Improved Discrimination Between Tone and Context During Fear Extinction in Chronically Stressed Rats Provided with a Post-Stress Rest Period

by

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ABSTRACT

The goal of the present study was to investigate whether a rest period following the end of chronic stress would impact fear extinction. Past research has indicated that chronic stress leads to impairments in the learning and recall of fear conditioning extinction. Moreover, the effects of chronic stress can return to levels similar to controls when a post-stress "rest" period (i.e., undisturbed except for normal husbandry) is given prior to testing. Male rats underwent chronic restraint stress for 6hr/day/21days (STR-IMM). Some rats, underwent a post-stress rest period for 6- or 3-weeks after the end of stress (STR-R6, STR-R3). Control (CON) rats were unrestrained for the duration of the experiment. In Experiment 1, following the stress or rest manipulation, all rats were acclimated to conditioning and extinction contexts, fear conditioned with 3 tone-foot shock pairings, and then had two days of extinction training. All groups froze similarly to the tone across all training sessions. However, STR-R6/R3 froze less in the non-shock context than did STR-IMM or CON. During extinction training, STR-IMM showed high levels of freezing to the non-shock context, leading to a concern they may be generalizing across contexts. Consequently, a follow-up experiment tested for context generalization. In Experiment 2, STR-IMM rats underwent a generalization test in an environment that was either different or the same as the conditioning environment, using STR-R6 as a comparison. STR-IMM and STR-R6 showed similar relative levels of freezing to tone and context, regardless of their conditioning environment to reveal that STR-IMM did not generalize and instead, maybe expressing hypervigilance. Thus, the present study demonstrated the novel finding that a rest period from chronic stress can lead to reduced fear responsiveness in a non-shock environment.

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Post-traumatic stress disorder (PTSD) is a psychiatric condition that affects 6-9% of the U.S. population and 40% of those exposed to extreme trauma, such as combat veterans (Hoge & Warner, 2014; Sareen, 2014). PTSD is characterized by persistent memories of the traumatic event, avoidance of things that are associated with or trigger memories of the traumatic event, and hyperarousal (American Psychiatric Association, 2013). Current therapies for the treatment of PTSD include antidepressants (the most common) and cognitive behavioral or exposure therapies. Unfortunately, antidepressants fail to provide complete remission in nearly 70 to 80% of patients and moreover, behavioral therapies are often inconsistent in effectiveness or are inaccessible (Difede, Olden, & Cukor, 2014). Consequently, many individuals continue to suffer from PTSD. A better understanding of the factors influencing the development and persistence of strong fear memories that are characteristic PTSD will aide in the development of novel treatments and/or therapies.

The ability to form a fear memory is a necessary function for long-term survival and danger avoidance (Boissy, 1995). However, in PTSD, fear memory is maladaptive and overly robust (Milad et al., 2008). Models of PTSD have investigated how individual differences, including early life and juvenile stress, sex differences, and chronic stress in adulthood, impact the development of PTSD-like phenotypes (Daskalakis, Yehuda, & Diamond, 2013). Models of PTSD often use fear conditioning as an easy and efficient way to create fear memories, which then can then be tested to determine the robustness of the fear memory using extinction or reconsolidation methods.

Fear conditioning is based on Pavlovian conditioning principles (Maren, Luan Phan, & Liberzon, 2013; Phillips & LeDoux, 1992). A stimulus (unconditioned stimulus or US), such as a foot shock or puff to the eye, that produces a response without training (unconditioned response or UR), is presented immediately following a neutral stimulus (conditioned stimulus or CS) that has no obvious significance, such as a tone or light. Repeated pairings between the CS and US can lead to an association in which the individual responds to the presentation of the CS alone. Such responses to the CS are called conditioned responses or CR, which is differentiated from the unconditioned response by when it occurs; a CR occurs in response to a CS, whereas a UR is in response to the US. In a typical fear conditioning set up, a tone (CS) is presented immediately prior to a mild foot shock (US). In rats and mice, freezing behavior (a species-typical fear response) is the CR to the presentation of the CS. Extinction training can be performed to test the strength of the memory and involves presenting the CS without the US. Extinction training is often done in a context different than the original fear conditioning to isolate the strength of the memory to the discrete (tone) CS. Repeated presentations of the CS without the US will result in a reduction of the CR (Bouton & Bolles, 1979). In models of PTSD that use fear conditioning, an impairment in extinction training is a frequent and translationally relevant finding (VanElzakker, Kathryn Dahlgren, Caroline Davis, Dubois, & Shin, 2014).

A history of chronic stress is a risk factor for the development of PTSD in humans (Breslau, Chilcoat, Kessler, & Davis, 1999; Sareen, 2014) and can create a PTSD-like phenotype in rodent models (Daskalakis et al., 2013). Compared to unstressed rats, chronically stressed rodents demonstrate faster and more robust acquisition of the CR (Conrad, LeDoux, Magariños, & McEwen, 1999; Cordero, Venero, Kruyt, & Sandi, 2003.; Hoffman et al., 2015), are more prone to generalize their fear response to other contexts (Hoffman, Lorson, Sanabria, Foster Olive, & Conrad, 2014; Radulovic, Kammermeier, & Spiess, 1998), show deficits in extinction training and recall of extinction (Baran, Armstrong, Niren, Hanna, & Conrad, 2009; Hoffman, Lorson, Sanabria, Foster Olive, & Conrad, 2014; Rau, DeCola, & Fanselow, 2005), and display heightened anxiety-like behaviors (Chiba et al., 2012; Vyas, Pillai, & Chattarji, 2004). These findings in rats parallel the clinical population's robust fear memories (American Psychiatric Association, 2013; Yehuda & LeDoux, 2007), generalization of fear responses to safe environments (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007), resistance to extinction-based therapies (Blechert et al., 2007; Milad et al., 2009), and increased anxiety (Grillon et al., 2009). Consequently, these rat models can be a useful tool to understand the underlying neurobiology of PTSD in human populations (Bryant et al., 2017).

Another factor to consider is that behavioral outcomes of chronic stress are dynamic. That is that some of the outcomes in response to chronic stress can change with the passage of time. For example, chronic stress leads to deficits in acquisition (Ghiglieri et al., 1997; Ortiz et al., 2015) and retention (Abidin et al., 2004; Song, Che, Min-wei, Murakami, & Matsumoto, 2006) of spatial navigation tasks, when testing occurs within days after chronic stress has ended. However, when chronically stressed rodents are permitted a rest period, where the animal is left undisturbed for weeks beyond the typical husbandry requirements, the spatial memory deficits disappear and animals show spatial abilities that are similar to or even better than their non-stressed counter-parts (Bian et al., 2012; Conrad, Ortiz, & Judd, 2016; Hoffman et al., 2011; Luine, Villegas, Martinez, & Mcewen, 1994; Ortiz et al., 2015). Chronic stress also leads to an increase in anxiety-like behaviors, when tested within days after the end of chronic stress (Vyas 2004, D'Aquila 1994, Kim 2006, Eiland 2010). In contrast to spatial memory outcomes, chronically stressed rats continue to show heightened anxiety-like behavior even following a post-stress rest period (Vyas, Pillai, & Chattarji, 2004). Both heightened anxiety and impairments in fear conditioning extinction training are seen in PTSD patients and in chronic stress models animal models of PTSD. Additionally, the basolateral amygdala is important in both of these behaviors. (Phillips & Ledoux, 1992; Vyas, Pillai, & Chattarji, 2004; Ajai Vyas, Bernal, & Chattarji, 2003). Taken together, this suggests chronic stress-induced fear conditioning extinction impairments may be observed even when there is a post-stress rest period, with a similar pattern to the elevated levels of anxiety-like behavior that persist following the post-stress rest period. Thus, the goal of the present study is to determine how a rest period following the end of chronic stress will impact the strength of fear memories to potentially resist extinction training.

