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Aromatized testosterone attenuates contextual generalization of fear in male rats

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

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

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ABSTRACT

Generalization is a common symptom of many anxiety disorders, and females are 60% more likely to suffer from an anxiety disorder than males. We have previously demonstrated that female rats display significantly accelerated rates of contextual fear generalization compared to male rats; a process driven, in part, by activation of ERβ. The current study was designed to determine the impact of estrogens on contextual fear generalization in male rats. For experiment 1, adult male rats were gonadectomized (GDX) and implanted with a capsule containing testosterone proprionate, estradiol, dihydrotestosterone proprionate (DHT), or an empty capsule. Treatment with testosterone or estradiol maintained memory precision when rats were tested in a different (neutral) context 1 day after training. However, male rats treated with DHT or empty capsules displayed significant levels of fear generalization, exhibiting high levels of fear in the neutral context. In Experiment 2, we used acute injections of gonadal hormones at a time known to elicit fear generalization in female rats (e.g. 24 h before testing). Injection treatment followed the same pattern of results seen in Experiment 1. Finally, animals given daily injections of the aromatase inhibitor, Fadrozole, displayed significant fear generalization. These data suggest that testosterone attenuates fear generalization likely through the aromatization testosterone into estradiol as animals treated with the non-aromatizable androgen, DHT, or animals treated with Fadrozole, displayed significant generalized fear. Overall, these results demonstrate a sex-dependent effect of estradiol on the generalization of contextual fear.

1. Introduction

Over the last several years, our lab has investigated gonadal hormone modulation of context fear generalization in female rodents. Fear generalization can be a temporally dynamic process where animals lose the ability to recognize contextual cues that were associated with an aversive stimulus over time. For example, when rodents receive foot-shocks in one (training) context and are tested in a different (neutral) context 24 h later, animals can discriminate the contexts and display less fear behavior. However, when tested after longer delays (14–36 days), rodents exhibit similar heightened fear responses to the training and neutral contexts (Cullen et al., 2015; Jasnow et al., 2012; Lynch et al., 2013, 2014; Wiltgen and Silva, 2007; Wiltgen et al., 2010). Thus, over time, rodents generalize their fear response to neutral contexts.

Fear generalization is a symptom of several anxiety disorders, in which females represent a disproportionately large percentage (Cloitre et al., 2004; Kessler et al., 1994; Lissek, 2012; Lissek et al., 2010; Wang et al., 2005). Unlike the temporally-dependent fear generalization described above, we found that female rats displayed a faster rate of fear generalization to a neutral context after passive avoidance training than male rats; an effect driven, in part, by estrogens (Lynch et al., 2013). We subsequently found that injections of 17β-estradiol induced fear generalization by affecting memory retrieval rather than acquisition or consolidation. Additionally, using estrogen receptor (ER) agonists, we found that estradiol-induced generalization in ovariectomized females occurs through activation of estrogen receptor-beta (ERβ) rather than estrogen-receptor-alpha (ERα) (Lynch et al., 2014). Finally, estradiol acts on the dorsal CA1 to induce generalization, whereas this region is not involved in temporally-dependent generalization (Cullen et al., 2015; Lynch et al., 2016). What remains unanswered is why male rats, which produce estrogens through aromatization of testosterone (Bon-chu and Meng-Chun, 2002; Simpson and Davis, 2001), are able to maintain memory specificity over a longer period of time compared to female rats. One possibility is that the level of estradiol produced by aromatization of testosterone in the brain is not sufficient to induce fear generalization in male rats. Exogenous administration of estradiol in male rats would, therefore, induce fear generalization as seen in females. Alternatively, aromatized testosterone may help maintain memory specificity over time in male rats, thereby reducing fear generalization. Similarly, testosterone can be converted to dihydrotestosterone (DHT) via 5α-reductase (Andriole et al., 2004) and DHT can be further converted into 5α androstane 3β, 17β diol (3β-diol) via

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

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several enzymes. 3β-diol can act upon ERβ, which may also maintain memory specificity over time in males (Gangloff et al., 2003; Jin and Penning, 2001; Kuiper et al., 1996, 1998; Steckelbroeck et al., 2004; Törn et al., 2003; Weihua et al., 2006). A final possibility is that androgens (testosterone or DHT) act directly on androgen receptors to help maintain memory specificity. Nothing is currently known about how gonadal hormones affect fear generalization in male rats. But, existing literature on anxiety, a related yet different behavioral phenomenon, suggests that low testosterone is correlated with the development of anxiety disorders. These data suggest that testosterone may serve a protective role in the development of anxiety disorders (Barrett-Connor et al., 1999; Cooper and Ritchie, 2000; Kaminetsky, 2005; Veras and Nardi, 2010). Additionally, post-pubertal castration increases anxiety-like behavior, and testosterone can reduce anxiety-like behavior in male and female rodents (Bitran et al., 1993; Edinger and Frye, 2004; Forman et al., 1989; McDermott et al., 2012; Molina et al., 1994; Romero et al., 1988). The evidence therefore, supports androgens such as testosterone reducing anxiety-like behavior in male and female rats, but the ultimate mechanisms responsible for these effects are not well understood. How the effects of testosterone on anxiety-like behavior are related to its potential role in fear generalization are also unknown.

Our data in female rats show that estradiol induces fear generalization through effects within the dorsal hippocampus and on memory retrieval (Lynch et al., 2013, 2014, 2016). We hypothesized that male rats show a slower rate of fear generalization compared to females due to the presence of testosterone and its actions on androgen receptors. Therefore, we predicted that gonadectomy and/or estradiol administration would induce fear generalization. Administration of testosterone or DHT through its actions on androgen receptors, however, would attenuate fear generalization. DHT cannot be metabolized into estradiol and, therefore, cannot act at estrogen receptors unlike testosterone (Andriole et al., 2004). In order to test our hypotheses, male gonadectomized rats were given silastic capsule implants to chronically administer testosterone proprionate, estradiol benzoate, or DHT. Rats were then tested in the training context or a neutral context for fear generalization 1 or 7 days later. In an additional experiment, animals were given peripheral injections of hormones to assess the acute effects of hormone treatment on fear generalization, similar to our previous study in females (Lynch et al., 2014). Finally, to assess the role of aromatized testosterone, DHT or fadrozole (FAD), an aromatase inhibitor, was administered to males prior to fear generalization testing.

