Olfactory-mediated fear conditioning in mice: Simultaneous measurements of fear-potentiated startle and freezing

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Abstract

This study demonstrates that mice display olfactory-cued fear as measured with both freezing and fear-potentiated startle. Following a pre-conditioning test to measure any unconditioned responses to odor, mice received five pairings of a 10-sec odor with a 0.25-s, 0.4-mA footshock. The next day, startle and freezing were measured in the presence and absence of the odor. Both fear measures increased after training with amyl acetate (Experiment 1) and acetophenone (Experiment 2). The enhancement of startle did not occur when the same number of odors and shocks were presented in an unpaired fashion (Experiment 3). Furthermore, mice were able to discriminate between an odor paired with shock and a non-reinforced odor (Experiment 4).

Cue-specific conditioned fear in rodents has been used as a powerful model for the study of learning and memory as well as anxiety disorders. In rodents, fear is often studied using Pavlovian fear conditioning, in which a previously neutral stimulus such as a light or tone (the conditioned stimulus, CS) is paired with an aversive stimulus, e.g. a footshock (the unconditioned stimulus, US). The resulting hypothetical state of fear can be assessed using several different behavioral measures (McAllister & McAllister, 1971). Two widely used measures of conditioned fear are fear-potentiated startle and freezing (Davis, 2000; Fendt & Fanselow, 1999). Fear-potentiated startle (FPS) is defined as an augmented startle response in the presence of the aversive conditioned cue, while freezing is defined as the absence of activity in the presence of the cue (D.C. Blanchard & Blanchard, 1969; Fendt & Fanselow, 1999). Usually these measures are taken independently, although techniques have been introduced to measure both simultaneously (Fendt, 2001; Gewirtz, Falls, & Davis, 1997; Leaton & Borscz, 1985). Numerous studies have demonstrated that both these fear responses are dependent upon the integrity of the amygdala complex (Davis, 2000; Fanselow & LeDoux, 1999; Fendt, 2001; Fendt & Fanselow, 1999). However, the exact nature of the behavioral relationship between conditioned freezing and FPS remains unclear.

Past studies examining conditioned fear responses in rodents have mainly utilized either auditory or visual stimuli as the CS. While these studies have elucidated some of the neural mechanisms involved in conditioned fear, many questions remain concerning the precise nature by which a specific stimulus acquires fear-eliciting properties. The use of olfactory CS may provide an additional tool to further understand the behavioral and neurobiological aspects of fear in

mammals for several reasons. Olfactory stimuli are particularly salient cues to rodents, and conditioning is most effective when more salient cues are used as CS during training (Rescorla & Wagner, 1972). For example, previous behavioral studies have shown that rats can acquire conditioned fear to an olfactory stimulus very quickly (Otto, Cousens, & Rajewski, 1997; Paschall & Davis, 2002). The use of olfactory CS may be especially useful for molecular studies which are optimized by training protocols that induce rapid and robust learning (Ressler, Paschall, Zhou, & Davis, 2002). Furthermore, anatomical and functional studies have revealed that the olfactory sensory system provides relatively immediate sensory projections to subcortical structures involved is processing of aversive stimuli, including the amygdala (Pitkänen, 2000). These sensory efferents appear to maintain an odorant-specific topographical organization that may be uniquely suited for functionally dissecting the circuitry changes that occur with discrete learning events (Zou, Horowitz, Montmayeur, Snapper, & Buck, 2001). In addition, the many commercially available synthetic and natural odors provide a wide array of discrete stimuli with individual topographical representations (Leon and Johnson, 2003).

Limited studies in olfactory fear conditioning have been done in rats. Examination of mice would be useful given the wide variety of transgenic and other genetically engineered mice with targeted disruptions in genes that are involved in olfactory-motivated behaviors and learning and memory in general. On one hand, olfactory fear conditioning in mice would be expected to have many of the same properties as those already seen in rats. However, species differences are important to investigate because inter-species performance variations have been

shown in other behavioral tasks such as the Morris water maze (D'Hooge & De Deyn, 2001) and defensive behaviors (D. C. Blanchard, Griebel, & Blanchard, 2003).

