Supplemental Information

Supplemental Methods & Materials

Animal Behavioral Apparatus and Procedure

Experimental procedures were performed in 25 x 29 x 29 cm^3 Plexiglas chambers (Coulbourn Instruments, Whitehall, PA) located inside sound-attenuated boxes (Med Associates, Burlington, VT), which also contained a single overhead house light, a speaker for conditioned stimulus (CS) presentations, and a video camera to monitor behavior throughout the sessions. The Plexiglas chambers were set up with a metal bar apparatus through which food pellets (Bioserve, Frenchtown, NJ) were delivered as the bar was pressed at a variable interval (GraphicState 3.03, Coulbourn Instruments, Whitehall, PA). The bottoms of the chambers were composed of stainless-steel bars through which a mild electric shock could be delivered. The CS was a 30 s presentation of a 4 kHz tone with an intensity of 80 dB. For the duration of the tone presentation, a light-emitting diode (LED) indicator would activate (this light was not visible to the animals and could only be seen on the video recordings used to monitor behavior).

Rats were trained to press a bar for a food pellet for approximately two weeks prior to the experiment. Successful bar-press learning is required to establish a consistent activity level against which to assess freezing, the measure for fear behavior (1). Rats that failed to establish operant conditioning at the predetermined learning criteria (12 presses per minute at the variable interval of 60 s) were excluded preemptively and did not undergo any phase of the experiment.

Vaginal epithelial cells were stained using DipQuick counter stain kit (Jorgensen Laboratories, Loveland, CO) and examined under a microscope daily over the course of at least two full

estrous cycles. Sample slides were randomly selected to be re-examined by collaborators to ensure consistency and accuracy of readings. For consistent cycle monitoring, vaginal swabs were taken between 8:00 and 10:00 am each day. To control for any effects of the handling process involved in swabbing, rats were returned to their home cages for at least 60 min before the onset of any experimental stage. Females who were cycling abnormally were also excluded from the experiment before it began.

Animal Experiment 3: Influence of estradiol on c-fos expression in the vmPFC and amygdala

RNA was extracted from approximately 20–30 mg of tissue using TRIzol® Reagent extraction process (Invitrogen, Carlsbad, CA). Total RNA quality was assessed by spectroscopy, and where deemed adequate, was reverse transcribed to cDNA using the Two Step RT-PCR Kit (Invitrogen) following the manufacturer's instructions in a Perkin Etus Thermal Cycler 480. The gene expression patterns were assessed using quantitative polymerase chain reaction PCR (qPCR). cDNA was analyzed by qPCR using the Stratagene mx3005P instrument (La Jolla, CA) with the following cycling conditions: step 1) 55°C for 2 min and 95°C for 2 min; step 2) amplification at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 50 cycles. A melting curve was used to confirm the specificity of each primer pair. Each sample was run in triplicate to exclude outliers. Primers used for amplification were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) (2) for amplicons between 100 and 200 base pairs (see Table S1 for primer sequences). Gene expression was analyzed using the ΔΔCT method, using b-actin as the normalizer gene. We computed the average gene expression for each experimental condition (estradiol, EST, $n = 5$) relative to the control condition (vehicle, VEH, $n = 5$).

Table S1. Entrez Gene ID Numbers and Primer Sequences of Genes Used for Quantitative Polymerase Chain Reaction Experiments

Gene	Entrez Gene No.	Forward Sequence	Reverse Sequence
cFos	2353	GAA GGA ACC AGA CAG GTC CA	TCA CCC TGC CTC TTC TCA AT
b-actin	450133	GTC GTA CCA CTG GCA TTG TG	TCT CAG CTG TGG TGG TGA AG

Human Conditioning and Extinction Procedure

Two digital photographs consisted of the visual contexts in which a lamp was switched from the off position (no color) to one of three colored lights, constituting the CS. All images were displayed on a computer monitor approximately two feet behind the subject and viewed on a mirror while the subject was in a 3T functional magnetic resonance imaging (fMRI) scanner (Siemens Medical Systems, Iselin, NJ). The unconditioned stimulus (US) was a 500 ms electric shock delivered through electrodes attached to the second and third fingers of the right hand, previously selected by the participant to be “highly annoying but not painful” (3). The electrodes were attached to the fingertips during each phase of the study, though the US was presented only during conditioning.

