Neurobiology of Learning and Memory 130 (2016) 83–92

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

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

Hippocampal cytosolic estrogen receptors regulate fear generalization in females

Joseph F. Lynch III, Patrick Winiecki, Tyler Vanderhoof, David C. Riccio, Aaron M. Jasnow *

Department of Psychological Sciences, Kent State University, Kent, OH 44242, United States

ARTICLE INFO

Article history:
Received 7 October 2015
Revised 27 December 2015
Accepted 25 January 2016
Available online 3 February 2016

Keywords:
Contextual memory precision
Fear generalization
Estradiol
Hippocampus
Contextual fear
Memory retrieval

ABSTRACT

Generalization of fear responses is a symptom of many anxiety disorders and we have previously demonstrated that female rats generalize fear to a neutral context at a faster rate compared to males. This effect is due in part, to activation of ER and modulation of memory retrieval mechanisms resulting in fear generalization. Given that the effects of estradiol on fear generalization required approximately 24 h, our data suggested possible genomic actions on fear generalization. To determine whether these actions were due to cytosolic versus membrane bound receptors, female rats were given infusions of ICI 182,780, a cytosolic estrogen receptor antagonist, into the lateral ventricle or dorsal hippocampus simultaneously with estradiol treatment or with an ER agonist (DPN). Infusions of ICI into the lateral ventricle or the dorsal hippocampus blocked fear generalization induced by peripheral or central treatment with estradiol or DPN, suggesting that estradiol acts through cytosolic ERβ receptors. In further support of these findings, intracerebroventricular or intra-hippocampal infusions of bovine serum conjugated estradiol (E2-BSA), activating membrane-bound estrogen receptors only, did not induce fear generalization. Moreover, rats receiving intra-hippocampal infusions of the ERK/MAPK inhibitor, U0126, continued to display estradiol-induced generalization, again suggesting that membrane-bound estrogen receptors do not contribute to fear generalization. Overall, these data suggest that estradiol-induced enhancements in fear generalization are mediated through activation of cytosolic/nuclear ER within the dorsal hippocampus. This region seems to be an important locus for the effects of estradiol on fear generalization although additional neuroanatomical regions have yet to be identified.

1. Introduction

Considerable research indicates that contextual fear generalization—the inability to discriminate between different contexts and, thus, recalling a fear memory in neutral contexts—increases over time (For review, see Jasnow, Cullen, & Riccio, 2012). Fear generalization can also be interpreted as a loss of memory precision for contextual cues; thus, mechanisms contributing to the establishment, maintenance, and recall of contextual memory are implicated in this process. Despite the importance of fear generalization as a fundamental component underlying many anxiety disorders, including PTSD (Brewin, 2001; Grillon & Morgan, 1999; Jovanovic et al., 2009), we do not fully understand how this phenomenon occurs. Moreover, females are 60% more likely than males to be diagnosed with an anxiety disorder such as PTSD (Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Kessler et al., 1994; Wang et al., 2005), and the exact cause of this sex difference remains unknown. Estrogens influence fear and anxiety behavior in rodents and humans (Díaz-Véliz, Alarcón, Espinoza, Dussaubat, & Mora, 1997; Frye, Petralia, & Rhodes, 2000; Frye & Walf, 2004; Morgan & Pfaff, 2001, 2002; Morgan, Schulkin, & Pfaff, 2004; Nofrey, Ben-Shahar, & Brake, 2008; Toufexis, Myers, Bowser, & Davis, 2007; Zuluaga et al., 2005), yet the contribution of estrogens to fear generalization has only recently been examined (Lynch, Cullen, Jasnow, & Riccio, 2013; Lynch et al., 2014).

Our previous research demonstrated that female rats displayed a faster rate of fear generalization to a neutral context after passive avoidance training compared to male rats; an effect driven, in part, by estradiol (Lynch et al., 2013). These findings were the first to show enhanced context fear generalization driven by estradiol and suggested a novel modulatory role for the steroid hormone on generalization mechanisms. Additionally, we utilized injections of estradiol during the learning and memory process and found that systemic injections of 17β-estradiol increased fear generalization only at a time point thought to effect memory retrieval (Lynch

* Corresponding author at: 600 Hilltop Drive, 230 Kent Hall, Department of Psychological Sciences, Kent State University, Kent, OH 44242, United States.
E-mail address: ajasnow@kent.edu (A.M. Jasnow).

http://dx.doi.org/10.1016/j.nlm.2016.01.010
1074-7427/

et al., 2014). However, the specific brain regions and downstream mechanisms through which estrogens modulate the precision of contextual memory retrieval and generalization have not been characterized.

One brain region implicated in memory generalization is the hippocampal formation. In addition, the hippocampus contains an abundance of estrogen receptors (ERs), and estrogens have effects on hippocampal neuronal morphology across the estrous cycle (Beltrán-Campos et al., 2011; Gould, Woolley, Frankfurt, & McEwen, 1990; Shors, Chua, & Falduto, 2001; Wallace, Luine, Arellanos, & Frankfurt, 2006; Woolley & McEwen, 1992). Considerable data indicate that estrogens have *classical* genomic effects occurring within a time frame of hours to days that are driven by cytosolic estrogen receptors (Couse & Korach, 1999; Etgen, 1984; Falkenstein, Tillmann, Christ, Feuring, & Wehling, 2000; McKenna & O'Malley, 2002; O'Malley & Means, 1974). In addition to *classical activation*, estrogens can also have rapid signaling through membrane-bound receptors with effects occurring within a time frame of seconds to minutes (Vasudevan & Pfaff, 2007). A number of recent studies have suggested that estradiol enhances object recognition through activation of membrane bound ERs within the hippocampus and through subsequent ERK/MPK and metabotropic glutamate receptor signaling (Fan et al., 2010; Fernandez et al., 2008; Gresack & Frick, 2006; Lewis, Kerr, Orr, & Frick, 2008; Zhao, Fan, & Frick, 2010). However, given the time frame in which estradiol induces fear generalization in our studies (i.e. between 6 and 24 h) (Lynch et al., 2013, 2014), we hypothesized that estradiol-induced generalization occurs via a genomic effect on retrieval, requiring the activation of cytosolic ERs within the hippocampus.

2. Methods

2.1. Animals and housing conditions

Adult female ovariectomized (OVX) Long Evans rats approximately 90 days old were used for all experiments. Eleven days prior to behavioral manipulations, animals were ovariectomized, cannulated, and then individually housed and maintained on a 14/10 h light/dark cycle (Lynch et al., 2013, 2014). Food and water were available *ad libtum* throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

2.2. Passive avoidance procedure

Behavior was conducted in a black/white passive avoidance chamber (52 × 30 × 35 cm, Passive Avoidance Apparatus 7550, Ugo Basil, Comerio, Italy). Female rats were trained in passive avoidance 11 days after ovariectomy. For training, animals were brought to Context A (training context), held on the experimenter's hand for 30 s, and placed on the white side of the shuttle box. The door was raised after 20 s and the initial latency to cross into the black compartment (all four paws) was recorded. Upon crossing, the sliding door closed and 5 s after closing, a 2-s, 1.0 mA scrambled footshock was delivered. Ten seconds after receiving the footshock, animals were removed from the chamber and returned to the main colony.

For testing, rats were brought back into the experimental room at the specific retention interval. Half of the rats were tested in Context A (training) and half in Context B (neutral). Context A was a 1.6 × 2.33 m room with house fluorescent lights and contained bare white walls and no artificial scents or sounds and was cleaned with 70% Ethanol; Context B was a 1.83 × 2.74 m room that was lit by a 25-w red light bulb with posters on the walls. Context B had White noise (70 db) and was cleaned with 60% quatricide. In each context, the experimenter wore different gloves (Rubber dish glove in A; vinyl lab glove in B) to handle rats. The test procedure was identical to training except the sliding door remained open for a maximum of 540 s and no shocks were delivered. The initial latency to cross was recorded as the dependent measure of fear behavior. Any animal that did not cross was given a score of 540 s. Upon crossing or at 540 s, animals were removed and returned to the main colony.

2.3. Surgical procedures

For ovarectomies, adult female rats were anesthetized with isoflurane vapors and received a bilateral ovariectomy through a dorsal incision (Lynch et al., 2013, 2014). Immediately after ovariectomy, rats were placed in a stereotaxic instrument for implantation of guide cannulas aimed at the lateral ventricle or dorsal CA1. Stereotaxic coordinates were derived from (Paxinos and Watson, 2005). The head was positioned in the stereotaxic instrument so that the skull was level between lambda and bregma before implantation of the guide cannulas. Rats were implanted with a unilateral cannula (Plastics One) aimed at the lateral ventricle (D/V: −3.4; A/P: −0.9; M/L: +1.6) or bilateral cannula aimed at the dorsal CA1 hippocampus (14°, D/V: −3.1; A/P: −4.0; M/L: +3.3). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 min a day for 2 consecutive days before passive avoidance training.

2.4. Site verification

Cannula placement was verified using 0.5 μl infusions of xylene cyanol FF at 0.25% in saline followed by rapid decapitation. Brains were fresh frozen and sliced on a cryostat and slices were mounted and observed for correct placement using an inverted microscope. Any animal with a misplaced cannula was not included in the final analysis (3% of animals).