In this study, we hypothesize that C57BL/6J mice can be fear conditioned to computer controlled odorant exposure and can learn to discriminate these odorants. The first two experiments in this study show that C57BL/6J mice can display FPS and freezing when shock is paired with two different odors, amyl acetate (Experiment 1) and acetophenone (Experiment 2). In contrast, FPS is not observed when odors and shocks are presented in an unpaired fashion (Experiment 3). Experiment 4 demonstrates that mice can discriminate between two odors that are either paired (CS+) or not paired (CS-) with shock, as indicated by selective freezing and FPS to the reinforced odor. Together, these findings show that olfactory stimuli can be effectively used in aversive and differential conditioning paradigms to elicit both freezing and FPS in C57BL/6J mice.

General Method

Animals

Adult (8-12 weeks) male C57BL/6J mice (Jackson Laboratories, Maine) housed in standard group cages ($\leq$5 / cage), were given *ad libitum* access to food and water, on a 12 hr on / 12 hr off light cycle. All experiments were performed during the light cycle (9 AM to 7 PM) and were approved by Emory University Institutional Review Board following NIH Internal Animal Care and Use Committee standards.

Olfactory stimuli

Odorants were prepared in advance and in a separate room. Mixtures of 5% amyl acetate and 10% acetophenone (Sigma-Aldrich, St. Louis) were dissolved in propylene glycol. A higher concentration of acetophenone was used on the basis of our pilot studies which indicated that lower concentrations were less effective - potentially due to the difference in volatility between the two odors (unpublished data, S. Jones). Odorants were placed in glass sample jars and attached to the odor delivery apparatus (see below).

Olfactory fear apparatus

Fear training and testing sessions were conducted using four identical startle response systems (SR-LAB, SDI, San Diego, CA). Each consisted of a nonrestrictive Plexiglas cylinder, 5.5 cm diameter,13 cm long, mounted on a Plexiglas platform which was located in a ventilated, sound-attenuated chamber. The floor of each cylinder was a cradle-shaped grid which contained seven 3.0 mm diameter stainless steel bars spaced 1 cm apart through which shock could be delivered. Cylinder movements were detected by a piezoelectric accelerometer mounted under each platform and were digitized and stored by an interfacing computer assembly as voltage output sampled each msec. Startle amplitude was defined as the peak accelerometer voltage that occurred during the first 100 ms after the onset of the startle stimulus. The voltage output was sampled every msec during a 5-s “activity window” starting 7 s before startle stimulus. For each cylinder, a voltage output “threshold” corresponding to mouse immobility was determined by recording the voltage output of the cylinder while empty (without animal). Voltage

readings above the average threshold response were used as evidence of mobility. Response sensitivities were calibrated (SR-LAB Startle Calibration System) to be nearly identical in each of the startle systems.

Startle and background stimuli were presented through a high-frequency speaker located 15 cm above the chambers. Startle was elicited by a 105-dB, 50-msec white noise burst. A continuous 65 dB white noise background was delivered through chamber speakers during training and testing. Sound intensities were measured by an audiometer (Radio Shack, #33-2055). The footshock US was generated by a programmable animal shocker (SDI, San Diego, CA) located outside the isolation chambers and was delivered through the cage floor bars. Footshock intensity was 0.4 mA. Startle, background, and US stimuli presentation and data acquisition were controlled by an IBM PC-compatible computer using SR-Lab software.

Odor stimuli were delivered to chambers in a manner similar to that described previously (Paschall & Davis, 2002). Briefly, a compressed air tank with a pressure regulator and flow meter delivered a constant flow rate of 40 L/min. The flow meter output was split with a Y-connector to create two separate delivery lines: a clean, odor-free line and an odor-delivery line that was connected to a solenoid(s) valve controlled by a computer running the SR-Lab software. PharMed Tygon tubing (3.2 mm id, Saint-Gobain, Akron OH) was used to form delivery lines because of its low permeability to vapors. When the valve opened, air flowed through the odor-delivery line into a sealed jar containing the dissolved odorant. Tubing from the jar merged with the odor-free line to form a single 80-cm delivery line that fed into the front of the Plexiglas cylinder. When the valve closed, air flowed thorough the odor-free line

only. Opening and closing of the solenoid valve did not produce any difference in rate of air flow to the cylinder. Backflow was prevented by one-way valves. The odor was rapidly removed from the back of the cylinder via an exhaust hose feeding into the room's ventilation fan.

