Brief Communication

Sex differences in the generalization of fear as a function of retention intervals

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In previous studies using male rodents, context change disrupted a fear response at a short, but not a long, retention interval. Here, we examined the effects of context changes on fear responses as a function of time in male and female rats. Males displayed context discrimination at all intervals, whereas females exhibited generalization by 5 d. Ovariectomized females with no hormone replacement displayed context discrimination at 5 d, whereas those receiving 17β-estradiol generalized their fear response to a neutral context. These results demonstrate that fear generalization for contextual cues occurs faster in female rats and is mediated, in part, by estrogens.

Anxiety disorders are the most prevalent mental disorder in the US (Kessler et al. 2005). More specifically, females are 60% more likely than males to be diagnosed with an anxiety disorder (Kessler et al. 1994; Wang et al. 2005) and the exact cause of this sex difference is unknown. For example, post-traumatic stress disorder (PTSD) is one specific anxiety disorder with a higher prevalence rate in females (Kessler et al. 1995; Olff et al. 2007). PTSD is marked by the persistence of fear and anxiety and the tendency to generalize fear to neutral cues and contexts (Grillon and Morgan 1999; Brewin 2001). These symptoms are possibly due to deficits in discriminatory fear learning (Grillon and Morgan 1999; Jovanovic et al. 2009, 2010) or the result of hyperarousal associated with anxiety disorders (Archer 1974; Bartolini et al. 1987; Armstrong and Hille 1998).

Studies of fear generalization with animals have demonstrated that a learned fear response is context-dependent; fear responses are significantly attenuated when testing occurs at a short interval (e.g., 1 d after training) in a neutral context (i.e., the context shift effect). However, when rodents are tested at long delays after training (e.g., 14 d after training), the context shift effect is eliminated; rodents freeze equivalently to the training and the neutral context (Riccio et al. 1984; Zhou and Riccio 1996; Biedenkapp and Rudy 2007; Wiltgen and Silva 2007). Over time, rodents generalize their fear response to neutral contexts. For example, Zhou and Riccio (1996) trained male rats in passive avoidance and tested 1 or 14 d after training in the same context as training or a neutral context. At 1 d, rats tested in the neutral context display little or no avoidance, whereas those tested in the same context demonstrate high levels of avoidance (i.e., the context shift effect). However, 14 d after training, the groups tested in a neutral context had performance comparable to the group in the same (training) context (Zhou and Riccio 1996). This effect has been replicated by several labs (for review, see Jasnow et al. 2012).

In recent years, several hypotheses have been developed to explain the phenomenon of fear generalization in animal models. Hippocampal-dependent memory, such as memory for context, undergoes a transfer from short-term hippocampal stores to more long-term cortical stores, a process known as systems consolidation (Kim and Fanselow 1992; Anagnostaras et al. 2001; Wiltgen et al. 2006; Jasnow et al. 2012). When the memory is transferred, the original memory trace is transformed from a context-specific hippocampal-dependent memory to a hippocampal-independent cortical memory that lacks context specificity (Winocur et al. 2009). Brain scans in humans during memory retrieval show differential activation of the hippocampus and prefrontal cortex (PFC) for newly acquired memories versus older memories. New memories have high hippocampal activation and low activation of the PFC, whereas older memories have low hippocampal activation and high PFC activation (Bontempi et al. 1999; Frankland et al. 2004; Maviel et al. 2004). The differences in activation are mirrored in studies assessing the effects of lesions of the hippocampus; lesions directly after training eliminate the context shift effect at short intervals. However, when the hippocampal lesions are delayed, performance is not affected in either context, suggesting the hippocampus is only required when the memory is still context-dependent or precise (Winocur et al. 2007, 2009; Wiltgen et al. 2010). Therefore, the hippocampus may be involved in processing a memory as long as that memory remains context-dependent. However, no evidence to date has examined whether this transformation hypothesis differs between males and females.