2. Methods

2.1. Animals and housing conditions

Adult male Long Evans rats approximately 90 days old at the time of surgery were used for all experiments. Eleven days prior to behavioral manipulations, animals were gonadectomized (GDX) or left intact, and then individually housed and maintained on a 14/10 h light/dark cycle. Food and water were available *ad libitum* throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

2.2. Surgical procedures

For gonadectomies, adult male rats were anesthetized with isoflurane and received a bilateral gonadectomy. Briefly, animals were given a small ventral incision in the scrotal skin and the testes were removed, the wound was sutured using absorbable gut suture and surgical staples. Animals were allowed to recover for 9 days and then were handled for 5 min a day for 2 consecutive days within the animal colony before passive avoidance training.

2.3. 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). Male rats were trained in passive avoidance 11 days after gonadectomy. 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 m room with house fluorescent lights and contained bare white walls and no artificial scents or sounds and was cleaned with 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 quatricide. In each context, the experimenter wore different gloves (Rubber dish glove in A; vinyl lab glove in B) to hold the rat. 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 memory. 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.

2.4. Drug administration

For chronic hormone exposure, animals were given silastic capsules containing hormone (estradiol, testosterone proprionate, or DHT) or an empty capsule (Sigma Aldrich). The silastic (polydimethylsiloxane) implants were constructed from silastic tubing (i.d. 0.078 in., o.d. 0.125 in.) and cut to a 5 mm length for estradiol (Bridges, 1984; Hiroi and Neumaier, 2006) and 14 mm length for testosterone proprionate or DHT (Edinger and Frye, 2004; Frye and Seliga, 2001). Each end of the capsule was filled with Factor II medical adhesive 1–2 mm in length. The hormone was packed into the remaining length of the capsule. Before implantation, all capsules were incubated in 0.9% saline solution for 24 h at 37 °C.

For acute hormone exposure to estradiol, animals received injections of estradiol dissolved in sesame oil (15 μg/0.1 mL) or vehicle (sesame oil, 0.1 mL) (Chang et al., 2009; Lynch et al., 2013, 2014; Zeidan et al., 2011). For acute exposure to androgens, animals received injections of testosterone proprionate (2 mg/0.1 mL), DHT (2 mg/0.1 mL), or vehicle (sesame oil, 0.1 mL). For distinct activation of ER subtypes, animals received injections of the ERα agonist, 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*-phenol; Caymen Chemical (PPT) (2.5 mg/0.1 mL), the ERβ agonist, 2,3-*bis*(4-hydroxyphenyl)-propionitrile, Caymen Chemical (DPN) (2.5 mg/0.1 mL) (Lynch et al., 2013, 2014; Meyers et al., 2001; Stauffer et al., 2000), or vehicle (DMSO, 0.1 mL). In order to block the conversion of testosterone into estradiol, GDX and intact male rats were given daily injections of the aromatase inhibitor, Fadrozole (FAD) (1 mg/kg) (Graham and Milad, 2014) for seven days prior to, and during, behavioral training.

2.5. Testosterone assays

Trunk blood was collected immediately after completion of testing. All blood samples were allowed to clot for 1 h at room temperature, then centrifuged at 3500 rpm for 1 h at 4 °C and stored at −80 °C until processed. Serum testosterone was measured using Enzo Life Sciences testosterone enzyme-linked immunosorbent assay (EIA) kits (Farmingdale, NY) according to the manufacturer's instructions. Each

sample was run in duplicate. The cross reactivity for the testosterone assay was 14.64% for 19-hydroxytestosterone, 7.20% for androstendione, and <0.01% for all other hormones and the inter-assay variability was <10% for all plates.

2.6. Statistical analyses

In each experiment, the effects of hormone treatments were examined by factorial ANOVAs and independent *t*-test analyses to make direct comparisons of vehicle-treated males tested in training or neutral context, estradiol-treated males tested in the training or neutral context, and vehicle-treated and estradiol-treated males in the neutral context. Statistical significance was set at $p < 0.05$. 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) for *t*-tests as well as eta squared (η^2) for ANOVAs.

3. Results

3.1. Chronic exposure to testosterone or estradiol prevents generalization in male rats

In order to assess the effects of gonadal hormones on fear generalization in male rats, gonadectomized males were given capsules containing either testosterone proprionate, estradiol benzoate, DHT, or an empty capsule (vehicle) and trained in passive avoidance (Fig. 1A). Animals were then tested in either the training context or a neutral context 1 day (Fig. 1A, B) or 7 days (Fig. 1A, C) after training. A factorial ANOVA of animals tested 1 day after training revealed a significant main effect for context, ($F_{(1,83)} = 32.82$, $p < 0.001$, $\eta^2 = 0.64$), 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, ($F_{(3,83)} = 3.27$, $p < 0.05$, $\eta^2 = 0.19$), indicating significant differences between animals given different