To test for the discrimination of odors, the odor delivery apparatus was modified to deliver two separate odors. Rather than two lines as above, there were three lines in this configuration: an odor-free line and two odor-delivery lines. Each odor-delivery line was configured to deliver one of two different odors via separately controlled solenoid valves. The two odor-delivery lines and the odor-free line were joined back together by Y-connectors and an 80 cm common line of tubing lead into the Plexiglas cylinder. When both valves were closed, air flowed through the odor-free line and directly to the Plexiglas cylinder.

Fear Conditioning

In each experiment, mice were given 3 days of pre-exposure to the startle cylinders to minimize contextual conditioning and to acclimate animals to handling. Two days prior to conditioning the animals received 15 startle stimulus presentations to habituate startle to a stable baseline. The next day, animals received a pre-conditioning test (pretest) session to assess whether mice displayed unconditioned effects to the odor prior to conditioning. During pretest, mice were placed in the cylinder and 5 min later presented with 12 startle-alone trials. The initial startle-alone trials were intended to habituate startle to a stable baseline and were not used in analyses. Mice were then presented with 10 odor-startle trials randomly intermingled with 10 startle-alone trials and separated by a 90-s intertrial interval (ITI). Odor-

startle trials consisted of a 10-s odor presentation that co-terminated with a 50-ms, 105 dB noise burst. This pretest is unlikely to result in any effects such as latent inhibition because such effects typically require a large number of pre-exposures to the CS (Schauz & Koch, 1998).The next day mice were placed in the startle cylinder and 5 min later received the first of 5 pairings of a 10-s odor CS co-terminating with a 0.25-s, 0.4-mA footshock, presented with an average 120-s ITI (range 90 – 150 s). Animals were then returned to their home cage. Twenty-four hours after training, mice were given a post-conditioning test (posttest) identical to the pretest.

Data analysis

Startle and immobility were measured in the presence (odor-startle trials) and absence (startle-alone trials) of the odor CS. For each animal, a percent FPS and a percent freezing were computed by first subtracting the mean of startle-alone trials from the mean of the odor-startle trials. This difference score was then divided by the mean of the startle-alone trials and multiplied by 100%. The presence of associative FPS and freezing were assessed by examining the change in behavior from pretest to posttest. This approach accounts for potential confounding non-associative effects of the CS on dependent behaviors (Falls, 2002; Heldt, Sundin, Willott, & Falls, 2000). As such, within-subject statistical analyses were performed for each experiment. In Experiments 1 and 2, simple paired-sample *t*-tests were performed. For Experiments 3 and 4, mixed-model analysis of variance (ANOVA) were performed with training (Paired, Unpaired) and as the between-subject factor and session (Pretest, Posttest) as the within-subject factor (Experiment 3) or trial type (CS+, CS-) and session

(Pretest, Posttest) as the within-subject factors (Experiment 4). Subsequent analyses were done with simple paired-sample and pair-wise *t*-tests.

Freezing scores were determined as follows: voltage outputs for each cage were first converted to the average voltage output for each second of the 5-s activity window. Averages above or below the mean voltage output of the cylinder while empty (without animal) were assigned an immobility score of 0 or 1 (0=mobile, 1=immobile) for each second of the 5-s activity window. A percent immobility score for each trial was computed by averaging the 5 immobility scores and multiplying by 100. This score was used as an index of freezing. Pilot studies have found a high correlation between this automated index of freezing and observational ratings of freezing (r's > .89).

