Estradiol levels in women predict skin conductance response but not cognitive beliefs in conditioned fear extinction.

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Abstract

Anxiety disorders are more prevalent in women than men. One contributing factor may be the sex hormone estradiol, which is known to impact conditioned fear extinction, a laboratory procedure that forms the basis of exposure therapy for anxiety disorders. To date, the literature examining estradiol and fear extinction in humans has focused on physiological measures of fear, such as skin conductance response (SCR) and fear potentiated startle. This is surprising, given that models of anxiety identify at least three important components: physiological symptoms, cognitive beliefs, and avoidance behavior. To help address this gap, we exposed women with naturally high (n = 20) or low estradiol (n = 19), women using hormonal contraceptives (n = 16), and a male control group (n = 18) to a fear extinction task, and measured SCR, US expectancy and CS valence ratings. During extinction recall, low estradiol was associated with high SCR, but was not related to US expectancy or CS evaluation. Importantly, women using hormonal contraceptives showed a dissociation between SCR and cognitive beliefs: they correctly reported that the extinguished cue was no longer dangerous, yet they continued to show increased SCR to this cue. This divergence underscores the importance of assessing multiple measures of fear when examining the role of estradiol in human fear extinction, especially when considering the potential of estradiol as an enhancement for psychological treatments for anxiety disorders.

Key Words: estrogen, sex hormones, menstrual cycle, fear extinction, anxiety, US expectancy

1. Introduction

Women are twice as likely as men to develop an anxiety disorder (Kessler, Petukhova, Sampson, Zaslavsky, and Wittchen, 2012). They also experience greater symptom severity and chronicity, and poorer treatment outcomes, relative to men (Pigott, 2003). While we do not fully understand the mechanisms underlying this gender disparity, one contributing factor may be the influence of sex hormones. We know that sex differences in anxiety prevalence do not emerge until after puberty, suggesting that activation of gonadal hormones may be playing a role (Paus, Keshavan, and Giedd, 2008). Furthermore, fluctuations in the sex hormone estradiol have been shown to impact conditioned fear extinction, a laboratory procedure that is widely used to understand the mechanisms underlying the acquisition and inhibition of fear. In this procedure, a previously neutral conditioned stimulus (CS) (e.g. a light) is paired with an aversive unconditioned stimulus (US) (e.g. a mild shock), until the CS evokes a conditioned fear response (e.g. increase in skin conductance). The conditioned fear is then extinguished by repeatedly presenting the CS without the US, until fear responses subside. Long-term extinction recall can be tested by presenting the extinguished CS after a delay: good extinction recall is evident by continual low levels of fear, while poor extinction recall is evident by an increase in fear responses (known as return of fear or ROF; Rachman, 1979; 1989).

Evidence from naturally-cycling humans and rodents suggests that females in the low estradiol phase of their cycle show significantly poorer recall of fear extinction than females in the high estradiol phase (Chang, Yang, Liang, Yeh, Huang, and Hsu, 2009; Graham and Milad, 2013; Gruene, Roberts, Thomas, Ronzio, and Shansky, 2015; Milad, Igoe, Lebron-Milad, and Novales, 2009; Milad, Zeidan, Contero, Pitman, Klibanski, Rauch, and Goldstein, 2010; Rey, Lipps, and Shansky, 2014; Zeidan, Igoe, Linnman, Vitalo, Levine, Klibanski, Goldstein, and Milad, 2011). Similarly, women with PTSD who have low estradiol levels exhibit heighted startle responses during extinction training (Glover, Jovanovic, Mercer, Kerley, Bradley, Ressler, and Norrholm, 2012). Additionally, estrogen agonists enhance, while estrogen antagonists impair, extinction recall in rodents (Chang et al., 2009; Milad et

al., 2009; Zeidan et al., 2011). Finally, poor recall of fear extinction is found when estradiol is blocked by hormonal contraceptives in humans, while pre-extinction estradiol treatment improves extinction recall in women with naturally low estradiol levels (Graham and Milad, 2013). These studies all support a crucial role of estradiol in the consolidation or maintenance of the extinction memory.

