Research

Learned together, extinguished apart: reducing fear to complex stimuli

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Pairing a previously neutral conditioned stimulus (CS; e.g., a tone) to an aversive unconditioned stimulus (US; e.g., a footshock) leads to associative learning such that the tone alone comes to elicit a conditioned response (e.g., freezing). We have previously shown that an extinction session that occurs within the reconsolidation window attenuates fear responding and prevents the return of fear in pure tone Pavlovian fear conditioning. Here we sought to examine whether this effect also applies to a more complex fear memory. First, we show that after fear conditioning to the simultaneous presentation of a tone and a light (T+L) coterminating with a shock, the compound memory that ensues is more resistant to fear extinction than simple tone-shock pairings. Next, we demonstrate that the compound memory can be disrupted by interrupting the reconsolidation of the two individual components using a sequential retrieval+extinction paradigm, provided the stronger compound component is retrieved first. These findings provide insight into how compound memories are encoded, and could have important implications for PTSD treatment.

[Supplemental material is available for this article.]

In Pavlovian fear conditioning, the pairing of an initially neutral conditioned stimulus (CS; e.g., a tone) to an aversive unconditioned stimulus (US; e.g., a footshock) leads to the formation of a fear memory such that later presentation of the tone without the footshock elicits a conditioned response (CR; e.g., freezing). Within hours post-conditioning, fear memories become consolidated and thereafter are thought to be extremely persistent. Much of research on fear memories involves Pavlovian conditioning to a single, simple conditioned stimulus (CS); yet, in most fear disorders (e.g., PTSD), memories are complex—they integrate elements from a number of sensory modalities. Combination laws and compound learning theory suggest that individuals respond differently to a pair of compound-conditioned stimuli than they do to separately conditioned stimuli that are presented simultaneously at a later time (Rescorla and Wagner 1972; Weiss 1972; Kamin and Gaioni 1974; Mackintosh 1976; Kehoe and Gormezano 1980; Pearce and Hall 1980). At the neural level, both the hippocampus and the amygdala are required for the encoding and retrieval (ret) of complex fear memories, whereas discrete cued fear conditioning relies heavily on the lateral amygdala for these functions (Kim and Fanselow 1992; Phillips and LeDoux 1992). Isolating the conditions that permit and/or inhibit targeting of complex memories will be important to translate basic findings to clinical settings.

Fear reduction after conditioning is generally achieved in one of two ways: extinction (ext) (Pavlov 1927) or reconsolidation blockade/update (Misanin et al. 1968; Nader et al. 2000; Sara 2000; Monfils et al. 2009; Schiller et al. 2010). When a memory is retrieved (such as with the presentation of a single CS), it enters a labile state that allows it to be updated with new information before becoming re-stored into long-term memory (LTM)—a phenomenon termed "reconsolidation" (Misanin et al. 1968; Nader et al. 2000). Reconsolidation blockade through pharmacological, surgical, or electroconvulsive shock intervention enables an enduring fear memory attenuation (Misanin et al. 1968; Land et al. 2000; Nader et al. 2000). In extinction, the repeated presentation of the CS in the absence of a reinforcer leads to the progressive decrease in CR. Recent evidence suggests that behavioral interference during reconsolidation can persistently update fear memories without pharmacological intervention (Monfils et al. 2009; Clem and Huganir 2010; Schiller et al. 2010; Rao-Ruiz et al. 2011; but see also Chan et al. 2010). By presenting an extinction session within the reconsolidation window after an isolated retrieval, the fear memory is reinterpreted as safe during the update period and reencoded into long-term storage as benign (Monfils et al. 2009). Reconsolidation-centered manipulations, particularly using behavioral paradigms, are a promising avenue to treat anxiety-related disorders.

In the current set of experiments, we examined the formation of multisensory compound (tone+light) fear memories in rats, and tested whether they could be attenuated through reconsolidation- and/or extinction-based manipulations of each, or both, elements of the compound over a series of five experiments. In experiment 1, the efficacy of ret+ext was examined after fear conditioning to a light CS and by testing animals for long-term memory and spontaneous recovery of freezing (SR). In experiment 2, we fear-conditioned animals to a tone+light (T+L) compound stimulus and measured freezing to the elements (tone alone, light alone, or tone+light) of the compound, as well as quantified c-Fos expression in order to discern the neural mechanisms engaged after the retrieval of the elements of the compound. In experiments 3, 4, and 5, different protocols were tested 24 h after conditioning to attenuate fear responding to the compound stimulus. Fear reduction was accomplished through either traditional extinction of the individual elements or of the entire compound, or through ret+ext where extinction

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Table 1. Experimental design for experiment 3

Day 1	Day 2	Day 3			Day 4
Context acclimation	FC T+L	T ret	10 min	T ext (18 CS)	T+L LTM T LTM L LTM
		Context	10 min	T ext (19 CS)	T+L LTM T LTM L LTM
Context acclimation	FC T+L	L ret	10 min	L ext (18 CS)	T+L LTM T LTM L LTM
		Context	10 min	L ext (19 CS)	T+L LTM T LTM L LTM
Context acclimation	FC T+L	T+L ret	10 min	T+L ext (18 CS)	T+L LTM T LTM L LTM
		Context	10 min	T+L ext (19 CS)	T+L LTM T LTM L LTM

All rats were handled 24 h prior to day 1. All experimental manipulations done on days 3 and 4 were in a different context (context B) than in days 1 and 2. For each retrieval group, $n = 12$. (T) Tone, (L) light, (T+L) tone+light, (FC) fear condition.

occurred 10 min after an isolated retrieval of either the entire compound or the individual elements (see Tables 1 and 2 for full experimental design).

