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Activation of ERβ modulates fear generalization through an effect on memory retrieval

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ABSTRACT

Women are 60% more likely to suffer from an anxiety disorder than men. One hypothesis for this difference may be that females exhibit increased rates of fear generalization. Females generalize fear to a neutral context faster than males, a process driven, in part, by estrogens. In the current study, ovariectomized adult female Long–Evans rats were given acute injections of estradiol benzoate (15 μg/0.1 mL sesame oil) or sesame oil during a passive avoidance procedure to determine if estrogens increase fear generalization through an effect on fear memory acquisition/consolidation or through fear memory retrieval. Animals injected 1 h prior to training generalized to the neutral context 24 h later but not 7 days after training. Generalization was also seen when injections occurred 24 h before testing, but not when tested at immediate (1 h) or intermediate (6 h) time points. In Experiment 3, animals were injected with estrogen receptor (ER) agonists, PPT or DPN, to determine which ER subtype(s) increased fear generalization. Only the ERβ agonist, DPN, increased fear generalization when testing occurred 24 h after injection. Our results indicate that estradiol increases fear generalization through an effect on fear memory retrieval mechanisms by activation of ERβ.

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Introduction

Posttraumatic stress disorder (PTSD) is an anxiety disorder that is marked by the persistence of fear and anxiety as well as the tendency to generalize fear to neutral cues and contexts (Grillon and Morgan, 1999; Jovanovic et al., 2009). Females are approximately 60% more likely than males to be diagnosed with an anxiety disorder such as PTSD (Kessler et al., 1994; Wang et al., 2005), implicating a role for estrogens in these disorders (see Hayward and Sanborn, 2002 for a review; Ressler et al., 2011). Therefore, determining how estrogens contribute to fear generalization in females may provide new insight into the mechanisms of fear generalization and explain the sex differences in anxiety disorder prevalence rates. However, research on fear memory and fear generalization to date has focused on mostly males, despite the increased prevalence of anxiety disorders in females (see Lebron-Milad and Milad, 2012 for a review).

Several decades of research has revealed that fear generalization—the inability to discriminate between contextual cues and, thus, recalling a fear memory in neutral contexts—increases over time (for a review, see Jasnow et al., 2012). When rodents receive footshocks in one context and are tested in a different (neutral) context after a short interval (24 h), a decrease in fear behavior is observed. However, when rodents are tested at long delays after training (14–36 days), they exhibit comparable fear responses to the training and neutral contexts (Jasnow et al., 2012; Lynch et al., 2013; Matynia et al., 2008; Wiltgen and Silva, 2007; Winocur et al., 2007). Thus, over time rodents generalize their fear response to neutral contexts, which can be interpreted as a loss of memory precision.

Recently, our laboratory demonstrated that female rats display accelerated fear generalization compared with male rats. When tested 7 days after training, males displayed reduced fear responses as measured by reduced latency to enter the black compartment in a passive avoidance chamber in a neutral context compared with animals tested in the training context. Females tested at 7 days displayed equivalent elevated avoidance when tested in either context, demonstrating sex differences in fear generalization (Lynch et al., 2013). To determine if this effect was attributable to estrogens, we tested fear generalization in ovariectomized (OVX) rats implanted with an estradiol benzoate capsule or an empty capsule. The accelerated fear generalization observed in intact females was expressed by OVX females receiving estradiol, but not control females, suggesting an influence of circulating estrogen levels on the rate of fear generalization (Lynch et al., 2013). These findings were the first to demonstrate enhanced context fear generalization driven by estrogens.

Although detailed information about the mechanisms through which estrogens have an effect on fear generalization is lacking, the

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effects of estrogens on other anxiety-like behaviors are well documented. Estrogens are believed to increase arousal, but the behavioral manifestation of that increased arousal is dependent upon context. In safe environments, estrogens increase overall activity levels and the likelihood of sexual behavior whereas in uncertain environments, estrogens increase fear or anxiety behavior (for a review, see Morgan et al., 2004). Despite several reports of reduced fear and anxiety in response to estrogen treatment (Frye et al., 2000; Kr żel et al., 2001; Walf and Frye, 2006), and reports of estrogens facilitating fear extinction (Milad et al., 2009), we and others have shown that estrogen treatment increases anxiety and fear responses in rodents in a variety of paradigms (Jasnow et al., 2006; Morgan and Pfaff, 2001; Morgan et al., 2004; Nofrey et al., 2008; Toufexis et al., 2007). The sex discrepancy in prevalence rates for anxiety disorders is seen following puberty, and is less pronounced following menopause in women (Bebbington et al., 2003), suggesting that endogenous estrogen levels have an impact on the expression of anxiety disorders. In humans, women at high estrogen stages during the menstrual cycle (preovulatory surge) display increased fear recognition compared with women at low estrogen stages (menstruation) (Pearson and Lewis, 2005) and recently, a connection has been made between estrogens and a polymorphism in the gene for the PAC1 receptor which predicts the severity of PTSD symptoms in females (Ressler et al., 2011). Taken together, these data suggest that estrogens have a facilitative effect on various forms of aversive learning and anxiety measures in both human and non-human animals.

Our previous data suggested that estrogens contribute to accelerated fear generalization in females without increasing overall fear and anxiety levels (Lynch et al., 2013). However, the use of chronic estradiol treatment did not allow us to determine if estrogens increase fear generalization through an effect on fear acquisition, consolidation, or retrieval. The consensus is that the processes involved during acquisition/consolidation and retrieval of fear memory involve distinct molecular and cellular mechanisms with some manipulations affecting either acquisition/consolidation or retrieval, but not necessarily both (Abel and Lattal, 2001; Antoniadis et al., 2009; Matus-Amat et al., 2007; Miserendino et al., 1990; Venable and Kelly, 1990; Walker and Davis, 2008). Many have demonstrated that estrogens can have enhancing effects on spatial learning when present during consolidation (Inagaki et al., 2010; Packard, 1998), but few studies have demonstrated the effects of estrogens on retrieval mechanisms, calling attention to the need for a more in depth examination of the effects of estrogens on retrieval.