2.5. Drug administration

Estradiol benzoate (E2) (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, & Watanabe, 2000). E2 for peripheral administration was dissolved in sesame oil (15 μg/0.1 mL) (Chang et al., 2009; Zeidan et al., 2011). To remove trace amounts of unconjugated estradiol, estradiol benzoate conjugated to BSA (E2-BSA) was centrifuged in a filter unit with a molecular weight cut-off of 3000 kDa (Millipore) and spun at 16,110*g* for 10 min. Filters were washed with 5% DMSO, spun for another 10 min at 16,110*g*, and washed again with 5% DMSO before being spun for 30 min at 16,110*g* (Santollo, Marshall, & Daniels, 2012; Taguchi, Koslowski, & Bodenner, 2004). The MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene; Sigma Aldrich) was dissolved in 50% DMSO to a concentration of 1 μg/μl for a final dose of 0.5 μg per hemisphere (Fernandez et al., 2008; Fortress, Fan, Orr, Zhao, & Frick, 2013; Zhao, Fan, Fortress, Boulware, & Frick, 2012). The cytosolic ER antagonist, ICI 182,780 was dissolved in DMSO at a concentration of 50 μg/μl for ICV and intrahippocampal infusions. The ERα specific agonist, PPT (4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*-phenol; Caymen Chemical) was dissolved in DMSO at a concentration of 0.2 pg/μl and infused at a dose of 0.1 pg per hemisphere. The ERβ specific agonist, DPN (2,3-*bis*(4-h ydroxyphenyl)-propionitrile, Caymen Chemical) was dissolved in DMSO at a concentration of 40 pg/μl (Boulware, Heisler, & Frick, 2013). At these low doses, PPT and DPN are specific for ERα and ERβ, respectively (Stauffer et al., 2000).

Table 1
Treatment groups and sample sizes for Experiment 1.

Peripheral	Central	Total *N*	Purpose
E2 or Veh	ICI or Veh	97	Determine if blocking cytosolic ERs would attenuate peripheral E2-induced generalization
N/A	E2 + ICI, E2, ICI, Veh	53	Determine if blocking cytosolic ERs would attenuate central E2-induced generalization
ERβ Agonist (DPN)	ICI or Veh	56	Determine if blocking cytosolic ERs would attenuate peripheral ERβ-induced generalization
N/A	E2, E2-BSA, Veh	57	Determine if membrane bound receptors contribute to estradiol-induced fear generalization

Table 2
Treatment groups and sample sizes for Experiment 2.

Agonist	Antagonist	Total N	Purpose
E2 or Veh	ICI or Veh	89	Determine if blocking hippocampal ERs would attenuate estradiol-induced fear generalization
ERβ (DPN), ERα (PPT), or Veh	ICI or Veh	107	Determine if blocking hippocampal cytosolic ERs would attenuate ERβ-induced generalization
E2, E2-BSA, or Veh	N/A	60	Determine if membrane bound ERs in the hippocampus contribute to E2-induced fear generalization
E2 or Veh	U0126 or Veh	68	Determine if blocking membrane bound ER signaling would attenuate E2-induced fear generalization

2.6. Experiment 1

The goal of Experiment 1 was to assess the role of cytosolic versus membrane bound estrogen receptors in estradiol-induced fear generalization. For all experiments, female rats were trained in passive avoidance and twenty-four hours after training, received drug treatments either through subcutaneous (SC) injections or ICV infusions into the lateral ventricle. Twenty-four hours after drug administration, animals were tested in passive avoidance retention in either the training or a neutral context. First, to determine if administering a cytosolic estrogen receptor antagonist would attenuate estradiol-induced fear generalization, female rats were administered SC injections of vehicle control (sesame oil) or estradiol benzoate (E2; 15 μg/0.1 mL) and received an ICV infusion of vehicle (DMSO) or ICI 182,780 (100 μg/2 μl) immediately after that injection. Second, another group of animals received ICV infusions of estradiol benzoate (10 mM, 2 μl) or in combination with ICI 182,780 (100 μg/2 μl). Third, to determine if a cytosolic estrogen receptor antagonist would attenuate fear generalization induced by an ERβ agonist, a group of animals received SC injections of DPN (2.5 mg/0.1 mL) or vehicle (DMSO) along with an ICV infusion of vehicle (DMSO) or ICI 182,780 (100 μg/2 μl). Finally, to determine if fear generalization could be induced through activation of membrane-bound ERs alone, a final group of animals was given ICV infusions of vehicle (DMSO), E2, or E2-BSA (10 mM, 2 μl), (Table 1).

2.7. Experiment 2

The goals of Experiment 2 were to assess the role of the dorsal CA1 hippocampus and confirm the importance of cytosolic ERβ in estradiol-induced fear generalization. For these experiments, female rats were trained in passive avoidance and twenty-four hours after training, received drug treatments through intra-hippocampal infusions. Twenty-four hours after drug administration, animals were tested in passive avoidance retention in either the training or a neutral context. First, we wanted to determine if fear generalization could be induced through local infusion of E2 into the CA1 hippocampus, and if this effect could be attenuated through intra-hippocampal infusions of the cytosolic ER antagonist, ICI 182,780. Therefore, animals received intra-hippocampal CA1 infusions of E2 (10 mM, 0.5 μl), ICI (25 μg/0.5 μL), a co-infusion of E2 and ICI, or vehicle (DMSO). Second, to determine if fear generalization is due to receptor specific activation within the hippocampus, animals received intra-hippocampal CA1 infusions of PPT (0.1 pg/0.5 μl), DPN (20 pg/0.5 μl), ICI (25 μg/0.5 μL), or a co-infusion of PPT and ICI, DPN and ICI, or vehicle. Third, to determine if membrane-bound receptors were involved in estradiol-induced generalization within the hippocampus, animals received infusions of E2, E2-BSA, or vehicle. In the final experiment, a cohort of animals received intra-hippocampal infusions of E2 (10 mM, 0.5 μl) alone, U0126 (0.5 μg/0.5 μl) alone, a combination of E2 and U0126, or vehicle (Table 2).

2.8. Statistical analyses

In each experiment, the effects of infusions were examined by two-way (Context × Treatment) ANOVAs. Independent *t*-tests were also performed to assess significant generalization between specific groups. Statistical significance was set at $p = 0.05$. Cohen's d effect size estimates were assessed by G*Power 3 (Faul, Erdfelder, Lang, & Buchner, 2007) and effect sizes were determined according to Cohen (1988).

3. Results

3.1. Experiment 1: Estradiol-induced generalization requires cytosolic estrogen receptor β

In order to evaluate the effects of estradiol on fear memory retrieval in the absence of a concurrent learning or testing episode, E2 administration was separated from passive-avoidance training by 24 h and also separated from testing by 24 h as described above (Fig. 1A). We previously used this method to demonstrate that estradiol induces generalization through an effect on memory retrieval (Lynch et al., 2014). When ICV infusions of the cytosolic receptor antagonist, ICI 182,780 were co-administered with SC injections of E2, estradiol-induced generalization was significantly attenuated (Fig. 1B). A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,88)} = 58.17$, $p < 0.001$), indicating longer latencies to cross to the black compartment when groups were tested in the training context versus the neutral context. The main effect for treatment was also significant, ($F_{(3,88)} = 4.06$, $p < 0.01$), indicating significant differences among animals injected with E2, ICI or vehicle control across both contexts. The interaction term was also significant, ($F_{(3,88)} = 3.06$, $p < 0.05$), suggesting a difference between treatments based on the context of testing. In addition to the ANOVA analysis, we were interested in direct comparisons of each condition tested in the training versus neutral context and direct comparisons between treatment groups tested in the neutral context versus vehicle-treated animals tested in the neutral context. Therefore, independent *t*-tests were conducted for those direct comparisons for all experiments. Independent *t*-test analyses revealed a significant difference between animals tested in the training versus neutral contexts for both the vehicle-treated group and ICI alone treated group (Veh + Veh:

