Chronic stress has lasting effects on improved cued discrimination early in extinction

Jessica M. Judd, Elliot A. Smith, Jinah Kim, Vrishti Shah, Federico Sanabria, and Cheryl D. Conrad

Department of Psychology, Arizona State University, Tempe, Arizona 85287-1104, USA

Chronic stress typically leads to deficits in fear extinction when tested soon after chronic stress ends. Given the importance of extinction in updating fear memories, the current study examined whether fear extinction was impaired in rats that were chronically stressed and then given a break from the end of chronic stress to the start of fear conditioning and extinction. Male rats were chronically stressed by restraint (6 h/d/21 d) and tested soon (termed immediate, STR-IMM), or 3 or 6 wk after a rest period from restraint (termed rest or “R,” STR-R3, STR-R6). In Experiment 1, STR-R3 and STR-R6 discriminated between the cue and nonshock context better than STR-IMM or control. Interestingly, STR-IMM showed high freezing to the nonshock context. Consequently, Experiment 2 investigated whether STR-IMM generalized across contexts, which was not supported. Experiment 3 determined whether STR-IMM were susceptible to second-order conditioning to a novel context, but showed that the level of second-order conditioning was similar for all groups. These findings reveal that rats exposed to chronic stress and then given a rest period of 3 or 6 wk, express unique fear extinction profiles compared to control and STR-IMM. Specifically, STR-R demonstrated excellent cue and context discrimination during extinction, and perhaps showed a stress inoculation effect. For STR-IMM, the heightened freezing under these extensive acclimation parameters was not attributed to generalization nor to second-order fear conditioning to “safe” contexts and, instead, may reflect hypervigilance.

[Supplemental material is available for this article.]
after chronic stress has ended (Sousa et al. 2000; Hoffman et al. 2011; Bian et al. 2012; Ortiz et al. 2015; Conrad et al. 2017). Consequently, the present study compared rats that had been recently chronically stressed with those that were remotely stressed 3 or 6 wk prior to fear conditioning (termed “stress-rest”), to determine whether the longer gap between the end of chronic stress and the start of fear conditioning was an important factor in the maintenance of robust fear memories.

Results

Experiment I: do rats resist fear extinction 3 or 6 wk after chronic stress ends?

Summary. All rats were fear conditioned (Supplemental Fig. 1) to three tone-footshock pairings in training Context A and then extinguished to the tone (30 trials, 15 trials/day) in the nonshock Context B over the next 2 d (Fig. 1A). On the first three trials of Extinction Day 1, chronically stressed rats given a poststress rest period (chronically stress-rest for three weeks (STR-R3), chronically stressed-rest for six weeks (STR-R6)) froze less to Context B than did CON and chronically stressed-immediate (STR-IMM), but all groups froze similarly to tone, which suggested a generalization of fear to context by CON and STR-IMM. Consequently, a difference score was calculated to determine how much freezing to tone occurred relative to the nonshock Context B; STR-R3 and STR-R6 discriminated between tone and context better than did CON and STR-IMM.

Specific results. During fear conditioning, freezing to tone increased in all groups (mixed factors analysis of variance (ANOVA) for group (CON, STR-IMM, STR-R3, STR-R6) across trials (1,2,3) for tone, \( F_{(2,66)} = 313.994, P < 0.001 \) with more freezing to tone as trials progressed \( (P < 0.05) \). Importantly, all groups froze similarly to tone during the third and last conditioning trial (one-way ANOVA for group, \( F_{(3,36)} = 0.834, P = 0.485 \). Supplemental Fig. 1). Over the course of the 2 d of extinction, freezing to tone decreased and was nearly extinguished by the last trial (Fig. 1B, mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by bins (3 trials/bin with 5 bins/day) revealed a significant effect of bin on Extinction Day 1 \( (F_{(4,132)} = 17.513, P < 0.001) \) and Extinction Day 2 \( (F_{(4,132)} = 25.312, P < 0.001) \) with no other significant effects. A week after extinction ended, spontaneous recovery was performed with three tone presentations in the nonshock context to determine whether the freezing was due to associative properties, which was confirmed. A return of freezing to tone was statistically similar across groups, although STR-R3 had a nonsignificant tendency toward less recovery than the other groups (Fig. 1C, one-way ANOVA for freezing to the first tone presentation). Freezing to tone ranged from \( 29.8 \pm 10.1 \% \) to \( 59.8 \pm 11.8 \% \).

