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

Jennifer J. Tuscher<sup>1</sup>, Julia S. Szinte<sup>1</sup>, Joseph R. Starrett<sup>2</sup>, Amanda A. Krentzel<sup>2</sup>, Ashley M. Fortress<sup>1</sup>, Luke Remage-Healey<sup>2</sup>, and Karyn M. Frick<sup>1,\*</sup>

<sup>1</sup>Department of Psychology, University of Wisconsin-Milwaukee, Milwaukee WI 53211

<sup>2</sup>Department of Psychological and Brain Sciences and Neuroscience and Behavior Program, Center for Neuroendocrine Studies, University of Massachusetts Amherst, Amherst, MA

### Abstract

The potent estrogen 17 $\beta$ -Estradiol (E<sub>2</sub>) plays a critical role in mediating hippocampal function, yet the precise mechanisms through which E<sub>2</sub> enhances hippocampal memory remain unclear. In young adult female rodents, the beneficial effects of E<sub>2</sub> on memory are generally attributed to ovarian-synthesized E<sub>2</sub>. However, E<sub>2</sub> 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<sub>2</sub> synthesis has been demonstrated in rodent hippocampal cultures, little is known about the functional role of local E<sub>2</sub> synthesis in mediating hippocampal memory function. Therefore, the present study examined the role of hippocampal E<sub>2</sub> synthesis in hippocampal memory consolidation. Using bilateral dorsal hippocampal infusions of the aromatase inhibitor letrozole, we first found that blockade of dorsal hippocampal E<sub>2</sub> synthesis impaired hippocampal memory consolidation. We next found that elevated levels of E<sub>2</sub> in 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<sub>2</sub> synthesis. Finally, aromatase inhibition did not prevent exogenous E<sub>2</sub> from enhancing hippocampal memory consolidation, indicating that hippocampal E<sub>2</sub> synthesis is not necessary for exogenous E<sub>2</sub> to enhance hippocampal memory. Combined, these data are consistent with the hypothesis that hippocampally-synthesized E<sub>2</sub> is necessary for hippocampus-dependent memory consolidation in rodents.

### Keywords

aromatase; letrozole; estradiol; object recognition

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\*Corresponding author: Karyn M. Frick, Ph.D., Department of Psychology, University of Wisconsin-Milwaukee, 2441 E. Hartford Ave., Milwaukee, WI 53211, Phone: 414-229-6615, Fax: 414-229-5219, frickk@uwm.edu.

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## Introduction

In recent years, the sex steroid hormone  $17\beta$ -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 suggests 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 demonstrates that  $E_2$  can be rapidly synthesized in the cortex during behavioral experiences including exposure to female conspecifics or conspecific song (Ramage-Healey et al., 2010; Ramage-Healey et al., 2012; Ramage-Healey et al., 2008). 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 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 post-synaptic terminals (Hojo et al., 2004; Hojo et al., 2011; Lephart, 1996). The presence of aromatase in hippocampal synaptic terminals suggests the potential for local  $E_2$  synthesis to rapidly mediate hippocampal plasticity and memory consolidation.

*De novo*  $E_2$  synthesis was first demonstrated in adult rodent hippocampal neurons *in vitro* (Prange-Kiel et al., 2003), and subsequent work has shown that levels of  $E_2$  are substantially higher in the hippocampus than in circulating plasma in both male and female rats (Hojo et al., 2004). *In vitro*, hippocampal  $E_2$  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  $E_2$  levels is accompanied by decreased spine synapse density and pre-synaptic bouton number in rats and mice, suggesting that the

