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The influence of gonadal hormones on conditioned fear extinction in healthy humans

Mohammed R Milad, PhD¹, Mohamed A. Zeidan, BA¹, Angelica Contero, BA¹, Roger K. Pitman, MD¹, Anne Klibanski, MD², Scott L. Rauch, MD^{1,3}, and Jill M. Goldstein, PhD^{1,4}

Geoffrey Schoenbaum

University of Maryland School of Medicine, Department of Anatomy and Neurobiology, 655 W. Baltimore Street, Baltimore MD 21201, USA

¹Department of Psychiatry, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA

²Neuroendocrine Unit, Massachusetts General Hospital, Boston, MA

³McLean Hospital, Belmont, MA

⁴Departments of Psychiatry and Medicine, Harvard Medical School, Brigham and Women's Hospital, and Connors Center for Women's Health and Gender Biology, Boston, MA

Abstract

Recent rodent studies suggest that gonadal hormones influence extinction of conditioned fear. Here we investigated sex differences in, and the influence of estradiol and progesterone on, fear extinction in healthy humans. Men and women underwent a two-day paradigm in which fear conditioning and extinction learning took place on day 1 and extinction recall was tested on day 2. Visual cues were used as the conditioned stimuli and a mild electric shock was used as the unconditioned stimulus. Skin conductance was recorded throughout the experiment and used to measure conditioned responses (CRs). Blood samples were obtained from all women to measure estradiol and progesterone levels. We found that higher estradiol during extinction learning enhanced subsequent extinction recall but had no effects on fear acquisition or extinction learning itself. Sex differences were only observed during acquisition, with men exhibiting significantly higher CRs. After dividing women into low- and high-estradiol groups, men showed comparable extinction recall to high-estradiol women, and both of these groups showed higher extinction recall than low-estradiol women. Therefore, sex differences in extinction memory emerged only after taking into account women's estradiol levels. Lower estradiol may impair extinction consolidation in women. These findings could have practical applications in the treatment of anxiety disorders through cognitive and behavioral therapies.

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Corresponding Author: Mohammed R. Milad, PhD Department of Psychiatry Harvard Medical School & Massachusetts General Hospital 149 13th St, CNY 2614 Charlestown, MA 02129 milad@nmr.mgh.harvard.edu Phone: 617-724-8533 Fax: 617-726-4078.

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Keywords

estrogen; progesterone; sex difference; fear; menstrual cycle; learning and memory

Failure to extinguish conditioned fear may play an important role in the pathogenesis of anxiety disorders (Davis et al., 2006; Milad et al., 2008; Milad and Rauch, 2007; Milad et al., 2006b; Orr et al., 2000; Rauch et al., 2006). Extinction forms the theoretical basis of treatment by exposure (Rothbaum and Davis, 2003). A substantial number of studies conducted in rodents and more recently in humans indicate that brain regions associated with the acquisition and consolidation of conditioned fear extinction are implicated in anxiety disorders. These areas include the amygdala, ventromedial prefrontal cortex (vmPFC), and hippocampus (for review, see Quirk and Mueller, 2008). These brain regions are sexually dimorphic and contain a high density of gonadal hormone receptors (Giedd et al., 1996; Goldstein et al., 2001; Ostlund et al., 2003). This may explain why measures of fear and arousal are associated with changes in hormonal levels over the menstrual cycle (Cahill, 2003; Goldstein et al., 2005; Gupta et al., 2001; Jasnow et al., 2005) and sex differences in arousal (Goldstein et al., 2010). Such findings also hold promise for clarifying important sex differences in anxiety disorders (Kinrys and Wygant, 2005; Pigott, 2003).

We recently showed that female rats undergoing extinction learning during the proestrous phase of the estrus cycle (when estradiol and progesterone levels are elevated) exhibited better extinction memory during subsequent extinction recall (i.e., retention) testing (Milad et al., 2009a). Moreover, exogenously administered estradiol and progesterone facilitated extinction recall, whereas estradiol and progesterone receptor antagonists impaired it (Milad et al., 2009a). A recent study found that estradiol administration into the hippocampus in female rats facilitated fear extinction (Chang et al., 2009). Collectively, these data suggest that gonadal hormones influence the consolidation of extinction memory.

