels. Delaying the infusion of E₂, perhaps by 30–60 min, may have better allowed for an observation of interactions between local and exogenous E₂.

In conclusion, the present study provides novel insights into the role of hippocampally-synthesized E₂ in mediating hippocampal memory consolidation in female mice. This is the first study to demonstrate that dorsal hippocampal E₂ synthesis is necessary for the consolidation of object recognition and spatial memories in rodents. Moreover, our finding that E₂ levels are elevated in the DH within 30 min of object training suggests that rodents synthesize E₂ in the hippocampus in an experience-dependent manner. However, the mechanisms that may

regulate dorsal hippocampal E₂ synthesis remain unclear at the present time. Finally, our data also suggest that dorsal hippocampal E₂ synthesis is not necessary for exogenous DH infusion of E₂ to enhance object recognition and spatial memory consolidation, at least at the dose of E₂ used here. Collectively, these findings shed new light into the contribution of dorsal hippocampal E₂ synthesis to learning and memory, and build a foundation for future studies to investigate the molecular mechanisms through which *de novo* E₂ mediates memory in rodents.

Acknowledgements

This project was supported by the University of Wisconsin-Milwaukee, a UWM Foundation Research Growth Initiative award to K.M.F, a National Institutes of Health R00NS066179 and National Science Foundation IOS1354906 awards to L.R.H, and a UWM Advanced Opportunity Program Fellowship and Department of Psychology Summer Research Fellowship to J.J.T. We would also like to thank the Office of Undergraduate Research at the University of Wisconsin-Milwaukee for their support of J.S.S.

References

- Abdelgadir, S.E., Resko, J.A., Ojeda, S.R., Lephart, E.D., McPhaul, M.J., Roselli, C.E., 1994. Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. *Endocrinology* 135, 395–401.
- Azcoitia, I., Yague, J.G., Garcia-Segura, L.M., 2011. Estradiol synthesis within the human brain. *Neuroscience* 191, 139–147.
- Bailey, D.J., Ma, C., Soma, K.K., Saldanha, C.J., 2013. Inhibition of hippocampal aromatization impairs spatial memory performance in a male songbird. *Endocrinology* 154, 4707–4714.
- Baker, K.B., Kim, J.J., 2002. Effects of stress and hippocampal NMDA receptor antagonism on recognition memory in rats. *Learn. Mem.* 9, 58–65.
- Balthazart, J., Ball, G.F., 2006. Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 29, 241–249.
- Bean, L.A., Kumar, A., Rani, A., Guidi, M., Rosario, A.M., Cruz, P.E., Golde, T.E., Foster, T.C., 2015. Re-opening the critical window for estrogen therapy. *J. Neurosci.* 35, 16077–16093.
- Boulware, M.L., Heisler, J.D., Frick, K.M., 2013. The memory-enhancing effects of hippocampal estrogen receptor activation involve metabotropic glutamate receptor signaling. *J. Neurosci.* 33, 15184–15194.
- Chamniansawat, S., Chongthammakun, S., 2012. A priming role of local estrogen on exogenous estrogen-mediated synaptic plasticity and neuroprotection. *Exp. Mol. Med.* 44, 403–411.
- Chao, A., Schlinger, B.A., Remage-Healey, L., 2011. Combined liquid and solid-phase extraction improves quantification of brain estrogen content. *Front. Neuroanat.* 5, 57.
- Cohen, S.J., Munchow, A.H., Rios, L.M., Zhang, G., Asgeirsdottir, H.N., Stackman Jr., R.W., 2013. The rodent hippocampus is essential for nonspatial object memory. *Curr. Biol.* 23, 1685–1690.
- Daniel, J.M., 2006. Effects of oestrogen on cognition: what have we learned from basic research? *J. Neuroendocrinol.* 18, 787–795.
- Daniel, J.M., Witty, C.F., Rodgers, S.P., 2015. Long-term consequences of estrogens administered in midlife on female cognitive aging. *Horm. Behav.* 74, 77–85.
- Duarte-Guterman, P., Yagi, S., Chow, C., Galea, L.A., 2015. Hippocampal learning, memory, and neurogenesis: effects of sex and estrogens across the lifespan in adults. *Horm. Behav.* 74, 37–52.
- Ervin, K.S., Lymer, J.M., Matta, R., Clipperton-Allen, A.E., Kavaliers, M., Choleris, E., 2015. Estrogen involvement in social behavior in rodents: rapid and long-term actions. *Horm. Behav.* 74, 53–76.
- Fan, L., Zhao, Z., Orr, P.T., Chambers, C.H., Lewis, M.C., Frick, K.M., 2010. Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation. *J. Neurosci.* 30, 4390–4400.
- Fernandez, S.M., Lewis, M.C., Pechenino, A.S., Harburger, L.L., Orr, P.T., Gresack, J.E., Schafe, G.E., Frick, K.M., 2008. Estradiol-induced enhancement of object memory consolidation involves hippocampal ERK activation and membrane-bound estrogen receptors. *J. Neurosci.* 28, 8660–8667.
- Fester, L., Prange-Kiel, J., Zhou, L., Blittersdorf, B.V., Bohm, J., Jarry, H., Schumacher, M., Rune, G.M., 2012. Estrogen-regulated synaptogenesis in the hippocampus: sexual dimorphism *in vivo* but not *in vitro*. *J. Steroid Biochem. Mol. Biol.* 131, 24–29.
- Fokidis, H.B., Adomat, H.H., Kharmate, G., Hosseini-Beheshti, E., Guns, E.S., Soma, K.K., 2015. Regulation of local steroidogenesis in the brain and in prostate cancer: lessons learned from interdisciplinary collaboration. *Front. Neuroendocrinol.* 36, 108–129.
- Fortress, A.M., Frick, K.M., 2014. Epigenetic regulation of estrogen-dependent memory. *Front. Neuroendocrinol.* 35, 530–549.
- Fortress, A.M., Fan, L., Orr, P.T., Zhao, Z., Frick, K.M., 2013. Estradiol-induced object recognition memory consolidation is dependent on activation of mTOR signaling in dorsal hippocampus. *Learn. Mem.* 20, 147–155.
- Fortress, A.M., Kim, J., Poole, R.L., Gould, T.J., Frick, K.M., 2014. 17β-estradiol regulates histone alterations associated with memory consolidation and increases Bdnf promoter acetylation in middle-aged female mice. *Learn. Mem.* 21, 457–467.