Methods

Subjects

Male Sprague-Dawley (Charles River Laboratories) rats weighing approximately 250 grams upon arrival were pair housed in standard laboratory cages (21-22 °C, corncob bedding). Except where noted below, animals were allowed food and water *ad libitum*. Animals were housed on a reverse 12:12 light cycle; lights off at 7AM. All procedures occurred during the dark phase of the light cycle and were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the approval of the Arizona State University Institutional Animal Care and Use Committee.

Chronic Stress Procedure

Rats were chronically stressed by restraint for 6 hours/day for 21 days. Our previous work demonstrated that these restraint parameters were the minimum required duration for restraint stress to produce behavioral and structural changes (Mclaughlin, Gomez, Baran, & Conrad, 2007). Restraint took place between 9AM and 3PM and occurred in the animal's home cage. Sound-attenuating chambers kept the animals undergoing restraint separated from animals not undergoing restraint. To keep food and water access similar between groups, the control group was yoked to the stress groups and their food and water was removed during restraint hours. Additionally, control rats were handled at the start of each day to keep daily handling from the investigator consistent. Animals were initially restrained using a wire mesh tube (6.4cm circumference ×26.7 in length) that was prepared using grip guard sealer (Flynn and Enslow, San Francisco, CA) to keep the wire ends coated, but were upgraded to a larger restrainer (7.6 in circumference× 29.2 in length) as they grew. Weights were recorded weekly.

Group Assignments and Timeline

In Experiment 1, rats were assigned to one of four groups (n=10/group, 40 rats total) in unstressed control, stress with a six-week rest period (STR-R6), stress with three-week rest period (STR-R3), or stress without a rest (i.e., tested within days or immediately, STR-IMM). Fear conditioning training occurred six weeks (STR-R6), three weeks (STR-R3) or within days (STR-IMM) from the last day of restraint (Figure 1a). The three-week rest duration was selected because under this timeframe, some behaviors, such as spatial ability, improve after chronic stress has ended (Bian et al., 2012; Conrad, Ortiz, & Judd, 2016; Hoffman et al., 2011; Luine, Villegas, Martinez, & Mcewen, 1994; Ortiz et al., 2015). The six-week rest duration was selected because it is double the length of time for spatial memory improvement and moreover, other studies report that four weeks can lead to high and maintained anxiety following the end of stress (Mikics, Baranyi, & Haller, 2008). Therefore, six weeks following the end of chronic stress was an extended rest duration that has not been investigated previously. In Experiment 2, rats underwent either the STR-IMM or STR-R6 stress timeline only. Rats were further grouped based on their conditioning environment, for four groups total (n=8/group, 36 rats total).

Fear Conditioning

Fear Conditioning Apparatus. Rat test cages were square and made of metal and plastic (12"Wx10"Dx12"H: Coulbourn Instruments, E10-18TC or H10-11R-TC) and were modified so that the top metal panel was replaced with clear Plexiglas for video recording. Both arenas were housed within a purchased sound-attenuating cabinet (Coulbourn, E10-23, white, 31.5” W x 21” D x 20” H) or a custom-made sound-attenuating cabinet (25” W x 24” D x 28”H: Melamine boards). Tones (75dB steady tone, 20 sec) were delivered through a speaker (Coulbourn, H12-01R) mounted on the inside of the sound-attenuating cabinet and were produced by a frequency generator (Coulbourn, E12-01 or H12-07). An animal shock generator (Coulbourn, H13-15) administered the shocks (0.8mA, 1 sec) through a shock floor (Coulbourn, E10-18RF or

H10-11RTC-NSF), with current equally distributed between parallel metal bars. Illumination was provided throughout testing by LED light bulbs in porcelain lamp-holders (Pass & Seymour, Legrand) mounted to the ceiling of the isolation cubicles.

All stimuli were controlled using Graphic State software (v 4.0 GS4-UP). Graphic State was installed on a Dell computer (3.19GHz, Intel i5 CPU, 64 bit) running Windows 7 Enterprise (2009, Microsoft Corp.). The computer was connected to a linc system (Coulbourn, H02-08) that controlled the stimuli output via an USB interface (Coulbourn, U90-11H). Infrared lights (Coulbourn, H27-91R) were positioned to be observed by the video and were programed to denote the context and tone. The infrared lights could not be visually detected unless viewed on video.

Behavioral Quantification. All behavior was digitally recorded on GoPro Hero 3 cameras (GoPro, Inc.) for offline analysis. Video from the GoPro cameras were monitored using a Quad Splitter Processor (Evertech, Amazon), which allowed four videos to be viewed on one monitor (Samsung, 24", Best Buy). The behavior from eight single chambers that were viewed on two monitors was backed up on a VCR recorder (Funai, Fry's Electronics). Behavior was manually scored by a trained observer. Freezing was defined as all movement except those associated with respiration (Blanchard & Blanchard, 1969). Freezing to tone was defined as any freezing that took place during the 20 second tone presentation and freezing to context was defined as any freezing that took place in the 20 seconds immediately prior to the presentation of the tone. A fear conditioning (FC) difference score was calculated as the amount of freezing to tone minus the amount of freezing to context 20 seconds prior to the tone in order. The FC difference score provides an understanding how much of the freezing to the tone was due to associative processes over a more generalize freezing response that occurs in the absence of the discrete cue. Intra-rater reliability was 95.7 ± 2.0%.

Fear Conditioning Contexts. For Context A, the testing cages were square metal and plastic and had a metal floor of parallel rods (Coulbourn, H10-11R-TC-SF), silver side panels (Coulbourn, H90-00R-M-KT01), and black and white striped panels on the clear plastic back wall. The sound-attenuating cabinet contained a 40-Watt equivalent LED bulb (450 Lumens; Osram Sylvania, Inc.) and a white LED computer fan (Thermaltake, CL-F020-PL12WT-A or Coulbourn, ACT-130). The cleaning solution used after each rat was an all-purpose, grapefruit scented cleaner (Method, Lowes) and the room lighting of the overall holding room was white-light. During transport of the rats, experimenters wore a yellow wrap gown and black gloves. Rats were transported from the colony room to the testing room by hand-carrying the rats in their home cages. For Context B, the testing cages were round, plastic blue buckets (14.5" H x 12" DIA, Lowes). A 3-Watt, Red LED bulb (91 Lumens; Feit Electric) was used as illumination in the isolation cubical. A 14in, red LED computer fan (Thermaltake, TT-1425) provided white noise/ventilation in the cubicle. The cleaning solution used after each rat was 70% isopropyl alcohol (Vi-Jon, Inc.). During transport of the rats, experimenters wore a white lab coat and blue gloves. The rats were transported from the colony room to the testing room in their home cages on a cart and the room lighting of the overall holding room was red-light. For Context C, the testing cages were modifications of the square testing cages (Coulbourn, H10-11R-TC-SF). A black semi-circular Plexiglas insert was placed in the testing cage to produce a curve in the back. The exposed side panels were covered in black plastic. Room lighting, transportation method, isolation cubical door positioning, chamber lighting, and computer fan used were the same as in Context B. The cleaning solution used after each rat was an all-purpose pine scented cleaner (Method, Inc.).