Fear extinction forms the basis of exposure therapy, which is an important component of Cognitive Behavioral Therapy (CBT), the gold standard treatment for anxiety disorders (Hofmann and Smits, 2008). Exposure therapy counteracts the pathological avoidance of feared stimuli and situations associated with anxiety disorders. However, established models of anxiety disorders (e.g. Beck, Emery, and Greenberg, 1985; Clark, 1986; Clark and Wells, 1995; Ehlers and Clark, 2000) also emphasize the crucial role of cognitions in the development and maintenance of clinical anxiety. According to these models, exaggerated beliefs regarding the likelihood and consequences of feared cues fuel both behavioral avoidance and the physiological sensations of anxiety (e.g. increased heart rate), which then reinforce exaggerated beliefs (Beck et al., 1985). Thus, anxiety disorders comprise at least three components: beliefs about the feared stimulus, physiological reactions, and behavioral responses. This is also in accordance with Lang's three-response system of fearful emotions, comprising verbal responses (cognitions and affect), physiological arousal, and avoidance behavior (Lang, 1985).

To date, the current literature examining sex hormones and fear extinction in humans has focused primarily on physiological measures of fear, such as skin conductance response (SCR) and fear-potentiated startle. While these measures are important, it is also crucial to investigate the link between sex hormones and alternative response systems, such as beliefs about extinguished cues. Research indicates that Lang's three emotional response systems do not always converge, with different patterns of responses found across contexts and populations (Lang, 1985; Lang, Bradley, and Cuthbert, 1998). Therefore, assessing multiple measures of fear will help to develop a more robust understanding of the role of

estradiol in fear extinction in humans. Such an understanding may help to shed light on the potential clinical utility of estradiol adjuncts to psychological treatments.

The aim of the current study was to investigate the relationship between estradiol and cognitive responses towards previously conditioned extinguished cues, in addition to its documented influence on physiological reactions to such cues. To achieve this, we exposed naturally cycling women with high and low levels of estradiol, women using hormonal contraceptives (which suppress endogenous estradiol and progesterone), plus a male control group, to a differential fear conditioning and extinction procedure. We measured SCR to cues, plus two verbal measures of fear responses: US expectancy (how much the CS predicts the occurrence of the US; Lovibond and Shanks, 2002), and CS evaluation (the change in the valence of the CS when it is paired with the US; De Houwer, Thomas, and Baeyens, 2001).

There is continued debate as to whether US expectancy and SCR are linked: some argue that SCR does not occur without contingency awareness of the CS-US relationship (e.g. Hamm and Vaitl, 1996; Hamm and Weike, 2005; Lovibond, 2004; Lovibond and Shanks, 2002; Purkis and Lipp, 2001; Sevenster, Beckers, and Kindt, 2014; Soeter and Kindt, 2010), while others suggest these two measures are dissociable (e.g. Esteves, Parra, Dimberg, and Ohman, 1994; Knight, Nguyen, and Bandettini, 2003; 2006; Schultz, Balderston, Geiger, and Helmstetter, 2013; Schultz and Helmstetter, 2010) and may reflect different neural circuitry (Bechara, Tranel, Damasio, Adolphs, Rockland, and Damasio, 1995; Cacciaglia, Pohlack, Flor, and Nees, 2015; Knight, Waters, and Bandettini, 2009). Similarly, debate exists regarding whether CS evaluation is independent of contingency awareness (for reviews, see De Houwer, Baeyens, and Field, 2005; Field, 2000), although research specifically examining the relationship between evaluative conditioning and SCR is limited. Interestingly, there is some evidence that residual negative evaluation of the CS predicts self-reported return of fear post extinction (Dirikx, Hermans, Vansteenwegen, Baeyens, and Eelen, 2004; 2007; Dirikx, Vansteenwegen, Eelen, and Hermans, 2009; Hermans, Dirikx, Vansteenwegenin, Baeyens, Van den Bergh, and Eelen, 2005; Zbozinek, Hermans,

Prenoveau, Liao, and Craske, 2015), though it is not yet clear whether these findings are applicable to SCR (Zbozinek et al., 2015).

Given the above, we predict that as per past research, women with low levels of estradiol (either naturally or due to hormonal contraceptive use) will exhibit an increase in SCR during extinction recall, relative to women with high levels of estradiol. Importantly, if SCR is dependent on contingency awareness, then women with low estradiol should also report an increase in US expectancy during extinction recall. Finally, if residual negative evaluation of the CS is a potential mechanism underlying the return of fear, then we would expect low estradiol women to rate the CS more negatively at the end of extinction, compared to women with high estradiol.

2. Methods

2.1 Participants

Eighty-three participants (18-35 years old, mean age 22.2 years; 21 men) were recruited from first-year psychology courses and community advertisements (see Table 1 for demographics). Women reported regular menstrual cycles (*n* = 45) or were using hormonal contraceptives (*n* = 17; see Table S1 in supplemental for details). Participants reported no history of DSM-V Axis I disorders and no history of endocrinologic conditions (e.g. polycystic ovary syndrome). Ten participants were excluded post data collection due to movement during CS trials interfering with SCR recording (4 naturally cycling, 1 Hrm-C, and 3 male participants), or skin conductance data greater than three standard deviations above the mean (2 naturally cycling participants), thus reducing the final sample to seventy-three participants. All procedures were approved by the University of New South Wales Human Research Ethics Committee, and written informed consent was obtained from all participants.

