Brief Communication

Sex differences in fear discrimination do not manifest as differences in conditioned inhibition

Allison R. Foilb, Julia Bals, Mary C. Sarlitto, and John P. Christianson

Department of Psychology, Boston College, Chestnut Hill, Massachusetts 02467, USA

Distinguishing safety from danger is necessary for survival, but is aberrant in individuals with post-traumatic stress disorder (PTSD). While PTSD is more prevalent in women than men, research on sex differences in safety learning is limited. Here, female rats demonstrated greater fear discrimination than males in a CS+/CS− paradigm. To determine if this sex difference transferred to fear inhibition, rats were tested for conditioned inhibition in a summation test with the CS+ and CS− presented in compound; no sex difference emerged. The results suggest sex differences in the neural mechanisms of discrimination learning but not recall of a fear inhibitor.

[Supplemental material is available for this article.]

Discrimination between safety and danger is necessary for survival. Incorrect evaluation of a stimulus as safe when it is dangerous could result in harm, while determining a stimulus as dangerous when it is safe results in unnecessary fear and anxiety. Further, when a safety cue is well learned, it can reduce fear in the presence of a danger cue, a learning phenomenon known as conditioned inhibition of fear (Kazama et al. 2013). Overgeneralization of fear-related cues and aberrant conditioned inhibition of fear are seen in individuals with post-traumatic stress disorder (PTSD) (Jovanovic et al. 2012; Costanzo et al. 2016; Jenewein et al. 2016). Females are more likely to be diagnosed with PTSD than males (Kilpatrick et al. 2013; Kessler et al. 1995) and safety learning and discrimination are sensitive to hormonal birth control and trauma history in females compared with males (Gamwell et al. 2015; Lornsdorf et al. 2015). Translational research regarding sex differences in rodent fear discrimination is in its infancy, but has indicated greater discrimination in females compared with males, with later generalization of fear (Day et al. 2016). Biological sex is a significant factor in the expression of fear-based psychoses (Shansky 2015) and a better understanding basic behavioral differences in fear discrimination is needed to more fully realize the impact of sex for an individual's health (Shansky and Woolley 2016).

Intact male and normally cycling female adult Sprague-Dawley rats were used as follows: ($n = 24$/sex) for both fear discrimination acquisition and later fear discrimination recall, ($n = 12$/sex) for fear discrimination acquisition and conditioned inhibition, and an additional ($n = 24$/sex) that were given fear discrimination acquisition only, for a total $N = 120$. CS+/CS− discrimination conditioning adapted from Myers and Davis (2004) was used (Chen et al. 2016; Foilb and Christianson 2016; Foilb et al. 2016). Conditioning trials began with a 5 sec, 1 kHz (75 dB) tone, followed by either the CS+ or CS− for 15 sec. CS+ trials coterminated with a 500 msec, 1.2 mA scrambled foot shock. Conditioning consisted of 15 presentations of each cue in quasi-random order, so that neither cue occurred more than twice in series with a 90 sec inter-trial interval. A flashing light or white noise pip were used as conditioning stimuli. Behavioral research has noted sex differences in fear expression, where many female rats exhibit an active response—termed darting—when presented with fear stimuli, as opposed to the passive freezing response typically observed in males (Gruene et al. 2015a). A trained observer screened videos for evidence of darting, but in our experimental conditions darting occurred too infrequently to analyze. Therefore, freezing was used as a behavioral measurement of fear (Fanselow 1980) as previously (Christianson et al. 2011; Chen et al. 2016; Foilb and Christianson 2016; Foilb et al. 2016). Freezing data were analyzed by analysis of variance (ANOVA) with sex as a between subjects factor and cue type and trial as within subjects factors with Sidak post hoc contrasts. Additional methodological details are provided in the online Supplemental Material.

Experiment 1 investigated sex differences in fear discrimination acquisition ($n = 60$/sex; Fig. 1) and recall ($n = 24$/sex; Fig. 2). Fear discrimination recall was tested the day after the initial conditioning session. The test began with 2 min of baseline context exposure, followed by 30 sec presentations of CS+ and CS− cues without presentation of shock. Each cue was presented 10 times in pseudorandom order with a 30-sec inter-trial-interval. Experiment 2 investigated sex differences in the acquisition and recall of conditioned inhibition ($n = 12$/sex; Fig. 3). Rats received five conditioning sessions as previously (Foilb et al. 2016). Summation tests were similar to the recall test used in Experiment 1 with CS+/− trials added in which CS+ and CS− were presented in compound. The tests began with 2 min of baseline context, followed by 30 sec of each cue—CS+, CS− and CS+/− —three times each in randomized order with a 30-sec inter-trial interval.