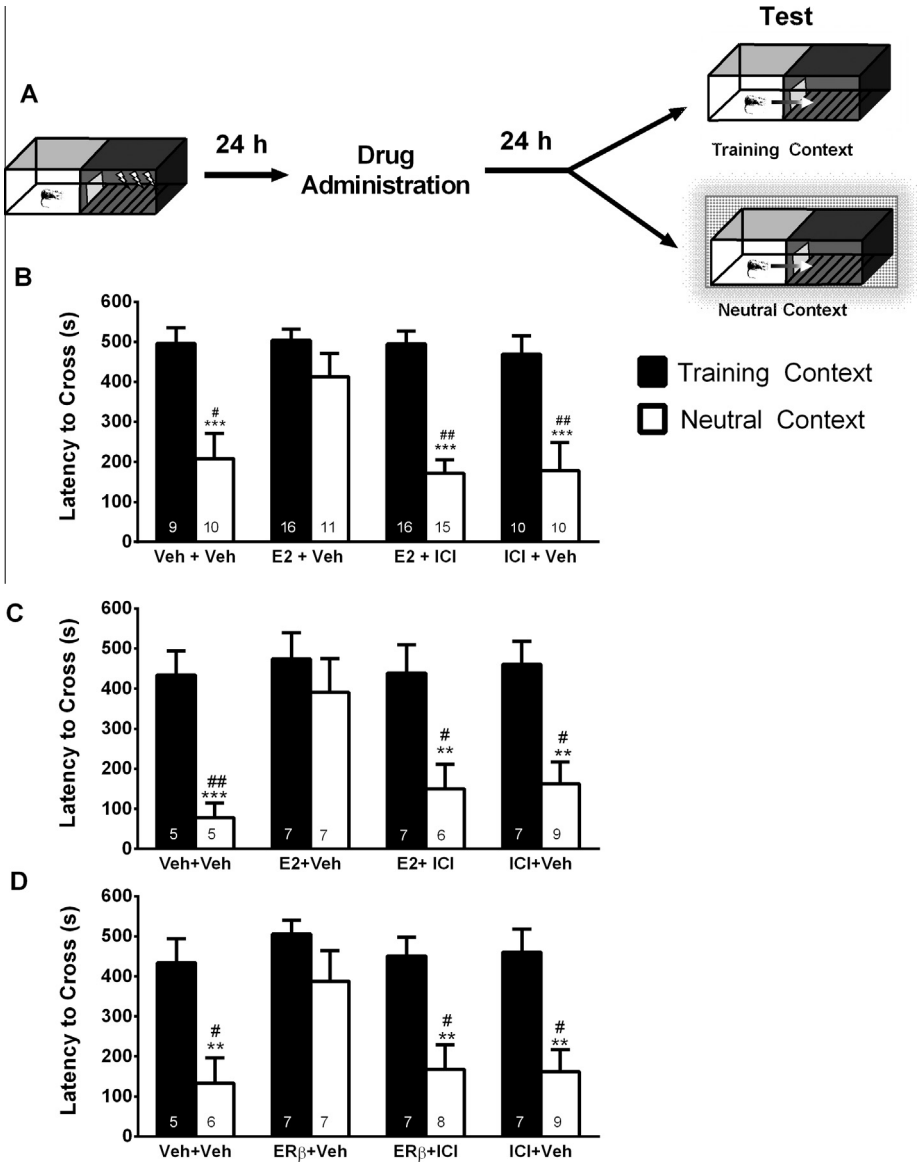

Fig. 1. Cytosolic estrogen receptors are necessary for estradiol-induced fear generalization. (A) Schematic of the experimental paradigm for all experiments. All animals were trained in passive avoidance and 24 h later received drug treatment. Twenty hours after drug treatment, animals were tested in either the training context or a neutral context. (B) Estradiol-treated animals displayed similar latency to cross scores in the training and neutral contexts, demonstrating estradiol-induced generalization. Infusions of ICI attenuated estradiol-induced generalization and vehicle-treated animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2 + ICI, ICI + Veh, and Veh + Veh treated animals. (C) Intracerebroventricular infusions of estradiol also resulted in generalized fear to the neutral context; an effect attenuated by co-infusion of ICI. ICI alone and vehicle infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2 + ICI, ICI + Veh, and Veh + Veh treated animals. (D) Peripheral injections of the ERβ agonist, DPN, induced generalized fear that was attenuated by infusions of ICI. ICI alone and vehicle infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, DPN-treated animals displayed significantly more fear in the neutral context compared to DPN (ERβ)+ICI, ICI + Veh, and Veh + Veh treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < .05$. (∗/# = $p < 0.05$, ∗∗/## = $p < 0.01$, ∗∗∗/### = $p < 0.001$). Numbers within each bar represent the sample size (n) of the group.

$t_{(17)} = 3.78$, $p < 0.01$; $d = 1.8$; Veh + ICI: $t_{(18)} = 3.46$, $p < 0.01$; $d = 1.55$). However, animals treated with E2 did not differ in latencies between contexts ($t_{(25)} = 1.55$, ns; $d = 0.58$). The estradiol-induced generalization was attenuated when animals were given simultaneous infusions of ICI 182,780 ($t_{(50)} = 5.05$, $p < 0.001$; $d = 1.63$). In addition, E2 treated animals tested in the neutral context displayed significantly more fear behavior as demonstrated by a longer latency to cross than vehicle-treated or ICI treated animals (veh + veh: $t_{(19)} = 2.38$, $p < 0.05$; $d = 1.04$; veh + ICI: $t_{(19)} = 2.58$, $p < 0.05$; $d = 1.12$; E2 + ICI: $t_{(45)} = 2.84$, $p < 0.01$; $d = 0.99$). These data suggest that activation of cytosolic estrogen receptors is necessary for estradiol-induced fear generalization; ICV infusions of ICI 182,780 attenuates estradiol-induced fear generalization when estradiol is given peripherally.

The initial results demonstrate that estradiol-induced generalization via peripheral injections could be attenuated with ICV infusions of ICI 182,780. To extend these findings, we gave ICV infusions of vehicle, E2 alone, ICI 182,780 alone, or a co-infusion of E2 and ICI 182,780 to determine if infusions of ICI 182,780 could attenuate estradiol-induced fear generalization when estradiol was given centrally rather than peripherally (Fig. 1C). A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,45)} = 30.16$, $p < .001$), indicating longer latencies between animals tested in the training context versus the neutral context.

The main effect for treatment, ($F_{(3,45)} = 2.66$, $p = 0.06$), was trending towards significance. The interaction term between treatment and context was not significant, ($p > 0.05$). Independent *t*-test analyses confirmed that estradiol induced fear generalization to the neutral context when infused centrally ($t_{(12)} = 0.78$, ns; $d = 0.42$). In comparison, vehicle-treated, ICI-treated, and E2 + ICI treated animals displayed a significant difference in fear between the training and neutral context (Veh: $t_{(8)} = 5.02$, $p < 0.001$; $d = 3.17$; ICI: $t_{(16)} = 3.71$, $p < 0.01$; $d = 1.88$; E2 + ICI: $t_{(11)} = 3.04$, $p < 0.01$; $d = 1.71$). Additionally, E2 treated animals displayed significantly more fear in the neutral context compared to all other treatment groups (E2 + ICI: $t_{(11)} = 2.24$, $p < 0.05$ $d = 1.26$; ICI: $t_{(14)} = 2.37$, $p < 0.05$; $d = 1.16$; Veh: $t_{(10)} = 2.97$, $p < 0.01$; $d = 1.87$). Together, these results demonstrate that centrally infused estradiol results in fear generalization similar to peripherally administered E2 and is attenuated by co-infusion of ICI 182,780.

Previously, we demonstrated that estradiol-induced generalization was driven by activation of ERβ (Lynch et al., 2014). To determine if ICI 182,780 infusions could attenuate generalization induced by specific activation of ERβ, animals were administered SC injections of the ERβ agonist, DPN, and co-administered ICV infusions of ICI 182,780 (Fig. 1D). A two way ANOVA analysis revealed a significant main effect for context, ($F_{(1,48)} = 35.63$, $p < 0.001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for treatment was significant, ($F_{(3,48)} = 3.09$, $p < 0.05$), but the interaction term was not significant, ($F_{(3,48)} = 1.14$, $p > 0.05$). The results replicate our previous finding that activation of ERβ induces fear generalization ($t_{(12)} = 1.41$, ns; $d = 0.75$). Additionally, infusions of ICI 182,780 attenuated fear generalization induced by infusions of DPN ($t_{(13)} = 3.58$, $p < 0.01$; $d = 1.88$). Animals treated with vehicle or ICI alone displayed a significant difference in fear between the training and neutral context (veh: $t_{(9)} = 3.39$, $p < 0.01$; $d = 2.07$; ICI: $t_{(14)} = 3.71$, $p < 0.01$; $d = 1.88$). When comparing animals tested in the neutral context, those treated with DPN displayed significantly more fear than all other groups (ERβ + ICI: $t_{(13)} = 2.27$, $p < 0.05$; $d = 1.17$; ICI: $t_{(14)} = 2.46$, $p < 0.05$; $d = 1.22$; Veh: $t_{(11)} = 2.51$, $p < 0.05$; $d = 1.41$). Taken together, these results replicate our previous findings that ERβ activation induces generalization to a neutral context, and further show that blocking activation of cytosolic ERs with ICI 182,780 attenuates this effect.

The experiments above demonstrated that cytosolic estrogen receptors were necessary for estradiol-induced fear generalization. Yet, we could not rule out the contribution of membrane bound receptors to this behavioral phenomenon. To determine if estradiol also acts through membrane-bound receptors, animals received ICV infusions of E2 or E2-BSA as described above (Fig. 1A). A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,51)} = 16.69$, $p < .001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for hormone treatment was not significant, ($F_{(2,51)} = 2.23$, $p > 0.05$) and the interaction terms between context and hormone treatment were also not significant, ($F_{(2,51)} = 2.19$, $p > 0.05$). Independent *t*-test analyses revealed a significant difference in fear response when tested in the training context or neutral context for vehicle-treated or E2-BSA-treated animals, but not E2-treated animals (veh: $t_{(38)} = 4.15$, $p < 0.001$; $d = 1.31$; E2-BSA: $t_{(16)} = 3.53$, $p < 0.01$; $d = 1.72$; E2: $t_{(17)} = 0.66$, ns; $d = 0.31$) (Fig. 2). In addition, vehicle-treated and E2-BSA animals were significantly different than E2-treated animals when tested in the neutral context (veh: $t_{(18)} = 2.18$, $p < 0.05$; $d = 0.94$; E2-BSA: $t_{(16)} = 2.30$, $p < 0.05$; $d = 1.03$). These data, in combination with the ICI experiments above suggest that activation of membrane-bound ERs alone is not sufficient to induce generalized responding to a neutral context in ovariectomized female rats.