2.2 Materials and Measures

2.2.1 Self-report measures

Participants were administered a number of self-report measures including the Emotion Regulation Questionnaire (ERQ; Gross and John, 2003), Beck Anxiety Inventory (BAI; Beck and Steer, 1993), Beck Depression Inventory (BDI; Beck, Steer, and Brown, 1996), and the State-Trait Anxiety Inventory (STAI; Spielberger, Gorsuch, Lushene, Vagg, and Jacobs, 1983) Trait (T) assessment (see Table 1 for outcomes).

2.2.2 Conditioned and unconditioned stimuli

Two black-and-white male faces with neutral facial expressions from the Center for Vital Longevity Face Database (Minear and Park, 2004; see Figure S1 in supplemental) served as the CS+ and CS- (counterbalanced across participants). The choice of faces was based on the fear conditioning procedure of Mason and Richardson (2010), in addition to research suggesting fear learning is facilitated by the use of evolutionary relevant stimuli (Dimberg and Ohman, 1996). The CSs were delivered using a Dell PC and Benq monitor. The US was a 0.2 sec mild electric shock delivered via a stainless steel bipolar electrode that was Velcro strapped to the distal phalanx of the index and middle fingers of the dominant hand. The US was conducted by a custom-made ADInstruments constant current stimulus isolator controlled by Labchart software. CS presentation and the timing of US onsets were controlled by E-Prime software.

2.2.3 Physiological assessment

An ADInstruments system was used to record skin conductance levels (SCLs) via an ADInstruments GSR amp (FE116) using constant voltage (22 mV_{rms} at 75 Hz) AC excitation through a stainless steel dry bipolar electrode (MLT116F) that was Velcro strapped to the distal phalanx of the index and middle fingers of the non-dominant hand. The analogue inputs were digitised by an ADInstruments Powerlab 8/35 data acquisition system (PL3508), and sampled using Labchart.

2.2.4 Serological assessment

A venous blood sample was drawn from each female participant on Day 1 of the experiment at a pathology service located within walking distance from the University.

Serum hormone concentrations were analyzed by Healthscope Pathology Services. Estradiol levels were analyzed using an ADVIA Centaur Enhanced Estradiol assay (Siemens), which is a competitive assay that measures serum estradiol concentrations up to 3000 pg/mL (11,010 pmol/L) with a limit of detection of 11.8 pg/mL (43.6 pmol/L). Progesterone levels were analyzed using an ADVIA Centaur Progesterone assay (Siemens), which is a competitive immunoassay that measures serum progesterone concentration up to 60 ng/mL (190.8 nmol/L) with a minimum detectable concentration of 0.21 ng/mL (0.67 nmol/L). Naturally cycling women were invited to participate in the experiment across all phases of the menstrual cycle to achieve wide variance in estradiol and progesterone levels. The experimenter remained blind to the results of serum analyses for each participant until after the completion of the two-day experimental procedure. Once all the data had been collected, naturally cycling women were divided into high and low estradiol (or progesterone) groups on the basis of a median split.

2.3 Procedure

All procedures took place within the same temperature-controlled room across two days. On Day 1, participants gave informed consent and completed the self-report measures. The skin conductance electrodes were attached, after which participants sat quietly for a 3 minute baseline measure of skin conductance. The shock electrodes were then attached, and participants underwent a shock threshold test to determine the shock level to be used for the experiment. Participants were advised to select a shock level that was “highly annoying but not painful”.

Participants subsequently underwent habituation, differential fear conditioning, and extinction training, in a procedure based on Mason and Richardson (2010). During habituation, participants received written instructions that they would be asked to provide ratings for some faces that would appear on the screen. They then received two presentations each of the CS+ and CS- in the absence of the US, and gave baseline fear and valence ratings. Fear ratings asked participants “How fearful did you find the face you just saw?” where 1 = really fearful and 9 = really pleasant. Valence ratings asked