Females displayed discrimination earlier in conditioning than males (Fig. 1A). A three-way ANOVA of sex by cue by trial block (five blocks of three cue trials) revealed a main effect of trial block, $F_{(4,472)} = 40.579$, $P < 0.001$, and interactions of trial by sex, $F_{(4,\ 472)} = 3.491$, $P = 0.008$, and cue by trial, $F_{(4,472)} = 45.474$, $P < 0.001$, but no significant interaction of trial block by cue by sex interaction, $F_{(4,473)} = 1.048$, $P = 0.382$. Post hoc comparisons showed that females significantly discriminated between the CS+ and CS− in the first-trial block ($P = 0.007$), while males failed to show this discrimination until the second trial block ($P = 0.769$ on trial block 1; $P = 0.004$ on trial block 2). Females also displayed significantly reduced freezing to the CS+ compared with males on

Corresponding author: foilb@bc.edu
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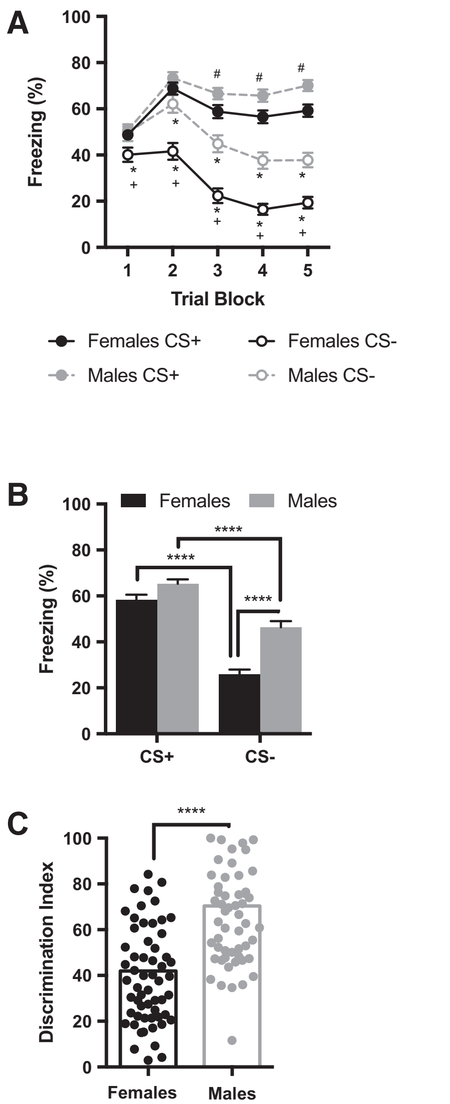

Figure 1. Sex differences in fear expression and discrimination during CS+/CS− conditioning. (*A*) Freezing averages (±SEM) to the CS+ and CS− across trial blocks of three trials in conditioning. Females significantly discriminated between the CS+ and CS− within the first trial block of each cue ($P = 0.001$), while males did not make this discrimination until the second trial blocks ($P = 0.002$). Females also displayed less freezing to the CS− than males on all trial blocks ($Ps < 0.05$). (*) Significant difference between CS+ and CS− within sex, ($^{+}$) significant difference between males and females to the CS−, and ($^{\#}$) significant difference between sexes on the CS+. (*B*) Freezing to CS+ and CS− cues during conditioning. Freezing to each cue was averaged over 15 presentations and converted to a percentage of time (+SEM). Both males and females significantly discriminated between the CS+ and CS− ($Ps < 0.0001$), but females displayed significantly less average freezing to both the CS+ ($P < 0.05$) and CS− ($P < 0.0001$) compared with the males. (*C*) Mean (and individual replicates) discrimination indices (time freezing to CS−/time freezing to CS+ × 100) during conditioning. An index *below* 100 signifies reduced freezing to the CS− compared with the CS+. (*) $P < 0.05$, (****) $P < 0.0001$.

trial blocks 3, 4, and 5 ($Ps = 0.039$, 0.018, 0.003, respectively) as well as less freezing to the CS− compared with males on all trial blocks ($P = 0.048$ on the first trial block, $Ps < 0.001$ on trial blocks 2, 3, 4, and 5).

