ESTROGENIC MODULATION OF FEAR GENERALIZATION

A dissertation submitted

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by

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I. Introduction

Anxiety disorders are the most prevalent class of mental disorders in the United States, highlighting the importance in understanding the characteristics of these disorders in order to provide better treatments (Kessler, Chiu, Demler, Merikangas, & Walters, 2005). In addition, females are 60% more likely than males to be diagnosed with an anxiety disorder, including generalized anxiety disorder, obsessive-compulsive disorder, panic disorder, specific phobias, and post-traumatic stress disorder (PTSD) (Cloitre, Chase Stovall-McClough, Miranda, & Chemtob, 2004; Kessler et al., 1994; Lissek, 2012; Lissek et al., 2010; Wang et al., 2005). Evidence suggests that changes in circulating levels of estrogens are linked to alterations in mood and emotion as well as to the development of mental illness in females (Hendrick, Altshuler, & Burt, 1996; ÖStlund, Keller, & Hurd, 2003; Sherwin, 2003; Sichel, Cohen, Robertson, Ruttenberg, & Rosenbaum, 1995; Walf & Frye, 2006). Despite the sex difference in prevalence, little research has focused on understanding sex differences on specific characteristics of these disorders. Understanding the mechanisms contributing to the differences between males and females on characteristics of anxiety disorders is important in understanding differences in the prevalence rates and ultimately designing effect treatments.

1.1.PTSD

One specific anxiety disorder with the largest sex difference in prevalence rates is PTSD(McLean, Asnaani, Litz, & Hofmann, 2011); females develop PTSD at approximately 2.5 times the rate of men, despite greater trauma exposure in men

(Breslau et al., 1998; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; McLean et al., 2011; Tanielian & Jaycox, 2008). In addition to higher rates of PTSD, females display greater symptom severity, longer symptom duration, and report a worse quality of life (Breslau, Davis, Andreski, Peterson, & Schultz, 1997; Holbrook, Hoyt, Stein, & Sieber, 2002; Seedat, Stein, & Carey, 2005) and these differences cannot be fully accounted for by risk of exposure to particular types of traumatic events (Tolin & Foa, 2006). Even though females are more likely to suffer from PTSD, sex-specific mechanisms underlying prevalence rate differences have remained understudied (Bekker & van Mens-Verhulst, 2007; Cameron & Hill, 1989). One possible explanation for the sex difference in prevalence rates is differences in reporting traumatic experiences as females are more likely to report abuse than males in certain situations (Ullman & Filipas, 2005). Another possible reason for the discrepancy in prevalence rates is because females may differ on particular characteristics that comprise PTSD, such as the tendency to generalize fear to neutral cues and contexts (Brewin, 2001; Grillon & Morgan, 1999; Jovanovic et al., 2009). In general, PTSD patients show heightened fear responses to contextual cues compared to healthy controls (Grillon & Morgan, 1999). During discrimination learning where they are presented with two discrete stimuli and only one is paired with an aversive stimulus, PTSD patients display heightened fear to the stimulus that was never paired with an aversive stimuli and should not elicit fearful responding (Grillon & Morgan, 1999; Lissek et al., 2008; Orr & Roth, 2000). Similarly, high symptom PTSD patients are unable to discriminate between danger and safety cues in a fear potentiated startle discrimination procedure (Jovanovic, Kazama, Bachevalier, & Davis, 2012; Jovanovic et al., 2010; Jovanovic et al., 2009). These results demonstrate a significant

increase in generalized responding to neutral discrete cues in individuals with PTSD. However, few have looked at sex differences in fear generalization where fear responses are elicited in contexts that are not associated with a fearful event.

1.2.Fear Generalization

Considerable research indicates that contextual fear generalization—the inability to discriminate between different contexts and, thus, recalling a fear memory in neutral contexts—increases over time (For review, see Jasnow, Cullen, & Riccio, 2012). When rodents are fear conditioned in a specific context and are tested in a different (neutral) context shortly after conditioning, animals can discriminate between the contexts and do not display fear behavior in the neutral context. However, when rodents are tested at long delays after conditioning, animals cannot discriminate between the contexts and display heightened fear in the neutral context demonstrating that the fearful response has generalization to distinct contextual cues (Jasnow et al., 2012; Lynch III, Cullen, Jasnow, & Riccio, 2013; Matynia et al., 2008; Wiltgen & Silva, 2007; Winocur, Moscovitch, & Sekeres, 2007). Fear generalization can also be interpreted as a loss of memory precision for contextual cues; mechanisms contributing to the establishment, maintenance, and recall of contextual memory are implicated in this process.

Several theories may explain why fear generalizes, or how memory precision is lost over time. According to the multiple trace theory, the medial prefrontal cortex (mPFC) is not involved in the storage of remote, older memories, but rather is required for recall at any time point and the older the memory, the more effortful the recall. When effortful recall is required, additional activation from the mPFC is required for proper retrieval (Rudy, Biedenkapp, & O'Reilly, 2005). Support for the multiple trace theory

comes from the findings of reminders, or reactivations, returning memory precision. Reminders and reactivations by exposing animals to the training context prior to a memory recall test may reduce the effort required to recall the memory, resulting in more hippocampal activation and less requirement from the mPFC. A brief (~90 second) re-exposure to the training context prior to testing in a neutral context at a retention interval that would normally elicit generalization can restore memory precision (Zhou & Riccio, 1994). Additionally, reactivation sessions given 3 times over the course of 28 days results in precise recall, and that precision is blocked by muscimol infusions into the dorsal hippocampus, suggesting that the reactivation returns the memory to being hippocampally-dependent (de Oliveira Alvares et al., 2012).

Another theory, the transformation hypothesis, suggests memories are transferred over time from the hippocampus to prefrontal cortical areas and upon transfer, the memory is transformed into a more schematic representation where specific contextual cues are forgotten (Nadel, Samsonovich, Ryan, & Moscovitch, 2000; Rosenbaum, Winocur, & Moscovitch, 2001; Winocur et al., 2007; see Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2006 for review). Some evidence supporting this hypothesis has looked at the involvement of the hippocampus and prefrontal cortex for recent and remote memories. Following training in context fear conditioning, a lesion of the hippocampus disrupts freezing when tested at a short interval in either the training or a neutral context. When lesions take place before a 28 day test, animals freeze equivalently in either context, demonstrating a loss in memory precision independent of the hippocampus. However, if animals undergo a reactivation session at a remote time point and then given hippocampal lesions, the reactivation restores memory precision and the

lesion of the hippocampus disrupts freezing, even at a long retention interval (Winocur, Frankland, Sekeres, Fogel, & Moscovitch, 2009). This finding suggests that the hippocampus is no longer involved in memory recall at longer retention intervals as inactivation of the hippocampus at a long time point after training does not affect generalization. As a result, the prefrontal cortex (PFC) may come online at these remote time points to recall the memory, and PFC involvement leads to the recall of a less precise memory. Evidence for the involvement of the PFC in remote memory recall comes from brain activation studies in humans where newly formed memories have low levels of activation within the PFC and high levels of activation within the hippocampus, whereas older memories have high PFC activation and low hippocampal activation (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Maviel, Durkin, Menzaghi, & Bontempi, 2004). In animal models, only recent memory recall is marked by increases in immediate early gene activity within the hippocampus. When tested at a remote time point in a neutral context, animals that generalize the most also show the least amount of immediate early gene activity within the hippocampus (Wiltgen et al., 2010). Additionally, blockade of synaptic transmission in the hippocampus only impairs recent memory recall, not remote memory recall (Xu et al., 2012). In comparison, blockade of synaptic transmission in the prefrontal cortex impairs the precision of recent and remote memory recall (Xu et al., 2012). Taken together, these data suggest a preferential role for the hippocampus for recent memory recall and, over time, the role of the hippocampus diminishes, suggesting that memories are transferred over time from the hippocampus to the PFC. However, hippocampal involvement for remote memory recall depends on the type of information;

certain behaviors require constant spatial updating, which requires hippocampal activity at recent and remote time points, whereas contextual fear learning does not appear to require the hippocampus at remote time points, initiating impaired memory precision during recall (Winocur, Sekeres, Binns, & Moscovitch, 2013). These findings suggest a complex role for hippocampal involvement in contextual memories in which the type of information being accessed during memory recall determines the role of the hippocampus. Recent data suggests a continued role for the hippocampus for contextual fear memory over time, but in a region-specific manner with more ventral hippocampal involvement at later time points (Cullen et al., 2015). Taken together, these theories provide possible explanations for why memories may generalize over time. However, the true mechanism of memory generalization remains largely unknown.

1.3.Estrogens and Generalization

The human menstrual cycle and the rodent estrus cycle are similar in the way that hormone levels change across the cycle, although the rodent estrus cycle takes place over a shorter period of time. The increased levels of estrogens and other gonadal hormones over the course of the cycle can have a profound impact on cognition and behavior.

Others have assessed the role of estrogens in discriminatory or inhibitory response procedures and found that estrogens have a detrimental impact on discrimination learning. In one such study, gonadectomized (GDX) male and female rats treated with estradiol were unable to inhibit responding to neutral stimuli (Toufexis, Myers, Bowser, & Davis, 2007). Estrogens also affect responding following latent inhibition (see Lubow, 1973 for review on latent inhibition). Specifically, ovariectomized (OVX) females treated with estradiol still respond fearfully to a stimulus they have been exposed to prior

to fear conditioning, whereas males and untreated OVX females inhibit fear responding to the pre-exposed stimulus (Nofrey, Ben-Shahar, & Brake, 2008).

1.4.General Scope

To date, no one has yet assessed sex differences in the generalization of fear. Given the discrepancy in prevalence rates for anxiety disorders, which are marked by a tendency to generalize fear to neutral cues, female rats are hypothesized to display a faster rate of generalization (i.e. generalize fear to a neutral context at a shorter retention interval than males). In addition, estradiol is predicted to contribute significantly to sex differences in fear generalization and act within specific neural circuits modulating memory retrieval. The first experiments demonstrated that intact female rats displayed a faster rate of fear generalization compared to intact male rats.

Specific Aim 1

The next set of experiments were designed to assess the mechanisms underlying the faster rate of generalization in females. To assess whether estrogens were mediating the faster rate of generalization, female rats were ovariectomized and given capsule implants containing estradiol. Chronic exposure to estradiol was hypothesized to induce generalization by 7 days (Experiment 2). In order to assess what aspect of learning and memory estradiol was affecting during fear generalization, female rats were ovariectomized and given acute injections at distinct time points during either acquisition/consolidation or memory retrieval.

Specific Aim 2

Given the time-dependent nature of generalization, animals were hypothesized to generalize 7 days after injections given during acquisition/consolidation (Experiment 4A-B). However, results revealed that estradiol affected fear generalization through memory retrieval. To determine how estradiol affected memory retrieval, ovariectomized females were given injections of estrogen receptor agonists for ERα or ERβ subtypes. The higher expression of ERβ with generalization circuitry and the effects of ERβ on certain behaviors led to the hypothesis that estradiol was inducing generalization through activation of ERβ (Experiment 5A).

Specific Aim 3

Next, animals were tested to determine if estrogen receptors within the cytoplasm (i.e. classical ERs) or membrane-bound receptors were responsible for estradiol-induced generalization. Given the results from Experiment 4B, where generalization took 24 hours to occur, genomic alterations through activation of cytosolic receptors were hypothesized to drive estradiol-induced generalization (Experiment 6). Then, animals were tested to see where estradiol is required within specific brains structures implicated in fear generalization in order to induce generalized responding. To test this, animals were given infusions of estradiol into the dorsal CA1 region of the hippocampus, the ventral CA1 region of the hippocampus, and the anterior cingulate cortex. All 3 structures are implicated in time-dependent generalization, leading to the hypothesis that estradiol within any of these regions would induce generalization (Experiment 7). Other

brain regions involved in estradiol-dependent generalization could not be determined via Arc activity (Experiment 8).

Specific Aim 4

Finally, in order to determine a mechanism by which estradiol may induce generalization, animals were tested for the role of enhanced glutamatergic transmission. Given the interaction of estradiol with glutamatergic receptor upregulation, and the notion the enhanced excitatory signaling may lead to generalization, animals undergoing estradiol-induced generalization via estradiol injection would display significantly reduced fear generalization if given an infusion of an NMDA or AMPA receptor antagonist before memory retrieval (Experiment 9). These experiments were designed to begin the process of determining the role of estradiol in generalization and determining the underlying mechanisms involved.

Specific Aim 5

The last set of experiments assessed the role of estradiol in male rats. Given the ability of estradiol to induce generalization in females, males given estradiol were hypothesized to generalize as well. To test this, males were given chronic exposure to estradiol via capsule (Experiment 3). Contrary to the hypothesis, estradiol exposure resulted in reduced generalization in gonadectomized males as did testosterone exposure through conversion of testosterone into estradiol (Experiment 3; Experiment 4D). In order to assess whether estradiol-*reduced* generalization followed a similar pattern as estradiol-*induced* generalization in females, males were given acute injections of gonadal hormones before memory retrieval (Experiment 4C:5B).

Despite the importance of fear generalization as a fundamental component underlying many anxiety disorders (Brewin, 2001; Grillon & Morgan, 1999; Jovanovic et al., 2009), understanding of how this phenomenon occurs and the role of sex in generalization is incomplete. Sex differences in fear generalization could be due, in part, to estrogens and, determining how estrogens contribute to fear generalization may provide new insights into the mechanisms of fear generalization to help explain the sex differences in anxiety disorder prevalence rates.

II. Experiment 1 – Sex Differences in Fear Generalization[1]

2.1.Introduction

The finding of contextual generalization over time is well established even if it remains poorly understood (For review, see Jasnow et al., 2012). However, experiments assessing contextual fear generalization focuses primarily on males and largely ignores females (see Lebron-Milad & Milad, 2012 for review). Therefore, experiment 1 was designed to assess whether females display a similar rate of generalization as males. Given the fact that females display higher prevalence rates of PTSD and generalization is a characteristic of the disorder, female rats were hypothesized to generalization at a faster rate (i.e. a shorter retention interval) than males.

2.2.Methods

Animals and Housing Conditions

Male and female adult Long-Evans rats approximately 90 days old provided by the breeding colony in the Department of Psychology at Kent State University were used in the experiment. Animals were maintained on a 14/10 hour light/dark cycle. Food and water were available *ad libtum* throughout the experiment. A week prior to beginning the experiment, all rats were individually housed. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

[1] Experiment describe here is published: Lynch III, J. F., Cullen, P. K., Jasnow, A. M., & Riccio, D. C. (2013). Sex Differences in the Generalization of Fear as a Function of Retention Intervals *Learning & Memory, 20*, 628-632.

Apparatus and Contexts

The training and testing apparatus were two identical 43.18 X 17.78 X 17.78 cm shuttle boxes placed on grid floors. The boxes were comprised of two chambers of equal size—one black and one white—that were divided by a guillotine door. Each box was placed on a table in one of two distinct contexts. Context A was a 1.6 X 2.33 meter room with house fluorescent lights, bare white walls, and no artificial scents or sounds. Context B was a 1.83 X 2.74 meter room lit by a 25-w red light bulb with posters on the walls. Context B contained white noise (70db) provided by a GPX AM/FM digital clock radio as well as a residual artificial scent via a Glade Plug-Ins Scented Oil Country Berry air freshener. In each context, the experimenter wore a different glove (Rubber *dish* glove in A; vinyl lab glove in B) to handle the rat. Therefore, the stimulus conditions in the two contexts differed in olfactory, visual, auditory, and cutaneous cues.

Passive Avoidance Procedure

For all experiments, animals were handled for 5 minutes on two consecutive days. For training, animals were brought to Context A (training context), held on the experimenter's hand for 30 seconds, and placed on the white side of the shuttle box. The door was raised after 20 seconds and the initial latency to cross into the black compartment (all four paws) was recorded. Upon crossing, the sliding door closed and 5 seconds after closing, 2 nonescapable, 1 second, 0.6 milliamp (mA) scrambled footshocks were delivered 5 seconds apart by a constant current AC shock generator (Model 5806, Lafayette Instruments, Lafayette, IN). Ten seconds after receiving the

second footshock, the animal was removed from the passive avoidance chamber and returned to the main colony.

For testing, animals were brought back into the experimental room after a specific retention interval (1, 3, 5, or 7 days) at the same time of day as training, which results in optimal retention (Holloway & Wansley, 1973). Half of the animals were tested in Context A (Training) and half in Context B (Neutral). The test procedure was identical to training, except that the guillotine door remained open for 600 seconds and no shocks were delivered. The initial latency to cross was recorded as the dependent measures. Upon crossing or after a total of 600 seconds had elapsed, the rat was removed and returned to the main colony.

Statistical Analysis

The role of sex and time on the generalization of contextual fear was analyzed with factorial ANOVA analyses and independent t-test analyses were used to make direct comparisons between animals tested in the training versus neutral context at different time points. Statistical significance was set at $p<0.05$. Cohen's d effect size estimates were assessed by G*Power 3 (Faul, Erdfelder, Lang, & Buchner, 2007) and effect sizes were determined according to Cohen (1988).

2.3.Results and Discussion

Animals were trained in passive avoidance and tested at different retention intervals in either the training or neutral context (Fig 1A). At 1 day (Fig 1B), the main effect of context was significant, ($F_{(1,42)} = 50.31$, $p<0.001$), but the main effect of sex was not significant, ($F_{(1,42)} = 0.56$, ns), suggesting that males and females did not differ in overall

latency scores. The interaction between context and sex was also not significant, ($F_{(1,42)}$ = 0.005, ns). Independent t-test analyses revealed that both males and females displayed significantly more fear in the training context compared to the neutral context (i.e. higher latencies to cross in the training context), (males: $t_{(20)} = 5.43$, $p<0.001$; $d = 2.38$; females: $t_{(22)} = 4.77$, $p<0.001$; $d = 1.95$). Additionally, animals tested in the neutral context did not display different levels of fear across sex, ($t_{(21)} = 0.57$, ns; $d = 0.24$). Therefore, at 1 day, males and females were able to discriminate between the contexts and did not display significant fear generalization.

At 3 days (Fig 1C), the main effect of context was significant, ($F_{(1,41)} = 31.14$, $p<0.001$), the main effect of sex was non-significant, ($F_{(1, 41)} = 0.17$, ns), and the interaction between context and sex was non-significant, ($F_{(1,41)} = 3.07$, ns). Independent t-test analyses revealed a similar pattern as seen at 1 day. Specifically, males and females displayed significantly more fear in the training context compared to the neutral context, (males: $t_{(19)} = 5.97$, $p<0.001$; $d = 2.67$; females: $t_{(22)} = 2.50$, $p<0.05$; $d = 1.02$). Additionally, animals tested in the neutral context did not display different levels of fear across sex, ($t_{(21)} = 1.26$, ns; $d = 0.53$). Therefore, at 3 day, males and females did not display significant fear generalization.

At 5 days (Fig 1D), the main effect for context, ($F_{(1,37)} = 26.55$, $p<0.001$), the main effect for sex, ($F_{(1,37)} = 15.50$, $p<0.001$), and the interaction between context and sex, ($F_{(1,37)} = 4.84$, $p<0.05$) were significantly different. Again, males and females had significantly higher fear levels in the training context, (males: $t_{(17)} = 4.38$, $p<0.001$; $d = 2.07$; females: $t_{(20)} = 2.53$, $p<0.05$; $d = 1.05$). Unlike the findings at 1 and 3 days, however, females displayed significantly higher levels of fear when tested in the neutral

context compared to males, ($t_{(16)} = 3.84$, $p<0.001$; $d = 1.83$). These results demonstrate that although females still displayed significant discrimination between the training and neutral contexts, females show more fear than males in the neutral context.

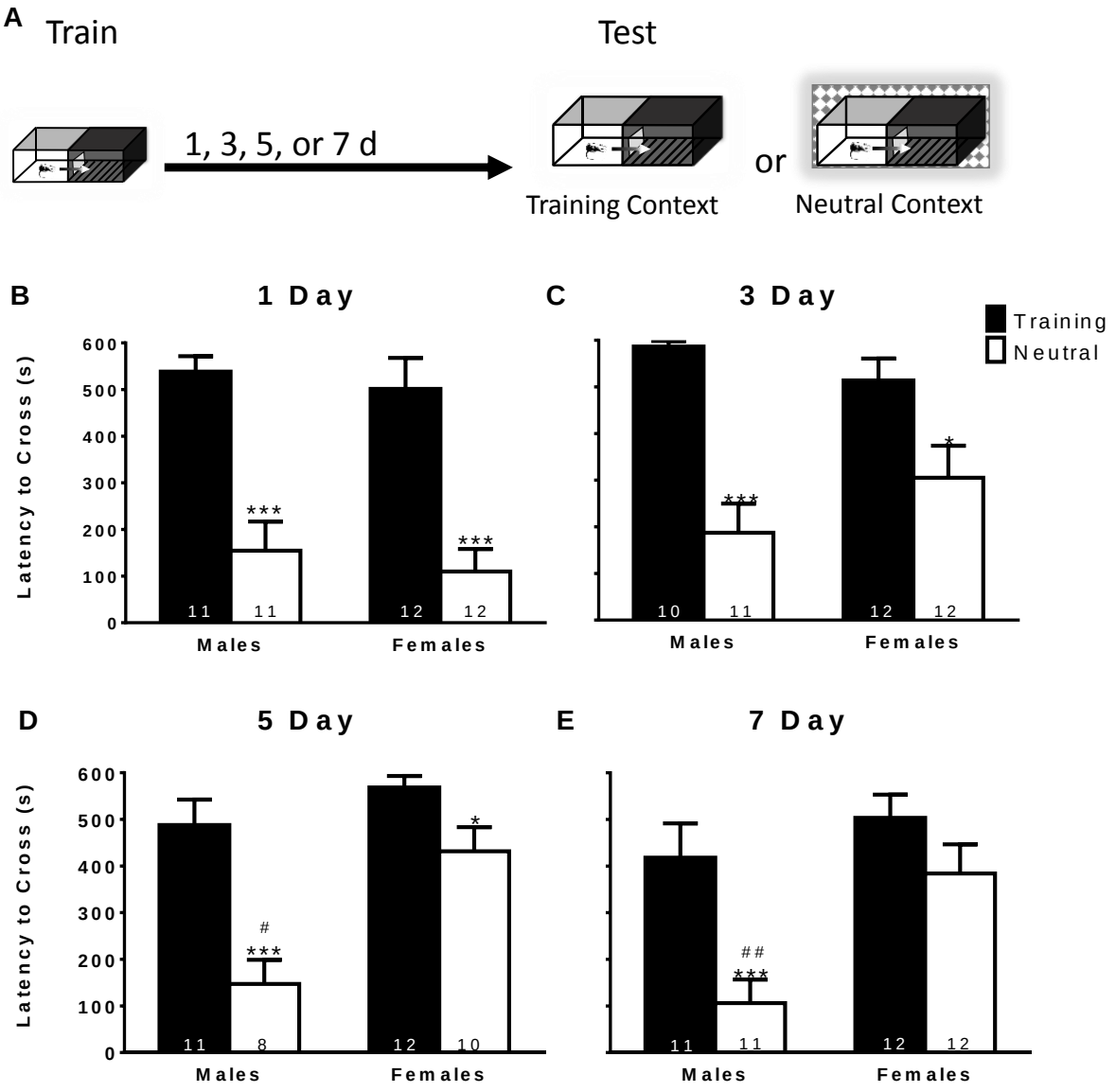
At 7 days (Fig 1E), the main effect for context, ($F_{(1,42)} = 13.08$, $p<0.001$), and sex, ($F_{(1,42)} = 9.26$, $p<0.01$) was significant, and the interaction between context and sex was non-significant, ($F_{(1,42)} = 2.60$, ns). Males rats tested at 7 days still maintained context discrimination, ($t_{(20)} = 3.50$, $p<0.01$; $d = 1.49$). However, females displayed equivalent levels of fear in either context, $t_{(22)} = 1.49$, ns; $d = 0.61$). Females also displayed significantly higher levels of fear when tested in the neutral context compared to males, ($t_{(21)} = 3.42$, $p<0.01$; $d = 1.43$). Therefore, unlike the 5 day retention interval group, females at 7 days demonstrated complete fear generalization to the neutral context.

Other explanations for the sex differences in fear generalization—such as differences in exploratory behavior (Archer, 1974), differential rates of acquisition in passive avoidance (Denti & Epstein, 1972), and shock sensitivity (Beatty & Beatty, 1970; Blizard, 1971; Snowdon, Bell, & Henderson, 1964)—can be ruled out due to intact males and females displaying equivalent behavioral responses at 1 day, as indicated by both sexes demonstrating equivalent discrimination between contexts. Overall, these data suggest that although females can perceive the contextual differences at short retention intervals, they generalize fear to neutral contextual cues at shorter retention intervals than males. Females had significantly longer cross latencies in the neutral context compared to males at the 5 and 7 day retention intervals, and displayed complete fear generalization by the 7 day interval. The findings of Experiment 1 demonstrate a sex difference in the generalization of fear. Experiment 2 was designed to determine whether or not the

reason for the faster rate of generalization seen in females is due to circulating levels of estrogens.

Figure 1. **Sex Differences in the Generalization of Fear. A)** Schematic of the experimental paradigm. All animals were trained in passive avoidance and tested 1, 3, 5, or 7 days later in the training or neutral context. **B)** Both sexes demonstrate significant context discrimination at 1 day. **C)** Both sexes demonstrate significant context discrimination at 3 days. **D)** By 5 days, males and females continue to display context discrimination although females tested in the neutral context have significantly higher fear than males. **E)** Females show higher fear compared to males tested in the neutral context. In addition, females display significantly generalization to the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 1. Sex Differences in the Generalization of Fear.[2]



[2] Figure adapted from: Lynch III, J. F., Cullen, P. K., Jasnow, A. M., & Riccio, D. C. (2013). Sex Differences in the Generalization of Fear as a Function of Retention Intervals *Learning & Memory*, *20*, 628-632

III. Experiment 2 – Role of Estrogens in Fear Generalization[3]

3.1. Introduction

A wealth of evidence has linked estrogens to alterations in fear and anxiety behavior in females (Morgan & Pfaff, 2001, 2002; Morgan, Schulkin, & Pfaff, 2004). However, whether estrogens contribute to the generalization of fear—a key symptom of anxiety disorders, including PTSD (Grillon & Morgan, 1999; Jovanovic et al., 2009)—has not been examined. In Experiment 1, no control over the estrus cycle was exerted. Therefore, Experiment 2 used ovariectomized (OVX) female rats to control for the levels of estrogens present during the experiment. Estrogens are a classification of gonadal steroid hormones that consists of several different subtypes. Of those subtypes, 17β-estradiol is the most active subtype of the estrogen family (Fiocchetti, Ascenzi, & Marino, 2012). 17β-estradiol can influence memory formation and cognitive processes by affecting areas of the brain not associated with reproductive behaviors, such as the hippocampus and cortex (Barha & Galea, 2010; McEwen, 2002; McEwen & Alves, 1999). In order to assess the role of estrogens in the generalization of fear, OVX females were given capsule implantations of 17β-estradiol benzoate. OVX females given estradiol capsules were expected to display generalized fear to a neutral context by 7 days following passive avoidance training.

[3] Experiment describe here is published: Lynch III, J. F., Cullen, P. K., Jasnow, A. M., & Riccio, D. C. (2013). Sex Differences in the Generalization of Fear as a Function of Retention Intervals *Learning & Memory, 20,* 628-632.

3.2.Methods

Animals

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

Passive Avoidance Procedure

Behavior was conducted in a black/white passive avoidance chamber (52x30x35 cm, Passive Avoidance Apparatus 7550, Ugo Basil, Comerio, Italy). For training, animals were brought in to Context A (training context), held on the experimenter's hand for 30 seconds, and placed on the white side of the shuttle box. The door was raised after 20 seconds, and the initial latency to cross into the black compartment (all four paws) was recorded. Upon crossing, the sliding door closed and 5 seconds 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 X 2.33 meter room with house fluorescent lights and contained bare white walls and no artificial scents or sounds and was cleaned with 70% Ethanol; Context B was a 1.83 X 2.74 meter room that was lit by a 25-w red light bulb with posters on the walls. Context B had White noise (70db) and was cleaned with 60%

quatricide. In each context, the experimenter wore different gloves (Rubber dish glove in A; vinyl lab glove in B) to handle the rat. The test procedure was identical to training except the sliding door remained open for a maximum of 540 seconds and no shocks were delivered. The initial latency to cross was recorded as the dependent measure of fear behavior. Any animal that did not cross was given a score of 540 seconds. Upon crossing or at 540 seconds, the animal was removed and returned to the main colony.

Surgical Procedures

Female rats were anesthetized with isoflurane and received a 5 mg/kg dose of Ketoprofen 5 minutes before bilateral ovariectomy through a dorsal incision. After removal of the ovaries, the incision was sutured using surgical staples and either an empty silastic capsule or a silastic capsule containing 17β-estradiol was inserted behind the shoulder blades of the animal. The silastic (polydimethylsiloxane) implants were constructed from silastic tubing (i.d. 0.078 inches, o.d. 0.125 inches) cut to a 5 mm length. Each end was filled with Factor II medical adhesive 1 mm in length. The hormone was packed into the remaining 3 mm length, which produces levels of 30-40 pg/ml of estradiol, which falls within the range of proestrus—the stage of the estrous cycle with the highest levels of estrogens (Bridges, 1984a; Hiroi & Neumaier, 2006). Before implantation, all capsules were incubated in saline solution for 24 hours at 37°C. Animals received another injection of Ketoprofen 24 hours post-surgery.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the role of hormone treatment and the effect of retention intervals on the generalization of contextual fear.

3.3.Results and Discussion

To determine if differences in fear generalization were mediated by estrogens, adult female rats were ovariectomized and half were given a capsule containing 17β-estradiol and the other half received an empty capsule and tested for fear generalization at 1, 5, or 7 days (Fig 2A). Factorial ANOVA analyses at 1 day (Fig 2B) revealed a significant main effect of context, ($F_{(1,39)} = 11.08$, $p<0.01$), but no main effect of hormone treatment, ($F_{(1,39)}= 0.56$, ns). The interaction between context and hormone treatment was also non-significant, ($F_{(1,39)}= 0.05$, ns). Independent t-test analyses revealed that animals given no hormone replacement (control) and animals treated with estradiol capsules, displayed significant discrimination between the contexts with higher overall levels of fear seen in animals tested in the training context, (control: $t_{(17)} = 2.11$, $p<0.05$; $d = 0.95$; estradiol: $t_{(22)} = 2.65$, $p<0.05$; $d = 1.08$). In addition, the level of fear was not significantly different between controls and estradiol-treated animals when testing occurred in the neutral context, ($t_{(19)} = 0.60$, ns; $d = 0.26$). Thus, 1 day after training, OVX females displayed a significant context shift effect, exhibiting less fear in the neutral context regardless of hormone treatment. Importantly, these results demonstrate that neither surgery nor capsule implantation had a negative impact on passive avoidance acquisition or the ability to discriminate contextual cues at a short retention interval.

When animals were tested 5 days after training (Fig 2C), factorial ANOVA analyses revealed a significant main effect of context, ($F_{(1,43)} = 9.04$, $p<0.01$), but a non-significant main effect for hormone treatment, ($F_{(1,43)} = 0.31$, ns) and interaction term, ($F_{(1,43)} = 0.97$, ns). Independent t-test analyses revealed that control animals were able to discriminate between contexts, displaying significantly higher levels of fear in the training context, ($t_{(22)} = 3.06$, $p<0.01$; $d = 1.25$). However, when OVX females were treated with estradiol, they displayed equivalent levels of fear in either context, suggesting generalization, ($t_{(21)} = 1.33$, ns; $d = 0.56$). Although these results suggest a significant difference between OVX females given estradiol and controls, the comparison of latency scores in the neutral context was not significant, ($t_{(22)} = 0.950$, ns; $d = 0.39$). Therefore, the generalization observed in estradiol-treated animals was not significantly greater than control animals at the 5 day retention interval.