In a study of healthy humans, we showed that phase of the menstrual cycle influences consolidation of fear extinction (Milad et al., 2006a), but gonadal hormones were not measured. The present goal was to explore the associations of estradiol and progesterone with physiological aspects of acquisition and extinction of conditioned fear in healthy women at different phases of their menstrual cycles, and to compare their responses to those of healthy men. Estradiol and progesterone levels were measured from blood samples obtained prior to the initiation of a previously described two-day fear conditioning and extinction experiment (Milad et al., 2005; Milad et al., 2007; Rauch et al., 2005). On Day 1, participants underwent fear conditioning in virtual context A and extinction learning in virtual context B. On Day 2, extinction recall (context B) was tested using skin conductance response (SCR) as a measure of fear (Milad et al., 2005; Rauch et al., 2005). Based on our recent findings in female rats and the literature reviewed above, we hypothesized that estradiol and progesterone would facilitate the extinction of conditioned fear and/or the consolidation thereof, such that women with higher levels of these hormones would display lower conditioned responses (CRs) during subsequent extinction recall. Further, these hormonal differences would, in part, account for sex differences in conditioned fear extinction.

Methods and Materials

Participants

Fifty-four participants (36 women, 18 men) ages 18-30 were recruited from the local community via advertisement. Women were divided by hormonal levels into two groups of 18 (see below). All participants were right-handed, without endocrinologic, neurologic, or other medical conditions, and without Axis I mental disorders, including substance dependence or

abuse, as determined by the Structured Clinical Interview for DSM-IV (First et al., 2002). No participant was using psychoactive or other potentially confounding drugs or medications. All female participants had regular menstrual cycles by history and had not been using oral contraceptives or hormone replacement for at least three months. After a complete description of the procedures, written informed consent was obtained from all participants in accordance with the requirements of the Partners Healthcare Human Research Committee.

Women were studied during the early follicular phase (days 3-5 of the cycle, $n = 6$), late follicular phase (days 10-12, $n = 10$), early luteal phase (days 18-20, $n = 11$), or late luteal phase (days 25-27, $n = 11$). Women were divided into low-estradiol group (LE) and high-estradiol (HE) groups, and separately into low-progesterone (LP) and high-progesterone (HP) groups based on median splits regardless of menstrual phase. Sixty one percent of women in the LE group were part of the LP group, and 66% of women in the HE group were part of the HP group.

Conditioning and Extinction Procedure

The fear conditioning and extinction procedures were identical to those previously described (Milad et al., 2006a; Milad et al., 2005). Digital photographs of two different rooms constituted the visual contexts. Each context contained a lamp that was shown first in the off position (no color) and then switched on to one of two different colors (blue or red), which constituted the conditioned stimuli (CSs) (see figure 1). The selection of the CS+ color (followed by shock) and CS- color (no shock) and the contexts were pseudorandom and counterbalanced across participants. Contexts and CSs were displayed on a computer monitor three feet in front of participants who were seated upright in a chair. The unconditioned stimulus (US) was a 500 ms electric shock previously selected by the participant to be “highly annoying but not painful” delivered through electrodes attached to the second and third fingers of the right hand (Milad et al., 2005; Orr et al., 2000). The shock electrodes remained attached during each session of the experiment, but the US was administered only during the Conditioning session.

The experimental protocol was conducted over two days. On Day 1, the Habituation session consisted of eight trials in which the to-be CS+ and to-be CS- (4 of each) were presented in a counterbalanced manner within both the to-be conditioning context and the to-be extinction context. The Conditioning session consisted of five CS+ and five CS- trials, presented within the conditioning context. The US occurred immediately following each CS+ offset (100% reinforcement). The Extinction session was divided into two sub-sessions: early and late, which were separated by an approximate one-minute rest period. Each sub-session consisted of five CS+ and five CS- trials, all presented within the extinction context. On Day 2, the Recall session was identical to an Extinction sub-session given on Day 1. The US was never presented on day 2. When questioned after completion of the study, all subjects were explicitly aware of the CS-US contingency.

Serological Measurements

All subjects underwent the experimental protocol in the morning and were instructed not to eat after midnight prior to participation. Blood samples were drawn on Days 1 and 2 20-30 minutes prior to the experiment. Estradiol levels were assessed using an RIA kit (Roche Diagnostics, Indianapolis, IN) with a sensitivity of 18.4 pmol/L and an intra-assay coefficient of variance (CV) of 1.6-5.7%. Progesterone levels were determined using an RIA kit (Roche Diagnostics, Indianapolis, IN) with a sensitivity of 0.095 nmol/L, and an intra-assay CV of 1.5 – 2.7%.

Psychometric Measures

On a day of psychiatric assessment prior to the experiment, each subject completed the Beck Anxiety and Beck Depression Inventories, Anxiety Sensitivity Index (ASI), STAI Trait (T) assessment, and NEO Five Factor Inventory (NEO-FFI). After undergoing conditioning and extinction on Day 1 of the experiment, subjects completed the STAI State (S) assessment.