Freezing to each cue during conditioning is summarized in Figure 1B. ANOVA revealed main effects of sex, $F_{(1,118)} = 23.42$, $P < 0.0001$, and cue, $F_{(1,118)} = 312.3$, $P < 0.0001$, and a cue by sex interaction, $F_{(1,118)} = 22.15$, $P < 0.0001$. Post hoc analyses showed that both sexes significantly discriminated between the CS+ and CS−, $Ps < 0.0001$, but that females displayed significantly less freezing to the CS−, $P < 0.0001$, compared with males. A discrimination index was calculated (freezing to CS+/freezing to CS− × 100) as a measure of animals' ability to discriminate between the CS+ and CS− (Fig. 1C). Males and females showed significantly different discrimination indices, $t_{(118)} = 6.343$, $P < 0.0001$, with females showing more discrimination compared with males. The sex difference was consistent across all cohorts of animals (Supplemental Fig. 1).

Sex differences were evident in fear discrimination recall tests. An ANOVA of the CS trials across test and sex (Fig. 2B), revealed a main effect of trial, $F_{(9,414)} = 14.856$, $P < 0.001$, and cue by trial

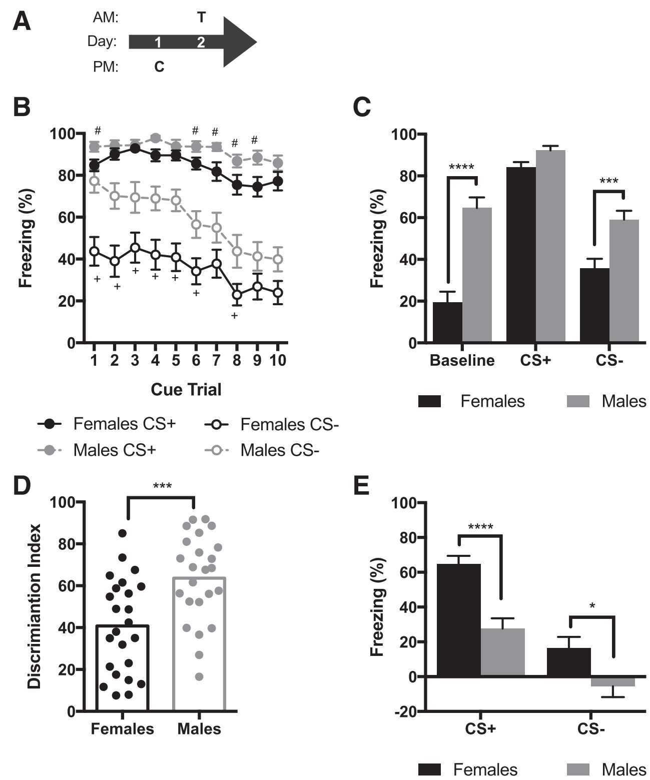

Figure 2. Sex differences in fear recall and discrimination. (*A*) Timeline of recall testing where conditioning (C) occurred in the afternoon of day 1 and recall testing (T) occurred the morning of day 2. (*B*) Mean freezing (±SEM) to the CS+ and CS− across each of the 10 cue presentations in testing. Both sexes significantly discriminated between the CS+ and CS− on all trials (significance not marked on graph). Females displayed significantly less freezing to the CS− compared with males at the start of the test, while males reduced freezing to the CS− throughout the test. ($^{+}$) Significant difference between sexes to the CS− ($Ps < 0.05$), and ($^{\#}$) significant difference between sexes to the CS+ ($Ps < 0.05$). (*C*) Average freezing (+SEM) to baseline context exposure, the CS+ and CS− during the recall test. Females displayed significantly less freezing to the baseline context and the CS− compared with males. There was no sex difference in freezing to the CS+. (*D*) Mean (individual replicates) discrimination indices (time freezing to CS−/time freezing to CS+ × 100) during recall testing. Females have a significantly lower discrimination index compared with males. (*E*) Average freezing (+SEM) to CS+ and CS− with baseline freezing subtracted. When comparing CS+ to CS− freezing, males and females showed significant discrimination (CS+ versus CS− $Ps < 0.0001$), while females showed greater freezing to the baseline subtracted CS+ and baseline subtracted CS− compared with males ($P < 0.0001$ and $P < 0.05$, respectively). (*) $P < 0.05$, (***) $P < 0.001$, (****) $P < 0.0001$.