Subjects were instructed to fast after midnight prior to participation. 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%.

Behavioral and Psychophysiological Data Analysis

Skin conductance responses (SCR) were calculated by subtracting the max response during cue presentation from the average response of the two seconds immediately preceding context onset (as previously described (3)). The SCR values were then square-root transformed to reduce heteroscedasticity. Skin conductance levels (SCL) were measured during the five seconds preceding the onset of each habituation session trial and then averaged across eight trials to yield a baseline SCL. To evaluate the unconditioned response (UCR) to the shock, the average response in the first second after the shock (before skin conductance increases) was subtracted from the peak level during the five seconds post-shock. Extinction retention index was measured by dividing the average SCR of the first two trials during recall by the peak response in the conditioning trial, and multiplying by 100 to yield a percentage of maximal conditioned responding. This was then subtracted from 100 to give the extinction retention index. All data are represented as means ± the standard error of the mean (SE).

Image Acquisition and Functional MRI Data Analysis

A Trio 3.0-Tesla whole-body, high-speed imaging device with a 12-channel gradient head coil was used (Siemens Medical Systems, Iselin, NJ). An automated scout image was obtained and shimming procedures were performed followed by high-resolution, three-dimensional magnetization prepared rapid gradient echo sequences (repetition time/echo time [TR/TE]/flip angle = 7.25 ms/3 ms/7^{o}; 1 mm X 1 mm in plane X 1.3 mm) were collected for spatial normalization and positioning the subsequent scans. Registration of individual functional scans was done using T1 (TR/TE/flip angle = 8 sec/39 msec/90^{o}) and T2 (TR/TE/flip angle = 10 sec/48 msec/120^{o}) sequences. Functional MRI images (i.e., blood oxygen level-dependent (BOLD))

were acquired with gradient echo T2*-weighted sequences (TR/TE/flip angle = 3 sec/30 msec/90^{o}) (4). The T1, T2, and gradient-echo functional images were collected in the same plane (45 coronal oblique slices parallel to the anterior-posterior commissure line, tilted 30^{o} anterior) with the same slice thickness (3 mm x 3 mm x 3 mm).

All fMRI data were analyzed with the Freesurfer Functional Analysis Stream (http://surfer.nmr.mgh.harvard.edu). Functional runs were motion-corrected, spatially smoothed (full-width-at-half-maximal = 5 mm) with a three-dimensional Gaussian filter, and intensity-normalized to the low-level baseline. Individual functional runs were individually registered to their anatomical volumes, and registrations were visually inspected for accuracy. Responses to the stimuli at each voxel were estimated with an event-related design, and by convolving the functional signal for each event with a canonical hemodynamic response function. The analysis included a linear correction to account for drift.

Supplemental Results

Additional analyses on freezing levels during conditioning and extinction learning

Animal Studies

Estrogen-receptor sub-types and fear extinction. During conditioning, a one-way ANOVA revealed no significant main effect of group ($F_{(2,34)}$ = 0.96, p = 0.39). During extinction, a repeated measures ANOVA revealed a significant main effect of trial, ($F_{(3,96)}$ = 4.35, $p < 0.01$), indicating that freezing was significantly lower by the end of extinction, but no significant main effect of group ($F_{(2,34)}$ = 2.79, p = 0.08), and no significant interaction ($F_{(6,96)}$ = 0.82, p = 0.56).