The first three trials in Extinction Day 1 were investigated to understand the tone-shock memory prior to extensive extinction presentations; they revealed that groups performed similarly (Fig. 1D). A group (CON, STR-IMM, STR-R3, STR-R6) \( \times \) trial \((1, 2, 3)\) ANOVA showed a significant effect of trial, \( F_{(2,66)} = 24.996, P < 0.001 \), with no other significant effects. Freezing to tone increased from Trial 1 to Trial 2 \((P < 0.001)\), and became statistically similar between Trials 2 and 3. Importantly, all groups performed similarly.

Freezing to Context B prior to the first tone (20 sec prior to the first tone presentation, i.e., Baseline freezing to Context B) was assessed separately from subsequent context measures to determine whether any a priori differences existed before tone presentation. Freezing to context was similar for all groups (Fig. 1E, one-way ANOVA for freezing to Context B, Baseline/Trial 1) and was
relatively low, ranging from 12.7 ± 10.0 to 19.3 ± 12.9%. For subsequent Trials 2 and 3, rats given a rest after the end of chronic stress froze less to Context B than did STR-IMM or CON (mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trial (2, 3) showed significant effect of group $F_{(3,33)} = 3.991, P < 0.05$, with no other significant effects). Specifically, STR-R3 froze significantly less to Context B than did STR-IMM ($P < 0.05$) or CON ($P < 0.05$) and STR-R6 froze significantly less to Context B than did STR-IMM ($P < 0.05$) and froze less to Context B than did CON, but the difference was not statistically significant ($P = 0.07$). This suggests that, compared to STR-IMM and CON, both STR-R3 and STR-R6 froze less to a context that never involved footshock and hence, could be considered a “safe” context.

To understand how much freezing to the tone was due to associative processes over the more generalized freezing responses occurring in the absence of a discrete cue, a fear conditioning difference score was computed and analyzed. The difference score in Trial 1 was much higher ($P < 0.001$) than in Trials 2 and 3 (Fig. 1F, mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trial (1, 2, 3) on the difference scores showed a significant effect of trial, $F_{(2,66)} = 28.631, P < 0.001$, which was followed by a one-way ANOVA for the difference score in the first trial). The groups were statistically similar and showed high freezing to the tone over the context during Trial 1 (lowest difference score = 43.8 ± 12.8%, highest difference score = 53.9 ± 8.6%). This indicated low Baseline freezing to Context B in Trial 1. For Trials 2 and 3, however, a mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trial (2, 3) on the difference score, revealed a significant main effect of group, $F_{(3,33)} = 5.557, P < 0.05$, but no significant trial or interaction. Stress groups given a rest period after chronic stress, STR-R3 and STR-R6, froze selectively to tone over the nonshock Context B compared to STR-IMM ($P < 0.05$) and CON ($P < 0.05$), suggesting that STR-R3 and STR-R6 were better than STR-IMM and CON at learning that Context B was safe.

**Experiment 2: comparison of STR-IMM with STR-R6 on context generalization**

In Experiment 1, an extended acclimation paradigm was implemented to reduce contextual conditioning and, consequently, reduce generalization between contexts. However, the STR-IMM group, but not the STR-R3 or STR-R6 groups, froze similarly to the tone and the nonshock context, suggesting that STR-IMM had facilitated generalization of fear conditioning. Consequently, Experiment 2 was performed to test for differences in context generalization between STR-IMM and STR-R6.