loss of local E<sub>2</sub> 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* E<sub>2</sub> regulates hippocampal synaptic plasticity, little is known about the role of hippocampal E<sub>2</sub> 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 E<sub>2</sub> synthesis in memory consolidation. Therefore, the present study sought to address the questions of whether hippocampally-synthesized E<sub>2</sub> is essential for either hippocampus-dependent memory consolidation or the memory enhancing effects of exogenous E<sub>2</sub>. Using bilateral dorsal hippocampal infusions of letrozole, we found that hippocampal E<sub>2</sub> 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 E<sub>2</sub> synthesis after object training. Finally, our data suggest that hippocampal E<sub>2</sub> synthesis is not necessary for exogenous E<sub>2</sub> to enhance hippocampal memory consolidation, at least at the doses used here. To our knowledge, this is the first demonstration that hippocampal E<sub>2</sub> 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 4 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 E<sub>2</sub> 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; Zhao et al., 2012; Boulware et al., 2013; Fortress et al., 2013). Here, we used triple cannulae to administer E<sub>2</sub> 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., 2012; Zhao et al., 2010b), resulting in letrozole doses of 0.005, 0.025, and 0.05 µg/hemisphere. For experiments also involving ICV infusion of E<sub>2</sub>, cyclodextrin-encapsulated E<sub>2</sub> (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 E<sub>2</sub> solution. ICV infusions were conducted at the same rate as DH infusions (0.5 µl/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<sub>2</sub> 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; Fortress et al., 2014; Pereira et al., 2014; Zhao et al., 2012; Zhao et al., 2010a) 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 x 60 cm x 47 cm) for 5 min/day. For the OR task, mice were then returned to the arena and allowed to accumulate 30 sec 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 hours later by measuring the amount of time spent with the novel and familiar object. A 24-hour 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-hour delay was used to measure whether letrozole blocked the memory-enhancing effects of E<sub>2</sub> because vehicle-infused females show impaired OR memory consolidation, whereas E<sub>2</sub>-infused females show intact OR memory consolidation, at this time point (Fortress et al., 2013, Boulware et al., 2013). Moreover, E<sub>2</sub>-infused females spend significantly more time with the novel object than vehicle-infused females 48 hours after infusion (Kim, 2016). Because most mice inherently prefer novelty, those that remember the training objects spend more time than chance (15 sec) exploring the novel object during testing. Chance was set at 15 sec 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-

hour delay was used to determine whether letrozole could block the memory-enhancing effects of E<sub>2</sub> on OP memory consolidation. At this time point, E<sub>2</sub>-infused, but not vehicle-infused, mice spend more time than chance with the moved object and E<sub>2</sub>-infused mice spend significantly more time with the moved object than vehicle-infused mice (Boulware et al., 2013; Kim, 2016).

### E<sub>2</sub> Measurement

E<sub>2</sub> 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 ddH<sub>2</sub>O 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<sub>2</sub> 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<sub>2</sub> (cross-reactivity: 14% for E<sub>2</sub>-3-glucuronide; 12% for estrone; 1% for E<sub>2</sub>-17-glucuronide; < 0.10% for other major steroids including testosterone; Cayman chemical). Data are expressed in pg E<sub>2</sub> 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 seconds; 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* < 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<sub>2</sub> synthesis immediately after training impairs object recognition and object placement memory consolidation

To determine if hippocampal E<sub>2</sub> 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 µg/hemisphere; n=9–13/group). Object recognition memory was tested 24 hours 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., 2010b). 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 µg/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 testing ( $F_{(3,40)} = 1.02$ ,  $p = 0.39$ ; Vehicle =  $649.7 \pm 84.61$ ; 0.005 µg =  $821.4 \pm 87.07$ ; 0.025 µg =  $669.7 \pm 46.51$ ; 0.05 µg =  $714.4 \pm 57.07$ ).