Experiments 1 and 2

Method

To test whether mice could acquire fear to an olfactory cue, mice were first given a pretest (Figure 1 a-b). The next day animals were presented with five odor-shock pairings and then given a posttest 24 hrs later. For Experiment 1, 5% amyl acetate was used as the CS; and for Experiment 2, 10% acetophenone was used as the CS. For each experiment, n=8, with 1 mouse was excluded from analysis in Experiment 1 because of an equipment malfunction.

Results

As seen in Figures 1 a-b, during the pretest the amyl acetate presentation produced nonsignificant changes in the percent FPS (-17%) and percent freezing (-

4%) prior to conditioning, *p*s>.05. After odor-shock pairing, animals showed 41% FPS in the presence of amyl acetate, which represented a significant increase from pretest, $t(6)=2.42$, $p<0.05$. Animals also showed a significant increase in freezing to the odor following training, $t(6)=3.09$, $p<0.03$.

In the Experiment 2 pretest (Figures 1 c-d) mice displayed nonsignificant increases in percentage of FPS (12%) and freezing (-19%) in the presence of acetophenone, *p*s>.05 (Figures 1c-d). After pairing the acetophenone with shock, the mice showed reliable increases in FPS (74%) and freezing (19%), $t(7)=3.3$, $p<0.02$ and $t(7)=2.54$ $p<0.05;$ respectively.

Experiment 3

Methods

To control for non-associative effects, we compared odor-shock paired animals to a group of animals in which the odor and shock were explicitly unpaired (Figure 2a). The paired group (n=14) received 5 amyl acetate-shock pairings on each of 2 training days. The unpaired group (n=14) received 5 shocks and 5 odor presentations on each day, but these stimuli were not paired. During unpaired training, the time between odor and shock averaged 90 sec but was randomized between 60 - 120 s.

Results

An analysis of the percent FPS with the mixed-model ANOVA revealed a significant Group x Session interaction, $F(1,22)=5.16$, $p<0.05$. Neither the group nor session main effects were significant, *p*s>.05. Within-subject analyses indicated that mice given paired training showed a significant increase in percent FPS after

conditioning, $t(11)=2.80$, $p<0.02$. In contrast, mice given unpaired training showed no difference from the pretest, $t(11)=0.30$, $p>0.05$ (Figure 2a).

For percent freezing, the overall analysis revealed only a significant session effect, $F(1,22)=4.88$, $p<0.04$, indicating an overall increase in freezing from the pretest to the posttest. Both the group main effect and Group x Session interaction were nonsignificant, *p*s>.05. Thus, as a combined group, mice given both paired and unpaired training displayed more posttest freezing when compared to the pretest. However, individual evaluations of each group indicated that the reliable session effect was primarily driven by a significant increase in mice given paired training, $t(11)=2.43$, $p<0.04$. Mice given unpaired training displayed no increase in percent freezing, $t(11)=0.73$, $p>0.05$ (Figure 2b).

Experiment 4

Methods

The purpose of Experiment 4 was to determine whether mice could learn to discriminate between an odor paired with shock and a non-reinforced odor. Prior to differential conditioning, mice were given a pretest. This test was similar to the pretest described above except that the animals received 10 odor-startle trials for each of the 2 odors (CS+, CS-) and 10 startle-alone trials. The order of the test trials was randomly assigned. Training was conducted on the following two days, and involved differential reinforcement of the 2 odors in a counterbalanced design. On each of two days Group 1 (n=10) received five presentations of acetophenone alone (CS-) interspersed with five pairings of amyl acetate and footshock (CS+). Group 2 (n=6) received acetophenone paired with the shock (CS+) and amyl acetate alone (CS-).

The following day, FPS and freezing were measured using the same test session used in the Experiment 4 pretest.