As in Experiment 1, STR-IMM and STR-R6 were acclimated to Contexts A and B for 6 d and then underwent fear conditioning (3 tone/footshock trials; Supplemental Fig. 2). Half the rats were fear conditioned in Context A and the other in Context B (which were counterbalanced between groups). On the next day, rats were presented with three tones without footshock in Context B, which gave rise to rats being trained and then tested in different contexts (A+/B) or in the same context (B+/B), Figure 2A.

![FIGURE 2.](image.png)

**FIGURE 2.** (A) Timeline of Experiment 2. Fear conditioning training consisted of 3 tone-footshock pairings in either Context A or B (A+ and B+, respectively). The following day, rats were placed in Context B and presented with three tones, giving rise to being tested in a different context from training (A+/B) or the same context as training (B+/B). No differences in freezing levels were found during acquisition of fear conditioning for either the stress condition (STR-IMM, STR-R6) or the contexts (data not shown). (B) Tone-alone presentation in Context B: freezing to tone. Differences among groups were observed in the first trial, with STR-IMM freezing more to tone than did STR-R6 (stress effect, $P < 0.05$), and groups trained and tested in the same Context B (B+/B) freezing more to tone than those trained and tested in a different context (A+/B) (context effect, $P < 0.05$). (C) Tone-alone presentation in Context B: freezing to context. Differences among groups were observed in the first trial (baseline freezing to Context B), with STR-IMM freezing more to Context B than did STR-R6 (stress effect, $P < 0.05$). (D) Tone-alone presentation in Context B: difference score for freezing to tone minus freezing to Context B. Groups performed similarly with no significant differences. (E) Tone-alone presentation in novel Context C: freezing to context. Freezing to novel Context “C” increased over trials for all groups, but rats trained and then tested in a different context (A+/B) froze more to the novel context prior to any tone presentation (baseline freezing to novel Context C) than did rats trained and tested in the same context (B+/B), which is illustrated in F. Freezing to tone in Context C was similar for all groups. Final number of subjects per group were n=7–8. For all graphs, ($§§$) $P < 0.01$ for significant effect of trial.
Whether trained and tested in a different (A+/B) or in the same (B+/B) context, STR-IMM froze more to tone and context in the first trial than did STR-R6 (Fig. 3B and 3B insert for tone, significant stress group by trial interaction, $F_{2,50} = 5.304, P < 0.05$, and significant trial effect, $F_{2,50} = 5.304, P < 0.05$; Fig. 2C and 2C insert for context, stress group on Trial 1 before tone presentation (baseline freezing to Context B), $F_{1,25} = 4.638, P < 0.05$). Also, freezing to tone decreased as trials progressed for all groups (Fig. 3B).

When the freezing to Context B was subtracted from the freezing to tone, no group differences were detected, and average difference scores were above chance levels (Fig. 2D). These data suggest that STR-IMM and STR-R6 discriminated between tone and context similarly, and that training context (A+/B or B+/B) did not influence tone-context discrimination. Consequently, the high freezing to tone and to Context B by STR-IMM is indicative of a potentiated freezing response and not necessarily attributed to generalization.

On the next day, rats were placed into a novel context that was unfamiliar to them, in order to ascertain freezing in a nonacclimated, nonshock environment (see timeline in Fig. 2A). Rats were presented with the tone cue (no shock) three times. Whereas the groups showed similar freezing to the tone across the trials (data not shown), differences were observed in baseline freezing to Context C, prior to the first tone presentation (Fig. 2E). Specifically, rats trained and tested in the same training condition (B+/B), froze significantly less to the novel Context C than did the rats who were trained and then tested in a different context (A+/B); stress history (STR-IMM, STR-R6) did not modify the outcome (Fig. 2F, significant training context, $F_{1,25} = 4.762, P < 0.05$). After the presentation of the tone, freezing to the novel Context C increased and was similar across all groups and across the remaining two trials ($F_{2,50} = 11.989, P < 0.001$), without any other significant main effects or interactions.