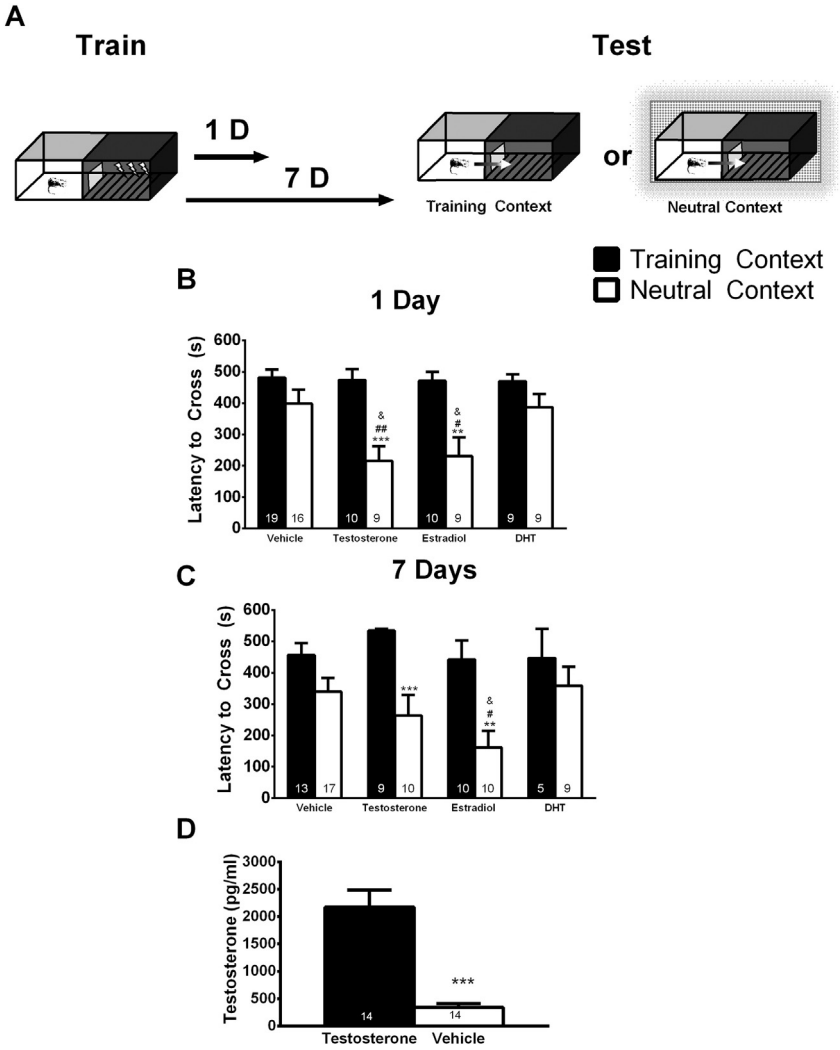

Fig. 1. Chronic testosterone or estradiol prevents fear generalization to a neutral context. A) Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and tested 24 h or 7 days later in either the training context or a neutral context. B) Animals implanted with testosterone or estradiol capsules displayed significant discrimination between the training and neutral context 1 day after training. However, animals treated with DHT or given no hormone replacement demonstrated significant generalization, suggesting that testosterone and estradiol attenuate generalized fear responding in gonadectomized males. C) Testing 7 days after training results in the same pattern as seen at 1 day. Testosterone and estradiol treated animals display no significant generalization whereas those treated with DHT or are given no hormone replacement display significant generalization. D) Serum testosterone concentrations were confirmed by collecting trunk blood from a subset of animals. Testosterone levels were significantly elevated in animals given testosterone capsules compared to control animals that received no hormone replacement. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < 0.05$. ($^*/\&/^{\#} = p < 0.05$, $^{**}/^{\#\#} = p < 0.01$, $^{***}/^{\#\#\#} = p < 0.001$). Numbers within each bar represent N values for each group. # compares groups to vehicle neutral; & compares groups to DHT neutral.

hormone treatments. The interaction term was also significant, ($F_{(3,83)} = 2.90$, $p < 0.05$, $\eta^2 = 0.17$), suggesting a difference between treatments based on the context of testing. For animals tested 7 days after training, ANOVA revealed a significant main effect for context, ($F_{(1,75)} = 23.07$, $p < 0.001$, $\eta^2 = 0.70$), a non-significant main effect for treatment, ($F_{(3,75)} = 1.65$, ns, $\eta^2 = 0.15$), and a non-significant interaction, ($F_{(3,75)} = 1.66$, ns, $\eta^2 = 0.15$). In addition to the ANOVA, 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 that animals treated with testosterone displayed significant discrimination between the training context and the neutral context at either retention interval (1 D; $t(17) = 4.48$, $p < 0.001$, d = 2.04; 7 D: $t(17) = 3.90$, $p < 0.001$, d = 1.85). Contrary to our hypothesis, estradiol treatment also resulted in significant discrimination between contexts at either retention interval (1 D: $t(17) = 3.72$, $p < 0.001$, d = 1.68; 7 D: $t(18) = 3.49$, $p < 0.01$, d = 1.56). Testosterone-treated and estradiol-treated animals tested in the neutral context displayed significantly less fear as demonstrated by lower latency to cross compared to control animals (empty capsule) when tested at 1 day (TP: $t(23) = 2.65$, $p < 0.01$, d = 1.14; estradiol: $t(23) = 2.25$, $p < 0.05$, d = 0.94). Empty capsule-treated (Control) and DHT-treated males, however, displayed significant fear generalization at both retention intervals (Control; 1 D: $t(33) = 1.67$, ns, d = 0.56; 7 D: $t(28) = 1.93$, ns, d = 0.72) (DHT; 1 D: $t(16) = 1.72$, ns, d = 0.81; 7 D: $t(12) = 0.83$, ns, d = 0.45). At 7 days, estradiol-treated animals had significantly lower generalization than controls tested in the neutral context although testosterone-treated animals were not different than controls (TP: $t(25) = 0.99$, ns, d = 0.39; estradiol: $t(25) = 2.53$, $p < 0.05$, d = 1.01). In addition, at 1 day, animals treated with DHT displayed significantly more fear in the neutral context compared to testosterone-treated and estradiol-treated animals tested in the neutral context (TP: $t(16) = 2.70$, $p < 0.05$,d = 1.27; estradiol: $t(16) = 2.11$, $p < 0.05$, d = 1.00). At 7 days, DHT-treated animals had higher fear generalization than estradiol-treated animals tested in the neutral context, but were not significantly different from testosterone-treated animals (TP: $t(17) = 1.05$, ns, d = 0.48; estradiol: $t(17) = 2.45$, $p < 0.05$, d = 1.12).