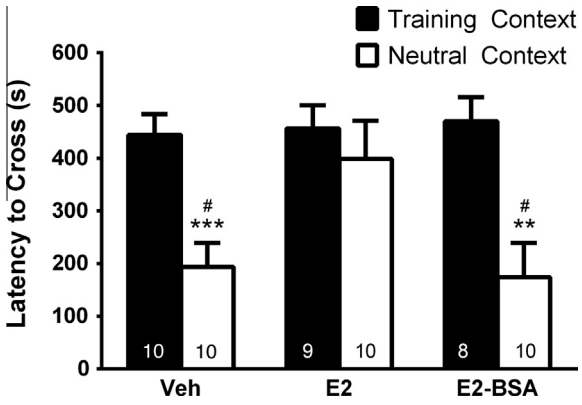

Fig. 2. Membrane bound ER's do not contribute to estradiol-induced fear generalization. Intracerebroventricular infusions of estradiol resulted in generalized fear to the neutral context. However, activation of membrane-bound receptors via infusions of E2-BSA did not induce generalized fear, suggesting that activation of membrane-bound receptors is not sufficient for estradiol-induced generalization. Vehicle-infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2-BSA and Veh treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < .05$. (∗/# = $p < 0.05$, ∗∗ = $p < 0.01$, ∗∗∗ = $p < 0.001$). Numbers within each bar represent the sample size (n) of the group.

3.2. Estradiol acts within the dorsal CA1 to induce fear generalization

The hippocampus is involved in context fear learning (Holland & Bouton, 1999; Kim & Fanselow, 1992; Phillips & LeDoux, 1992) and implicated in the process of fear generalization (Nadel, Samsonovich, Ryan, & Moscovitch, 2000; Rosenbaum, Winocur, & Moscovitch, 2001; Winocur, Moscovitch, & Sekeres, 2007; see Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2006 for review; Xu et al., 2012). In addition, estrogens act within the hippocampus to affect hippocampal neuronal morphology (Beltrán-Campos et al., 2011; Gould et al., 1990; Wallace et al., 2006; Woolley & McEwen, 1992) Given the role of the hippocampus in fear generalization, we characterized the role of the dorsal CA1 in estradiol-induced fear generalization. To test the role of cytosolic ERs within the hippocampus, animals received infusions of E2 alone, or co-infusions of E2 with ICI 182,780 (Fig. 3A). A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,81)} = 54.10$, $p < 0.001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for treatment, ($F_{(3,81)} = 3.91$, $p < 0.01$) was also significant and the interaction term between context and treatment was significant, ($F_{(3,81)} = 3.18$, $p < 0.05$). Independent *t*-test analyses reveal that local infusions of E2 into the dorsal CA1 alone induced generalized responding to the neutral context ($t_{(25)} = 1.69$, ns; $d = 0.70$). The estradiol-induced generalization was attenuated by co-infusions of ICI ($t_{(15)} = 5.71$, $p < 0.001$; $d = 3.71$). Additionally, animals given vehicle or ICI only infusions showed significantly higher levels of fear in the training context compared to the neutral context (Veh: $t_{(23)} = 5.61$, $p < 0.001$; $d = 2.25$; ICI: $t_{(18)} = 2.77$, $p < 0.01$; $d = 1.31$). Animals treated with estradiol showed significantly higher fear when tested in the neutral context compared to all other treatment groups (E2 + ICI: $t_{(27)} = 4.50$, $p < 0.001$; $d = 1.71$; ICI: $t_{(26)} = 2.20$, $p < 0.05$; $d = 0.83$; Veh: $t_{(27)} = 3.93$, $p < 0.001$; $d = 1.48$). These results demonstrate that the dorsal CA1 hippocampus is an important locus for actions of estradiol on fear generalization. Additionally, the actions of estradiol within the hippocampus depend upon activation of cytosolic receptors; ICI can block fear generalization induced by hippocampal administered E2.

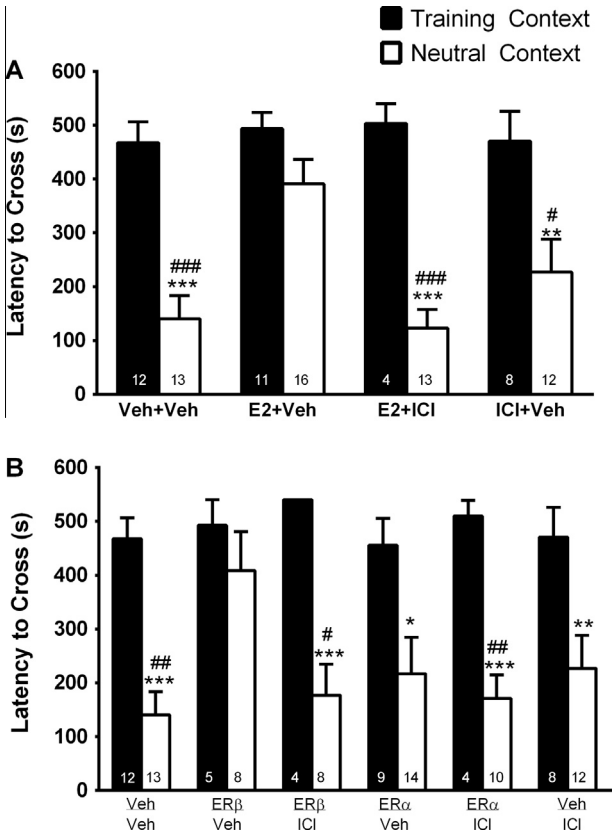

Fig. 3. The dorsal CA1 hippocampus is an important locus for the actions of estradiol on fear generalization. (A) Dorsal hippocampal infusions of estradiol resulted in generalized fear to the neutral context; an effect attenuated with simultaneous infusions of ICI. ICI alone and vehicle infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2 + ICI, ICI + Veh, and Veh + Veh treated animals. (B) Dorsal Hippocampal infusions of the ERβ agonist, DPN, induced generalized fear that was attenuated by co-infusion of ICI. Infusions of the ERα agonist, PPT, did not induce generalized fear. Animals receiving PPT (ERα) and ICI, ICI alone, or vehicle infusions displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, DPN-treated animals displayed significantly more fear in the neutral context compared to DPN (ERβ) + ICI, ERα, PPT (ERα) + ICI, ICI + Veh, and Veh + Veh treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < .05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). Numbers within each bar represent the sample size (n) of the group.

Our previous findings demonstrated that peripheral administration of an ERβ agonist, but not an ERα agonist, could induce fear generalization (Lynch et al., 2014). Previous reports have demonstrated differential effects of ER agonists given intracranially versus when administered systemically (Boulware et al., 2013). Therefore, to confirm that ERβ, but not ERα activation within the dorsal CA1 could also induce fear generalization, animals were infused with the specific ERα agonist, PPT, or ERβ agonist, DPN into the hippocampus with or without co-infusions of ICI 182,780, as described above (Fig. 1A).

Activation of ERβ specifically within the dorsal hippocampus replicated systemic effects of DPN administration. A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,95)} = 52.60$, $p < 0.001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for treatment, ($F_{(5,95)} = 1.21$, $p > 0.05$) and the interaction term were not significant, ($F_{(5,95)} = 1.14$, $p > 0.05$). Independent t-test analyses revealed that animals treated with DPN displayed equivalent levels of fear in either context ($t_{(11)} = 0.84$, ns; $d = 0.51$). Co-infusions of the ERβ agonist and ICI 182,780 attenuated the generalization seen with ERβ activation ($t_{(11)} = 4.35$, $p < 0.001$; $d = 3.15$). Unlike ERβ activation, activation of ERα did not induce generalized responding to the neutral context ($t_{(21)} = 2.55$, $p < 0.05$; $d = 1.15$), confirming our previous findings using peripheral infusions (Lynch et al., 2014). Animals given co-infusions of the ERα agonist and ICI 182,780, ICI 182,780 alone, or vehicle treatment also displayed significantly more fear in the training context compared to the neutral context (ERα + ICI: $t_{(12)} = 4.67$, $p < 0.001$; $d = 3.21$; ICI: $t_{(18)} = 2.77$, $p < 0.01$; $d = 1.31$; Veh: $t_{(23)} = 5.61$, $p < 0.001$; $d = 2.25$). Additionally, animals given the ERβ agonist displayed more fear in the neutral context compared to all other treatment groups (ERβ + ICI: $t_{(14)} = 2.51$, $p < 0.05$; $d = 1.25$; ERα: $t_{(20)} = 1.83$, $p < 0.08$; $d = 0.83$; ERα + ICI: $t_{(16)} = 2.95$, $p < 0.01$; $d = 1.36$; ICI: $t_{(18)} = 1.90$, $p < 0.07$; $d = 0.87$; Veh: $t_{(19)} = 3.41$, $p < 0.01$; $d = 1.48$) (Fig. 3B). These results extend the findings from the previous experiment with systemic injections of the ERβ agonist. Activation of ERβ within the dorsal hippocampus is necessary and sufficient to induce fear generalization; an effect that is attenuated by blockade of cytosolic ERs via infusions of ICI 182,780. In addition, activation of ERα within the dorsal hippocampus is not sufficient to induce fear generalization.