Estradiol and extinction memory consolidation. During habituation and conditioning, an independent samples *t*-test revealed no significant difference between the VEH and EST groups ($t_{(28)}$ = -1.05, p = 0.30, $t_{(28)}$ = 0.27, p = 0.79, respectively). During extinction, a repeated measures ANOVA revealed a significant main effect of trial ($F_{(3,84)}$ = 19.74, $p < 0.01$), indicating that freezing was significantly lower by the end of extinction, but no significant main effect of group ($F_{(1,28)}$ = 0.80, p = 0.38), and no significant interaction ($F_{(3,84)}$ = 0.17, p = 0.91).

In the 4 hr-post experiment, in which the injection took place four hours post-extinction training as opposed to immediately following, no significant difference between the VEH and EST groups was found during habituation ($t_{(15)}$ = 0.14, p = 0.89) or conditioning ($t_{(15)}$ = 0.05, p = 0.96). During extinction, a repeated measures ANOVA revealed a significant main effect of trial, ($F_{(3,45)}$ = 5.00, $p < 0.01$), indicating that freezing was significantly lower by the end of extinction, but no significant main effect of group ($F_{(1,15)}$ = 1.35, p = 0.26), and no significant interaction ($F_{(3,45)}$ = 1.60, p = 0.20).

Human Studies

Evidence for extinction learning. Extinction learning did occur and did not differ between groups. To specifically examine the extinction learning between groups, we conducted an additional analysis in which we compared the maximum acquisition levels during fear conditioning to the average of the last 2 trials of extinction learning. This analysis revealed that significant extinction occurred in both groups, and that both groups did not differ in the level of extinction training. There was a main effect of trial (drop from acquisition to end of extinction) but no main effect of group and no interaction (a drop of 0.46 $\pm$ 0.09 to 0.07 $\pm$ 0.06 μS in the H-

EST group, and 0.44 ± 0.09 to 0.04 ± 0.06 μS in the L-EST group, $p < 0.05$ for main effect of trial, and $p > 0.05$ for main effect of group and interaction).

Psychometric and behavioral tests

Table S2. High vs. low estradiol comparisons in demographics, anxiety measures, psychophysiological measures, and NEO-FFI personality measures.

	High Estrogen	**Low Estrogen**	***t*-value**	***P*-value**
Demographics				
Age (yrs)	23.41 ± 0.75	23.35 ± 0.61	-0.06	0.95
Years of Education	15.94 ± 0.27	15.94 ± 0.27	0.00	1.00
Pyschophysiological Measures				
Shock (mA)	1.84 ± 0.21	2.04 ± 0.22	0.67	0.51
UCR (μs)	1.60 ± 0.36	1.45 ± 0.36	-0.29	0.77
Baseline SCL (μs)	0.45 ± 0.14	0.22 ± 0.06	-1.44	0.16
Psychometric Measures				
Spielberger Trait	31.11 ± 1.34	31.82 ± 1.60	0.34	0.74
Spielberger State	28.67 ± 1.90	31.81 ± 2.60	0.97	0.34
Anxiety Sensitivity Index	11.82 ± 1.97	14.06 ± 2.23	.755	0.46
Beck Anxiety	2.00 ± .57	2.06 ± .46	.085	0.93
Beck Depression	2.76 ± .82	0.81 ± .45	-2.04	0.05*
Mindfulness	65.00 ± 2.22	65.29 ± 2.33	0.09	0.93
NEO-FFI Personality				
Neuroticism	38.76 ± 2.93	42.25 ± 2.00	0.97	0.34
Extraversion	58.41 ± 3.02	59.69 ± 2.31	0.33	0.74
Openness	61.64 ± 3.64	58.00 ± 2.56	-0.81	0.42
Agreeableness	57.18 ± 2.97	56.38 ± 2.90	-0.19	0.85
Conscientiousness	47.18 ± 3.45	47.00 ± 2.59	-0.04	0.97

*$p \leq .05$

NEO-FFI, NEO Five Factor Inventory; SCL, skin conductance levels; UCR, unconditioned response.