At 7 days (Fig 2D), the main effect for context was significant, ($F_{(1,36)} = 13.63$, $p<0.001$). The main effect for hormone treatment was non-significant by the conventional standards of $p<0.05$, although it approached significance, ($F_{(1,36)} = 3.78$, $p = 0.06$). The interaction between context and hormone replacement was non-significant, ($F_{(1,36)} = 2.51$, ns). Independent t-tests revealed that control animals continued to discriminate between the contexts, ($t_{(18)} = 3.32$, $p <0.01$). In contrast, estradiol-treated animals displayed equivalent levels of fear in either context, ($t_{(18)} = 1.74$, ns; $d = 0.78$). Additionally, control animals displayed significantly lower levels of fear in the neutral context compared to estradiol-treated animals, ($t_{(18)} = 2.13$, $p<0.05$; $d = 0.95$). Thus, OVX females given estradiol demonstrated significant fear generalization as evidenced by similar latency to cross scores in either context, and also had significantly longer

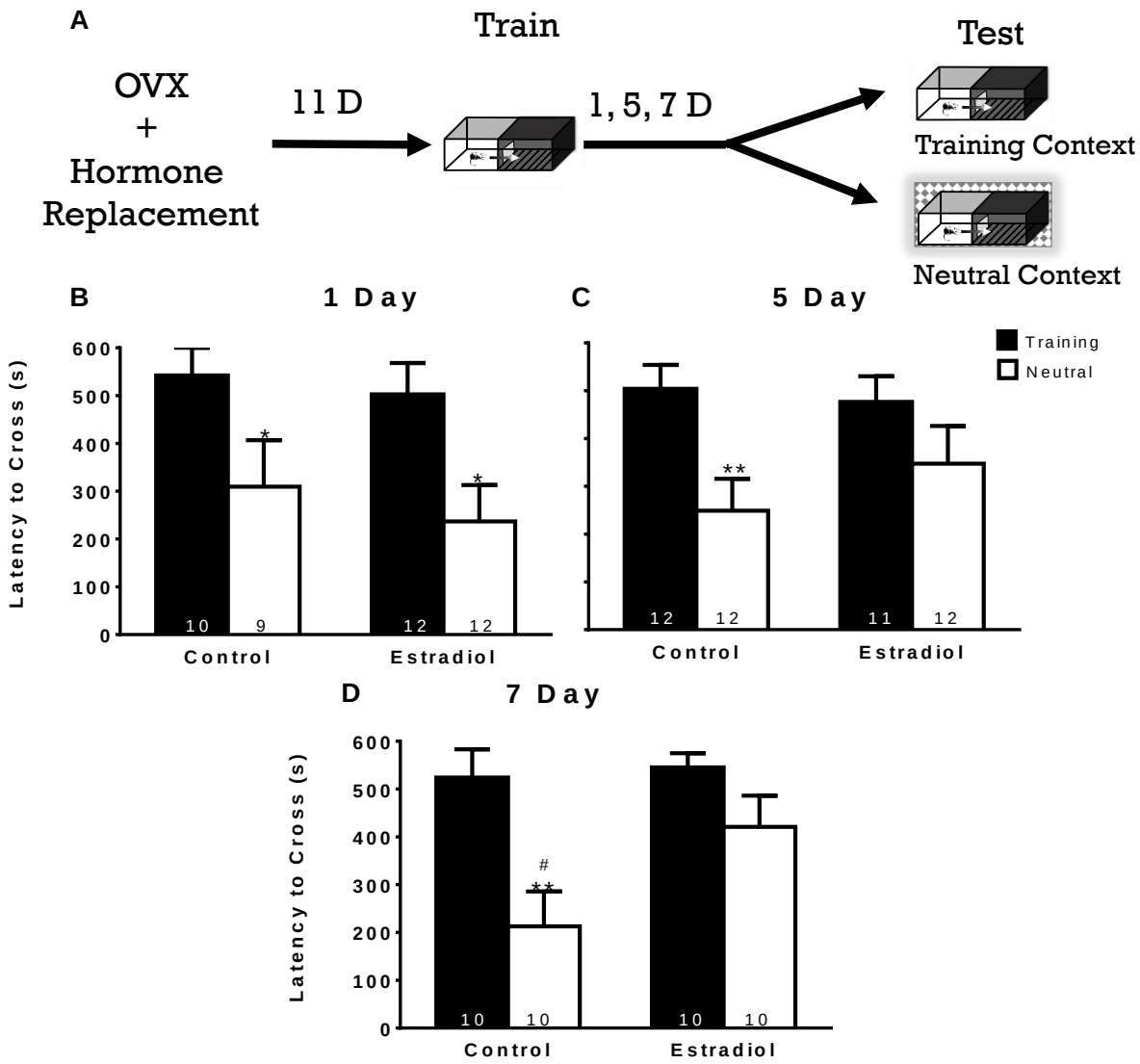
latencies in the neutral context compared to controls. Taken together, these data suggest that fear generalization in female rodents is regulated, in part by, estrogens.

In general, estrogens appear to increase arousal either by increasing overall activity levels in a safe environment or increasing fear or anxiety in an uncertain environment (For review, see Morgan et al., 2004). However, the effect of estrogens on fear learning has yielded conflicting results. Some experiments have demonstrated decreased fear in rodent models after estrogen treatment (Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; Markus & Zecevic, 1997), whereas others have reported an increase in fear (Jasnow, Schulkin, & Pfaff, 2006; Morgan & Pfaff, 2001). Although not specifically examined, estrogens did not increase or decrease overall avoidance levels. Rather, estrogens affected the ability to discriminate between contexts at a longer retention interval (i.e. 5 days). Therefore, the results may be best discussed in terms of selective attention. Estrogens in females—either endogenous or exogenous—appear to affect selective attention. For instance, estradiol benzoate given to ovariectomized rats results in decreases latent inhibition (Nofrey et al., 2008) and also disrupts fear inhibition in a discrimination learning task (Toufexis et al., 2007). Thus, estrogens seem to enhance attention capacity, resulting in negative consequences when the task in question requires focus on specific stimuli or ignoring specific stimuli. Animals respond fearfully to neutral contextual cues instead of inhibiting the fear response in the presence of those irrelevant stimuli. Another way to conceptualize the enhanced generalization seen with estradiol treatment is that estrogens are affecting the ability of females to inhibit responding. When animals are tested in the neutral context following estradiol treatment, females do not inhibit the fear response, resulting in generalized fear. Experiment 2 demonstrated

that estradiol is sufficient to induce generalized responding in ovarectomized females. The next experiment was designed to determine if estradiol acts to affect generalization the same way in male rats.

Figure 2. **Chronic Exposure to Estradiol Induces Generalization in OVX Females. A)** Schematic of the experimental paradigm. All animals were ovariectomized, given hormone replacement capsules, trained in passive avoidance, and tested 1, 5, or 7 days later. **B)** Both groups displayed significant context discrimination at 1 day, regardless of estradiol treatment. **C)** Control animals did not display significant generalization whereas estradiol-treated animals did. However, groups display no significant difference in fear levels when tested in the neutral context at 5 days. **D)** When tested 7 days after training, estradiol-treated animals display significant generalization and more fear than control animals in the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 2. Chronic Exposure to Estradiol Induces Generalization in OVX Females[4]



[4] Figure adapted from: Lynch III, J. F., Cullen, P. K., Jasnow, A. M., & Riccio, D. C. (2013). Sex Differences in the Generalization of Fear as a Function of Retention Intervals *Learning & Memory*, *20*, 628-632

IV. Experiment 3 - Chronic Hormone Exposure in GDX Males[5]

4.1. Introduction

Male rats produce estrogens through the aromatization of testosterone (Bon-chu & Meng-Chun, 2002; Simpson & Davis, 2001). Given that estradiol induces generalization in females, how males are able to maintain memory specificity over a longer period of time compared to females remains unknown. One possibility is that the level of estradiol produced by aromatization of testosterone in the brain is not sufficient to induce fear generalization in males. Exogenous administration of estradiol in males could induce fear generalization as seen in females. Alternatively, aromatized testosterone may help maintain memory specificity over time in males, thereby reducing fear generalization. A final possibility is that androgens act directly on androgen receptors to help maintain memory specificity. Nothing is currently known about how estradiol affects fear generalization in males. But, existing literature on anxiety—a related, yet different behavioral phenomenon—shows 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, von Mühlen, & Kritz-Silverstein, 1999; Cooper & Ritchie, 2000; Kaminetsky, 2005; Veras & Nardi, 2010). Additionally, post-pubertal castration increases anxiety-like behavior, and testosterone can reduce anxiety-

[5] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

like behavior in male and female rodents (Bitran, Kellogg, & Hilvers, 1993; Edinger & Frye, 2004; Forman, Tingle, Estilow, & Cater, 1989; McDermott, Liu, & Schrader, 2012; Molina, Bedran-de-Castro, & Bedran-de-Castro, 1994; Romero, Cooper, Komisaruk, & Bodnar, 1988). The evidence therefore, supports androgens, such as testosterone, reducing anxiety-like behavior in males and females, but the mechanisms responsible for these effects are not well understood. How the effects of testosterone on anxiety-like behavior are related to fear generalization are also unknown. Testosterone can be converted into several different metabolites including estradiol and dihydrotestosterone (DHT). DHT is a potent androgen that has higher affinity and efficacy on androgen receptors than testosterone (Deslypere, Young, Wilson, & McPhaul, 1992; Wilbert, Griffin, & Wilson, 1983). DHT is metabolized from testosterone through 5α-reductase and DHT cannot be metabolized into estradiol (Andriole et al., 2004). Given the role of testosterone in anxiety-like behavior, males in Experiment 3 were hypothesized to show a slower rate of fear generalization compared to females due to the presence of testosterone and its actions on androgen receptors. Thus, gonadectomy and/or estradiol administration would induce fear generalization. Administration of testosterone or DHT through actions on androgen receptors, however, would attenuate fear generalization**.** In order to test this hypothesis, male gonadectomized (GDX) 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.

4.2.Methods

Animals

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 and then individually housed and maintained on a 14/10 hour light/dark cycle with food and water available *ad libtum* throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 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 and a silastic capsule was inserted behind the shoulder blades of the animal. All pre-operative and post-operative care was conducted as described in Experiment 2

Drug Administration

Animals were given silastic capsules containing hormone (estradiol benzoate, testosterone proprionate, or DHT) or an empty capsule (Sigma Aldrich). The silastic (polydimethylsiloxane) implants were constructed from silastic tubing (i.d. 0.078 inches, o.d. 0.125 inches) and cut to a 5 mm length for estradiol (Bridges, 1984b; Hiroi & Neumaier, 2006) and 14 mm length for testosterone proprionate or DHT (Edinger & Frye,

2004; Frye & 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 hours at 37°C.

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 immunosorbant 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.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone treatments.

4.3. Results and Discussion

In order to assess the effects of gonadal hormones on fear generalization in male rats, GDX males were given capsules containing either testosterone proprionate, estradiol benzoate, DHT, or an empty capsule (control) and trained in passive avoidance (Fig 3A).

Animals were then tested in either the training context or a neutral context 1 day (Fig 3B) or 7 days (Fig 3C) after training A factorial ANOVA analysis of animals tested 1 day after training revealed a significant main effect for context, ($F_{(1,83)} = 32.82$, $p < 0.001$), indicating longer latencies to cross to the black compartment when groups were tested in the training context versus the neutral context. The main effect for treatment was also significant, ($F_{(3,83)} = 3.27$, $p < 0.05$), indicating significant differences between animals given different hormone treatments. The interaction term was also significant, ($F_{(3,83)} = 2.90$, $p < 0.05$), suggesting a difference between treatments based on the context of testing. For animals tested 7 days after training, ANOVA analyses revealed a significant main effect for context, ($F_{(1,75)} = 23.07$, $p < 0.001$), a non-significant main effect for treatment, ($F_{(3,75)} = 1.65$, ns), and a non-significant interaction, ($F_{(3,75)} = 1.66$, ns). Independent t-test analyses revealed that animals treated with testosterone did not display significant generalization 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 the hypothesis, estradiol treatment also resulted in no generalization 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, (testosterone: $t_{(23)} = 2.65$, $p < 0.01$, $d = 1.14$; 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, (testosterone: $t_{(25)} = 0.99$, 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, (testosterone: $t_{(16)} = 2.70$, $p < 0.05$,$d = 1.27$; 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, (testosterone: $t_{(17)} = 1.05$, 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 reduced testosterone levels in GDX males treated with a blank capsule and that testosterone capsules significantly elevated testosterone levels, ($t_{(26)} = 5.62$, $p < 0.001$, $d = 2.12$), (Fig 3D).

These results demonstrate that testosterone and estradiol attenuate generalized fear in gonadectomized males. Treatment with testosterone in the current study resulted in reduced fear generalization. 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 males. 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.

One reason why estradiol may act to reduce generalization in males 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 & Breedlove, 1985; McCarthy, 2006; McEwen, 1992; Schwarz & McCarthy, 2008). Differentiation requires aromatization in neonatal rat brain (McEwen, Lieberburg, Chaptal, & Krey, 1977). Thus, organizational effects of estradiol on regions controlling contextual fear memory in males may establish adult responses resulting in a sex-dependent effect of estradiol on fear generalization. That the hippocampus responds distinctly in males and females in response to estradiol is evidence that generalization occurs on a physiological level that could translate to behavior. Dendritic spine density in the CA1 changes 30% across the stages of the estrous cycle in females rats. The highest density of dendritic spines is seen during the proestrus stage, where estrogen levels are highest, and spine density levels surpass the levels seen in males at this stage (Shors, Chua, & Falduto, 2001; Woolley & McEwen, 1992). Within males, GDX reduces CA1 synapse density by ~50%, which is reversed by 2 days of androgens (Testosterone or DHT), but not estradiol (Kovacs, MacLusky, & Leranth, 2003; Leranth, Petnehazy, & MacLusky, 2003). Thus, dendritic remodeling in females is highly sensitive to changes in estrogens, whereas dendritic remodeling is unresponsive to estrogens in males. These sex-specific differences in the hippocampal response to gonadal hormones may explain why animals display a sex-dependent response to gonadal hormones in fear generalization.

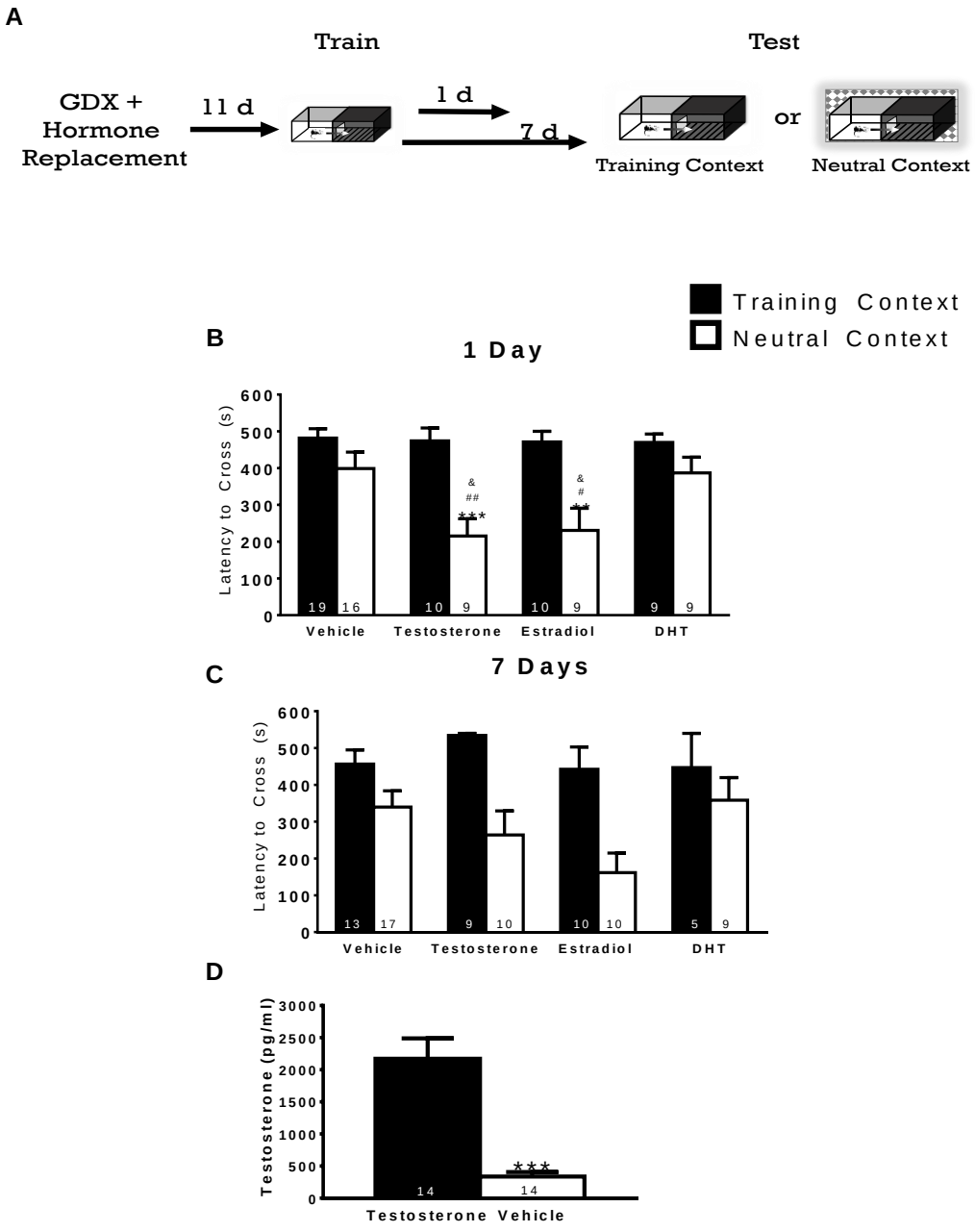
One alternative possibility is that the sex difference in generalization is related to sex differences in spatial task performance. The hippocampus is larger, thicker, and heavier in male rats (Diamond, 1987; Madeira, Sousa, Cadete-Leite, Lieberman, & Paula-

Barbosa, 1993; Pfaff, 1966) and these differences have been correlated with the sex-dependent behavior seen in spatial memory tasks. Specifically, males display better performance than females in a variety of spatial memory tasks (Barrett & Ray, 1970; Beatty, 1984; Beiko, Lander, Hampson, Boon, & Cain, 2004; Davenport, Hagquist, & Rankin, 1970; Dawson, 1972; Dawson, Cheung, & Lau, 1975; Einon, 1980; Gaulin & FitzGerald, 1986; Gresack & Frick, 2003; Perrot-Sinal, Kostenuik, Ossenkopp, & Kavaliers, 1996). This sex difference in spatial memory may also be a result of distinct strategies utilized by males and females. Males and proestrus females are more likely to utilize extramaze cues compared to estrous females (Korol & Kolo, 2002). These distinctions in learning strategies and spatial memory may translate to the sex-specific effects seen in contextual generalization.

Data from Experiment 2 showed that estrogens contribute to accelerated fear generalization in females without increasing overall fear and anxiety levels. However, the use of chronic estradiol treatment does not allow the determination of whether estrogens increase fear generalization through an effect on fear acquisition, consolidation, or retrieval. Therefore, Experiment 4 was designed to assess which aspect of the learning and memory process estrogens act upon to effect fear generalization.

Figure 3. **Chronic Exposure to Testosterone or Estradiol Prevents Generalization in GDX Males. A)** Schematic of the experimental paradigm. All animals were trained in passive avoidance and tested 24 hours 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 resulted in the same pattern as seen at 1 day. Testosterone- and estradiol-treated animals displayed no significant generalization whereas those treated with DHT or given no hormone replacement displayed 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 or testosterone concentration (pg/ml). Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 3. Chronic Exposure to Testosterone or Estradiol Prevents Generalization in GDX Males[6]



[6] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

V. Experiment 4 - Effects of Estradiol on Memory Acquisition/Consolidation or Retrieval

5.1.Experiment 4A - Pre-Training Acute Hormone Exposure in OVX Females[7]

The consensus is that the processes involved during acquisition/consolidation and retrieval of fear memory initiate distinct molecular and cellular mechanisms and manipulations can affect either acquisition/consolidation or retrieval, but not necessarily both (Abel & Lattal, 2001; Antoniadis, Winslow, Davis, & Amaral, 2009; Matus-Amat, Higgins, Sprunger, Wright-Hardesty, & Rudy, 2007; Miserendino, Sananes, Melia, & Davis, 1990; Venable & Kelly, 1990; Walker & Davis, 2008). Many have demonstrated that estrogens can have enhancing effects on spatial learning when present during consolidation (Inagaki, Gautreaux, & Luine, 2010; Packard, 1998), but few studies have demonstrated effects of estrogens on retrieval mechanisms, calling attention to the need for a more in depth examination of the effects of estrogens on retrieval. Given that the chronic treatment in Experiment 2 was present during the time of acquisition/consolidation, estradiol was expected to would increase fear generalization through an effect on fear acquisition/consolidation. To test this hypothesis, OVX female rats were given injections of estradiol or vehicle at different time points before passive avoidance training.

[7] Experiment describe here is published: Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429.

5.2.Methods

Animals

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 was available *ad libtum* throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Surgical Procedures

Ovarectomies were conducted as described in Experiment 2.

Drug Administration

OVX females were administered either vehicle control (sesame oil, 0.1 mL) or estradiol benzoate (estradiol) dissolved in sesame oil (15 μg/0.1 mL) by subcutaneous (SC) injection. This dose of estradiol 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 estradiol 24 hours, 6 hours, or 1 hour before passive avoidance training and tested 24 hours or 7 days later.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone treatments.

5.3.Results and Discussion

OVX females were given injections of either vehicle or estradiol prior to passive avoidance training and then tested at different retention intervals (Fig 4A). When injections were given 24 hours before training and testing occurred 24 hours after training, both estradiol-treated rats and vehicle-treated rats exhibited significantly reduced latencies in the neutral context compared to the training context (Fig 4B). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1, 36)} = 143.2$, $p<0.001$), indicating longer latencies to cross to the black compartment when groups were tested in the training context versus the neutral context. The main effect for hormone treatment was not significant, ($F_{(1, 36)} = 0.1309$, ns), indicating no differences between animals injected with estradiol or vehicle control in latencies across both contexts. The interaction term was also not significant, ($F_{(1, 36)} = 1.877$, ns), suggesting no differences between estradiol-treated and vehicle-treated females. 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 estradiol-treated rats tested in the training versus neutral context, ($t_{(18)} = 5.91$, $p < 0.001$; $d = 2.64$). In addition, vehicle-treated and estradiol-treated rats did not differ in latency to cross when

tested in the neutral context, ($t_{(18)} = 0.6$, ns; $d = 0.26$) (Figure 2A). These data demonstrate that when estradiol administration occurred 24 hours before testing, females were able to discriminate between contexts at a 24-hour test interval.

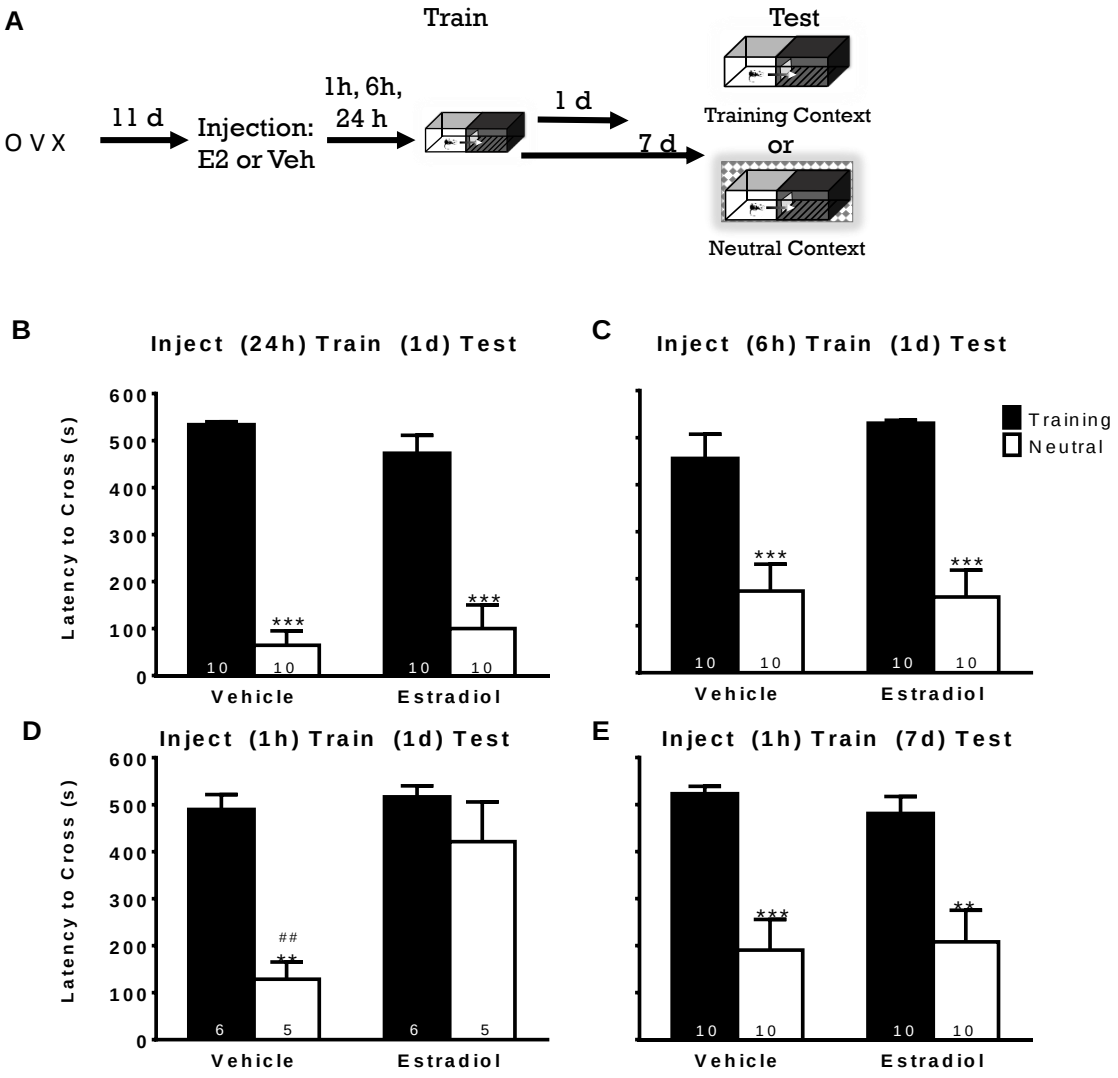
Given that estradiol did not have an impact when administered 24 hours before training, another group of was injected with either vehicle or estradiol 6 hours before training and tested 24 hours later (Fig 4C). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1, 36)} = 45.72$, $p<0.001$), whereas the main effect for hormone treatment, ($F_{(1, 36)} = 0.416$, ns), and the interaction term were not significant, ($F_{(1, 36)} = 0.825$, ns). These data mirror the findings observed when rats were administered estradiol 24 hours before training. Indeed, Independent t-test analyses revealed that estradiol injections 6 hours before training did not influence fear generalization to the neutral context. Animals tested in the neutral context exhibited significantly less fear compared to animals tested in the training context, regardless of treatment, (vehicle: $t_{(18)} = 3.65$, $p < 0.001$; $d = 1.63$; estradiol: $t_{(18)} = 6.4$, $p<0.001$; $d = 2.86$). In addition, vehicle-treated rats were not significantly different than estradiol-treated rats when tested in the neutral context, ($t_{(18)} = 0.156$, ns; $d = 0.07$).

Another group of animals were given injections 1 hour prior to training, and were tested either 24 hours after training (Fig 4D) or 7 days after training (Fig 4E). When tested 24 hours after training, factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1, 17)} = 25.46$, $p<0.001$), a main effect for hormone treatment, ($F_{(1, 17)} = 13.00$, $p<0.01$), and a significant interaction, ($F_{(1, 17)} = 6.50$, $p<0.05$). Independent t-test analyses revealed a significant difference between vehicle-treated females but not estradiol-treated females, (vehicle: $t_{(9)} = 7.47$, $p < 0.001$; $d = 4.52$; estradiol: $t_{(8)} = 1.40$,

ns, $d = 0.68$). In addition, vehicle-treated rats were significantly different than estradiol-treated rats when tested in the neutral context, ($t_{(8)} = 3.1732$, $p < 0.01$, $d = 2.00$). These findings suggest that estradiol affects fear acquisition/consolidation and increased fear generalization. However, when rats were tested 7 days after training, the context fear generalization was absent. A factorial ANOVA revealed a significant main effect for context, ($F_{(1, 36)} = 35.51$, $p<0.001$), a non-significant main effect for hormone, ($F_{(1, 36)} = .057$, ns), and interaction between context and hormone treatment, ($F_{(1, 36)} = 0.34$, ns). 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, (vehicle: $t_{(18)} = 4.95$, $p<0.001$; $d = 2.22$; estradiol: $t_{(18)} = 3.58$, $p < 0.01$; $d = 1.60$). In addition, vehicle-treated rats were not significantly different than estradiol-treated rats when tested in the neutral context, ($t_{(18)} = 0.187$, ns; $d = 0.08$). The findings at 7 days suggests that estradiol is not altering or affecting acquisition or consolidation of memory because the effects of estradiol appear to be transient. 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. In order to directly test the role of estrogens on memory retrieval, OVX females were trained on passive avoidance, administered estradiol 24 hours later, and tested at various retention intervals following injections in Experiment 4B.

Figure 4. **Acute Exposure to Estradiol Has a Transient Effect on Fear Generalization When Given 1 Hour Before Training in OVX Females. A)** Schematic of the experimental paradigm. The timing of estradiol injections was varied in relation to passive avoidance training. Testing occurred at varying retention intervals. **B)** Estradiol was administered 24 hours before passive avoidance training and animals were tested 24 hours after training. Latency to cross was significantly higher in the training context compared to the neutral context, regardless of estradiol treatment. When injected 24 hours before training, estradiol did not increase fear generalization to the neutral context. **C)** Estradiol was administered 6 hours before training and animals were tested 24 hours after training. Latency to cross was significantly higher in the training context compared to the neutral context, regardless of estradiol treatment. Estradiol did not increase fear generalization when injected 6 hours before training. **D)** Estradiol was administered 1 hour before training and animals were tested 24 hours after training. Estradiol-treated animals displayed similar latency to cross scores in the training and neutral contexts. Thus, estradiol significantly increased fear generalization to the neutral context when injected 1 hour prior to training. **E)** When estradiol was administered 1 hour before training and animals were tested 7 day after training, fear generalization was absent. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 4. Acute Exposure to Estradiol Has a Transient Effect on Fear Generalization When Given 1 Hour Before Training in OVX Females[8]



[8] Figure adapted from Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429.

5.4.Experiment 4B - Post-Training Acute Hormone Exposure in OVX Females[9]

In Experiment 4B, all animals were injected with estradiol or vehicle 24 hours after training and tested at different retention intervals after injections to assess whether estrogens are required during memory retrieval in order to affect fear generalization or if they have a more long-term effect on contextual fear memory retrieval.