The experiments above demonstrate that activation of cytosolic ERβ within the dorsal CA1 region of the hippocampus is sufficient to induce generalized fear responses to a neutral context. In order to determine if activation of membrane-bound estrogen receptors specifically within the dorsal hippocampus could also result in generalized responding, animals received infusions of E2, E2-BSA, or vehicle into the dorsal CA1 as described above (Fig. 1A). A two-way ANOVA analysis revealed a significant main effect for context, ($F_{(1,54)} = 54.96$, $p < .001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for hormone treatment was also significant, ($F_{(2,54)} = 8.59$, $p < 0.001$), and the interaction terms between context and hormone treatment was significant, ($F_{(2,54)} = 6.76$, $p < 0.01$). Independent t-tests reveal that E2 infusions resulted in fear generalization to the neutral context ($t_{(25)} = 0.10$, ns; $d = 0.70$) whereas infusions of vehicle or E2-BSA did not induce generalized responding (veh: $t_{(15)} = 6.49$, $p < 0.001$; $d = 3.15$; E2-BSA: $t_{(14)} = 4.80$, $p < 0.001$; $d = 2.4$) (Fig. 4A). Additionally, E2-treated animals displayed significantly more fear in the neutral context compared to vehicle-treated and E2-BSA-treated animals (Veh: $t_{(22)} = 4.20$, $p < 0.001$; $d = 1.93$; E2-BSA: $t_{(22)} = 3.85$, $p < 0.001$; $d = 1.66$). These results confirm the above findings of ICV administered E2-BSA, further demonstrating that activation of membrane-bound estrogen receptors within the hippocampus is not sufficient to induce generalized fear responding. Overall, these results demonstrate the dorsal CA1 hippocampus is an important locus for actions of estradiol on fear generalization. Additionally, the actions of estradiol within the hippocampus depend upon activation of cytosolic receptors; ICI can block hippocampal administered E2 and infusions of E2-BSA are not sufficient to induce generalization.

To further confirm that hippocampal membrane bound estrogen receptors are not necessary for estradiol-induced fear generalization, we also administered estradiol alone or estradiol given simultaneously with an ERK/MAPK pathway inhibitor, U0126, into the dorsal CA1 using the same timing as above (Fig. 1A). A major second messenger pathway activated by membrane bound estrogen receptors is the ERK/MAPK pathway, which is important for some of the learning effects of estradiol within the hippocampus (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013). If estradiol-induced generalization is due, at least in part, to activation of membrane-bound ERs, then blocking activation of the ERK/MAPK pathway should attenuate estradiol-induced generalization. A two-way ANOVA analysis revealed a significant main

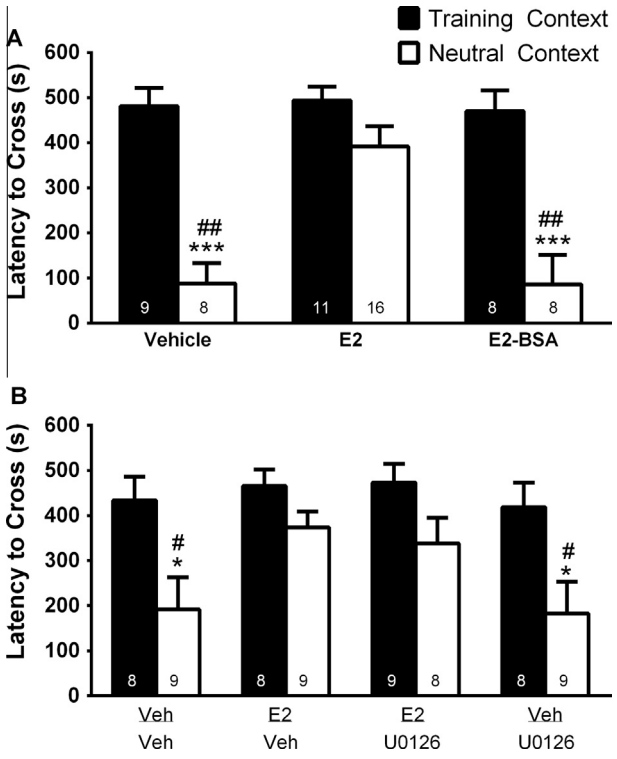

Fig. 4. Cytosolic estrogen receptors within the dorsal hippocampus regulate estradiol-induced fear generalization. (A) Direct infusions of estradiol into the dorsal CA1 hippocampus resulted in generalized fear to the neutral context. However, activation of membrane-bound receptors within the dorsal CA1 via infusions of E2-BSA did not induce generalized responding, suggesting that activation of membrane-bound receptors within the dorsal hippocampus is not sufficient for estradiol-induced generalization. Vehicle infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2-BSA and Veh treated animals. (B) Dorsal hippocampal infusions of estradiol resulted in generalized fear to the neutral context that was not attenuated by co-infusion of the MEK inhibitor, U0126. U0126 alone and vehicle infused animals displayed significantly longer latency to cross in the training context compared to the neutral context. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to U0126 + Veh and Veh + Veh treated animals. These data further suggest that membrane bound ERs alone do not contribute to estradiol-induced fear generalization. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < .05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). Numbers within each bar represent the sample size (n) of the group.

effect for context, ($F_{(1,60)} = 20.68$, $p < 0.001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for treatment trended towards significance, ($F_{(3,60)} = 2.55$, $p = 0.06$), and the interaction was not significant, ($F_{(3,60)} = 0.93$, $p > 0.05$). Independent t-tests revealed that estradiol infusions into the hippocampus induced significant generalization as animals displayed equivalent levels of fear in either context ($t_{(15)} = 1.81$, ns; $d = 0.88$) (Fig. 4B). Infusions of U0126 alone did not affect fear generalization ($t_{(15)} = 2.56$, $p < 0.05$; $d = 1.26$) and did not block estradiol-induced fear generalization ($t_{(15)} = 1.95$, ns, $d = 0.94$) (Fig. 4B). Animals given vehicle treatment also displayed significantly more fear in the training context compared to the neutral context (Veh: $t_{(15)} = 2.68$, $p < 0.51$; $d = 1.32$). Additionally, animals given estradiol displayed significantly more fear in the neutral context compared to U0126 and vehicle treated animals (U0126: $t_{(18)} = 2.40$, $p < 0.05$; $d = 1.13$; Veh: $t_{(16)} = 2.29$, $p < 0.05$; $d = 1.08$). These results demonstrate that blocking activation of the ERK/MAPK pathway through infusions of a MEK inhibitor into the dorsal hippocampus does not attenuate estradiol-induced generalization. Taken together with the results of E2-BSA infusions, these data suggest that estradiol-induced generalization is not a direct result of activation of membrane-bound estrogen receptors.

4. Discussion

In the present study, we demonstrate that the dorsal CA1 is an important locus of estradiol actions on fear generalization. Moreover, we extended our previous findings (Lynch et al., 2014), showing that cytosolic ERs within the dorsal CA1 mediate the actions of estradiol; DPN- and E2-induced fear generalization was attenuated by blockade of cytosolic ERs through infusions of the antagonist, ICI 182,780 into the dorsal CA1. Further, activation of membrane-bound ERs alone through infusions of E2-BSA was not sufficient to induce generalization. Finally, blocking membrane-bound activation of the ERK/MAPK pathway with infusions of U0126 did not attenuate estradiol-induced fear generalization. Overall, these results reveal mechanisms by which estradiol induces generalization to a neutral context. Specifically, estradiol acts through activation of cytosolic ERβ within the hippocampus, affecting memory retrieval for context. Despite several reports of reduced fear and anxiety in response to estrogen treatment (Frye et al., 2000; Krężel, Dupont, Krust, Chambon, & Chapman, 2001; Walf & Frye, 2006), and reports of estrogens facilitating fear extinction (Graham & Daher, 2015; McDermott, Liu, Ade, & Schrader, 2015; Milad, Igoe, Lebron-Milad, & Novales, 2009), we and others have shown that estrogen treatment increases anxiety and fear responses in rodents in a variety of paradigms (Jasnow, Schulkin, & Pfaff, 2006; Morgan & Pfaff, 2001; Morgan et al., 2004; Nofrey et al., 2008; Toufexis et al., 2007). The current findings add to the growing literature on the effects of estrogens on the inhibition of fear to neutral or safety cues (Nofrey et al., 2008; Toufexis et al., 2007). Overall, these findings suggest that high levels of estrogens disrupt the ability of animals to inhibit a fear response to a neutral environment or a discrete neutral stimulus. Unlike the well-established effect of estrogens enhancing extinction retention, where new learning occurs about the relationship between the conditioned stimulus and absence of the unconditioned stimulus (Graham & Daher, 2015; McDermott et al., 2015; Milad et al., 2009), the present findings are not a result of estrogens enhancing memory formation as has been demonstrated frequently (Daniel & Dohanich, 2001; Daniel, Hulst, & Lee, 2005; Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Frye, Duffy, & Walf, 2007; Frye & Rhodes, 2002; Gibbs, 2002; Packard, 1998; Packard, Kohlmaier, & Alexander, 1996; Packard & Teather, 1997; Rhodes & Frye, 2004; Sandstrom & Williams, 2004; Walf, Koonce, & Frye, 2008; Walf, Rhodes, & Frye, 2006; Zhao et al., 2010), but rather changing what cues elicit the fear memory response.