Psychophysiological Measures

In each trial, the context image was presented for 18 seconds: six seconds alone (light off) followed by 12 seconds in combination with the CS+ or CS- (light on). The mean inter-trial interval was 16 seconds and the range 12-21 seconds. The SCR was calculated during each CS by subtracting the mean skin conductance level (SCL) during the two seconds immediately preceding context onset from the highest SCL recorded during the 12-second CS presentation. To reduce heteroscedasticity, each SCR was square-root transformed prior to analysis (for a negative SCR, the square root of the absolute value was calculated and given a negative sign). Differential responses were calculated by subtracting the SCRs to the CS- from those of the CS+, and these values were used to conduct all statistical analyses. Skin conductance level was measured during the five seconds preceding the onset of each habituation session trial and then averaged across all eight trials to yield baseline SCL. Unconditioned response (UCR) levels were calculated by subtracting the average SCR during the one second immediately following the shock (before the SCR starts to rise) from the maximum SCR during the five seconds after the shock.

To quantify the amount of extinction memory expressed during the recall test on Day 2, we calculated an “extinction retention index”, which takes into account the level of fear acquired during conditioning by each subject when measuring the recall of extinction. Each subject's average differential SCR during the first two trials of the Extinction Recall session was divided by their largest differential SCR during the Conditioning session and then multiplied by 100, yielding a percentage of maximal conditioned responding. This was subtracted from 100% to yield an extinction retention index. We chose the highest SCR during the Conditioning session as a reference to assess the extinction performance on Day 2. This contrasts with using the last few conditioning trials as is commonly done in animal studies (Quirk et al., 2000) because human CRs typically decline towards the end of conditioning. Unless specified, all data are presented as means \pm standard error of the mean (S.E.). Analysis of variance (ANOVA) with repeated measures was used to analyze the data across experimental sessions, and analysis of covariance (ANCOVA) was used to adjust for potentially confounding variables such as baseline SCL, shock intensity, UCR, and anxiety sensitivity index when comparing men versus women. The statistical software was SPSS (Version 17.0, SPSS Inc., Chicago, IL 2008); the Tukey Honestly Significant Difference method was used in making post hoc comparisons. Student t-test was used when appropriate.

Results

Demographics, Personality Measures, and Baseline Physiological Measures

Men and women did not differ in age or years of education. Men exhibited higher mean baseline SCL, higher mean unconditioned SCR to the US, and higher mean shock level selection. Men scored lower on the NEO-FFI Agreeableness survey than women (for full statistics, see supplemental table 1).

Comparisons between the low-estradiol (LE, $n = 18$, mean 57.5 ± 5.7 pg/ml; median split 112 pg/ml) and high-estradiol (HE, $n = 18$, mean 256.7 ± 34.4 pg/ml) female groups revealed no differences in age, years of education, nor ethnicity. The HE group showed lower baseline SCL and scored lower on the NEO-FFI Extroversion survey than the LE group (supplemental table

2). Comparisons between the low-progesterone (LP, $n = 18$, mean 0.58 ± 0.04 pg/ml; median split 0.9 pg/ml) and high-progesterone (HP, $n = 18$, mean 8.3 ± 1.6 pg/ml) female groups revealed no differences in any demographic, physiological, or personality measure (supplemental table 3).

Fear Conditioning and Extinction

Group differences in differential responses (CS+ minus CS-) during Habituation, Conditioning, Extinction Learning, and Recall comparing women with high estradiol (HE) and women with low estradiol (LE) are shown in Figure 2. Repeated-measures ANOVA with two factors (experimental Session \times Group) revealed significant main effects of Session ($F_{(1,34)} = 21.1$, $p < 0.001$) and Group ($F_{(1,34)} = 4.18$, $p < 0.05$) and no significant interaction ($F_{(3,102)} = 0.98$, $p = 0.40$). Post-hoc analysis showed that the two groups did not differ significantly during Habituation, Conditioning, or Extinction Learning on day 1 (p values > 0.05). During Recall, the HE group exhibited significantly lower CRs relative to the LE group ($p < 0.05$ see figure 2). A further examination of the relationship between estradiol levels and extinction retention in women, operationalized as correlating estradiol levels on day 1 and extinction retention index, revealed a significant positive correlation ($r = 0.39$, $p = 0.03$).

Group differences during each experimental session (CS+ minus CS-) in high progesterone (HP) versus low progesterone (LP) women are shown in Figure 3. ANOVA revealed a significant main effect of Session ($F_{(1,34)} = 20.8$, $p < 0.001$), but not of Group ($F_{(1,34)} = .19$, $p = 0.67$), or interaction ($F_{(3,102)} = 0.40$, $p = 0.84$), indicating that the LP and HP groups did not differ at any experimental session on day 1 or day 2.