Next, to determine if local E<sub>2</sub> 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 =  $569.8 \pm 61.30$ ; 0.005 µg =  $408.8 \pm 43.65$ ; 0.025 µg =  $442.3 \pm 50.43$ ; 0.05 µg =  $564.0 \pm 63.16$ ). Together, these data provide the first evidence that local E<sub>2</sub> 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<sub>2</sub> treatment are restricted to between 1–3 hours 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<sub>2</sub> delayed 3 hours 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-hour 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 hours later in OR, or 4 hours 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 µg/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<sub>2</sub> levels 30 minutes after object training

We next measured levels of E<sub>2</sub> in the DH at various points after object training to determine: 1) the extent to which letrozole reduced E<sub>2</sub> levels within the DH, and 2) if letrozole could block a learning-induced increase in DH E<sub>2</sub> 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 µg/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: 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> levels were similar in absolute quantity to those reported in other studies of hippocampal and brain DH E<sub>2</sub> 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<sub>2</sub> content between regions or time periods. These data suggest that letrozole suppresses an increase in DH E<sub>2</sub> levels 30 min after infusion at a dose shown to impair OR and OP memory consolidation *in vivo*.



## Dorsal hippocampal E<sub>2</sub> synthesis is not necessary for exogenous E<sub>2</sub> to enhance hippocampal memory consolidation