The present results build upon our previous and novel finding that estradiol accelerates the rate of fear generalization in females (Lynch et al., 2013). In our previous study, acute administration of estradiol through systemic injections suggested that estradiol modulated memory retrieval to induce generalized responding to a neutral context. Specifically, estradiol given 24 h after training, presumably after the consolidation window was no longer open, still resulted in generalization 24 h after administration, suggesting an effect on fear memory retrieval. Additionally, estradiol given 24 h after training did not result in generalized responding if testing occurred 1 or 6 h after administration (Lynch et al., 2014). However, these results only suggested that estradiol acted through a long term mechanism, likely through genomic changes to induce generalization. In the current study, we used ICI 182,760 to block cytosolic ERs, but this compound can also act as an estrogen receptor agonist in certain tissues. Specifically, *in vitro*, ICI can increase

ERK1/2 phosphorylation and spinophilin expression to a similar level as estradiol and can act at the G-protein estrogen receptor, GPER, as an agonist. These findings suggest that ICI may activate membrane-bound estrogen receptors similar to estradiol itself (Filardo, Quinn, Frackelton, & Bland, 2002; Zhao, O'Neill, & Brinton, 2006). However, we found no effect of ICI treatments alone on fear generalization, and when considered in combination with the consistent lack of membrane-bound receptor involvement in this behavioral phenomenon, the results suggest that estradiol-induced generalization is not due to membrane bound receptor activation. Taken together, the current results support our original hypothesis that estradiol-induced generalization is due to an effect on memory retrieval through activation of cytosolic ERs.

The current study also extended our previous findings assessing which ER subtype is required for estradiol-induced generalization. Previously, we found that systemic injections of the ERβ agonist, DPN, but not the ERα agonist, PPT, induced fear generalization (Lynch et al., 2014). Here, we found that the ERβ-induced generalization was attenuated by simultaneous ICV or intra-hippocampal infusions of ICI 182,780 (Figs. 1D and 3B). These results demonstrate that activation of cytosolic ERβ, but not ERα, within the hippocampus induces generalized fear responses to a neutral context.

Several studies demonstrate the importance of the hippocampus in the generalization of fear and in context memory precision (Ruediger et al., 2011; Wiltgen & Silva, 2007; Wiltgen et al., 2010; Winocur et al., 2007). Generally, as a context memory ages, the memory is transferred from the hippocampus to a distributed cortical network for long-term storage (Frankland, O'Brien, Ohno, Kirkwood, & Silva, 2001; Frankland et al., 2006; Kim & Fanselow, 1992; McGaugh, 1966; Vetere et al., 2011; Zola-Morgan & Squire, 1990). Similarly, with the passage of time, animals generalize fear to neutral contexts (Jasnow et al., 2012) and this may be due to some modulation of memory storage within the hippocampus or its potential interaction with cortical regions (Cullen, Gilman, Winiecki, Riccio, & Jasnow, 2015). Although the precise mechanisms through which estrogens influence fear generalization remain unknown, estrogen receptors are widely distributed throughout the hippocampus (Li, Schwartz, & Rissman, 1997; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2000a,b; Österlund, Kuiper, Gustafsson, & Hurd, 1998), putting them in an ideal location to modulate contextual memory precision. Indeed, estradiol enhances memory consolidation for novel object recognition memory through activation of membrane-bound estrogen receptors and subsequent ERK/MAPK pathway activation (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013. However, these studies differ from the present study; we have not observed effects of estradiol on fear generalization during the consolidation of passive-avoidance memory (Lynch et al., 2014). Rather, we consistently observe effects of estradiol on memory retrieval, and that membrane-bound estrogen receptors and the subsequent ERK/MAPK pathway are not sufficient to induce generalized responding to a neutral context. Additionally, we have previously shown that estradiol does not induce fear generalization through an effect on memory consolidation (Lynch et al., 2014). The differences among these studies suggest that estradiol has very specific modulatory control over hippocampal functioning that is dependent upon learning demands and stimulus modality.

In addition to impacting learning and memory, as well as synaptic plasticity in the hippocampus and prefrontal cortex, estrogens act in regions controlling neuroendocrine and behavioral stress responses and this may account for increased fear generalization in estradiol treated females. For example, pituitary adenylate cyclase activating peptide (PACAP) is implicated in anxiety-like behaviors (e.g. Hammack et al., 2010), especially within the bed nucleus of the stria terminalis (BNST) (Hammack et al., 2009; Lezak et al., 2014; Roman et al., 2014), which also has an abundance of estrogen receptors (Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998; Simerly, Swanson, Chang, & Muramatsu, 1990). In addition, high levels of PACAP are associated with greater PTSD symptoms and greater fear responses in a fear discrimination task (Ressler et al., 2011). Alternatively, the effects in the present study and in previous studies by our lab suggest that estradiol may be modulating the inhibition of fear. Others have demonstrated that estradiol-treated animals are unable to inhibit fear responses to a neutral cued stimulus (Toufexis et al., 2007) and display significantly less latent inhibition (Nofrey et al., 2008). These results are in line with results from human patients with PTSD who also show an inability to inhibit fear responding to a safety cue (Brewin, 2001; Grillon & Morgan, 1999; Jovanovic et al., 2009). Taken together with our data on estradiol-induced fear generalization, these results suggest that estrogens impact the ability to inhibit responding to irrelevant cues, whether those cues are discrete or contextual in nature.

Overall, these experiments extend our previous findings of estradiol-induced generalization through an effect on memory retrieval. Importantly, the relatively rapid induction of fear generalization seen with estradiol treatment provides an additional mechanism by which memory can be affected, resulting in generalized recall. To date, theories on the process of fear generalization all share the idea that the passage of time is required in order for generalized responding to occur (Biedenkapp & Rudy, 2007; Jasnow et al., 2012; Lynch et al., 2013; Matynia et al., 2008; Wiltgen & Silva, 2007; Winocur et al., 2007). The current data suggest, in some cases, generalized responding does not require a significant passage of time (i.e., several or more days) and can be dependent upon memory retrieval mechanisms rather than alterations to consolidation as traditionally thought. Taken together, these experiments lead to a better understanding of the primary mechanisms through which estrogens enhance fear generalization. Future studies will determine the precise mechanisms underlying how estrogens increase fear generalization, which may help explain the discrepancy in prevalence rates for anxiety disorders seen between males and females and is crucial for developing more effective treatments for anxiety disorders such as PTSD.

Acknowledgments

Partial funding of this research was provided by a Kent State GSS Research Award to JFL. Funding was also provided by a Farris Family Foundation Award to AMJ and Whitehall Foundation Award to AMJ. We thank Dr. T. Lee Gilman and Maeson S. C. M. Latsko for helpful comments on the manuscript and for the helpful contributions of Madison Tasker, Emily Oullette, Jeremy London, Sarah Adkins, Jordan Adkins, and Michael Gray.

References

Beltrán-Campos, V., Prado-Alcalá, R., Leon-Jacinto, U., Aguilar-Vazquez, A., Quirarte, G., Ramírez-Amaya, V., & Díaz-Cintra, S. (2011). Increase of mushroom spine density in CA1 apical dendrites produced by water maze training is prevented by ovariectomy. *Brain Research, 1369*, 119–130.

Biedenkapp, J. C., & Rudy, J. W. (2007). Context preexposure prevents forgetting of a contextual fear memory: Implication for regional changes in brain activation patterns associated with recent and remote memory tests. *Learning & Memory, 14*, 200–203.

Boulware, M. I., Heisler, J. D., & Frick, K. M. (2013). The memory-enhancing effects of hippocampal estrogen receptor activation involve metabotropic glutamate receptor signaling. *The Journal of Neuroscience, 33*, 15184–15194.

Brewin, C. R. (2001). Memory processes in post-traumatic stress disorder. *International Review of Psychiatry, 13*, 159–163.

Chang, Y. J., Yang, C. H., Liang, Y. C., Yeh, C. M., Huang, C. C., & Hsu, K. S. (2009). Estrogen modulates sexually dimorphic contextual fear extinction in rats through estrogen receptor β. *Hippocampus, 19*, 1142–1150.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences*. Psychology Press.

Couse, J. F., & Korach, K. S. (1999). Estrogen receptor null mice: What have we learned and where will they lead us? *Endocrine Reviews, 20*, 358–417.