Experiment 3: are STR-IMM showing second-order conditioning during extinction?

Compared to STR-R6, STR-IMM showed heightened fear responses early in extinction in Experiment 1 and fear generalization was excluded as a possible interpretation in Experiment 2. Another possible explanation for the more robust fear responding in STR-IMM is that they more readily formed a strong second-order tone-context association, which is most likely to happen when the original tone-shock association is strong. Indeed, individuals with PTSD are more likely to form second-order associations between trauma-related and neutral cues (Wessa and Flor 2007). In Experiment 3, we tested for second-order conditioning with context as the second-order cue (Fig. 3A).

On Day 1, rats (CON, STR-IMM, STR-R6) underwent fear conditioning (first-order) as described in Experiment 1 (Supplemental Fig. 3A,B). On the next day, rats were returned back to Context A, the environment where they had been exposed to tone and footshock. Half the rats were presented with three tones and the other half exposed to Context A without tones. Approximately 4 h later, the rats were brought to Context B, which was used as the second order cue. The rats exposed to Context A earlier without tone presentations were now presented with three tones in Context B, while the remainder who received tones earlier in Context A were placed in Context B without the tone presentations. Therefore, all rats had equal exposure to both contexts and tones, but with different pairings of tone and environment: rats that received tone only presentations on Day 2 in the same context as training were designated as A+/B and those that received the second-order conditioning paradigm on Day 2 with tone in Context B were designated as A+B+. Rats in the A+B+ showed similar and high freezing to Context B by the end of the second-order conditioning session (one-way ANOVA for freezing to Context Trial 3, $F_{2,21} = 0.668, P = 0.524$; Supplemental Fig. 3C). On the third day, rats were returned to Context B to assess their potential for second order conditioning.

When freezing to Context B was assessed for second-order conditioning on Day 3, groups trained in the A+B+ order showed more freezing to Context B than did rats trained in the A+/B order, but stress history did not modify performance (Fig. 3B, mixed factors ANOVA for group (CON, STR-IMM, STR-R6) by training history (A+/B, A+B+) for freezing to Context B showed a significant effect of training history, $F_{1,44} = 24.536, P < 0.001$, with no other significant effects).

Discussion

The goal of this study was to investigate whether chronically stressed rats provided with a poststress rest period would show facilitated fear extinction learning compared to chronically stressed rats without a rest period. We used an extended acclimation model (6 d) to the training and testing contexts because it leads to similar...
danger-related cues that are paired with otherwise safe environments. In this view, freezing during Trials 2 and 3 of Extinction may have reflected the combination of an inhibitory response to Context B and an excitatory response to the tone, a test of which would be similar to a summation test for latent inhibition (Rescorla 1972; Gewirtz and Davis 2000). Indeed, results from human studies suggest that second-order conditioning in a safe context contributes to the maintenance of responding to trauma-related cues (Wessa and Flor 2007). In Experiment 3, we demonstrated that, when presented with the tone (the first-order conditioning stimulus) in a nonshock environment, the nonshock environment functions as a second-order conditioning cue for recently chronically stressed rats. However, recently chronically stressed rats, chronically stressed rats with a 6 wk rest period, and nonstressed rats all performed similarly. Our results did not support the idea that second-order conditioning contributed to the more robust freezing responses in recently chronically stressed rats. We suspect that instead of solely achieving second-order conditioning, we might have also encountered some other compound-stimuli phenomena, such as latent inhibition (discussed next, see also Rauhut et al. 1999; Brems and Heisenberg 2001; Urcelay and Miller 2009).