Experimental Procedures

Experiment 1: Influence of a Rest Period Following the End of Chronic Stress on Fear Extinction. Six days before the chronic restraint procedure ended for the last cohort of rats (STR-IMM), acclimation to the contextual environments commenced to reduce potential generalization of contexts with novel or few exposures (Hoffman et al., 2015, 2014) (Figure 1b). Rats were acclimated by being placed in the context for 10 minutes daily. Exposure to Contexts A and B alternated over the six days for a total of three exposures to each context. The day after the last acclimation session (Day 7), fear conditioning training in Context A occurred. Training consisted of three tone-foot shock pairings (inter trial interval (ITI) range between pairings = 80-170 sec). After 114 secs, the first tone was presented and the training session lasted eight minutes and 55 seconds. One and two days after training, rats underwent extinction training sessions in Context B. Extinction training consisted of 15 presentations of the tone (ITI range=85-120 sec). Six days after the second extinction session, rats were exposed to three more presentations of the tone in Context B to assess spontaneous recovery (ITI range=90-120 sec).

Experiment 2: Comparison of STR-IMM with STR-R6 on Context Generalization. In Experiment 1, there were indications that STR-IMM might be generalizing, so a second experiment for to test for generalization was performed. STR-R6 was used as a comparison group due to low freezing to context seen in this group. Acclimation occurred as described above, but differed in that acclimation was to Contexts A and C so that foot shocks could be administered in two different environments (Figure 2). One day after acclimation ended (Day 7) fear conditioning occurred in either Context A or C to produce a 2 x 2 design for stress and training environment. Training consisted of three tone-foot shock pairings as in Experiment 1. On day after training (Day 8), rats were tested for cued and contextual freezing behavior in Context C. This test consisted of three presentations of the tone only (ITI=320 sec). Contextual freezing was assessed in 20 second blocks, each 40 seconds apart, so that six measures of contextual freezing occurred prior to each tone. One day later (Day 9), the rats received another three-tone presentation in Context B.

Euthanasia and Tissue Collection

Three days following spontaneous recovery in Experiment 1, rats were euthanized using CO_2 gas and rapidly decapitated. Adrenal glands were excised and weighed for a secondary measure of stressor effectiveness.

Statistical Analysis

Results were analyzed using ANOVA (Analysis of Variance). Results that were significant at the $p<0.05$ level were additionally analyzed using the LSD (least significant difference) post-hoc test. In some cases, plan comparisons were performed based on results from previous published studies. Rats were excluded from further analysis if freezing to context exceeded 25% of the total freezing prior to the first presentation of tone during training (i.e., before tone or foot shock presentation). Three rats were excluded from both Experiment 1 (1 CON, 2 STR-IMM) and Experiment 2 (1 STR-R6_SAME, 1 STR-IMM_DIFF, 1 STR-IMM_SAME). When variances were unequal, data was transformed using $\sqrt{x+1}$ (Tabachnick & Fidell, 2007). Data analysis was done using SPSS Version 24.

Results

Experiment 1: Influence of a Rest Period Following the End of Chronic Stress on Fear Extinction

Conditioning

Summary. All groups increased freezing to tone and context across the three conditioning trials and these reached similar maximum levels by the third trial. While the STR-R6 group showed significantly less freezing to the second tone, their overall freezing levels were lower, leading to a FC difference score that was similar to all groups (CON, STR-IMM, STR-R3, STR-R6; Figure 3).

Specific Results. A mixed factors ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) across trials (1,2,3) was performed for tone and context. A significant effect of trial was observed for tone, $F(2, 66)=313.994$, $p<0.001$ and context, $F(2, 66)=301.298$, $p<0.001$. As trials progressed, freezing increased ($p<0.05$) until groups were freezing similarly and at near maximum levels during the third trial.

In addition, a significant group by trial interaction was found for freezing to tone, $F(6, 66)=2.637$, $p<0.05$, which was further probed by one-way ANOVAs at each trial. No group differences in freezing to tone were found for trials 1 or 3, but groups differed in their freezing to tone at trial 2, $F(3, 36)=2.857$, $p=0.05$. Post-hoc analysis revealed that STR-R6 froze less than did CON ($p=0.05$), STR-IMM ($p<0.05$), and STR-R3 ($p<0.05$). Freezing to tone by CON and STR-IMM also corroborated past findings that differences between STR-IMM and CON were minimized with extended acclimation to contexts (Hoffman et al., 2015). No other significant main effects or interactions were observed.

For the FC difference score, a mixed factors ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) across trials (1, 2, 3) was performed and revealed a significant effect of trial, $F(2, 66)=3.710$, $p<0.05$, but no significant main effects or interactions. The FC difference score for trial 1 (3.1± 2.1) was statistically higher than for trial 3 (-8.8 ± 3.4, $p<0.001$). Groups tended to freeze more to tone than to the context in trial 1, as shown by the positive FC difference score, but as trials progressed, the groups began to freeze to both context and tone by trial 3. Importantly, groups showed similar FC difference scores and the STR-R6 lower freezing to tone most likely reflected lower overall freezing to tone and context.

Extinction 1

Summary. Across binned trials, all groups decreased freezing to tone and context similarly. However, when analyzing the first three trials (bin 1), the groups given a post-stress rest period (STR-R3, STR-R6) froze less to context (but similarly to tone) than did CON and STR-IMM. The resulting FC difference scores during trials 2 and 3 showed that STR-R3 and STR-R6 froze more to tone than they did to context, whereas CON and STR-IMM froze similarly to tone and context.

Specific Results. Extinction training results were grouped into five bins of three trials each (Figure 4). A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x binned trial (1, 2, 3, 4, 5) was performed for tone and context. A significant effect of binned trial was found for tone, $F(4, 132)=17.513$, $p<0.001$, and context, $F(4, 132)=17.513$, $p<0.001$, with no other significant main effects or interactions. As more presentations were given, freezing to tone or context decreased and performance was similar across groups.

For the FC difference score, a significant effect of bin was found $F(4, 132)=4.783$, $p=0.001$, with no significant main effect for group or group x bin interaction. The FC difference score was lower in bins 2 and 3 compared to bins 1, 4 and 5 ($p<0.01$ for all comparisons). This appeared to be driven by the STR-IMM, which showed similar freezing to tone and context or even less freezing to tone than to context. The CON, STR-R3 and STR-R6 demonstrated FC difference score with means ± SEM that were above zero, indicating more freezing to tone than to context.

We also looked at the first three trials to understand freezing behaviors before many tone presentations were given (Figure 5). A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trial (1, 2, 3) was performed for tone and context. A significant effect of trial was found for both tone, $F(2, 66)=24.996$, $p<0.001$, and context, $F(2, 66)=71.377$, $p<0.001$, with freezing to both tone and context increasing from trial 1 to trial 2 ($p<0.001$), and becoming statistically similar between trials 2 and 3. There were no significant main effects or interactions for freezing to tone.

To determine whether any *a priori* differences existed before the start of extinction, a 1-way ANOVA for freezing tone and context for the first trial showed no statistical differences among the groups. On trial 1, percent freezing to tone group means ranged from 62.5 ± 9.7 to 69.0 ± 7.2% (Figure 5A) and percent freezing to context group means ranged from 12.7 ± 10.0 to 19.3 ± 12.9% (Figure 5B).