Previous work from our laboratory has demonstrated that 5 µg E<sub>2</sub> infused bilaterally into the dorsal hippocampus or 10 µg 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., 2010b). However, these studies did not take into account the potential contribution of local E<sub>2</sub> synthesis to the observed memory enhancements. In the hypothalamus of ovariectomized monkeys, exogenous estradiol benzoate infusion increases the release of local E<sub>2</sub> (Kenealy et al., 2013), suggesting potentially important interactions between exogenous and local E<sub>2</sub>. To determine if local and exogenous E<sub>2</sub> may synergistically facilitate memory consolidation, we next investigated the role of local E<sub>2</sub> synthesis in the memory-enhancing effects produced by exogenous E<sub>2</sub>. 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 µg/hemisphere), followed immediately by an ICV infusion of HBC vehicle or 10 µg E<sub>2</sub>. OR and OP memory were then tested 48 or 24 hours later, respectively, because exogenous E<sub>2</sub> 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<sub>2</sub>, spent more time than chance with the novel object 48 h after OR training (Veh/E<sub>2</sub>:  $t_{(11)} = 4.75$ ,  $p = 0.0006$ ; Let/E<sub>2</sub>:  $t_{(13)} = 3.40$ ,  $p = 0.005$ ; Fig. 3A), and with the moved object 24 h after OP training (Veh/E<sub>2</sub>:  $t_{(11)} = 3.20$ ,  $p = 0.01$ ; Let/E<sub>2</sub>:  $t_{(11)} = 3.73$ ,  $p = 0.003$ ; 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<sub>2</sub> mice spent significantly more time with the novel object than Veh/Veh or Let/Veh mice ( $p < 0.05$ ; Fig. 3A). Let/E<sub>2</sub> 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<sub>2</sub> mice spent significantly more time with the moved object than Veh/Veh mice ( $p < 0.05$ ; Fig. 3B). Veh/E<sub>2</sub> mice also spent significantly more time with the moved object relative to Veh/Veh or Let/Veh mice. 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 =  $746.1 \pm 67.01$ ; Let/Veh =  $748.8 \pm 69.77$ ; Let/E<sub>2</sub> =  $785.0 \pm 78.24$ ; Veh/E<sub>2</sub> =  $837.2 \pm 67.40$ ). 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<sub>2</sub> mice ( $F_{(3,43)} = 4.33$ ,  $p = 0.01$ ; Veh/Veh =  $649.4 \pm 65.93$ ; Let/Veh =  $588.0 \pm 62.02$ ; Let/E<sub>2</sub> =  $767.5 \pm 72.10$ ; Veh/E<sub>2</sub> =  $901.1 \pm 55.86$ ). 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 that inhibition of local E<sub>2</sub> synthesis in the DH does not prevent exogenous E<sub>2</sub> from enhancing object recognition or spatial memory consolidation, and suggest that hippocampal *de novo* E<sub>2</sub> synthesis is not necessary for exogenous E<sub>2</sub> to enhance memory, at least at the 10 µg 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<sub>2</sub> synthesis immediately after training is necessary for object recognition and spatial memory consolidation in young ovariectomized mice. Next, we found that DH E<sub>2</sub> 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 at a dose that impairs recognition and spatial memory consolidation *in vivo*. Together, these data suggest E<sub>2</sub> 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<sub>2</sub> synthesis is not necessary for exogenous E<sub>2</sub> to enhance object recognition and spatial memory consolidation, at least at the 10 µg dose of E<sub>2</sub> we used. Collectively, these findings are the first to demonstrate that *de novo* E<sub>2</sub> synthesis in the dorsal hippocampus is necessary for the consolidation of hippocampus-dependent memories in female rodents. Further, these experiments provide novel insights into the role of dorsal hippocampal E<sub>2</sub> synthesis in the memory-enhancing effects of exogenous E<sub>2</sub> 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<sub>2</sub> synthesis regulates the expression of synaptic proteins, synaptic spine density, and LTP (Fester et al., 2012; Kretz et al., 2004; Vierk et al., 2014; Vierk et al., 2012). Our present findings are also consistent with recently published *in vivo* studies showing that *de novo* E<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> synthesis in this brain region may play a role. Other recent data supports the importance of local E<sub>2</sub> synthesis by showing that lentiviral delivery of estrogen receptor  $\alpha$  (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<sub>2</sub> (Foster et al., 2008; Witty et al., 2012). These data suggest that endogenously synthesized E<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> treatment drives p42 ERK activation (Boulware et al., 2013; Fernandez et al., 2008; Srivastava et al., 2008), the mechanisms through which *de novo* E<sub>2</sub> facilitates memory consolidation in rodents are currently unknown. Therefore, future studies should address whether local E<sub>2</sub> 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<sub>2</sub> synthesis in the rodent hippocampus. The fact that the elevated E<sub>2</sub> levels in vehicle-treated mice observed 30 min after training was not observed at 60 or 120 min later, suggests a transient increase in hippocampal E<sub>2</sub> synthesis driven by object training. Although the high E<sub>2</sub> levels at 30 min cannot be definitively attributed to learning in the absence of samples collected at 0 min, the fact that hippocampal E<sub>2</sub> levels were suppressed following 30 min of 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<sub>2</sub> synthesis, degradation, and conjugation vis-à-vis our quantification of dynamic DH E<sub>2</sub> levels are still unclear. Nevertheless, the 30-min data serve to confirm that DH infusion of letrozole suppresses local E<sub>2</sub> synthesis in the DH at a dose that impairs recognition and spatial memory consolidation *in vivo*. The putative experience-induced increase in hippocampal E<sub>2</sub> 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 (Ramage-Healey et al., 2010; Ramage-Healey et al., 2011; Ramage-Healey et al., 2008). Also, our finding that DH delivery of the aromatase inhibitor letrozole suppresses local E<sub>2</sub> synthesis is consistent with zebra finch studies showing suppression of acute changes in E<sub>2</sub> in the caudomedial nidopallium (NCM) of male zebra finches after retrodialysis of the aromatase inhibitor fadrozole (Ramage-Healey et al., 2010; Ramage-Healey et al., 2008). In the zebra finch forebrain, it has also been established that rapid changes in E<sub>2</sub> synthesis are dependent upon Ca<sup>2+</sup> influx, much like classical neurotransmitters (Ramage-Healey et al., 2011). Evidence from zebra finches also shows that blocking *de novo* E<sub>2</sub> synthesis in the NCM disrupts neuronal response properties, such as spike rate and burst firing activity, during the processing of auditory stimuli (Ramage-Healey et al., 2010). Furthermore, suppressing *de novo* E<sub>2</sub> 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 E<sub>2</sub> impacts auditory processing in other downstream target regions that receive input from the NCM (Ramage-Healey and Joshi, 2012). Although the present study did not measure the electrophysiological consequences of blocking local E<sub>2</sub> 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 E<sub>2</sub> synthesis influences cognitive function.