Results

Experiment 1: ret+ext of a light CS prevents spontaneous recovery

Previous research has shown that the ret+ext paradigm can persistently attenuate the fear response to a tone cue (Monfils et al. 2009); thus, we first sought to replicate this result in a light CS before proceeding with a compounded cue. We found that both ret+ext and ext-only of the light resulted in extinction of the fear memory, as evidenced by a significant within-subjects reduction in freezing revealed by a repeated measures ANOVA with extinction cue as the repeated factor and retrieval group as the between-subjects factor (within subjects effect of cue, $F_{(17,153)} = 10.928$, $P < 0.001$). We then tested consolidation of extinction by measuring freezing to the tone 24 h after extinction for a long-term memory (LTM) test, and the return of fear by assessing spontaneous recovery (SR) of freezing 1 mo later. A repeated measures ANOVA with mean LTM freezing and mean SR freezing as the within-subjects factor and retrieval group as the between-subjects factor revealed a significant within-subjects effect of test ($F_{(1,19)} = 6.699$, $P = 0.018$) and a significant test × retrieval group interaction ($F_{(1,19)} = 15.041$, $P < 0.001$). These results indicate that retrieval group significantly influenced freezing between the testing time points. Follow-up *t*-tests were performed to determine specific effects of ret+ext or ext-only. In order to investigate the return of fear for each group individually, a paired *t*-test was performed between SR and LTM time points. Consistent with previous research on tone fear conditioning (Monfils et al. 2009), ret+ext of the light prevented the spontaneous recovery of fear ($P = 0.193$), whereas ext-only of the light resulted in a significant spontaneous recovery of freezing ($P = 0.011$) using a within-subjects comparison of freezing during the SR test compared to the LTM test (Fig. 1). Additionally, an independent samples *t*-test showed that although ret+ext and ext-only of the light resulted in similar levels of freezing during the LTM test ($P = 0.75$), ret+ext of the light cue resulted in significantly less freezing during the spontaneous recovery test 24 d after extinction than ext-only of the light ($P < 0.001$) (Fig. 1).

Experiment 2: retrieval of the components after compound fear conditioning

To examine complex fear memories, we used a compound cue that consisted of auditory (tone) and visual (light) components to create a memory that was comprised of elements of different intensities. This memory incorporates aspects of how learning occurs outside the laboratory while allowing us to maintain significant experimental control. We first verified that, after fear conditioning to the compound T+L, rats had a differential freezing response to the individual components of the compound memory (tone, light, and T+L). Initial investigation of the two cues showed that although rats froze less to the light than to the tone (Fig. 2), the amount of freezing to the individual components after compound conditioning did not differ significantly from freezing to the components after fear conditioning to that individual component (Fig. 2). Thus, there was no evidence of overshadowing after our conditioning paradigm. Additionally, because rats can freeze no more than 100% during this test, the high levels of freezing evoked during testing to the tone would not allow for a summation effect where the rat would freeze to

Table 2. Experimental design for experiment 4

Day 1	Day 2	Day 3							Day 4		
Context acclimation	FC T+L	T ret Context	10 min	T ext (18 CS) T ext (19 CS)	2–3 h	L ret Context	10 min	L ext (18 CS) L ext (19 CS)	T+L LTM	T LTM	L LTM
Context acclimation	FC T+L	L ret Context	10 min	L ext (18 CS) L ext (19 CS)	2–3 h	T ret Context	10 min	T ext (18 CS) T ext (19 CS)	T+L LTM	T LTM	L LTM

Experiments done on days 3 and 4 were in a different context than the experiments performed on days 1 and 2. For each retrieval group, $n = 12$. (T) Tone, (L) light, (T+L) tone+light, (FC) fear condition.

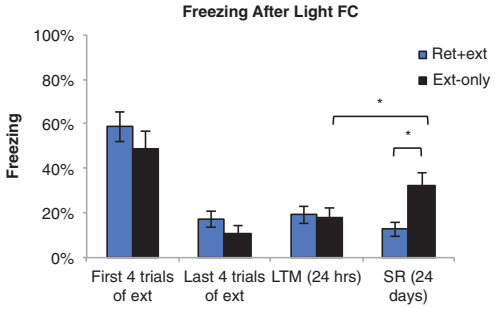

Figure 1. Effect of extinction or ret+ext of a light CS after fear conditioning to the light on long-term memory (LTM) 24 h after extinction and spontaneous recovery (SR) tests 24 d after extinction. Both ret+ext ($n = 12$) and ext-only ($n = 10$) groups extinguish to the same levels, presented as the last four trials of extinction averaged together, and freeze at similar levels during LTM tests. Ret+ext does not show spontaneous recovery when tested 24 d after extinction compared to LTM ($P = 0.193$), but ext-only ($n = 10$) does show spontaneous recovery compared to LTM ($P = 0.011$) as evidenced by increased freezing during spontaneous recovery tests. Additionally, during SR tests ret+ext rats froze significantly less than ext-only rats. (*) $P < 0.05$.

the T+L compound at a level equal to that of summing the freezing levels of the tone alone and the light alone. Finally, there was minimal unconditioned freezing to the components as measured by freezing during the first cue of fear conditioning to each of the cues (Supplemental Table 1).

Before attempting to reduce freezing to the compound fear memory, we wanted to understand potential mechanistic differences underlying partial retrieval of components of a fear memory. In order to look at brain regions activated after a single retrieval session, we performed immunohistochemistry for c-Fos protein, a measure of brain activity (Hoffman et al. 1993), following tone, light, T+L, or context-only retrieval in rats fear conditioned to the T+L compound.

Rats freeze less to the light than to the tone or T+L

Rats were fear conditioned to the T+L compound, received a T+L, light-only, or tone-only retrieval the next day, and were perfused 1 h after retrieval. Rats froze significantly less to the light component than to the T+L or tone-only component and freezing to the context was minimal (Fig. 3A). One-way ANOVA with cue retrieved as a fixed factor revealed a significant effect ($F_{(3,25)} = 32.454$, $P < 0.001$). Post-hoc Tukey HSD tests revealed that the rats froze significantly less during context retrieval ($n = 5$) when compared to retrieval of any of the elements ($P < 0.001$ for L [$n = 8$], T [$n = 9$], and T+L [$n = 9$]). Additionally, the rats froze significantly less during light retrieval than retrieval of the full T+L compound ($P < 0.001$) and less than during tone retrieval ($P = 0.03$). Rats did not freeze significantly less to the tone than they did to the T+L during retrieval ($P = 0.632$).

c–Fos expression in LA and PrL mirrors freezing behavior

We examined c-Fos expression in the lateral amygdala with a one-way ANOVA contrasts to compare activation after retrieval of the individual components. One-way ANOVA with retrieval group as the factor revealed a significant effect of retrieval group on c-Fos activation in the lateral amygdala ($F_{(3,27)} = 25.135$, $P < 0.001$). Tukey post-hoc comparisons showed that retrieval of the full T+L compound resulted in significantly more activation in the lateral amygdala compared to that in the light ($P < 0.001$), context ($P < 0.001$), and tone ($P = 0.015$), and retrieval of the tone resulted in significantly more c-Fos activation compared to that in the context ($P < 0.001$) and the light ($P = 0.048$) (Fig. 3B).