To understand freezing to context after the introduction of the first tone, we looked at freezing to contexts during trials 2 and 3 in a mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x 2 trials (2, 3). There was a significant main effect of group, $F(3, 33)=3.991$, $p<0.05$. STR-R3 froze significantly less to context than did STR-IMM (p<0.05) or CON (p<0.05). STR-R6 froze significantly less to context than did STR-IMM and bordered on freezing less to context than did CON (p=0.07, Figure 5B). No significant effect of trial or interaction was observed for freezing to the second and third tones. This suggests that, compared to STR-IMM and CON, STR-R3 and STR-R6 froze less to a context that never involved foot shock and hence, could be considered a "safe" context.

For the FC difference scores of the first three trials, a mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trial (1, 2, 3) showed a significant effect of trial, F(2, 66)=28.631, p<0.001, with more positive difference scores in trial 1 than in trials 2 and 3 (p<0.001). A 1-way ANOVA for the difference score in the first trial showed no significant differences among the groups. Moreover, the FC difference scores in trial 1 favored more freezing to tone than to context (lowest = 43.8±12.8, highest = 53.92±8.6). For trials 2 and 3, however, a mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trial (2, 3) there was a significant main effect of group, F(3, 33)=0.5.557, p<0.05, but no significant trial or interaction. STR-R3 and STR-R6 froze selectively to tone over context compared to STR-IMM (p<0.05) and CON (p<0.05, Figure 5C). Consequently, stress groups given a rest period after chronic stress (STR-R3, STR-R6) froze more to the discrete CS than they did to the context and suggests that they maintain an understanding of the safe, non-training environment.

Extinction 2.

Specific Results. Extinction training results were binned into five bins of three trials each (Figure 6). A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x binned trial (1, 2, 3, 4, 5) was performed for tone and context. A significant effect of binned trial was found for tone, $F(4, 132)=25.312$, $p<0.001$, and context, $F(4, 132)=2.789$, $p<0.05$, but there were no significant main effects or interactions. The all groups froze less to tone and context as trials progressed, specifically showing statistically less freezing to tone and context during bin 2 than bin 1 ($p<0.05$) and bin 4 compared to bin 3 ($p<0.05$), and less freezing to tone from bin 3 to bin 2 ($p<0.05$). By the last bin, all rats showed a similar level of extinction, freezing only about 10% of the duration of the tone.

Groups also show similar levels of freezing when we look at just the first three trials (Figure 7). A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trials (1, 2, 3) was performed for tone and context. A significant effect of trial was found for tone, $F(2, 66)=3.980$, $p<0.05$, and context, $F(2, 66)=15.443$, $p<0.001$, but there were no significant main effects or interactions. A 1-way ANOVA showed that there were no a priori differences in freezing to tone or context for the first trial.

FC Difference score. Extinction training results were binned into five bins of three trials each. A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x binned trial (1, 2, 3, 4, 5) was performed and revealed a significant effect of binned trial, $F(4, 132)=2.789$, $p<0.05$, with more freezing to tone than context in bin 1 was significantly different than bins 3 ($p<0.05$) and 4 ($p<0.05$). This appeared to be due to more freezing to the tone than context in bin 1 than in bins 3 and 4. There were no significant main effect or interaction. As in Extinction 1, we looked at the first three trials individually. A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trial (1, 2, 3) showed a significant effect of trial, $F(2, 66)=4.890$, $p<0.05$, with no significant main effect or interaction. The first and second trials were significantly different (*p*<0.05); there appeared to be more freezing to tone than context in the first trial compared to the second and third (*p*<0.05). There were no differences amongst the groups in a 1-way ANOVA for 1st difference score.

Spontaneous Recovery.

Summary. All groups demonstrated spontaneous recovery to the CS and there were no statistical differences between the groups on presentation of the tone. All groups had a reduction in responding during the extinction sessions. This return of the freezing response indicates that the original memory of the CS-US association remained intact (Quirk, 2002).

Specific Results. All groups were statistically similar in freezing to tone and context during spontaneous recovery (Figure 8). A mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) x trial (1, 2, 3) was performed for freezing to tone, context, and difference score. There was a significant trial effect for tone, $F(2, 66)=15.023$, $p<0.001$, context, $F(2, 66)=8.594$, $p<0.001$, and difference score, $F(2, 66)=11.141$, $p<0.001$, but no significant main effects or interactions. A 1-way ANOVA for the first trial showed no differences in freezing amongst the groups.

Experiment 2: Comparison of STR-IMM with STR-R6 on Context Generalization

In Experiment 1, an extended acclimation paradigm was implemented to reduce generalization to contexts. However, the STR-IMM froze similarly to the tone CS and the non-shock context, suggesting they might be generalizing. Experiment 2 was performed to test for generalization.

Conditioning.

Summary. All groups acquired freezing response to tone and context

Specific Results. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) across trials (1, 2, 3) was performed for both tone and context. There was a significant effect of trial for freezing to tone, $F(2, 54)= 135.037$, $p<0.001$, and context, $F(2, 54)= 333.397$, $p<0.001$, and no significant main effects or interactions. Freezing increased across trials ($p<0.001$). A 1-way ANOVA for freezing to the first trial showed no baseline differences between the groups and both expressed low levels of freezing (4 seconds or less) prior to the first tone and foot shock presentation. A planned 1-way ANOVA for the second trial showed no differences in freezing to tone or context between groups (Figure 9A, B).

FC Difference score. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) x trial (1, 2, 3) showed that the difference scores significantly changed across trials, $F(2, 54)= 15.338$, $p<0.001$, but there was not a significant main effect or interaction (Figure 9C). A 1-way ANOVA showed that both groups had a statistically similar FC difference scores on trial 1 (give both). The FC difference scores were marginally different on trials 2 and 3 ($p=0.06$), with STR-IMM having a more positive score than did STR-R6 (trial 2, give each difference score, and trial 3 again).

Context Test 1: Comparison of Freezing to Tone when Presented in the SAME or DIFF Context as Training

Summary. STR-IMM froze more to tone and context than did STR-R6, regardless of whether test 1 occurred in the SAME or DIFF environment as in conditioning. However, STR-IMM and STR-R6 discriminated similarly, as the FC difference scores were similar. Hence, high freezing by STR-IMM to both tone and context was indicative of a potentiated freezing response and within this heightened responsivity STR-IMM demonstrated discrimination between their freezing responses to tone and context. As would be expected, rats trained and tested in the SAME environment showed more freezing to context prior to the first tone presentation than did rats trained in the DIFF environment (Figure 10).

Specific Results. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) x training environment (SAME, DIFF) x trial (1, 2, 3) was performed for both tone and context. There was a significant effect of trial for tone, $F(2, 50)=5.304$, $p<0.05$. There was similar freezing to tone during trials one and two, but freezing decreased on trial three ($p<0.05$). The ANOVA also revealed an interaction of trial and stress condition, $F(2, 50)=3.670$, $p<0.05$, with STR-IMM freezing more across trials than STR-R6, but no significant main effects. For freezing to context across trials there were no significant trial effects, interactions, or main effects. A 1-way ANOVA for first trial showed group differences on the first trial. For the first tone presentation, STR-IMM froze significantly more to than STR-R6 ($p<0.05$), but there were no significant differences in freezing between training environment groups. For the first context presentation, STR-IMM froze significantly more than STR-R6 ($p<0.01$) and rats trained in the SAME environment froze more than rats trained in the DIFF environment ($p<0.05$).