To confirm that capsule implantation produced significant levels of testosterone, trunk blood was collected from a subset of animals. Serum testosterone concentrations confirmed gonadectomy in blank capsule-treated males and were significantly elevated in testosterone-treated males ($t(26) = 5.62$, $p < 0.001$, d = 2.12), (Fig. 1D).

These results demonstrate that testosterone and estradiol attenuate generalized fear in gonadectomized male rats. Further, these data suggest that aromatization, rather than androgen receptors, is the likely route through which testosterone acts to attenuate fear generalization because DHT-treated and control males displayed significant fear generalization.

3.2. Acute exposure to testosterone and estradiol prevents fear generalization in male rats

In a separate experiment, animals were gonadectomized and given injections of testosterone, estradiol, DHT, or vehicle to assess the effects of acute hormone exposure on fear generalization. Previously, we demonstrated that estradiol injections induced generalization to a neutral context in ovariectomized females when the hormone was administered 24 h before a retrieval test (Lynch et al., 2014, 2016). Therefore, we administered hormones to gonadectomized male rats at this same time. Animals were trained in passive avoidance, injected 24 h later, and tested 24 h after injection in either the training or neutral context (Fig. 2A). ANOVA revealed a significant main effect for context, ($F_{(1,89)} = 21.39$, $p < 0.001$, $\eta^2 = 0.63$), a non-significant main effect for treatment, ($F_{(3,89)} = 2.374$, $p = 0.08$, $\eta^2 = 0.21$), and a non-significant interaction, ($F_{(3,89)} = 1.70$, ns, $\eta^2 = 0.15$). Similar to chronic exposure, animals injected with testosterone displayed significant discrimination between the training context and the neutral context ($t(13) = 2.49$, $p < 0.05$, d = 1.43). Additionally, estradiol-treated animals discriminated significantly between contexts ($t(16) = 3.329$, $p < 0.01$, d = 1.57) (Fig. 2B). Testosterone treated animals displayed significantly lower latencies in the neutral context compared to vehicle- and DHT-treated animals (Veh: $t(24) = 2.49$, $p < 0.05$, d = 1.01; DHT: $t(28) = 2.23$, $p < 0.05$, d = 1.05) and estradiol-treated animals trended towards significantly lower latencies compared to the other two groups (Veh: $t(23) = 1.82$, $p = 0.08$, d = 0.72; DHT: $t(26) = 1.97$, $p = 0.06$, d = 0.76). Similar to the chronic treatments, vehicle-treated and DHT-treated males displayed significant generalization to the neutral context (Veh: $t(29) = 1.83$, ns, d = 0.66; DHT: $t(31) = 1.33$, ns, d = 0.48), again suggesting that testosterone reduces generalization through conversion to estradiol.

3.3. ERα and ERβ mediate reduced fear generalization in male rats

Estradiol-induced generalization in females is mediated by activation of cytosolic estrogen receptor β (ERβ), but not ERα (Lynch et al., 2014, 2016). In order to determine which ER subtype mediates attenuated fear generalization in males, animals were given injections of ER agonists, PPT or DPN, which activate ERα or ERβ, respectively (Fig. 2A). ANOVA revealed a significant main effect for context, ($F_{(1,85)} = 36.98$, $p < 0.001$, $\eta^2 = 0.79$), a non-significant main effect for treatment, ($F_{(3,85)} = 0.69$, ns, $\eta^2 = 0.04$), and a trending interaction, ($F_{(3,85)} = 2.50$, $p = 0.07$, $\eta^2 = 0.16$) (Fig. 2C). Again, estradiol injections reduced generalization whereas vehicle treated animals displayed significant generalization (Estradiol: $t(15) = 3.992$, $p < 0.001$, d = 1.88; Veh: $t(24) = 0.9799$, ns, d = 0.39). Additionally, activation of either ERα or ERβ reduced generalization (ERα: $t(23) = 3.296$, $p < 0.01$, d = 1.65; ERβ: $t(23) = 3.666$, $p < 0.001$, d = 1.74). Animals given the ERα or ERβ agonist and tested in the neutral context were not significantly different from vehicle-treated animals whereas estradiol-treated animals were significantly different (ERα: $t(27) = 1.545$, ns, d = 0.60; ERβ: $t(22) = 0.704$, ns, d = 0.69; estradiol: $t(18) = 2.338$, $p < 0.05$, d = 1.02). These results suggest that the reduction of generalization through estradiol in male rats is mediated by either estrogen receptor subtype.

3.4. Fadrozole prevents testosterone-reduced generalization

We next wanted to directly determine if the reduction in generalization produced by testosterone is due to the aromatization of testosterone into estradiol. Gonadectomized animals were implanted with capsules containing testosterone or no hormone and were administered daily injections of the aromatase inhibitor, FAD, or vehicle (Veh), for 7 days continuing through passive avoidance training. A group of animals were left intact and also received FAD or Veh injections for 7 days. Animals were then tested 1 day after training in either the training or a neutral context (Fig. 3A). An ANOVA revealed a significant main effect for context, ($F_{(1,38)} = 10.44$, $p < 0.01$, $\eta^2 = 0.49$), a non-significant main effect for treatment, ($F_{(3,38)} = 1.82$, ns, $\eta^2 = 0.25$), and a non-significant interaction, ($F_{(3,38)} = 1.86$, ns, $\eta^2 = 0.26$) (Fig. 3B). Animals given testosterone capsules and vehicle injections displayed significant discrimination as seen previously ($t(9) = 3.292$, $p < 0.001$, d = 2.05). Animals given no hormone replacement and injections of vehicle or FAD displayed significant generalization, demonstrating that FAD by itself does not impact generalization (Veh: $t(8) = 0.6286$, ns, d = 0.40; FAD: $t(7) = 1.18$, ns, d = 1.00). When gonadectomized male rats given testosterone were administered daily injections of FAD, they displayed significant generalized fear to a neutral context ($t(11) = 2.016$, ns, d = 1.27). However, the latency to cross in the neutral context only approached significance compared to control rats given testosterone only, ($t(13) = 1.986$, $p = 0.07$, d = 0.82). This was possibly due to testosterone capsule treatment competing with FAD. In order to