Estrogens can interact with several different receptor subtypes with each contributing to a different behavioral output. The two major receptor subtypes—ERα and ERβ—are structurally distinct and regulate different target genes upon activation (Tee et al., 2004). In general, ERα activation underlies estradiol-mediated effects on sexual and exploratory behaviors (Luine et al., 2003; Mazzucco et al., 2008; Morgan et al., 2004; Rhodes and Frye, 2006), ERβ activation underlies estradiol-mediated effects on spatial learning and anxiety (Bodo and Rissman, 2006; Lephart et al., 2002; Lund et al., 2005; Walf et al., 2004), whereas both receptors are thought to play a role in emotional memory (Rhodes and Frye, 2006). Currently, the receptor subtype(s) responsible for estradiol-induced fear generalization is unknown (Lynch et al., 2013).

Given that the chronic treatment in our previous findings was present during the time of acquisition/consolidation, we hypothesized that acute estradiol treatment would increase fear generalization through an effect on fear acquisition/consolidation. To test this hypothesis, OVX female rats were injected at different time points before passive avoidance training in Experiment 1. In contrast, females were injected at different time points before testing to examine the effects of estradiol on retrieval mechanisms in Experiment 2. To determine the estrogen receptor subtype(s) responsible for fear generalization, animals were injected with an ERα agonist or an ERβ agonist in Experiment 3. Because ERβ activation has effects on spatial learning and anxiolytic behaviors, we hypothesized that ERβ activation would increase fear generalization.

Methods

Animals and housing conditions

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

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-second, 1.0 mA scrambled footshock was delivered. Ten seconds after receiving the footshock, the animal was 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 meter room with house fluorescent lights and contained bare white walls and no artificial scents or sounds; Context B was a 1.83 × 2.74 meter room that was lit by a 25-W red light bulb with posters on the walls. Context B had white noise (70 dB) and artificial scent via a Glade Plug-Ins Scented Oil Country Berry air freshener at all times. In each context, the experimenter wore different gloves (rubber dish glove in A; vinyl lab glove in B) to handle the rat. The test procedure was identical to training except that 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. Any animal that did not cross was given a score of 540 s. Upon crossing or at 540 s, the animal was removed and returned to the main colony.

Experiment 1: effects of estradiol on fear acquisition/consolidation

In Experiment 1, 141 adult female rats were used. Rats were anesthetized with isoflurane and received a 1 mg/kg dose of ketoprofen 5 min before bilateral ovariectomy through a dorsal incision. Animals were allowed to recover for 9 days and then they were handled for 5 min a day for 2 consecutive days before passive avoidance training. In Experiment 1, ovariectomized female rats were administered either vehicle control (sesame oil, 0.1 mL) or estradiol benzoate (EB) dissolved in sesame oil (15 μg/0.1 mL) by subcutaneous (SC) injection. This dose of EB is common in research involving estrogens and provides animals with a plasma concentration of estradiol around the levels seen during the proestrus stage of the estrous cycle (Chang et al., 2009; Zeidan et al., 2011). Injections were given prior to passive avoidance training to assess if estradiol increases fear generalization through modulation of fear acquisition/consolidation. Animals were injected with vehicle control or EB 24 h, 6 h, or 1 h before passive avoidance training and tested 24 h or 7 days later (n = 5–10) (Fig. 1A).

Experiment 2: effects of estradiol on fear memory retrieval

In Experiment 2, 194 adult female rats were used. Rats were ovariectomized as described above and allowed to recover for 9 days prior to handling. In Experiment 2, ovariectomized female rats were administered either vehicle control or EB (15 μg/0.1 mL) by SC injection.