5.5. Methods

All behavior and surgical procedures were conducted as described in Experiment 4A.

Open Field

To test for effects on locomotor activity, animals were given injections of estradiol and were placed into an open field chamber 24 hours later (122 cm diameter) and allowed to explore the open field for 10 minutes. Locomotor activity was measured via AnyMaze 4.99 software (Stoelting, Wood Dale, IL).

5.6. Results and Discussion

Animals were given injections of estradiol or vehicle 24 hours after training and were tested at different retention intervals following injection (Fig 5A). When estradiol or vehicle was administered 24 hours before testing, female rats given estradiol displayed

[9] Experiment describe here is published: Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429.

significant fear generalization to the neutral context compared to females administered vehicle (Fig 5B). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1, 34)} = 15.09$, $p<0.001$), a main effect for hormone trending towards significance, ($F_{(1, 34)} = 3.956$, $p = 0.054$), and a non-significant interaction, ($F_{(1, 34)} = 2.866$, ns). Independent t-test analyses revealed a significant difference between vehicle-treated females but not estradiol-treated females, (vehicle: $t_{(16)} = 4.23$, $p < 0.001$; $d = 1.99$; estradiol: $t_{(18)} = 1.48$, ns; $d = 0.66$,). In addition, vehicle-treated rats were significantly different than estradiol-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.

To determine how long fear generalization would persist following estradiol treatment, animals were injected with estradiol and tested 48 hours later (Fig 5C). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1, 36)} = 19.55$, $p<0.001$), a non-significant main effect for hormone, ($F_{(1, 36)} = 0.312$, ns), and an interaction trending towards significance, ($F_{(1, 36)} = 3.881$, $p = 0.056$). Independent t-test analyses revealed a significant difference between vehicle-treated females but not estradiol-treated females, (vehicle: $t_{(16)} = 5.88$, $p < 0.001$; $d = 2.63$; estradiol: $t_{(18)} = 1.45$, ns; $d = 0.65$). However, vehicle-treated rats were not significantly different from estradiol-treated rats when tested in the neutral context, ($t_{(18)} = 1.60$, ns; $d = 0.72$). These results suggest that estradiol injections administered 48 hours before testing results in fear generalization in the neutral context. However, this generalization is not as robust as that seen at a 24-hour test. Although estradiol-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. Thus, estradiol has a transient effect on fear generalization through modulation of fear memory retrieval that lasts at least 48 hours.

To confirm that estradiol-treatment would not impact generalization at longer retention intervals, females were injected with estradiol or vehicle and tested 6 days later (Fig 5D). Factorial ANOVA analyses revealed a significant main effect of context, ($F_{(1, 36)} = 17.55$, $p<0.001$), a non-significant main effect of hormone, ($F_{(1, 36)} = 0.015$, ns), and a non-significant interaction, ($F_{(1, 36)} = 0.001$, ns). 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, (vehicle: $t_{(18)} = 3.01$, $p < 0.01$; $d = 1.35$; estradiol: $t_{(18)} = 2.91$, $p < 0.01$; $d = 1.30$). In addition, vehicle-treated rats were not significantly different than estradiol-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 hours.

Although the 24 hour and 48 hour test suggests a transient effect on retrieval, the 24 hour delay between estradiol administration and behavioral testing does not rule out potential immediate effects of estradiol on fear memory retrieval and generalization. Thus, to determine if estradiol has immediate effects on fear generalization, OVX females were injected with estradiol and tested 1 hour later (Fig 5E). Factorial ANOVA analyses revealed a significant main effect of context, ($F_{(1, 32)} = 354.1$,$p<0.001$), a non-significant main effect of hormone, ($F_{(1, 32)} = 1.173$, ns), and a non-significant interaction, ($F_{(1, 32)} = 0.007$, ns). 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, (vehicle: $t_{(16)} = 15.206$, $p < 0.001$; $d = 7.17$; estradiol: $t_{(16)} = 11.96$, $p < 0.001$; $d = 5.64$). In addition, vehicle-treated rats were not significantly different from estradiol-treated rats when tested in the neutral context, ($t_{(16)} = 0.60$, ns; $d = 0.28$). This suggests that estradiol is acting to affect fear generalization on memory retrieval, but estradiol takes time to induce generalized responding to a neutral context; no effects are seen when testing occurs shortly after estradiol treatment.

Finally, to rule out intermediate effects of estradiol, animals were injected with estradiol and tested 6 hours following the injection (Fig 5F). Factorial ANOVA analyses revealed a significant main effect of context, ($F_{(1, 34)} = 39.67$, $p<0.001$), a non-significant main effect of hormone, ($F_{(1, 34)} = 0.001$, ns), and a non-significant interaction, ($F_{(1, 34)} = 0.11$, ns). 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, (vehicle: $t_{(16)} = 4.95$, $p < 0.001$; $d = 2.19$; estradiol: $t_{(18)} = 4.07$, $p < 0.001$; $d = 1.82$). In addition, vehicle-treated rats were not significantly different than estradiol-treated rats when tested in the neutral context, ($t_{(17)} = 0.15$, ns; $d = 0.07$). Taken together, these data suggest that estradiol increases fear generalization through an effect on fear memory retrieval.

To make sure that estradiol at this time point was not resulting in generalized fear due to locomotion differences between estradiol-treated and vehicle-treated animals, animals were given injections and 24 hours later were placed into an open field for 10 minutes and assessed for locomotor activity. Independent t-test analyses reveal no differences in distance travelled between estradiol-treated and vehicle-treated animals,

($t_{(6)}$ = 0.69, ns; d = 0.49), demonstrating that estradiol given 24 hours beforehand is not affecting generalized responding through alterations in locomotor activity (Fig 5G).

Estradiol has an enhancing effect on passive avoidance retention (Rhodes & Frye, 2004), and could have enhanced retention in the current study rather than having effects on fear generalization. However, in the current study, no differences were found between vehicle-treated and estradiol-treated animals tested in the training context, suggesting no enhancement of fear retention. 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. These results are in agreement with other spatial memory tasks where memory improvements are seen at 24 hours—but not 8 hours—following estradiol treatment (Sandstrom & 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 estrogens are required during memory consolidation of object placement task (Inagaki et al., 2010). In the present study, estradiol given 24 hours after training, presumably after the consolidation window is no longer open, still resulted in generalization 24 hours 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 & Pfaff, 2001; Morgan et al., 2004). However, injections given 1 hour before training only elicited fear generalization 24 hours later, not 7 days later,

suggesting that the consolidation process was not altered, as no generalization was seen at the later retention interval.

Although not explicitly tested here, the time course of the effects of estradiol on fear generalization suggests 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 & 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 & Levin, 2001; Levin, 2005; Vasudevan, Kow, & Pfaff, 2001). Although the immediate (1 hour test) and intermediate (6 hour test) time points 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 estradiol 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.

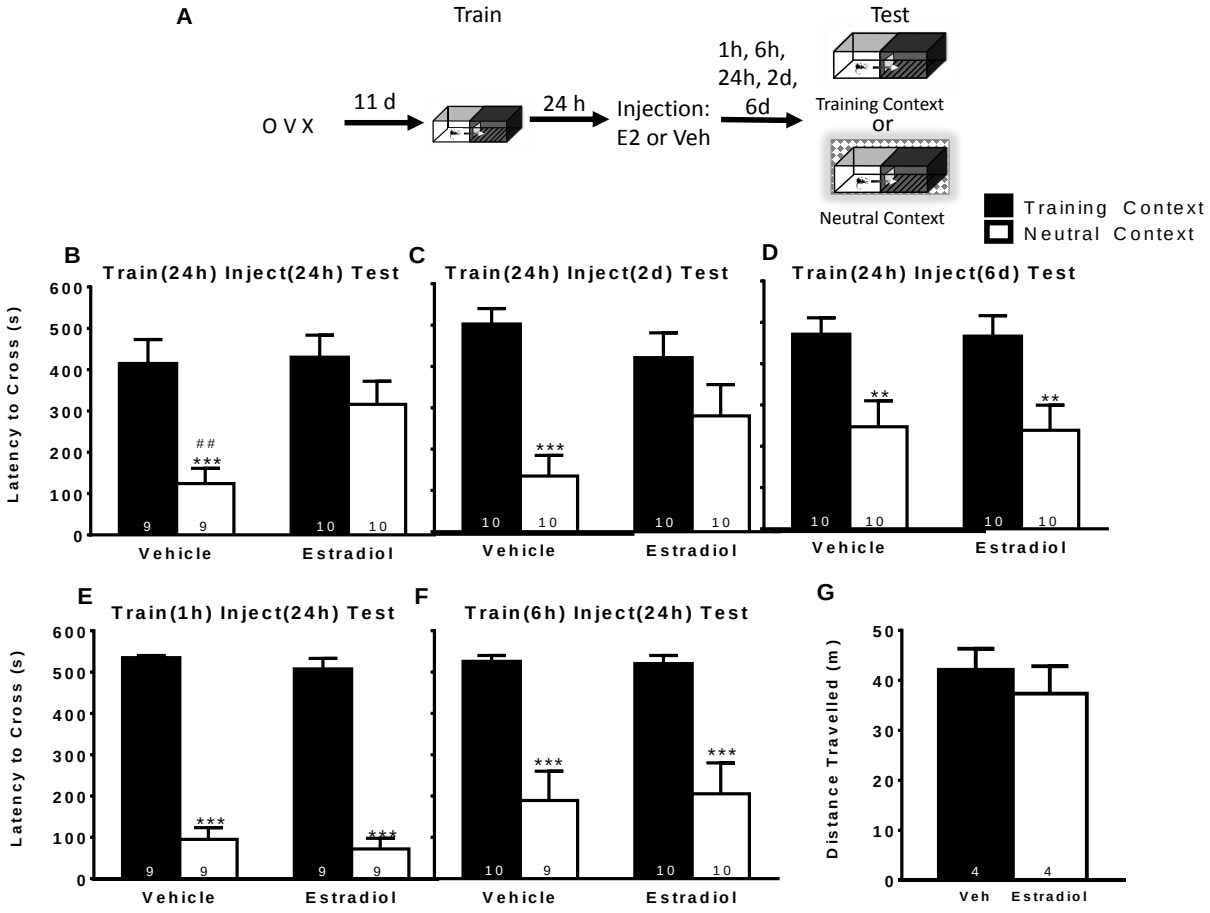
The generalization seen here 24 hours after estradiol administration rather than 7 days after estradiol treatment is different from what was observed with chronic estradiol in Experiment 2. This finding is not surprising given that several reports suggest differences in effects of estradiol on behavior with chronic versus acute exposure (Luine, Richards, Wu, & Beck, 1998; Walf & Frye, 2006). In fact, the length of exposure to estradiol (5 days vs. 35 days) dramatically alters estradiol's anxiolytic effects on the open field task (Luine et al., 1998) and chronic estradiol exposure can alter the expression of

estrogen receptors by up-regulating ERβ, but not ERα, after 12 days of treatment with estradiol valerate (M. Jin, Jin, Zhang, Chen, & Huang, 2005). Therefore, the differences between Experiment 2 and Experiment 4 could be due to compensatory mechanisms of chronic estradiol exposure versus acute treatment. Acute estradiol treatment may more accurately reflect the endogenous surge of estrogens during the estrous cycle as compared to chronic estradiol implants, and thus, may more accurately represent the effects of estrogens on fear generalization as would be seen in intact females.

Overall, Experiment 4A and 4B demonstrates a role for estradiol to induce context generalization by affecting memory retrieval. In order to determine if estradiol acts within the same time frame to attenuate fear generalization in GDX males, the next experiment used acute hormone injections in males.

Figure 5. **Estradiol has a Transient Effect on Memory Retrieval in OVX Females**. **A)** Schematic of the experimental paradigm. The timing of injections occurred 24 hours after training and testing occurred at varying retention intervals. **B)** Estradiol-treated animals displayed significant fear generalization whereas vehicle-treated animals did not. **C)** When testing 48 hours after injection, estradiol-treated animals continued to display significant fear generalization. Generalization was not as robust as that seen at the 24 hour test as estradiol-treated animals did not show significantly higher latencies than vehicle-treated animals in the neutral context. **D)** When testing occurred 6 days after injection, vehicle-treated and estradiol-treated animals displayed significant context discrimination. **E)** Testing 1 hour after injection did not elicit fear generalization, regardless of estradiol treatment. **F)** Testing 6 hour after injection did not elicit fear generalization, regardless of estradiol treatment. **G)** When estradiol injections were given and animals were tested 24 hours later in an open field, no differences were seen in distance travelled compared to vehicle-treated animals, demonstrating that effects on generalization are not due to activity level changes in estradiol-treated animals. Values are displayed as mean (±SEM) latency to cross in seconds or distance travelled (m). Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 5. Estradiol has a Transient Effect on Memory Retrieval in OVX Females[10]



[10] Figure adapted from Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429.

5.7.Experiment 4C - Post-Training Acute Hormone Exposure in GDX Males[11]

In order to assess whether estradiol acted on memory retrieval to attenuate fear generalization in GDX males, animals were gonadectomized, trained in passive avoidance, given injections of testosterone, estradiol, DHT, or vehicle 24 hours later and tested 24 hours after the injections.

5.8.Methods

Animals

Adult male Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 3.

Surgical Procedures

Surgical Procedures were conducted as described in Experiment 3.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

[11] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

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 III et al., 2013; Lynch III et al., 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).

5.9. Results and Discussion

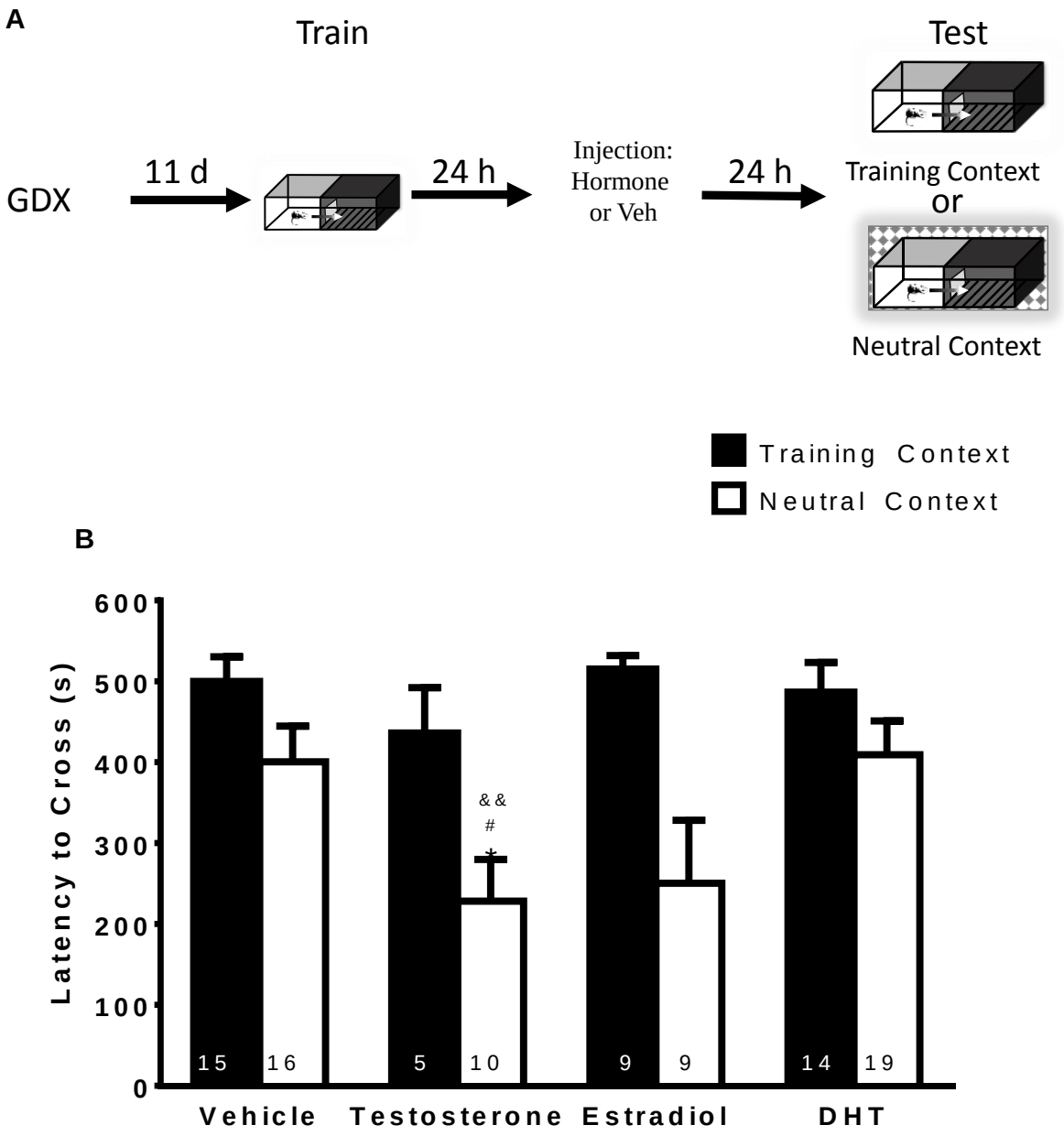
Animals were trained in passive avoidance, injected 24 hours later, and tested 24 hours after injection in either the training or neutral context (Fig 6A). ANOVA analyses revealed a significant main effect for context, ($F_{(1,89)} = 21.39$, $p < 0.001$), a non-significant main effect for treatment, ($F_{(3,89)} = 2.374$, $p = 0.08$), and a non-significant interaction, ($F_{(3,89)} = 1.70$, ns). 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 6B). Testosterone treated animals displayed significantly lower latencies in the neutral context compared to vehicle- and DHT-treated animals, (vehicle: $t_{(24)} = 2.49$, $p <0.05$, $d = 1.01$; 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, (vehicle: $t_{(23)} = 1.82$, $p = 0.08$, $d = 0.72$; 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, (vehicle: $t_{(29)} = 1.83$, ns, $d = 0.66$; DHT: $t_{(31)} = 1.33$, ns, $d = 0.48$), again suggesting that testosterone reduces generalization through conversion to estradiol. Together with results from experiment 4B, findings suggest estradiol acts through

memory retrieval to impact fear generalization in a sex-specific manner. In order to confirm that testosterone affects generalization through conversion into estradiol, Experiment 4D directly manipulated aromatase activity.

Figure 6. **Acute Exposure to Estradiol Affects Memory Retrieval by Attenuating Generalization in GDX Males**. **A)** Schematic of the experimental paradigm. Male rats were gonadectomized and trained in passive avoidance. Twenty four hours after training, animals were given hormone injections and tested 24 hours later. **B)** Animals given acute injections of testosterone or estradiol do not displayed significant generalization whereas those treated with DHT or are given no hormone replacement displayed significant generalization.

Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/##/&& = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 6. Acute Exposure to Estradiol Affects Memory Retrieval by Attenuating Generalization in GDX Males[12]



[12] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

5.10. Experiment 4D - Effects of Aromatase Inhibition on Generalization[13]

Thus far, experiments assessing the effects of hormones on fear generalization in males has revealed that testosterone and estradiol reduce fear generalization in GDX males compared to GDX males given no hormone replacement. Given the fact that testosterone can be converted into estradiol, the findings suggest that the reduction in generalization produced by testosterone is due to the aromatization of testosterone into estradiol. In order to directly determine the role of aromatization, male rats were treated with an aromatase inhibitor, Fadrozole, throughout the passive avoidance procedure.

5.11. Methods

Animals

Adult male Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 3.

Surgical Procedures

Surgical Procedures were conducted as described in Experiment 3.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

[13] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

Drug Administration

GDX and intact male rats were given daily injections of the aromatase inhibitor, Fadrozole (FAD) (1 mg/kg) (Graham & Milad, 2014) for seven days prior to and during behavioral training. GDX males received capsule implantations containing testosterone proprionate or a control capsule as described in Experiment 3.

5.12. Results and Discussion

GDX males were implanted with capsules containing testosterone or no hormone and were administered daily injections of the aromatase inhibitor, FAD, or vehicle, for 7 days continuing through passive avoidance training. A group of animals were left intact and also received FAD or vehicle injections for 7 days. Animals were then tested 2 days after training in either the training or a neutral context (Fig 7A). ANOVA analyses revealed a significant main effect for context, ($F_{(1,38)} = 10.44$, $p < 0.01$), a non-significant main effect for treatment, ($F_{(3,38)} = 1.82$, ns), and a non-significant interaction, ($F_{(3,38)} = 1.86$, ns) (Fig 7B). 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, (vehicle: $t_{(8)} = 0.6286$, ns, $d = 0.40$; FAD: $t_{(7)} = 1.18$, ns, $d = 1.00$). When gonadectomized males 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 test the effects of FAD with natural

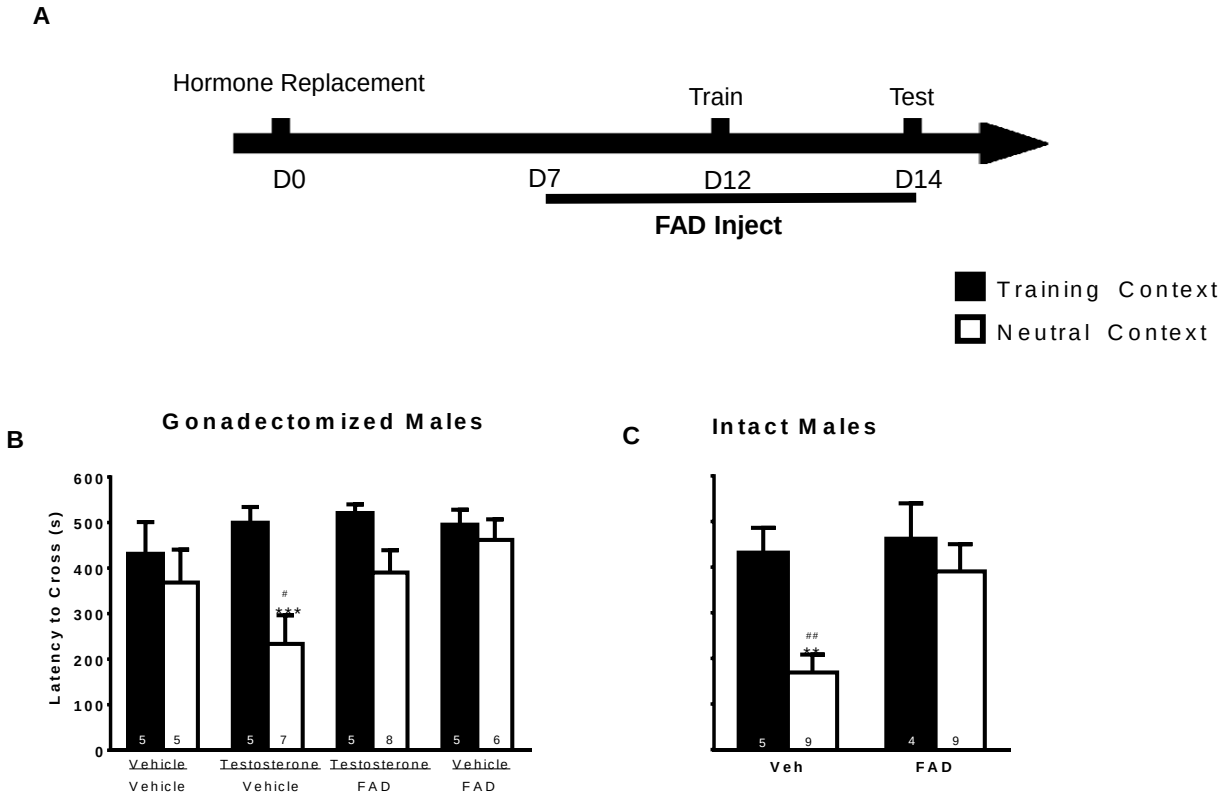
levels of testosterone present, intact males were given daily injections of FAD or vehicle and trained and tested in passive avoidance (Fig 7C). ANOVA analyses revealed a significant main effect for context, ($F_{(1,23)} = 7.65$, $p < 0.01$), a significant main effect for treatment, ($F_{(1,23)} = 4.35$, $p<0.05$), and a non-significant interaction, ($F_{(1,23)} = 2.46$, ns). Intact males given vehicle injections displayed significant discrimination between contexts, ($t_{(12)} = 3.951$, $p < 0.01$, $d = 2.19$). In contrast, intact males treated with FAD injections 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 the aromatization into estradiol; FAD treatment results in fear generalization to a neutral context.

The enzyme aromatase is responsible for the final step of estradiol biosynthesis, through the metabolism of testosterone (Bon-chu & Meng-Chun, 2002; Simpson & Davis, 2001). The conversion of testosterone into estradiol is implicated in affecting several types of behavior in males including sex behavior (Vagell & McGinnis, 1997), spatial memory (Packard, Kohlmaier, & Alexander, 1996), and extinction learning (Graham & Milad, 2014). When the synthesis of estradiol was blocked with FAD, gonadectomized males treated with testosterone and intact males displayed significant generalization. Overall, these results suggest that estradiol has significant effects on fear generalization in males, but in the opposite direction as seen in females. Removing testosterone via gonadectomy results in induced generalization as does treating intact males 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 males. Intact males generalize contextual fear in a time window of 14-28 days (Jasnow et al., 2012). Intact females generalize fear at a much faster rate of 5-7 days as seen in Experiment 1 and this faster rate of generalization is due to estradiol, which can also rapidly induce fear generalization in females. Thus, high levels of estrogens appear to disrupt the ability of female rats to inhibit a fear response to a neutral environment, but maintain appropriate inhibitory responses in males. These findings demonstrate the role of estrogens in fear generalization, but the mechanisms by which estrogens have an effect remain unknown. To begin to assess the mechanisms involved, Experiment 5 was designed to test specific receptor activation involved in fear generalization.

Figure 7. **Testosterone Prevents Generalization in Males Through Aromatization in Estradiol. A)** Schematic of the experimental paradigm for the experiment. All animals were given injections of Fadrozole (FAD) or vehicle for 7 days up through passive avoidance training and drug administration. Animals were trained in passive avoidance and 24 hours later, they were injected with testosterone or vehicle. Twenty four hours after treatment, animals were tested in the training or neutral context. **B)** Animals injected for 7 days with vehicle and treated with testosterone 24 hours prior to testing did not display significant generalization. In comparison, animals treated with FAD for 7 days and injected with testosterone displayed significant generalization, suggesting that testosterone prevents generalization through 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 vehicle through passive avoidance training. Animals were tested 48 hours after training and those animals treated with FAD displayed significant generalization whereas intact males injected with vehicle did not generalize fear to the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 7. Testosterone Prevents Generalization in Males Through Aromatization in Estradiol[14]



[14] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior*.

VI. Experiment 5 - Activation of Specific Estrogen Receptor (ER) Subtypes

6.1. Experiment 5A – Estrogen Receptor Agonist Injections to OVX Females[15]

In order to affect cellular processes, estrogens must bind to specific receptors. Estrogens can interact with two main receptor subtypes: ERα and ERβ. These receptors have nearly identical DNA sequences, but each receptor subtype has distinct N-terminals regions; the region that mediates receptor interactions with signaling pathways (Tee et al., 2004; Tremblay et al., 1997). Throughout the brain, ERα and ERβ are found within the amygdala, hippocampus, and prefrontal cortex—structures implicated in fear generalization—and ERβ is more widely distributed throughout these regions (Li, Schwartz, & Rissman, 1997; Österlund, Kuiper, Gustafsson, & Hurd, 1998; Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2000a, 2000b). The activation of each receptor subtype is associated with different behavioral responses. In general, ERα activation underlies sexual and exploratory behaviors (Luine, Jacome, & Maclusky, 2003; Mazzucco, Walker, Pawluski, Lieblich, & Galea, 2008; Morgan et al., 2004; Rhodes & Frye, 2006). For example, ovariectomized (OVX) rats treated with PPT, an ERα agonist (Stauffer et al., 2000), displayed increases in proceptive and receptive sexual behaviors comparable to animals treated with estradiol. However, DPN, an ERβ agonist (Meyers et al., 2001), did not have an effect on reproductive behaviors at any

[15]Experiment describe here is published: Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429.

dose (Mazzucco et al., 2008). In contrast, ERβ activation is linked to alterations in spatial learning and mediates the anxiolytic effects of estradiol (Bodo & Rissman, 2006; Lephart et al., 2002; Lund, Rovis, Chung, & Handa, 2005; Walf, Rhodes, & Frye, 2004) whereas both receptors are thought to play a role in emotional memory (Rhodes & Frye, 2006). Due to the diverse effects of ER activation on behavior, estrogens can affect several different behaviors in different ways based on levels of estrogens present and the type of receptor that is activated.

In order to determine the estrogen receptor subtype(s) responsible for fear generalization, animals were injected with an ERα agonist or an ERβ agonist at the time intervals established in Experiment 4B. Specifically, animals were given injections of the ER agonists 24 hours after training and were tested 24 hours later in either the training or neutral context. Given that ERβ activation has effects on spatial learning and anxiolytic behaviors, ERβ activation was hypothesized to increase fear generalization.

6.2.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

Surgical Procedures were conducted as described in Experiment 2.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Injections consisted of the ERα agonist, ,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*-phenol; Caymen Chemical (PPT) (2 mg/0.1 mL), the ERβ agonist, 2,3-*bis*(4-hydroxyphenyl)-propionitrile, Caymen Chemical (DPN), or vehicle injections (DMSO, 0.1 mL) (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 behavior, respectively, similar to those observed with estradiol treatment (Frasor et al., 2003; H. A. Harris, Katzenellenbogen, & Katzenellenbogen, 2002; Mazzucco et al., 2008).

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of ER agonists.

6.3.Results and Discussion

Animals were injected with PPT (ERα agonist), DPN (ERβ agonist), or vehicle, 24 hours after training and tested 24 hours after injection (Fig 8A). When 1 mg/kg of PPT, DPN, or vehicle was administered 24 hours before testing, no treatment elicited generalized responding to the neutral context (Fig 8B). ANOVA analyses revealed a main effect for context, ($F_{(1,59)} = 74.94$, $p<0.001$), but the main effect for treatment, ($F_{(2,59)} = 0.56$, ns), and the interaction term, ($F_{(2,59)} = 0.25$, ns), were not significant. 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, (vehicle: $t_{(16)} = 4.39$, $p < 0.001$; $d = 2.07$; ERα: $t_{(22)} = 5.94$, $p < 0.001$; $d = 2.43$;

ERβ: $t_{(21)} = 4.75$, $p < 0.001$; $d = 2.00$). Additionally, activation of either ERα or ERβ did not induce more fear in the neutral context compared to vehicle-treated animals (ERα: $t_{(19)} = 0.039$, ns; $d = 0.02$; ERβ: $t_{(19)} = 0.21$, ns; $d = 0.09$). When animals were injected with 2.5 mg of PPT, DPN, or both, only activation of ERβ increased fear generalization (Fig 8C). Factorial ANOVA analyses revealed a main effect for context, ($F_{(1,72)} = 23.67$, $p<0.001$), a trending main effect for treatment, ($F_{(3,72)} = 2.59$, $p=0.06$), and a non-significant interaction term, ($F_{(3,72)} = 1.62$, ns). 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, (vehicle: $t_{(18)} = 3.43$, $p < 0.001$; $d = 1.53$; ERα: $t_{(18)} = 2.91$, $p < 0.01$; $d = 1.30$; ERβ: $t_{(18)} = 0.97$, ns; $d = 0.43$). Therefore, activation of ERβ, but not ERα, resulted in significant generalization to the neutral context. However, animals given PPT and DPN combined 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, (ERα: $t_{(18)} = 0.28$, ns; $d = 0.16$; ERβ: $t_{(18)} = 2.61$, $p < 0.05$; $d = 1.72$; ERα+ERβ: $t_{(18)} = 0.26$, ns; $d = 0.14$). These results demonstrate that activation of ERβ increases fear generalization in a dose-dependent manner, suggesting that the effects of estradiol on memory retrieval seen in Experiment 4B are due to activation of ERβ. Interestingly, when PPT and DPN were given simultaneously, the increased generalization was attenuated. This finding is similar to that seen in Mazzucco et al. (2008) with proceptive and receptive sexual behavior, suggesting a modulatory effect of ERα on ERβ.