In the vmPFC, we found that the prelimbic and infralimbic cortices showed opposite patterns of activity after retrieval of either the light or the T+L component. We found significantly more activation in the infralimbic cortex after light compared to T+L retrieval (Fig. 3B). In the prelimbic cortex, however, retrieval of the T+L compound resulted in significantly more activation compared to retrieval of the light (see Supplemental Fig. 1 for representative image). One-way ANOVA with retrieval group as the factor revealed a significant effect of group ($F_{(3,26)} = 27.592$, $P < 0.001$) in the infralimbic cortex. Post-hoc Tukey HSD mean comparison tests revealed significantly greater c-Fos activation in the IL after light retrieval compared to retrieval of each of the other components (context, tone, and T+L, $P < 0.001$). Additionally, retrieval of T+L resulted in significantly less c-Fos expression in the IL than retrieval of the tone ($P = 0.049$). One-way ANOVA with retrieval group as the factor revealed a significant effect of group ($F_{(3,26)} = 10.723$, $P < 0.001$) in the prelimbic cortex. Post-hoc Tukey HSD mean comparisons revealed significantly greater c-Fos activation after T+L retrieval compared to context ($P < 0.001$) and light ($P = 0.003$), but not when compared to retrieval of the tone ($P = 0.206$). Based on the freezing behavior during the retrieval session, an a priori planned comparison revealed significantly less c-Fos expression in the PrL during retrieval of the light compared to retrieval of the tone ($P = 0.04$).

Light and T+L retrieval induce increase in CA1 c–Fos expression

In the CA1 subfield of the hippocampus, retrieval of any combination of components containing the light (light only as well as T+L) resulted in significantly more c-Fos expression than retrieval of the tone component, suggesting the possibility that the light carries a contextual component, given the well-documented role of the CA1 in the retrieval of contextual memories (Hall et al. 2001; Strekalova et al. 2003; Lee and Kesner 2004; Hunsaker and Kesner 2008). One-way ANOVA with retrieval group as the factor revealed a significant effect of group ($F_{(3,24)} = 12.572$, $P < 0.001$) in the CA1 subfield of the hippocampus (Fig. 3B). Post-hoc Tukey HSD mean comparisons revealed significantly greater c-Fos activation after light and T+L retrieval when compared to that of tone ($P < 0.001$ and $P = 0.002$, respectively) and the context ($P = 0.001$ and $P = 0.005$, respectively). c-Fos expression in the CA3 and dentate gyrus (DG) regions of the hippocampus did not reveal any differences after retrieval of the separate components (data not shown). All retrievals took place in a context different than the one used during fear conditioning. Therefore, the activity seen in the CA1 subfield of the hippocampus after the context-only retrieval provides a baseline of activity for neural activation after exposure to a new contextual environment in fear conditioned rats.

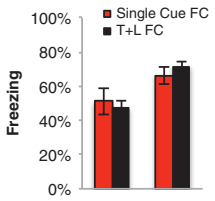

Figure 2. Freezing to the individual component of the compound 24 h after either single cue fear conditioning or compound fear conditioning shows no significant differences in freezing to the cues based on the method of fear conditioning (single cue or compound cue). For the single cue FC groups, tests were performed on the same cue as the one used in FC.

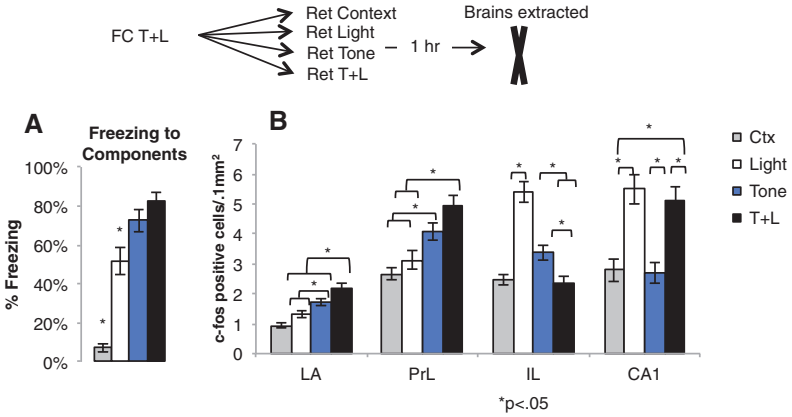

Figure 3. Counts of c-Fos positive cells in the LA and vmPFC (IL and PrL) after an isolated retrieval of one of the components (context, $n = 5$; light, $n = 5$; tone, $n = 6$; or T+L, $n = 5$) and freezing to each component. (*A*) Freezing during a single retrieval of the elements of the compound 1 h prior to perfusion. Rats froze significantly less during light retrieval than during retrieval of either the full T+L compound or retrieval of the tone ($P < 0.05$). Rats showed significantly less freezing during context retrieval ($n = 5$) when compared to retrieval of any of the elements ($P < 0.001$ for L [$n = 8$], T [$n = 9$], and T+L [$n = 9$]). Rats did not freeze significantly less to the tone than they did to the T+L during retrieval ($P = 0.632$). (*B*) c-Fos activity in the lateral amygdala mirrored the pattern of expression seen in freezing where retrieval of the T+L results in significantly more activation than that of the light ($P = 0.001$) and context ($P < 0.001$) and retrieval of the tone results in significantly more activation than that of the context ($P = 0.026$) but does not reach significance when compared to the light ($P = 0.063$). Expression in the PrL showed that retrieval of the T+L compound resulted in significantly more c-Fos activity than retrieval of the context alone ($P = 0.037$). The IL showed the opposite pattern of expression than that seen in the PrL, consistent with the idea that the PrL and IL have opposing functions in fear conditioning, where retrieval of the light resulted in significantly more activity than retrieval of any of the other components ($P < 0.001$). Additionally, retrieval of the tone resulted in significantly more c-Fos activity than retrieval of the full compound ($P = 0.049$) in the IL. In the CA1 subfield of the hippocampus, a region involved in contextual fear conditioning, retrieval of the light and T+L resulted in significantly more activation than retrieval of the tone ($P = 0.033$ and $P = 0.048$, respectively). Error bars represent ± SEM.