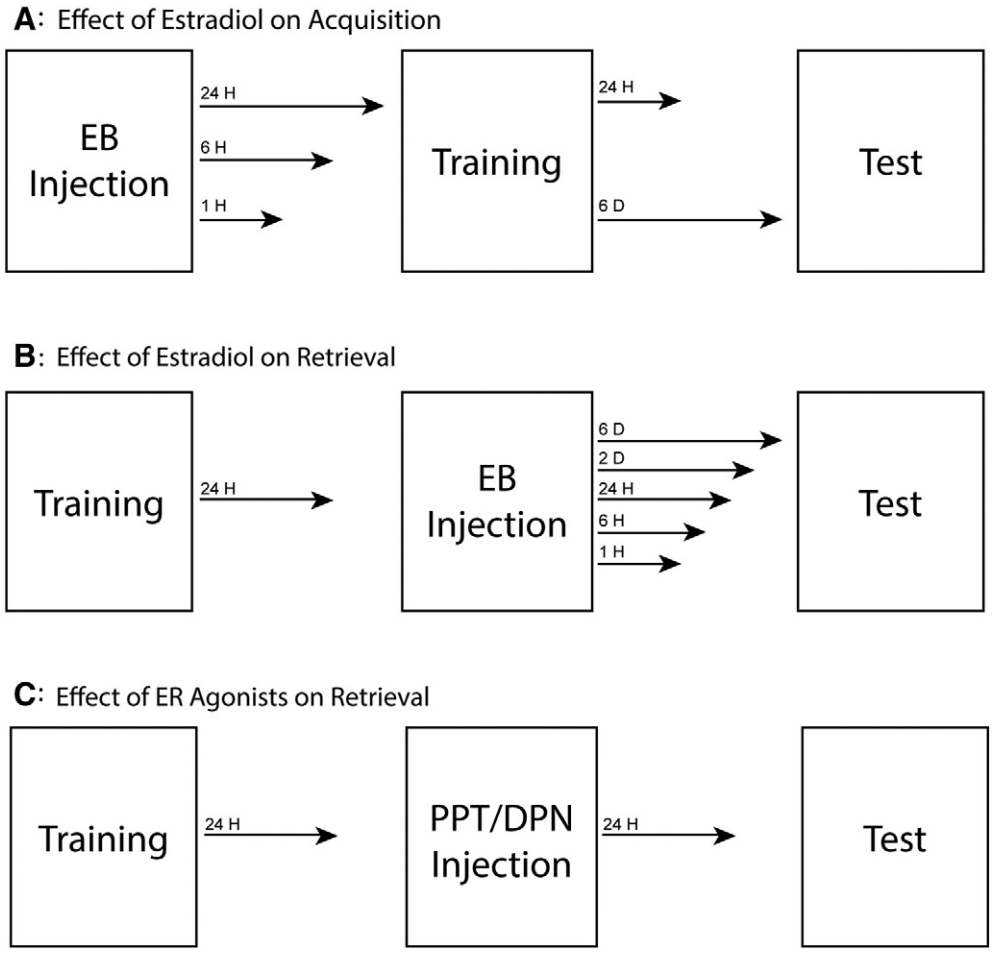

Fig. 1. Diagram of experimental procedure. A) Schematic of the experiment paradigm for Experiment 1. The timing of estradiol benzoate (EB) injections was varied in relation to passive avoidance training. Testing occurred at varying retention intervals. B) Schematic of the experiment paradigm for Experiment 2. The timing of injections occurred 24 h after training and testing occurred at varying retention intervals. C) Schematic of the experiment paradigm for Experiment 3. The timing of injections of PPT (ERα agonist) or DPN (ERβ agonist) occurred 24 h after training and testing occurred 24 h after injections.

Animals were injected before passive avoidance testing to determine if estradiol increases fear generalization through effects on fear memory retrieval. Rats were trained on the passive avoidance procedure then 24 h later, animals were injected with EB or vehicle control and tested 1 h, 6 h, 24 h, 48 h, or 6 days later (n = 9–10) (Fig. 1B).

Experiment 3: effects of ERα and ERβ selective agonists on fear generalization

In Experiment 3, 146 adult female rats were ovariectomized as described above and allowed 9 days of recovery prior to handling. All animals were injected 24 h after training and tested 24 h after injection in the same procedure as Experiment 2. Injections consisted of the ERα agonist, PPT, or ERβ agonist, DPN (Meyers et al., 2001; Stauffer et al., 2000). These injections were given at two different doses (1 mg/kg or 2.5 mg), which were chosen because they have effects on uterine weight and sexual proceptive and receptive behaviors similar to those observed with estradiol treatment (Frasor et al., 2003; Harris et al., 2002; Mazzucco et al., 2008).

Statistical analyses

In each experiment, the effects of EB or ER agonists were examined by independent *t*-test analyses to make direct comparisons of vehicle-treated females tested in training or neutral context, EB-treated or ER agonist-treated females tested in the training or neutral context, and vehicle-treated and EB-treated or ER agonist-treated females in the neutral context. Statistical significance was set at $p \leq 0.05$. Cohen's d effect size estimates were assessed by G*Power 3 (Faul et al., 2007) and effect sizes were determined according to Cohen (1988).

Results

Experiment 1: estradiol does not increase fear generalization through an effect on fear acquisition/consolidation

In Experiment 1, when injections were given 24 h before training and testing occurred 24 h after training, both EB-treated rats and vehicle-treated rats exhibited significantly reduced latencies in the neutral context (Context B) compared to the training context (Context A) (Fig. 2A). Independent *t*-test analyses revealed a significant difference between vehicle-treated animals tested in the training versus neutral context ($t_{(18)} = 15.0$, $p < 0.001$; $d = 6.71$), and EB-treated rats tested in the training versus neutral context ($t_{(18)} = 5.91$, $p < 0.001$; $d = 2.64$). In addition, vehicle-treated and EB-treated rats did not differ in latency to cross when tested in the neutral context, ($t_{(18)} = 0.6$, ns; $d = 0.26$) (Fig. 2A). These data demonstrate that when EB administration occurred 24 h before testing, females discriminated between contexts at the 24-hour test interval.

Given that estradiol did not have an impact when administered 24 h before training, another group of OVX females was injected with either vehicle or EB 6 h before training and tested 24 h later (Fig. 2B). Independent *t*-test analyses revealed that EB injections 6 h before training did not influence fear generalization to the neutral context. Animals tested in the