Latent inhibition is a phenomenon in which a neutral cue is paired with a meaningful stimulus, leading to two competing interpretations, neutral or meaningful. As it pertains to the current study, rats received extensive nonreinforced exposure to the nonshock Context B during acclimation, and so Context B likely had a "neutral" or "safe" meaning (see Fig. 1E, Baseline, low freezing to Context B prior to the introduction of the first tone). Consequently, when the shock-paired tone was introduced to Context B, the rat freezing response to Context B may have reflected a combination of information: the previous information that Context B was neutral and the new information coming from a predictive tone. In this view, freezing during Trials 2 and 3 of Extinction may have reflected the combination of an inhibitory response to Context B and an excitatory response to the tone, a test of which would be similar to a summation test for latent inhibition (Rescorla 1969; Foilb et al. 2018). While we did not explicitly test for latent inhibition, it is possible that the strength of latent inhibition was enhanced in chronically stressed rats provided with a rest period. Future studies should investigate the degree to which latent inhibition is able to control behavior in chronically stressed rodent models, as this has important clinical implications for patients with PTSD (Grillon et al. 1998; Jovanovic et al. 2012).

A defining feature of PTSD is a failure to recognize or appropriately respond to safety signals, cues that should indicate safety even in the presence of a trauma related cue (Grillon et al. 1998; Jovanovic et al. 2012). Extinction training, which here consisted of the shock-paired tone presented in a nonshock environment, should lead to a suppression of the fear response to the tone because of the new association that the tone in this environment does not predict shock. As such, the nonshock environment becomes a safety signal for when shock will not occur (Rothbaum and Davis 2003). In the present study, we found that recently chronically stressed rats froze to the "safe" context in extinction more than chronically stressed rats with a rest period of 3 or 6 wk, as seen in the first three trials of extinction in Experiment 1 and during the first context of Test 1 in Experiment 2. Perhaps the recently chronically stressed group had difficulty in Learning the safety signals as quickly as the chronically stressed rats with a rest period cohorts. A similar argument would suggest that chronically stressed rats with a rest period were better able to learn safety signals because of their lower freezing levels during extinction sessions. Future work should continue to investigate the behavioral
mechanisms that lead to better, more flexible outcomes in the aftermath following chronic stress.

Taken together, the freezing responses to tone reflected associative learning in the present study. In Experiment 1, all groups showed spontaneous recovery seven days after the last extinction session, with freezing to tone ranging from 32.4 ± 9.8% to 52.7 ± 10.7% in the first two trials. Moreover, groups showed statistically similar freezing to the tone during spontaneous recovery. The return of freezing response in chronically stressed rats with a 3 wk rest period showed a tendency to be less robust than the other groups, but this was unlikely to be attributed to a priori differences, as all groups showed similar and low levels of freezing by the end of Extinction 2. Consequently, the freezing to tone during the spontaneous recovery session likely reflected associative processes, as opposed to carry-over effects from extinction or nonassociative effects (Ji and Maren 2007). Additional supporting evidence was that, in Experiment 1, the freezing response was nearly absent in all groups prior to the first tone presentation in extinction using a nonshock context (i.e., Baseline freezing), in which footshock would be least expected. After the tone was presented in the nonshock context, freezing subsequently increased and this phenomenon was replicated in Experiment 2 with the two different contexts. Hence, this evidence suggests that freezing to tone reflected an association formed between tone and footshock during conditioning.

In the present studies, most key findings arose from freezing to the context or the difference score that incorporated freezing to context, rather than from freezing to the tone cue. In Experiment 1 specifically, the introduction of the tone to the extinction context led to enhanced freezing to context in all groups, as well as significant group differences in freezing to context (Fig. 1E, trials 2 and 3). However, a potential concern is that freezing to tone may have contributed to the freezing to context directly (e.g., carry-over from prior tone presentation) or indirectly (e.g., through a context-tone association activated by tone presentation). Nonetheless, by incorporating a measure of freezing to tone, the difference score partially addresses these concerns. In particular, if significant effects on freezing to context were merely due to differences in freezing to tone that spilled over to the context through carry-over, higher-order associations, generalization, or any other mechanism, then these spill-over effects could be eliminated by subtracting freezing to tone from freezing to context (or context from tone, as was conducted here). However, if treatment selectively affected the magnitude of such tone-to-context spill-over without significantly affecting freezing to tone itself, then significant treatment effects on difference scores may still arise. As unlikely as the latter possibility might be, it cannot yet be ruled out.