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. Results with specific ER agonists demonstrate that ERβ activation drives the increased fear generalization produced by acute injections of estradiol. The following experiment was designed to assess which receptor subtype estradiol acts through to affect fear generalization in male rats.

6.4.Experiment 5B - Estrogen Receptor Agonist injections to GDX Males[16]

Estradiol acts in a sex-dependent manner on fear generalization, inducing generalization in OVX females and attenuating generalization in GDX males. However, the mechanisms by which the sex-dependent effect occurs remains unknown. The current experiment was designed to determine if activation of ERα or ERβ was necessary for male-specific attenuation of generalization. Given that ERβ drives the induction of generalized responding in females, activation of ERβ was hypothesized to be required for attenuation of generalization in GDX males.

6.5. Methods

Animals

Adult male Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 3.

Surgical Procedures

Surgical Procedures were conducted as described in Experiment 3.

[16] Manuscript has been submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior.*

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Injections consisted of the ERα agonist, ,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*-phenol; Caymen Chemical (PPT) (2 mg/0.1 mL), the ERβ agonist, 2,3-*bis*(4-hydroxyphenyl)-propionitrile, Caymen Chemical (DPN), or vehicle injections (DMSO, 0.1 mL) (Meyers et al., 2001; Stauffer et al., 2000).

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of ER agonists in males.

6.6.Results and Discussion

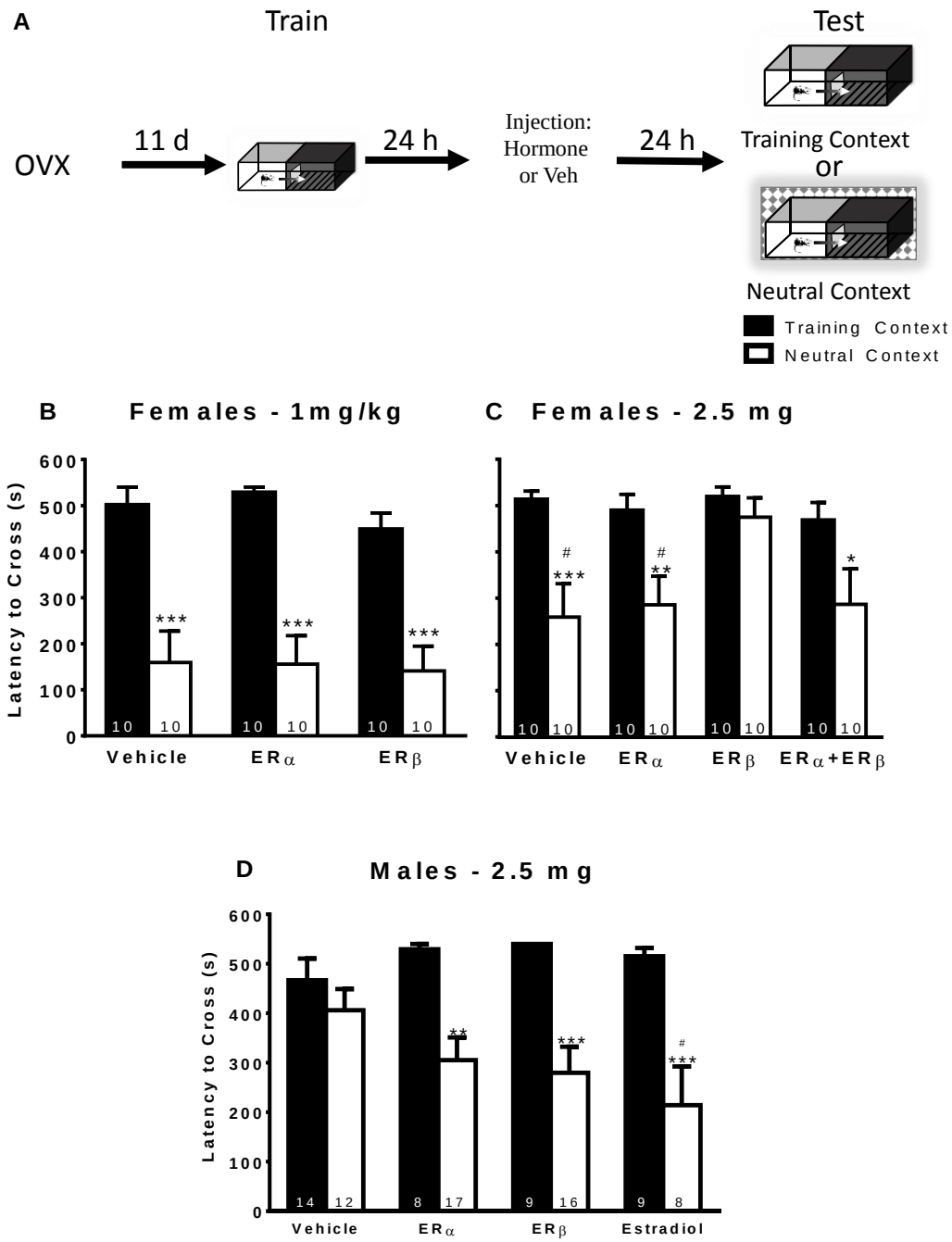
In order to determine which ER subtype mediates attenuated fear generalization in males, animals were given injections of ER agonists (Fig 8A). ANOVA analyses revealed a significant main effect for context, ($F_{(1,85)}$ = 36.98, $p < 0.001$), a non-significant main effect for treatment, ($F_{(3,85)}$ = 0.69, ns), and a trending interaction, ($F_{(3,85)}$ = 2.50, $p = 0.07$) (Fig 8D). Again, estradiol injections reduced generalization whereas vehicle treated animals displayed significant generalization, (estradiol: $t_{(15)} = 3.992$, $p < 0.001$, $d = 1.88$; vehicle: $t_{(24)} = 0.9799$, 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 males is mediated by either estrogen receptor subtype.

The influence of estradiol on fear generalization in males and females is dependent upon sex, and also diverges on receptor specificity. Estradiol induces fear generalization in females solely through activation of ERβ as seen in Experiment 5A, whereas estradiol attenuates fear generalization in males through actions at either receptor subtype. This difference in ER requirement could be due to differences in receptor distribution within the contextual fear memory circuit or organizational effects of hormones on brain and behavior (Arnold & Breedlove, 1985; McCarthy, 2006; McEwen, 1992; Schwarz & McCarthy, 2008). For example, the hippocampus and prefrontal cortex play an important role in the generalization of fear (Cullen, Gilman, Winiecki, Riccio, & Jasnow, 2015; Ruediger et al., 2011; Wiltgen & Silva, 2007; Wiltgen et al., 2010; Winocur et al., 2007), and within this region, adult female rats display higher levels of ERβ, but not ERα, compared to males within the hippocampus (Weiland, Orikasa, Hayashi, & McEwen, 1997; J. Q. Zhang, Cai, Zhou, & Su, 2002). This sex difference in ER expression within the hippocampus may explain the lack of receptor specificity seen in males, although it does not explain the directional nature of the effect.

Figure 8. **Activation of ERβ Induces Generalization in Females, Activation of Either Subtype Prevents Generalization In Males. A)** Schematic of the experimental paradigm. Twenty four hours after training, animals were given ER agonist injections and tested 24 hours later. **B)** In females, 1 mg/kg of ERα (PPT) or ERβ agonists (DPN) did not increase fear generalization 24 hours after injection. **C)** 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. **D)** In 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 $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 8. Activation of ERβ Induces Generalization in Females, Activation of Either Subtype Prevents Generalization In Males[17]



[17] Figure B/C adapted from Lynch, J. F., Dejanovic, D., Winiecki, P., Mulvany, J., Ortiz, S., Riccio, D. C., & Jasnow, A. M. (2014). Activation of ERβ modulates fear generalization through an effect on memory retrieval. *Hormones and behavior*, *66*(2), 421-429. Figure D submitted for publication: Lynch III, J. F., Vanderhoof, T., Winiecki, P., Latsko, M.S., Riccio, D. C., & Jasnow, A. M. (under review). Aromataized testosterone attenuates contextual generalization of fear in male rats. *Hormones and Behavior.*

VII. Experiment 6 - Assessing Cytosolic Versus Membrane-Bound Estrogen Receptors[18]

7.1.Experiment 6A – Assessing Cytosolic ERs With Peripheral Estradiol Administration

Classical estrogen receptors (ERs) are located within the cytoplasm and estrogens, which are lipid-soluble steroids, can pass through the cell membrane to bind to cytosolic receptors, resulting in the translocation of the receptor-ligand complex into the nucleus where the complex can affect specific gene transcription by binding to Estrogen Response Elements (EREs) located within the promoter regions of target genes, and these effects occur within a time frame of hours to days (Couse and Korach, 1999; Etgen, 1984; Falkenstein, Tillmann, Christ, Feuring, and Wehling, 2000; McKenna and O'Malley, 2002; O'Malley and Means, 1974). In addition to classical activation, which can take time to affect gene transcription, estrogens can also have rapid signaling through membrane-bound receptors by increasing calcium influx, which can activate several different molecular signaling pathways. Activation of these pathways may lead to changes in gene transcription and protein synthesis, but the effects can occur much quicker than what is normally seen with signaling through cytosolic ERs (Vasudevan & Pfaff, 2007). A number of recent studies have suggested that estradiol enhances object recognition through activation of membrane bound ERs within the hippocampus and

[18] Experiment describe here is published: Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

through subsequent ERK/MPK and metabotropic glutamate receptor signaling (Fan, Zhao, Orr, Chambers, Lewis, and Frick, 2010; Fernandez, Lewis, Pechenino, Harburger, Orr, Gresack, Schafe, and Frick, 2008; Gresack and Frick, 2006; Lewis, Kerr, Orr, and Frick, 2008; Zhao, Fan, and Frick, 2010). However, the results from Experiment 4 suggests that estradiol induces fear generalization between 6 and 24 hours, suggesting that estradiol is acting through genomic mechanisms to affect memory retrieval, resulting in generalized responding. In order to test if estradiol induces generalization through genomic effects on memory retrieval, female rats were trained in passive avoidance and twenty-four hours after training, received drug treatments either through subcutaneous (SC) injections or ICV infusions into the lateral ventricle. In order to test the role of cytosolic receptor activity, animals received infusions of the cytosolic receptor antagonist, ICI 182,780 (ICI). ICI has a similar binding affinity as estradiol in ER competition assays and inhibits ER activation by impairing ER dimerization (Chen et al., 2002; Wade, Blaustein, Gray, & Meredith, 1993). In order to assess the effects of membrane-bound receptors, animals received infusion of estradiol-bovine serum albumin (E2-BSA). BSA conjugates of steroids can be used as tool for distinguishing genomic and nongenomic effects (Zheng, Ali, & Ramirez, 1996). Thus, E2-BSA allows the assessment of membrane-bound ERs as the drug cannot pass through the membrane to activate cytosolic receptors. Twenty-four hours after drug administration, animals were tested in passive avoidance retention in either the training or a neutral context.

7.2.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Immediately after ovariectomy, rats were placed in a stereotaxic instrument for implantation of guide cannulas aimed at the lateral ventricle. Stereotaxic coordinates were derived from (Paxinos and Watson, 1986). The head was positioned in the stereotaxic instrument so that the skull was level between lambda and bregma before implantation of the guide cannulas. Rats were implanted with a unilateral cannula (Plastics One) aimed at the lateral ventricle (D/V: -3.4; A/P: -0.9; M/L: +1.6). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 minutes a day for 2 consecutive days before passive avoidance training. Animals received 2 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Site Verification

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

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol for peripheral administration was dissolved in sesame oil (15 µg/0.1 mL) (Chang, Yang, Liang, Yeh, Huang, and Hsu, 2009; Zeidan, Igoe, Linnman, Vitalo, Levine, Klibanski, Goldstein, and Milad, 2011). The cytosolic ER antagonist, ICI 182,780 was dissolved in DMSO at a concentration of 50 µg/µl for intracerebroventricular (ICV) infusions.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone treatments and antagonist infusions.

7.3.Results and Discussion

To determine if administering a cytosolic estrogen receptor antagonist would attenuate estradiol-induced fear generalization, female rats were administered SC injections of vehicle (sesame oil) or estradiol benzoate (15 µg/0.1 mL) and received an ICV infusion of vehicle (DMSO) or ICI 182,780 (100 µg/2 µl**)** immediately following the injection (Fig 9A). When ICV infusions of the cytosolic receptor antagonist, ICI 182,780 were co-administered with SC injections of estradiol, estradiol-induced generalization was significantly attenuated (Fig 9B). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,88)} = 58.17$, $p < 0.001$), indicating longer latencies to cross to the black compartment when groups were tested in the training context versus

the neutral context. The main effect for treatment was also significant, ($F_{(3,88)} = 4.06$, $p < 0.01$), indicating significant differences between animals injected with estradiol, ICI, or vehicle control in latencies across both contexts. The interaction term was also significant, ($F_{(3,88)} = 3.06$, $p<0.05$), suggesting a difference between treatments based on the context of testing. Independent t-test analyses revealed a significant difference between animals Independent t-test analyses revealed a significant difference between animals tested in the training versus neutral contexts for both the vehicle-treated group and ICI alone treated group, (Veh+Veh: $t_{(17)} = 3.78$, $p < 0.01$; $d = 1.8$; Veh+ICI: $t_{(18)} = 3.46$, $p < 0.01$; $d = 1.55$). However, animals treated with estradiol did not differ in latencies between contexts, ($t_{(25)} = 1.55$, ns; $d = 0.58$). The estradiol-induced generalization was attenuated when animals were given simultaneous infusions of ICI 182,780, ($t_{(50)} = 5.05$, $p < 0.001$; $d = 1.63$). In addition, estradiol treated animals tested in the neutral context displayed significantly more fear behavior as demonstrated by higher latency to cross in the neutral context than vehicle-treated or ICI treated animals, (Veh+Veh: $t_{(19)} = 2.38$, $p < 0.05$; $d = 1.04$; Veh+ICI: $t_{(19)} = 2.58$, $p < 0.05$; $d = 1.12$; estradiol+ICI: $t_{(45)} = 2.84$, $p < 0.01$; $d = 0.99$). These data suggest that activation of cytosolic estrogen receptors is necessary for estradiol-induced fear generalization; ICV infusions of ICI 182,780 attenuates estradiol-induced fear generalization when estradiol is given peripherally.

7.4.Experiment 6B - Assessing Cytosolic ERs With Central Infusions of Estradiol

The results from Experiment 6A demonstrate that estradiol-induced generalization via peripheral injections could be attenuated with ICV infusions of ICI 182,780. To extend these findings, animals received ICV infusions of vehicle, estradiol alone, ICI

182,780 alone, or a co-infusion of estradiol and ICI 182,780 to determine if infusions of ICI 182,780 could attenuate estradiol-induced fear generalization when estradiol was given centrally rather than peripherally.

7.5.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 6A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, and Watanabe, 2000). The cytosolic ER antagonist, ICI 182,780, was dissolved in DMSO

at a concentration of 50 µg/µl. Animals received 2 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone and antagonist infusions.

7.6. Results and Discussion

Animals received infusions of estradiol, estradiol+ICI, ICI, or vehicle and were tested 24 hours after the infusion (Fig 9A). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,45)} = 30.16$, $p<0.001$), indicating longer latencies between animals tested in the training context versus the neutral context. The main effect for treatment, ($F_{(3,45)} = 2.66$, $p = 0.06$), was trending towards significance. The interaction term between treatment and context was not significant, ($F_{(3,45)} = 1.66$, ns). Independent t-test analyses confirmed that estradiol induced fear generalization to the neutral context when infused centrally, ($t_{(12)} = 0.78$, ns; $d = 0.42$). In comparison, vehicle-treated, ICI-treated, and estradiol+ICI treated animals displayed a significant difference in fear between the training and neutral context, (Veh: $t_{(8)} = 5.02$, $p < 0.001$; $d = 3.17$; ICI: $t_{(16)} = 3.71$, $p < 0.01$; $d = 1.88$; estradiol+ICI: $t_{(11)} = 3.04$, $p < 0.01$; $d = 1.71$). Additionally, estradiol treated animals displayed significantly more fear in the neutral context compared to all other treatment groups, (estradiol+ICI: $t_{(11)} = 2.24$, $p < 0.05$ $d = 1.26$; ICI: $t_{(14)} = 2.37$, $p < 0.05$; $d = 1.16$; Veh: $t_{(10)} = 2.97$, $p < 0.01$; $d = 1.87$) (Fig 9C). Together, these results demonstrate that centrally infused estradiol results in fear generalization similar to peripherally administered estradiol and is attenuated by co-

infusion of ICI 182,780, again implicating that activation of cytosolic receptors is required for estradiol-induced generalization.

7.7.Experiment 6C - Assessing Cytosolic ERs With Peripheral ERβ Activation

Experiment 5A demonstrated that estradiol-induced generalization was driven by activation of ERβ. To determine if ICI 182,780 infusions could attenuate generalization induced by specific activation of ERβ, animals were administered SC injections of the ERβ agonist, DPN, and co-administered ICV infusions of ICI 182,780

7.8.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 6A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Injections of the ERβ agonist, DPN, were given at a dose of 2.5 mg/0.1 ml in DMSO as described in Experiment 5A. The cytosolic ER antagonist, ICI 182,780 was dissolved in DMSO at a concentration of 50 µg/µl. Animals received 2 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of Agonist treatments and antagonist infusions.

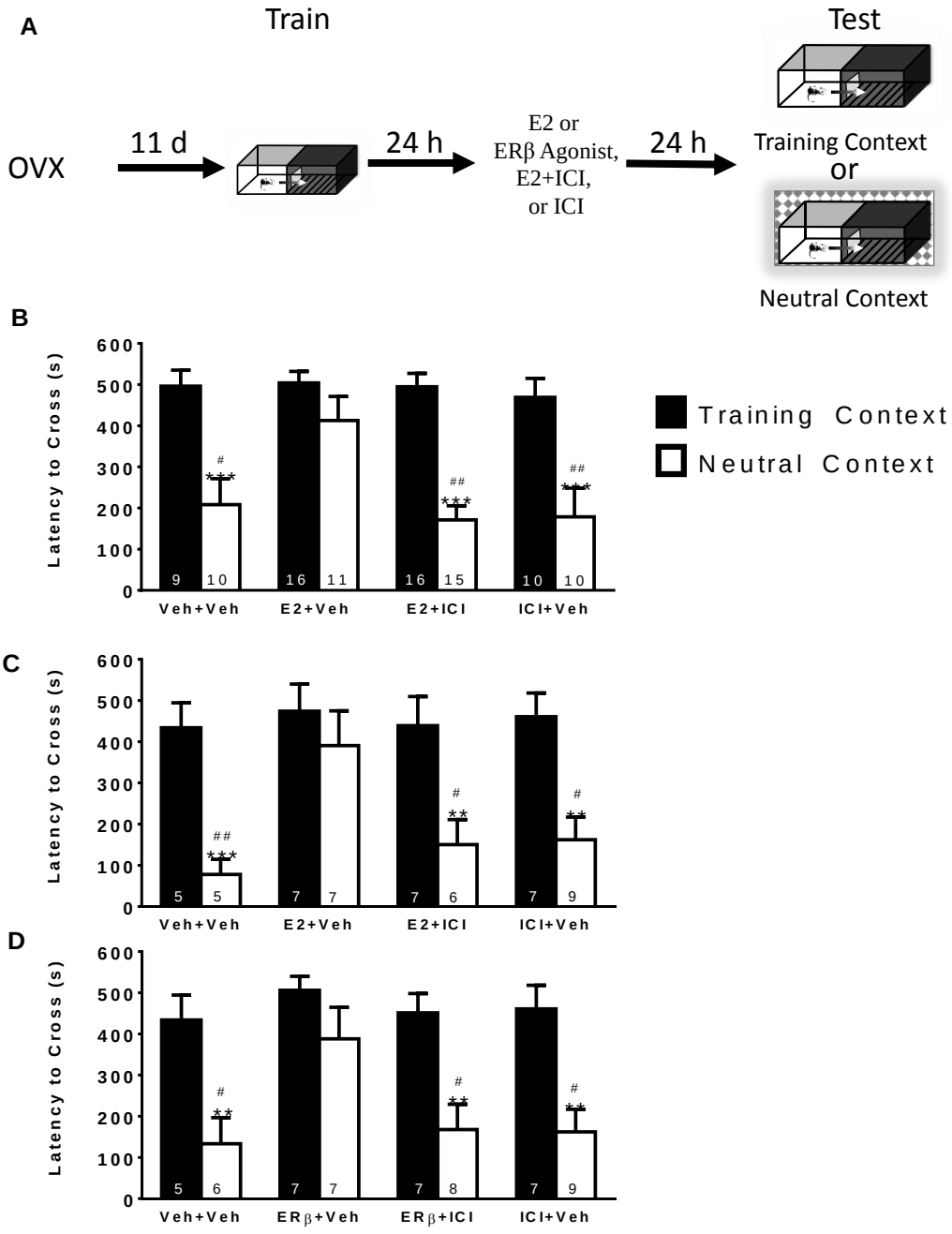
7.9.Results and Discussion

To determine if a cytosolic estrogen receptor antagonist would attenuate fear generalization induced by an ERβ agonist, a group of animals received SC injections of DPN or vehicle (DMSO) along with an ICV infusion of vehicle (DMSO) or ICI 182,780 (Fig 9A). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,48)} = 35.63$, $p < 0.001$), a significant main effect for treatment, ($F_{(3,48)} = 3.09$, $p < 0.05$), and a non-significant interaction term, ($F_{(3,48)} = 1.14$, $p > 0.05$). The results replicate our previous finding that activation of ERβ induces fear generalization, ($t_{(12)} = 1.41$, ns; $d = 0.75$). Additionally, infusions of ICI 182,780 attenuated fear generalization induced by infusions of DPN, ($t_{(13)} = 3.58$, $p < 0.01$; $d = 1.88$). Animals treated with vehicle or ICI alone displayed a significant difference in fear between the training and neutral context, (Veh: $t_{(9)} = 3.39$, $p < 0.01$; $d = 2.07$; ICI: $t_{(14)} = 3.71$, $p < 0.01$; $d = 1.88$). When comparing animals tested in the neutral context, those

treated with DPN displayed significantly more fear than all other groups, (ERβ+ICI: $t_{(13)} = 2.27$, $p < 0.05$; $d = 1.17$; ICI: $t_{(14)} = 2.46$, $p < 0.05$; $d = 1.22$; Veh: $t_{(11)} = 2.51$, $p < 0.05$; $d = 1.41$) (Fig 9D). Taken together, these results replicate the previous findings that ERβ activation can induce generalization to a neutral context, and further show that this effect is attenuated by blocking activation of cytosolic ERs with ICI 182,780.

Figure 9. **Estradiol Induces Generalization Through Activation of Cytosolic ERβ. A**) Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug treatment. Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. **B**) Estradiol-treated animals displayed significant generalization. Infusions of ICI attenuated estradiol-induced generalization and vehicle-treated animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2+ICI, ICI+Veh, and Veh+Veh treated animals. **C**) Intracerebroventricular infusions of estradiol resulted in generalized fear to the neutral context; an effect attenuated by co-infusion of ICI. ICI alone and vehicle infused animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2+ICI, ICI+Veh, and Veh+Veh treated animals. **D**) Peripheral injections of the ERβ agonist, DPN, induced generalized fear that was attenuated by infusions of ICI. ICI alone and vehicle infused animals displayed significant context generalization. Additionally, DPN-treated animals displayed significantly more fear in the neutral context compared to DPN (ERβ)+ICI, ICI+Veh, and Veh+Veh treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 9. Estradiol Induces Generalization Through Activation of Cytosolic ERβ[19]



[19] Figure adapted from Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

7.10. Experiment 6D - Assessing Membrane-Bound ERs

The previous experiments demonstrated that cytosolic estrogen receptors are necessary for estradiol-induced fear generalization. However, these data do not eliminate the possibility of genomic effects initiated by membrane-bound receptor activation. Therefore, to determine if fear generalization could be induced through activation of membrane-bound ERs alone using a membrane-bound ER agonist, E2-BSA.

7.11. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 6A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate conjugated to BSA (E2-BSA) was centrifuged in a centrifugal filter unit with a molecular weight cut-off of 3,000 kDa (Millipore) and spun at 16,110 x g for 10 min. Filters were washed with 5% DMSO, spun for another 10 min at 16,110 x g, and washed again with 5% DMSO before being spun for 30 min at 16,110 x g (Santollo, Marshall, and Daniels, 2012; Taguchi, Koslowski, and Bodenner, 2004). Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, and Watanabe, 2000). Animals received 2 μl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

7.12. Results and Discussion

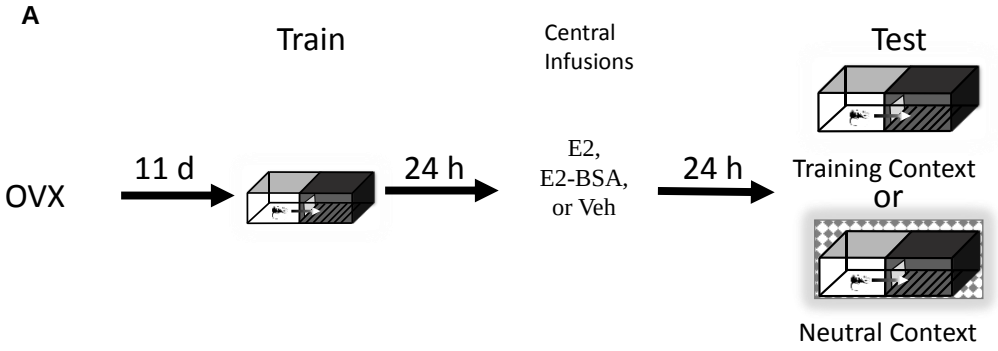
To determine if estradiol also acts through membrane-bound receptors, animals received ICV infusions of estradiol or E2-BSA (Fig 10A). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,51)} = 16.69$, $p<0.001$), a non-significant main effect for hormone treatment, ($F_{(2,51)} = 2.23$, ns), and a non-significant interaction term between context and hormone treatment, ($F_{(2,51)} = 2.19$, ns). Independent t-test analyses revealed a significant difference in fear response when tested in the training context or neutral context for vehicle-treated or E2-BSA-treated animals, but not estradiol-treated animals, (vehicle: $t_{(38)} = 4.15$, $p < 0.001$; $d = 1.31$; E2-BSA: $t_{(16)} = 3.53$,

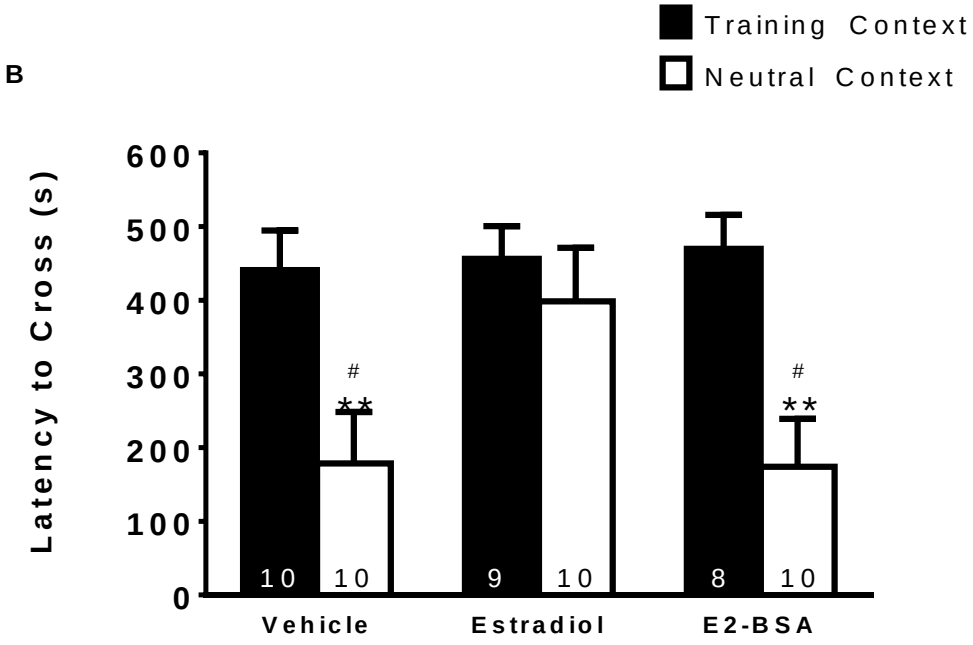
$p < 0.01$; $d = 1.72$; estradiol: $t_{(17)} = 0.66$, ns; $d = 0.31$) (Fig 10B). In addition, vehicle-treated and E2-BSA animals were significantly different than estradiol-treated animals when tested in the neutral context, (vehicle: $t_{(18)} = 2.18$, $p < 0.05$; $d = 0.94$; E2-BSA: $t_{(16)} = 2.30$, $p < 0.05$; $d = 1.03$). These data, in combination with the ICI experiments suggests that activation of membrane-bound ERs alone is not sufficient to induce generalized responding to a neutral context in OVX female rats.

In these experiments, infusions of ICI 182,760 were used to block cytosolic ERs, but this compound can also act as an estrogen receptor agonist in certain tissues. Specifically, *in vitro*, ICI can increase ERK1/2 phosphorylation and spinophilin expression to a similar level as estradiol and can act at the G-protein estrogen receptor, GPER, as an agonist. These findings suggest that ICI may activate membrane-bound estrogen receptors similar to estradiol itself (Filardo, Quinn, Frackelton Jr, and Bland, 2002; Zhao, O'Neill, and Brinton, 2006). However, no effect of ICI treatments alone were found to affect fear generalization, and when considered in combination with the consistent lack of membrane-bound receptor involvement in this behavioral phenomenon, the results suggest that estradiol-induced generalization is not due to membrane-bound receptor activation. Taken together, the current results support the original hypothesis that estradiol-induced generalization is due to an effect on memory retrieval through activation of cytosolic ERs, resulting in genomic effects.