Differences in fear and anxiety between males and females have been well established in both humans and nonhuman animals (Archer 1975; Stewart et al. 1997; Frye et al. 2000; Zorawski et al. 2005; Stark et al. 2006). In addition, a wealth of evidence demonstrates that estrogens play a major role in sex differences in behavior and that estrogens act directly on the hippocampus to affect synaptic plasticity and learning and memory (Beatty and Beatty 1970; Frye et al. 2000; Li et al. 2004; Baufreton et al. 2005; González-Burgos et al. 2005; Bekker and van Mens-Verhulst 2007; Biedenkapp and Rudy 2007; Liu et al. 2008; Arias et al. 2009). Thus, estrogens may mediate differences in memory generalization between males and females, although this has not yet been examined. A serendipitous finding in our lab found that male rats exhibited a context shift effect 5 d after training, whereas females exhibited high levels of avoidance in either context (PK Cullen, L Pickens, SF Fountain, DC Riccio, in prep.). The present study investigated sex differences in fear generalization more systematically. In each experiment, animals were trained in passive

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avoidance and then tested at different retention intervals in the same context as training or a neutral context. First, we established a temporal gradient of fear generalization for male and female rats across different retention intervals. Second, we examined the role of estrogens on the generalization of fear by testing ovariectomized (OVX) female rats given Silastic capsule implants containing 17β-estradiol.

Analyses of latency-to-cross scores at training revealed no significant differences ($P > 0.09$). At 1 d (Fig. 1A), the main effect of context was significant ($F_{(1,42)} = 50.309$, $P < 0.001$), but the main effect of sex ($F_{(1,42)} = 0.556$, ns) and the interaction between context and sex was not significant ($F_{(1,42)} = 0.005$, ns). Although the interaction term was not significant, we were interested in differences between males tested in the same or neutral context, females tested in the same or neutral context, and males and females tested in the neutral context. Therefore, independent *t*-tests were conducted for those direct comparisons at all retention intervals. Latency-to-cross scores for Male-Same vs. Male-Neutral ($t_{(20)} = 5.432$, $P < 0.001$) and Female-Same vs. Female-Neutral were significantly different ($t_{(22)} = 4.765$, $P < 0.001$). In addition, Male-Neutral and Female-Neutral did not differ ($t_{(21)} = 0.569$, ns). Thus, a change in context attenuated avoidance, regardless of sex, at 1 d.

At 3 d (Fig. 1B), the main effect of context was significant ($F_{(1,41)} = 31.140$, $P < 0.001$), the main effect of sex was nonsignificant ($F_{(1,41)} = 0.174$, ns), and the interaction between context and sex was nonsignificant ($F_{(1,41)} = 3.066$, ns). Male-Same vs. Male-Neutral were significantly different ($t_{(19)} = 5.974$, $P < 0.001$) as were Female-Same vs. Female-Neutral ($t_{(22)} = 2.504$, $P < 0.05$). In addition, Male-Neutral vs. Female-Neutral were not significantly different ($t_{(21)} = 1.262$, ns). These results demonstrate that males and females continued to display the context shift effect at the 3-d retention interval.

At 5 d (Fig. 1C), the main effects for context ($F_{(1,37)} = 26.550$, $P < 0.001$), sex ($F_{(1,37)} = 15.504$, $P < 0.001$), and the interaction between context and sex ($F_{(1,37)} = 4.839$, $P < 0.05$) were significantly different. Male-Same vs. Male-Neutral ($t_{(17)} = 4.38$, $P < 0.001$), Female-Same vs. Female-Neutral ($t_{(20)} = 2.528$, $P < 0.05$), Male-Neutral vs. Female-Neutral were significantly different ($t_{(16)} = 3.837$, $P < 0.001$). These results demonstrate that females did not display complete generalization to the neutral context at 5 d. However, females did display higher latencies compared to males in the neutral context, suggesting that females begin to generalize fear responses to the neutral context at an earlier time point compared to males.

At 7 d (Fig. 1D), the main effects for context ($F_{(1,42)} = 13.075$, $P < 0.001$) and sex ($F_{(1,42)} = 9.26$, $P < 0.01$) were significant. However, the interaction between context and sex was nonsignificant ($F_{(1,42)} = 2.603$, ns). Male-Same vs. Male-Neutral were significantly different ($t_{(20)} = 3.504$, $P < 0.01$). However, Female-Same vs. Female-Neutral were not significantly different ($t_{(22)} = 0.149$, ns). In addition, Male-Neutral vs. Female-Neutral were significantly different ($t_{(21)} = 3.416$, $P < 0.01$). Therefore, these data suggest that females generalized fear responses to the neutral context at an earlier time point than males.

Overall, these data suggest that, although females can perceive the contextual differences at short retention intervals, they tend to generalize fear to neutral contextual cues at a shorter retention interval than males. Females had significantly higher latencies in the neutral context compared to males at the 5 and 7 d retention intervals, displaying complete fear generalization by the 7-d interval.