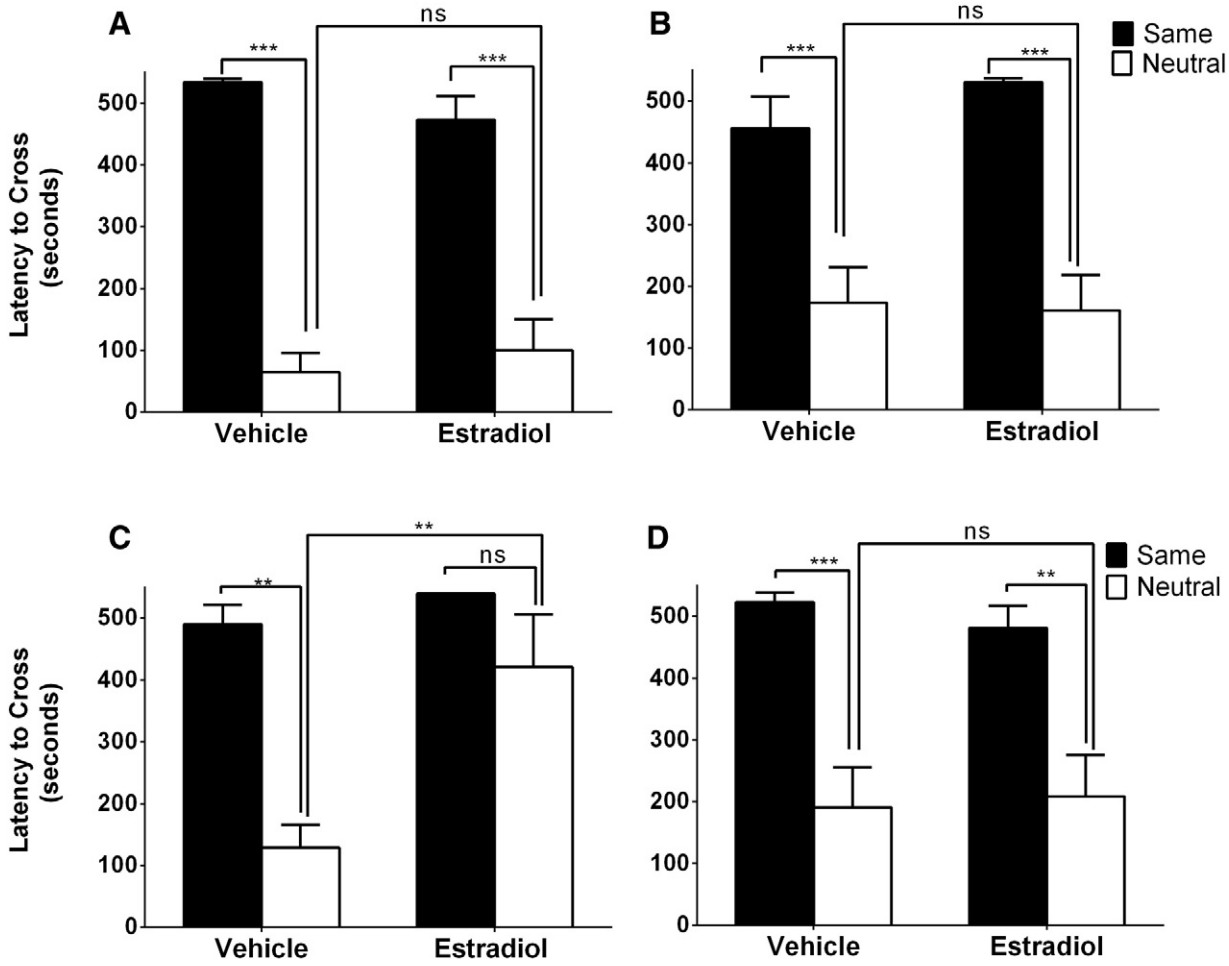

Fig. 2. Estradiol does not increase fear generalization through an effect on acquisition/consolidation. A) Estradiol was administered 24 h before passive avoidance training and animals were tested 24 h after training. Latency to cross was significantly higher in the training context compared to the neutral context, regardless of estradiol treatment. When injected 24 h before training, estradiol did not increase fear generalization to the neutral context. B) Estradiol was administered 6 h before training and animals were tested 24 h after training. Latency to cross was significantly higher in the training context compared to the neutral context, regardless of estradiol treatment. Again, estradiol did not increase fear generalization when injected 6 h before training. C) Estradiol was administered 1 h before training and animals were tested 24 h after training. Latency to cross remained higher in the training context for vehicle-treated animals. However, estradiol-treated animals displayed similar latency to cross scores in the training and neutral contexts. Thus, estradiol appeared to significantly increase fear generalization to the neutral context when injected 1 h prior to training. D) However, when estradiol was administered 1 h before training and animals were tested 7 days after training, fear generalization was absent. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < .05$ (** = $p < 0.01$, *** = $p < 0.001$).

neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(18)} = 3.65$, $p < 0.001$; $d = 1.63$, vehicle-treated; $t_{(18)} = 6.4$, $p < .001$; $d = 2.86$, EB-treated). In addition, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(18)} = 0.156$, ns; $d = 0.07$) (Fig. 2B). These data mirrored the findings observed when rats were administered EB 24 h before training.

We next tested whether more rapid effects of estradiol would increase fear generalization through an effect on fear acquisition/consolidation. Animals were injected with estradiol 1 h prior to training, and were tested either 24 h after training (Fig. 2C) or 7 days after training (Fig. 2D). When tested 24 h after training, independent *t*-test analyses revealed a significant difference between vehicle-treated females but not the EB-treated females ($t_{(9)} = 7.47$, $p < 0.001$; $d = 4.52$, vehicle-treated; $t_{(8)} = 1.40$, ns, $d = 0.68$, EB-treated). In addition, vehicle-treated rats were significantly different from EB-treated rats when tested in the neutral context ($t_{(8)} = 3.1732$, $p < 0.01$, $d = 2.00$).

The results presented in Fig. 2C are in contrast with Fig. 2A where no generalization is seen after estradiol treatment, suggesting that estradiol had an effect on fear acquisition/consolidation and increased fear generalization. However, when rats were tested 7 days after training, the increase in context fear generalization was absent. Independent *t*-test analyses revealed that animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(18)} = 4.95$, $p < 0.001$; $d = 2.22$, vehicle-treated; $t_{(18)} = 3.58$, $p < 0.01$; $d = 1.60$, EB-treated). In addition, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(18)} = 0.187$, ns; $d = 0.08$). Taken together, these results suggest that estradiol may increase fear generalization through fear memory retrieval processes rather than through an effect on acquisition or consolidation. To examine this possibility in Experiment 2, we trained OVX females on passive avoidance, administered estradiol 24 h later, and varied the interval between injection and test.

Experiment 2: estradiol increases fear generalization through an effect on fear memory retrieval

In Experiment 2, all animals were injected with EB or vehicle 24 h after training and tested at different retention intervals after injections. This methodology allowed us to assess whether estrogens are required during training in order to affect fear generalization or if they have a more long-term effect on contextual fear memory retrieval.

When EB or vehicle was administered 24 h before testing, female rats given EB displayed significant fear generalization to the neutral context compared to females administered vehicle (Fig. 3A). Independent *t*-test