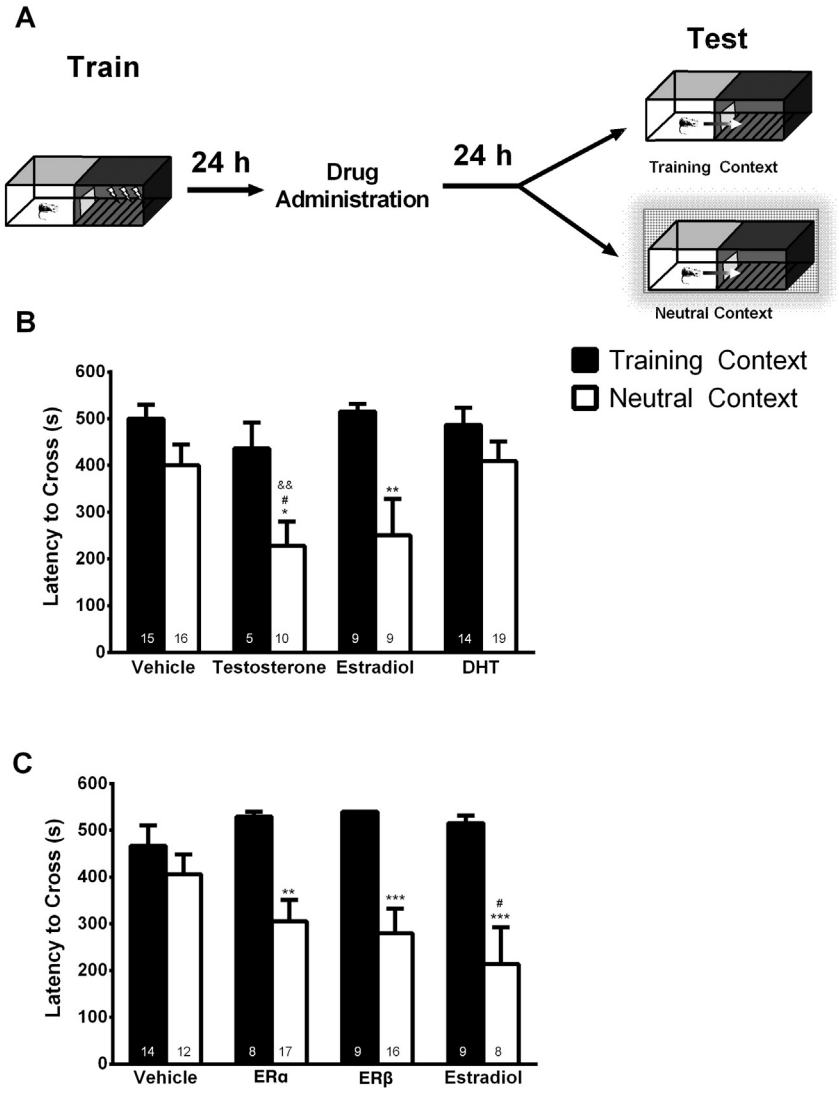

Fig. 2. Acute injections of testosterone or estradiol prevent fear generalization through activation of either ERα or ERβ. A) Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 h later were injected with drug treatment. Twenty-four hours after drug administration, animals were tested in either the training or neutral context. B) Animals given acute injections of testosterone or estradiol do not display significant generalization whereas those treated with DHT or are given no hormone replacement display significant generalization. C) Animals were injected with an ERα (PPT) or ERβ (DPN) agonist to assess which estrogen receptor prevents generalized fear in gonadectomized males. Activation of either estrogen receptor subtype prevented generalized fear as did injections of estradiol. Vehicle treated animals displayed significant generalization to the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < 0.05$. ($^{*}/^{\#} = p < 0.05$, **/&&/$^{\#\#} = p < 0.01$, $^{***}/^{\#\#\#} = p < 0.001$). Numbers within each bar represent N values for each group. # compares groups to vehicle neutral; & compares groups to DHT neutral.

test the effects of FAD with natural levels of testosterone present, intact males were given daily injections of FAD or vehicle and trained and tested in passive avoidance (Fig. 3C). An ANOVA revealed a significant main effect for context, ($F_{(1,23)} = 7.65$, $p < 0.01$, $\eta^2 = 0.53$), a significant main effect for treatment, ($F_{(1,23)} = 4.35$, $p < 0.05$, $\eta^2 = 0.30$), and a non-significant interaction, ($F_{(1,23)} = 2.46$, ns, $\eta^2 = 0.17$). Intact male rats given vehicle displayed significant discrimination between contexts ($t(12) = 3.951$, $p < 0.01$, $d = 2.19$). In contrast, intact males treated with FAD displayed significant generalization ($t(11) = 0.6897$, ns, $d = 0.43$), and displayed significantly more fear in the neutral context compared to vehicle-treated males ($t(16) = 3.097$, $p < 0.01$, $d = 1.46$). These results demonstrate that endogenous and exogenous testosterone attenuates fear generalization in male rats through its aromatization into estradiol; FAD treatment results in fear generalization to a neutral context.