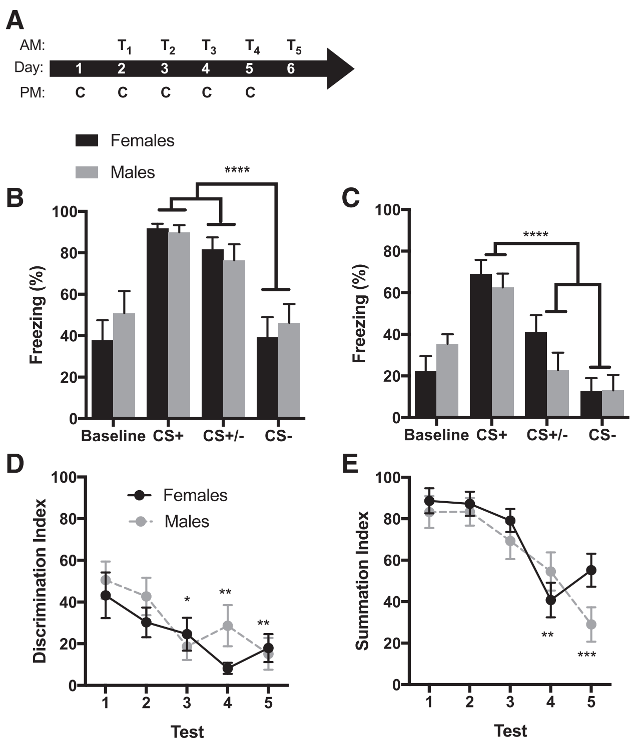

Figure 3. Sex differences were not evident in conditioned inhibition of fear. (A) Timeline of experiment where conditioning (*C*) occurred each afternoon and recall testing (T) occurred the morning after each conditioning session. (*B*) Average freezing (+SEM) to baseline context exposure, the CS+, CS+/− and CS− during the recall test on day 1. Animals significantly discriminated between CS+ and CS− ($P < 0.0001$), but did not discriminate between the CS+ and CS+/− ($P = 0.33$). (*C*) Average freezing (+SEM) to baseline context exposure, the CS+, CS+/− and CS− during the recall test on day 5. Animals significantly discriminated between all cues, with reduced freezing to the CS+/− and the CS− compared with the CS+ ($Ps < 0.0001$). (*D*) Discrimination indices (±SEM) across each of the five tests. While there was no sex difference on any test day, discrimination improved on tests 3 ($P < 0.05$), 4 and 5 ($Ps < 0.01$) compared with tests 1 and 2. (*E*) Summation indices (time freezing to CS+/−/time freezing to CS+ × 100; ±SEM) across each of the five tests. There were no sex differences in summation, but improved inhibitory summation on tests 4 and 5 compared with tests 1, 2, and 3 ($Ps < 0.01$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$.

interaction, $F_{(9,414)} = 3.333$, $P = 0.001$, but no significant interactions of sex by trial, $F_{(9,414)} = 0.421$, $P = 0.924$, or cue by trial by sex, $F_{(9,\ 414)} = 1.668$, $P = 0.095$. Females significantly discriminated between the CS+ and CS− on the first trial ($P < 0.001$), and continued this level of discrimination throughout the test. Males also displayed immediate discrimination on trial 1 ($P = 0.004$) and match the discrimination level of the females throughout the remainder of the test ($P < 0.001$). While females' response to CS− was stable across trials, males reduced freezing on CS− presentations 7, 8, and 9 compared with trial 1 ($Ps = 0.004$, 0.003, and <0.001, respectively), as well as on trial 10 compared with trials 2 ($P = 0.048$) and 3 ($P = 0.037$), and on CS+ presentations 8, 9, and 10 compared with presentation 4 ($Ps = 0.034$, 0.03, and 0.007, respectively). Males and females significantly differed in their response to the CS+ on trials 1 ($P = 0.019$), 6 ($P = 0.038$), 7 ($P = 0.019$), 8 ($P = 0.049$), and 9 ($P = 0.020$) and showed even more differential responding to the CS−, with significantly different freezing on trials 1–6 and trial 8 ($Ps < 0.032$).