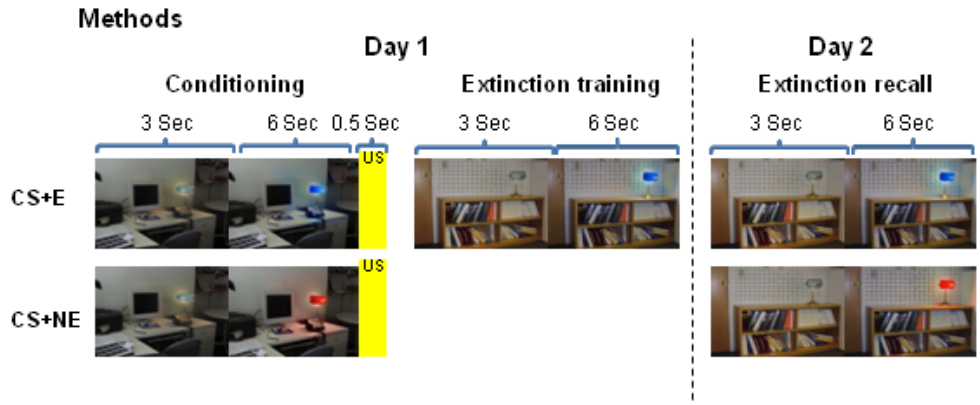

Figure S1. Schematic of experimental paradigm. Images shown display visual contexts used in the experiment. Three conditioned stimuli (CS) were presented: Two CS+ lights followed by shock (unconditioned stimulus, US) and a CS- (no shock, light not shown in figure). The CS+ lights were presented in the conditioning context (office) and after a 1-minute break, one CS+ was extinguished (CS+E) in the safe context (conference room) while the other was not extinguished (CS+NE). Extinction recall was tested on day 2 in the extinction context in which all CSs were presented without presentation of the US (adapted from Milad *et al.* (5)).

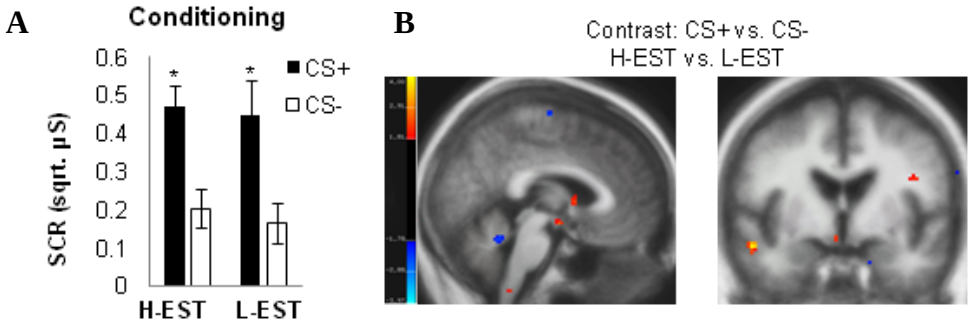

Figure S2. Estradiol does not affect fear conditioning. **(A)** Women in the high estradiol group (H-EST, n = 17) and the low estradiol group (L-EST, n =17) show increased skin conductance response to the CS+ relative to the CS- during fear conditioning ($F_{(1,32)} = 29.47$, $p < 0.01$). There was no significant main effect of group ($F_{(1,32)} = 0.16$, $p = 0.69$) and no significant Group x Stimulus interaction ($F_{(1,32)} = 0.44$, $p = 0.51$). **(B)** Group x Stimulus interaction contrast compares H-EST vs. L-EST, functional activation during first four CS+ trials versus first four CS- trials. Threshold for display image is set at $p = 0.01$. No significant differences were found in our *a priori* regions of interest (vmPFC and amygdala). CS, conditioned stimuli; SCR, skin conductance responses; vmPFC, ventromedial prefrontal cortex.

Supplemental References

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5. Milad MR, Zeidan MA, Contero A, Pitman RK, Klibanski A, Rauch SL, *et al.* (2010): The influence of gonadal hormones on conditioned fear extinction in healthy humans. *Neuroscience*. 168:652-658.