4. Discussion

In the present study, we assessed the effects of gonadal hormones on fear generalization in male rats. We found that estradiol and testosterone both reduce fear generalization in male rats. Gonadectomized male rats treated with testosterone or estradiol chronically or through acute injections, displayed significant contextual discrimination whereas control animals receiving no hormone replacement displayed significant generalization to a neutral context. These results are in direct contrast with our previous findings in females, in which estradiol *induces* generalized fear (Lynch et al., 2013, 2014, 2016). Additionally, we demonstrated that the actions of testosterone are the result of its aromatization into estradiol; animals treated with non-aromatizeable DHT or empty capsules displayed significant generalization. Also, contextual discrimination in testosterone-treated or intact male rats was blocked by aromatase inhibition with FAD. Taken together with our previous data, these results reveal a sex-dependent effect of estradiol on the generalization of fear—estradiol *induces* generalized fear in female rats whereas estradiol *attenuates* generalized fear in male rats (Lynch et al., 2013, 2014).

Testosterone can be converted into several different metabolites including estradiol and DHT. DHT is a potent androgen that has higher affinity and efficacy on androgen receptors than testosterone (Deslypere

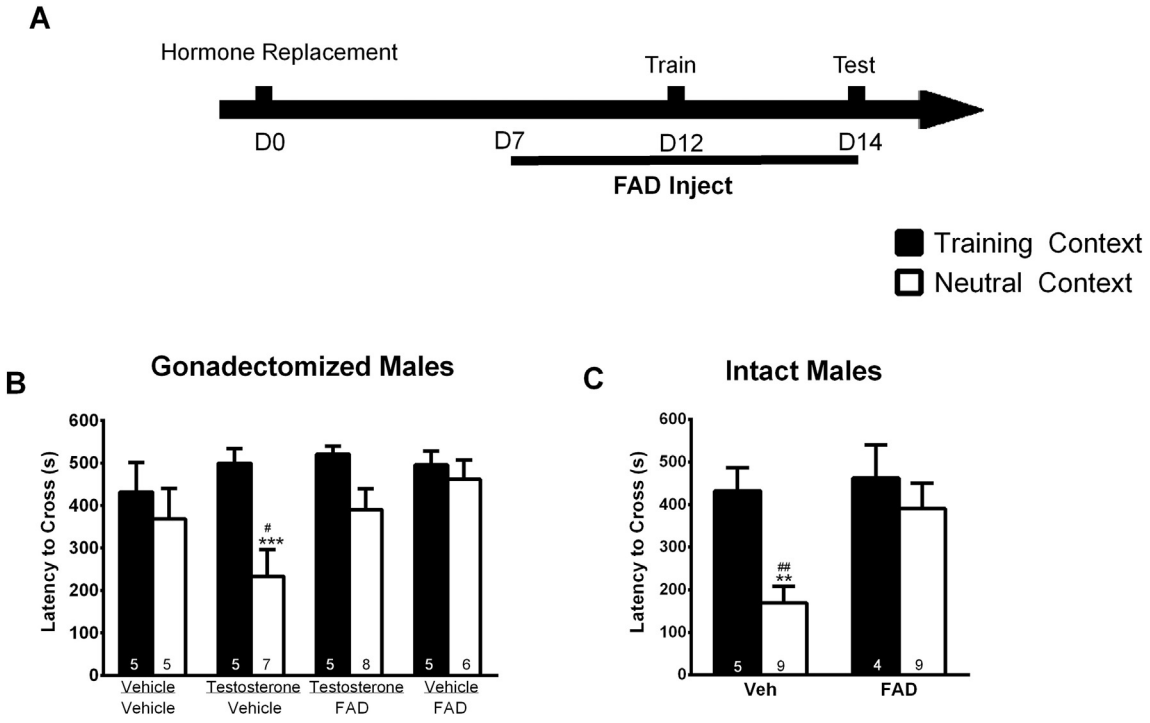

Fig. 3. Pre-treatment with the aromatase inhibitor, Fadrozole, blocks the prevention of generalization from acute testosterone treatment. A) Schematic of the experimental paradigm for the experiment. All animals were given injections of Fadrozole (FAD) or Veh for 7 days up through passive avoidance training and drug administration. Animals were trained in passive avoidance and 24 h later, they were injected with testosterone or vehicle. Twenty fours after treatment, animals were tested in the training or neutral context. B) Animals injected for 7 days with vehicle and treated with testosterone 24 h prior to testing did not display significant generalization. In comparison, animals treated with FAD for 7 days and injected with testosterone display significant generalization, suggesting that testosterone prevents generalization through its conversion into estradiol. Animals treated with vehicle displayed significant generalization as did animals injected for seven days with FAD. C) Animals were left intact and given daily injections of FAD or Veh through passive avoidance training. Animals were tested 48 h after training and those animals treated with FAD display significant generalization whereas intact males injected with vehicle do not generalize fear to the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p < 0.05$. ($^{*/\#} = p < 0.05$, $^{**/\#\#} = p < 0.01$, $^{***/\#\#\#} = p < 0.001$). Numbers within each bar represent N values for each group. # compares groups to vehicle neutral.

et al., 1992; Wilbert et al., 1983). DHT is metabolized from testosterone through 5α-reductase, and DHT cannot be metabolized into estradiol (Andriole et al., 2004). Treatment with testosterone in the current study resulted in reduced generalized fear. However, when rats were treated with DHT, they displayed significant generalization, similar to control animals without hormone replacement. Taken together with the effects of estradiol, these data suggest that testosterone acts primarily through aromatization to estradiol to influence contextual fear generalization in male rats. Although we did not block androgen receptors directly, DHT did not reduce generalization similar to testosterone, making androgen receptors an unlikely mediator of this effect. The finding that DHT does not reduce fear generalization in male rats suggests that actions of testosterone on generalization are mediated via activation of ERs rather than ARs. However, DHT can be converted into 5α androstane 3β, 17β diol (3β-diol) via several enzymes and 3β-diol can act upon ERβ (Gangloff et al., 2003; Jin and Penning, 2001; Kuiper et al., 1996, 1998; Steckelbroeck et al., 2004; Törn et al., 2003; Weihua et al., 2006) potentially reducing generalized fear in GDX male rats. Although an intriguing possibility, DHT does not likely modulate fear generalization through conversion into 3βdiol and actions at ERβ because DHT alone did not reduce fear generalization in GDX male rats; an effect we observed with estradiol treatment or treatment with the ERβ agonist, DPN.