Figure 2C shows average freezing to each cue during recall. ANOVA revealed main of effects of cue, $F_{(2,92)} = 93.01$, $P <$ 0.0001, sex, $F_{(1,46)} = 40.33$, $P < 0.0001$, and cue by sex interaction, $F_{(2,92)} = 12.74$, $P < 0.0001$. Post hoc analyses showed that females continued to freeze significantly less than males to the CS− ($P =$ 0.0003), as well as to the baseline context at the start of the test ($P < 0.0001$). Females froze significantly less to context than all other cues ($Ps < 0.01$) and froze significantly less to the CS− than the CS+ ($P < 0.0001$). Males also froze significantly less to the CS− and baseline context compared with the CS+ ($Ps <$ 0.0001), but freezing to the CS− and baseline context did not significantly differ ($P = 0.641$). This sex by cue interaction is summarized by significant difference in discrimination index (Fig. 2D, $t_{(46)} = 3.63$, $P = 0.0007$). To indicate that the sex differences in cue response were not simply artifacts of differential baseline fear, we subtracted baseline freezing from average freezing to each cue. Here we find main effects of cue, $F_{(1,46)} = 248.6$, $P <$ 0.0001, sex, $F(1,46) = 14.05$, $P = 0.0005$, and a cue by sex interaction, $F_{(1,46)} = 8.692$, $P = 0.005$. These differences indicate that the sex difference in discrimination is not only due to the sex difference in baseline fear, but that there is a general sex difference in discrimination between the CS and the conditioning context (Supplemental Fig. 2).

In Experiment 2 conditioned inhibition of fear was assessed in summation tests. On test 1, analysis on average freezing to each cue (Fig. 3B) in a 4 (cue) by 2 (sex) ANOVA revealed a significant effect of cue $F_{(3,66)} = 30.06$, $P < 0.0001$, with significantly reduced freezing to CS− compared with the CS+ and CS+/− ($Ps <$ 0.0001) and significantly increased freezing to the CS+ and CS +/− compared with baseline context ($Ps < 0.0001$). Animals did not discriminate between the CS+ and CS+/− in this test ($P =$ 0.33). There was no main effect of sex, $F_{(1,22)} = 0.1599$, $P =$ 0.6936, or cue by sex interaction, $F_{(3,66)} = 0.8828$, $P = 0.4547$. The difference in baseline context freezing that was observed in Experiment 1 was present in this smaller sample as a trend, but did not reach significance. This may reflect an effect of different estrous status on the test day between experiments, or sampling error and the intrinsic variability in this dependent measure, which is discussed in the online Supplemental Material. The lack of sex difference in simple CS+/CS− discrimination here is likely the result of differences in cue presentation in the summation test compared with the recall test. Specifically, the first presentation of the CS− in the summation test is as a compound with the CS+. In test 5 (Fig. 3C), the same analysis revealed a significant main effect of cue, $F_{(3,66)} = 35.72$, $P < 0.0001$, and a significant cue by sex interaction, $F_{(3,66)} = 3.15$, $P = 0.0307$, but no main effect of sex, $F_{(1,22)} =$ 0.1521, $P = 0.7003$. Post hoc analyses showed that animals displayed greater fear to the CS+ compared with the baseline context exposure, CS− and CS+/− compound ($Ps < 0.0001$). Animals also froze less to the CS− compared with the CS+/− and baseline context ($Ps < 0.05$).

Comparing discrimination indices across all five summation tests (Fig. 3D) with a 5 (test day) × 2 (sex) ANOVA revealed a main effect of test day, $F_{(4,88)} = 5.603$, $P = 0.0005$, but no main effect of sex, $F_{(1,22)} = 1.328$, $P = 0.2615$, and no test day by sex interaction, $F_{(4,88)} = 0.9538$, $P = 0.4371$. Discrimination between the CS + and CS− was significantly improved on tests 3, 4, and 5 compared with test 1 ($Ps < 0.05$). A summation index was calculated as freezing to CS+/−/freezing to CS+ × 100 as a measurement of conditioned inhibition (Fig. 3D). ANOVA revealed a main effect of test day, $F_{(4,88)} = 14.9$, $P < 0.0001$, but no main effect of sex, $F_{(1,22)} =$ 1.949, $P = 0.1767$, and no test day by sex interaction, $F_{(4,88)} =$ 1.751, $P = 0.1460$. Rats showed greater inhibition on tests 4 and 5 compared with tests 1, 2, and 3 ($Ps < 0.01$).