When women were divided into LE and HE groups, mean progesterone levels significantly differed (LE $1.6 + 0.63$ vs. HE $7.2 + 1.8$, $t_{(35)} = 2.95$, $p = 0.005$). Mean estradiol levels in the LP and HP groups, however, were not statistically different (LP 131.3 ± 35.5 vs. HP 182.8 ± 38.2 , $t_{(35)} = 1.07$, $p = 0.29$). Two-factor ANCOVA for LE versus HE with progesterone level as a covariate yielded a comparably significant group main effect ($p < 0.05$) to that noted above, suggesting that progesterone was not influencing fear extinction.

However, as mentioned previously, 61% of women in the LE group were also part of the LP group, and 66% of women in the HE group were part of the HP group. Thus, while our data suggest no progesterone effects on extinction recall, we did not obtain sufficient differential variance between the two groups to fully examine the effects of progesterone independent of estradiol. In addition, we conducted analyses to examine the impact of estradiol either opposed or unopposed by progesterone, operationalized as an E:P ratio. Here we again conducted a median split and a correlation analysis, based on the E:P ratio, that revealed no significant effects (data not shown).

Women were pooled to assess sex differences in differential responses (CS+ minus CS-) during each experimental session (see Figure 4). Repeated-measures ANOVA (Session \times Sex) revealed a significant main effect of Session ($F_{(1,52)} = 6.1$, $p < 0.05$) and significant interaction ($F_{(3,156)} = 4.6$, $p < 0.01$). Post-hoc analysis revealed that men exhibited significantly higher conditioned responses during the Conditioning phase relative to women ($p < 0.05$).

Regarding extinction retention index, Student's t -test revealed no significant difference between men and women in extinction retention ($85.9\% \pm 7.7\%$ and $74.6\% \pm 7.1\%$ respectively, $t = -1.01$, $p = 0.32$). However, as shown in Figure 5, mean extinction retention indices for male, HE female, and LE females were significantly different ($F_{(2,48)} = 3.5$, $p < 0.04$). Post-hoc analysis revealed that men and HE women did not differ ($p = 0.69$), whereas both of these groups showed significantly higher extinction retention (i.e. less fear) than LE women ($p < 0.02$ for HE vs. LE women, and $p = 0.04$ for men vs. LE women).

Discussion

We examined the influence of natural fluctuations of gonadal hormones on the acquisition, extinction learning, and extinction recall of conditioned fear in women. Estradiol levels did not affect the acquisition or extinction of conditioned fear but facilitated extinction memory recall. These results suggest a possible role of estradiol in the consolidation of extinction memories. With regard to sex differences, when gonadal hormones were not considered, sex differences were observed during the acquisition of conditioned fear, with men showing elevated CRs relative to women, replicating our previous report (Milad et al., 2006a). When taking the women's estradiol levels into consideration, however, sex differences were observed during the extinction retention test. Men and women with high estradiol levels exhibited significantly more extinction memory than women with low estradiol levels.

Several studies previously examined sex differences in fear conditioning in humans (van der Molen et al., 1988; Zorawski et al., 2005). However, the influence of estradiol and on fear extinction and extinction memory has not been addressed. Our findings here suggest that estradiol appears to significantly facilitate extinction recall in women. Such a role for estradiol in extinction retention has recently received support from the rodent literature. In an auditory fear conditioning paradigm, we recently showed that administration of estradiol to female rats significantly enhanced extinction recall whereas injection of estradiol receptor antagonists impaired it (Milad et al., 2009a). Chang et al., (2009) have shown that injections of estradiol into the hippocampus facilitate extinction of contextual fear. Moreover, estradiol has anxiolytic effects in rodents (Lund et al., 2005; Walf and Frye, 2005) and appears to facilitate memory formation in several behavioral paradigms (Leuner et al., 2004), including extinction of passive avoidance (Rivas-Arancibia and Vazquez-Pereyra, 1994) and conditioned-taste aversion (Yuan and Chambers, 1999). Previously, we assessed the influence of menstrual phase on extinction recall by focusing on two phases of the menstrual cycle: early follicular, where estradiol and progesterone levels are low, and late follicular, where estradiol levels are high and progesterone relatively low (Milad et al., 2006a). However, hormonal levels were not directly measured in that study, and therefore estimates relied solely on the reports of the participants regarding their menstrual phase in contrast to the study reported here.