Cullen, P. K., Gilman, T. L., Winiecki, P., Riccio, D. C., & Jasnow, A. M. (2015). Activity of the anterior cingulate cortex and ventral hippocampus underlie increases in contextual fear generalization. *Neurobiology of Learning and Memory*.

Daniel, J. M., & Dohanich, G. P. (2001). Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. *The Journal of Neuroscience, 21*, 6949–6956.

Daniel, J. M., Hulst, J. L., & Lee, C. D. (2005). Role of hippocampal M2 muscarinic receptors in the estrogen-induced enhancement of working memory. *Neuroscience, 132*, 57–64.

Díaz-Véliz, G., Alarcón, T., Espinoza, C., Dussaubat, N., & Mora, S. (1997). Ketanserin and anxiety levels: Influence of gender, estrous cycle, ovariectomy and ovarian hormones in female rats. *Pharmacology Biochemistry and Behavior, 58*, 637–642.

Etgen, A. M. (1984). Progestin receptors and the activation of female reproductive behavior: A critical review. *Hormones and Behavior, 18*, 411–430.

Falkenstein, E., Tillmann, H.-C., Christ, M., Feuring, M., & Wehling, M. (2000). Multiple actions of steroid hormones—A focus on rapid, nongenomic effects. *Pharmacological Reviews, 52*, 513–556.

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. *The Journal of Neuroscience, 30*, 4390–4400.

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods, 39*, 175–191.

Fernandez, S. M., Lewis, M. C., Pechenino, A. S., Harburger, L. L., Orr, P. T., Gresack, J. E., ... Frick, K. M. (2008). Estradiol-induced enhancement of object memory consolidation involves hippocampal extracellular signal-regulated kinase activation and membrane-bound estrogen receptors. *The Journal of Neuroscience, 28*, 8660–8667.

Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr, & Bland, K. I. (2002). Estrogen action via the G protein-coupled receptor, GPR30: Stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Molecular Endocrinology, 16*, 70–84.

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 the dorsal hippocampus. *Learning & Memory, 20*, 147–155.

Frankland, P. W., Ding, H.-K., Takahashi, E., Suzuki, A., Kida, S., & Silva, A. J. (2006). Stability of recent and remote contextual fear memory. *Learning & Memory, 13*, 451–457.

Frankland, P. W., O'Brien, C., Ohno, M., Kirkwood, A., & Silva, A. J. (2001). α-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature, 411*, 309–313.

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. *Neurobiology of Learning and Memory, 88*, 208–216.

Frye, C. A., Petralia, S. M., & Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha, 5alpha-THP. *Pharmacology Biochemistry and Behavior, 67*, 587–596.

Frye, C. A., & Rhodes, M. E. (2002). Enhancing effects of estrogen on inhibitory avoidance performance may be in part independent of intracellular estrogen receptors in the hippocampus. *Brain Research, 956*, 285–293.

Frye, C. A., & Walf, A. A. (2004). Estrogen and/or progesterone administered systemically or to the amygdala can have anxiety-, fear-, and pain-reducing effects in ovariectomized rats. *Behavioral Neuroscience, 118*, 306.

Gibbs, R. B. (2002). Basal forebrain cholinergic neurons are necessary for estrogen to enhance acquisition of a delayed matching-to-position T-maze task. *Hormones and Behavior, 42*, 245–257.

Gould, E., Woolley, C. S., Frankfurt, M., & McEwen, B. S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *The Journal of Neuroscience, 10*, 1286–1291.

Graham, B. M., & Daher, M. (2015). Estradiol and progesterone have opposing roles in the regulation of fear extinction in female rats. *Neuropsychopharmacology*.

Gresack, J. E., & Frick, K. M. (2006). Post-training estrogen enhances spatial and object memory consolidation in female mice. *Pharmacology Biochemistry and Behavior, 84*, 112–119.

Grillon, C., & Morgan, C. A. (1999). Fear-potentiated startle conditioning to explicit and contextual cues in Gulf War veterans with posttraumatic stress disorder. *Journal of Abnormal Psychology, 108*, 134–142.

Hammack, S. E., Cheung, J., Rhodes, K. M., Schutz, K. C., Falls, W. A., Braas, K. M., & May, V. (2009). Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): Roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology, 34*, 833–843.

Hammack, S. E., Roman, C. W., Lezak, K. R., Kocho-Shellenberg, M., Grimmig, B., Falls, W. A., ... May, V. (2010). Roles for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) in mediating the behavioral consequences of chronic stress. *Journal of Molecular Neuroscience, 42*, 327–340.

Holland, P. C., & Bouton, M. E. (1999). Hippocampus and context in classical conditioning. *Current Opinion in Neurobiology, 9*, 195–202.

Jasnow, A. M., Cullen, P. K., & Riccio, D. C. (2012). Remembering another aspect of forgetting. *Frontiers in Psychology, 3*, 175.

Jasnow, A. M., Schulkin, J., & Pfaff, D. W. (2006). Estrogen facilitates fear conditioning and increases corticotropin-releasing hormone mRNA expression in the central amygdala in female mice. *Hormones and Behavior, 49*, 197–205.

Jovanovic, T., Norrholm, S. D., Fennell, J. E., Keyes, M., Fiallos, A. M., Myers, K. M., ... Duncan, E. J. (2009). Posttraumatic stress disorder may be associated with impaired fear inhibition: Relation to symptom severity. *Psychiatry Research, 167*, 151–160.

Kessler, R. C., McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., ... Kendler, K. S. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Archives of General Psychiatry, 51*, 8–19.

Kessler, R. C., Petukhova, M., Sampson, N. A., Zaslavsky, A. M., & Wittchen, H. U. (2012). Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. *International Journal of Methods in Psychiatric Research, 21*, 169–184.

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of General Psychiatry, 52*, 1048–1060.

Kim, J. J., & Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science, 256*, 675–677.

Krężel, W., Dupont, S., Krust, A., Chambon, P., & Chapman, P. F. (2001). Increased anxiety and synaptic plasticity in estrogen receptor β-deficient mice. *Proceedings of the National Academy of Sciences, 98*, 12278–12282.

Kuroki, Y., Fukushima, K., Kanda, Y., Mizuno, K., & Watanabe, Y. (2000). Putative membrane-bound estrogen receptors possibly stimulate mitogen-activated protein kinase in the rat hippocampus. *European Journal of Pharmacology, 400*, 205–209.

Laflamme, N., Nappi, R. E., Drolet, G., Labrie, C., & Rivest, S. (1998). Expression and neuropeptidergic characterization of estrogen receptors (ERa and ERb) throughout the rat brain: Anatomical evidence of distinct roles of each subtype. *Journal of Neurobiology, 36*, 357–378.

Lewis, M. C., Kerr, K. M., Orr, P. T., & Frick, K. M. (2008). Estradiol-induced enhancement of object memory consolidation involves NMDA receptors and protein kinase A in the dorsal hippocampus of female C57BL/6 mice. *Behavioral Neuroscience, 122*, 716.

Lezak, K., Roelke, E., Harris, O., Choi, I., Edwards, S., Gick, N., ... Braas, K. (2014). Pituitary adenylate cyclase-activating polypeptide (PACAP) in the bed nucleus of the stria terminalis (BNST) increases corticosterone in male and female rats. *Psychoneuroendocrinology, 45*, 11–20.

Li, X., Schwartz, P. E., & Rissman, E. (1997). Distribution of estrogen receptor-β-like immunoreactivity in rat forebrain. *Neuroendocrinology, 66*, 63–67.

Lynch, J. F., III, Cullen, P. K., Jasnow, A. M., & Riccio, D. C. (2013). Sex differences in the generalization of fear as a function of retention intervals. *Learning & Memory, 20*, 628–632.

Lynch, J. F., III, Dejanovic, D., Winiekci, P. A., Mulvany, J. L., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and Behavior*.

Matynia, A., Anagnostaras, S. G., Wiltgen, B. J., Lacuesta, M., Fanselow, M. S., & Silva, A. J. (2008). A high through-put reverse genetic screen identifies two genes involved in remote memory in mice. *PLoS ONE, 3*, e2121.

McDermott, C. M., Liu, D., Ade, C., & Schrader, L. A. (2015). Estradiol replacement enhances fear memory formation, impairs extinction and reduces COMT expression levels in the hippocampus of ovariectomized female mice. *Neurobiology of Learning and Memory, 118*, 167–177.

McGaugh, J. L. (1966). Time-dependent processes in memory storage. *Science, 153*, 1351–1358.

McKenna, N. J., & O'Malley, B. W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell, 108*, 465–474.

Milad, M. R., Igoe, S. A., Lebron-Milad, K., & Novales, J. E. (2009). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience, 164*, 887–895.

Morgan, M. A., & Pfaff, D. W. (2001). Effects of estrogen on activity and fear-related behaviors in mice. *Hormones and Behavior, 40*, 472–482.

Morgan, M. A., & Pfaff, D. W. (2002). Estrogen's effects on activity, anxiety, and fear in two mouse strains. *Behavioural Brain Research, 132*, 85–93.