Finally, the present findings also suggest that local E<sub>2</sub> synthesis is not essential for exogenous E<sub>2</sub> 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 E<sub>2</sub> 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 E<sub>2</sub> from enhancing hippocampal memory consolidation. Second, the synaptic proteins measured in the *in vitro* studies discussed above may not be necessary for E<sub>2</sub> to enhance object recognition or spatial memory consolidation, as the necessity of these specific proteins for E<sub>2</sub>-induced memory enhancement has not been directly evaluated in our behavioral paradigms. Third, the dose of exogenous E<sub>2</sub> used in the present study may have been too high for local inhibition to matter, as much lower doses of E<sub>2</sub> were used in the aforementioned *in vitro* studies (10<sup>-7</sup> – 10<sup>-12</sup> M E<sub>2</sub> compared to 10 μg E<sub>2</sub> in this study). Finally, the timing of aromatase inhibitor and E<sub>2</sub> administration may have played an important role in our findings. That is, local E<sub>2</sub> synthesis may not have been sufficiently suppressed by the time exogenous E<sub>2</sub> was administered. Thus, exogenous E<sub>2</sub> may have activated the cell-signaling cascades (i.e., ERK, PI3K, mTOR) necessary for E<sub>2</sub> to enhance memory (Boulware et al., 2013; Fortress et al., 2013) before letrozole had time to suppress local E<sub>2</sub> levels. Delaying the infusion of E<sub>2</sub>, perhaps by 30–60 min, may have better allowed for an observation of interactions between local and exogenous E<sub>2</sub>.

In conclusion, the present study provides novel insights into the role of hippocampally-synthesized E<sub>2</sub> in mediating hippocampal memory consolidation in female mice. This is the first study to demonstrate that dorsal hippocampal E<sub>2</sub> synthesis is necessary for the consolidation of object recognition and spatial memories in rodents. Moreover, our finding that E<sub>2</sub> levels are elevated in the DH within 30 min of object training suggests that rodents synthesize E<sub>2</sub> in the hippocampus in an experience-dependent manner. However, the mechanisms that may regulate dorsal hippocampal E<sub>2</sub> synthesis remain unclear at the present time. Finally, our data also suggest that dorsal hippocampal E<sub>2</sub> synthesis is not necessary for exogenous DH infusion of E<sub>2</sub> to enhance object recognition and spatial memory consolidation, at least at the dose of E<sub>2</sub> used here. Collectively, these findings shed new light into the contribution of dorsal hippocampal E<sub>2</sub> synthesis to learning and memory, and build a foundation for future studies to investigate the molecular mechanisms through which *de novo* E<sub>2</sub> mediates memory in rodents.