Unlike estradiol, progesterone did not appear to have a significant effect on fear extinction. This is interesting given that we have recently shown that progesterone does appear to have a significant role in facilitating recall of fear extinction in female rats (Milad et al., 2009a). Moreover, it has also been shown that progesterone administration to rodents facilitates cognitive performance in a variety of behavioral tasks such as object recognition (Frye and Walf, 2008). Allopregnanolone, a metabolite of progesterone, has anxiolytic effects that are mediated through brain regions involved in fear extinction such as the amygdala and the vmPFC (Engin and Treit, 2007). The substantial overlap of estradiol and progesterone in our sample did not allow us to fully examine the effects of progesterone independent of estradiol. Thus, it remains possible that progesterone may have an effect on fear extinction consolidation in women directly or perhaps by interacting with estradiol. Additional studies are needed to further examine the role of progesterone in fear extinction in women.

One study of healthy human subjects reported no sex differences in fear conditioning (Zorawski et al., 2005), whereas another reported elevated CRs in women relative to men (Guimaraes et al., 1991). The apparent discrepancy between the results of the above studies and our data presented herein may be due, in part, to at least two factors. First, these studies did not control for menstrual cycle phase in their sample. Second, these studies included an unidentified number of women on birth control (who by definition would not be cycling), with other women who were cycling, thus introducing potential confounds with regard to investigating sex effects. However, men showed elevated conditioned responses during the acquisition phase relative to

women, which was consistent with our previous results (Milad et al., 2006a), and with finding increased freezing in male relative to female rodents in cued and contextual fear conditioning (Aguilar et al., 2003; Gupta et al., 2001; Wiltgen et al., 2001). Our findings are also consistent with a recent functional MRI study that demonstrated increased brain activity in stress response circuitry in men compared with women during ovulation (when estradiol is high relative to progesterone) in contrast to men compared with women during the early follicular period (during which estradiol and progesterone are low) (Goldstein et al., 2010).

In the present study, similar to the stress response in our previous work (Goldstein et al., 2010), men consistently displayed elevated expression of CRs during fear conditioning relative to both HE and LE groups in women (data not shown). Thus, these data indicate that sex differences during fear acquisition may be mediated by other mechanisms, including other hormonal systems that may or may not interact with estrogen and progesterone. For example, it has been previously shown that cortisol levels are associated with facilitated memory recall during acute stress in women (Andreano et al., 2008). Pre-conditioning injections of cortisol reduced conditioned fear in men while it had the opposite effect in women (Merz et al., 2010; Stark et al., 2006), thus suggesting that sex differences during fear learning may be mediated via the impact of cortisol (Andreano and Cahill, 2009). Another possibility for the observed sex differences during fear conditioning may be due to influences of gonadal hormones during early development on brain regions mediating fear conditioning (Dalla and Shors, 2009).

The level of extinction memory in men was comparable to that in women with high estradiol, whereas women with low estradiol showed significantly lower extinction memory during the recall test relative to men. This finding is counterintuitive given that the levels of estradiol in men would be most comparable to those found in low-estradiol women, and therefore one would have expected comparable extinction between these two groups. We speculate that the observed differences may be due to the elevated levels of testosterone in men. It has been shown that testosterone injections into male rats facilitated learning of inhibitory avoidance, reduced anxiety levels, and facilitated extinction of conditioned fear in mice (Edinger and Frye, 2007; Frye et al., 2008). Testosterone may have direct effects on facilitating extinction in men and may have indirect effects through its aromatization into estrogen. The role of testosterone in men and women on extinction memory consolidation should be further examined given that there are few studies investigating this issue.

It is worth noting that we observed a significant group difference in the extroversion trait between the HE and LE groups and significant group difference in the agreeableness factor between men and women. In a previous study, we found that only extroversion was positively correlated with fear extinction (Rauch et al., 2005). Yet, in the present sample, extroversion was higher in women with lower extinction memory (the low-estradiol group) and was lower in the women that showed enhanced extinction memory (the high estradiol group). Thus, these differences are unlikely to have influenced the between-group differences we observed in fear extinction. In addition, we observed a marginal difference between men and women in the ASI measure (supplemental table 1). This difference does not appear to influence group psychophysiological differences as such differences remained significant when co-varying for the ASI values.