participants “How much do you like or dislike the face you just saw?” where 1 = really dislike and 9 = really like. The conditioning and extinction phases followed habituation, as indicated by the instructions: “In the next part of the experiment, faces will again appear on the screen, and you may or may not receive shock. If you do receive shock, just notice if there is any pattern.” The CS+ and CS- were then presented 8 times each, with the CS+ 62.5% partially reinforced with the US. The CS- was never paired with the US. Extinction training immediately followed conditioning, in which the CS+ and CS- were presented 7 times without the US. Participants provided online US valence and expectancy ratings on the first, fourth and eighth conditioning trials, and on every trial during extinction training and extinction recall. Valence ratings were as per habituation, while expectancy ratings asked participants “How much do you expect the face you just saw to be followed by a shock the next time you see it?” where 0 = certain no shock would occur and 100 = certain shock would occur. Participants were taken to the pathology service to provide a blood sample within 15 min of the end of extinction training. On Day 2 (approximately 24 hours after extinction training) participants underwent extinction recall, which was prefaced with the instructions: “In the next part of the experiment, you’ll see the same faces as yesterday, and, the same as yesterday, you may or may not receive shock. If you do receive shock, just notice if there is any pattern”. The remainder of the extinction recall phase was identical to extinction training.

Across all phases, each CS trial consisted of a 2-second presentation of a grey rectangle, signaling the participant to fixate on the center of the screen, followed immediately by a 6-second presentation of the CS. Reinforced trials co-terminated with a 0.2 s US. The inter-trial interval (ITI) was 10 seconds. CSs were always presented in a pseudo-random order such that no CS appeared more than two consecutive times. The skin conductance and shock electrodes remained attached during each session of the experiment, but the US was only administered during the conditioning phase. All instructions and ratings were presented on a Benq monitor and keypad responses were recorded using E-Prime software.

2.4 Psychophysiological Data Analysis

To evaluate participants' unconditioned response to the shock (UCR), the average SCL during the first 1.5 sec after the shock (before skin conductance increases) was subtracted from the peak SCL during the first 5 sec after the US.

Skin conductance responses to the CSs (SCRs) were calculated by subtracting the maximum SCL during each 6 sec CS presentation from the average SCL during the two-second grey rectangle immediately preceding the CS, such that SCR values represent changes above and beyond those produced by orienting/visual stimulation. This method also ensures that the maximal increase in SCL at any point during the 6 sec CS will be detected, and has been validated by previous studies (e.g. Milad, Orr, Pitman, and Rauch, 2005). All SCR values were square-root transformed to reduce heteroscedasticity.

Conditioning strength was indexed as average differential SCRs across conditioning trials (average SCRs to the CS+ minus average SCRs to the CS-). Extinction acquisition strength was assessed by comparing average differential SCRs during conditioning to average differential SCRs during the last three extinction trials on day 1. Extinction recall was assessed by calculating the percent recovery of fear, controlling for individual differences in conditioning strength. To do this, each participant's average SCRs to the CS+ across the first four extinction recall trials were divided by their largest SCR to the CS+ during conditioning. The resulting product was multiplied by 100, yielding a percent recovery of fear.

2.5 Statistical Analysis

Analysis of variance was used to examine overall effects, with repeated measures used when appropriate to analyze data within experimental phases. Post hoc comparisons used Tukey's Honestly Significant Difference test. Homogeneity of variance was assessed using Levene's Test, and when this assumption was violated group differences were analyzed using the non-parametric Welch's test, followed by Games-Howell post hoc comparisons as appropriate. The role of hormones as predictors of the dependent measures was assessed via post-hoc regression. Greenhouse-Geisser corrections are reported when the assumption of sphericity was violated. All analyses used SPSS version 22.0.

3. Results

3.1 Menstrual Cycle and Hormone Levels

Naturally cycling female participants reported a mean cycle length of 28.4 days (*SD* = 3.12). On Day 1 of the experiment, 17 naturally cycling participants (43.6%) reported being in the early follicular phase of their cycle, 7 (17.9%) reported being in the late follicular phase, and 15 (38.5%) reported being in the luteal phase. In order to compare naturally cycling women with women using contraceptives and males, naturally cycling women were split into high and low groups based on a median split for both estradiol (*Md* = 143 pmol/L) and progesterone (*Md* = 0.8 nmol/L). While both estradiol and progesterone were correlated with the dependent variable, only estradiol emerged from a regression analysis as a significant predictor of the dependent variable (see below for details). Thus, all subsequent analyses compare high and low estradiol groups with women using contraceptives and males (see supplement for a similar analysis comparing high and low progesterone). Estradiol levels were significantly different between the three female groups [Welch's $F(2,31.91) = 20.012$, $p < .01$]. Post-hoc Games-Howell tests indicated that all three comparisons were significant (all *p's* < .01). Hrm-C women had the lowest estradiol levels, followed by L-EST women, followed by H-EST women (Figure 1). Progesterone levels were not significantly different between estradiol groups [Welch's $F(2,30.61) = 2.28$, $p = .12$], although estradiol and progesterone were highly correlated [$r(55) = .73$, $p < .01$].