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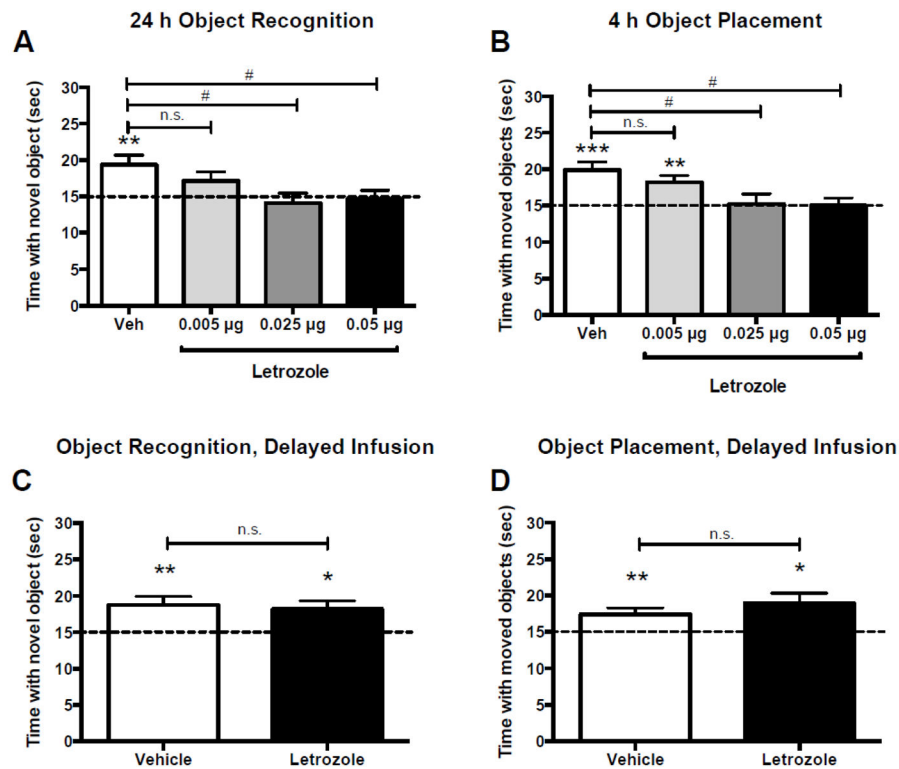
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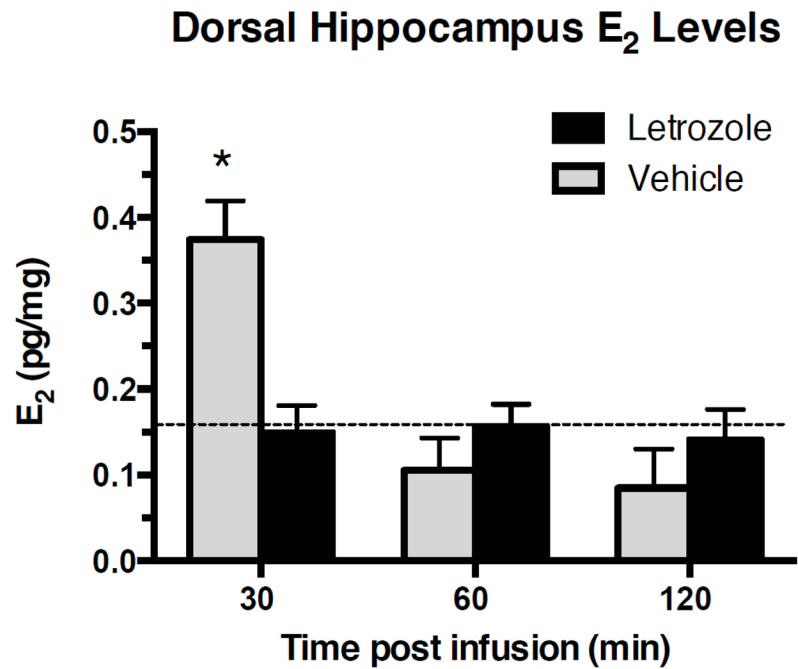


### Highlights

- Hippocampal estradiol synthesis is necessary for object recognition memory
- Letrozole blocks elevated estradiol levels observed after object learning
- Hippocampal estradiol is not necessary for exogenous estradiol to enhance memory