Figure 10. **Activation of Membrane-Bound Receptors is Not Sufficient to Induce Generalization**. **A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug treatment. Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. **B)** Intracerebroventricular infusions of estradiol resulted in generalized fear to the neutral context. However, activation of membrane-bound receptors via infusions of E2-BSA did not induce generalized fear. Activation of membrane-bound receptors is not sufficient for estradiol-induced generalization. Vehicle-infused animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2-BSA and vehicle treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 10. Activation of Membrane-Bound Receptors is Not Sufficient to Induce Generalization[20]





[20] Figure adapted from : Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

VIII. Experiment 7 - Circuitry Involved in Estradiol-Induced Fear Generalization

8.1.Introduction

Several decades of research has tried to determine the neural circuitry involved in the time-dependent generalization of fear (For review, see Jasnow et al., 2012). Several studies demonstrate the importance of the hippocampus in the generalization of fear and in context memory precision (Ruediger et al., 2011; Wiltgen & Silva, 2007; Wiltgen et al., 2010; Winocur et al., 2007). Generally, as a context memory ages, the memory is transferred from the hippocampus to a distributed cortical network for long-term storage (Frankland, Ding, Takahashi, Suzuki, Kida, and Silva, 2006; Frankland, O'Brien, Ohno, Kirkwood, and Silva, 2001; Kim and Fanselow, 1992; McGaugh, 1966; Vetere, Restivo, Cole, Ross, Ammassari-Teule, Josselyn, and Frankland, 2011; Zola-Morgan and Squire, 1990) although others find prolonged activity for remote memory recall for generalized responding within the dorsal CA1 region of the hippocampus (Cullen et al., 2015). In order to determine what regions may be involved in imprecise memory recall at long delays, a recent study assessed time-dependent generalization in mice and saw increased activity levels within cortical regions, such as the anterior cingulate cortex (ACC), and the ventral CA1 region of the hippocampus at the time of generalized fear responding. Additionally, inactivation of either region at a time point when generalization occurs (i.e. 21 days post-training) restored memory precision (Cullen et al., 2015). Other studies have demonstrated that blockade of synaptic transmission in the prefrontal cortex or

blocking transmission in the nucleus reuniens—a relay for connections between the mPFC and the hippocampus—impairs the precision of recent and remote memory recall (Xu et al., 2012; Xu & Südhof, 2013). Taken together, these data indicate a possible circuitry of fear generalization where the dorsal hippocampus supports recall of precise memories and the ACC supports recall of imprecise memories at long delays. Additionally, the activity of ACC resulting in imprecise memory recall is either driven by, or results in increased activation, of the ventral hippocampus (Fig 16A).

One issue with the above circuitry is that generalized memory, or imprecise memory recall, requires the passage of time whereas estradiol-induced generalization, as seen in the previous experiments, does not require a long delay. In fact, estradiol-induced generalization is seen 24 hours post-injection (Fig 5B). Therefore, the circuitry involved in estradiol-induced generalization may be distinct from that of time-dependent generalization. The current set of experiments was designed to assess the role of estradiol within the dorsal CA1 (EX 7A-D), ventral CA1 (EX 7E), and ACC (EX 7F).

8.2.Experiment 7A - Assessing Cytosolic ERs Within the Dorsal Hippocampus[21]

Although the precise mechanisms through which estrogens influence fear generalization remain unknown, estrogen receptors are widely distributed throughout the hippocampus (Li, Schwartz, and Rissman, 1997; Österlund, GJM Kuiper, Gustafsson, and Hurd, 1998; Shughrue, Lane, and Merchenthaler, 1997; Shughrue and Merchenthaler, 2000a; b), putting them in an ideal location to modulate contextual memory precision. Additionally, estrogens have effects on hippocampal neuronal

[21] Experiment describe here is published: Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

morphology across the estrous cycle, including alterations in dendritic spine density (Beltrán-Campos et al., 2011; Gould, Woolley, Frankfurt, & McEwen, 1990; Shors et al., 2001; Wallace, Luine, Arellanos, & Frankfurt, 2006; Woolley & McEwen, 1992). Overall, given the role of the hippocampus in contextual fear memory and the direct impact estradiol can have on synaptic morphology, the first set of experiments targeted the dorsal CA1 as a potential mediator for estradiol-induced generalization. To test the effects of cytosolic ER activation, animals received animals received intra-hippocampal CA1 infusions of estradiol, ICI, a co-infusion of estradiol and ICI, or vehicle.

8.3.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. For cannulation surgeries, rats were implanted with bilateral cannula aimed at the dorsal CA1 hippocampus (14^{o}, D/V: -3.1; A/P: -4.0; M/L: +3.3). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 minutes a day for 2 consecutive days before passive avoidance training.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, and Watanabe, 2000). The cytosolic ER antagonist, ICI 182,780 was dissolved in DMSO at a concentration of 50 µg/µl for intrahippocampal infusions. Animals received 2 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

8.4.Results and Discussion

To test the role of cytosolic ERs within the hippocampus, animals received infusions of estradiol alone, ICI alone, co-infusions of estradiol with ICI 182,780, or vehicle (Fig 11A). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,81)} = 54.10, p < 0.001$), a significant main effect for treatment, ($F_{(3,81)} = 3.91$, $p < 0.01$), and a significant interaction term between context and treatment, ($F_{(3,81)} = 3.18$, $p < 0.05$). Independent t-test analyses revealed that local infusions of estradiol into the dorsal CA1 alone induced generalized responding to the neutral context, ($t_{(25)} = 1.69$, ns; $d = 0.70$). The estradiol-induced generalization was attenuated by co-infusions of ICI, ($t_{(15)} = 5.71$, $p < 0.001$; $d = 3.71$). Additionally, animals given vehicle or

ICI only infusions showed significantly higher levels of fear in the training context compared to the neutral context, (Veh: $t_{(23)} = 5.61$, $p < 0.001$; $d = 2.25$; ICI: $t_{(18)} = 2.77$, $p < 0.01$; $d = 1.31$). Animals treated with estradiol showed significantly higher fear when tested in the neutral context compared to all other treatment groups, (E2 + ICI: $t_{(27)} = 4.50$, $p < 0.001$; $d = 1.71$; ICI: $t_{(26)} = 2.20$, $p < 0.05$; $d = 0.83$; Veh: $t_{(27)} = 3.93$, $p < 0.001$; $d = 1.48$). These results demonstrate that the dorsal CA1 hippocampus is an important locus for the actions of estradiol on fear generalization. Additionally, the actions of estradiol within the hippocampus depend upon activation of cytosolic receptors; ICI can block fear generalization induced by hippocampal administered estradiol (Fig 11B).

8.5.Experiment 7B - Assessing Cytosolic Estrogen Receptors With Dorsal Hippocampus Infusions of ER Agonists[22]

Findings from Experiment 5A and 6C demonstrate that peripheral administration of an ERβ agonist, but not an ERα agonist, could induce fear generalization. Within the hippocampus, ERα and ERβ are found within the CA1, CA3, and dentate gyrus (DG), and ERβ are more abundant throughout the entire hippocampus (Azcoitia, Sierra, & Miguel Garcia-Segura, 1999; Blurton-Jones, Kuan, & Tuszynski, 2004; Mitra et al., 2003; Nishio, Kuroki, & Watanabe, 2004; Orikasa, McEwen, Hayashi, Sakuma, & Hayashi, 2000; Shughrue et al., 1997; Weiland, Orikasa, Hayashi, & McEwen, 1997; Woolley, Wenzel, & Schwartzkroin, 1998; Zhang, Cai, Zhou, & Su, 2002). Previous reports have demonstrated differential effects of ER agonists given intra-cranially versus

[22] Experiment describe here is published: Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

systemically (Boulware et al., 2013). Therefore, to confirm that ERβ, but not ERα, activation within the dorsal CA1 could also induce fear generalization, animals were infused with the specific ERα agonist, PPT, or ERβ agonist, DPN, into the hippocampus with or without infusions of ICI 182,780.

8.6.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 7A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

The ERα specific agonist, PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol; Caymen Chemical) was dissolved in DMSO at a concentration of 0.2 pg/µl and infused at a dose of 0.1 pg per hemisphere. The ERβ specific agonist, DPN (2,3-bis(4-

hydroxyphenyl)-propionitrile, Caymen Chemical) was dissolved in DMSO at a concentration of 40 pg/µl (Boulware, Heisler, and Frick, 2013). At these low doses, PPT and DPN are specific for ERα and ERβ, respectively (Stauffer, Coletta, Tedesco, Nishiguchi, Carlson, Sun, Katzenellenbogen, and Katzenellenbogen, 2000). Animals received 0.5 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

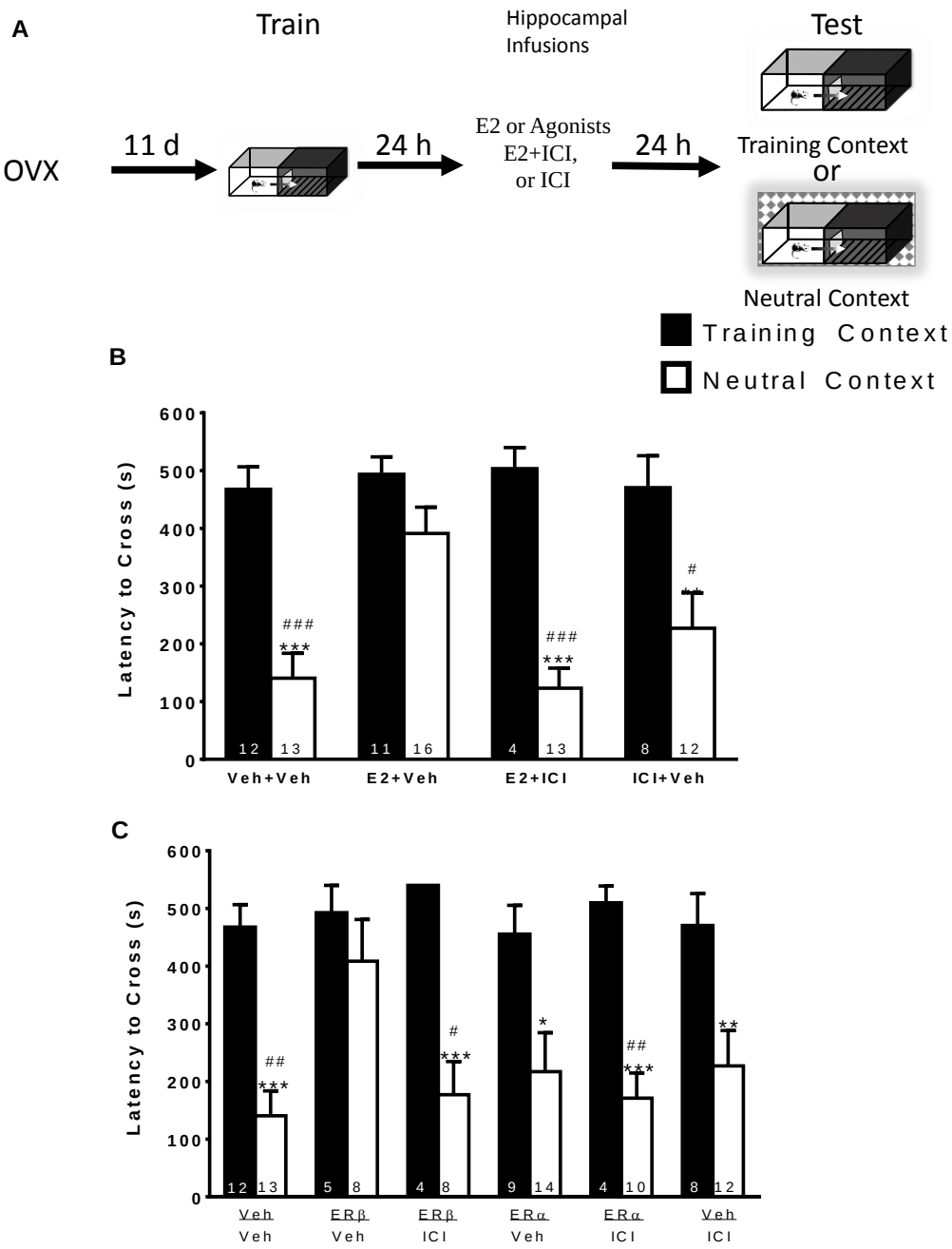
8.7.Results and Discussion

To determine if fear generalization is due to receptor specific activation within the hippocampus, animals received intra-hippocampal CA1 infusions of PPT (0.1 pg/0.5 µl), DPN (20 pg/0.5 µl), ICI (25 µg/0.5 µL), a co-infusion of PPT and ICI, DPN and ICI, or vehicle (Fig 11A). Activation of ERβm specifically within the dorsal hippocampusm replicated systemic effects of DPN administration. A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,95)} = 52.60$, $p < 0.001$), but a non-significant main effect for treatment, ($F_{(5,95)} = 1.21$, $p > 0.05$), and interaction term, ($F_{(5,95)} = 1.14$,$p > 0.05$). Independent t-test analyses revealed that animals treated with DPN displayed equivalent levels of fear in either context, ($t_{(11)} = 0.84$, ns; $d = 0.51$). Co-infusions of the DPN and ICI 182,780 attenuated the generalization seen with ERβ activation, ($t_{(11)} = 4.35$, $p < 0.001$; $d = 3.15$). Unlike ERβ activation, activation of ERα did not induce generalized responding to the neutral context, ($t_{(21)} = 2.55$, $p < 0.05$;

d = 1.15), confirming previous findings using peripheral injections. Animals given co-infusions of PPT and ICI 182,780, ICI 182,780 alone, or vehicle treatment, also displayed significantly more fear in the training context compared to the neutral context, (ERα + ICI: $t_{(12)} = 4.67$, $p < 0.001$; $d = 3.21$; ICI: $t_{(18)} = 2.77$, $p < 0.01$; $d = 1.31$; Veh: $t_{(23)} = 5.61$, $p < 0.001$; $d = 2.25$). Additionally, animals given DPN displayed more fear in the neutral context compared to all other treatment groups, (ERβ + ICI: $t_{(14)} = 2.51$, $p < 0.05$; $d = 1.25$; ERα: $t_{(20)} = 1.83$, $p < 0.08$; $d = 0.83$; ERα + ICI:$t_{(16)} = 2.95$, $p < 0.01$; $d = 1.36$; ICI: $t_{(18)} = 1.90$, $p < 0.07$; $d = 0.87$; Veh: $t_{(19)} = 3.41$,$p < 0.01$; $d = 1.48$) (Fig 11C). These results extend the findings from the previous experiments with systemic injections of DPN. Activation of ERβ within the dorsal hippocampus is necessary and sufficient to induce fear generalization; an effect that is attenuated by blockade of cytosolic ERs via infusions of ICI 182,780. In addition, activation of ERα within the dorsal hippocampus is not sufficient to induce fear generalization.

Figure 11. **Activation of Cytosolic ERβ Within The Dorsal CA1 Results in Generalization.** **A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug treatment in the dorsal CA1. Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. **B)** Dorsal hippocampal infusions of estradiol resulted in generalized fear to the neutral context; an effect attenuated with simultaneous infusions of ICI. ICI alone and vehicle infused animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2+ICI, ICI+Veh, and Veh+Veh treated animals. **C**) Dorsal Hippocampal infusions of the ERβ agonist, DPN, induced generalized fear that was attenuated by co-infusion of ICI. Infusions of the ERα agonist, PPT, did not induce generalized fear. Animals receiving PPT (ERα) and ICI, ICI alone, or vehicle infusions displayed significant context discrimiation. DPN-treated animals displayed significantly more fear in the neutral context compared to DPN (ERβ)+ICI, ERα, PPT (ERα) + ICI, ICI+Veh, and Veh+Veh treated animals. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 11. Activation of Cytosolic ERβ Within The Dorsal CA1 Results in Generalization[23]



[23] Figure adapted from Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92

8.8.Experiment 7C - Assessing Membrane-Bound Estrogen Receptors Within the Dorsal Hippocampus[24]

The experiments above demonstrate that activation of cytosolic ERβ within the dorsal CA1 region of the hippocampus is sufficient to induce generalized fear responses to a neutral context. In order to determine if activation of membrane-bound estrogen receptors specifically within the dorsal hippocampus could also result in generalized responding, animals received infusions of estradiol, E2-BSA, or vehicle into the dorsal CA1.

8.9.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 7A.

Site Verification

Site verification was conducted as described in Experiment 6A.

[24] Experiment describe here is published: Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, and Watanabe, 2000). Estradiol benzoate conjugated to BSA (E2-BSA) was centrifuged in a centrifugal filter unit with a molecular weight cut-off of 3,000 kDa (Millipore) and spun at 16,110 x g for 10 min. Filters were washed with 5% DMSO, spun for another 10 min at 16,110 x g, and washed again with 5% DMSO before being spun for 30 min at 16,110 x g (Santollo, Marshall, and Daniels, 2012; Taguchi, Koslowski, and Bodenner, 2004). Animals received 0.5 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

8.10. Results and Discussion

To determine if fear generalization could be induced through activation of membrane bound ERs within the hippocampus, animals received infusions of estradiol, E2-BSA, or vehicle into the dorsal CA1 (Fig 12A). A factorial ANOVA analysis revealed a significant main effect for context, ($F_{(1,54)} = 54.96$, $p<0.001$), a significant main effect for hormone treatment, ($F_{(2,54)} = 8.59$, $p < 0.001$), and a significant interaction

term between context and hormone treatment, ($F_{(2,54)} = 6.76$, $p < 0.01$). Independent *t*-tests revealed that estradiol infusions resulted in fear generalization to the neutral context, ($t_{(25)} = 0.10$, ns; $d = 0.70$), whereas infusions of vehicle or E2-BSA did not induce generalized responding, (vehicle: $t_{(15)} = 6.49$, $p < 0.001$; $d = 3.15$; E2-BSA: $t_{(14)} = 4.80$, $p < 0.001$; $d = 2.4$). Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to vehicle-treated and E2-BSA-treated animals, (vehicle: $t_{(22)} = 4.20$,$p < 0.001$; $d = 1.93$; E2-BSA: $t_{(22)} = 3.85$, $p < 0.001$; $d = 1.66$) (Fig 12B). These results confirm the above findings of ICV administered E2-BSA, further demonstrating that activation of membrane-bound estrogen receptors within the hippocampus is not sufficient to induce generalized fear responding. Overall, these results demonstrate the dorsal CA1 hippocampus is an important locus for actions of estradiol on fear generalization. Additionally, the actions of estradiol within the hippocampus depend upon activation of cytosolic receptors; ICI can block hippocampal administered estradiol and infusions of E2-BSA are not sufficient to induce generalization.

8.11. Experiment 7D - Assessing Membrane-Bound Estrogen Receptors With Dorsal Hippocampus Infusions of U0126[25]

Estrogens can induce rapid signaling through membrane-bound receptors by increasing calcium influx, which can activate several different molecular signaling pathways such as MAPK, ERK, PI3K/Akt, and PKA. Activation of these pathways may ultimately lead to changes in gene transcription and protein synthesis, but the effects can

[25] Experiment describe here is published: Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

occur much quicker than what is normally seen with signaling through cytosolic ERs (Vasudevan & Pfaff, 2007). The above experiments demonstrate that activation of membrane-bound receptors through infusions of E2-BSA is not sufficient to induce generalized responding. However, the current experiment was designed to assess the possible contributions of second-messenger pathways activated by estradiol on the generalization of fear. The ERK/MAPK pathway is a major second messenger pathway activated by membrane-bound estrogen receptors and is important for some of the learning effects of estradiol within the hippocampus (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013). Indeed, estradiol enhances memory consolidation for novel object recognition memory through activation of membrane-bound estrogen receptors and subsequent ERK/MAPK pathway activation (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013). In order to test the role of activation the ERK/MAPK pathway for estradiol-induced generalization, animals were given infusions of the MEK inhibitor, U0126. U0126 selectively binds to MEK1 and MEK2 (Duncia et al., 1998), and can impair memory retrieval for spatial learning (H. Zhang et al., 2004). If estradiol-induced generalization is due, at least in part, to activation of membrane-bound ERs, then blocking activation of the ERK/MAPK pathway should attenuate estradiol-induced generalization.

8.12. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 7A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

The MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene; Sigma Aldrich) was dissolved in 50% DMSO to a concentration of 1µg/µl for a final dose of 0.5 µg per hemisphere (Fernandez et al., 2008; Fortress, Fan, Orr, Zhao, and Frick, 2013; Zhao, Fan, Fortress, Boulware, and Frick, 2012). Animals received 0.5 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion. Animals were then tested 5 minutes after the completion of the infusion.

Statistical Analysis

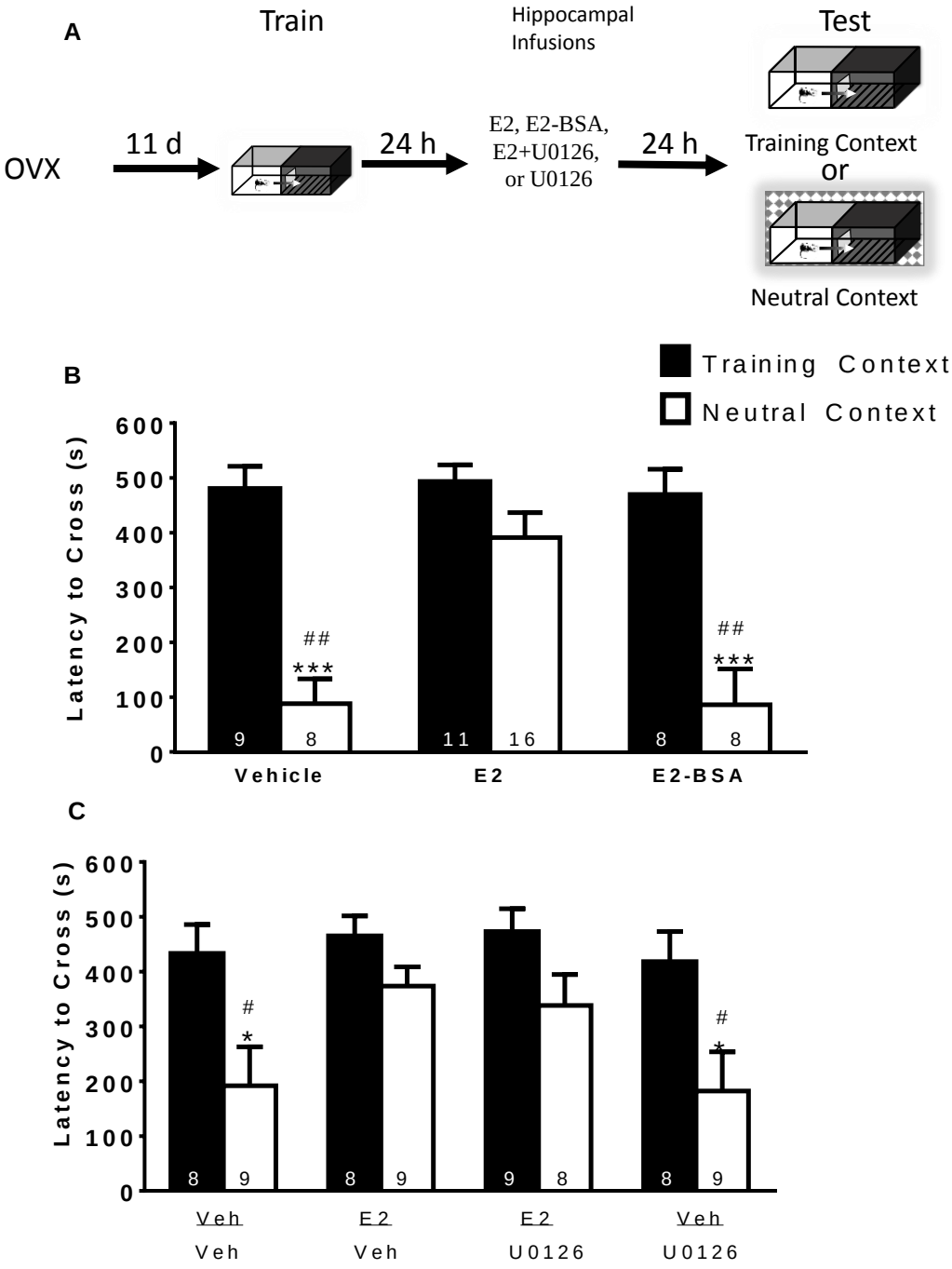
Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

8.13. Results and Discussion

Animals received intra-hippocampal infusions of estradiol (10 mM, 0.5 µl) alone, U0126 (0.5µg/0.5µl) alone, a combination of estradiol and U0126, or vehicle (Fig 12A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,60)} = 20.68$, $p < 0.001$). The main effect for treatment trended towards significance, ($F_{(3,60)} = 2.55$, $p=0.06$), and the interaction was not significant, ($F_{(3,60)} = 0.93$, ns). Independent t-tests revealed that estradiol infusions into the hippocampus induced significant generalization as animals displayed equivalent levels of fear in either context, ($t_{(15)} = 1.81$, ns; $d = 0.88$) (Fig 12C). Infusions of U0126 alone did not affect fear generalization, ($t_{(15)} = 2.56$, $p <0.05$; $d = 1.26$) and did not block estradiol-induced fear generalization, ($t_{(15)} = 1.95$, ns, $d = 0.94$). Animals given vehicle treatment also displayed significantly more fear in the training context compared to the neutral context, (Veh: $t_{(15)} = 2.68$, $p < 0.05$; $d = 1.32$). Additionally, animals given estradiol displayed significantly more fear in the neutral context compared to U0126 and vehicle treated animals, (U0126: $t_{(18)} = 2.40$, $p < 0.05$; $d = 1.13$; Veh: $t_{(16)} = 2.29$, $p < 0.05$; $d = 1.08$). These results demonstrate that blocking activation of the ERK/MAPK pathway through infusions of a MEK inhibitor into the dorsal hippocampus does not attenuate estradiol-induced generalization. Taken together with the results of E2-BSA infusions, these data suggest that estradiol-induced generalization is not a direct result of activation of membrane-bound estrogen receptors.

Figure 12. **Activation of Membrane-Bound Receptors in the Dorsal CA1 is Not Sufficient to Induce Generalization**. **A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug treatment into the dorsal CA1. Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. **B)** Direct infusions of estradiol into the dorsal CA1 hippocampus resulted in generalized fear to the neutral context. However, activation of membrane-bound receptors within the dorsal CA1 via infusions of E2-BSA did not induce generalized responding. Vehicle infused animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to E2-BSA and vehicle treated animals. **C**) Dorsal hippocampal infusions of estradiol resulted in generalized fear to the neutral context that was not attenuated by co-infusion of the MEK inhibitor, U0126. U0126 alone and vehicle infused animals displayed significant context discrimination. Additionally, estradiol-treated animals displayed significantly more fear in the neutral context compared to U0126+Veh and Veh+Veh treated animals. Thus, membrane bound ERs alone do not contribute to estradiol-induced fear generalization. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 12. Activation of Membrane-Bound Receptors in the Dorsal CA1 is Not Sufficient to Induce Generalization[26]



[26] Figure adapted from Lynch, J. F., Winiecki, P., Vanderhoof, T., Riccio, D. C., & Jasnow, A. M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of learning and memory*, *130*, 83-92.

8.14. Experiment 7E - Role of Estradiol Within the Ventral Hippocampus

Another region implicated the process of time-dependent fear generalization is the ventral hippocampus, presumably through connections from the ventral hippocampus to the ACC (Cullen et al., 2015). In addition to being implicated in time-dependent fear generalization, the ventral hippocampus is thought to associate contextual information with a learning experience by acting as a relay between the dorsal hippocampus and the amygdala (Maren, 2001). Additionally, the ventral hippocampus may be involved in memory retrieval when animals are presented with ambiguous or neutral stimuli (J. Jin & Maren, 2015). Therefore, estradiol may act within this region to affect fear generalization. In order to assess this, animals were given infusions of estradiol or vehicle directly into the ventral CA1.

8.15. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. For cannulation surgeries, rats were implanted with bilateral cannula aimed at the ventral CA1 hippocampus (5º, D/V: -5; A/P: -5.8; M/L: +5.8). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 minutes a day for 2 consecutive days before passive avoidance training.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, and Watanabe, 2000). Animals received 0.5 μl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion. Animals were then tested 5 minutes after the completion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

8.16. Results and Discussion

In order to assess the effects of estradiol within the ventral CA1 region of the hippocampus, animals were given infusions of estradiol or vehicle into the ventral CA1 (Fig 13A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,21)} = 17.89$, $p < 0.001$), and a non-significant main effect for treatment, ($F_{(1,21)} = 2.07$, ns), and interaction term, ($F_{(1,21)} = 0.46$, ns). Independent t-tests revealed that estradiol infusions into the ventral hippocampus did not induce significant generalization, ($t_{(11)} =$

2.13, $p<0.05$; $d = 1.54$) (Fig 13B). Animals given vehicle treatment also displayed significantly more fear in the training context compared to the neutral context, ($t_{(11)} = 4.28$, $p < 0.001$; $d = 2.73$). Additionally, animals given estradiol did not display significantly more fear in the neutral context compared to vehicle treated animals, ($t_{(15)} = 1.63$, ns; $d = 0.78$). These results demonstrate that estradiol within the ventral CA1 is not sufficient to induce generalized responding. This finding is distinct from that seen with time-dependent generalization, which shows the involvement of the ventral CA1 in generalized fear after a long delay (Cullen et al., 2015). However, the distinction between these findings may be due to the relatively rapid generalization that is induced by estradiol compared to time-dependent generalization, which may indicate distinct mechanisms are involved in either process. Overall, these findings suggest that estradiol-induced generalization may utilize a different neural circuit than that involved in time-dependent generalization.

8.17. Experiment 7F – Role of Estradiol Within the ACC

Another region implicated in fear generalization is the ACC (Cullen et al., 2015), including generalization in humans (Lissek et al., 2014). Estradiol also acts within the prefrontal cortex (PFC) to affect synaptic morphology. Specifically, dendritic spine density fluctuates across the estrus cycle, or with estradiol treatment, within the PFC (Hajszan, MacLusky, Johansen, Jordan, & Leranth, 2007; Hao et al., 2006; Wallace, Frankfurt, Arellanos, Inagaki, & Luine, 2007; Wallace et al., 2006). Given the role of the ACC in generalization and the findings of estradiol-induced synaptic changes within PFC areas, estradiol may be mediating alterations in generalization through actions exerted

within the ACC. To test this, animals were given infusions of estradiol or vehicle directly into the ACC.

8.18. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. For cannulation surgeries, rats were implanted with bilateral cannula aimed at the ACC (D/V: -1.75; A/P: +1.5, M/L: +0.4). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 minutes a day for 2 consecutive days before passive avoidance training.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Estradiol benzoate (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno,

and Watanabe, 2000). Animals received 0.5 µl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of infusions.

8.19. Results and Discussion

In order to assess the effects of estradiol within the ACC, animals were given infusions of estradiol or vehicle into the ACC (Fig 13A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,27)} = 11.49$, $p < 0.01$), a non-significant main effect for treatment, ($F_{(1,27)} = 1.57$, ns), and an interaction term that trended towards significance, ($F_{(1,27)} = 3.21$, $p = 0.08$). Independent t-tests revealed that estradiol infusions into the ACC induced significant generalization as animals displayed equivalent levels of fear in either context, ($t_{(14)} = 1.28$, ns; $d = 0.72$) (Fig 13C). Animals given vehicle treatment displayed significantly more fear in the training context compared to the neutral context, ($t_{(13)} = 3.28$, $p < 0.01$; $d = 2.11$). Additionally, animals given estradiol displayed significantly more fear in the neutral context compared to vehicle treated animals, ($t_{(18)} = 2.14$, $p < 0.05$; $d = 0.96$). These results demonstrate that estradiol does act within the ACC to affect fear generalization.