3.2 Baseline Skin Conductance and Shock Levels

A one-way ANOVA revealed no significant group differences for the level of shock selected [$F(3,69) = 1.41$, $p = .24$] or preconditioning baseline SCL [$F(3,69) = .60$, $p = .61$] (Table 1). These measures indicate that the group differences reported below are not a function of prior differences in shock levels or baseline skin conductance.

3.3 Conditioned Fear Extinction

Skin conductance responses to conditioned and extinguished stimuli were compared across groups. No group differences were observed during conditioning [$F(3,69) = .51$, $p =$

.68]. Skin conductance responses at the end of extinction training were significantly less than those during conditioning [$F(1,69) = 10.94$, $p < .01$], with no differences between group [$F(3,69) = .51$, $p = .68$] and no group-by-phase interaction [$F(3,69) = .03$, $p = .99$], suggesting all groups obtained comparable extinction on Day 1 (Figure 2A). However, there were group differences in the percent recovery of fear at extinction recall the next day [$F(3,72) = 2.89$, $p = .042$]. Post hoc Tukey tests confirmed that Hrm-C women exhibited significantly more recovery of fear than H-EST women ($p = .033$), while L-EST women and males were not significantly different to either Hrm-C or H-EST women (Figure 2B). These data imply that while conditioning and acquisition of extinction is unaffected in women using hormonal contraceptives, the consolidation or maintenance of extinction across time is impaired.

Regarding participants' unconditioned response to the shock, there was a significant main effect of trial [$F(4,276) = 6.96$, $p < .01$], due to unconditioned responses habituating across the task (data not shown). However there were no group differences [$F(3,69) = .097$, $p = .96$] and no interaction between group and trial [$F(12,276) = 1.74$, $p = .079$), indicating that the differences in extinction recall were not due to pre-existing differences in unconditioned responses to the shock.

Shock expectancy ratings (Figure 3) were initially examined with a 4 (Group) x 2 (CS + vs. CS-) x N (trials) repeated measures ANOVA for each experimental phase. The interaction between CS type and trial was significant for conditioning [$F(2,138) = 42.92$, $p < .01$], extinction training [$F(6, 414) = 45.21$, $p < .01$], and extinction recall [$F(6,414) = 10.98$, $p < .01$], indicating differential expectancy learning for the CS+ and CS- across the experiment. Each CS type was then examined separately. For the CS+, there was a main effect of trial for conditioning [$F(2,138) = 11.05$, $p < .01$], extinction training [$F(6,414) = 82.27$, $p < .01$], and extinction recall [$F(6, 414) = 57.35$, $p < .01$], due to shock expectancy for the CS+ rising during conditioning and falling during both extinction training and recall. There was a significant group difference for shock expectancy during conditioning [$F(3,69) = 3.08$, $p = .03$], due to Hrm-C women having higher shock expectancy than Males ($p = .038$).

There was also a significant group-by-trial interaction at extinction recall [$F(18,414) = 2.08$, $p = .04$], due to Males exhibiting no change in expectancy ratings across the first four trials of extinction recall (lowest $p = .115$). There were no significant group differences or interactions in expectancy ratings for extinction training (lowest $p = .42$; Figure 3A). For the CS-, there was a main effect of conditioning [$F(2,138) = 43.41$, $p < .01$], extinction training [$F(6,414) = 6.50$, $p < .01$], and extinction recall [$F(6, 414) = 28.81$, $p < .01$], due to shock expectancy for the CS- falling sharply during conditioning, and then again gradually during extinction training and recall. There were no significant group differences or interactions in shock expectancy for the CS- in any phase.

As per shock expectancy ratings, valence ratings (Figure 4) were initially examined with a 4 (Group) x 2 (CS + vs. CS-) x N (trials) repeated measures ANOVA for each separate phase. The interaction between CS type and trial was significant for conditioning [$F(2,138) = 34.09$, $p < .01$], and extinction training [$F(6, 414) = 14.16$, $p < .01$], but not for extinction recall [$F(6,414) = .16$, $p = .98$]. This indicates that participants evaluated the CS+ and CS- differentially only during conditioning and extinction training. Each CS type was then examined separately. For the CS+, there was a main effect of trial for conditioning [$F(2,138) = 21.00$, $p < .01$], extinction training [$F(6,414) = 15.10$, $p < .01$], and extinction recall [$F(6, 414) = 2.62$, $p = .049$], due to valence ratings for the CS+ falling during conditioning and rising during extinction training and recall. For the CS-, there was a main effect of trial for conditioning [$F(2,138) = 21.83$, $p < .01$], a trend for extinction training [$F(6,414) = 2.40$, $p = .051$], and a main effect for extinction recall [$F(6, 414) = 2.73$, $p = .044$], due to valence ratings for the CS- rising during conditioning, remaining unchanged during extinction training, and then again rising gradually during extinction recall. There were no significant group differences or interactions in valence ratings for either CS across any of the experiment phases (lowest $p = .118$).