Experiment 3: differential effects of retrieval+extinction of complete, vs. partial elements of, compound on long-term memory of fear

The results of experiment 2 confirm that fear is greater to the T+L compound than to the light, as evidenced both by increased freezing and by increased expression of c-Fos protein in the LA and PrL. Experiment 2 also showed that the CA1 subfield of the hippocampus is involved in retrieval of the light and T+L components but not the tone. Since the rats both freeze at varying levels and show different patterns of neural activation depending on the component retrieved, we next sought to reduce freezing with either a retrieval+extinction or extinction-only procedure targeting either the full compound or the individual elements of the compound.

Experiment 3a: fear returns 24 h after ret+ext/ext–only of T+L

Fear conditioning rats to a T+L compound and following with a ret+ext or ext-only of this compound T+L (see Table 1 for experimental design) revealed that compound fear memories are resistant to a single extinction session, as evidenced by spontaneous recovery of freezing 24 h after extinction or ret+ext (Fig. 4A). LTM was analyzed with mixed factor ANOVAs with retrieval group (ret+ext, ext-only, or no ext) as the between subjects factor and LTM cue as a repeated factor. For T+L LTM, there was a main effect of retrieval group ($F_{(2,33)} = 4.318$, $P = 0.022$). Post-hoc Tukey HSD mean comparisons revealed that ret+ext of the TL compound resulted in a significant decrease in freezing compared to the no ext group ($P = 0.028$) and the T+L ext-only group showed a trend toward freezing reduction compared to the no ext group that was not significant ($P = 0.063$). During T+L extinction, both ret+ext ($n = 12$) and ext-only ($n = 12$) groups showed extinction to the T+L compound ($P < 0.001$); however, just 24 h later both groups showed spontaneous recovery of freezing to the T+L compound ($P < 0.001$) relative to the last four trials of extinction (see Fig. 5A for LTM graphs to individual cues).

Experiment 3b: ret+ext of the tone reduces freezing to tone and light components

Ret+ext with the tone is the only manipulation that resulted in a persistent decreased freezing to the tone (Fig. 4B). Both ret+ext ($n = 12$) and ext-only ($n = 12$) groups induced a reduction in freezing to the tone component during the extinction session ($P < 0.001$); however, the ext-only group showed spontaneous recovery of freezing ($P = 0.026$) during LTM tests 24 h after extinction, whereas the ret+ext group did not show recovery ($P = 0.427$). Additionally, the ret+ext group showed significantly less freezing than the no ext group during LTM tests to the tone ($P < 0.0001$, $n = 12$) as well as the ext-only group ($P = 0.002$). Within-compound associations were next examined by extinguishing one component of the compound and then testing to LTM of the other component (Rescorla and Cunningham 1978). Interestingly, ret+ext of the tone led to reduced freezing of the light component compared to the no ext group ($n = 12$, $P = 0.005$) (Fig. 5B) and reduced freezing to the T+L compound compared to no ext ($n = 12$, $P < 0.05$) (Fig. 5B) as shown through post-hoc Tukey HSD mean comparisons.

Experiment 3c: ret+ext/ext–only with the light reduce freezing to light only

For light extinction (Fig. 4C) after T+L fear conditioning, both ret+ext ($n = 12$) and ext-only ($n = 12$) groups showed extinction to the light component ($P < 0.001$), and neither group showed spontaneous recovery 24 h later (ret+ext, $P = 0.008$; ext-only, $P = 0.002$). Within-compound associations were next examined, and neither ret+ext nor ext-only of the light reduced freezing to the tone component or the full compound (Fig. 5C).

Together, these results suggest that targeting the more salient component of the compound (i.e., tone) through the ret+ext paradigm allows for targeting the within-compound association of the memory. Despite this reduction in freezing, there was a return of fear just 24 h after extinction for the T+L extinction groups and the overall levels of freezing to the compound after extinction of individual components was high enough to suggest that ret+ext did not completely update the compound fear memory as safe (>40%). Since the compound memory consists of an additional component of the within-compound association, which the results of experiment 3b suggest can be targeted through ret+ext of the tone, we next sought to target the compound fear memory with two extinction (or ret+ext) sessions, one for each of the individual components. By targeting each component separately, we aimed to reduce the overall CS–US association as well as the T–L within-compound association.

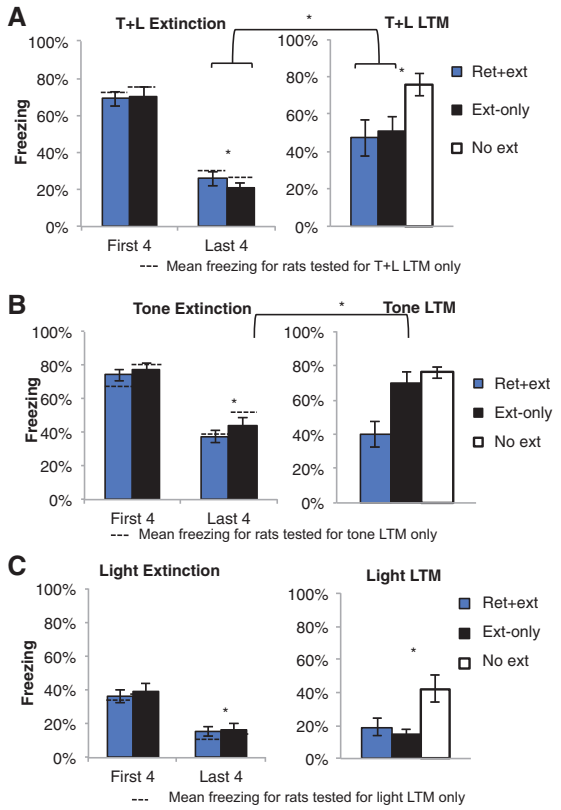

Figure 4. Freezing during the first four and last four trials of extinction after either an isolated retrieval session of context-only retrieval followed by 10 min in the home cage, along with LTM tests to a single component of the T+L compound after T+L fear conditioning. For each panel, the dotted lines indicate the mean freezing of the rats that were later tested for LTM of the same component as extinction. (*A*) Ext-only or ret+ext of the T+L compound results in a significant reduction in freezing during the extinction session ($P < 0.001$); however, during LTM tests 24 h after extinction, there is recovery of freezing with both extinction treatments ($P < 0.001$) suggesting that compound memories are resistant to extinction. Nonetheless, ret+ext of the T+L compound resulted in a significant reduction in freezing ($P > 0.05$) compared to the no ext group. (*B*) During tone extinction, both groups showed extinction to the tone component ($P < 0.001$). However, the ext-only group showed spontaneous recovery of freezing ($P = 0.026$) during LTM tests 24 h after extinction, whereas the ret+ext group maintained a reduction in freezing compared to the no ext group ($P < 0.0001$) and ext-only group ($P = 0.002$). (*C*) During light extinction, both groups (ret+ext and ext-only) showed extinction to the light component ($P < 0.001$). Additionally, neither group showed spontaneous recovery during the light LTM test ($P = 0.33$) and maintained a significant reduction in freezing compared to the no ext group (ret+ext, $P = 0.008$; ext-only, $P = 0.002$).