FC Difference score. The STR-IMM and STR-R6 performed similarly when the FC difference scores were compared. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) x training environment (SAME, DIFF) x trial (1, 2, 3) showed no significant trial effects, interactions, or main effects. A 1-way ANOVA for the first trial showed no differences between the groups for stress condition or training environment. Importantly, the average means ± SEM showed that, as a group, both STR-IMM and STR-R6 froze more to tone than they did to context and this was unaffected by training environment.

Test 2. Comparison of Freezing to Tone in a Completely Novel Environment

Summary. All groups were tested in a completely novel environment to ascertain their behavior in a non-acclimated, non-shock environment (Figure 11). Freezing behavior was similar between the stress groups and the environmental training groups.

Specific Results. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) x training environment (SAME, DIFF) x trial (1, 2, 3) was performed for tone and context. For tone, there were no significant effect of trial, interactions, or main effects. There was an effect of trial for context, $F(2, 50)=11.989$, $p<0.001$, but no significant interactions or main effects. There was less freezing to context for trial one than trials two ($p=0.001$) or three ($p<0.001$). A 1-way ANOVA for the first trial indicated that rats trained in the SAME environment froze more than rats trained in the DIFF environment ($p<0.05$) for the first context, prior to any tone presentations.

FC Difference score. A mixed factors ANOVA for stress group (STR-R6, STR-IMM) x training environment (SAME, DIFF) x trial (1, 2, 3) for the FC difference score showed a trial effect, $F(2, 50)=4.693$, $p<0.05$, with significant difference between freezing to tone over context in trial one and three ($p<0.05$), but no significant main effects or interactions for the difference scores. Again, the average means and ± SEM of FC difference score for all groups demonstrated higher freezing to tone than context regardless of stress history or training environment. The FC difference score for the first trial showed no significant difference between the groups based on their stress group or the previous training history.

Physiological Measures

Summary. Each stress group showed an attenuation of weight gain during the stress manipulation. At the experiment's end, adrenal weights were elevated in the STR-IMM compared to CON, STR-R6, and STR-R3.

Experiment 1. Body weights were analyzed in four-week blocks corresponding to three weeks of chronic stress for each stress group and the week before as a baseline measure. A mixed factors ANOVA group (CON, STR-IMM, STR-R3, STR-R6) by week for body weights revealed a significant interaction during block 1 (the weeks of stress for STR-R6), $F(9, 108)=13.894$, $p<0.001$; block 2 (the weeks of stress for STR-R3) $F(9, 108)=66.601$, $p<0.001$; and block 3 (the weeks of stress for STR-IMM) $F(9, 108)=77.831$, $p<0.001$. Over the course of the experiment, there was a significant increase in weight for all groups ($p<0.001$). During the stress manipulation, the stressed group showed attenuated weight gain, compared to the other groups. By the final block, STR-IMM was a lower weight than CON ($p<0.01$), STR-R6 ($p<0.05$), and STR-R3

($p<0.01$). CON, STR-R6, and STR-R3 were statistically similar. Similarly, a one-way ANOVA for adrenal weight as percent of body weight by group (CON, STR-IMM, STR-R3, STR-R6) revealed an significant effect of group $F(3, 36)=4.149$, $p<0.05$, with STR-IMM showing higher adrenal weights than CON ($p<0.01$), STR-R3 ($p<0.05$), STR-R6 ($p<0.01$). Lastly, Prior to the stress manipulation, there were no group differences in body weights.

Experiment 2. Each stress group showed an attenuation of weight gain during the stress manipulation. Body weight was analyzed in four-week blocks corresponding to three weeks of chronic stress for each stress group and the week before as a baseline measure. A mixed factors ANOVA group (STR-IMM, STR-R6) by week for body weights revealed a significant interaction during block 1 (the weeks of stress for STR-R6), $F(3, 90)= 138.623$, $p<0.001$, and block 2 (the weeks of stress for STR-IMM), $F(3, 90)= 5.746$, $p<0.01$. Both groups showed an increase in weigh over blocks ($p<0.01$). During block 1, STR-R6 had attenuated weight gain compared to STR-IMM ($p<0.05$). For block 2, STR-IMM showed attenuated weight gain compared to STR-R6 ($p<0.01$).

Discussion

The goal of this study was to investigate whether chronically stressed rats provided with a post-stress rest period would differ in fear extinction learning. We used an extended acclimation model (6 days) to the training and testing contexts because it leads to similar fear conditioning acquisition (Hoffman et al., 2015). Consequently, differences in learning were minimized, allowing us to focus on extinction processes. In Experiment 1, the two chronically stressed groups given a post-stress rest period (STR-R3 and STR-R6) discriminated between the discrete CS from the context better than did

CON and STR-IMM. When the amount of freezing to the context was subtracted from the amount of freezing to the tone, producing a FC difference score, the STR-R3 and STR-R6 consistently demonstrated positive and high FC difference scores during the first extinction day compared to CON and STR-IMM. This reveals that STR-R3 and STR-R6 were able to better discriminate the tone from the context by freezing less to the context that never included a foot shock than did STR-IMM or CON.

Interestingly, the STR-IMM appeared to freeze similarly and robustly to tone and context during the first extinction day to suggest that they were generalizing. Consequently, Experiment 2 was performed to assess potential generalization to context, by testing STR-IMM rats in an environment that is different (DIFF) or the same as (SAME) the conditioning environment, using STR-R6 as a comparison. The results revealed that STR-IMM discriminated, but showed higher freezing to both tone and contexts (whether or not the context was associated with *a priori* exposure to foot shocks) than did STR-R6. Taken together, the STR-IMM may be exhibiting higher freezing to reflect hypervigilance, but not necessary higher freezing due to generalization.

In the present study, freezing to tone reflected associative learning. In Experiment 1, all groups showed spontaneous recovery (Ji & Maren, 2007). Specifically, groups showed a return of the CR, ranging from 32.4 ± 9.82% to 52.7 ±10.7% in the first two trials, when assessed six days after the end of extinction. Moreover, the CR was statistically similar amongst the groups. Last, the CR expressed during spontaneous recovery was unlikely attributed to *a priori* differences, as all groups showed similar and low levels of freezing by the end of day 2 extinction. Consequently, the freezing to tone during spontaneous recovery reflects a return of CR, as opposed to carry-over from extinction or non-associated effects. Additional supporting evidence is that the freezing response was nearly absent in all groups prior to the first tone presentation in extinction using a non-shock context, where foot shock would be least expected in Experiment 1. After the tone was presented in the non-shock context, freezing subsequently increased and this phenomenon was replicated in Experiment 2 with the two different contexts. Together, this evidence suggests that freezing to tone reflected an association formed between tone and foot shock during conditioning.

In Experiment 1, a possibility existed for *a-priori* differences in the acquisition of fear conditioning, which could have confounded interpretation for treatment influences on extinction. Specifically, STR-R6 froze less during the second tone-foot shock presentation than did the other groups. However, when the level of freezing to context was subtracted from freezing to tone, STR-R6 freezing was relatively similar as the other groups. Consequently, acquisition was similar across groups after factoring out the freezing to context. Therefore, the differences in freezing to tone during the second trial can be attributed to lower baseline freezing by the STR-R6 group, an effect that was replicated in Experiment 2.