We observed a marked difference in fear discrimination between male and female rats. Differential freezing to the fear CS+ and safe CS− was greater in females compared with males during the initial conditioning and in a recall test one day later, furthermore, females exhibited greater initial discrimination between the context and the CS. These results are consistent with recent findings (Day et al. 2016), although in contrast to studies on fear discrimination in humans where females show less discrimination than males (Gamwell et al. 2015; Lornsdorf et al. 2015). Females also displayed lower contextual freezing, consistent with several prior reports in rodents (Pryce et al. 1999; Daviu et al. 2014; Pettersson et al. 2016, although see Keiser et al. 2017 for exception). When this difference in contextual freezing was subtracted from freezing to each cues, there was a larger difference in freezing to CS+, whereas the main difference in the uncorrected data was primarily in freezing to the CS−. This indicates that the sex difference may be in discrimination per se, rather than freezing to a particular cue.

The discrimination test required that rats flexibily transition between fear and safe states, which may favor the inherently more active female rats (Gruene et al. 2015a,b). However, after several days of conditioning, females may accrue more fear to the CS− (Day et al. 2016) which could manifest as a transition from more active behavior early in conditioning to more passive, i.e., male-like, with additional conditioning and stress (Foilb and Christianson 2016) and account for the lack of sex difference in conditioned inhibition summation tests. This outcome contrasts some of the results of Day et al. (2016) in which after repeated discrimination conditioning the CS− failed to pass a retardation test of conditioned inhibition. Whether these different empirical results are a consequence procedural differences or of different neural mechanism underlying summation and retardation phenomena remains unknown.

The sex difference in discrimination indicates that there may be sex differences in the neural circuitry underlying discrimination learning. Interactions between the amygdala and prefrontal cortex are critical for CS+/CS− discrimination (Likhtik et al. 2014) and sexual dimorphisms observed in humans include sex differences in amygdala anatomy (Ruigrok et al. 2014), amygdala response to negative or stressful emotions (Stevens and Hamann 2012; Kogler et al. 2015), and amygdala functional connectivity (Lopez-Larson et al. 2011; Engman et al. 2016). Sex differences have been found in the rodent medial prefrontal cortex (Baran et al. 2010; Fenton et al. 2014, 2016), a brain region critical to fear expression, extinction, and discrimination (Sotres-Bayon and Quirk 2010; Milad et al. 2014; Sangha et al. 2014). Similarly, basolateral amygdala projecting neurons of the infralimbic region of the medial prefrontal cortex appear to differently mediate fear expression in males and females (Gruene et al. 2015b). That sex differences in safety learning do not persist in conditioned inhibition of fear also suggests that conditioned inhibition of fear occurs in a neural circuit that is distinguishable from the fear discrimination circuitry.