Morgan, M. A., Schulkin, J., & Pfaff, D. W. (2004). Estrogens and non-reproductive behaviors related to activity and fear. *Neuroscience & Biobehavioral Reviews, 28*, 55–63.

Moscovitch, M., Nadel, L., Winocur, G., Gilboa, A., & Rosenbaum, R. S. (2006). The cognitive neuroscience of remote episodic, semantic and spatial memory. *Current Opinion in Neurobiology, 16*, 179–190.

Nadel, L., Samsonovich, A., Ryan, L., & Moscovitch, M. (2000). Multiple trace theory of human memory: Computational, neuroimaging, and neuropsychological results. *Hippocampus, 10*, 352–368.

Nofrey, B. S., Ben-Shahar, O. M., & Brake, W. G. (2008). Estrogen abolishes latent inhibition in ovariectomized female rats. *Brain and Cognition, 66*, 156–160.

O'Malley, B. W., & Means, A. R. (1974). Female steroid hormones and target cell nuclei the effects of steroid hormones on target cell nuclei are of major importance in the induction of new cell functions. *Science, 183*, 610–620.

Österlund, M., Kuiper, G. G. J. M., Gustafsson, J.-Å., & Hurd, Y. L. (1998). Differential distribution and regulation of estrogen receptor-α and-β mRNA within the female rat brain. *Molecular Brain Research, 54*, 175–180.

Packard, M. G. (1998). Posttraining estrogen and memory modulation. *Hormones and Behavior, 34*, 126–139.

Packard, M. G., Kohlmaier, J. R., & Alexander, G. M. (1996). Posttraining intrahippocampal estradiol injections enhance spatial memory in male rats: Interaction with cholinergic systems. *Behavioral Neuroscience, 110*, 626–632.

Packard, M. G., & Teather, L. A. (1997). Posttraining estradiol injections enhance memory in ovariectomized rats: Cholinergic blockade and synergism. *Neurobiology of Learning and Memory, 68*, 172–188.

Paxinos, G., & Watson, C. (2005). *The rat brain in stereotaxic coordinates*. Academic Press.

Phillips, R. G., & LeDoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience, 106*, 274–285.

Ressler, K. J., Mercer, K. B., Bradley, B., Jovanovic, T., Mahan, A., Kerley, K., ... Myers, A. J. (2011). Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature, 470*, 492–497.

Rhodes, M. E., & Frye, C. A. (2004). Estrogen has mnemonic-enhancing effects in the inhibitory avoidance task. *Pharmacology Biochemistry and Behavior, 78*, 551–558.

Roman, C. W., Lezak, K. R., Hartsock, M. J., Falls, W. A., Braas, K. M., Howard, A. B., ... May, V. (2014). PAC1 receptor antagonism in the bed nucleus of the stria terminalis (BNST) attenuates the endocrine and behavioral consequences of chronic stress. *Psychoneuroendocrinology, 47*, 151–165.

Rosenbaum, R. S., Winocur, G., & Moscovitch, M. (2001). New views on old memories: Re-evaluating the role of the hippocampal complex. *Behavioural Brain Research, 127*, 183–197.

Ruediger, S., Vittori, C., Bednarek, E., Genoud, C., Strata, P., Sacchetti, B., & Caroni, P. (2011). Learning-related feedforward inhibitory connectivity growth required for memory precision. *Nature, 473*, 514–518.

Sandstrom, N. J., & Williams, C. L. (2004). Spatial memory retention is enhanced by acute and continuous estradiol replacement. *Hormones and Behavior, 45*, 128–135.

Santollo, J., Marshall, A., & Daniels, D. (2012). Activation of membrane-associated estrogen receptors decreases food and water intake in ovariectomized rats. *Endocrinology, 154*, 320–329.

Shors, T. J., Chua, C., & Falduto, J. (2001). Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus. *The Journal of Neuroscience, 21*, 6292–6297.

Shughrue, P. J., Lane, M. V., & Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *Journal of Comparative Neurology, 388*, 507–525.

Shughrue, P. J., & Merchenthaler, I. (2000a). Estrogen is more than just a "sex hormone": Novel sites for estrogen action in the hippocampus and cerebral cortex. *Frontiers in Neuroendocrinology, 21*, 95–101.

Shughrue, P. J., & Merchenthaler, I. (2000b). Evidence for novel estrogen binding sites in the rat hippocampus. *Neuroscience, 99*, 605–612.

Simerly, R., Swanson, L., Chang, C., & Muramatsu, M. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *Journal of Comparative Neurology, 294*, 76–95.

Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., ... Katzenellenbogen, J. A. (2000). Pyrazole ligands: Structure–affinity/activity relationships and estrogen receptor-α-selective agonists. *Journal of Medicinal Chemistry, 43*, 4934–4947.

Taguchi, Y., Koslowski, M., & Bodenner, D. L. (2004). Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). *Nuclear Receptor, 2*.

Toufexis, D. J., Myers, K. M., Bowser, M. E., & Davis, M. (2007). Estrogen disrupts the inhibition of fear in female rats, possibly through the antagonistic effects of estrogen receptor α (ERα) and ERβ. *The Journal of Neuroscience, 27*, 9729–9735.

Vasudevan, N., & Pfaff, D. W. (2007). Membrane-initiated actions of estrogens in neuroendocrinology: Emerging principles. *Endocrine Reviews, 28*, 1–19.

Vetere, G., Restivo, L., Cole, C. J., Ross, P. J., Ammassari-Teule, M., Josselyn, S. A., & Frankland, P. W. (2011). Spine growth in the anterior cingulate cortex is necessary for the consolidation of contextual fear memory. *Proceedings of the National Academy of Sciences, 108*, 8456–8460.

Walf, A. A., & Frye, C. A. (2006). A review and update of mechanisms of estrogen in the hippocampus and amygdala for anxiety and depression behavior. *Neuropsychopharmacology, 31*, 1097–1111.

Walf, A. A., Koonce, C. J., & Frye, C. A. (2008). Estradiol or diarylpropionitrile administration to wild type, but not estrogen receptor beta knockout, mice enhances performance in the object recognition and object placement tasks. *Neurobiology of Learning and Memory, 89*, 513–521.

Walf, A. A., Rhodes, M. E., & Frye, C. A. (2006). Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. *Neurobiology of Learning and Memory, 86*, 35–46.

Wallace, M., Luine, V. N., Arellanos, A., & Frankfurt, M. (2006). Ovariectomized rats show decreased recognition memory and spine density in the hippocampus and prefrontal cortex. *Brain Research, 1126*, 176–182.

Wang, P. S., Lane, M., Olfson, M., Pincus, H. A., Wells, K. B., & Kessler, R. C. (2005). Twelve-month use of mental health services in the United States – Results from the national comorbidity survey replication. *Archives of General Psychiatry, 62*, 629–640.

Wiltgen, B. J., & Silva, A. J. (2007). Memory for context becomes less specific with time. *Learning & Memory, 14*, 313–317.

Wiltgen, B. J., Zhou, M., Cai, Y., Balaji, J., Karlsson, M. G., Parivash, S. N., ... Silva, A. J. (2010). The hippocampus plays a selective role in the retrieval of detailed contextual memories. *Current Biology, 20*, 1336–1344.

Winocur, G., Moscovitch, M., & Sekeres, M. (2007). Memory consolidation or transformation: Context manipulation and hippocampal representations of memory. *Nature Neuroscience, 10*, 555–557.

Woolley, C. S., & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat [published erratum appears in J Neurosci 1992 Oct; 12 (10): following table of contents]. *The Journal of Neuroscience, 12*, 2549–2554.

Xu, W., Morishita, W., Buckmaster, P. S., Pang, Z. P., Malenka, R. C., & Südhof, T. C. (2012). Distinct neuronal coding schemes in memory revealed by selective erasure of fast synchronous synaptic transmission. *Neuron, 73*, 990–1001.

Zeidan, M. A., Igoe, S. A., Linnman, C., Vitalo, A., Levine, J. B., Klibanski, A., ... Milad, M. R. (2011). Estradiol modulates medial prefrontal cortex and amygdala activity during fear extinction in women and female rats. *Biological Psychiatry, 70*, 920–927.

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. *The Journal of Neuroscience, 32*, 2344–2351.

Zhao, Z., Fan, L., & Frick, K. M. (2010). Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation. *Proceedings of the National Academy of Sciences, 107*, 5605–5610.

Zhao, L., O'Neill, K., & Brinton, R. D. (2006). Estrogenic agonist activity of ICI 182,780 (Faslodex) in hippocampal neurons: Implications for basic science understanding of estrogen signaling and development of estrogen modulators with a dual therapeutic profile. *Journal of Pharmacology and Experimental Therapeutics, 319*, 1124–1132.

Zola-Morgan, S., & Squire, L. R. (1990). The neuropsychology of memory. Parallel findings in humans and nonhuman primates. *Annals of the New York Academy of Sciences, 608*, 434–450. discussion 450–436.

Zuluaga, M., Agrati, D., Pereira, M., Uriarte, N., Fernandez-Guasti, A., & Ferreira, A. (2005). Experimental anxiety in the black and white model in cycling, pregnant and lactating rats. *Physiology & Behavior, 84*, 279–286.