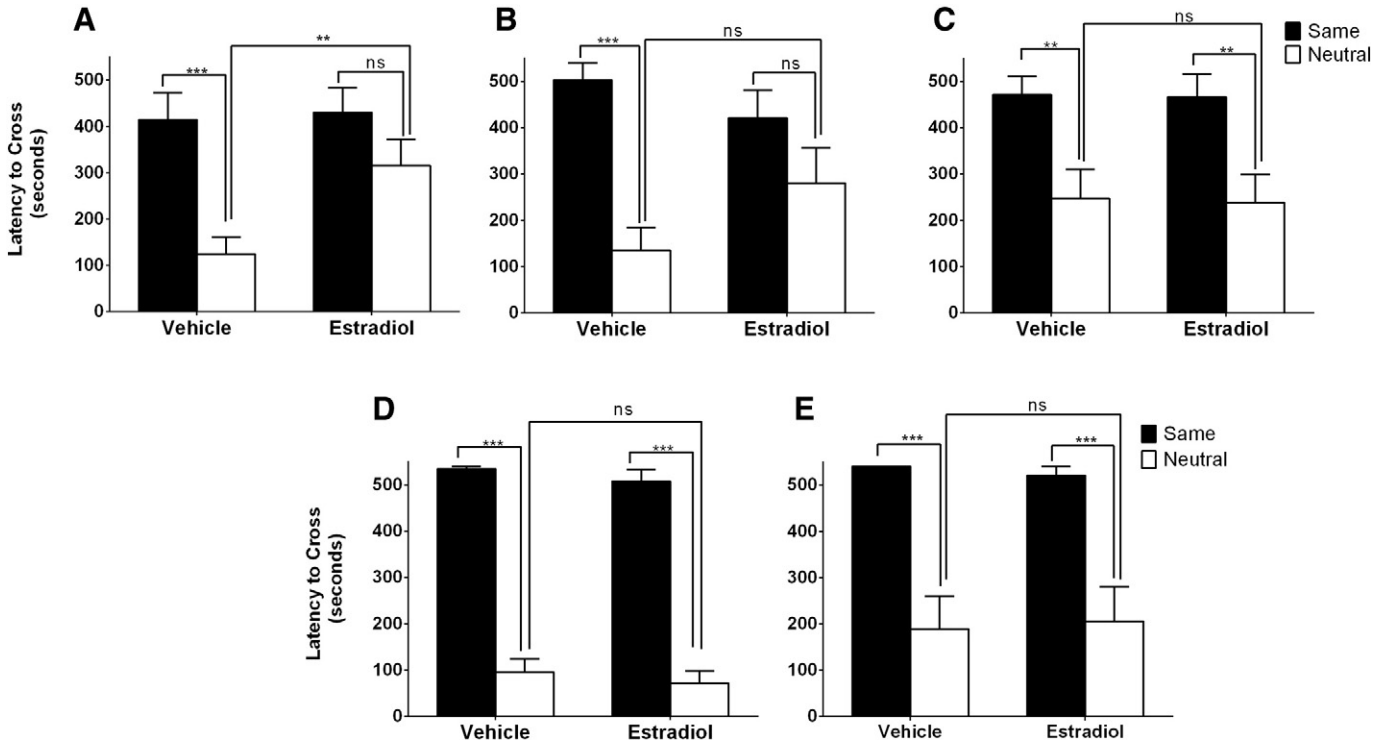

Fig. 3. Estradiol increases fear generalization through an effect on memory retrieval. A) Estradiol was administered 24 h after passive avoidance training and animals were tested 24 h after injection. As seen with injections 1 h before training, estradiol-treated animals displayed significant fear generalization whereas vehicle-treated animals did not as they showed significantly higher latencies to cross in the training context compared to the neutral context. B) When testing 48 h after injection, estradiol-treated animals continued to display significant fear generalization with comparable latencies to cross in either context. However, this generalization was not as robust as that seen at the 24 hour test as estradiol-treated animals did not show significantly higher latencies than the vehicle treated animals in the neutral context. C) When testing occurred 6 days after injection, vehicle-treated and estradiol-treated animals had higher latency to cross scores in the training context compared to the neutral context. D) Testing 1 h after injection did not elicit fear generalization, regardless of estradiol treatment. E) The same pattern of results was seen when testing occurred 6 h after injection with neither treatment group displaying significant generalization. Values are displayed as mean (±SEM) latency-to-cross in seconds. Significance values were set at $p < .05$ (** = $p < 0.01$, *** = $p < 0.001$).

analyses revealed a significant difference between vehicle-treated females but not in EB-treated females ($t_{(16)} = 4.23$, $p < 0.001$; $d = 1.99$, vehicle-treated; $t_{(18)} = 1.48$, ns; $d = 0.66$, EB-treated). In addition, vehicle-treated rats were significantly different from EB-treated rats when tested in the neutral context ($t_{(17)} = 2.80$, $p < 0.01$; $d = 1.30$). These data suggest that estradiol increases fear generalization through an effect on retrieval of contextual fear memories, and also explain why EB administration 1 h before testing resulted in fear generalization only when animals were tested 24 h later (Fig. 2D).

To determine how long fear generalization would persist following EB treatment, animals were injected with EB and tested 48 h later (Fig. 3B). Independent *t*-test analyses revealed a significant difference between vehicle-treated females but not in EB-treated females ($t_{(16)} = 5.88$, $p < 0.001$; $d = 2.63$, vehicle-treated; $t_{(18)} = 1.45$, ns; $d = 0.65$, EB-treated). However, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(18)} = 1.60$, ns; $d = 0.72$). These results suggest that EB injections administered 48 h before testing result in fear generalization in the neutral context. However, this generalization is not as robust as that seen at a 24-hour test (Fig. 3A). Although EB-treated females displayed significant generalization as indicated by similar latencies across contexts, they did not differ significantly from the vehicle-treated females tested in the neutral context (Fig. 3B). Thus, estradiol has a transient effect on fear generalization through modulation of fear memory retrieval that lasts for approximately 48 h.

To confirm that EB-treatment would not impact generalization at longer retention intervals, females were injected with EB or vehicle and tested 6 days later (Fig. 3C). Independent *t*-test analyses revealed that animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(18)} = 3.01$, $p < 0.01$; $d = 1.35$, vehicle-treated; $t_{(18)} = 2.91$, $p < 0.01$; $d = 1.30$, EB-treated). In addition, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(18)} = 0.098$, ns; $d = 0.04$). These results further suggest that estradiol increases fear generalization through a transient effect on fear memory retrieval that lasts for up to 48 h.