This study is one of the first to investigate fear extinction processes based upon the timing from the end of chronic stress. In a prior study, rats given a single prolonged stressor and then tested for fear conditioning and extinction seven days later show poor retention of fear extinction (Knox et al. 2012). Another report used chronic variable stress and then tested rats on fear conditioning and extinction seven days later and found resistance to fear extinction (McGuire et al. 2010). The present study adds to this literature and revealed that chronically stressed rats given a 3 or 6 wk rest period after chronic stress ended, show fear extinction that differs from either controls or stressed rats tested soon thereafter. Our results support the interpretation that exposure to a traumatic event 3 or 6 wk after a chronic stress history leads to a different fear extinction profile than had a traumatic event occurred soon after chronic stress ended.

A possible interpretation for the chronically stressed rats with a rest period improved discrimination of the conditioned tone from context is an inoculation effect. For example, stressor exposure early in life can lead to less anxiety and better cognitive flexibility when faced with stressors later in life (Katz et al. 2009; Lyons et al. 2009, 2010). In the present study, when young adult rats were chronically stressed, they demonstrated better discrimination between a conditioned cue and the safe context when they were permitted 3 or 6 wk of rest following the end of chronic stress, compared to rats tested soon after stress ended. A similar finding has been observed for spatial memory in that a rest period following the end of chronic stress leads to better performance compared to a delay of a few days (Hoffman et al. 2011). This suggests that, under some circumstances, an earlier chronic stress experience can be helpful in navigating a later stressful experience.

A significant amount of PTSD patients do not seek therapy until much later after the traumatic event, giving the traumatic memory a chance to strengthen (Bryant 2017b). Indeed, symptoms must be present for at least one month before PTSD can be diagnosed (American Psychiatric Association 2013). In the present study, however, extinction training occurred in the days after fear conditioning (i.e., the traumatic experience). This was performed because it allowed for early assessment, as some patients exhibit PTSD-associated symptoms immediately after a traumatic event, called acute stress disorder, or ASD (American Psychiatric Association 2013). If left untreated, ASD could become PTSD (Bryant 2017a). Our current study suggests that it is possible that early extinction therapies in individuals showing ASD symptoms might lead to reduced PTSD rates. This is supported by the observation that the recently chronically stressed group was able to eventually extinguish their elevated freezing to the cue tone and context to a similar level as the other groups as extinction continued. Further supporting evidence is that recently chronically stressed performed similarly to the rest of the conditions during the measurement of spontaneous recovery. Future studies should investigate fear responding to extinction in weeks after fear conditioning to determine whether early extinction therapies could inoculate against PTSD.

The results of the present study suggest that when contexts are familiar, such as with our paradigm that included an extensive acclimation prior to training, chronically stressed individuals perform differently during the initial fear extinction acquisition process, depending upon whether extinction occurs soon after chronic stress ends or after a rest period. Chronically stressed individuals soon thereafter exposed to fear extinction discriminated between the conditioned stimulus and safe environment, but exhibited high freezing, perhaps due to being hypervigilant. Follow-up studies ruled out the possibility that chronically stressed subjects were generalizing or forming a stronger second-order context-tone association than the other groups. When chronically stressed individuals were exposed to fear extinction after a 3 or 6 wk period, they also discriminated between the conditioned stimulus and safe environment, but without showing hypervigilance. Typically, clinical populations with PTSD show heightened responses to trauma related cues and impaired improvement with extinction-based therapies. In the present study, the stress group that best modeled these PTSD-like characteristics was the recently chronically stressed group, which displayed more freezing to context during extinction training than either the chronically stressed with a rest period groups. This suggests that the time between chronic stress and the trauma exposure is a factor that may influence PTSD development.