To determine if differences in fear generalization were mediated by estrogens, adult female rats were ovariectomized (OVX) and half were given a Silastic capsule containing 17β-estradiol and the other half received an empty capsule and tested for fear generalization at 1, 5, or 7 d. Analyses of latency-to-cross scores at training revealed no significant differences ($P > 0.29$). At 1 d (Fig. 2A), the main effect of context was significant ($F_{(1,39)} = 11.08$, $P < 0.01$), but not the main effect of hormone ($F_{(1,39)} = 0.562$, ns). The interaction between context and hormone was also nonsignificant ($F_{(1,39)} = 0.049$, ns). OVX-C Same vs. OVX-C Neutral ($t_{(17)} = 2.105$, $P < 0.05$) and OVX-ES Same vs. OVX-ES Neutral ($t_{(22)} = 2.647$, $P < 0.05$) were significantly different and OVX-C Neutral vs. OVX-ES Neutral were not significantly different ($t_{(19)} = 0.598$, ns). Thus, 1 d after training, ovariectomized females displayed a significant context shift effect, exhibiting less fear in the neutral context, regardless of estrogen treatment.

At 5 d (Fig. 2B), the main effect of context was significant ($F_{(1,43)} = 9.039$, $P < 0.01$). The main effect for hormone ($F_{(1,43)} = 0.305$, ns) and the interaction between context and hormone ($F_{(1,43)} = 0.969$, ns) were not significant. OVX-C Same vs. OVX-C Neutral were significantly different ($t_{(22)} = 3.059$, $P < 0.01$). However, OVX-ES Same vs. OVX-ES Neutral were not ($t_{(21)} = 1.327$, ns). The latency scores for OVX-C Neutral vs. OVX-ES Neutral were nonsignificant ($t_{(22)} = 0.950$, ns). Therefore, the generalization

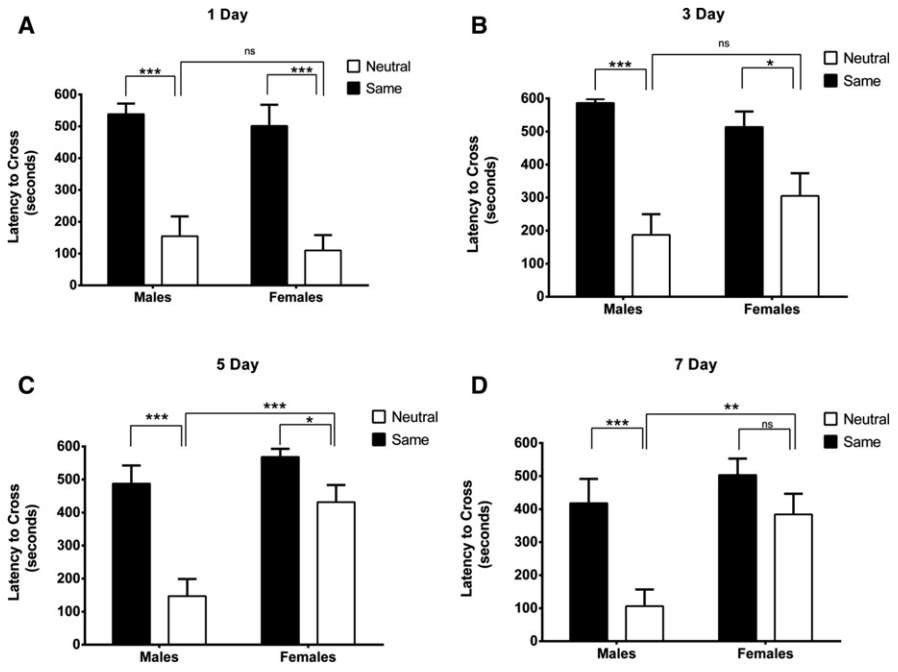

Figure 1. Mean (± SEM) latency-to-cross in seconds. (*A*) Both sexes demonstrate significant context discrimination at 1 d. (*B*) Both sexes demonstrate significant context discrimination at 3 d. (*C*) By 5 d, males and females continue to display context discrimination, although females tested in the neutral context have significantly higher fear than males. (*D*) Females continue to show higher fear compared to males tested in the neutral context. In addition, females no longer show significant context discrimination between the same and neutral context. Significant differences were ascribed at (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