A possible explanation for the heightened freezing to context in the non-shock context in Experiment 1 is second-order conditioning. In second-order conditioning, another association is developed from a pre-existing CS_1-US association. Specifically, after a CS_1-US association is formed, the presentation of the CS_1 results in a CR. If the CS_1 is then subsequently paired with a CS_2, such that CS_2 predicts the presentation of the CS_1, then the presentation of the CS_2 alone can then lead to the CR, particularly when the original CS-US association is strong or the US is of high intensity (Gewirtz, 2000; Rizley

& Rescorla, 1972). In the present study, it is possible that when presented with the tone (CS_1) in the non-shock environment, the non-shock environment came to function as a CS_2, resulting in freezing to context that may not necessary be reflective of background freezing, but rather the context could be a CR_2. Indeed, results from human studies of PTSD support this interpretation. Wessa & Flor (2007) compared PTSD patients, non-PTSD trauma exposed subjects, and healthy individuals on a differential delay-conditioning paradigm. Of these groups, only PTSD patients showed both impairments in extinction and successful second-order conditioning for trauma related cues. This suggests that the pairing of trauma-related cues in a neutral environment may be the reason extinction is difficult.

Our acquisition paradigm may further help explain why STR-R3 and STR-R6 showed better freezing discrimination between tone and context in the non-shock environment, because it may have caused latent inhibition to the context. Latent inhibition is pre-exposure to a CS prior to conditioning to the US, and this makes forming an association between the CS and US, and development of a CR, more difficult or slower than if pre-exposure had not occurred (Lubow, 1965). In the present study, we utilized extensive contextual acclimation. Acclimation minimizes non-specific freezing to context in chronically stressed rats (Hoffman et al., 2015), but the rats may have learned that the contexts are non-threatening and became less likely to freeze in them later. Indeed, this may be the case with STR-R3 and STR-R6 because they froze minimally to the context during extinction, whereas CON and STR-IMM froze at higher levels to context during extinction when compared to STR-R3 and STR-R6. Consequently, any lower freezing to context in the non-shock environment may reflect suppression of the

CR in a context where latent inhibition to the context had occurred. This suggests that the effects of context latent inhibition might be stronger in STR-R3/R6 than in CON or STR-IMM.

As described in the introduction, fear memories are robust and resistant to extinction training in the days after chronic stress ends (Baran et al., 2009; Hoffman et al., 2014; Miracle, Brace, Huyck, Singler, & Wellman, 2006) There are some reports that have investigated fear extinction after some time has passed following the end of chronic stress. Seven days after exposure to an single prolonged stressor, rats show impairments of in retention of fear extinction (Knox et al., 2012). Additionally, seven days after chronic variable stress rats continue to show a heighten post-extinction fear response, that is similar to the response seen in recently stressed rats (McGuire, Herman, Horn, Sallee, & Sah, 2010). In contrast, the present study found that either a 3- or 6-week rest after the end of chronic stress, rats show less freezing to the extinction context than rats who had been recently stressed or unstressed controls. The current study differed importantly from the other studies with a rest period in that the rest period in the present study was three times as long as in the previous research. Indeed, in other areas of chronic stress research, the 3-week rest timeline used in the present study reflects the rest period where a return to control-like conditions is observed, at least for conditions pertaining to spatial ability (Conrad et al., 2016; Radley et al., 2005). This longer rest period could be an important variable to the rest-mediated improvements from chronic stress seen in the current study.

Chronic stress-induced changes in behavior often correspond to changes in the morphological features of neurons, especially in dendritic complexity. For spatial navigation that requires hippocampal function (Moser, Moser, & Andersen, 1993), chronic stress impairs spatial ability and leads to hippocampal dendritic atrophy (Conrad, 2010; Mclaughlin et al., 2007). Then, after chronic stress ends and a post-stress rest period ensues, spatial ability improves and hippocampal dendrites become more complex (Bian et al., 2012; Conrad et al., 2016; Hoffman et al., 2011; Luine et al., 1994; Ortiz et al., 2015; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000). For problem solving strategies, chronic stress leads to a shift from goal-directed to habit-based problem solving (Schwabe & Wolf, 2011) and there is a corresponding increase in dendritic complexity in the dorsolateral striatum and nucleus accumbens core (Dias-ferreira et al., 2009; Taylor et al., 2014). Chronic stress produces deficits in working memory and behavioral flexibility that corresponds to dendritic retraction the medial prefrontal cortex (Holmes & Wellman, 2009). Following a post-stress rest period, medical prefrontal cortex dendritic complexity has been found to return to baseline levels (Goldwater et al., 2009; Radley et al., 2005). For fear conditioning and anxiety, the basolateral amygdala is critical (Maren, 2001; Sierra-Mercado, Padilla-Coreano, & Quirk, 2010; Vazdarjanova & McGaugh, 1999). Chronic stress facilitates the acquisition of fear conditioning and enhances anxiety (Chiba et al., 2012; Conrad et al., 1999; Cordero et al., 2003; Hoffman et al., 2015; Vyas et al., 2004), which correspond with basolateral amygdala dendritic hypertrophy (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002). If a post-stress rest period ensures following the end of chronic stress or a single stressor, anxiety is maintained and basolateral amygdala dendritic hypertrophy remains (Hoffman et al., 2017; Vyas et al., 2004). In the current study, the post-stress rest periods used (i.e., 3-weeks and 6-weeks after stress ended for STR-R3, STR-R6) were within the timeline that observed dendritic hypertrophy of the basolateral amygdala (Vyas, Pillai, & Chattarji, 2004). Consequently, it would be expected that STR-IMM, STR-R3 and STR-R6 would express similar dendritic hypertrophy in the basolateral amygdala and corresponding behavior on fear conditioning extinction. Yet, STR-R3/STR-R6 differed from STR-IMM in fear extinction, despite the potential for similar levels of dendritic hypertrophy in the basolateral amygdala. Consequently, changes in basolateral amygdala dendritic complexity may not always correspond with fear extinction. All of the regions listed are important for fear extinction (Myers & Davis, 2007). Thus, fear extinction and the results of the present study are likely determined by the interwoven circuity of these brain regions, where some regions show a return to control-like conditions and others maintain their stress-induced modifications.

The results of the present study suggest that impaired fear extinction seen in chronically stressed rats is dependent on whether a rest period from chronic stress precedes fear conditioning. Typically, clinical populations with PTSD show limited improvement from extinction-based therapies. In the present study, the only stress group that showed this PTSD-like characteristic was the STR-IMM group, which displayed more freezing to context during extinction training than either the STR-R3 or STR-R6 groups. This suggests that, in adults, the ability of chronic stress to be a risk factor for the development of PTSD may diminish with the passage of time from when chronic stress ends and when the trauma manifests. Thus, chronic stress models of PTSD need to take into account the amount of time since the chronic stress ended in experimental design and interpretation of the results.