Materials and Methods

Subjects
Male Sprague-Dawley (Charles River Laboratories) rats weighing ~250 g upon arrival were pair housed in standard laboratory cages (21°C–22°C, corn cob bedding). Except where noted below,
animals were allowed food and water ad libitum. Animals were housed on a reverse 12:12 light cycle, with lights off at 7 a.m. 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 h/day for 21 d. Our previous work demonstrated that these restraint parameters were the minimum required duration for restraint stress to produce behavioral and structural changes (McLaughlin et al. 2007); Restraint took place between 9 a.m. and 3 p.m. 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 by the investigator consistent. Animals were initially restrained using a wire mesh tube (6.4 cm DIA × 26.7 cm L) that was made using grip guard sealer (Flynn and Enslow) to keep the wire ends coated, but were upgraded to a larger restrainer (7.6 cm DIA × 29.2 cm L) as the rats grew. Weights were recorded weekly to confirm stressor effectiveness.

Group assignments and timeline

In Experiment 1, rats were assigned to one of four groups (n = 10/group, 40 rats total): control (CON), chronic stress with a 6 wk rest period (STR-R6), chronic stress with a 3 wk rest period (STR-R3), or chronic stress without a rest (i.e., tested within days or immediately, STR-IMM). Training on fear conditioning occurred 6 wk (STR-R6), 3 wk (STR-R3), or within days (STR-IMM) from the last day of restraint. The 3 and 6 wk rest durations were selected because some behaviors, such as spatial ability, improve 3 wk after the rats grew. Weights were recorded weekly to confirm stressor effectiveness.

Fear conditioning

Fear conditioning apparatus. Rat test cages were square and made of metal and plastic (30.5 cm W × 25.4 cm D × 30.5 cm 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, E10-12MR) mounted on the inside of the sound-attenuating cabinet and were produced by a frequency generator (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, Microsft Corp.). The computer was connected to a line system (Coulbourn, H27-91R) that controlled the stimulus output via an USB interface (Coulbourn, U90-11H). Infrared lights (Coulbourn, H27-91R) were positioned to be observed by the video and were programmed 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 Processors (Evertech), which allowed four videos to be viewed on one monitor (Samsung, 24”). The behavior from eight single chambers that were viewed on two monitors was also backed up on a VCR/DVD recorder (Funai). Behavior was manually scored by a trained observer. Freezing was defined as the lack of all movement, except those associated with respiration (Blanchard and Blanchard 1969). Freezing to tone was defined as any freezing that took place during the 20 sec tone presentation and freezing to context was defined as any freezing that took place in the 20 sec immediately prior to the presentation of the tone. A fear conditioning score was calculated in order to assist in understanding how much of the freezing to the tone was due to associative processes over a more generalized, nonassociative freezing response that may occur in the absence of the discrete cue. This was calculated as the amount of freezing to tone minus the amount of freezing to context 20 sec prior to the tone (similar to Majchrzak et al. 2006). Inter-rater reliability was 97.3 ± 6.4% and intra-rater reliability was 95.7 ± 2.0%.