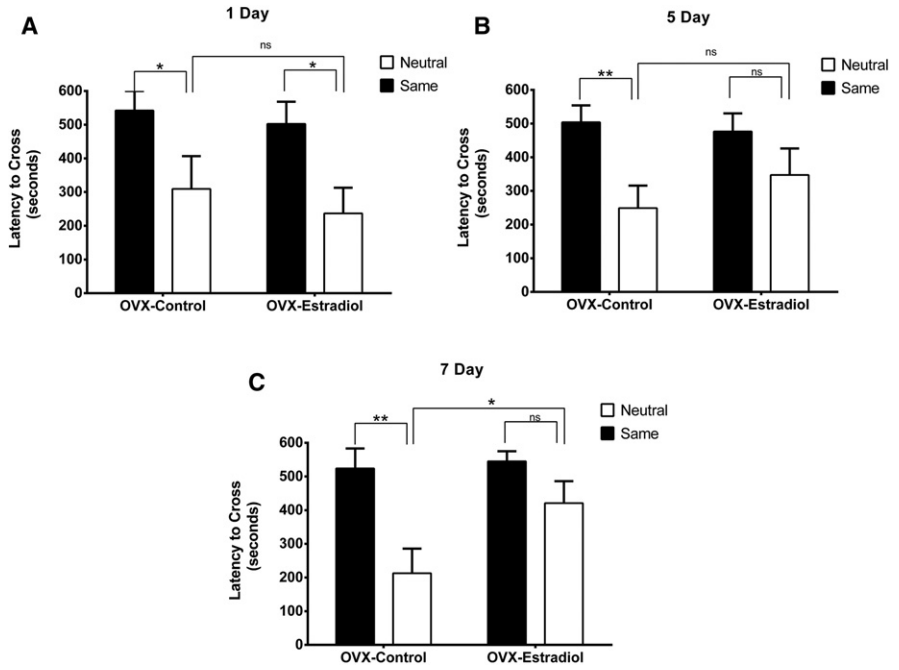

Figure 2. Mean (±SEM) latency-to-cross in seconds. (*A*) Both groups display significant context discrimination at 1 d, regardless of estradiol treatment. (*B*) OVX-C females display significant context discrimination whereas OVX-ES females do not. However, no significant difference between the groups tested in the neutral context at 5 d is seen. (*C*) Only females receiving no estradiol display context discrimination at 7 d. Those females receiving 17β-estradiol replacement demonstrated context generalization and higher fear in the neutral context compared to OVX-C females. Significant differences were ascribed at (*) $P < 0.05$, (**) $P < 0.01$.

observed in OVX-ES at the 5-d retention interval was not significantly greater than that for OVX-C animals.

At 7 d, the main effect for context was significant ($F_{(1,36)} = 13.627$, $P < 0.001$). The main effect for hormone treatment was nonsignificant by the conventional standards of $P < 0.05$, although it approached significance ($F_{(1,36)} = 3.779$, $P = 0.060$). The interaction between context and hormone replacement was also nonsignificant ($F_{(1,36)} = 2.508$, ns). OVX-C Same vs. OVX-C Neutral were significantly different ($t_{(18)} = 3.319$, $P < 0.01$). In contrast, OVX-ES Same vs. OVX-ES Neutral were not significantly different ($t_{(18)} = 1.736$, ns). Comparison of OVX-C Neutral vs. OVX-ES Neutral was significantly different ($t_{(18)} = 2.128$, $P < 0.05$). Thus, ovariectomized females given 17β-estradiol demonstrated significant fear generalization as evidenced by similar latency-to-cross scores in either context and also show significantly longer latencies in the neutral context compared to females given no estrogen following ovariectomy.

Taken together, these data suggest that fear generalization in female rodents is regulated, in part, by estrogens. Alternative explanations for the sex differences in fear generalization, such as differences in exploratory behavior (Archer 1974), differential rates of acquisition in passive avoidance (Denti and Epstein 1972), and shock sensitivity (Snowdon et al. 1964; Beatty and Beatty 1970; Blizard 1971), can be ruled out due to intact males and females displaying equivalent behavioral responses at 1 d, as indicated by the context shift effect. Similarly, the context shift effect exhibited by both OVX-C and OVX-ES at 1 d suggests that neither surgery nor capsule implantation had a negative impact on passive avoidance acquisition or the ability to discriminate contextual cues at a short interval. One potential issue with our findings is the use of a 10-min time limit at test, which may have generated an arbitrary ceiling effect. However, such cut-off times are a standard in the field and we do not believe that the results of the present study are due to an arbitrary ceiling effect.