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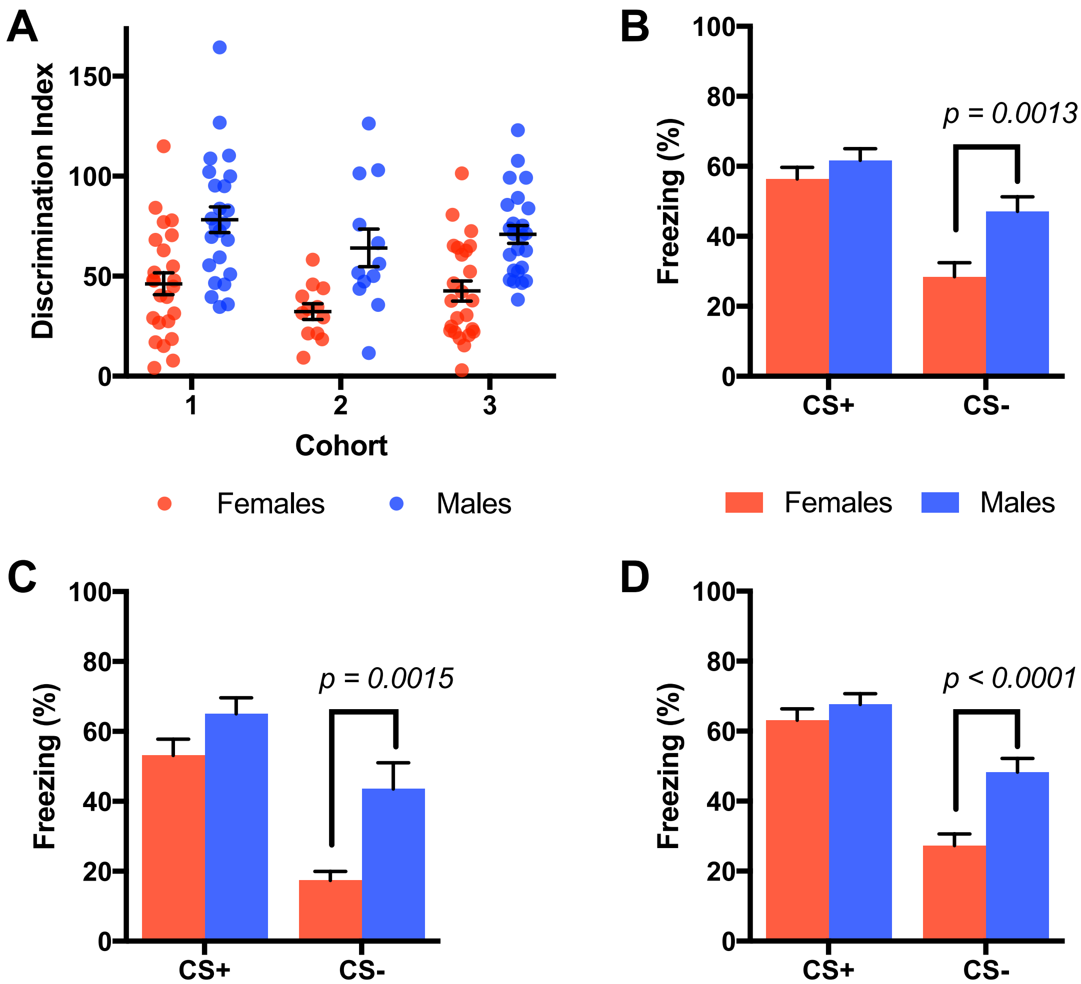
Supplementary Methods:

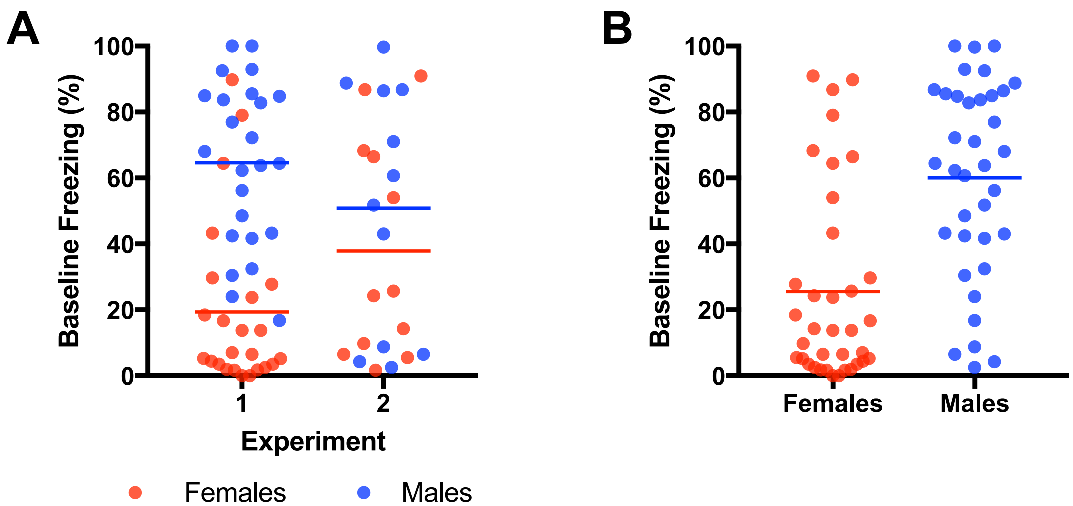
Rats were obtained from Taconic Biosciences (Hudson, NY) weighing 200-250g upon arrival. Rats were same-sex pair housed in plastic tub cages with free access to food and water, and maintained on a 12h light/dark cycle. Males and females were housed in the same colony room, and were run through behavioral procedures simultaneously. All animals were given 7 days to acclimate to colony housing before behavioral procedures. All experimental procedures were reviewed and approved by the Boston College Institutional Animal Care and Use Committee.