The conversion of testosterone into estradiol is implicated in affecting several types of behavior in males including sex behavior (Vagell and McGinnis, 1997) and spatial memory (Packard et al., 1996). Similar to estradiol's role in facilitating fear extinction in females (Graham and Milad, 2013; Lebron-Milad and Milad, 2012; Milad et al., 2009, 2010), aromatase activity is linked to enhanced fear extinction in males; blocking aromatase impairs extinction learning (Graham and Milad, 2014). Here, we demonstrate that testosterone reduces generalization and does so through metabolism into estradiol. Treatment with DHT, which cannot be aromatized into estradiol, does not reduce generalization. Additionally, when the synthesis of estradiol was blocked with FAD, gonadectomized male rats treated with testosterone and intact males displayed significant generalization. Overall, these results suggest that estradiol has significant effects on fear generalization in male rats, but in the opposite direction to what we see in females.

The studies here are the first to explore the effects of gonadal hormones in male rats on fear generalization. Our previous work has focused on uncovering the mechanisms underlying the estradiol-induced fear generalization in females. The reasons for the sex-dependent effects of estradiol on fear generalization remain unknown. Other behaviors that may be related to generalization also have sex differences including anxiety-like behavior and spatial learning. Additionally, sex differences in specific hormone levels, sexually dimorphic structures within the brain, differential receptor distributions, or involvement of distinct brain structures could help to explain the sex differences in generalization. However, the connection between these behaviors and physiological differences and fear generalization is currently uncertain.

Existing literature on anxiety-like behaviors suggests that low testosterone is correlated with the development of anxiety disorders (Barrett-Connor et al., 1999; Cooper and Ritchie, 2000; Kaminetsky, 2005; Veras and Nardi, 2010). Some additional support for these findings is derived from reports of increased anxiety-like behavior following post-pubertal castration. Furthermore, testosterone can reduce anxiety-like behavior in male and female rodents (Bitran et al., 1993; Edinger and Frye, 2004; Forman et al., 1989; McDermott et al., 2012; Molina et al., 1994; Romero et al., 1988). The evidence therefore, supports androgens, such as testosterone, reducing anxiety-like behavior in male and female rodents, but the ultimate mechanisms responsible for these effects are not well understood. Although some reports

suggest testosterone acts upon androgen receptors to reduce anxiety-like behavior (Carrier et al., 2015; Chen et al., 2014; Edinger and Frye, 2006), the effects observed in the present study are not due to androgen receptor activation because DHT treatment did not reduce fear generalization. One possibility is that estradiol and testosterone may reduce anxiety-like behavior through activation of ERβ. For example, administration of an ERβ, but not ERα, agonist reduces anxiety-like behavior in gonadectomized male and female rats as measured by the elevated-plus maze and open field (Kudwa et al., 2014; Lund et al., 2005). We previously found that ERβ, but not ERα, mediates estradiol-induced fear generalization in females (Lynch et al., 2014, 2016). The current results show that fear generalization is ameliorated in male rats through activation of either ER, demonstrating a sex difference in the direction of the response and in ER specificity for fear generalization. Together, these data suggest that estrogens act through dissociable mechanisms to influence non-associative behaviors versus associative learning. However, the direct relationship between anxiety-like behavior and fear memory—whether generalized or specific—is uncertain because these two theoretical constructs have different precipitating conditions, behavioral outputs, and are controlled by distinguishable neuroanatomical circuits (Perusini and Fanselow, 2015). Within the circuit that controls contextual memory, the hippocampus and prefrontal cortex play an important role in the generalization of fear and memory precision (Cullen et al., 2015; Ruediger et al., 2011; Wiltgen and Silva, 2007; Wiltgen et al., 2010; Winocur et al., 2007). We have also shown that the dorsal CA1 hippocampus is an important locus for the actions of estradiol on contextual fear generalization, and this is due to actions at ERβ (Lynch et al., 2016). ERα and ERβ are expressed in the hippocampus and cortex, and ERβ is more widely distributed throughout these regions (Li et al., 1997; Österlund et al., 1998; Shughrue et al., 1997; Shughrue and Merchenthaler, 2000a, 2000b). However, adult female rats display higher levels of ERβ, but not ERα, compared to males within the hippocampus (Weiland et al., 1997; Zhang et al., 2002). This sex difference in ER expression within the hippocampus may explain the lack of receptor specificity seen in males, but does not explain the directional nature of the effect.