- Foster, T.C., 2012. Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging. *Hippocampus* 22, 656–669.
- Foster, T.C., Rani, A., Kumar, A., Cui, L., Semple-Rowland, S.L., 2008. Viral vector-mediated delivery of estrogen receptor-alpha to the hippocampus improves spatial learning in estrogen receptor-alpha knockout mice. *Mol. Ther.* 16, 1587–1593.
- Frick, K.M., 2012. Building a better hormone therapy? How understanding the rapid effects of sex steroid hormones could lead to new therapeutics for age-related memory decline. *Behav. Neurosci.* 126, 29–53.
- Frick, K.M., 2015. Molecular mechanisms underlying the memory-enhancing effects of estradiol. *Horm. Behav.* 74, 4–18.
- Frick, K.M., Gresack, J.E., 2003. Sex differences in the behavioral response to spatial and object novelty in adult C57BL/6 mice. *Behav. Neurosci.* 117, 1283–1291.
- Frick, K.M., Kim, J., Tuscher, J.J., Fortress, A.M., 2015. Sex steroid hormones matter for learning and memory: estrogenic regulation of hippocampal function in male and female rodents. *Learn. Mem.* 22, 472–493.
- Frye, C.A., Duffy, C.K., Walf, A.A., 2007. Estrogens and progestins enhance spatial learning of intact and ovariectomized rats in the object placement task. *Neurobiol. Learn. Mem.* 88, 208–216.
- Graham, B.M., Milad, M.R., 2014. Inhibition of estradiol synthesis impairs fear extinction in male rats. *Learn. Mem.* 21, 347–350.
- Heimovics, S.A., Prior, N.H., Ma, C., Soma, K.K., 2016. Rapid effects of an aggressive interaction on dehydroepiandrosterone, testosterone and estradiol levels in the male song sparrow brain: a seasonal comparison. *J. Neuroendocrinol.* 28. <http://dx.doi.org/10.1111/jne.12345>.
- Hojo, Y., Hattori, T.A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H.T., Mukai, H., Morrison, J.H., Janssen, W.G., Kominami, S., Harada, N., Kimoto, T., Kawato, S., 2004. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc. Natl. Acad. Sci. U. S. A.* 101, 865–870.
- Hojo, Y., Higo, S., Kawato, S., Hatanaka, Y., Ooishi, Y., Murakami, G., Ishii, H., Komatsuzaki, Y., Ogiue-Ikeda, M., Mukai, H., Kimoto, T., 2011. Hippocampal synthesis of sex steroids and corticosteroids: essential for modulation of synaptic plasticity. *Front. Endocrinol. (Lausanne)* 2, 43.
- Ivanova, T., Beyer, C., 2000. Ontogenetic expression and sex differences of aromatase and estrogen receptor-alpha/beta mRNA in the mouse hippocampus. *Cell Tissue Res.* 300, 231–237.
- Kenealy, B.P., Kapoor, A., Guerriero, K.A., Keen, K.L., Garcia, J.P., Kurian, J.R., Ziegler, T.E., Terasawa, E., 2013. Neuroestradiol in the hypothalamus contributes to the regulation of gonadotropin releasing hormone release. *J. Neurosci.* 33, 19051–19059.
- Kim, J., Szinte, J.S., Boulware, M.I., Frick, K.M., 2016. 17 β -estradiol and agonism of G-protein Coupled Estrogen Receptor (GPER) enhance hippocampal memory via different cell-signaling mechanisms. *J. Neurosci.* 36, 3309–3321.
- Korol, D.L., Pisani, S.L., 2015. Estrogens and cognition: friends or foes?: an evaluation of the opposing effects of estrogens on learning and memory. *Horm. Behav.* 74, 105–115.
- Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., Prange-Kiel, J., Naumann, T., Jarry, H., Frotscher, M., Rune, G.M., 2004. Hippocampal synapses depend on hippocampal estrogen synthesis. *J. Neurosci.* 24, 5913–5921.
- Lephart, E.D., 1996. A review of brain aromatase cytochrome P450. *Brain Res. Brain Res. Rev.* 22, 1–26.
- Luine, V.N., 2014. Estradiol and cognitive function: past, present and future. *Horm. Behav.* 66, 602–618.
- Luine, V.N., Frankfurt, M., 2012. Estrogens facilitate memory processing through membrane mediated mechanisms and alterations in spine density. *Front. Neuroendocrinol.* 33, 388–402.
- McGaugh, J.L., 1989. Dissociating learning and performance: drug and hormone enhancement of memory storage. *Brain Res. Bull.* 23, 339–345.
- Overk, C.R., Perez, S.E., Ma, C., Taves, M.D., Soma, K.K., Mufson, E.J., 2013. Sex steroid levels and AD-like pathology in 3xTgAD mice. *J. Neuroendocrinol.* 25, 131–144.
- Packard, M.G., Teather, L.A., 1997. Intra-hippocampal estradiol infusion enhances memory in ovariectomized rats. *Neuroreport* 8, 3009–3013.
- Pereira, L.M., Bastos, C.P., de Souza, J.M., Ribeiro, F.M., Pereira, G.S., 2014. Estradiol enhances object recognition memory in Swiss female mice by activating hippocampal estrogen receptor alpha. *Neurobiol. Learn. Mem.* 114, 1–9.
- Prange-Kiel, J., Wehrenberg, U., Jarry, H., Rune, G.M., 2003. Para/autocrine regulation of estrogen receptors in hippocampal neurons. *Hippocampus* 13, 226–234.
- Remage-Healey, L., Joshi, N.R., 2012. Changing neuroestrogens within the auditory forebrain rapidly transform stimulus selectivity in a downstream sensorimotor nucleus. *J. Neurosci.* 32, 8231–8241.
- Remage-Healey, L., Maidment, N.T., Schlinger, B.A., 2008. Forebrain steroid levels fluctuate rapidly during social interactions. *Nat. Neurosci.* 11, 1327–1334.
- Remage-Healey, L., Coleman, M.J., Oyama, R.K., Schlinger, B.A., 2010. Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. *Proc. Natl. Acad. Sci. U. S. A.* 107, 3852–3857.
- Remage-Healey, L., Dong, S., Maidment, N.T., Schlinger, B.A., 2011. Presynaptic control of rapid estrogen fluctuations in the songbird auditory forebrain. *J. Neurosci.* 31, 10034–10038.
- Remage-Healey, L., Dong, S.M., Chao, A., Schlinger, B.A., 2012. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *J. Neurophysiol.* 107, 1621–1631.
- Rensel, M.A., Salwiczek, L., Roth, J., Schlinger, B.A., 2013. Context-specific effects of estradiol on spatial learning and memory in the zebra finch. *Neurobiol. Learn. Mem.* 100, 41–47.
- Roselli, C.E., Resko, J.A., 1989. Testosterone regulates aromatase activity in discrete brain areas of male rhesus macaques. *Biol. Reprod.* 40, 929–934.
- Roselli, C.E., Horton, L.E., Resko, J.A., 1985. Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology* 117, 2471–2477.
- Saldanha, C.J., Remage-Healey, L., Schlinger, B.A., 2011. Synaptocrine signaling: steroid synthesis and action at the synapse. *Endocr. Rev.* 32, 532–549.