Both conditioning and recall testing occurred in the same, dark context: a 10 x 11 x 6in (L x W x H) chamber made of black plastic with wire mesh lids with a stainless steel grid floor. The chamber was housed within a 15 x 12 x 27in (L x W x H) light and sound-attenuating chamber with a fan for ventilation and background noise (~55dB). We have previously shown the fear discrimination and conditioned inhibition develop equally when tested in the conditioning context or a distinct context. To focus on cue discrimination here, the same context was used for all treatments. Infrared illuminators allowed for video observation through an overhead camera. Scrambled foot shock was delivered via shocker Model H13-15, Coulbourn Instruments. The CS+ and CS- stimuli were distinct cues of different modalities: a flashing white LED light cue was 264.0 Lux, 20ms on/off and the white noise pip was 10ms duration, 3 Hz interval, 75 dB. Assignment of stimuli as CS+ or CS- was counterbalanced, and no effect of cue was observed, as reported previously (Chen et al. 2016, Foilb and Christianson 2016, Foilb et al. 2016). Freezing behavior was detected with ANY-Maze computer software (version 4.99, Stoelting, Wood Dale, IL).



Supplementary Figure 1. *Sex differences in acquisition of discrimination in three separate cohorts.* Acquisition of discrimination was established in 3 cohorts of animals. Cohort 1 are animals that received fear discrimination recall (n = 24; Figure 2), cohort 2 received conditioned inhibition recall testing (n = 12; Figure 3) and cohort 3 were animals run through the same conditioning procedures with all the same procedures, but do not have recall data included here (n = 24). **(A)** Mean (± SEM, individual replicates) discrimination indices (time freezing to CS- / time freezing to CS+ X 100) during conditioning of females (red) and males (blue) in each of the 3 cohorts included in Figure 1. An index below 100 signifies reduced freezing to the CS- compared to the CS+. Females displayed significantly lower discrimination indices compared to males, indicating more discrimination, in all 3 cohorts, *p*s < 0.05. **(B)** Mean (+ SEM) freezing to the CS+ and CS- during conditioning in cohort 1. A two-way ANOVA revealed a main effects of sex, F (1, 46) = 6.51, $p = 0.014$, cue, F (1, 46) = 76.74, $p < 0.0001$, and a cue by sex interaction, F (1, 46) = 7.569, $p = 0.008$. Both males and females significantly discriminated between the CS+ and CS- (*p*s < 0.001), but females displayed significantly less average freezing to the CS- ($p = 0.0013$) compared to the males. **(C)** Mean (+ SEM) freezing to the CS+ and CS- during conditioning in cohort 2. A two-way ANOVA found significant main effects of sex, F (1, 22) = 8.785, $p = 0.007$, cue, F (1, 22) = 73.96, $p <$

0.0001, and a cue by sex interaction, $F (1, 22) = 4.572$, $p = 0.0439$. Both sexes significantly discriminated between the CS+ and CS- (*p*s < 0.001), but females displayed significantly less freezing to the CS- ($p = 0.0015$) compared to the males. **(D)** Mean (+ SEM) freezing to the CS+ and CS- during conditioning in cohort 2. Again, a 2-way ANOVA showed significant main effects of sex, $F (1, 46) = 9.164$, $p = 0.004$, cue, $F (1, 46) = 137.2$, $p < 0.0001$, and a cue by sex interaction, $F (1, 46) = 12.25$, $p = 0.001$. And both sexes in this cohort also significantly discriminated between the CS+ and CS- (*p*s $<$ 0.0001), and females froze less to the CS- compared to males ($p < 0.0001$).



Supplementary Figure 2. *Baseline freezing in experiments 1 and 2.* **(A)** Mean (individual replicates) baseline freezing in recall tests of experiment 1 and the first test of experiment 2. Looking at baseline freezing across both experiments in a two-way ANOVA, there was a main effect of sex, $F (1, 68) = 16.38$, $p < 0.001$, and a experiment by sex interaction, $F (1, 68) = 5.028$, $p = 0.0282$. There was no main effect of experiment, $F (1, 68) = 0.1098$, $p = 0.7414$. Although freezing levels changed in males and females between experiments 1 and 2, the mean values did not differ from each other ($p = 0.141$ and $p = 0.3294$, respectively). **(B)** Mean (individual replicates) baseline freezing by sex in test 1 or experiments 1 and 2 combined. Males and females displayed significantly different baseline freezing across the two tests, $t (70) = 4.975$, $p < 0.0001$.