Although the 24 h and 2 day tests suggest a transient effect on retrieval, the 24 hour delay between EB administration and behavioral testing does not rule out potential immediate effects of EB on fear memory retrieval and generalization. Thus, to determine if estradiol has immediate effects on fear generalization, females were injected with EB and tested 1 h later (Fig. 3D). Independent *t*-test analyses revealed that animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(16)} = 15.206$, $p < 0.001$; $d = 7.17$, vehicle-treated; $t_{(16)} = 11.96$, $p < 0.001$; $d = 5.64$, EB-treated). In addition, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(16)} = 0.60$, ns; $d = 0.28$) (Fig. 3D).

Next, to rule out intermediate effects of estradiol, animals were injected with EB and tested 6 h following the injection (Fig. 3E). Independent *t*-test analyses revealed that animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(16)} = 4.95$, $p < 0.001$; $d = 2.19$, vehicle-treated; $t_{(18)} = 4.07$, $p < 0.001$; $d = 1.82$, EB-treated). In addition, vehicle-treated rats were not significantly different from EB-treated rats when tested in the neutral context ($t_{(17)} = 0.15$, ns; $d = 0.07$) (Fig. 3E). Taken together, these data suggest that estradiol increases fear generalization through an effect on fear memory retrieval.

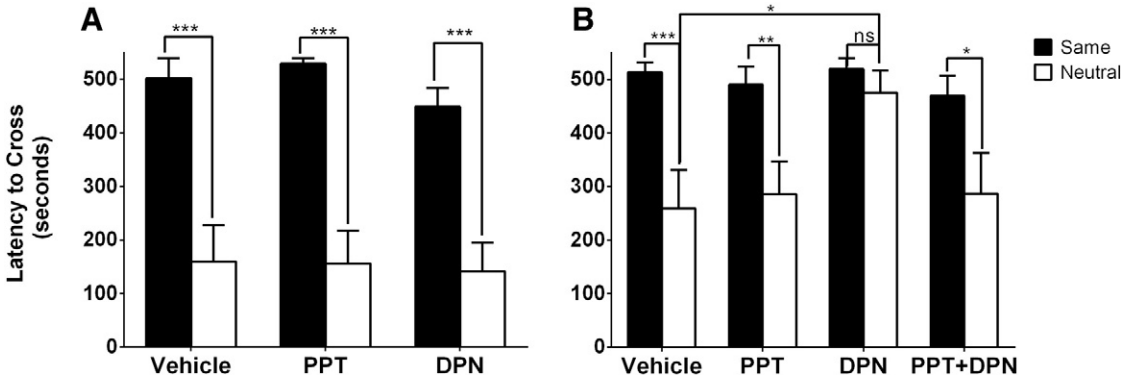

Fig. 4. Activation of ERβ increases fear generalization. A) 1 mg/kg of ERα (PPT) or ERβ agonists (DPN) did not increase fear generalization 24 h after injection. B) At a larger dose (2.5 mg), DPN increased fear generalization whereas PPT had no effect on fear generalization. The combination of DPN and PPT treatment attenuated the fear generalization elicited by DPN alone, suggesting an interaction between the two receptors. Values are displayed as mean (±SEM) latency-to-cross in seconds. Significance values were set at $p < .05$ (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Experiment 3: activation of ERβ increases fear generalization

In Experiment 3, animals were injected with PPT (ERα agonist), DPN (ERβ agonist), or vehicle, 24 h after training and tested 24 h after injection (Fig. 1C)—the time point in which estradiol increased fear generalization (Fig. 3A). Animals were injected with a 1 mg/kg or 2.5 mg dose, which affects uterine weight and sexual proceptive and receptive behaviors respectively to a similar extent as estradiol (Frasor et al., 2003; Harris et al., 2002; Mazzucco et al., 2008). When 1 mg/kg of PPT, DPN, or vehicle was administered 24 h before testing, no treatment elicited generalized responding to the neutral context (Fig. 4A). Independent *t*-test analyses revealed that animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment ($t_{(16)} = 4.39$, $p < 0.001$; $d = 2.07$, vehicle-treated; $t_{(22)} = 5.94$, $p < 0.001$; $d = 2.43$, PPT-treated; $t_{(21)} = 4.75$, $p < 0.001$; $d = 2.00$, DPN-treated). Additionally, neither PPT-treated nor DPN-treated animals differed significantly from vehicle-treated animals tested in the neutral context ($t_{(19)} = 0.039$, ns; $d = 0.02$, PPT-treated; $t_{(19)} = 0.21$, ns; $d = 0.09$, DPN-treated). When animals were injected with 2.5 mg of PPT, DPN, or both, only DPN injections increased fear generalization (Fig. 4B). Independent *t*-test analyses revealed a significant difference between vehicle-treated and PPT-treated females, but not DPN-treated females tested in the training versus neutral context ($t_{(18)} = 3.43$, $p < 0.001$; $d = 1.53$, vehicle-treated; $t_{(18)} = 2.91$, $p < 0.01$; $d = 1.30$, PPT-treated; $t_{(18)} = 0.97$, ns; $d = 0.43$, DPN-treated). However, animals given PPT and DPN treatment did not display significant fear generalization ($t_{(18)} = 2.13$, $p < 0.05$; $d = 0.95$). In addition, only DPN-treated animals differed from vehicle-treated animals tested in neutral context ($t_{(18)} = 0.28$, ns; $d = 0.16$, PPT-treated; $t_{(18)} = 2.61$, $p < 0.05$; $d = 1.72$, DPN-treated; $t_{(18)} = 0.26$, ns; $d = 0.14$, PPT + DPN-treated). These results demonstrate that DPN injections increase fear generalization to the neutral context at a 2.5 mg dose, suggesting that the effects of estradiol on memory retrieval seen in Experiment 2 (Fig. 3A) are due to activation of ERβ. Interestingly, when PPT and DPN were given simultaneously, the increased generalization was attenuated (Fig. 4B). Taken together, these data suggest that estradiol increases fear generalization through activation of ERβ.