Findings in the study reported here may have clinical implications given that several epidemiological studies have reported elevated incidence of anxiety disorders in women relative to men (Kinrys and Wygant, 2005; Pigott, 2003). Other studies have demonstrated sex differences in brain activity in processing emotional stimuli (Andreano and Cahill, 2009; Cahill, 2003; Goldstein et al., 2010; Protopopescu et al., 2005). Importantly, women diagnosed with premenstrual dysphoric disorder (PMDD) show increased anxiety and depression

symptoms that are recurrent during every cycle, and that have recently been associated with genetic variation of the estrogen receptor alpha (Huo et al., 2007). Moreover, women diagnosed with PMDD exhibited increased amygdala and decreased vmPFC activation in the context of a behavioral inhibition paradigm (Protopopescu et al., 2008). Results of the present study suggest that there may be an association between natural variation of gonadal hormones, estradiol in particular, and the brain circuitry involved in fear inhibition. Collectively, therefore, it appears that low levels of gonadal hormones may be related to impaired fear extinction and impaired processing of emotional stimuli in women with various psychiatric disorders. Future studies of the neural circuitry of fear extinction, such as those conducted with healthy humans and patients with anxiety disorders such as PTSD (Bremner et al., 2005; Milad et al., 2009b), should consider obtaining information regarding gonadal hormone levels and examining their associations with activity in brain regions involved in fear inhibition. Such studies could potentially lead to novel ways to enhance the efficacy of extinction-based therapies for anxiety disorders. For example, concentrating exposure therapy at a particular phase of the menstrual cycle in women and/or administration of estradiol in conjunction with exposure therapy may strengthen extinction consolidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

CR	Conditioned Response
vmPFC	ventromedial Prefrontal Cortex
SCR	Skin Conductance Response
LE	Low Estrogen
HE	High Estrogen
LP	Low Progesterone
HP	High Progesterone
CS	Conditioned Stimulus
US	Unconditioned Stimulus
ASI	Anxiety Sensitivity Index
SCL	Skin Conductance Level
UCR	Unconditioned Response
PTSD	Post-traumatic Stress Disorder

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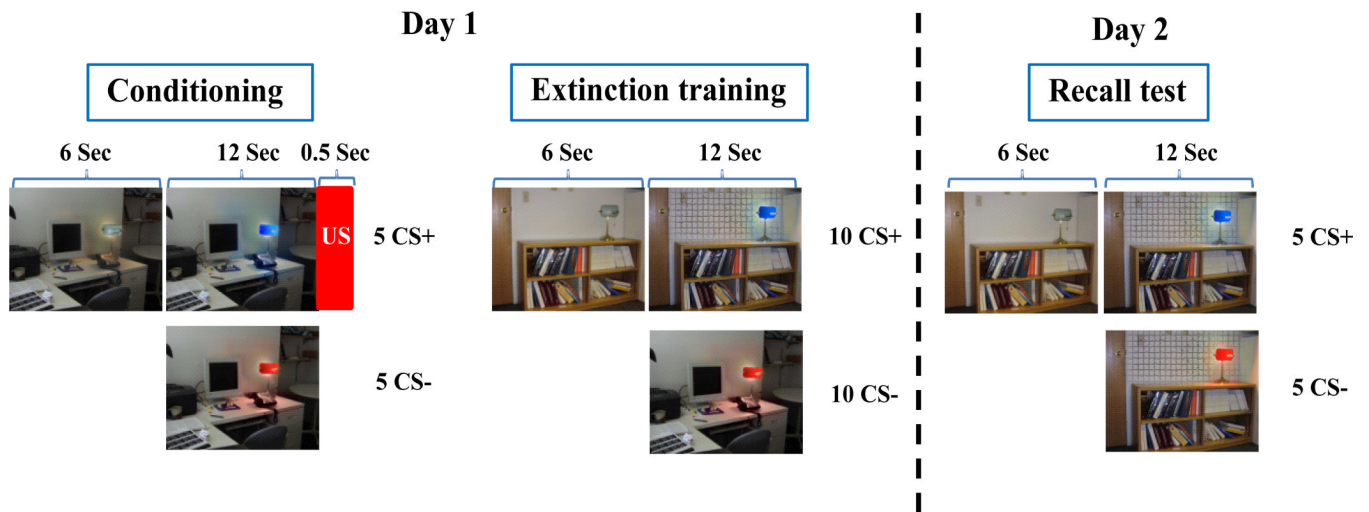


Figure 1. Fear conditioning and extinction protocol. CS+: conditioned stimulus, CS-: conditioned stimulus never paired with shock (unconditioned stimulus, US). Adapted from Milad et al., 2005.