8.20. Experiment 7 – Conclusions

The findings from Experiment 7 demonstrate that the dorsal CA1and ACC is an important locus of estradiol actions on fear generalization. Specifically, cytosolic ERβ within the dorsal CA1 mediate the actions of estradiol. Further, activation of membrane-

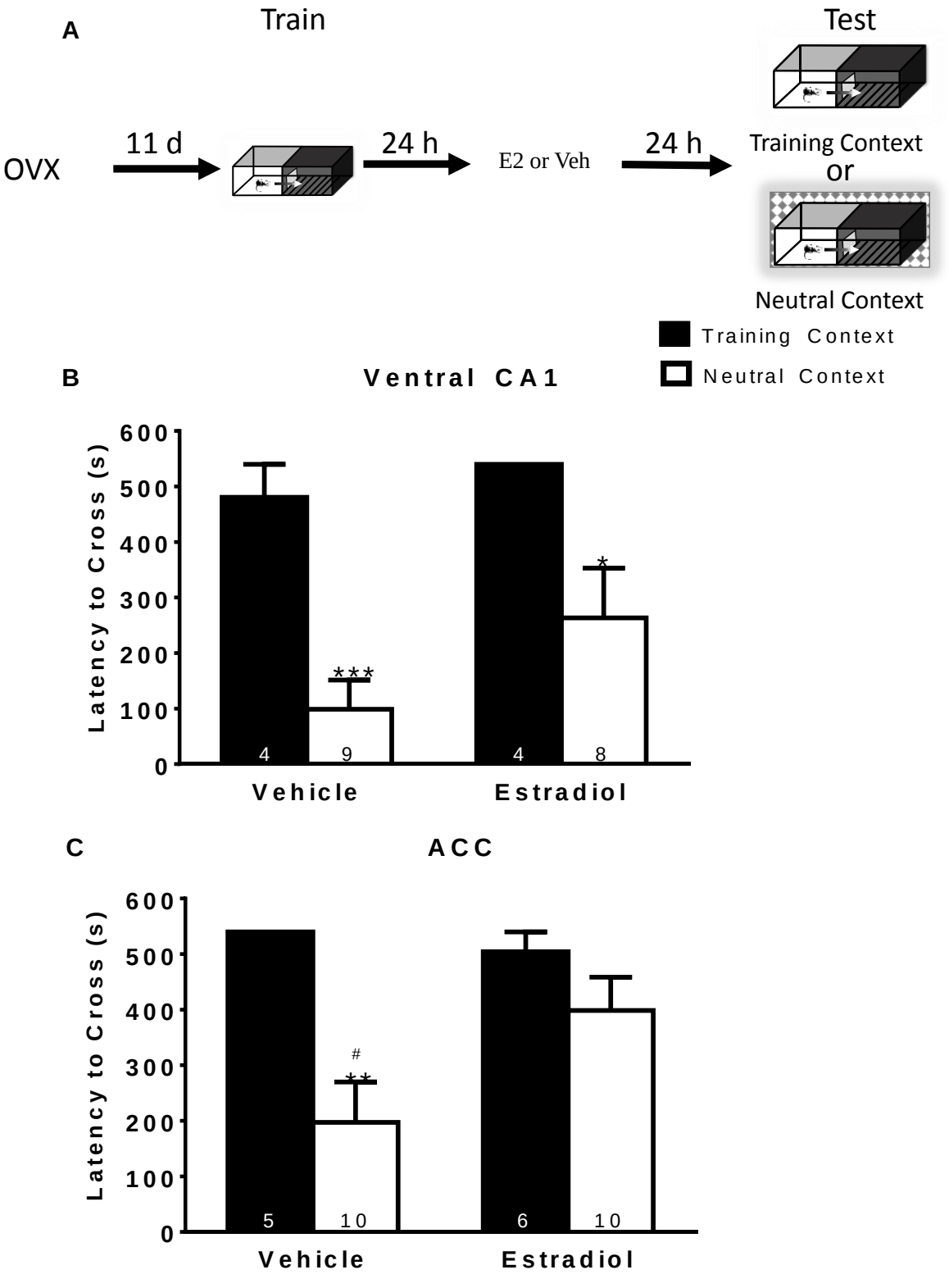
bound ERs alone through infusions of E2-BSA was not sufficient to induce generalization nor was blocking membrane-bound activation of the ERK/MAPK pathway with infusions of U0126. To date, theories on the process of fear generalization all share the idea that the passage of time is required in order for generalized responding to occur (Biedenkapp and Rudy, 2007; Jasnow et al., 2012; Lynch III et al., 2013; Matynia, Anagnostaras, Wiltgen, Lacuesta, Fanselow, and Silva, 2008; Wiltgen and Silva, 2007; Winocur et al., 2007). The current data suggest, in some cases, generalized responding does not require a significant passage of time (i.e., several or more days), and can be dependent upon memory retrieval mechanisms rather than alterations to consolidation as traditionally thought. Additionally, the data from Experiment 7 suggests that estradiol-induced generalization also acts through a distinct neural circuit compared to that of time-dependent generalization. Specifically, infusions of estradiol into the ventral CA1 did not result in generalized responding to the neutral whereas the ventral CA1 appears to be involved in inducing generalized responding during time-dependent generalization (Cullen et al., 2015). These findings demonstrate a need to determine the circuitry underlying estradiol-induced generalization as it appears to differ from that of time-dependent generalization.

Other brain regions are implicated in time-dependent generalization, including the nucleus reuniens within the thalamus. This structure has projections from the mPFC to the hippocampus and is considered a critical link between the two structures, responsible for the flow of information between the two regions (Vertes, 2006; Vertes, Hoover, Szigeti-Buck, & Leranth, 2007). In addition, the nucleus reuniens is implicated in hippocampal-dependent memory (Dolleman-van der Weel, Morris, & Witter, 2009;

Loureiro et al., 2012). When the propagation of signals from the nucleus reuniens is suppressed, animals overgeneralize fear two weeks later, suggesting a role of the nucleus reuniens in fear generalization. More specifically, if signal propagation is impaired following conditioning, no effect is seen, suggesting that the nucleus reuniens is involved in encoding the memory through a closed loop between the mPFC, which initially encodes memory precision, to the nucleus reuniens, to the hippocampus, and back to the mPFC. Thus, the nucleus reuniens may control memory precision by controlling excitability of hippocampal neurons and if excitability is increased in the hippocampus, more contextual cues can elicit a fear response. Given the dramatic effects of estrogens on hippocampal function and excitability (Beltrán-Campos et al., 2011; Gould et al., 1990; Shors et al., 2001; Wallace et al., 2006; Woolley & McEwen, 1992), estrogens may be acting through the mPFC-nucleus reuniens-hippocampal circuit to enhance excitability and allow more cues to elicit a fear response. One caveat to this idea that is the current experiments described here suggests the effects of estradiol occurs during the retrieval process of a fear memory, rather than on the acquisition. Future experiments will need to assess whether or not the nucleus reuniens is involved in estradiol-induced generalization, providing an explanation for how estradiol acts within the hippocampus and ACC to induce generalized fear responding.

Figure 13. **Estradiol within the ACC, But Not the Ventral CA1 Results in Generalization**. **A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug treatment in the ventral CA1(B) or ACC (C). Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. **B)** Animals receiving vehicle or estradiol infusions into the ventral CA1 did not display significant generalization to the neutral context. **C)** Animals receiving vehicle infusions into the ACC did not display significant generalization whereas animals given estradiol infusions generalized significantly to the neutral context and displayed higher fear than vehicle treated animals tested in the neutral context. Values are displayed as mean (±SEM) latency to cross in seconds. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 13. Estradiol within the ACC, But Not the Ventral CA1 Results in Generalization



IX. Experiment 8 - Assessment of Arc Activity in OVX Females Given Estradiol

9.1. Introduction

Estradiol appears to act through the ACC and dorsal CA1 to affect fear generalization, but other brain regions may be involved in the process. As another way to fully assess the brain regions that estradiol may act within to affect fear generalization, animals were analyzed for levels of activity-regulated cytoskeleton-associated protein (Arc) mRNA. Arc is an immediate-early gene that is expressed when a neuron becomes active. Therefore, Arc can serve as an indicator of neuronal activity in that region (Verde, Lee-Osbourne, Worley, Malinow, & Cline, 2006). If estradiol is enhancing overall levels of neuronal activation, then regions implicated in fear generalization will display higher levels of Arc activity following estradiol treatment including the dorsal CA1 region of the hippocampus, the ventral CA1 region of the hippocampus, the dorsal CA3 region of the hippocampus, and the ACC. Animals underwent passive avoidance training, received estradiol injections, and were tested in the training or neutral context. Ten minutes after the completion of the test, animals were sacrificed and brains were extracted for *in situ* hybridization analysis of Arc mRNA levels.

9.2.Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2. Pseudo-trained animals were placed into the passive avoidance chamber during training and allowed to cross, but received no footshocks upon crossing. Home cage control animals remained in their home cage within the colony, received injections of estradiol or vehicle, but were never placed into the passive avoidance apparatus.

Drug Administration

OVX females were administered either vehicle control (sesame oil, 0.1 mL) or estradiol benzoate (estradiol) dissolved in sesame oil (15 μg/0.1 mL) by subcutaneous (SC) injection.

In Situ Hybridization

Following brain extraction, brains were immediately placed in dry ice to freeze. For production of the ARC riboprobe, a pBluescript SK vector containing a 1.2-kb fragment

of the rat ARC cDNA was linearized with *Xho*I to make antisense cRNA probes. Labeled RNA probes was synthesized by *in vitro* transcription of linearized, gel-purified DNA templates using the appropriate T3 polymerase with S35-labeled UTP. Full-length probes was separated from labeling reactions via size-exclusion columns before being mixed with hybridization buffer and measured for specific activity. *In situ* hybridization was performed on 20μm sections sliced on a cryostat and mounted on Superfrost Plus slides (Fisher Scientific). A standard hybridization procedure was used with slight modifications (Jasnow et al., 2013). Briefly, slides were fixed in 4% paraformaldehyde for 30 minutes, rinsed through a series of phosphate-buffered saline (PBS) washes, followed by rinses in triethanolamine with acetic anhydride. Slides were then washed in PBS and dH_2O and dehydrated through a series of graded alcohols. For prehybridization, slides were exposed to hybridization solution in the absence of labeled probe for 2 hours at 55°C in a humidified chamber. Following the prehybridization procedure, the sections were hybridized with S^{35}-labeled CRH probes at 55°C for 16 h in a humidified chamber and then underwent a series of rigorous washes, exposure to RNase, and dehydration through a series of ethanol washes containing NaOAc. Slides were then air dried and exposed to Kodak BioMax MR film for 14 days to generate autoradiograms.

Relative densities were measured from the autoradiograms using standard computerized image analysis software (NIH Image; ImageJ). The area of brain regions including the dorsal CA1 region of the hippocampus, the ventral CA1 region of the hippocampus, and the anterior cingulate cortex (ACC) were traced and mean densities were collected for the left and right hemisphere for each animal and averaged together. Data was then calculated as a percent change from that of home cage control animals.

Statistical Analysis

Analyses for behavior was conducted as described in Experiment 1 to assess the effects of hormone treatment. The effects of estradiol on Arc activity was examined by factorial ANOVAs and independent t-tests.

9.3.Results and Discussion

Animals were trained in passive avoidance and given injections of estradiol or vehicle 24 hours later. Testing occurred 24 hours after the injection and animals were sacrificed and had brains extracted 10 minutes after the test (Fig 14A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,41)} = 6.53$, $p < 0.01$), a significant main effect for treatment, ($F_{(2,41)} = 14.46$, $p<0.001$), and a non-significant interaction, ($F_{(2,41)} = 1.72$, ns). Independent t-tests revealed that acute injections of estradiol induced significant generalization; animals displayed equivalent levels of fear in either context, ($t_{(17)} = 1.21$, ns; $d = 0.54$) (Fig 14B). Vehicle injections did not induce fear generalization, ($t_{(19)} = 3.52$, $p <0.01$; $d = 1.52$). Pseudo-trained animals (N.S.) injected with estradiol did not display differences in either context due to very short latencies in either context, ($t_{(4)} = 1.14$, ns, $d = 0.93$). Additionally, animals given estradiol displayed significantly more fear in the neutral context compared to vehicle treated animals and pseudo-trained animals, (vehicle: $t_{(17)} = 2.47$, $p < 0.05$; $d = 1.14$; pseudo: $t_{(10)} = 3.59$, $p < 0.01$; $d = 3.02$). These results replicate the findings from Experiment 4B and demonstrate significant estradiol-induced generalization.

All regions of the hippocampus and the ACC were analyzed for Arc density. The percent change from home cage controls was calculated where home cage controls

represents 100%. When assessing the dorsal CA1 region of the hippocampus (Fig 14C), factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,28)} = 0.09$, ns), or treatment, ($F_{(2,28)} = 0.54$, ns), and the interaction was also not significant, although it trended towards significance, ($F_{(2,28)} = 2.62$, $p = 0.09$). Independent t-test analyses revealed no significant difference between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 0.36$, ns; $d = 0.22$; pseudo: $t_{(4)} = 1.63$, ns; $d = 1.33$). Estradiol-treated animals displayed significantly more Arc activity when tested in the training context, ($t_{(14)} = 2.24$, $p < 0.05$; $d = 1.12$) (Fig 14C). Additionally, no groups differed from estradiol-treated animals when tested in the neutral context although differences between estradiol-treated and pseudo-trained animals was trending, (vehicle: $t_{(13)} = 0.11$, ns; $d = 0.05$; pseudo: $t_{(9)} = 1.97$, $p = 0.08$; $d = 1.16$). These findings suggest more activation within the dorsal CA1 when animals receive estradiol and are tested in the training context.

When assessing the CA2 region (Fig 14C), factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,28)} = 0.07$, ns), or treatment, ($F_{(2,28)} = 0.41$, ns), and the interaction was also not significant, although it trended towards significance, ($F_{(2,28)} = 2.64$, $p = 0.09$). Independent t-test analyses revealed no significant differences between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 0.09$, ns; $d = 0.006$; pseudo: $t_{(4)} = 0.27$, ns; $d = 1.05$). Estradiol-treated animals displayed significantly more Arc activity when tested in the training context, ($t_{(14)} = 2.24$, $p < 0.05$; $d = 1.16$). Again, no groups differed from estradiol-treated animals when tested in the neutral context, (vehicle: $t_{(13)} = 0.43$, ns; $d = 0.03$; pseudo: $t_{(9)} = 1.48$, ns; $d = 0.90$). These results are similar to those found in the CA1 where estradiol-

treated animals have higher levels of Arc activity within the dorsal CA2 when tested in the training context.

When assessing the CA3 region (Fig 14C), factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,28)} = 4.01$, $p<0.05$), a non-significant effect of treatment, ($F_{(2,28)} = 1.16$, ns), and a non-significant interaction, ($F_{(2,28)} = 1.65$, $p = 0.09$). Independent t-test analyses revealed no significant difference between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 0.06$, ns; $d = 0.04$; pseudo: $t_{(4)} = 0.95$, ns; $d = 0.78$). Estradiol-treated animals displayed significantly more Arc activity when tested in the training context, ($t_{(14)} = 2.64$, $p < 0.05$; $d = 1.32$). Additionally, no groups differed from estradiol-treated animals when tested in the neutral context, (vehicle: $t_{(13)} = 0.25$, ns; $d = 0.13$; pseudo: $t_{(9)} = 0.18$, ns; $d = 0.11$). Finally, estradiol-treated animals had higher levels of activation in the CA3 when tested in the training context compared to vehicle-treated animals, ($t_{(11)} = 2.19$, $p < 0.05$; $d = 1.39$). Overall, these findings are similar to those seen within the CA1 and CA2 with higher levels of activation in estradiol-treated animals tested in the training context.

When assessing the dentate gyrus (DG) (Fig 14C), factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,28)} = 2.41$, ns), or treatment, ($F_{(2,28)} = 0.47$, ns), and the interaction was also not significant, ($F_{(2,28)} = 1.19$, ns). Independent t-test analyses revealed no significant difference between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 1.50$, ns; $d = 0.93$; pseudo: $t_{(4)} = 0.25$, ns; $d = 0.20$). Estradiol-treated animals displayed significantly more Arc activity when tested in the training context, ($t_{(14)} = 2.37$, $p < 0.05$; $d = 1.19$). No groups differed from estradiol-treated animals when tested in the neutral context,

(vehicle: $t_{(13)} = 0.05$, ns; d = 0.03; pseudo: $t_{(9)} = 0.60$, ns; d = 0.33). Taken together, the results reveal that estradiol treatment results in significantly more activation within the training context versus the neutral context within all 4 subregions of the dorsal hippocampus.

Next, animals were analyzed for activity levels within subregions of the ventral hippocampus (Fig 14D). When assessing the ventral CA1 region, factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,26)} = 0.41$, ns), or treatment, ($F_{(2,26)} = 2.13$, ns), and the interaction was also not significant, although it trended towards significance, ($F_{(2,26)} = 2.53$, $p = 0.09$). Independent t-test analyses revealed no significant difference between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(9)} = 1.26$, ns; d = 0.80; pseudo: $t_{(3)} = 0.84$, ns; d = 0.86). Estradiol-treated animals displayed significantly more Arc activity when tested in the training context, ($t_{(14)} = 2.62$, $p < 0.05$; d = 1.31). Additionally, vehicle-treated did not differ from estradiol-treated animals when tested in the neutral context, ($t_{(12)} = 0.81$, ns; d = 0.41), but estradiol-treated animals did differ from pseudo-trained animals, ($t_{(9)} = 2.36$, $p<0.05$; d = 1.18). These findings are similar to those seen within the dorsal CA1 region with higher activation within the training context in estradiol-treated animals.

When assessing the ventral CA3 region (Fig 14D), factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,27)} = 0.002$, ns), or treatment, ($F_{(2,27)} = 1.40$, ns), but a significant interaction, ($F_{(2,27)} = 3.78$, $p<0.05$). Independent t-test analyses revealed no significant differences between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 1.06$, ns; d = 0.66; pseudo: $t_{(3)} = 1.18$, ns; d = 1.17). Estradiol-treated animals displayed significantly

more Arc activity when tested in the training context, ($t_{(14)} = 2.86$, $p < 0.01$; $d = 1.42$). Additionally, vehicle-treated did not differ from estradiol-treated animals when tested in the neutral context, ($t_{(13)} = 0.28$, ns; $d = 0.22$), but estradiol-treated animals did differ from pseudo-trained animals, ($t_{(9)} = 2.84$, $p<0.05$; $d = 1.42$). These findings are similar to those seen within the dorsal CA1 and CA3 regions with higher activation within the training context in estradiol-treated animals.

Finally, when assessing the ventral DG (Fig 14D), factorial ANOVA analyses revealed no significant main effect for context, ($F_{(1,25)} = 2.04$, ns), or treatment, ($F_{(2,25)} = 0.14$, ns), and the interaction was also not significant, ($F_{(2,25)} = 1.04$, ns). However, unlike previous regions, independent t-test analyses revealed that vehicle-treated animals trended towards more significant activation in the training context, ($t_{(9)} = 2.09$, $p = 0.06$; $d = 1.32$), whereas estradiol treated animals did not have differences in activation, ($t_{(13)} = 1.61$, ns; $d = 0.83$). Again, no difference was seen in pseudo-trained animals, ($t_{(3)} = 0.50$, ns; $d = 0.46$). No differences were seen between estradiol-treated animals and the other groups tested in the neutral context, (vehicle: $t_{(11)} = 0.50$, ns; $d = 0.28$; pseudo: $t_{(8)} = 0.57$, ns; $d = 0.43$). These findings are different from those of other hippocampal regions, including the dorsal DG where estradiol treatment resulted in increased activation when testing occurred in the training context. Overall, these findings do not demonstrate a significant difference in activation levels within hippocampal subregions between vehicle-treated and estradiol-treated animals tested in the neutral context as hypothesized.

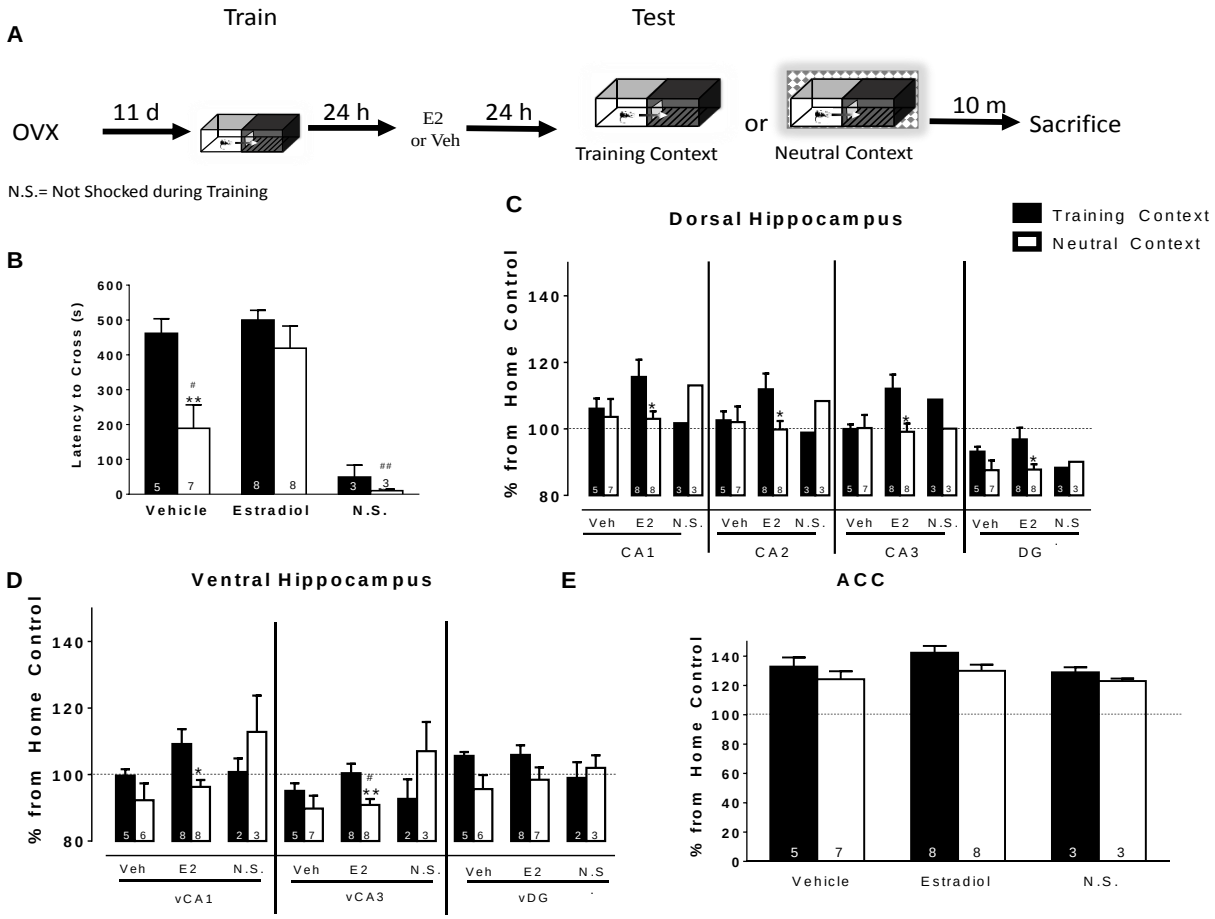
The final region that was assessed for overall Arc activity was the ACC (Fig 14E). Factorial ANOVA analyses revealed a trending main effect for context, ($F_{(1,28)} = 3.75$, $p = 0.06$), a non-significant effect of treatment, ($F_{(2,28)} = 2.06$, ns), and a non-

significant interaction, ($F_{(2,28)} = 0.17$, ns). Independent t-test analyses revealed no significant difference between vehicle-treated or pseudo-trained control animals tested in the training or neutral contexts, (vehicle: $t_{(10)} = 1.04$, ns; d = 0.61; pseudo: $t_{(4)} = 1.56$, ns; d = 1.24). Estradiol-treated animals trended towards more Arc activity when tested in the training context, ($t_{(14)} = 1.96$, p = 0.06; d = 0.99). No groups differed from estradiol-treated animals when tested in the neutral context, (vehicle: $t_{(13)} = 0.84$, ns; d = 0.44; pseudo: $t_{(9)} = 0.97$, ns; d = 0.80). These findings are similar to those seen within the hippocampus with higher activation only seen in estradiol-treated animals tested in the training context.

The lack of differences between estradiol-treated and vehicle-treated animals for Arc mRNA suggests that the overall level of regional activation was not significantly different. The difference between these findings and the findings of estradiol-induced generalization when estradiol is delivered directly into brain regions such as the dorsal CA1 and ACC suggests that Arc mRNA, which has been shown to be a sensitive measure for detecting differences in brain activation during time-dependent generalization in mice trained in context fear conditioning (Cullen et al., 2015), is not a sensitive measure to detect effects of estradiol-induced generalization. The differences in the viability of Arc may be due to the use of rats instead of mice or the use of passive avoidance instead of context fear conditioning. Perhaps passive avoidance behavior requires more overall neuronal activation than context fear conditioning, which masks any differences in activation that may be produced during estradiol-induced generalization.

Figure 14. **Arc mRNA Activity Levels Within the Hippocampal Formation and ACC. A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug injections. Twenty four hours after drug treatment, animals were tested in either the training context or a neutral context. Ten minutes following test, animals were sacrificed and brains were extracted for Arc mRNA analysis via *in situ* hybridization. No shock (N.S.) animals were pseudo-trained and received no shock during training. **B)** Estradiol-treated animals displayed significant fear generalization whereas vehicle-treated animals did not. N.S. animals displayed low levels of fear in either context. **C)** Arc activity data displayed as percent increase from home-cage controls for dorsal hippocampal regions. Estradiol-treated animals had significantly more Arc activity when tested in the training context compared to the neutral context for each region. **D)** Arc activity for ventral hippocampal regions. Estradiol-treated animals had higher activity levels when tested in the training context for the ventral CA1 and ventral CA3 regions. **E)** Arc activity levels within the ACC. No differences were seen among the groups. Values are displayed as mean (±SEM) percent change from home-cage controls. Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p < 0.01$, ***/### = $p < 0.001$). N values for each group displayed within bar graph.

Figure 14. Arc mRNA Activity Levels Within the Hippocampal Formation and ACC



X. Experiment 9 - Effect of Glutamatergic Transmission on Fear Generalization

10.1. Introduction

One of the neural correlates of learning and memory is long-term potentiation (LTP) (Bliss & Collingridge, 1993; Martin, Grimwood, & Morris, 2000; Morris, 2003). Therefore, understanding the effects of estrogens on LTP is crucial to understanding the effects of estradiol on learning and memory. LTP in females is regulated by levels of estrogens and is increased during proestrus and in OVX rats treated with estradiol (Córdoba Montoya & Carrer, 1997; Warren, Humphreys, Juraska, & Greenough, 1995). The increase in LTP magnitude with increased levels of estrogens is mediated by estradiol-induced increases in NMDA receptor (NMDAR) expression and transmission (Bi, Foy, Vouimba, Thompson, & Baudry, 2001; Córdoba Montoya & Carrer, 1997; Cyr et al., 2001; Daniel & Dohanich, 2001; Gould et al., 1990; Maren, 2001; McEwen, 1994; Smith & McMahon, 2005, 2006; Woolley, Weiland, McEwen, & Schwartzkroin, 1997). In fact, estradiol has dramatic impacts on NMDAR expression levels and function. For example, OVX animals have decreased NR1 and NR2B subunit expression, and this effect is reversed with hormone treatment (Cyr et al., 2001). Again, classical estrogenic effects are thought to be mediated by binding of cytosolic receptors to estrogen response elements (ERE) within specific genes. Even though the NMDA subunit genes do not have EREs, other motifs, such as SP1, are found within NR1 and NR2C genes, and these motifs can also respond to the estrogen-receptor ligand

(Bai & Kusiak, 1993, 1995; Katzenellenbogen, O'Malley, & Katzenellenbogen, 1996; Suchanek, Seeburg, & Sprengel, 1995). In addition to estradiol increasing protein expression of NMDA subunits, estradiol also increases binding of NMDA agonists within the CA1 region (Gazzaley, Weiland, McEwen, & Morrison, 1996; Paolo, 2000; Weiland, 1992). Estrogens may have effects on hippocampal-dependent memory through upregulation of NMDAR function, specifically through increases in NR2B expression (Adams, Fink, Janssen, Shah, & Morrison, 2004; Cyr et al., 2001; Smith & McMahon, 2006).

NMDA and AMPA transmission is linked to memory formation, although the determination of when glutamatergic transmission is required during learning and memory remains uncertain. One early study found that NMDA antagonists given before passive avoidance training impaired memory performance, but the antagonists did not impact memory if given after training or prior to testing, suggesting that NMDAR transmission is only required during memory acquisition (Parada-Turska & Turski, 1990). Another study found that when infusions of APV, an NMDA antagonist, were given directly into the amygdala pre- and post-training, memory recall was impaired, but not when given prior to pre-test, suggesting that NMDAR transmission can impact memory acquisition and consolidation, but not memory retrieval (Liang, Hon, & Davis, 1994). Similarly, blocking AMPARs via NBQX prior to training within the BLA impairs fear-potentiated startle (Walker & Davis, 1997). In contrast, recent studies show that blocking NMDAR transmission within the retrosplenial cortex prior to either recent or remote retrieval impairs memory recall, suggesting a role for NMDAR transmission on memory retrieval (Corcoran et al., 2011). Additionally, blocking either AMPARs overall

or specific AMPAR subunits within the hippocampus impairs remote context fear memory (Thoeringer et al., 2012). Therefore, studies over the years have differing results showing the involvement of either glutamatergic receptor being involved in every aspect of the learning and memory process.

The effects of estradiol on glutamatergic transmission also impact learning and memory tasks. For example, blocking either NMDARs overall, specifically blocking NR2B activity, or blocking metabotropic glutamate receptors attenuates estradiol-enhanced object recognition memory (Boulware, Heisler, & Frick, 2013; Lewis, Kerr, Orr, & Frick, 2008; Vedder, Smith, Flannigan, & McMahon, 2013). Given the role of glutamatergic transmission in learning and memory and the direct modulatory role estradiol has on aspects of glutamatergic signaling, the current experiments were designed to test if estradiol-induced generalization was a result of increased glutamatergic transmission. In order to assess this, animals were trained in passive avoidance, injected with estradiol 24 hours later, and tested 24 hours after the injection. Five minutes before the test, animals were given infusions of either the NMDAR antagonist, APV, or the AMPAR antagonist, NBQX, into the dorsal CA1 of the hippocampus (EX9A), or the ACC (EX9B)—regions where estradiol can induce generalized responding as seen in Experiment 7.

10.2. Experiment 9A - Role of Glutamatergic Signaling Within the Dorsal Hippocampus

As noted above, the dorsal hippocampus is a major site of learning and memory, NMDAR-dependent LTP, and estrogenic modulation. For instance, selectively blocking the NR2B subunit-expressing NMDARs in the CA1 also blocks the enhancing effect of estradiol on novel object memory and suggests that the improvement of object

recognition induced by estradiol treatment is due to the total number and number of activated NMDARs (Vedder et al., 2013). Additionally, the effects of estradiol on learning and memory within the hippocampus may be ERβ-dependent—ERβ activation enhances LTP, increases dendritic branching, and upregulates markers of synaptic plasticity, such as GluR1 and synaptophysin (Liu et al., 2008). These findings are in line with the previous data demonstrating that estradiol-induced generalization requires activation of ERβ. In order to test the role of increased glutamatergic transmission inducing generalized responding following estradiol treatment, animals were given infusions of an NMDAR or AMPAR antagonist 5 minutes before memory recall.

10.3. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 7A.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Open Field

To test for effects on locomotor activity, animals were given infusions of APV or NBQX and were placed into an open field chamber 5 minutes later (122 cm diameter) and allowed to explore the open field for 10 minutes. Locomotor activity was measured via AnyMaze 4.99 software (Stoelting, Wood Dale, IL).