Results

Overall ANOVA's for both percent FPS and freezing revealed no main effect for group, F's(1,13)<1.88, *p*'s>0.05. There were also no significant two-way or three-way interactions by group, F's(1,13)<3.057, *p*'s>.05. Therefore, the data obtained from Groups 1 and 2 were collapsed and analyzed with a mixed-model ANOVA including session (Pretest, Posttest) and trial type (CS+,CS-) as within-subject variables. For percent FPS, this overall analysis revealed a significant trial type effect, $F(1,15)=7.50$, $p<0.02$, and Session x Trial Type interaction, $F(1,15)=8.79$, $p<0.01$. Subsequent paired-sample *t*-tests indicated that after conditioning, percent FPS to the CS+ increased from a pretest level of –7% to a posttest level of 29%, a significant change, $t(15)=2.63$, $p<0.02$ (Figure 2c). On the other hand, percent FPS to the CS- increased nonsignificantly from –8% in the pretest to 8% in the posttest, $t(15)=0.26$, $p>0.05$.

The overall analysis of percent freezing resulted in a significant main effect of session, $F(1,15)=8.83$, $p<0.01$, and a Session x Trial Type interaction, $F(1,15)=5.00$, $p<0.05$. Follow-up statistics indicated that after training mice froze significantly more to the trained odor (CS+) than they did in the pretest, $t(15)=3.87$, $p<0.02$ (Figure 2d). Freezing to the non-reinforced odor (CS-) increased slightly, but nonsignificantly, $t(15)=1.54$, $p>0.05$.

General Discussion

These experiments demonstrate that olfactory cues can reliable elicit conditioned fear in mice after aversive classical conditioning, as measured with both FPS and freezing in the same test session. Using rats, past research has demonstrated that an olfactory CS elicits a number of Pavlovian conditioned responses, including conditioned freezing (Richardson & McNally, 2003) (Cousens & Otto, 1998; Otto et al., 1997), FPS (Paschall & Davis, 2002), analgesia, and cardiac responses (Hunt, Hess, & Cambell, 1997; Richardson & McNally, 2003). Our results show that olfactory CS can reliably be used in mice and extend the finding of past studies showing learned-fear responses in mice using either an auditory and/or visual stimuli CS (Falls, Carlson, Turner, & Willott, 1997; McCaughran, Bell, & Hitzemann, 2000; Risbrough, Brodkin, & Geyer, 2003; Willott et al., 1998).

In Experiments 1 and 2, significant freezing and FPS were observed after pairing of the odor with shock, but not before. However, these results could be due to a number of non-associative or pseudoconditioning effects, including a generalized increase in anxiety or vigilance, sensitization, or context conditioning (Rescorla & Wagner, 1972).

Therefore, we tested two groups in Experiment 3: one in which the odor was paired with shock, and a second group in which the same number of odors and shocks were presented separately. As shown in Figures 2a-b, animals in the paired group learned the association between odor and shock, while the unpaired group did not when assessed using FPS. In contrast, the evaluation of freezing behavior in our paradigm revealed that as a combined group, both paired and unpaired animals displayed conditioned freezing. Posthoc analyses, however, showed that this overall

effect was primarily driven by a significant increase in freezing in paired animals. Unpaired animals displayed only a mild, nonsignificant increase in freezing. The evidence of a low level of freezing acquisition in the unpaired group may be due to the lack of complete odor clearance prior to shock. This explanation is consistent with a recent finding that unpaired odor + shock conditioning in rats produces mild CS conditioning (Sorg, Swindell, & Tschirgi, 2004). Efforts to increase the time between CS and shock presentations in the present study may have eliminated signs of acquisition in the unpaired group**.** Nevertheless, the possibility that mild conditioning produced evidence of conditioned freezing but not FPS may be an indication of differential behavioral thresholds.

To exclude the possibility that the mice were using some cue produced by the mechanism of the odor delivery apparatus as the CS, mice were given differential conditioning in which one odor was paired with shock (CS+), and the presentation of another odor was non-reinforced (CS-, Experiment 4). During the test session, mice showed a significant increase in both FPS and freezing to the trained odor (CS+) but not to the non-reinforced odor (CS-). This finding suggests that mice acquired odor-specific fear and that differences between reinforced and non-reinforced odors were unlikely due to other non-olfactory factors.