Experiment 4: effects of sequential ret+ext/ext-only of components

In experiment 4, we investigated the effect of two separate ret+ext/ext-only sessions on freezing to the individual, as well as combined, components of the compound. In order to discern any effects of the presentation order of the two extinction sessions, experiment 4 was divided into two parts. In part one, 24 h after T+L fear conditioning, rats received a light retrieval+light extinction (or light extinction only) followed 3 h later by a tone retrieval+tone extinction (or tone extinction only). The 3-h time point was chosen to ensure that both extinction sessions occurred within the reconsolidation window, as it is presumed that manipulations performed within this window of opportunity take advantage of targeting the memory during reconsolidation before the memory is fully consolidated into long-term storage (Nader et al. 2000). In part two, rats underwent the same procedures as in part one, however they received the tone ret+tone extinction (or tone extinction only) prior to the light extinction. Twenty-four hours after extinction, LTM tests were performed for each cue (T+L, tone, and light, three CSs per test) in sequence (see Table 2 for experimental design). Freezing levels for the no ext group did not differ significantly ($P > 0.05$) when LTM tests were performed in sequence (each rat tested for all three cues) from freezing of the no ext groups in experiment 3 where each rat was only tested to one cue.

Sequential ret+ext reduces freezing more than ext–only if tone is targeted first

Targeting the tone and light separately and leading with the more salient of the two (the tone) resulted in a significant reduction in freezing to each component, as well as the compound as a whole. In this case, ret+ext of tone followed by ret+ext of light lead to significant decreases in freezing during LTM tests the next day compared both to FC-only control rats (no ext) and ext-only groups (Fig. 6A). Ret+ext of the tone component followed 3 h later by ret+ext of the light component ($n = 12$) resulted in significantly reduced freezing compared to both the ext-only ($n = 12$) and no ext groups ($n = 12$) during sequential LTM tests performed 24 h after extinction for the T+L ($P = 0.032$) and tone components ($P = 0.047$) and compared to the no ext group for the T+L ($P = 0.026$), tone ($P = 0.009$), and light components ($P < 0.001$). Long-term memory data were analyzed with separate two-way ANOVAs with retrieval group and CS cue (as a repeated measure, three cues per LTM test) as the factors. There was a main effect of group

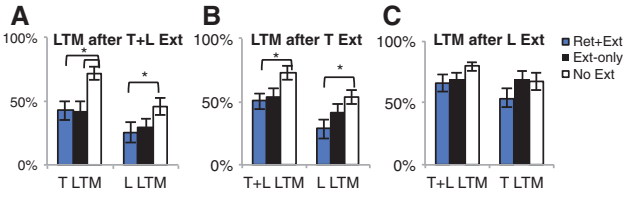

Figure 5. Freezing during LTM tests to the cues that were not the same as the cue extinguished in experiment 3. (*A*) Retrieval+extinction ($n = 12$) as well as extinction only ($n = 12$) of the full T+L compound resulted in a significant decrease in freezing to the tone component compared to the no ext group ($n = 12$) (ret+ext, $P = 0.007$; ext-only, $P = 0.009$). Additionally, retrieval+extinction of the full T+L compound ($n = 12$) resulted in significant reductions in freezing to the light compared to the no ext group ($n = 12$; T+L, $P = 0.045$), whereas the extinction-only group ($n = 12$) did not quite reach significance ($P = 0.135$). (*B*) Freezing during LTM to T+L showed that retrieval+extinction of the tone ($n = 12$) resulted in a significant decrease in freezing compared to the control group ($n = 12$) during T+L LTM ($P = 0.040$), whereas the tone extinction only ($n = 12$) did not result in a significant reduction in freezing during T+L LTM ($P = 0.122$). Retext of the tone component ($n = 12$) resulted in reduced freezing to the light during light LTM tests ($P = 0.005$). Post-hoc Tukey HSD mean comparison tests showed that retrieval+extinction of the tone component resulted in significant reductions in freezing to the light compared to the no ext group ($n = 12$, $P = 0.005$), whereas the ext-only group did not reach significance ($n = 12$, $P = 0.169$). (C) Extinction of the light component did not result in any significant decreases in freezing for either the ret+ext ($n = 12$) or the ext-only ($n = 12$) group during T+L LTM ($P = 0.088$ and $P = 0.185$, respectively). Post-hoc Tukey HSD mean comparison tests showed that neither ret+ext ($n = 12$) nor extinction only of the light ($n = 12$) resulted in significant reduction in freezing to the tone component (ret+ext, $P = 0.253$; ext-only, $P = 0.985$). Error bars represent ± SEM. (*) $P < 0.05$.