Discussion

In the present study, we demonstrated that estradiol increases fear generalization through an effect on fear memory retrieval mechanisms by activation of ERβ. Whereas several studies have demonstrated estrogenic effects on learned fear responses (Jasnow et al., 2006; Morgan and Pfaff, 2001; Morgan et al., 2004; Nofrey et al., 2008; Toufexis et al., 2007), the present findings demonstrate that estradiol specifically increases generalization of fear to neutral contexts. These 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 findings of estrogens enhancing extinction retention, where new learning occurs about the relationship between the conditioned stimulus and absence of the unconditioned stimulus (Milad et al., 2009), the present findings are not a result of estrogens enhancing memory formation, but rather changing what cues elicit the fear memory response.

The present findings build upon our previous and novel finding that estradiol accelerates the rate of fear generalization in females (Lynch et al., 2013). Here, our initial experiments examined if estradiol increased fear generalization through an effect on fear acquisition or on consolidation. When EB was administered 6 h or 24 h before training, no effect on fear generalization was seen when rats were tested 24 h later. When EB was administered 1 h before training, females generalized fear responses to the neutral stimulus when tested 24 h later. Initially, these data suggested that estradiol increases fear generalization through an immediate effect on fear acquisition or memory consolidation. However, when EB was administered 1 h before training, but testing occurred 7 days later, no fear generalization was observed. This suggested that EB might increase fear generalization through an effect on fear memory retrieval. To test this possibility, we separated training and EB administration by 24 h and then tested rats 24 h, 48 h or 7 days after EB administration. These experiments demonstrated that estradiol increased fear generalization when administered 24 h or 48 h before memory retrieval, but not 7 days before memory retrieval. We further demonstrated that estradiol did not increase fear generalization when administered 1 h or 6 h before testing (Figs. 2D, 3D), ruling out an immediate effect of estradiol on fear memory retrieval. Estradiol has been reported to have an enhancing effect on passive avoidance retention (Rhodes and Frye, 2004), and could have enhanced retention in the current study rather than having effects on fear generalization. However, in the current study, we saw no differences between vehicle-treated and estradiol-treated animals tested in the training context, suggesting no enhancement of fear retention. In Experiment 1, the lack of differences observed between vehicle-treated and estradiol-treated animals tested in the training context may be due to a ceiling effect. However, we observed clear evidence of generalized fear in Experiment 2 (Figs. 3A, B) that cannot be attributed to a fear enhancing effect or to a ceiling effect. Taken together, these data suggest that acute estradiol has a transient effect on retrieval mechanisms to produce fear generalization to neutral contextual cues observed 24 h after an injection.

Although estradiol can have immediate effects on brain morphology and long-term potentiation (LTP) (Foy et al., 1999; Srivastava et al., 2008)—a process critical for learning and memory—those effects do not appear to elicit generalized responding to a neutral context (Figs. 2D, 3D). These results are in agreement with other spatial memory tasks where memory improvements are seen at 24 h—but not 8 h—following estradiol treatment (Sandstrom and Williams, 2004). Others have demonstrated that estrogens facilitate object placement memory only when given immediately after training; a 45 minute delay in treatment did not result in facilitated memory, suggesting that estrogens are required during memory consolidation of an object placement task (Inagaki et al., 2010). In the present study, estradiol given 24 h after training, presumably after the consolidation window is no longer open, still resulted in generalization 24 h after administration, suggesting an effect on fear memory retrieval.

One possibility to account for the present findings is that estradiol may have altered activity levels, perhaps influencing performance or consolidation of the fear memory (Morgan and Pfaff, 2001; Morgan et al., 2004). However, injections given 1 h before training only elicited fear generalization 24 h later (Fig. 2C), not 7 days later (Fig. 2D), suggesting that the consolidation process was not altered, as no generalization was seen at the later retention interval. Furthermore, injections 24 h before training demonstrated that EB treatment had no effect on memory consolidation, as we observed no generalization when testing occurred 24 h after training (Fig. 2A). These data suggest that EB does not have immediate effects on fear acquisition/consolidation. Rather, enhanced fear generalization observed following EB treatment is due to an effect on fear memory retrieval and these results may have implications for the role of genomic effects on fear memory generalization. Furthermore, few, if any, studies have assessed the effect of estrogens on memory retrieval mechanisms by treating with estrogens well after the consolidation window for memory is presumably closed.

In addition to testing the effects of estradiol on fear generalization, we also examined the effects of specific ER activation on fear generalization. ERα and ERβ receptors are 97% homologous with distinct N-terminal regions that mediate receptor interactions with different signaling pathways (Tremblay et al., 1997). ERα and ERβ are activated equally by estradiol (Kuiper et al., 1997), making it unclear which estrogen receptor subtype contributes to enhanced fear generalization when animals are given injections of estradiol. Therefore, we explicitly tested the role of each receptor subtype by injecting animals with ERα and ERβ agonists. Results demonstrate that ERβ activation drives the increased fear generalization produced by acute injections of estradiol (Fig. 4B). Interestingly, the effects of DPN were attenuated by the simultaneous delivery of PPT at the 2.5 mg dose. This finding is similar to that seen in Mazzucco et al. (2008) with proceptive and receptive sexual behaviors, suggesting a modulatory effect of ERα on ERβ. Although not explicitly tested here, the time course of the effects of EB and DPN on fear generalization suggests that estradiol acts through a genomic effect on fear memory retrieval.

The genomic effects of estrogens are regulated by cytosolic estrogen receptors (ERs), which bind to estrogen response elements (EREs) located within the promoter regions of several genes to impact transcription of those genes (Jensen and Jacobson, 1962; Levin, 2005). In addition to the classical effects of estrogens via nuclear receptors, estrogens can also initiate more rapid effects via membrane-bound receptors, which activate distinct second messenger systems that have diverse effects on cells (Kelly and Levin, 2001; Levin, 2005; Vasudevan et al., 2001). Although our immediate (1 hour test) and intermediate (6 hour test) time points explored in Experiment 2 do not directly test membrane-bound versus cytosolic ERs, the lack of fear generalization at those early time points suggests that the fear generalization seen as a result of EB treatment is not due to rapid effects of membrane-bound receptors. However, these data do not eliminate the possibility of genomic effects initiated by membrane-bound receptor activation.