FPS and freezing are arguably the most commonly used measures of conditioned fear and have been used independently by different labs with slightly varying procedural protocols. In these experiments, immobility measurements were assessed from automated recordings of cage cylinder movements which correlates well with freezing behavior measured by a trained observer. Video recordings from pilot studies revealed that presentation of the odor CS elicited an orientation

response toward the odor source accompanied by sniffing. A past report using similar protocol methodology indicated that auditory and visual CS do not induce prominent orienting responses (Sundin, Heldt, Willott, Buck, & Falls, 1998). Thus, as seen in appetitive conditioning (Holland, 1977), it appears that stimulus modality influences the topography of the conditioned fear response in mice. The initial orientation reaction subsided after about 2 s. To avoid confounding our freezing measurement with this orienting activity we recorded activity 3-7 sec after odor CS onset.

Olfactory cue conditioning may provide a powerful approach to dissecting the functional neurocircuitry of fear. From a sensory standpoint, odors provide discrete cues that are detected by individual receptors on sensory neurons dedicated to that receptor (Ressler, Sullivan, & Buck, 1993). This molecular discrimination is maintained at the level of the olfactory bulb, and there is some evidence that functional topography representing different odors is present in the olfactory piriform cortex (Illig & Haberly, 2003). Thus, there exists a topographical representation of olfactory sensory inputs in brain regions only one synapse away from the amygdala. This organization may be uniquely suited for functionally dissecting the circuitry changes that occur with discrete learning events.

Acetophenone was used as one CS odorant due to findings that it may activate only a small number of olfactory bulb glomeruli and that a putative odorant receptor has been identified that is activated by this odorant (Bozza, Feinstein, Zheng, & Mombaerts, 2002). Future studies comparing olfactory fear learning with acetophenone to other odorants that do not activate the same receptor may allow for the combination of molecular anatomical approaches with sophisticated learning

paradigms. For example, transgenic mice containing manipulations of the M71 odorant receptor can be examined in a stimulus-specific way comparing odorant ligands that activate this receptor to other odors that do not. The choice of amyl acetate as another CS was based on the effectiveness of this stimulus in other rodent olfactory learning paradigms using this concentration (5%) (Paschall & Davis, 2002, Yuan et al. 2002).

In summary, the current study demonstrates olfactory-mediated FPS and freezing measured simultaneously in C57 mice. Although both measures are wildly accepted indices of a central state of fear, the exact relationship between these behavioral responses is presently unclear. It is generally assumed that freezing and potentiated startle co-occur (Leaton & Borscz, 1985). However, few studies have closely investigated this relationship, and in some cases manipulations of a fear state affect only one of these two fear responses. The establishment and use of a protocol which measures both freezing and FPS in the same animals may shed further light on the correlation of these two fear-related behaviors. Finally, this study lays the groundwork for experimental manipulations using transgenic mice to functionally dissect the olfactory system's role in learning as well as to further examine the role of brain areas that mediate fear conditioning.