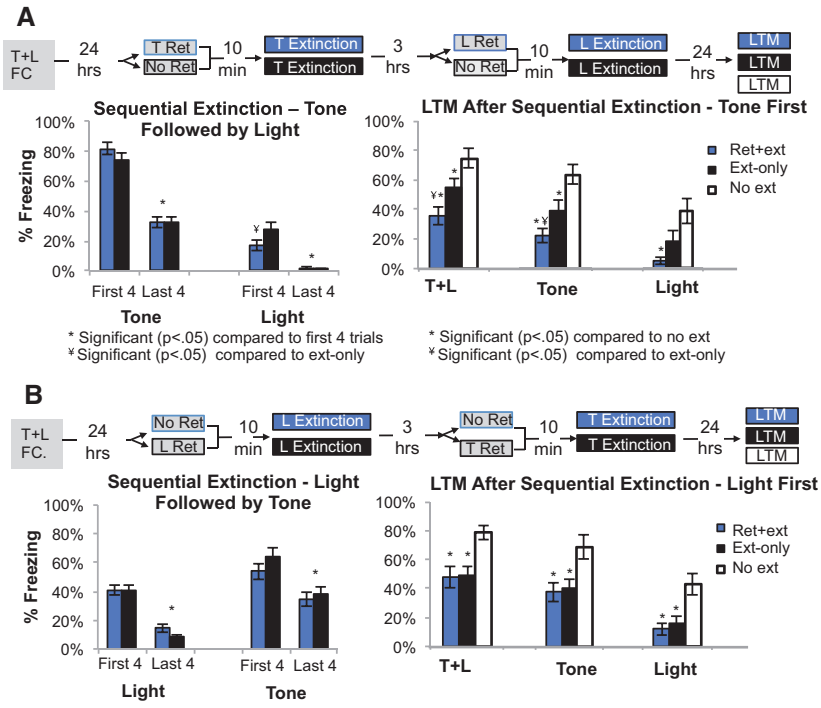

Figure 6. Effects of sequential extinction (or ret+ext) of each component of the compound on LTM. (*A*) Extinction or ret+ext of the light component followed 3 h later by extinction or ret+ext of the tone component reduced freezing to the T, the L, or the full T+L compound during sequential LTM tests compared to no ext groups, but did not result in significantly different amounts of freezing between ret+ext and ext-only groups. (*B*) Ret+ext of the tone component followed 3 h later by ret+ext of the light component resulted in significantly reduced freezing compared to both the ext-only and no ext groups during sequential LTM tests performed 24 h after extinction for the T+L and tone components and compared to the no ext group for the T+L, tone, and light components. Error bars represent ± SEM.

for T+L, tone, and light LTM tests ($F_{(2,33)} = 14.484$, $P < 0.001$; $F_{(2,33)} = 16.710$, $P < 0.0001$; and $F_{(2,33)} = 9.403$, $P = 0.001$, respectively) indicating a reduction in freezing for all components of the compound. Post-hoc Tukey's honestly significant difference mean comparisons showed that tone retrieval+one extinction followed by light retrieval+light extinction resulted in a significant decrease in freezing during LTM tests across three CS presentations compared to extinction only for T+L LTM ($P = 0.032$) and tone LTM ($P = 0.047$) and no extinction groups for T+L LTM ($P < 0.001$), tone LTM ($P < 0.001$), and light LTM ($P < 0.001$). The difference in freezing between ret + ext and ext-only during light LTM tests was not significant ($P = 0.149$). Extinction only also showed a significant reduction of freezing compared to controls for T+L LTM ($P = 0.026$) and tone LTM ($P = 0.009$) but not for light LTM ($P = 0.055$). Additionally, an independent sample *t*-test comparing ret+ext and ext-only during the first four trials of the light extinction showed a significant decrease in freezing in the group that had previously undergone ret+ext of the tone ($P = 0.05$). Alternatively, when the light is extinguished first, there is a main effect of group for T+L, tone, and light LTM tests ($F_{(2,33)} = 11.695$, $P < 0.001$; $F_{(2,33)} = 6.855$, $P = 0.003$; and $F_{(2,33)} = 13.822$, $P < 0.001$, respectively) but that there are no significant differences in freezing between ret+ext group and ext-only group in any of the LTM tests ($n = 12$; T+L, $P = 0.984$; tone, $P = 0.975$; light, $P = 0.777$) (Fig. 6B) but ret+ext results in significant decreases in freezing compared to no extinction group for all LTM tests (T+L, $P < 0.001$; tone, $P = 0.006$; light, $P < 0.001$) along with ext-only compared to no ext (T+L, $P = 0.001$; tone, $P = 0.011$; and light, $P = 0.001$).

Experiment 5: compound fear conditioning to auditory and visual cues of equal saliences

To determine whether the sequential ret+ext effect found in experiment 4 was a result of targeting the more salient cue of the compound or a result of targeting the auditory cue, we next attempted to equate the saliences of the auditory and visual cues and repeat the sequential extinction/ret+ext performed in experiment 4. This was accomplished by reducing the amplitude of the tone CS (from 80 dB to 70 dB) and changing the light from a constant cue to a blinking light (500 msec on/500 msec off). Saliency of a cue was determined by measuring freezing to each of the components after compound fear conditioning. Twenty-four hours after fear conditioning to the 70-dB tone and blinking light compound (70T+BL) long-term memory tests to either the full compound, 70-dB tone alone (70T), or blinking light (BL) alone revealed equivalent freezing to the 70-dB tone alone and blinking light alone revealed a main effect of cue tested (one-way ANOVA $F_{(2,30)} = 15.801$, $P < 0.001$). Post-hoc Tukey HSD mean comparisons indicated that the rats froze equivalently to the 70-dB tone as the blinking light ($P = 0.464$) and that freezing to either of these cues alone was significantly less than freezing to the full compound ($P < 0.001$). When rats were fear conditioned to a single cue (either the 70-dB tone or the blinking light) and then tested to that cue the following day, no significant differences were found in freezing to either the 70-dB tone or the blinking light ($t_{(25)} = 0.558$, $P = 0.582$) (Fig. 7).

Sequential ret+ext of matched intensity cues reduces freezing to compound and prevents reinstatement of fear regardless of presentation order

Twenty-four hours after fear conditioning to the matched intensity 70T+BL compound, rats underwent either ret+ext with the

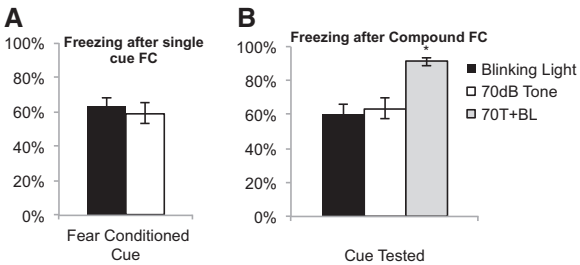

Figure 7. Freezing to individual components when saliency was matched. (*A*) Rats were fear conditioned to a single cue (either the 70-dB tone or the blinking light) and then tested to that cue the following day. No significant differences were found in freezing to either the 70-dB tone or the blinking light ($t_{(25)} = 0.558$, $P = 0.582$). (*B*) After compound fear conditioning, long-term memory tests to either the full compound, 70-dB tone alone, or blinking light alone indicated that the rats froze equivalently to the 70-dB tone as to the blinking light ($P = 0.464$) and that freezing to either of these cues alone was significantly less than freezing to the full compound ($P < 0.001$).