To the best of our knowledge, our findings are the first to show sex differences in fear generalization. Several studies demonstrate estrogenic effects on fear and anxiety in both human and nonhuman animals. For example, naturally cycling women with high estrogen levels demonstrate better extinction retention than those with low estrogen levels (Milad et al. 2010). In animals, extinction retention is facilitated by high levels of estrogens (Milad et al. 2009) and this effect may be regulated via estradiol actions on the hippocampus (Chang et al. 2009). Similarly, the hippocampus plays an important role in the generalization of fear (Ruediger et al. 2011). Estrogens may act upon ERα and ERβ in the CA3 region (Azcoitia et al. 1999; Shughrue and Merchenthaler 2000; Milner et al. 2005) to modulate the connections made between the CA3 and CA1 regions by increasing the ability of CA3 neurons to synchronize with CA1 targets (Woolley et al. 1998; Yankova et al. 2001). Indeed, the presence of estrogens results in increased apical dendritic spines within the CA1 (Gould et al. 1990; Woolley et al. 1990, 1998; Woolley and McEwen 1993; McEwen et al. 1995). Thus, estrogens may act directly on hippocampal regions to modulate contextual memory storage, resulting in alteration in the generalization of fear responses to neutral contexts. Taken together, the present data suggest that estrogens may modulate the transfer of contextual memories to neocortical sites from the hippocampus, resulting in fear generalization at earlier time points in females compared to males. These findings potentially underlie the greater incidence of PTSD in females than in males, as the more rapid loss of precision of contextual memory impairs the compartmentalization of fear-eliciting cues ordinarily afforded by different contexts.

Male and female adult Long-Evans rats ~90 d old were used for all experiments ($n = 8$–12). A week prior to beginning the experiment, animals were individually housed and were maintained on a 14/10-hr light/dark cycle. Food and water were available ad libitum throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

For all experiments, the training and testing apparatus were two identical 43.18 × 17.78 × 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 in one of two distinct contexts. Context A was a 1.6 × 2.33-m room with house fluorescent lights and contained bare white walls and no artificial scents or sounds; Context B was a 1.83 × 2.74-m room that was lit by a 25-W red light bulb with posters on the walls. Context B had white noise (70 db) and artificial scent via a Glade Plug-Ins Scented Oil Country Berry air freshener at all times. In each context, the experimenter wore a different glove (rubber dish glove in A; vinyl lab glove in B) to handle the rat.

Prior to training, animals were handled for 5 min on two consecutive days. For training, animals were brought to Context A,

held on the experimenter's hand for 30 sec and placed on the white side of the shuttle box. The door was raised after 20 sec and the initial latency to cross into the black compartment (all four paws) was recorded. Upon crossing, the guillotine door was lowered and two nonescapable 1-sec, 0.6-mA scrambled footshocks were delivered 5 sec apart by a constant current AC shock generator (Model 5806, Lafayette Instruments). Ten seconds after receiving the second footshock, the animal was removed from the chamber and returned to the main colony. Animals with a latency to cross longer than 100 sec during training—<1% of the animals—were removed from the final analysis (e.g., Zarrindast et al. 2002, 2005; Ahmadi et al. 2007a,b; Khakpai et al. 2012).

For testing, rats were brought back into the experimental room at 1, 3, 5, or 7 d after training. Half of the rats were tested in Context A (same) and half in Context B (neutral). The test procedure was identical to training except that the guillotine door remained open for 600 sec and no shocks were delivered. The initial latency to cross was recorded as the dependent measure. After a total of 600 sec had elapsed, the rat was removed and returned to the main colony.

In the second experiment, female rats were anesthetized with isoflurane and received a 5 mg/kg dose of ketoprofen 5 min before bilateral ovariectomy through a dorsal incision. After removal of the ovaries, the incision was sutured using surgical staples and a Silastic capsule containing 17β-estradiol or an empty capsule was inserted behind the shoulder blades of the animal. The Silastic (polydimethylsiloxane) implants were constructed from Silastic tubing (i.d. 0.078 in, o.d. 0.125 in) 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 has been shown to produce levels of ~30–40 pg/mL of estradiol (Bridges 1984; Hiroi and Neumaier 2006). Before implantation, capsules were incubated in saline for 24 h at 37°C. Animals were handled 7 d following surgery and began training 9 d after surgery. Animals were tested 1, 5, or 7 d after training in either the same context or a neutral context. Animals were split into groups based on the retention interval of test and separate two-way ANOVAs were conducted at each retention interval for both experiments. To make direct comparisons, independent *t*-tests were conducted.

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