Drug Administration

Animals received injections of estradiol dissolved in sesame oil (15 μg/0.1 mL) or vehicle (sesame oil, 0.1 mL). For infusions of the glutamate antagonists, the NMDA receptor antagonist, (2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate (APV) was dissolved in 0.9% saline to a concentration of 6.25 mM**.** The AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dion (NBQX) was dissolved in NaOH to a concentration of 7.8 mM. The doses of APV and NBQX were reduced from those used in other experiments that found memory impairments as the goal of the current set of experiments was not to induce memory deficits for the passive avoidance procedure, but to attenuate generalization of contextual fear to a neutral context (Burman & Gewirtz, 2007; Hou et al., 2009; Walker & Davis, 1997; Walker, Paschall, & Davis, 2005). Animals received 0.5 μl infusions over 5 minutes and the infusion needle was left in place for 1 minute following the conclusion of the infusion. Animals were then tested 5 minutes after the completion of the infusion.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone treatments and antagonist infusions.

10.4. Results and Discussion

Animals were trained in passive avoidance and given injections of estradiol or vehicle 24 hours later. Twenty-four hours after injection, animals were given infusions of either APV, NBQX, or vehicle 5 minutes before a memory recall test (Fig 15A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,89)} = 55.83$, $p < 0.001$), a non-significant main effect for treatment, ($F_{(5,89)} = 1.74$, ns), and a non-significant interaction term, ($F_{(5,89)} = 1.85$, ns). Independent t-tests revealed that vehicle-treated animals were able to distinguish between the two contexts, ($t_{(7)} = 3.49$, $p<0.01$; d 2.93) whereas estradiol-treated animals displayed significant fear generalization, ($t_{(23)} = 0.26$, ns; d = 0.45), and trended towards higher levels of fear in the neutral context compared to vehicle treated animals, ($t_{(15)} = 1.951$, $p = 0.07$; d = 1.02). When vehicle-treated animals were given infusions of either the NMDAR or AMPAR antagonist, animals did not show generalization, (APV: $t_{(14)} = 6.9$, $p<0.001$; d = 3.55; NBQX: $t_{(16)} = 2.88$, $p<0.01$; d = 1.44). These data demonstrate that the antagonist doses used here did not have a negative impact on overall memory retention nor did the antagonists impact generalized responding. When estradiol-treated animals were given infusions of either the NMDAR or AMPAR antagonist, estradiol-induced generalization was attenuated, (APV: $t_{(16)} = 3.85$, $p<0.001$; d = 1.93; NBQX: $t_{(13)} = 2.41$, $p<0.05$; d = 1.35). These findings suggest that the induction of generalization produced by estradiol injections is reduced by blocking either NMDA or AMPA receptors within the dorsal CA1 just prior

to memory recall (Fig 15B). Additionally, estradiol-treated animals tested in the neutral context were significantly different from estradiol-treated animals given the NMDAR antagonist, ($t_{(21)} = 3.23$, $p<0.01$; $d = 1.34$), and trended towards more generalized responding compared to estradiol-treated animals given the AMPAR antagonist, ($t_{(18)} = 1.85$, $p=0.08$; $d = 0.83$). These results demonstrate that inactivation of either NMDARs or AMPARs within the dorsal CA1 is sufficient to attenuate estradiol-induced generalization.

To make sure that the glutamatergic antagonists were not attenuating estradiol-induced generalization through alterations in locomotor activity, animals were given infusions of either APV or NBQX and 5 minutes later were placed into an open field for 10 minutes and assessed for locomotor activity. One-way ANOVA analyses revealed no significant difference in distance travelled between the groups, ($F_{(2,9)} = 0.19$, ns). These results demonstrate that the glutamate receptor antagonists given 5 minutes beforehand does not affect generalized responding through alterations in locomotor activity (Fig 15D).

10.5. Experiment 9B - Role of Glutamatergic Signaling Within the ACC

The other brain region in which estradiol induced generalization was within the ACC. Some studies suggest that NMDA binding is reduced in the frontal cortex of OVX females given hormone replacement (Cyr et al., 2001), but can increase AMPAR expression within the cortex (Cyr et al., 2001). Thus, estrogens may also be acting within the ACC to induce generalization through an increase in glutamatergic transmission. In order to test this, animals were treated the same way as described in Experiment 9A

except that infusions of the glutamate receptor antagonists were given in the ACC instead of the hippocampus.

10.6. Methods

Animals

Adult female Long Evans rats approximately 90 days old at the time of surgery were used for all experiments and maintained as described in Experiment 2.

Surgical Procedures

OVX Surgical Procedures were conducted as described in Experiment 2. Cannulation surgical procedures were conducted as described in Experiment 7F.

Site Verification

Site verification was conducted as described in Experiment 6A.

Passive Avoidance Procedure

Passive avoidance behavior was conducted as described in Experiment 2.

Drug Administration

Animals received drug treatment as described in Experiment 9A.

Statistical Analysis

Analyses were conducted as described in Experiment 1 to assess the effects of hormone treatment and antagonist infusions.

10.7. Results and Discussion

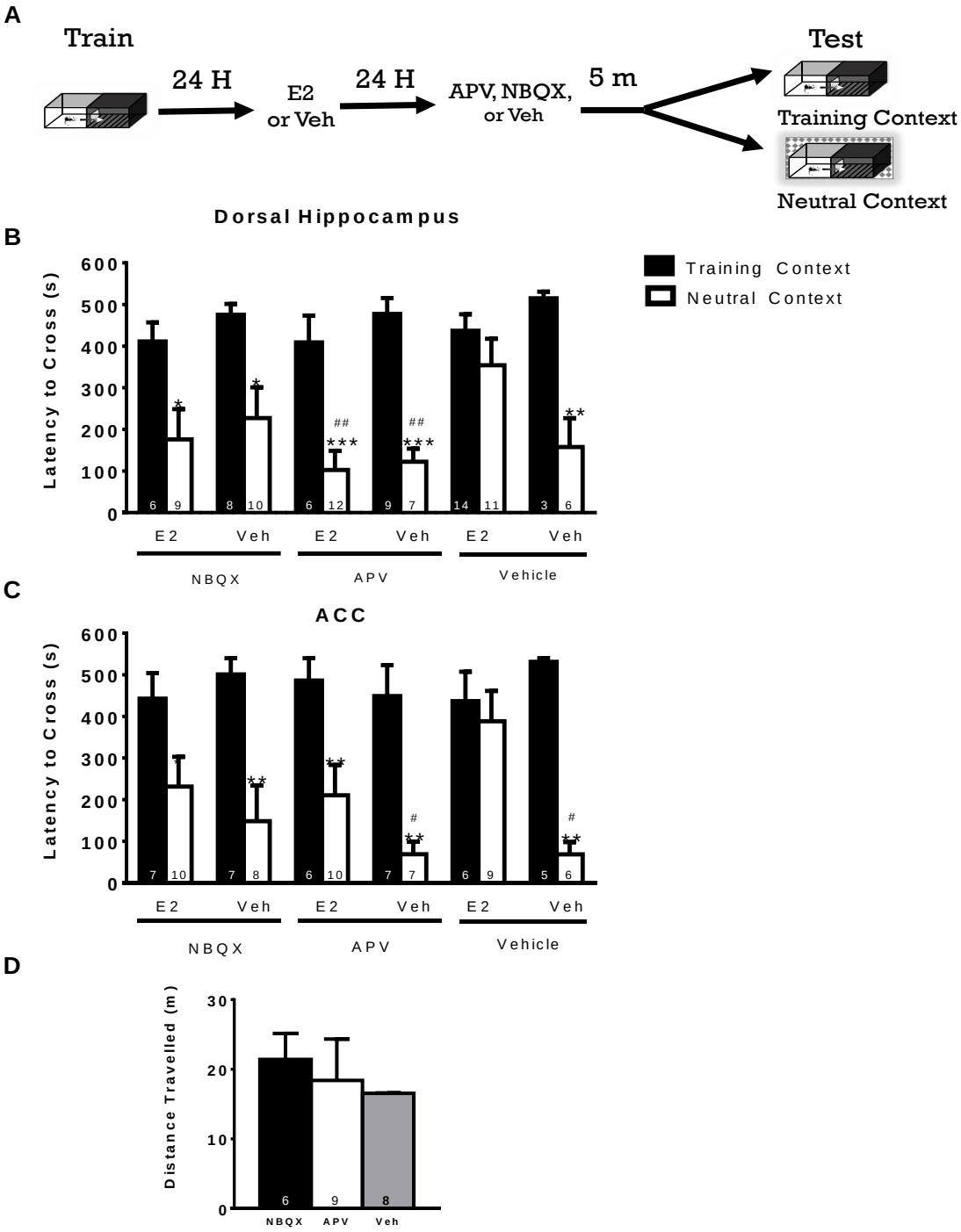
Animals were trained in passive avoidance and given injections of estradiol or vehicle 24 hours later. Twenty-four hours after injection, animals were given infusions of either APV, NBQX, or vehicle 5 mintues before a memory recall test (Fig 15A). Factorial ANOVA analyses revealed a significant main effect for context, ($F_{(1,76)} = 53.43$, $p < 0.001$), a non-significant main effect for treatment, ($F_{(5,76)} = 1.13$, ns), and an interaction term that trended towards significance, ($F_{(5,76)} = 2.18$, $p=0.07$). Independent t-tests revealed that vehicle-treated animals were able to distinguish between the two contexts, ($t_{(11)} = 4.30$, $p<0.001$; $d = 8.80$) whereas estradiol-treated animals displayed significant fear generalization, ($t_{(14)} = 0.46$, ns; $d = 0.27$), and trended towards higher levels of fear in the neutral context compared to vehicle treated animals, ($t_{(16)} = 1.85$, $p = 0.08$; $d = 1.96$). When vehicle-treated animals were given infusions of either the NMDAR or AMPAR antagonist, animals did not show generalized fear, (APV: $t_{(13)} = 3.28$, $p<0.01$; $d = 2.53$; NBQX: $t_{(15)} = 2.98$, $p<0.01$; $d = 1.90$). As before, these data demonstrate that the antagonist doses used here did not have a negative impact on overall memory retention nor did the antagonists impact generalized responding by themselves. When estradiol-treated animals were given infusions of either the NMDAR or AMPAR antagonist, animals displayed significant discrimination, (APV: $t_{(15)} = 2.95$, $p<0.01$; $d = 1.46$; NBQX: $t_{(15)} = 2.49$, $p<0.05$; $d = 1.07$). These findings suggest that the induction of generalization produced by estradiol injections is attenuated by blocking either NMDA or AMPA receptors within the ACC (Fig 15C). However, estradiol-treated animals tested in the neutral context were not significantly different compared to either estradiol-treated

animals given the NMDAR antagonist or the AMPAR antagonist, (APV: $t_{(18)} = 1.35$, ns; $d = 0.79$; NBQX: $t_{(18)} = 1.42$, ns; $d = 0.64$).

These results demonstrate that inactivation of either NMDARs or AMPARs within the ACC or dorsal CA1 is sufficient to attenuate estradiol-induced generalization. These experiments provide clues that estradiol is acting to enhance glutamatergic transmission resulting in animals responding fearfully to neutral contexts. Future experiments will need to determine if increased glutamatergic signaling in the dorsal CA1 changes signaling within the ACC or vice versa. Additionally, future experiments will need to determine which type of NMDA and AMPA receptor is driving the effect of estradiol-induced generalization by using subunit-specific antagonist infusions rather than global receptor antagonists.

Figure 15. **Blocking Glutamatergic Signaling Attenuates Estradiol Induced Generalization in the Dorsal CA1 and ACC. A)** Schematic of the experimental paradigm for the experiment. All animals were trained in passive avoidance and 24 hours later received drug injections. Twenty four hours after drug treatment, animals were given infusions of APV, NBQX, or vehicle into the dorsal CA1 (B) or ACC (C) and tested 5 minutes later in either the training context or a neutral context. **B)** Estradiol injections induced generalization compared to vehicle-treated animals. Infusions into the dorsal CA1 to block either NMDARs (APV) or AMPARs (NBQX) were effective in reducing estradiol-induced generalization. **C)** Estradiol injections induced generalization compared to vehicle-treated animals. Infusions into the ACC to block either NMDARs (APV) or AMPARs (NBQX) were effective in reducing estradiol-induced generalization. **D)** Infusions of either APV or NBQX did not affect locomotor activity as measured in a 10 minute open field test compared to vehicle-treated animals. Values are displayed as mean (±SEM) latency to cross in seconds or distance travelled (m). Significance values were set at $p<0.05$. (*/# = $p < 0.05$, **/## = $p <0.01$, ***/### = $p <0.001$). N values for each group displayed within bar graph.

Figure 15. Blocking Glutamatergic Signaling Attenuates Estradiol-Induced Generalization in the Dorsal CA1 and ACC



XI. General Discussion

11.1. Main Findings

The present findings demonstrate a sex difference in the generalization of contextual fear; females display a faster rate of generalization than males (EX 1; Fig 1). In addition, the results reveal a sex-dependent effect of estradiol on fear generalization. Specifically, estradiol acts to induce generalization in OVX females (EX 2; Fig 2) whereas estradiol acts to prevent, or attenuate, fear generalization in GDX males (EX3, 4D; Fig 3). When assessing when estradiol acts to modulate generalization, acute exposure experiments reveal estradiol acts on memory retrieval in females (EX4B; Fig 5) and males (EX4C; Fig 6). In addition to estradiol acting in a sex-dependent manner to affect generalization, estradiol also acts through distinct ER subtypes in males and females. Within OVX females, only activation of ERβ results generalization (EX5A; Fig 8C). However, in GDX males, activation of either ERα or ERβ attenuates generalization (EX5B; Fig 8D). Site-specific infusion analyses suggest that estradiol acts through cytosolic ERβ rather than membrane-bound ERs in OVX females to induce generalization (EX6; Fig 9, 10), and that estradiol is required within the dorsal CA1 (EX7A-D; Fig 11) and the ACC (EX7F; Fig 13C), but not the ventral CA1 (EX7E; Fig 13B). Analysis of neuronal activity through measurements of Arc mRNA levels did not reveal any specific increase in estradiol-induced generalizing animals compared to vehicle controls, suggesting that Arc levels is not a sensitive measure for detecting

differences during generalization of fear perhaps as a result of the type of behavioral task used (EX8; Fig 14). Finally, estradiol appears to induce generalized responding by enhancing glutamatergic signaling; blocking either AMPA or NMDA receptors with infusions of antagonists into either the dorsal hippocampus (EX9A; Fig 15B) or the ACC (EX9B; Fig 15C) attenuates estradiol-induced generalization. Taken together, these experiments demonstrate for the first time a sex differences in the generalization of fear and they begin to parse apart the mechanisms by which estradiol exerts sex-dependent effects on fear generalization.

Several differences between male and female behavior may explain the differential rates of fear generalization seen in Experiment 1. For instance, males and females differ in exploratory behavior. Several studies have shown that female rats have higher values on active exploration measures (e.g. sniffing, rearing, walking, orienting toward a novel object). Similarly, females emerge sooner than males from a familiar environment to a novel one (Archer, 1974, 1975). These differences in exploration do not explain the faster rate of generalization seen in females, however; higher rates of exploratory behavior should result in females crossing faster to the novel context compared to males at all retention intervals. Additionally, females tested 1 day after training show a similar pattern of behavior as the males, which suggests that the results are not due to differences in other behavioral measures such as active exploration or a general inability of females to perceive differences in contexts.

Another possible behavioral sex difference that may explain the current findings is differences in learning strategy during spatial memory tasks. Males focus more on spatial and geometric cues in the environment, which is a hippocampus-dependent

strategy, whereas females rely more on landmark cues, which activates other systems such as the striatum (Miranda, Blanco, Begega, Rubio, & Arias, 2006; Williams, Barnett, & Meck, 1990; Williams & Meck, 1991). Males are also more efficient when choosing a place strategy when solving a spatial task, resulting in better overall spatial memory in males (van Gerven, Schneider, Wuitchik, & Skelton, 2012). Together, these findings suggest that males may display slower rates of fear generalization compared to females as a result of better spatial processing, although the correlation between spatial learning and the generalization of fear remains unknown.

Any differences in behavioral output between males and females may be due to specific hormone levels, sexually dimorphic structures within the brain, differential receptor distributions, or involvement of distinct brain structures. For instance, estradiol concentration is reduced in males and females in the hippocampus following long term gonadectomy, but only males given exogenous estradiol treatment display higher levels of estradiol in the hippocampus compared to intact males. The current results give an early indication of the brain structures involved in estradiol-*induced* generalization seen in females, but the circuitry underlying estradiol-*reduced* generalization seen in males has yet to be analyzed. Therefore, one possibility is that estrogens interact within a distinct sex-specific circuit. 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 several sexually dimorphic behaviors, such as aggression, stress, and anxiety behavior, and is also involved in maintaining responses that are long in duration; once the BNST is activated, it can influence behaviors for a long time following the termination of the learning event (Walker, Toufexis, & Davis, 2003). The BNST is also sexually dimorphic

with a 97% higher volume and more cells in male versus female rats and mice (Hines, Allen, & Gorski, 1992; Murray, Hien, de Vries, & Forger, 2009). In addition to overall differences in volume, the expression of aromatase mRNA within the BNST is higher in males in terms of amount of aromatase mRNA and number of cells expressing aromatase (Foidart, De Clerck, Harada, & Balthazart, 1994; Lauber, Sarasin, & Lichtensteiger, 1997; Roselli, Ellinwood, & Resko, 1984; Wagner & Morrell, 1997). The regulation of aromatase mRNA levels within males, but not females, is mediated by gonadal hormones; gonadectomized males have reduced levels of aromatase mRNA compared to intact males whereas OVX females and OVX females treated with exogenous estradiol do not have differing levels of aromatase mRNA (Tabatadze, Sato, & Woolley, 2014; Wagner & Morrell, 1997).

The BNST also has sex-specific expression differences in estrogen receptors. Specifically, males display higher levels of ERβ expression within the BNST whereas females display higher expression levels within the hippocampus (Zhang et al., 2002) . Thus, ERβ expression within the BNST is most likely upregulated through estrogenic signaling due to the high levels of aromatase activity in this region.

Although the circuitry involved in estradiol-*reduced* generalization in gonadecotmized males has not been explored yet, the BNST appears ideally placed to be involved due sex differences in ER expression and estradiol-sensitive levels of aromatase activity. Additionally, the BNST also receives input from the ventral hippocampus (Dong, Petrovich, & Swanson, 2001), which is a structure implicated in time-dependent fear generalization (Cullen et al., 2015). Although the current experiments found no involvement of the ventral hippocampus in estradiol-*induced* generalization, the ventral

hippocampus still plays a role in contextual information relay, making the connections between the ventral hippocampus and the BNST a viable avenue of exploration for estradiol-*reduced* generalization in males. Future studies will need to assess the role of the BNST and the connections between the BNST and ventral hippocampus. If this circuitry is involved, it would suggest that the sex-dependent effects of estradiol on fear generalization are indeed due to different circuitry involvement in males and females. In addition to understanding the sex-dependent effects of estradiol on fear generalization, a full understanding of what underlies estradiol-*induced* generalization remains unknown. Several possible explanations and other behavioral phenomenon could help explain why estradiol acts to induce generalization in females including effects of estradiol on anxiety-like behavior, stress activity, and spatial learning.

11.2. Estrogens and Anxiety-like behavior

Several researchers have demonstrated a dynamic role of estrogen in anxiety-like behavior. When an organism is in a fearful situation, estrogens increase the fear response, whereas when in a less stressful situation, estrogens increase activity levels (Morgan & Pfaff, 2001, 2002; Morgan et al., 2004). For example, estradiol treatment in OVX mice increases activity on a running wheel in the home cage, but produces more anxiety-like behavior and fear behavior in an open field (Morgan & Pfaff, 2002). Despite several reports of reduced fear and anxiety in response to treatment with estrogens (Frye, Petralia, & Rhodes, 2000; Krężel, Dupont, Krust, Chambon, & Chapman, 2001; Walf & Frye, 2006), others have shown that treatment with estrogens increases anxiety and fear responses in rodents in a variety of paradigms (Jasnow et al., 2006; Morgan & Pfaff, 2001; Morgan et al., 2004; Nofrey et al., 2008; Toufexis et al., 2007). One possible

explanation for the different effects of estrogens on anxiety-like behavior may be differential activation of ER subtypes. In general, activation of ERβ is thought to have anxiolytic effects (Bodo & Rissman, 2006; Lephart et al., 2002; Lund et al., 2005; Walf et al., 2004). For example, ERβ knockout mice (BERKO) mice generally show increases in anxiety-like behavior (Imwalle, Gustafsson, & Rissman, 2005; Krężel et al., 2001; Oyola et al., 2011; Rocha, Fleischer, Schaeffer, Rohrer, & Hickey, 2005; Walf & Frye, 2006) and more anxiety-like behavior in the open field than wild type littermates and ERα knockout mice (AERKO) mice (Krężel et al., 2001). However, the role of ERβ activation in anxiety-like behavior has not been confirmed; others find either no effects or opposite effects with DPN treatment or activation of ERα resulting in less anxiety-like behavior in the elevated plus maze (Byrnes, Casey, & Bridges, 2012; Jacome et al., 2010). Overall, the effects of estrogens on anxiety-like behavior may be contributing to the findings of estradiol-induced generalization in females. When put into a stressful environment—a similar, but distinct, context (neutral) from one in which fear conditioning occurred—estradiol treatment increases overall fear. But, the connection between increased anxiety-like behavior and fear generalization is not established and the current findings suggest that estradiol-induced generalization in females is due to activation of ERβ, which is mostly associated with reduced anxiety-like behavior. Additionally, no differences were seen in levels of overall fear between animals treated with estradiol compared to vehicle-treated animals as indicated by equivalent levels of fear in the training context. The lack of differences in overall levels of fear during testing in the training context could be a result of a behavioral ceiling effect—animals are removed at a specific time following initiation of the test—but, other data using passive

avoidance suggests that a longer test would not elicit differences between females treated with vehicle or estradiol. When comparing studies using 300 second tests instead of 540-600 second tests, animals display ceiling-level fear responses in the training context (Drago, Bohus, Scapagnini, & De Wied, 1980; Zhou & Riccio, 1996), suggesting that longer tests (i.e. 1800 seconds) at the shock level used in the current set of experiments would result in near-ceiling levels of avoidance when testing occurs in the training context. Even with the limited understanding of the effects of estrogens on anxiety-like behavior and the connection of anxiety-like behavior and generalization behavior, the two behaviors may be related, providing a possible explanation for estradiol-induced generalization.

11.3. Estrogens and Stress

The effects estrogens have on anxiety-like behavior may be a result of interactions with the stress system. The main stress pathway, the hypothalamic pituitary adrenal (HPA) axis, can be affected by estrogens, and dysregulation of the stress system is implicated in psychiatric disorders, such as PTSD (Baker et al., 1999; De Souza, 1995; Holsboer & Barden, 1996). In addition to impacting learning and memory and synaptic plasticity in the hippocampus and prefrontal cortex, estrogens act in regions controlling neuroendocrine and behavioral stress responses, and this may account for increased fear generalization in estradiol treated females. For example, pituitary adenylate cyclase activating peptide (PACAP) is implicated in anxiety-like behaviors (e.g. Hammack et al., 2010), 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)—the differences in results may be due to the timing of estradiol exposure during the learning and memory process—emphasizing the complex 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 estradiol benzoate leads to an increase in CRH mRNA expression in the central nucleus of the amygdala. Thus, CRH may serve as an intermediary between estrogens and alterations in fear responses (Jasnow et al., 2006). Overall, 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 modulation of the hippocampal functioning and fear memory retrieval.

The interactions of estradiol with the stress system may also help explain the sex-dependent effects of estrogens on fear generalization. In response to different stressors, females can exhibit higher levels of corticosterone than males (Haleem, Kennett, & Curzon, 1988; Kant et al., 1983; Kitay, 1963; Le Mevel, Abitbol, Beraud, & Maniey, 1979; Livezey, Miller, & Vogel, 1985; Wilson & Biscardi, 1994). Following the presentation of a stressor, female rats have a faster increase in corticosterone levels (Kant et al., 1983). These sex differences within the stress system and in response to stress, are a result of organizational effects of gonadal hormones; neonatal estrogen exposure converts females to male-like stress system patterns by increasing CRH and decreasing

glucocorticoid receptor expression (Patchev, Hayashi, Orikasa, & Almeida, 1995). Overall, female rats appear to have a greater magnitude of response and duration of the HPA axis after stressors, suggesting that females may have a less responsive negative feedback loop with glucocorticoid secretion compared to males (see Rhodes & Rubin, 1999 for review). Structures such as the BNST are altered by stressful experiences (i.e. footshock), and these changes may underlie changes in learning and memory following a stressful experience (Bangasser, Santollo, & Shors, 2005). In fact, the stress response within the BNST is highly regulated by PACAP signaling, possibly through interactions between PACAP and corticosterone levels (Lezak et al., 2014; Roman et al., 2014). Given the sexually dimorphic nature of the BNST, the interaction of stress within this structure may underlie sex differences in fear generalization seen in the present experiments.

Sex differences in response to stressors may also explain the sex difference seen in fear generalization. Perhaps females are more sensitive to the footshock given to induce fear of the black compartment during passive avoidance training. Evidence for differences in shock sensitivity comes from findings demonstrating that females respond with more escape-like behaviors to shock than males (Beatty & Beatty, 1970) and exhibit higher heart rates following shock (Archer, 1975). Therefore, the sex difference in reaction to the shock could explain why intact females displayed significantly faster rates of fear generalization compared to males (EX1). However, if this were the case, one would expect to see differences in fear at shortest retention interval in females; testing at one day should result in sex differences in generalization to the neutral context if females are responding with higher levels of stress to the shock and this increased stress level is

directly related to generalization. Therefore, it seems unlikely that any differences between males and females seen in the neutral context during testing at long retention intervals is due to the shock being more salient or more fear-evoking for females than males.

11.4. Spatial Memory

In addition to affecting anxiety-like behaviors and interacting with the stress system, estrogens can also affect spatial learning and memory. Spatial learning may be related to fear generalization due to the need to orient and remember spatial environments and cues in which a learning event occurs. In humans, males generally outperform females in tasks that require spatial skills (Halpern, 2011; L. J. Harris, 1978; Maccoby & Jacklin, 1974; McGee, 1979; Postma, Jager, Kessels, Koppeschaar, & van Honk, 2004). However, some find no sex differences in spatial working memory and object location (Postma et al., 2004). In rodents, males tend to perform better than females in a variety of spatial tasks (Barrett & Ray, 1970; Beatty, 1984; Beiko et al., 2004; Davenport et al., 1970; Dawson, 1972; Dawson et al., 1975; Einon, 1980; Gaulin & FitzGerald, 1986; Gresack & Frick, 2003; Perrot-Sinal et al., 1996). In general, proestrus female rats have poorer performance in the Morris water maze (MWM), a spatial navigation task, compared to estrus females, suggesting that the high levels of estrogens present during proestrus impairs spatial learning and memory (Warren & Juraska, 1997). Similarly, estradiol treatment in OVX females impairs performance compared to OVX control animals in the MWM and other maze tasks (Fader, Hendricson, & Dohanich, 1998; Frye, 1995; Galea et al., 2001; Luine et al., 1998). Taken together, these data suggest that high

levels of estrogens, either through exogenous delivery or during proestrus, produces deficits in spatial memories.

Estradiol-induced spatial memory impairment could be a result of alterations in the strategy used in spatial orientation tasks, such as the MWM. In a version of the MWM where the room contains a landmark cue that animals can use to orient themselves in relation to the hidden platform, OVX females use a cue learning strategy where the landmark cue is used to help locate the platform. If OVX females are treated with estradiol, animals undergo random searching during the early trials of the MWM, suggesting that with high levels of estrogens, animals no longer attend to the landmark cue and, therefore, the deficits in spatial memory performance is a result of a switch from a cue learning strategy to a random search strategy (Daniel & Lee, 2004). The difference in strategy is also seen when comparing proestrus and estrus females (Sava & Markus, 2005). This change in strategy choice may be a result of estrogens increasing attention to more stimuli, whereas lower levels of estradiol are more likely to attend only to cues that are salient to them (Sava & Markus, 2005).

The excessive attention and lack of focus on specific cues in spatial learning with high levels of estrogens is also seen in cued fear conditioning and may explain the lack of latent inhibition and discrimination in estradiol-treated rats (Nofrey et al., 2008; Toufexis et al., 2007). The increase in attention during high levels of estrogens may explain the enhanced fear generalization to a neutral context seen in estradiol-treated female rats. When treated with estrogens, female rats are unable to focus attention to relevant stimuli and, therefore, respond fearfully in the presence of cues that should not elicit a fear

response. When levels of estrogens are low (e.g. OVX controls), animals are able to focus attention and do not respond fearfully in the neutral context (EX2).

One way to alleviate the estradiol-impairing effects on spatial learning is through pretraining, which provides animals with more time to focus more on relevant cues and reduces random search time seen in proestrus animals (Beiko et al., 2004; Berry, McMahan, & Gallagher, 1997; Warren & Juraska, 1997). Similarly, in context fear conditioning, 3 days of pre-exposure in males reduces generalized responding, suggesting that pretraining exposure allows for more focus on the training context details, resulting in less generalization after a long retention interval (Biedenkapp & Rudy, 2007). Additionally, re-exposure to the training context prior to testing in a neutral context can reduce generalization (Zhou & Riccio, 1994). The process of re-exposure could function to enhance attention to relevant cues in a similar fashion as pre-exposures prior to training. Of note, males and females are equally affected by a pre-test cue and display significant discrimination at a 14 day retention interval following the pre-test cue (Lynch III et al., unpublished observation). If enhanced rates of fear generalization in female rats is a result of increased attention to irrelevant cues similar to that seen in other spatial tasks, then pre-exposures to the training context prior to training or re-exposures prior to testing in a neutral context should reduce the enhancement in fear generalization seen after estradiol treatment.

Another hypothesis for the impaired spatial learning during times of high estrogen levels suggested by Warren and Juraska (1997) is that estradiol-induced production of so many synapses in a short time span produces noise in the system and disrupts the learning ability of the animal (e.g. Woolley, Gould, Frankfurt, & McEwen, 1990; Woolley &

McEwen, 1992, 1993; Woolley et al., 1997; Woolley, Wenzel, & Schwartzkroin, 1996). This interpretation may also explain differences in fear generalization; high levels of estrogens produces an increased number of synapses that are producing too much signaling in the hippocampus and other brain regions, which impacts the retrieval of the precise memory, resulting in fear expression in inappropriate (i.e. neutral) contexts. However, not all report estradiol-induced impairments in spatial memory (e.g. Packard, 1998; Packard et al., 1996; Packard & Teather, 1997) so caution must be taken when considering the effects of estrogens on spatial memory as a possible interpretation for estradiol-induced fear generalization.

Overall, the complex interaction between estrogens, stress, and spatial memory may be involved in the increasing in fear generalization seen with high levels of estrogens. In the procedure used in the present experiments, animals are placed into a specific context and trained under a stressful condition (i.e. footshock). The animals then have to be able to discriminate between the training context and a neutral context. Therefore, estradiol may be interacting to increase the stress response to shock, affecting spatial recognition, which results in increased fear generalization to the neutral context. All of these interactions remain unexplored, however.

11.5. Extinction Learning

The current findings of estradiol-induced generalization 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). Others have assessed sex differences in other forms of inhibitory learning such as extinction learning. In extinction learning, participants learn to no longer fear a conditioned stimulus previously paired with an

aversive stimulus (i.e. shock) through repeated exposure to that stimulus in the absence of the aversive stimulus (Sotres-Bayon, Cain, & LeDoux, 2006). During fear extinction, PTSD patients have higher dorsal ACC activation after fear conditioning and during the beginning of extinction and have reduced activation in the ventromedial prefrontal cortex (vmPFC) during the end of the extinction compared to healthy controls (Milad et al., 2007). These results suggest that PTSD patients display unique brain activation patterns during the process of fear conditioning and extinction that may be related to their pathology and the persistence of symptoms.