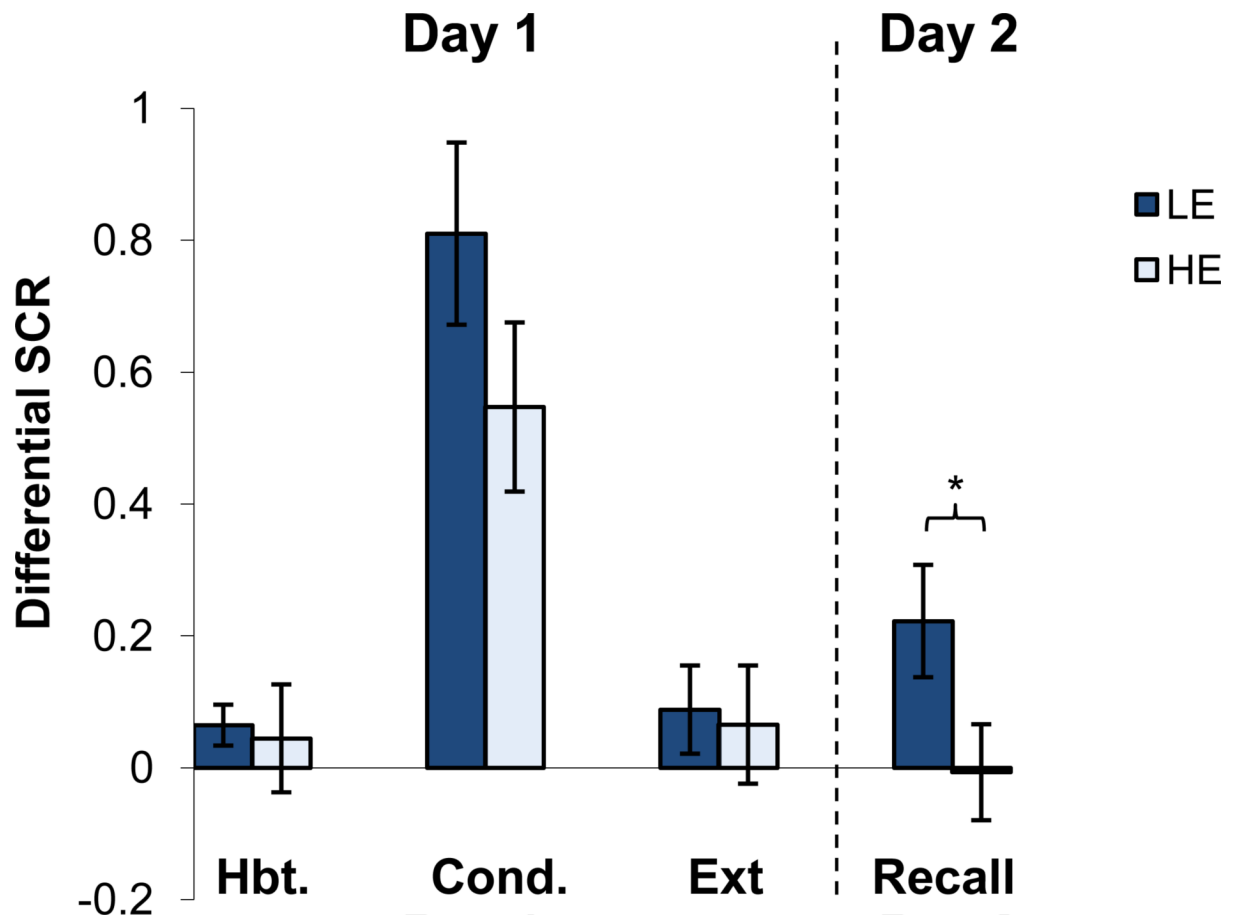


Figure 2.

Conditioned responses (SCRs) of high and low estradiol (HE and LE respectively) females across each session of the experiment. Habituation shows the mean of five trials, Conditioning displays the mean max response, Extinction shows the mean of the last two trials of each subject, Recall shows the mean of the first two trials. All data are shown based on differential response (CS+ minus CS-). Significant differences ($p < .05$) are indicated by *.