3.4 Regression Analysis

Given that estradiol and progesterone can also be treated as continuous variables, additional analyses were carried out to examine the relationship between hormones and fear

extinction. A correlational analysis revealed that SCR percent fear recovery was negatively correlated with estradiol, progesterone, BDI, BAI, STAI-T, and UCR (see Table S2 in supplemental). However, only estradiol and STAI-T emerged from a stepwise regression analysis as significant predictors of SCR fear recovery [$F(2,52) = 17.11$, $p < .01$]. The combined estradiol and STAI-T model accounted for approximately 37.4% of the variance in fear recovery, which was less than ten percent over and above the variance accounted for by an estradiol-only model (29.6%) [$F(1,53) = 23.7$, $p < .01$].

Estradiol and progesterone levels were unrelated to baseline SCL, shock level, or unconditioned SCRs (see Table S2 in supplemental), again suggesting that the relationship between SCR fear recovery and hormones was not a function of prior differences in baseline SCL levels, shock tolerance levels, or unconditioned responses. There was also no relationship between estradiol and progesterone with US expectancy or CS valence during extinction recall (smallest $p = .29$), which is in accordance with the group analysis above. Finally, there was no correlation between the valence rating of the CS+ at the end of extinction and SCR percent fear recovery ($p = .31$) or US expectancy during extinction recall ($p = .41$).

4. Discussion

These findings demonstrate yet again that estradiol levels negatively predict return of fear, consistent with reports from other laboratories using variations of learned fear paradigms (Chang et al., 2009; Graham and Milad, 2013; Milad et al., 2009; Milad et al., 2010; Rey et al., 2014; Wegerer, Kerschbaum, Blechert, and Wilhelm, 2014; Zeidan et al., 2011). Women using hormonal contraceptives, who had the lowest estradiol levels, displayed higher SCR at extinction recall relative to women with naturally high estradiol, while women with naturally low estradiol were not significantly different from either group, with their mean percent recovery of SCRs at extinction recall falling between these groups. Crucially, our regression analysis across all three groups indicated almost one third of the variance in extinction recall was predicted by estradiol levels.

Interestingly, our hypotheses regarding beliefs about extinguished cues were not supported: there was no evidence that estradiol levels were related to US expectancy or CS valence during extinction recall. Instead, women using hormonal contraceptives (with chronically low estradiol) displayed a dissociation between physiological and cognitive responses. That is, they correctly reported that the extinguished cue no longer predicted shock and was no longer aversive, yet continued to display increased SCR to this cue during extinction recall. This result contradicts research implying that SCR reflects declarative knowledge of CS-US contingencies (e.g. Hamm and Vaitl, 1996; Hamm and Weike, 2005; Lovibond, 2004; Lovibond and Shanks, 2002; Purkis and Lipp, 2001; Sevenster et al., 2014; Soeter and Kindt, 2010), and instead supports the notion that these two response systems are dissociable (e.g. Bechara et al., 1995; Esteves et al., 1994; Knight et al., 2003; 2006; Knight et al., 2009; Schultz et al., 2013; Schultz and Helmstetter, 2010), at least in specific populations or contexts.

One explanation for the divergence between SCR and contingency awareness could be that these response systems are controlled by separate brain regions that are differentially affected by estradiol. There is some evidence that contingency awareness is linked to hippocampal activity, while SCR is associated with amygdala response (Bechara et al., 1995; Cacciaglia et al., 2015; Knight et al., 2009). In fear extinction, the hippocampus activates the ventromedial prefrontal cortex (vmPFC), which then exerts top-down inhibition on the amygdala, preventing the expression of conditioned fear (Graham and Milad, 2011). Critically, high estradiol levels are associated with increased vmPFC and reduced amygdala activation during fear extinction recall (Zeidan et al., 2011), implying that estradiol may boost the ability of the vmPFC to exert top-down control over the amygdala. This is supported by evidence that low estradiol impairments in extinction can be rescued by application of D1 dopamine agonists, suggesting that estradiol may also modulate prefrontal dopaminergic mechanisms (Rey et al., 2014). Therefore, it is possible that the return of SCR in the present study may be due to reduced ability to recruit vmPFC top-down control over the

amygdala in women using contraceptives. Future studies utilizing fMRI will be necessary to assess this possibility.