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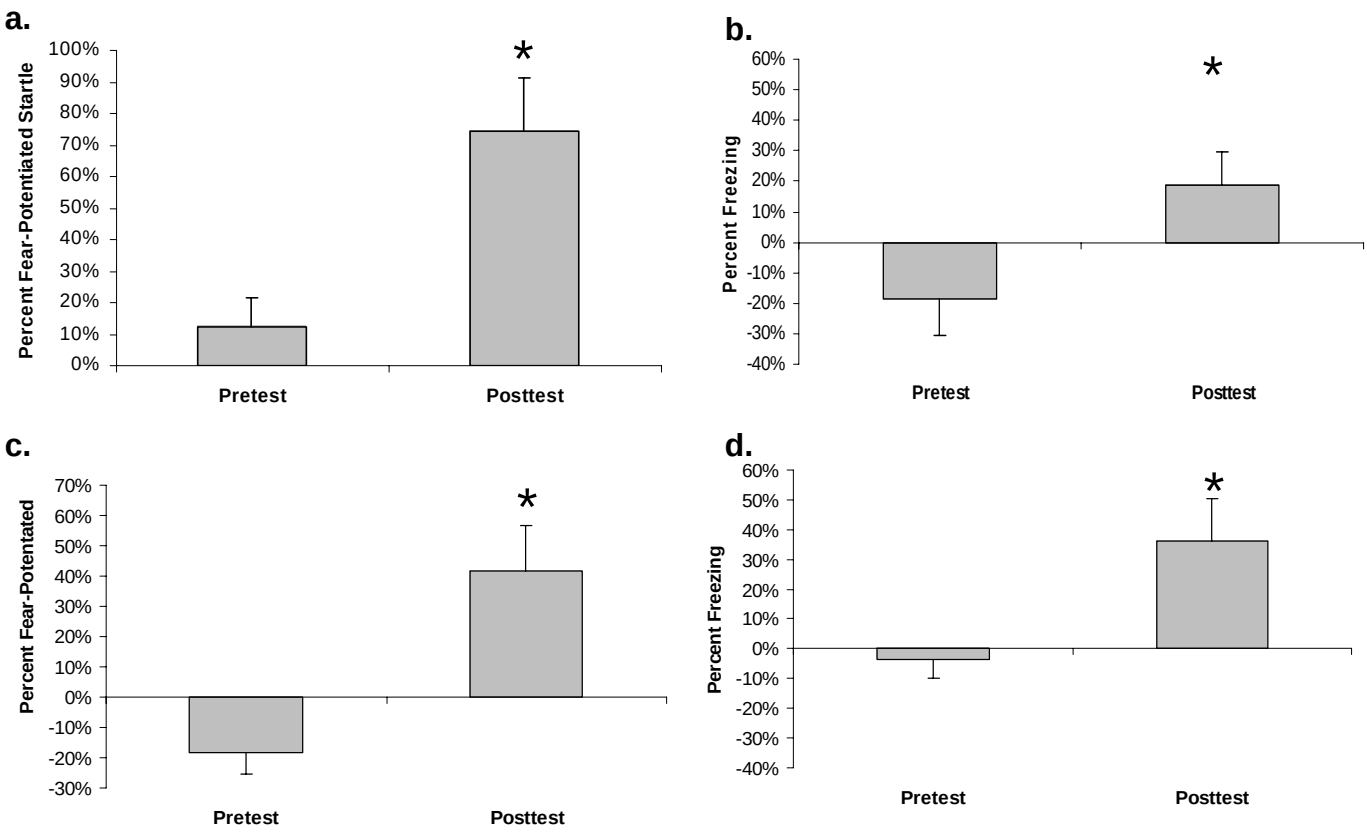
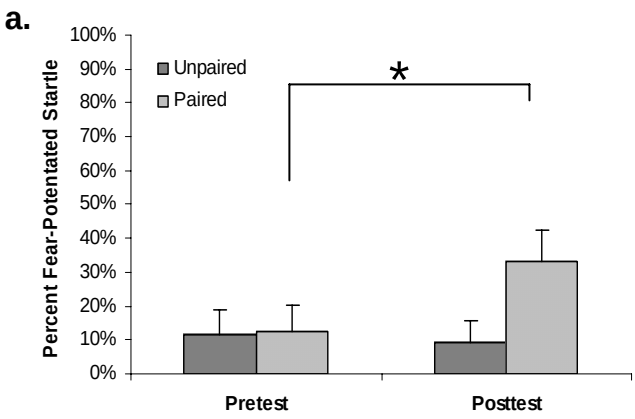
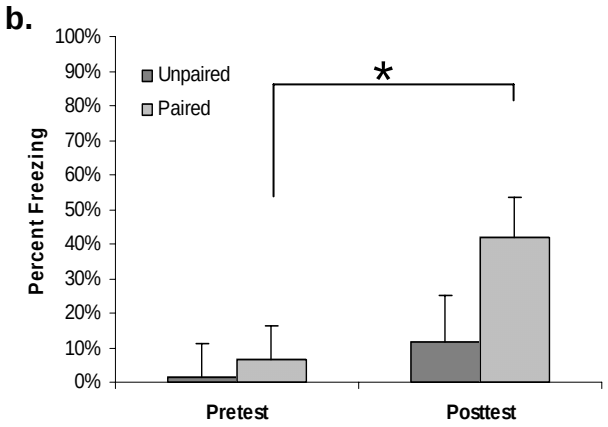
Figure Legends