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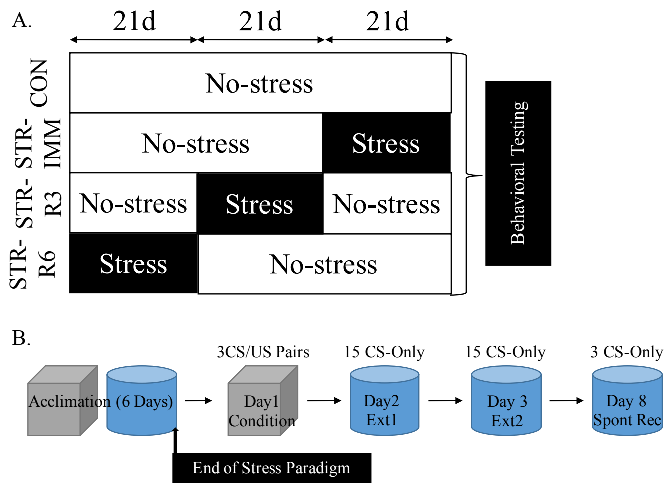

Figure 1. Timeline of Experiment 1. Animals were exposed to chronic restraint stress (6hr/day/21days) at various time points prior to the start of fear conditioning (A). Acclimation overlapped with the last six days of chronic stress and consisted of 10-minute exposure to one of the two contexts, alternating over the six days. Fear conditioning training consisted of 3 tone-foot shock pairings and was followed extinction trials and spontaneous recovery over the next eight days (B).

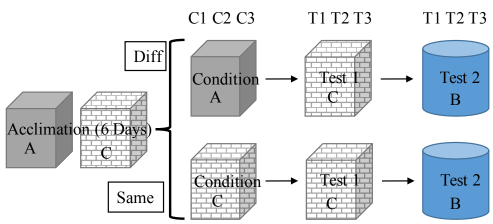

Figure 2. Timeline of Experiment 2. Acclimation overlapped with the last six days of chronic stress and consisted of 10-minute exposure to one of the two contexts, alternating over the six days. Fear conditioning training consisted of 3 tone-foot shock pairings in either Context A or C. Testing on Days 8 and 9 occurred in Contexts C and B, respectively, and consisted of 3 presentations of the tone alone.

Table 1

Differences Between Contexts

Environmental Aspects	**Context** *A*	*B*	*C*
Room Appearance			
Lighting	White	Red	Red
Isolation Cubical Doors	Closed	Open	Open
Transport	Hand	Cart	Cart
Chamber			
Arena	Test Cage	Bucket	Test Cage
Side Walls	Silver Panels	NA	Black insert and panels
Back Wall	Stripes	NA	Black insert
Drop Pan	White	NA	Dark Grey
Floor	Shock	NA	Shock
Lighting	White	Red	Red
Fan/Ambient Sound	White Computer	Red Computer	Red Computer
Cleaner	Grapefruit Scent	70% Isopropyl Alcohol	Pine Scented
Attire			
Lab Coat	Wrap Gown	White Lab Coat	Wrap Gown
Gloves	Black	Blue	Black

Note. Context A was the environment used for training. Context B was used in extinction and spontaneous recovery. Context C was an alternative training and testing environment used in Experiment 2 only.

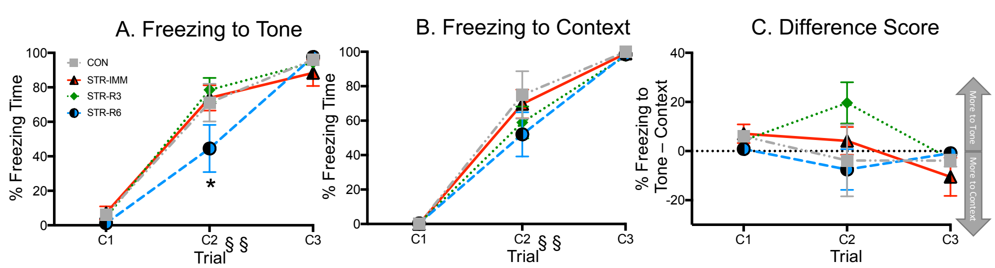

Figure 3. Freezing behavior during conditioning in Experiment 1. A. As trials progressed, all groups increased freezing to tone ($^{§§}p<0.01$), reaching peak levels by the third conditioning trial (C3). While CON and STR-IMM froze similarly to each other (as expected), the STR-R6 group froze significantly less to the second tone than did the other conditions ($^{*}p<0.05$ compared to CON, STR-IMM, STR-R3). B. As trials progressed, all groups increased freezing to context ($^{§§}p<0.01$) at a similar rate. C. When freezing to context (20 sec prior to each tone) was subtracted from the freezing to tone, no group differences were detected. This latter finding supports the interpretation that the freezing to tone on trial 2 by STR-R6 reflected a lower level of baseline freezing, but that STR-R6 showed a similar level of (or lack thereof) for tone/context discrimination as observed from the other groups during the actual training day, which can happen with few trials.

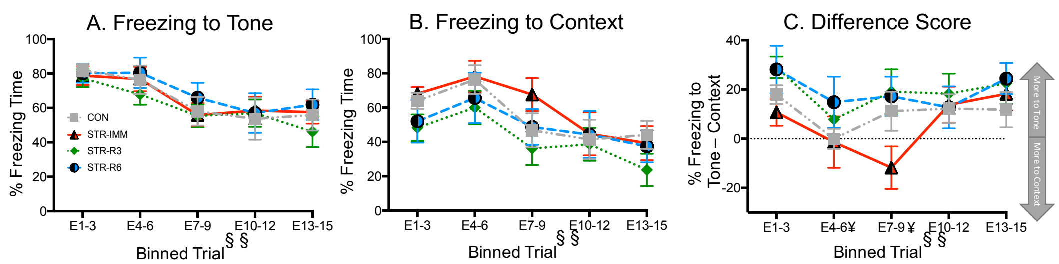

Figure 4. Over the binned Extinction 1 trials, freezing to both tone (A) and context (B) decreased ($^{§§}p<0.01$), with groups performing similarly. C. For the Difference Scores, a significant effect of bin was found with no statistical differences among groups. The Difference Score for bins E4-6, E7-9 were significantly lower than for bins E1-3, E10-12, E13-15 ($^{¥}p<0.01$). However, this appeared to be driven by STR-IMM showing lower discrimination, than the other groups (CON, STR-R3, STR-R6).

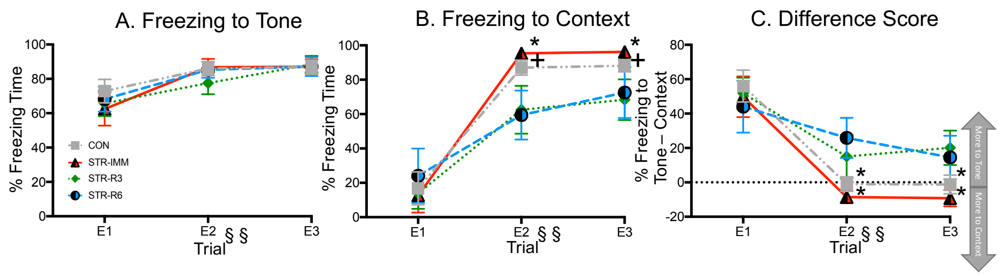

Figure 5. The first three trials of Extinction 1 were investigated to further understand the freezing behavior and potential discrimination differences. A. In the very first presentation of tones without foot shock, all groups increased freezing to tone over the first three trials ($^{§§}p<0.01$), with no group effects. B. For freezing to context prior to each tone, group differences became apparent during Trials 2 and 3. STR-IMM froze more to context than did STR-R3 and STR-R6 ($^{*}p<0.05$). Also, CON froze more to context than did STR-R3 ($^{+}p<0.05$), but did not reach significance for differing from STR-R6. C. For the difference score, both STR-IMM and CON showed lower difference scores than both STR-R3 and STR-R6 during E2 and E3 ($^{*}p<0.05$). Moreover, the difference scores for STR-R3 and STR-R6 were above chance; they froze more to tone than to context.