Interestingly, while estradiol has been linked with prefrontal areas, it has also been shown to enhance plasticity in the hippocampus via increased dendritic spine density (Chen, Yan, Wang, Chen, Wang, and Tseng, 2009; Hao, Rapp, Leffler, Leffler, Janssen, Lou, McKay, Roberts, Wearne, Hof, and Morrison, 2006; Woolley and McEwen, 1993). If high estradiol increases plasticity in the hippocampus, and this region is important for contingency awareness, then it is surprising that we did not find an association between estradiol and this measure. It is possible that our conditioning procedure was too simple to reveal a differential association between estradiol and contingency learning. Beckers, Krypotos, Boddez, Effting, and Kindt (2013) argue that basic fear conditioning paradigms reflect adaptive learning, and thus should exhibit little variation between different populations. Instead, Beckers et al. (2013) recommend using procedures such as blocking, where ambiguous stimuli could potentially reveal more subtle differences in contingency learning. Following this reasoning, it is also possible that the cognitive measures we used in the current study were not sensitive enough to detect differences in women using contraceptives, or measured something slightly different than SCR. While our expectancy and valence ratings were assessed online throughout each experimental phase, they were taken at the end of each CS trial (so as to avoid interference with skin conductance), whereas SCR was measured continuously throughout the CS. Therefore, it is possible that the cognitive ratings may reflect anticipatory anxiety of the next trial, while SCR may reflect acute fear of the current trial, and the potential influence of estradiol on each may differ. Future studies could improve these limitations by using longer CS durations and a moment-by-moment measure of US expectancy (e.g. Lovibond, 1992), or by examining the link between estradiol and fear conditioning using more complex, ambiguous procedures.

With respect to valence ratings, while we found clear evidence of evaluative conditioning, there was no evidence that this failed to extinguish, and no evidence that this component of conditioning extinguished differently in women with different estradiol levels.

We also found no indication that residual negative evaluation of the CS+ at the end of extinction predicted the return of SCR during extinction recall, which is in accordance with previous work (Zbozinek et al., 2015). Taken together, this suggests that CS evaluation may not be a mechanism underlying the return of physiological responding seen in women using contraceptives.

It should also be considered that the increase in SCR observed during extinction recall may reflect something other than return of fear, such as changes in arousal, or greater reactivity of SCR. However, if this was the case, we would expect to see similar differences during acquisition and extinction, and perhaps differences during baseline SCL, none of which the current data supported. This interpretation also contradicts findings from animal literature, which show clear effects of estradiol on alternative measures of extinction recall, such as freezing (Chang et al., 2009; Graham and Milad, 2013; Milad et al., 2009; Zeidan et al., 2011). Moreover, even if the rise in SCR during extinction recall was not directly related to fear responses, we speculate that an increase in physiological arousal in the presence of an extinguished cue may still lead to behavioral avoidance of that cue, despite knowledge that it is no longer dangerous. Given the central role of behavior both in models of emotion (e.g. Frijda, 2004; Lang, 1985; Lang et al., 1998), and models of anxiety disorders (e.g. Barlow, 2002; Wells, 1997), future studies on sex hormones and fear extinction should also measure avoidance behavior, the final component of the three-system model of emotions (Lang, 1985; Lang et al., 1998). This is particularly important given that avoidance and safety behaviors are seen as key maintaining factors in anxiety disorders (Barlow, 2002; Wells, 1997).

It was somewhat surprising that women with naturally low estradiol were not significantly different to women with high estradiol, given past research showing differences in fear extinction recall between high and low naturally cycling females (Chang et al., 2009; Graham and Milad, 2013; Milad et al., 2009; Milad et al., 2010; Rey et al., 2014; Zeidan et al., 2011). One explanation for our results may be that estradiol levels for low estradiol women fell between high estradiol women and women using contraceptives. This account is

in accordance with our regression analysis which treated estradiol as a continuous variable across all three groups. Alternatively, it is possible that chronically suppressed estradiol has a differential effect to transiently reduced estradiol. Furthermore, while the regression analysis implies our findings are due to endogenous estradiol levels (which are suppressed by hormonal contraceptives), we cannot rule out any additional influences of hormonal contraceptives that may be contributing to the relationship, such as the presence of synthetic hormones. Future studies could examine these questions by investigating populations where endogenous hormone levels are chronically low (such as low-weight or postmenopausal women), or by systematically examining the effects of contraceptives with varying concentrations of synthetic hormones.