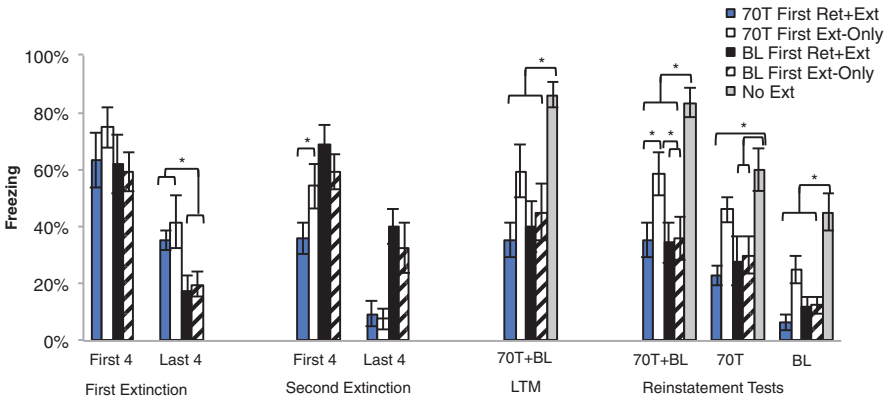

Figure 8. Freezing during sequential extinction and retrieval+extinction, LTM, and reinstatement tests when compound CS consists of matched intensity blinking light and 70-dB tone. Rats were fear conditioned to a matched intensity 70T+BL compound, and underwent either ret+ext with the 70-dB tone followed 3 h later by ret+ext of the blinking light (70T first ret+ext, $n = 8$), ret+ext with the blinking light first followed 3 h later by ret+ext of the 70-dB tone (BL first ret+ext, $n = 14$), ext-only with the 70-dB tone followed 3 h later by ext-only of the blinking light (BL first ext-only, $n = 8$), ext-only with the blinking light first followed 3 h later by ext-only of the 70-dB tone (70T first ext-only, $n = 14$), or remained in their home cages (no ext, $n = 15$). Ret+ext of the 70-dB tone component reduced freezing to the blinking light during the second ret+ext compared to ext-only ($P = 0.048$). All forms of sequential extinction with individual cues matched on saliency resulted in a significant decrease in freezing to the compound 24 h after extinction or retrieval+extinction compared to no ext (ext-only 70-dB tone first, $P = 0.03$; all other P's < 0.001). There was no reinstatement of freezing to the compound for any of the extinction groups (all P's > 0.05) when compared to their freezing during LTM and all forms of sequential ext-only or ret ext resulted in a significant decrease in freezing to the compound compared to the no ext groups ($P < 0.01$). Additionally, both forms of ret+ext and ext-only with the blinking light first significantly reduced freezing during tests for reinstatement to the compound compared to ext-only with the tone first (all P's < 0.05). The tone only reinstatement test revealed that all forms of extinction or retrieval+extinction reduced freezing to the tone compared to the no extinction group (all P's < 0.001) with the exception of the tone first ext-only ($P = 0.322$). Ret+ext with the tone first significantly reduced freezing to the tone during reinstatement tests compared to ext-only with the tone first ($P = 0.047$). All extinction groups showed reduced freezing to the blinking light test for reinstatement (all P's < 0.05).

70-dB tone followed 3 h later by ret+ext of the blinking light (70T first ret+ext, $n = 8$), ret+ext with the blinking light first followed 3 h later by ret+ext of the 70-dB tone (BL first ret+ext, $n = 14$), ext-only with the 70-dB tone followed 3 h later by ext-only of the of the blinking light (BL first ext-only, $n = 8$), ext-only with the blinking light first followed 3 h later by ext-only of the 70-dB tone (70T first ext-only, $n = 14$), or remained in their home cages (no ext, $n = 15$). Consistent with the results of experiment 4, ret+ext of the tone component in the first extinction session resulted in a significant reduction in freezing to the light component during the first three trials of the second extinction session ($t_{(14)} = 2.171$, $P = 0.048$). Despite similar initial levels of freezing to the individual components, as evidenced by no significant differences in freezing during the first three cues of extinction ($F_{(3,37)} = 1.072$, $P = 0.373$), the rats froze less to the blinking light during the last three cues of the first extinction than they did to the 70-dB tone during the last three cues of the first extinction, for both ret+ext and ext-only ($F_{(3,37)} = 7.090$, $P < 0.001$; all post-hoc Tukey HSD $P < 0.05$) (Fig. 8), suggesting the blinking light was more efficiently targeted with ext-only or ret+ext than the 70-dB tone. The following day, long-term memory tests to the matched intensity full compound revealed that there was a main effect of extinction group ($F_{(4,54)} = 13.689$, $P < 0.001$). Post-hoc Tukey HSD mean comparisons revealed that all forms of sequential extinction with individual cues matched on saliency resulted in a significant decrease in freezing to the compound 24 h after extinction or retrieval+extinction compared to no ext (ext-only 70-dB tone first, $P = 0.03$; all other P's < 0.001) (Fig. 8). After receiving an unsignaled footshock, rats were tested for reinstatement to each of the components sequentially. Paired t-tests comparing the mean freezing during the 70T+BL reinstatement test to the 70T+BL LTM test revealed no reinstatement of freezing for any of the extinction groups (all P's > 0.05) suggesting that each form of matched sequential extinction (ext-only and ret+ext) prevented the reinstatement of freezing to the 70T+BL compound. One-way ANOVAs to investigate between group differences of freezing during the reinstatement tests for each cue revealed a main effect of extinction group for the cues (70T+BL reinstatement, $F_{(4,52)} = 25.882$, $P < 0.001$; 70T reinstatement, $F_{(4,52)} = 10.645$, $P < 0.001$; BL reinstatement, $F_{(4,52)} = 13.51$, $P < 0.001$). Post-hoc Tukey HSD mean comparisons revealed that in the T+L reinstatement test, all forms of sequential ext-only or ret+ext resulted in a significant decrease in freezing to the compound compared to the no ext groups ($P < 0.01$). Additionally, both forms of ret+ext and ext-only with the blinking light first significantly reduced freezing during tests for reinstatement to the compound compared to ext-only with the tone first (all P's < 0.05). The tone-only reinstatement test revealed that all forms of extinction or retrieval+extinction reduced freezing to the tone compared to the no extinction group (all P's < 0.001) with the exception of the tone first ext-only ($P = 0.322$). Ret+ext with the tone first significantly reduced freezing to the tone during reinstatement tests compared to ext-only with the tone first ($P = 0.047$). All extinction groups showed reduced freezing to the blinking light test for reinstatement (all P's < 0.05).