Environments for fear conditioning procedures. Over the course of the three experiments, three different contexts were used. In one context, the testing cages were square metal and plastic and had a metal floor of parallel rods (Coulbourn, H10-11RTC-SC), silver side panels (Coulbourn, H90-00MR-MKT01), 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-lit 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, Inc.) and the room lighting of the overall holding room was white light. 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 a second context, the testing cages were round, plastic blue buckets (37 cm H × 30.5 cm DIA, Lowes). A 3-Watt, Red LED bulb (91 Lumens; Feit Electric) was used as illumination in the isolation cubical. A 356 cm, computer fan with red LED light (Thermaltake, TT-1425) provided white noise/ventilation in the cubicle. The cleaning solution used after each rat was 70% isopropyl alcohol (Vi-Jon, Inc.). 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 the third context, the testing cages were modifications of the square testing cages (Coulbourn, H10-11RTC-SC). A black semi-circular Perspex insert was placed in the testing cage to provide 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 the second context. 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. The goal of the acclimation sessions was to reduce conditioning to the environment and decrease possibilities for generalization between the contexts allowing extinction processes to be studied without a priori differences in baseline freezing (Jacobs et al. 2010; Hoffman et al. 2014, 2015). Acclimation occurred ~1 h after restraint ended each day so they would have ample opportunity to access food and water prior to acclimation sessions. Rats were acclimated by being placed in a context for 10 min daily. Exposure to the two contexts alternated over the 6 d for a total of three exposures to each context. The day after the last acclimation session...
(Day 7), fear conditioning training occurred in Context “A.” Training consisted of three tone-footshock pairings (intertrial interval (ITI) range between pairings=80–170 sec), with the first tone was presented after 114 sec. The training session lasted 535 sec. 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). Seven 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 their fear responses to the non-shock environment, so a second experiment to test for generalization was performed. STR-R6 was used as a comparison group due to low freezing to context seen in this group, and because a goal of Experiment 2 was to better understand the differences between the chronic stress groups. A nonstressed group was not included here because the comparison in generalization between CON and STR-IMM has been previously reported (Hoffman et al. 2014). Acclimation and fear conditioning occurred as described in Experiment 1, whereby rats were acclimated to both environments over 6 days and then fear conditioned (three tone and footshock pairings) the following day in either Context “A” or “B,” which were counter-balanced across groups. A day after fear conditioning, all rats were given three tone-alone presentations in Context B (ITI = 320 sec). One day later, the rats were placed in a novel Context “C,” where they had no prior acclimation experience and then presented with three tone alone presentations.

Experiment 3: investigation as to whether STR-IMM is more likely to form second-order conditioning than CON or STR-R6. This experiment was done to test whether STR-IMM were more likely to form a second-order association between the tone and the extinction context compared to STR-R6 or CON. Acclimation occurred over 6 days as described in Experiment 1. One day after acclimation ended, fear conditioning occurred in Context “A.” Training consisted of three tone-footshock pairings as in Experiment 1. One day after training, all the rats were reexposed to Context A in the morning, with half of them receiving three-tone presentations (A/B). In the afternoon, all rats were exposed to Context “B,” with the half that did not get exposed to the tone in the morning receiving three-tone presentations (A/B) for the second-order conditioning manipulation. This led to a 3 × 2 design for stress group (CON, STR-IMM, STR-R6) and second-order conditioning or not (A/B+, A/B). The following day, all groups were tested for contextual freezing behavior in Context B.

Statistical analysis
Results were analyzed using ANOVA. Results that were significant at the P < 0.05 level were additionally analyzed using the LSD (least significant difference) post-hoc test. 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 footshock presentation). Three rats were excluded from both Experiment 1 (1 CON, 2 STR-R6) and Experiment 2 (1 STR-R6 +/B, 1 STR-IMM A+/B, 1 STR-IMM B+/B). Please note that due to equipment malfunction, some data were lost in Experiment 3 to produce n = 7 or 8/group (n = 7 for STR-R6 A+/B for Training Day 2 a.m. and p.m. sessions, for STR-R6 A+/B for Training Day 2 a.m. and Test Day, for STR-IMM A+/B for Training Day 2 a.m. and p.m. sessions, for STR-IMM A+/B for Training Day 2 a.m. and p.m. sessions, and for CON-A+/B for Training Day 2 a.m. and p.m. sessions, and for CON-A+/B for Training Day 2 a.m. session.) To correct for unequal variances, data was transformed using √x + T (Fiddell and Tabachnick 2007). Data analysis was done using SPSS Version 24 on an Apple iMac running macOS Sierra (v 10.12.6).

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