Like low estradiol women, the outcomes for men during extinction recall did not differ from high estradiol women or women using contraceptives. This is not surprising, given the reference range for estradiol levels in young males is similar to that of the low estradiol women in the current study (Leifke, Gorenoi, Wichers, Von Zur Muhlen, Von Buren, and Brabant, 2000). Regardless, it is likely that any effects of hormones in males are modulated by testosterone levels, given recent evidence that blocking the conversion of testosterone to estradiol in male rodents impairs fear extinction recall (Graham and Milad, 2014). Future studies examining this issue in males would benefit from measuring both estradiol and testosterone levels in addition to assessing fear extinction.

Conclusions

The current findings add to previous research showing estradiol influences the extinction of conditioned fear (Chang et al., 2009; Graham and Milad, 2013; Milad et al., 2009; Milad et al., 2010; Rey et al., 2014; Zeidan et al., 2011). Importantly, we found no evidence of a link between estradiol and cognitive beliefs about extinguished cues; in fact women using contraceptives showed a dissociation between their SCR and cognitive beliefs. This highlights the value of examining multiple indexes of fear responses when investigating the role of estradiol in conditioned fear extinction, particularly with a view to identifying the potential influence of sex hormones on exposure therapy.

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Tables

Table 1. Psychometrics and baseline psychophysiological results.

	H-EST (*n* = 20) Mean (SD)	**L-EST (*n* = 19)** Mean (SD)	**Hrm-C (*n* = 16)** Mean (SD)	**Males (*n* = 18)** Mean (SD)	*p*-value
Age	21.9 (3.44)	21.5 (3.74)	21.7 (3.02)	23.3 (3.77)	.45
ERQ Reappraisal	30.8 (4.10)	29.5 (4.42)	29.5 (4.30)	29.3 (4.81)	.69
ERQ Suppression	13.8 (4.36)	14.8 (3.68)	14.5 (4.21)	17.0 (4.99)	.15
BAI	10.9 (6.91)	7.4 (4.86)	9.6 (6.25)	7.4 (4.96)	.18
BDI	7.4 (3.39)	6.5 (5.26)	6.3 (5.04)	6.2 (5.62)	.86
STAI-T	38.0 (7.74)	40.5 (7.33)	35.3 (8.56)	37.4 (10.78)	.36
Shock Level	9.7 (5.68)	13.5 (8.45)	10.2 (6.26)	13.6 (9.14)	.24
Baseline SCR	0.43 (1.13)	0.52 (0.95)	0.36 (1.24)	0.83 (1.13)	.61

Figure Legends

Fig 1. Mean (± SEM) serum estradiol levels (pmol/L) for the three female groups. *n* = 20 for high estradiol (H-EST), *n* = 19 for low estradiol (L-EST), and *n* = 16 for hormonal contraceptive users (Hrm-C). *$p < .05$; H-EST vs. L-EST, and L-EST vs. Hrm-C.

Fig. 2. (A) Mean (±SEM) differential skin conductance responses (SCRs) during conditioning (average across trials) and the end of extinction training (average of last three trials) on Day 1. *$p < .05$; conditioning vs. end of extinction. **(B)** Mean (±SEM) percent recovery of SCRs at extinction recall on Day 2. *n* = 20 for high estradiol (H-EST), *n* = 19 for low estradiol (L-EST), *n* = 16 for hormonal contraceptive users (Hrm-C), and *n* = 18 for Males. * = $p < 0.05$; H-EST vs. Hrm-C.

Fig. 3. Mean (±SEM) shock expectancy ratings for the CS+ and CS- trials across conditioning (Cond.), extinction (Ext.) training, and extinction (Ext.) recall. *n* = 20 for high estradiol (H-EST), *n* = 19 for low estradiol (L-EST), *n* = 16 for hormonal contraceptive users (Hrm-C), and *n* = 18 for Males. * = $p < 0.05$; Hrm-C vs. Males (CS+ Cond.), and first four trials vs. last three trials (CS+ Ext. Recall, Males only).

Fig. 4. Mean (±SEM) valence (like/dislike) ratings for the CS+ and CS- trials across conditioning (Cond.), extinction (Ext.) training, and extinction (Ext.) recall. *n* = 20 for high estradiol (H-EST), *n* = 19 for low estradiol (L-EST), *n* = 16 for hormonal contraceptive users (Hrm-C), and *n* = 18 for Males. There were no group differences or interactions.