One alternative possibility is that the sex difference in generalization is related to sex differences in spatial task performance. Specifically, male rodents display better performance than females in a variety of spatial memory tasks (Barrett and Ray, 1970; Beatty, 1984; Beiko et al., 2004; Davenport et al., 1970; Dawson, 1972; Dawson et al., 1975; Einon, 1980; Gaulin and FitzGerald, 1986; Gresack and Frick, 2003; Perrot-Sinal et al., 1996). This sex difference in spatial memory may also be a result of distinct strategies utilized by males and females. Male and proestrus female rats are more likely to utilize extramaze cues (Korol and Kolo, 2002) compared to estrous females. Furthermore, estradiol enhances spatial learning, but disrupts performance learning (Korol and Kolo, 2002), and the latter is dependent on the striatum. Similarly in fear generalization, estradiol may differentially modulate the ability to inhibit a previously learned response despite the shift in context. If so, then estradiol may act on the dorsomedial striatum, a region critical to action-outcome learning and active and passive avoidance learning (Mazzucchelli et al., 2002; Yin et al., 2004, 2005). Alternatively, estradiol may differentially modulate pattern completion or pattern separation in male and female rodents; two processes that depend on the hippocampus (Besnard and Sahay, 2016; Matus-Amat et al., 2004; Nakashiba et al., 2008, 2012; Rudy, 2009; Rudy et al., 2004). In pattern completion, a subset or portion of the contextual cues present during the initial learning can activate the original memory trace as a whole. This could explain estradiol-induced fear generalization in females in which a similar, yet distinct, context activates the fear memory. On the other hand, pattern separation allows similar, yet overlapping, cortical memory traces to be distinctly activated, enabling precise memory recall. If estradiol administration interferes with the process of pattern separation in females, a similar, yet distinct, context may activate the original fear memory trace, promoting fear generalization. In male rats, estradiol may not interfere with pattern separation, enabling distinction between the two contexts, suggesting that estradiol acts on dissociable processes in male and female rats. These possibilities have yet to be systematically tested, which will be required to determine the exact mechanisms through which estradiol promotes generalized fear in females and ameliorates generalization in male rats.

One region that may be mediating estradiol effects in a sex-specific way is the bed nucleus of the stria terminalis (BNST). In general, the BNST is involved in several sexually dimorphic behaviors, such as aggression, stress, and anxiety-like behavior, and is also involved in maintaining responses that are long in duration (Walker et al., 2003). In addition, the expression of aromatase mRNA within the BNST is higher in males compared to females (Foidart et al., 1994; Lauber et al., 1997; Roselli et al., 1984; Wagner and Morrell, 1997). Also, the regulation of aromatase mRNA levels in the BNST of males, but not females, is sensitive to gonadal hormones; gonadectomized males have approximately 224% reduced levels of aromatase mRNA compared to intact males, whereas OVX females and OVX females treated with exogenous estradiol do not have different levels of aromatase mRNA (Tabatadze et al., 2014). Although the circuitry involved in estradiol-*reduced* generalization in gonadectomized males has not been explored yet, the BNST appears ideally placed to be involved due to sex differences in estradiol-sensitive levels of aromatase activity. Additionally, the BNST also receives input from the ventral hippocampus (Dong et al., 2001), which is a structure implicated in temporally-dependent fear generalization (Cullen et al., 2015). If this circuitry is involved, it would suggest that the sex-dependent effects of estradiol on fear generalization are indeed due to dissociable circuitry in males and females.

A final reason why estradiol may act to reduce generalization in male rats is due to organizational effects of gonadal hormones during development. In rats and mice, the critical period for sexual differentiation begins before birth and ends approximately 10 days after birth (Arnold and Breedlove, 1985; McCarthy, 2006; McEwen, 1992; Schwarz and McCarthy, 2008). Differentiation requires aromatization in neonatal rat brain (McEwen et al., 1977). Thus, organizational effects of estradiol on regions controlling contextual fear memory in male rodents may establish adult responses resulting in a sex-dependent effect of estradiol on fear generalization.

Taken together, these experiments lead to a better understanding of the primary mechanisms through which gonadal hormones affect fear generalization. We demonstrated that testosterone acts through metabolism into estradiol to reduce generalization in male rats. These results, when taken together with our previous studies demonstrating that estradiol *induces* fear generalization in females (Lynch et al., 2013, 2014), reveal a complex role of estradiol on contextual memory retrieval. Removing testosterone via gonadectomy results in generalization as does treating intact male rats with the aromatase inhibitor, FAD, suggesting a distinct sex-dependent effect of estradiol on fear generalization. Thus, testosterone—and the subsequent conversion into estradiol—maintains contextual memory precision at recent time points, but does not alter the time-dependent nature of fear generalization in male rats. Intact male rats generalize contextual fear in a time window of 14–28 days. Intact females generalize fear at a much faster rate of 5 days (Lynch et al., 2013), and this faster rate of generalization is due to estradiol, which can also rapidly induce fear generalization in females (Lynch et al., 2014, 2016).

Unlike the findings of estrogens enhancing extinction retention, where new learning occurs about the relationship between the conditioned stimulus and the absence of the unconditioned stimulus (Graham and Daher, 2015; McDermott et al., 2015; Milad et al., 2009), our findings in male and female rats are not a result of estrogens enhancing or reducing memory formation, but rather changing what cues elicit the fear memory response. 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).

Our findings suggest that high levels of estrogens disrupt the ability of female rats to inhibit a fear response to a neutral environment, but maintain appropriate inhibitory responses in males. Fear generalization is a major characteristic of many anxiety disorders including PTSD, eliciting a need to understand factors that affect fear generalization in a sex-dependent manner (Jovanovic, Kazama, Bachevalier, & Davis, 2012; Jovanovic et al., 2010; Jovanovic et al., 2009). Future studies will determine precisely how estradiol acts in opposing ways in male and female rodents on fear generalization including the possible contribution of organizational effects of hormones on this behavior (Arnold and Breedlove, 1985; McCarthy, 2006; McEwen, 1992; Schwarz and McCarthy, 2008) and differences in receptor distribution within fear memory circuits. It will also be important to determine the functional relevance of these findings to other species, including humans. Unlike rats and mice, castrated adult males of Guinea Pigs and Rhesus Macaques show little sexual behavior in response to estradiol treatment (Michael et al., 1990; Phoenix and Chambers, 1982; Wallen, 2005). Although fear generalization is most likely controlled by a different neural circuit than male sexual behavior, these data raise the possibility of species differences in the response to estrogen metabolites on fear generalization. These findings and future studies are crucial for developing more effective, potentially sex-specific, treatments for anxiety disorders such as PTSD.

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