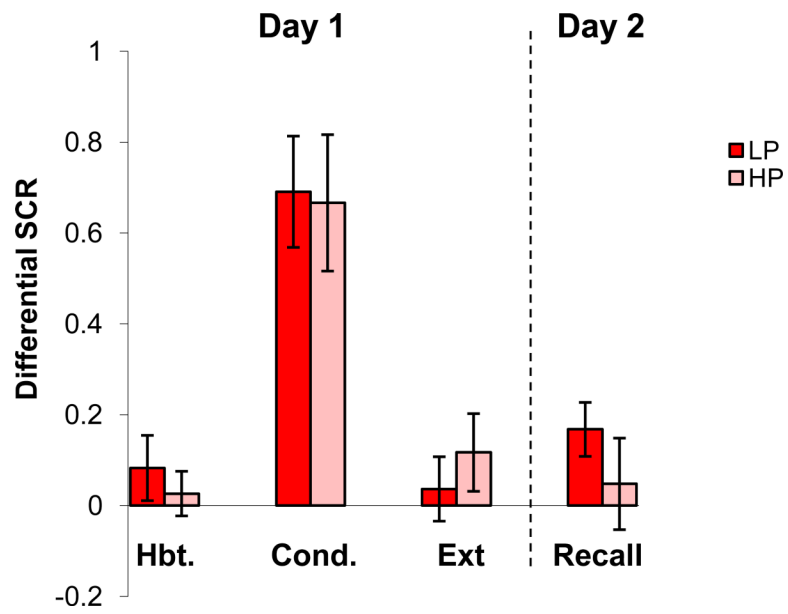


Figure 3. Conditioned responses (SCRs) of high (HP) and low progesterone (LP) women across each session of the experiment. See Figure 2 footnote for details. No significant differences were found.

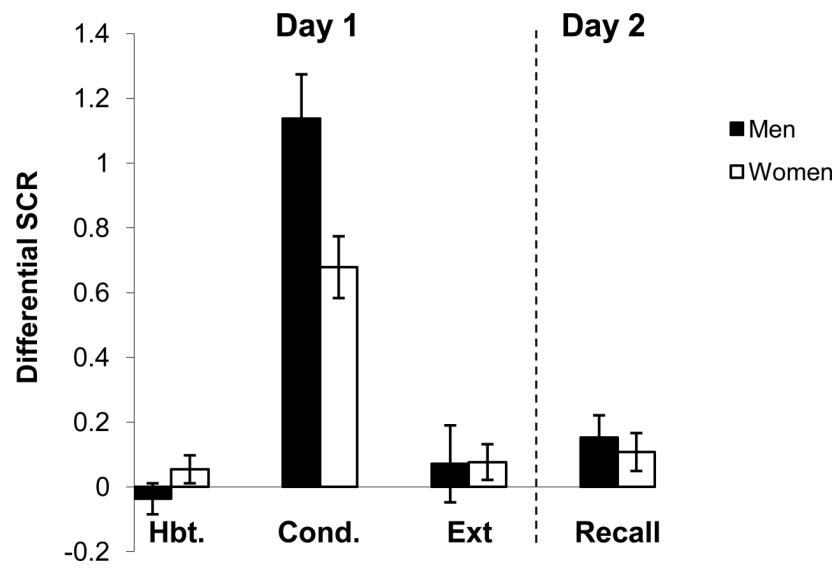


Figure 4. Conditioned responses (SCRs) of men and all women across each session of the experiment. See Figure 2 footnote for details.

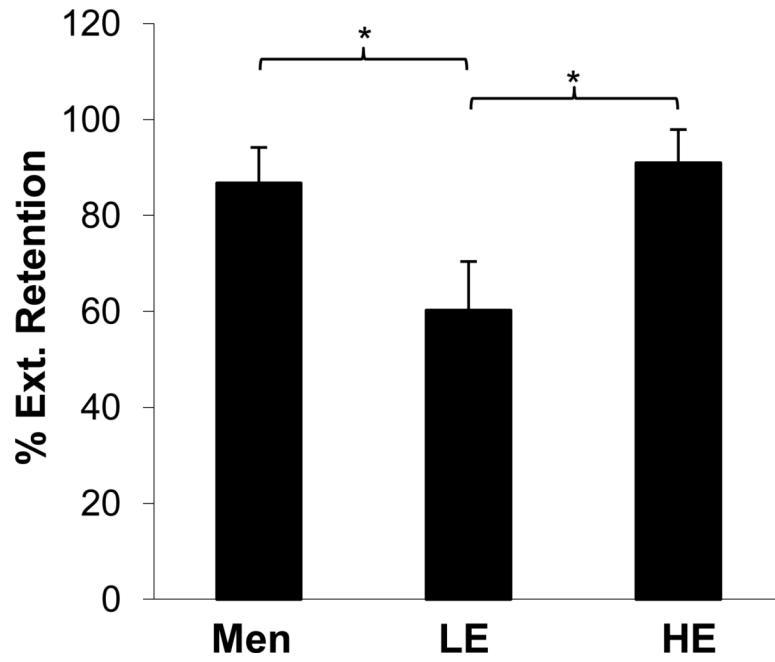


Figure 5. Percent extinction retention for men, women with high estradiol (HE) levels, and women with low estradiol (LE) levels. Significant differences ($p < .05$) are indicated by *.