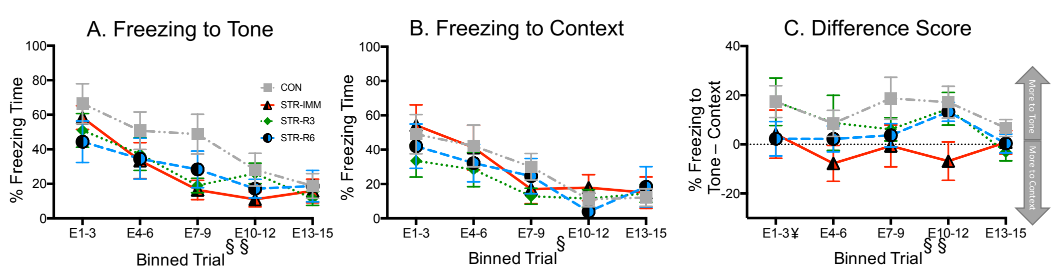

Figure 6. Binned trials in Extinction 2 for Experiment 1. Freezing to tone (A) and context (B) decreased across binned trials ($^{§§}p<0.01$), with groups performing similarly. C. For the FC Difference score, it appeared that STR-IMM showed lower discrimination, than did the other groups (CON, STR-R3, STR-R6) to produce this difference. Interestingly,

the CON discrimination means were consistently above chance, whereas STR-IMM discrimination scores were at chance.

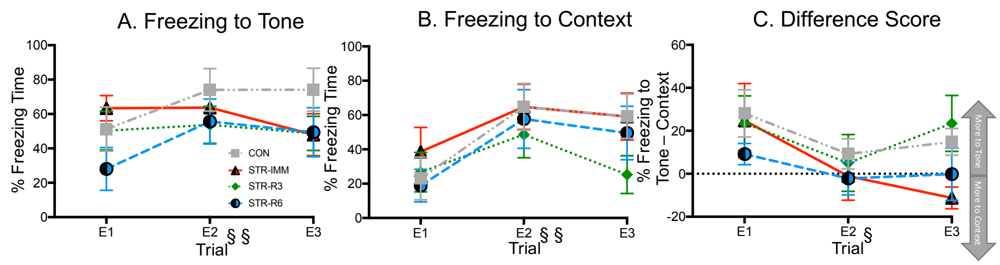

Figure 7. Freezing during the first three trials of Extinction 2 in Experiment 1. During the first three trials of Extinction 2, freezing to tone (A) and context (B), and the FC difference scores were statistically similar amongst groups.

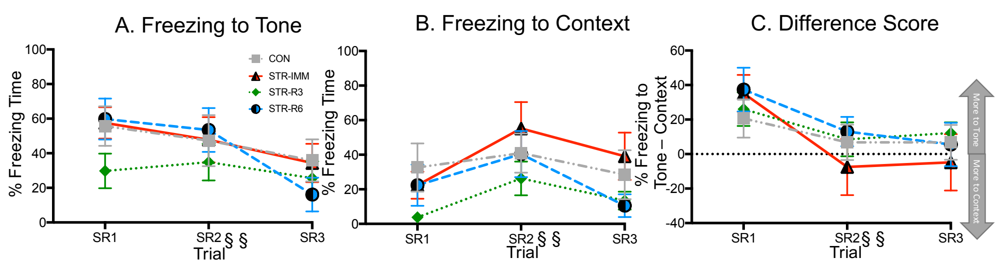

Figure 8. Freezing during spontaneous recovery in Experiment 1. All groups demonstrated spontaneous recovery. Freezing to tone (A) and context (B), and the FC difference scores were statistically similar amongst groups.

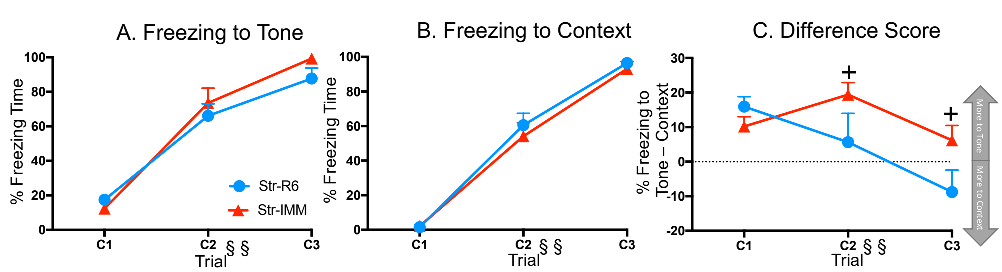

Figure 9. Freezing behavior during fear conditioning in Experiment 2. Freezing to both tone (A) and context (B) increased over conditioning trials. For the difference score, STR-IMM had higher freezing to the tone than the context compared to the STR-R6.

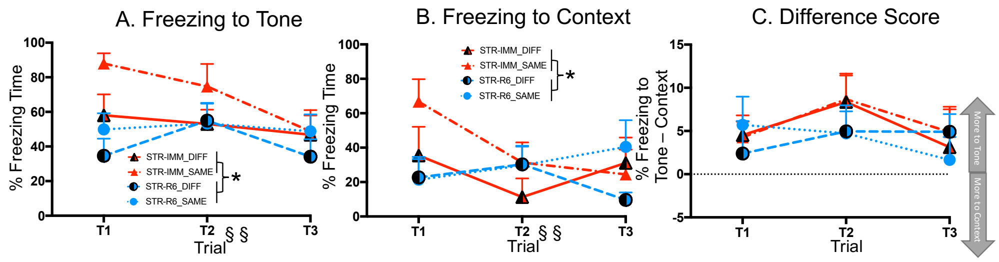

Figure 10. Freezing during Test 1 (no shock) in Experiment 2. (A) All groups decreased freezing to tone across trials (§§ p<0.05). Moreover, for trial 1, STR-IMM froze more to tone than did STR-R6 (Stress Effect * p<0.05) and training environment was significant for tone 1 (*p < 0.05), with higher freezing in the SAME environment than in the DIFF environment. (B) Freezing to context stayed the same across trials and STR-IMM (DIFF and SAME) froze more to the first context prior to tone 1 than did the STR-R6 (DIFF and SAME, *p < 0.05). (C) No differences amongst groups were found for the FC difference score; all groups froze more to tone than to context.

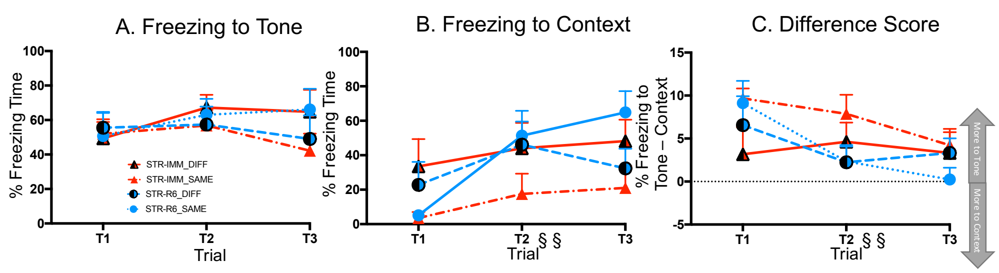

Figure 11. Freezing during Test 2. Groups froze similarly to Tone (A) & Context (B) and freezing to context increased over trials (p<0.01). (C) The FC difference scores were similar across groups and in the positive range (more freezing to tone than context), but the difference score decreased as trials progressed (§§ p<0.05).