- Sanghera, M.K., Simpson, E.R., McPhaul, M.J., Kozlowski, G., Conley, A.J., Lephart, E.D., 1991. Immunocytochemical distribution of aromatase cytochrome P450 in the rat brain using peptide-generated polyclonal antibodies. *Endocrinology* 129, 2834–2844.
- Srivastava, D.P., Evans, P.D., 2013. G-protein oestrogen receptor 1: trials and tribulations of a membrane oestrogen receptor. *J. Neuroendocrinol.* 25, 1219–1230.
- Srivastava, D.P., Woolfrey, K.M., Jones, K.A., Shum, C.Y., Lash, L.L., Swanson, G.T., Penzes, P., 2008. Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14650–14655.
- Vierk, R., Glassmeier, G., Zhou, L., Brandt, N., Fester, L., Dudzinski, D., Wilkars, W., Bender, R.A., Lewerenz, M., Gloger, S., Graser, L., Schwarz, J., Rune, G.M., 2012. Aromatase inhibition abolishes LTP generation in female but not in male mice. *J. Neurosci.* 32, 8116–8126.
- Vierk, R., Brandt, N., Rune, G.M., 2014. Hippocampal estradiol synthesis and its significance for hippocampal synaptic stability in male and female animals. *Neuroscience* 274, 24–32.
- Vockel, A., Prove, E., Balthazart, J., 1990. Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. *Brain Res.* 511, 291–302.
- Walf, A.A., Rhodes, M.E., Frye, C.A., 2006. Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. *Neurobiol. Learn. Mem.* 86, 35–46.
- Wehrenberg, U., Prange-Kiel, J., Rune, G.M., 2001. Steroidogenic factor-1 expression in marmoset and rat hippocampus: co-localization with StAR and aromatase. *J. Neurochem.* 76, 1879–1886.
- Witty, C.F., Foster, T.C., Semple-Rowland, S.L., Daniel, J.M., 2012. Increasing hippocampal estrogen receptor alpha levels via viral vectors increases MAP kinase activation and enhances memory in aging rats in the absence of ovarian estrogens. *PLoS One* 7, e51385.
- Woolley, C.S., 2007. Acute effects of estrogen on neuronal physiology. *Annu. Rev. Pharmacol. Toxicol.* 47, 657–680.
- Yague, J.G., Azcoitia, I., DeFelipe, J., Garcia-Segura, L.M., Munoz, A., 2010. Aromatase expression in the normal and epileptic human hippocampus. *Brain Res.* 1315, 41–52.
- Zeidan, M.A., Igoe, S.A., Linnman, C., Vitalo, A., Levine, J.B., Klisanski, A., Goldstein, J.M., Milad, M.R., 2011. Estradiol modulates medial prefrontal cortex and amygdala activity during fear extinction in women and female rats. *Biol. Psychiatry* 70, 920–927.
- Zhao, Z., Fan, L., Frick, K.M., 2010. Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation. *Proc. Natl. Acad. Sci. U. S. A.* 107, 5605–5610.
- Zhao, Z., Fan, L., Fortress, A.M., Boulware, M.I., Frick, K.M., 2012. Hippocampal histone acetylation regulates object recognition and the estradiol-induced enhancement of object recognition. *J. Neurosci.* 32, 2344–2351.
- Zhou, L., Fester, L., von Blittersdorff, B., Hassu, B., Nogens, H., Prange-Kiel, J., Jarry, H., Wegscheider, K., Rune, G.M., 2010. Aromatase inhibitors induce spine synapse loss in the hippocampus of ovariectomized mice. *Endocrinology* 151, 1153–1160.