In the current study, we found generalization 24 h after estradiol administration rather than 7 days after estradiol treatment, which is different from what was observed with chronic estradiol. This finding is not surprising given that several reports suggest differences in effects of estradiol on behavior with chronic versus acute exposure (Luine et al., 1998; Walf and Frye, 2006). Therefore, the differences between the two studies could be due to compensatory mechanisms of chronic estrogen exposure versus acute treatment. Acute estradiol treatment may more accurately reflect the endogenous estrogen surge during the estrous cycle as compared to chronic estradiol implants, and thusly, may more accurately represent the effects of estrogens on fear generalization. In the present study, the main focus was to manipulate the interval between estradiol administration and training in Experiment 1 or estradiol administration and testing in Experiment 2. These manipulations allowed us to determine when estradiol is required to elicit fear generalization. However, maintaining these intervals led to different intervals between training and testing in the two experiments. Therefore, one could argue that estradiol might have an effect on fear generalization when it is present during fear acquisition or memory consolidation if testing occurred 48 h after training. We did not explicitly examine 48 hour test intervals in Experiment 1, but the fact that fear generalization was not observed 7 days after training when estradiol was present during the memory consolidation period strongly suggests that estradiol is not required during training to enhance fear generalization.

Several studies have demonstrated the importance of the hippocampus in the generalization of fear and memory precision (Ruediger et al., 2011; Wiltgen and Silva, 2007; Wiltgen et al., 2010; Winocur et al., 2007). ERα and ERβ are found in the hippocampus, and ERβ is more widely distributed throughout the hippocampus (Li et al., 1997; Österlund et al., 1998; Shughrue and Merchenthaler, 2000a,b; Shughrue et al., 1997). A wealth of evidence demonstrates that estrogens play a major role in sex differences in behavior and that estrogens act directly on the hippocampus to affect synaptic plasticity and learning and memory (Frye et al., 2000; Spencer-Segal et al., 2012). Acquisition of hippocampal-dependent memories is accompanied by increases in dendritic spines (for a review see Urbanska et al., 2012) in the CA1 region of male and female rats (Beltrán-Campos et al., 2011; Leuner et al., 2003), and dendritic spine density in the hippocampus is sensitive to estrogen levels. For instance, in the CA1 region of the hippocampus, dendritic spine density changes 30% across the stages of the estrous cycle in female rats with the highest density of dendritic spines seen during the proestrus stage, when estrogen levels are highest (Woolley and McEwen, 1992). In addition, ovariectomy, which reduces endogenous levels of estrogens, leads to a decrease in dendritic spine density (Beltrán-Campos et al., 2011; Gould et al., 1990). In ERβ knockout mice (BERKO), LTP is reduced and hippocampal-dependent context fear conditioning is impaired (Day et al., 2005) whereas activation of ERβ increases LTP and dendritic branching in the hippocampus (Liu et al., 2008), suggesting that ERβ activation may be involved in estrogenic effects in synaptic plasticity and hippocampal-dependent learning and memory.

In addition to the CA1 region of the hippocampus, estrogens may act on the CA3 region to influence fear generalization. Hippocampal-dependent learning involves structural rearrangements in mossy fiber terminals involving feedforward excitation and inhibition (Ruediger et al., 2011, 2012). Specifically, contextual fear conditioning results in a large, yet transient, increase in feedforward inhibitory synapses. These feedforward inhibitory synapses originate from large mossy fiber filopodia onto CA3 parvalbumin-expressing, GABAergic interneurons. The time-dependent decline in feedforward inhibitory synapses is correlated with the time-dependent increase in fear generalization (Ruediger et al., 2011). ERs are expressed in CA3 principle neuron dendrites and terminals of large mossy fibers, suggesting that they modulate plasticity in the CA3 (Milner et al., 2005). More specifically, within the CA3 region, ERβ is highly expressed in dendrites and axons of mossy fiber projections, which has led researchers to speculate that

activation of ERβ within this region may elicit non-genomic actions (Milner et al., 2005). However, some ERβ are localized to the cytosol and membrane-bound receptors can elicit genomic changes normally seen with activation of classical estrogen receptors through the initiation of molecular cascades (Milner et al., 2005; Vasudevan and Pfaff, 2007). In addition, estrogens may modulate the connections made between the CA3 and CA1 regions of the hippocampus by increasing the ability of CA3 neurons to synchronize with CA1 targets (Woolley et al., 1998; Yankova et al., 2001). Thus, estrogens may be acting within this circuit to drive fear generalization through modulations to the CA3 region.

In addition to impacting synaptic plasticity in the hippocampus, estrogens interact with the stress response system. For example, pituitary adenylate cyclase activating peptide (PACAP) has been implicated in anxiety-like behaviors (e.g. Hammack et al., 2010), and high levels of PACAP are associated with greater PTSD symptoms and greater fear responses in a fear discrimination task (Ressler et al., 2011). Moreover, a polymorphism in the receptor for PACAP (PAC1), which contains an estrogen response element (ERE), is highly associated with PTSD only in females, suggesting a link between estrogens, the PACAP system and PTSD (Ressler et al., 2011). However, a study by the same group found that low estrogen levels may be a vulnerability factor for PTSD (Glover et al., 2012), emphasizing the complexity in the interaction between anxiety disorders and sex hormones. Another stress neurohormone associated with fear and anxiety-like behavior, corticotropin-releasing hormone (CRH), also contains EREs. Treatment with EB leads to an increase in CRH mRNA expression in the central nucleus of the amygdala, and may provide a mechanism where CRH serves as an intermediary between estrogens and alterations in fear responses (Jasnow et al., 2006). Thus, the interaction of estrogens with PACAP and downstream elements of the stress response may be another mechanism through which estrogens modulate fear generalization in addition to its modulation of hippocampal functioning and fear memory retrieval.

Taken together, these experiments lead to a better understanding of the primary mechanisms through which estrogens enhance fear generalization. We demonstrated that estradiol, through activation of ERβ enhances fear generalization by modulating fear memory retrieval mechanisms. 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.

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