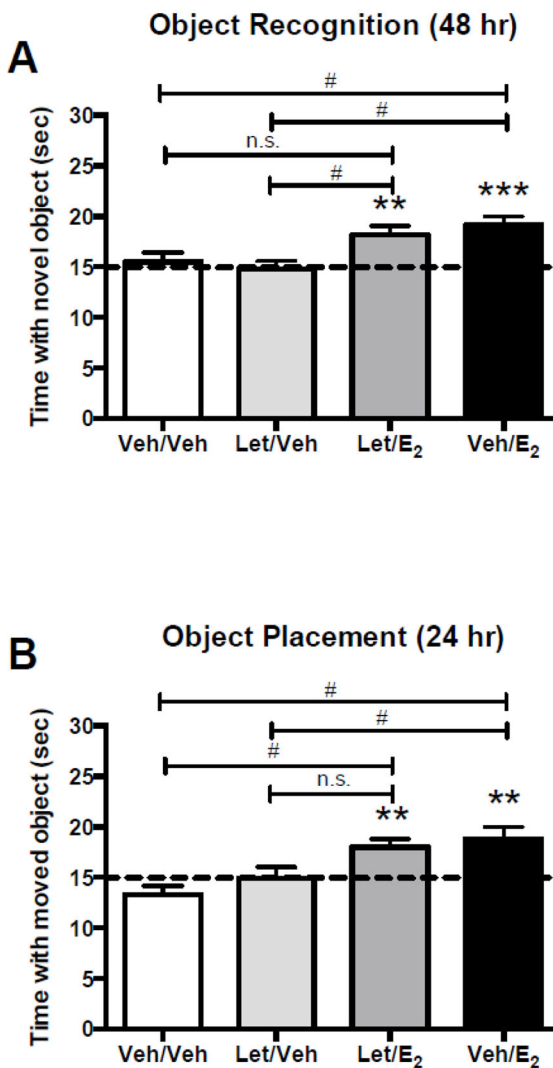


**Figure 1. Letrozole impairs object recognition and object placement memory consolidation**  
**(A)** Mice receiving bilateral dorsal hippocampal infusion of vehicle ( $n=13$ ), but not  $0.005 \mu\text{g}$  ( $n=9$ ),  $0.025 \mu\text{g}$  ( $n=10$ ), or  $0.05 \mu\text{g}$  ( $n=12$ ) letrozole, immediately after training spent significantly more time with the novel object 24 h after training relative to chance (dashed line at 15 sec). Vehicle-infused mice also spent significantly more time with the novel object than mice infused with  $0.025$  or  $0.05 \mu\text{g}$  letrozole. These data suggest that the  $0.025$  and  $0.05 \mu\text{g}$  doses of letrozole blocked object recognition memory consolidation. **(B)** Mice receiving bilateral dorsal hippocampal infusions of vehicle ( $n=12$ ) or  $0.005 \mu\text{g}$  ( $n=9$ ) letrozole, but not  $0.025 \mu\text{g}$  ( $n=9$ ) or  $0.05 \mu\text{g}$  ( $n=9$ ) 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  $0.05 \mu\text{g}$  letrozole, suggesting that the  $0.025$  and  $0.05 \mu\text{g}$  doses of letrozole blocked also spatial memory consolidation. This effect was limited to within the first 2–3 hours after training, as mice receiving a bilateral infusion of vehicle ( $n=11$ ) or  $0.025 \mu\text{g}$  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



**Figure 2. Letrozole reduces hippocampal E<sub>2</sub> 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<sub>2</sub> levels than vehicle-treated mice 30 min after infusion ( $*p < 0.05$ ; vehicle  $n=3$ ; letrozole  $n=6$ ). Dorsal hippocampal E<sub>2</sub> 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 estradiol concentration as reported by EIA for control wells; all estradiol levels above this concentration are validated as detectable levels on the EIA.



**Figure 3. Dorsal hippocampal E<sub>2</sub> synthesis is not necessary for exogenous DH infusion of E<sub>2</sub> to enhance object recognition or spatial memory consolidation**  
 Mice receiving infusions of vehicle or 0.025  $\mu$ 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  $\mu$ g letrozole into the dorsal hippocampus, followed by ICV infusion of E<sub>2</sub> (Veh/E<sub>2</sub>, Let/E<sub>2</sub>) spent significantly more time than chance (dashed line at 15 sec, \* $p < 0.05$ ) 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<sub>2</sub> 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<sub>2</sub> group (n=14) spent more time with the novel object relative to the Let/Veh control group. For the OP task, the Veh/E<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> synthesis in the dorsal hippocampus is not sufficient to prevent exogenous E<sub>2</sub> 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

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