In humans, females with PTSD display greater skin conductance responses during fear conditioning (Inslicht et al., 2013). Despite increased reactivity during fear conditioning, females also display enhanced fear extinction learning in an estrogen-dependent fashion. In animal models, extinction retention is facilitated by high levels of estrogens. Blockade of estrogens via antagonists results in a higher fear response following extinction learning in rats (Milad, Igoe, Lebron-Milad, & Novales, 2009). Extinction learning is best retained by naturally cycling females with high levels of estrogen compared to those with low estrogen levels (Milad et al., 2010; Zeidan et al., 2011), and high estrogen females have higher activation in the vmPFC, ACC, left amygdala, and hippocampus during extinction recall (Zeidan et al., 2011). Additionally, estradiol can improve extinction retention in males. Specifically, blocking aromatase impairs extinction learning, suggesting that without conversion of testosterone into estradiol, animals cannot retain extinction learning as efficiently (Graham & Milad, 2014).

Levels of estrogens have an opposite effect when females have PTSD, resulting in heightened fear expression during extinction learning when levels of estrogens are low compared to females without PTSD (Glover et al., 2012). These findings suggest that extinction learning is altered in patients with PTSD, and females in particular, may display differential responding in fear extinction depending on the levels of estrogens present during extinction learning.

Unlike the well-established effect of estrogens enhancing extinction retention, where new learning occurs about the relationship between the conditioned stimulus and absence of the unconditioned stimulus (Graham & Daher, 2015; McDermott, Liu, Ade, & Schrader, 2015; Milad et al., 2009), the present findings are not a result of estrogens enhancing memory formation as has been demonstrated frequently (Daniel & Dohanich, 2001; Daniel, Hulst, & Lee, 2005; Fan et al., 2010; Fernandez et al., 2008; Fortress, Fan, Orr, Zhao, & Frick, 2013; Frye, Duffy, & Walf, 2007; Frye & Rhodes, 2002; Gibbs, 2002; Packard, 1998; Packard et al., 1996; Packard & Teather, 1997; Rhodes & Frye, 2004; Sandstrom & Williams, 2004; Walf, Koonce, & Frye, 2008; Walf, Rhodes, & Frye, 2006; Zhao, Fan, & Frick, 2010). Enhancement of fear generalization is not a result of altered memory formation, but rather an alteration in what cues elicit the fear memory response.

11.6. Hippocampal Involvement

The hippocampus is considered a critical locus of contextual fear conditioning (Maren, 2001; Maren & Holt, 2004; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Matus-Amat et al., 2007; Rudy & Matus-Amat, 2005) and is implicated in the generalization of fear (Cullen et al., 2015; Ruediger et al., 2011; Wiltgen & Silva, 2007;

Wiltgen et al., 2010; Winocur et al., 2007). The hippocampus is also the structure that has received the most attention for the effect of estrogens on synaptic plasticity, even though other areas of the brain are sensitive to fluxes in estrogens, including the amygdala and prefrontal cortex (de Castilhos, Forti, Achaval, & Rasia-Filho, 2008; Hajszan et al., 2007; Hao et al., 2006; Nishizuka & Arai, 1982; Rasia-Filho, Fabian, Rigoti, & Achaval, 2004; Wallace et al., 2007; Wallace et al., 2006). Although estradiol affects synaptic plasticity throughout the brain, the effect of these changes on memory remains unknown. One possible mechanism by which estradiol-mediated changes in synaptic plasticity may be linked to alterations in memory is through changes to LTP. LTP is driven by activation of the two main glutamatergic receptors: AMPARs and NMDARs. Here, blockade of either glutamate receptor in the dorsal CA1 or ACC attenuated estradiol-induced generalization (EX9), providing a possible mechanism for estradiol-induced generalization. LTP in females is regulated by levels of estrogens and is increased during proestrus as well as in OVX rats treated with estradiol (Córdoba Montoya & Carrer, 1997; Warren et al., 1995). The increase in LTP magnitude with increased levels of estrogens is mediated by estradiol-induced increases in NMDAR expression and glutamate transmission (Bi et al., 2001; Córdoba Montoya & Carrer, 1997; Cyr et al., 2001; Daniel & Dohanich, 2001; Gould et al., 1990; Maren, 2001; McEwen, 1994; Smith & McMahon, 2005, 2006; Woolley et al., 1997). More specifically, the effect of estrogens on LTP and spine density may be a result of estradiol-induced increases in NR2B subunits, resulting in an increase in NR2B-containing silent synapses—synapses that only contain NMDARs and lack AMPARs. The increase in silent synapses stimulates mechanisms that allow the insertion of AMPARs into the

synapse, which leads to the synaptic plasticity changes seen after estradiol treatment. The decline of estradiol following injections or during the estrus cycle, results in a return of the NMDAR:AMPAR ratio to baseline levels, reducing the enhancements in spine density and LTP (Smith, Vedder, & McMahon, 2009). Even though the effects of estradiol on LTP and synaptic plasticity are robust, other researchers see no effect of chronic estradiol treatment on LTP induction in the Schaffer collateral-CA1 pathway (Barraclough, Ingram, & Brown, 1999). NR2B transgenic mice that have upregulated levels of NR2B display better LTP within the hippocampus and improved retention of object recognition tasks, context fear conditioning, and cued fear conditioning (Tang, Wang, Feng, Kyin, & Tsien, 2001). When combined with the finding that estradiol increases NR2B mRNA, the localization of NR2B-containing receptors, and that increases in LTP via estradiol treatment are prevented with NR2B-specific antagonists, it suggests that estrogens may have effects on hippocampal-dependent memory through upregulation of NMDAR function, specifically through increases in NR2B expression (Adams et al., 2004; Cyr et al., 2001; Smith & McMahon, 2006). Future experiments will need to test for subunit-specific roles of AMPAR and NMDAR involvement in estradiol-induced generalization through subunit-specific antagonists.

Another important aspect to the hypothesis of estradiol-induced generalization resulting from enhanced glutamatergic signaling comes from the findings that activation of ERβ increases LTP and dendritic branching in the hippocampus (Liu et al., 2008). Additionally, ERβ knockout mice (BERKO) mice display reduced LTP and impairment in hippocampal-dependent context fear conditioning (Day, Sung, Logue, Bowlby, & Arias, 2005). Finally, the sex differences seen in NMDA-dependent LTP also provides a

possible explanation for the sex-dependent effects of estradiol on generalization. Male and female rats demonstrate sex differences in LTP due to the effect of estrogens on NMDARs. Males display higher rates of LTP resulting from greater overall activation of NMDARs (Maren, De Oca, & Fanselow, 1994). These findings could help explain why males react differently to estradiol exposure when tested in a neutral context. Future studies will need to assess the involvement of enhanced glutamatergic signaling in estradiol-*reduced* generalization seen in GDX males. Overall, the findings from the present experiments suggest that estradiol-induced generalization is a result of enhanced glutamatergic signaling. These experiments are not the first to suggest that generalization is a result of increased excitability (Xu & Südhof, 2013). One problem with the role of estradiol affecting LTP to induce generalization in females is that the main finding from the current set of experiments utilizes injections or infusions of estradiol well outside the traditional window for consolidation (i.e. 24 hours following fear conditioning; EX4B). Given that estradiol is exerting effects on memory retrieval, it may be unlikely that these effects are due to direct interactions between estradiol and LTP.

One interesting finding from the present experiments was that the ventral hippocampus is not involved in estradiol-induced generalization (EX7E). Other studies on time-dependent generalization have shown that activity within the ventral hippocampus results in generalized fear at a long retention interval (Cullen et al., 2015). Additionally, the ventral hippocampus is a relay for contextual information processed by the dorsal hippocampus (Maren, 2001). However, estradiol does not appear to act within this region to induce generalization. These findings are important as they suggest that time-dependent generalization and estradiol-induced generalization may occur via

distinct mechanisms and the neural circuit involved may be distinct for each form of generalization.

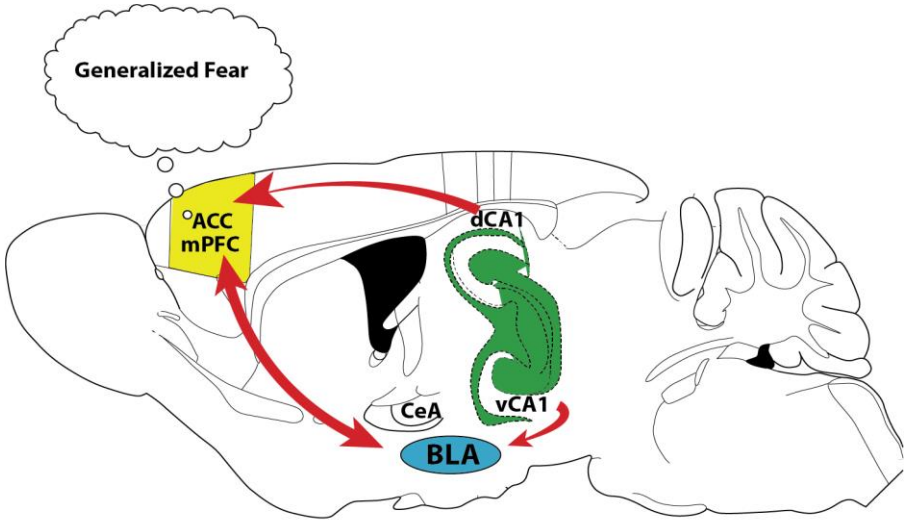
11.7. Prefrontal Cortex Involvement

The dorsal hippocampus is not the only region implicated in time-dependent generalization nor was it the only region in which estradiol appears to exert an effect to influence generalization. The PFC has long been connected to memory precision (Bontempi et al., 1999; Cullen et al., 2015; Frankland et al., 2004; Maviel et al., 2004), although the main theory behind PFC involvement in memory precision requires the passage of time and, as mentioned above, estradiol-induced generalization may utilize a different neural circuit than the circuit involved in time-dependent generalization. Here, estradiol infused directly into the ACC induced generalized responding to the neutral context within 24 hours (EX7F). One of the only other studies that shows such an early involvement of the cortex used pre-training inactivation of the ACC and found impaired memory precision (Xu & Südhof, 2013). Taken together with the hippocampal data, the present results suggest the need for a new model of circuitry. Time-dependent generalization may be a result of communication from the dorsal hippocampus to the ventral hippocampus, which interacts bi-directionally with the ACC to maintain memory precision (Fig 16A) (Cullen et al., 2015). Estradiol-dependent generalization in females appears to result from enhanced glutamatergic signaling within the hippocampus and possibly between the dorsal hippocampus and the ACC (Fig 16B). Future experiments will need to continue to parse out the details of each circuit to provide a better understanding of the unique aspects of estradiol-induced generalization compared to that of time-dependent generalization.

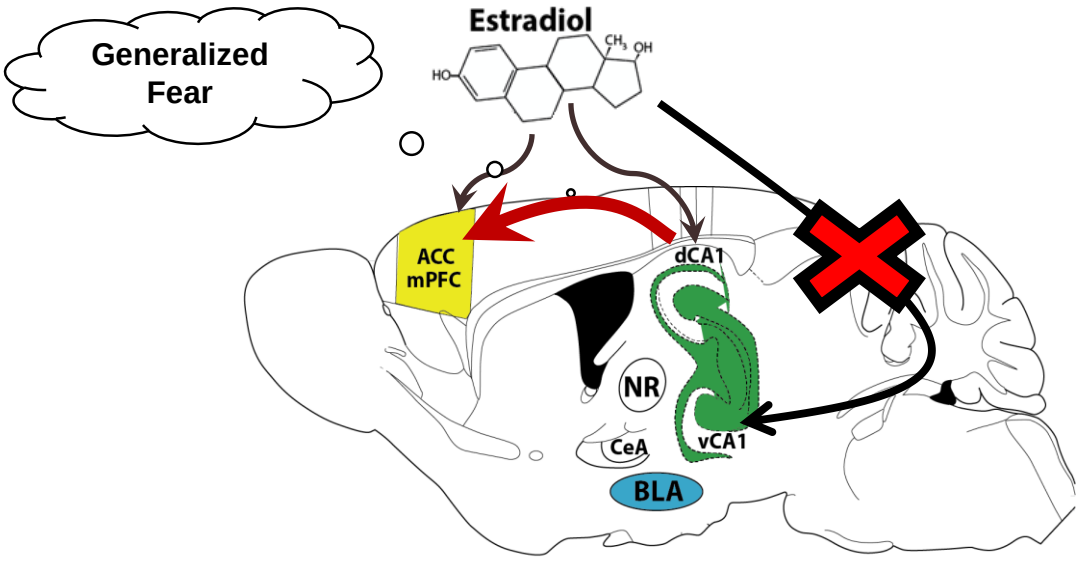
Figure 16. **Models of Fear Generalization. A)** Diagram of time-dependent generalization circuitry. The dorsal CA1 may support the precision of remote memory recall, preventing generalized responding at long intervals whereas the ACC is involved in generalized fear responding at remote time points. The role of the ACC may mediate or be mediated by the involvement of the ventral CA1, resulting in generalized responding. **B)** Unlike time-dependent generalization, estradiol acts within 24 hours to affect memory retrieval and acts within the dorsal CA1 and ACC, but not the ventral CA1, to induce generalization. Additionally, estradiol appears to be acting through activation of cytosolic ERβ to enhance glutamatergic transmission to induce generalization.

Figure 16. Models of Fear Generalization

A **Time Dependent Generalization**



B **Estradiol-Induced Generalization**



11.8. GABA

In addition to glutamatergic signaling, estradiol may be altering GABAergic transmission. GABA is the main inhibitory neurotransmitter in the nervous system (Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002) and has two main receptor subtypes: $GABA_A$, an ionotropic receptor, and $GABA_B$, a metabotropic receptor (Bettler, Kaupmann, Mosbacher, & Gassmann, 2004; Bowery & Enna, 2000). Estrogens can have direct effects on GABAergic neurons as ERs are often co-expressed within these neurons. Specifically, ERβ-expressing neurons within the cortex also express parvalbumin (PV), a marker for GABAergic transmission (Blurton-Jones & Tuszynski, 2002). GABAergic neurotransmission plays a large role within the hippocampus, and accounts for over 90% of terminal connections on pyramidal neurons within the CA1 (Mugnaini & Oertel, 1985). In fact, control of inhibitory tone is considered a potential mechanism by which estrogens modulate memory (Blurton-Jones & Tuszynski, 2002).

Females treated with an estradiol benzoate pellet for 5 weeks display reduced expression of $GABA_{B1}$ and $GABA_{B2}$ mRNA and protein levels in the pituitary and hypothalamus (Rey-Roldán et al., 2006). The effects of estrogens on $GABA_B$ expression and activation is of importance because evidence suggests a role of $GABA_{B(1a)}$ receptors in the precision of memory. Within the $GABA_{B1}$ family, receptors can be classified as $GABA_{B(1a)}$ or $GABA_{B(1b)}$ based on the presence of two sushi domains within the N terminus (Bettler et al., 2004; Gassmann & Bettler, 2012). $GABA_{B(1b)}$ is predominately post-synaptic whereas the $GABA_{B(1a)}$ subunit is predominately pre-synaptic on glutamatergic terminals (Jacobson, Kelly, Bettler, Kaupmann, & Cryan, 2007), contributing to presynaptic inhibition of glutamate release. Global $GABA_{B(1a)}$ knockout

mice display higher freezing responses to a CS never paired with shock (CS-) and generalized responding to a neutral context 24 hours following context fear conditioning (Cullen, Dulka, Ortiz, Riccio, & Jasnow, 2014; Shaban et al., 2006). Overall, these results suggest a role of presynaptic inhibition in the preservation of memory precision and lacking these receptors or inhibition of these receptors results in generalized responding. Given the effects of estradiol on $GABA_{B(1)}$ expression (Rey-Roldán et al., 2006), estrogens may interact with these receptors within areas of interest, such as the hippocampus and PFC, to affect fear generalization. In fact, the increased glutamatergic signaling hypothesized to induce generalization (EX9) may actually be a result of decreased inhibition within the hippocampus, resulting in increased excitatory transmission compared to baseline. Through this mechanism, estradiol may not affect changes in spine density by acting directly on excitatory pyramidal neurons. Instead, estrogens may bind to interneurons, reducing GABA synthesis, which reduces overall inhibition within the hippocampus. The reduction in inhibitory signaling results in increased excitation, which results in increased spine density (McEwen, 2002; Murphy, Cole, Greenberger, & Segal, 1998). Whether or not estradiol acts to enhance excitatory signaling above basal levels or if excitatory transmission is increased through reduction in inhibitory signaling remains unknown. Additionally, the effects of estrogens on spine density appears to be modulated mainly by ERα located within interneurons (McEwen, 2002; Murphy et al., 1998), making this an unlikely mechanism to explain estradiol-induced generalization found in the current experiments as that effect is driven by activation of ERβ, not ERα (EX5A). Studies are needed to determine the effects of

estradiol treatment on GABAergic receptor activity, and the effects of reduced GABA expression on fear generalization.

Alternatively, GABAergic transmission has been linked to generalization via another mechanism known as feedforward inhibition. In feed-forward inhibition, afferent excitatory connections activate inhibitory neurons, which, in turn, reduce the probability of firing of principle cells (Freund & Buzsáki, 1996). After context fear training, filopodial contacts on GABAergic interneurons in the CA3 decrease, suggesting that increased feedforward inhibitory connectivity by large mossy fiber terminals in the CA3 is critical for memory precision or preventing generalization (Ruediger et al., 2011). The CA3 is also a site of estrogenic modulation. Immunoreactivity and *in situ* hybridization studies show the presence of the two major estrogen receptor subtypes—ERα and ERβ—in the CA3 region of the hippocampus (Azcoitia, Sierra, & Miguel Garcia-Segura, 1999; Milner et al., 2005; Shughrue & Merchenthaler, 2000b). Specifically, CA3 neurons express the greatest abundance of membrane-bound and cytosolic ERβ (Mitra et al., 2003; Shughrue et al., 1997; Shughrue & Merchenthaler, 2000b; J. Q. Zhang et al., 2002). Following estradiol treatment, ERα, ERβ, and synaptophysin levels are higher in CA3 compared to CA1 (Rune et al., 2002). Given the more dramatic impact of estradiol on the CA3, the changes within the CA1 mediated by estradiol may be a result of activation of ERs within the CA3 increasing Schaffer collateral synapses into the CA1 (Rune et al., 2002). In addition, estrogens may modulate the connections made between the CA3 and CA1 region of the hippocampus by increasing the ability of CA3 neurons to synchronize with CA1 targets (Woolley, Wenzel, & Schwartzkroin, 1998; Yankova, Hart, & Woolley, 2001). The activity of estrogens in the CA3 may contribute to alterations in fear

generalization perhaps through memory updating, which is a process that allows newly encountered information to be incorporated into a previously learned memory trace. For example, estradiol in hippocampal slices reduces the frequency threshold for Long-term depression (LTD) induction at CA3-CA1 synapses, which could allow for removal of older memories to be replaced by newer, updated memories (Zamani, Desmond, & Levy, 2000). The decrease in frequency threshold for LTD could result in updating memories from precise memories to imprecise memories, resulting in fear generalization. Overall, estrogens may increase feedforward inhibition and simultaneously decrease expression of $GABA_B$ receptors—two factors that are associated with fear generalization. As a result, treatment with estradiol could be increasing fear generalization through the modulation of GABAergic transmission.

11.9. Pattern Completion

One proposed model for fear generalization is the idea of pattern completion. In pattern completion, hippocampal cells, specifically within the CA3 region can undergo an all-or-none process of remapping (Colgin, Moser, & Moser, 2008; J. K. Leutgeb, Leutgeb, Moser, & Moser, 2007; S. Leutgeb et al., 2005). Remapping refers to changes in the firing rate and response field of particular neurons, called place cells, that are activated upon exploration of a novel environment (Colgin et al., 2008). The CA3 receives inputs from several different brain regions and integrates the information to form an episodic memory of an event. When placed into the environment in which the learning occurred, the CA3 is activated by a portion of the original elements present at the time of retrieval. If placed in a distinct, novel environment, the CA3 may be activated by a subset of original elements present in that distinct environment and the

learned response occurs as a result of pattern completion (Colgin et al., 2008; S. Leutgeb, Leutgeb, Treves, Moser, & Moser, 2004). Alternatively, subjects can utilize a pattern separation strategy. Pattern separation is the process by which subjects form distinct, contextual representations of new information, and the process is mediated by hippocampal circuitry. For example, if animals are exposed to two contexts and only one is reinforced, animals will separate out the details of the contexts and only respond fearfully to the conditioned context. Another example of pattern separation is object location procedures where animals learn the location of two objects over several trials and, at test, one object is moved to a new location. Estrogens can affect behaviors like object location (Fan, Zhao, Orr, Chambers, Lewis, and Frick, 2010; Fernandez, Lewis, Pechenino, Harburger, Orr, Gresack, Schafe, and Frick, 2008; Gresack and Frick, 2006; Lewis, Kerr, Orr, and Frick, 2008; Zhao, Fan, and Frick, 2010), suggesting that sex hormone are involved in modulation the process of pattern separation and does so possibly through enhancing the processing of information within the hippocampus.

The process of pattern completion is very similar to the effects seen in fear generalization—a similar, but distinct, context is able to elicit a fearful response. In the fear generalization procedure used to assess sex differences, the apparatus the animal is tested in for memory recall remains constant regardless of the context of testing; only the context in which the apparatus is located is altered. Therefore, estradiol treatment, through increases in synaptic transmission within the fibers from the CA3 to the CA1 (Rune et al., 2002; Woolley et al., 1998; Yankova et al., 2001), may enhance the retrieval function of CA3 so fewer contextual elements of the original fear memory would be required in order for the whole memory to be retrieved (Kim et al., 2006). Thus,

estrogens may be facilitating fear generalization by enhancing the process of pattern completion through modulation of the CA3.

Pattern completion via remapping is related to NMDAR functioning and inputs from the DG into the CA3. For instance, knockout mice lacking NR1 subunits within the DG or the CA3 do not display remapping (McHugh et al., 2007; Nakazawa et al., 2002). Additionally, when tested for the ability to discriminate between distinct contexts, NR1 knockout mice are unable to do so (McHugh et al., 2007). Previous research has seen an increase in glutamate transmission through NMDA receptors following estradiol treatment. However, these studies focused on the CA1 and little is known about the effects of estradiol on NMDA function within the DG (Weiland, 1992; Woolley et al., 1997), although estradiol does decrease density of noncompetitive NMDA antagonist sites (Weiland, 1992). Although speculative, these data suggest that estrogens may be acting within the DG to change NMDA receptor-dependent synaptic transmission, resulting in modified connections to the CA3, altering the process of hippocampal remapping, and thereby affecting rates of fear generalization.

The mPFC is also involved in remapping. Evidence suggests that the mPFC is not involved in the encoding of a spatial memory, but is involved in the retrieval and pattern completion (Jo et al., 2007). When animals are trained in a Morris water maze (MWM) spatial task and tested under conditions in which the majority of extra-maze cues are removed, lesions to the mPFC reduces performance. These results suggest that when animals are placed within an environment that does not contain enough cues to engage the hippocampus fully, the mPFC becomes engaged and helps to recall a less 'precise' memory. When tested in the MWM, the number of extramaze cues available does not

affect performance as long as the mPFC is intact (Jo et al., 2007). The involvement of the mPFC in pattern completion is also NMDA receptor-dependent; blocking these receptors within the mPFC via APV infusions disrupts performance in a partial cue memory retrieval trial (Jo & Choi, 2014). In relation to the fear generalization procedure, these results suggest that when animals are placed in a neutral context, which lacks the same 'extramaze cues' present during training, the mPFC is engaged and the fear memory is recalled, resulting in generalized responding. Therefore, estrogens may increase the ability of the mPFC to complete patterns by increasing NMDAR-dependent synaptic transmission within the mPFC, allowing the few cues present in the neutral context that are the same as those found in the training context to elicit the fear memory, thus increasing fear generalization.

Although estrogen-induced modulation of fear generalization could be a result of pattern completion, the idea may be unlikely given the behavior of intact control animals. If pattern completion was involved, one would predict that control animals presented with distinct context would elicit CA3 responding and those animals would then be able to use the cues that are similar between the contexts to complete the pattern, which would be displayed behaviorally through a fear response in the distinct context (i.e. generalization). However, control animals when tested 1 day after passive avoidance training, do not complete the pattern in the neutral context; animals do not display fear generalization at the short retention interval. Therefore, it may be unlikely that pattern completion is the underlying mechanism of estrogen-induced fear generalization. Additionally, the enhancement of pattern separation by estradiol cannot explain estradiol-induced generalization. First, pattern separation would predict animals are able to

distinguish between two distinct contexts better, resulting in reduced generalization. Second, the effects of estradiol on pattern separation tasks are due to estradiol enhancing the consolidation of the task, whereas estradiol-induced generalization is due to effects on memory retrieval. Overall, the current findings suggest that pattern completion or pattern separation strategies are unlikely explanations for estradiol-induced generalization

11.10. Conclusions

To date, theories on the process of fear generalization all share the idea that the passage of time is required in order for generalized responding to occur (Biedenkapp and Rudy, 2007; Jasnow et al., 2012; Lynch III et al., 2013; Matynia, Anagnostaras, Wiltgen, Lacuesta, Fanselow, and Silva, 2008; Wiltgen and Silva, 2007; Winocur et al., 2007). The current data suggest that in some cases, generalized responding does not require a significant passage of time (i.e., several days) and can be dependent upon memory retrieval mechanisms rather than alterations to consolidation as traditionally thought. Taken together, these experiments lead to a better understanding of the primary mechanisms through which estrogens enhance fear generalization.

Understanding exactly how estrogens interact within the brain to affect emotional behavior and memory is important to determining the efficacy of using hormonal therapies and other treatments in women. More importantly, understanding how estrogens interact to affect emotional behavior can provide useful information on the discrepancies in prevalence rates for anxiety disorders seen between males and females (Kessler et al., 1994; Valentino, Reyes, Van Bockstaele, & Bangasser, 2012; Wang et al., 2005). Estrogens do induce fear generalization—a characteristic of anxiety disorders—in females, but not males, and this finding may help explain why females are more

susceptible to anxiety disorders. Research now needs to focus on determining how estrogens are enhancing fear generalization. As pointed out in a review by Frick and colleagues (2010), using only global sex steroid treatments may limit the scope of findings assessing sex differences in learning and memory; these techniques remain unrefined and could elicit non-specific effects. Therefore, future studies of fear generalization and other sex steroid-mediated behaviors should attempt to discover more detailed mechanisms of action by manipulating downstream effects of estradiol and other sex steroids through protein blocking, genetic manipulations, and epigenetic regulations (Frick, Fernandez, & Harburger, 2010).

In general, estradiol-induced generalization in females appears to function through a distinct neural circuit when compared to time-dependent generalization. But, the current findings only begin to explain how estradiol acts within females to induce generalization. Here, the findings establish that estradiol acts in a sex-specific way to affect the generalization of fear to a neutral context. One possible explanation for the effects of estradiol is sex-specific hormonal organization during development (Arnold & Breedlove, 1985; McCarthy, 2006; McEwen, 1992; Schwarz & McCarthy, 2008). The alterations in neuronal circuitry may allow estradiol to act within the same brain areas and yet have opposing effects on generalization. One hypothesis is that altering sexual differentiation through hormonal modulation during gestation could change the way estradiol acts in adulthood to activate the circuitry involved in generalization. Specifically, feminizing males through blocking aromatization during the neonatal period will result in the induction of generalization in adulthood in the presence of exogenous

estradiol and vice versa if the effects seen in the currents experiments are a result of organizational hormone effects.

Alternatively, the circuit that estradiol acts within to alter generalization may be distinct across sex. Estradiol acts within the dorsal hippocampus and ACC to induce generalization, but the details of the circuit remain incomplete. Given the connections within the subregions of the hippocampus and the role of glutamatergic signaling in estradiol-induced generalization, one hypothesis for the complete circuit underlying estradiol-induced generalization is connections from the CA3 project to the CA1 where estradiol acts to increase glutamatergic transmission through upregulation of NMDA/AMPA receptor expression. Connections from the dorsal CA1 to the ACC would follow a similar pattern. The increased excitatory transmission within these regions is hypothesized to affect generalization by altering the ability of the animal to inhibit responding. During conditioning, animals learn to associate an aversive footshock with the black compartment of the shuttlebox. When tested in a neutral context, animals should be able to inhibit the learned fear response upon recognizing the context they are in is not associated with the original learning experience. When animals are tested following estradiol treatment, females do not inhibit the fear response while in the neutral context. This hypothesis also explains the enhanced fear to discrete neutral cues following estradiol exposure found in other studies (Nofrey et al., 2008; Toufexis et al., 2007).

Given this hypothesis, the sex-dependent effects of estradiol on generalization may be due to estradiol acting within a different circuit in males compared to females. Although the circuit involved in male subjects has yet to be analyzed, one possible

candidate for a sex-specific brain structure discussed above is the BNST, a sexually dimorphic structure involved in stress and long-term learning that has sex-specific expression of aromatase. If true, experiments assessing the effects of estradiol directly within the BNST will help to elucidate the circuit involved in estradiol-*reduced* generalization in males.

The current experiments establish a novel form of generalization that does not require the passage of time. These findings are not the only data suggesting a distinct time-*independent* mechanism of generalization. Recent data indicates that presynaptic inhibition through $GABA_{B(1a)}$ receptors is important for preventing fear generalization—when absent, as in knockout animals, or blocked pharmacologically, animals display generalization to a neutral context within 24 hours (Cullen et al., 2014; Lynch et al., in prep). Additionally, presynaptic inhibition is required within the dorsal hippocampus, similar to what is seen with estradiol-induced generalization. Finally, the loss of presynaptic inhibition through blocking of $GABA_{B(1a)}$ receptors has a net result of increased glutamatergic signaling, indicating another common element between these two forms of generalization. Current theories for time-dependent generalization suggest that memories are modified, or transformed, as they age and details about contextual information is lost during the transformation, allowing non-specific cues to elicit a fear response over time. However, estradiol-induced generalization and generalization due to the loss of presynaptic inhibition, do not require the passage of time, suggesting that these two mechanisms of generalization act in a distinct manner compared to time-dependent generalization. Indeed, it seems unlikely that estradiol is inducing generalization by speeding up the transfer of the memory trace from hippocampal sites to cortical sites,

resulting in a faster transformation of the memory, which means these two forms of generalization require a new theory to explain how generalized responding occurs in such a short amount of time. As mentioned before, it seems more likely that both forms of time-*independent* generalization occur through alterations in inhibitory control.

Although the finding of a discrepancy in prevalence rates for disorders that are marked by the inability to inhibit fearful responding to neutral stimuli is well established, studies assessing potential mechanisms to explain this phenomenon are scarce. The present experiments establish a role for estradiol in the process of fear generalization in a sex-dependent manner. Indeed, the current findings are the first steps in determining the neural mechanisms underlying sex differences in the generalization of fear. But, these findings only begin to explain how estradiol acts within females to induce generalization. Future work will need to continue to assess the connectivity and directionality of the circuit as well as determine how estradiol acts within the circuit to induce generalization. Overall, the current findings provide a strong foundation on which future experiments can build upon to better understand how fear generalization occurs in a sex-specific manner. Further understanding of these mechanisms is necessary in order to gain a better understanding of how sex differences in generalized responding is related to the develop of anxiety disorders, and is crucial for developing more effective, potentially sex-specific, treatments for these disorders.

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