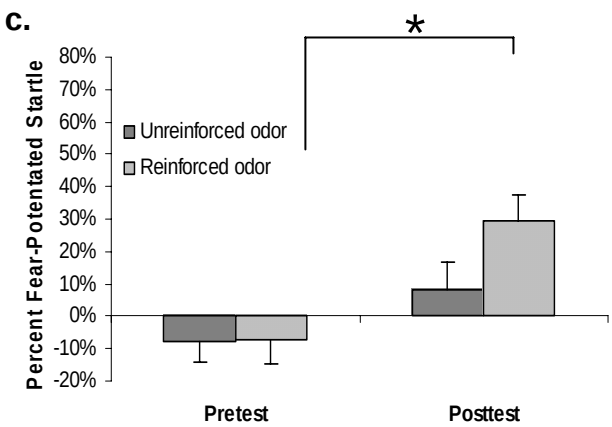
Figure 1: a) In Experiment 1, mice showed more fear-potentiated startle with an amyl acetate cue after the odor was paired with shock. b) Mice showed increased freezing in the presence of amyl acetate after the odor was paired with a shock. c) In Experiment 2, mice showed an increase in fear-potentiated startle after the odor acetophenone was paired with a shock. d) Mice showed increased freezing in the presence of acetophenone after the odor was paired with a shock. ($* p < .05$)

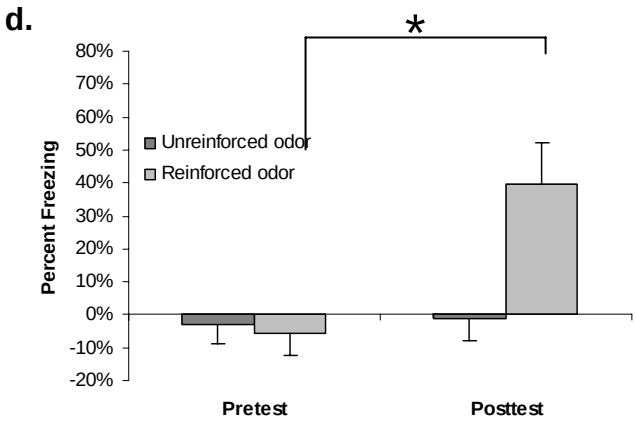
Figure 2: a) To control for non-associative effects in Experiment 3, odor-shock paired animals were compared to animals in which the odor and shock were explicitly unpaired. In the posttest, the paired mice showed an increase in fear - potentiated startle, but the unpaired group did not. b) Mice in the paired, but not the unpaired group showed increased freezing from the pretest to posttest. c) Experiment 4 tested for discrimination of two odors in fear conditioning. Some mice received amyl acetate paired with shock and acetophenone as the odor-alone while others received the opposite. When tested, the mice showed an increase in fear-potentiated startle to the reinforced odor but not to the non-reinforced odor. d.) Mice also showed an increase in freezing to the odor that was paired with shock but not to the non-reinforced odor. ($* p < .05$)


a.
Percent Fear-Potentiated Startle
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
Pretest
Posttest
b.
Percent Freezing
60%
50%
40%
30%
20%
10%
0%
-10%
-20%
-30%
-40%
*
Pretest
Posttest
c.
Percent Fear-Potentated
70%
60%
50%
40%
30%
20%
10%
0%
-10%
-20%
-30%
*
Pretest
Posttest
d.
Percent Freezing
60%
50%
40%
30%
20%
10%
0%
-10%
-20%
-30%
-40%
*
Pretest
Posttest


a.
Percent Fear-Potentiated Startle
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Unpaired
Paired
*
Pretest
Posttest


b.
Percent Freezing
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Unpaired
Paired
*
Pretest
Posttest


c.
Percent Fear-Potentiated Startle
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
-20%
Unreinforced odor
Reinforced odor
*
Pretest
Posttest


d.
Percent Freezing
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
-20%
Unreinforced odor
Reinforced odor
*
Pretest
Posttest