Discussion

Pavlovian fear conditioning to a single cue CS, frequently a tone, is a commonly used and effective paradigm to investigate the basic mechanisms of fear learning. Behavioral interference during the reconsolidation window (ret+ext) has been shown to reduce fear responding and prevent the return of fear to a tone CS (Monfils et al. 2009). Outside the laboratory setting, however, signs that predict or signal danger can consist of multiple different cues, potentially from more than one sensory modality. In the present set of experiments, we sought to expand the application of the ret+ext paradigm to slightly more complex fear memories consisting of a compound tone+light cue. First, we tested the ret+ext procedure in Light+shock fear conditioning paradigm. We found that, consistent with the previous application of this paradigm in tone cued fear conditioning (Monfils et al. 2009), in light cued fear conditioning, ret+ext of the light CS prevented the spontaneous recovery of fear. Given that ret+ext prevented the return of fear of both a light CS (experiment 1) and a tone CS (Monfils et al. 2009), we created a complex CS of the tone and light presented in compound in order to investigate the neural mechanisms involved in retrieval of the components (experiment 2) and used a modified version of the ret+ext paradigm as a means of reducing fear to the compound CS (experiments 3, 4, and 5).

In experiment 2, we examined the neural regions activated by retrieval of the components in order to understand the contribution of each element of the compound. The expressions of c-Fos protein in the LA and vmPFC confirm that fear expression to the T+L component is greatest as shown by increased expression in the LA and PrL and that the rat perhaps suppresses fear to the light component as evidenced by increased c-Fos expression in the IL cortex, an area shown to suppress the fear response by inhibiting the amygdala output (Vidal-Gonzalez et al. 2006). Previous research has shown that the PrL and IL regions of the vmPFC have opposing effects on the expression of conditioned fear where the IL inhibits and the PrL excites amygdala output, resulting in either decreased or increased freezing, respectively (Milad and Quirk 2002; Vidal-Gonzalez et al. 2006). Recalling a cue that was previously extinguished has been shown to activate the IL but not the PrL, suggesting that the IL plays a role in inhibiting the fear response after extinction training (Milad and Quirk 2002; Santini et al. 2008). In this experiment, the increased activity seen in the IL after retrieval of the light is consistent with the low levels of freezing induced by the light portion of the compound and could be the result of engaging a mechanism similar to extinction (Milad and Quirk 2002; Mueller et al. 2008; Sierra-Mercado et al. 2011) or fear inhibition (Vidal-Gonzalez et al. 2006; Laurent and Westbrook 2009). Additionally, c-Fos expression in the dorsal CA1 subfield of the hippocampus, a region known to play an important role in the retrieval of contextual fear memories (Hall et al. 2001; Strekalova et al. 2003; Lee and Kesner 2004; Hunsaker and Kesner 2008) shows that both retrieval of the light and T+L leads to increased activation. If the tone and light components contain information that is coded for differently at the neural level, where the tone carries a strong cued component, as evidenced by increased lateral amygdala activation, and the light may represent a more complex contextual environment separate from the context provided by the conditioning chambers, as evidenced by increased CA1 activation. This suggests that both components must be targeted in order to reduce fear response to the compound.

The purpose of this set of experiments was not to confirm or propose a specific configural or elemental theory based on previous research on combination laws and compound learning theories (Rescorla and Wagner 1972; Weiss 1972; Kamin and Gaioni 1974; Mackintosh 1976; Rescorla and Cunningham 1978; Durlach and Rescorla 1980; Kehoe and Gormezano 1980; Pearce and Hall 1980; Wagner and Brandon 2001), but rather to find a behavioral paradigm that could reduce fear expression to compound stimuli.

In experiments 3, 4, and 5, rats were fear conditioned to a compound stimulus that consisted of the concurrent presentation of a tone and a light CS coterminating with a footshock and then received a retrieval+extinction protocol previously found to enable updating of a memory trace and persistently reduce freezing. In order to fully understand the limits of this memory updating paradigm, we performed a series of experiments comparing extinction and retrieval+extinction of each individual component of the compound as well as of the compound as a whole.

Our results suggest that compound fear memories are resistant to a single extinction session, as evidenced by spontaneous recovery of freezing 24 h after extinction or retrieval+extinction. This set of experiments also provides some information on the boundary conditions surrounding behavioral methods of reconsolidation update (ret+ext). Although ret+ext of the compound fear memory significantly reduces freezing to the compound compared to rats that did not undergo any extinction, the amount of freezing displayed by the rats is still substantial; this suggests that the fear memory has not been completely updated to a safe memory. We further show that a compound memory consisting of a loud tone and a less salient light can be targeted behaviorally through the retrieval and subsequent extinction of individual elements instead of the entire combination of events, provided the tone is activated first. When the saliency of the tone and light components are equated, we show that both ret+ext and ext-only of the individual components in sequence reduce freezing to the entire combination of events regardless of which component is targeted first compared to no ext. However, if the equally salient tone is targeted first, sequential ret+ext reduces freezing during long-term memory and reinstatement tests to the full compound compared to ext-only. This suggests that, depending on the combination of cues, the retrieval+extinction paradigm promotes efficient targeting of the stored compound memory, allowing for a more persistent fear reduction without ever presenting the compound as a whole.

One important theoretical concept is that not only do the components of the compound form an association with the US, but they also form an association with each other (Rescorla and Cunningham 1978; Durlach and Rescorla 1980; Speers et al. 1980; Holland and Ross 1981; Heth 1985). These within-compound associations were nicely demonstrated in an eloquent set of taste aversion experiments by Rescorla and Cunningham (1978) where rats were first exposed to two distinct flavors (hydrochloric acid and sucrose) presented in compound. Following this compound exposure, one of the solutions was aversively paired with lithium chloride and rats were later tested for consumption to the opposite component (in this case sucrose, which a rat would naturally prefer). Confirming the presence of within-compound associations, the rats no longer preferred to ingest the sucrose (Rescorla and Cunningham 1978). This phenomenon has since been replicated in a number of other paradigms, including fear conditioning, where within-compound associations have been found not only between cues presented in compound (Rescorla 1980; Cunningham 1981; Rescorla and Colwill 1983; Williams et al. 1986), but also between the fear conditioning context and a single cue used as the CS (Marlin 1982).

In the present set of experiments, we found that the ret+ext paradigm allowed for targeting the within-compound associations whereas ext-only did not. Rats that were fear conditioned to the compound T+L but then underwent tone ret+ext showed decreases in freezing to the light component when tested for LTM 24 h after extinction whereas the ext-only group did not. In experiment 3, the group of rats that underwent tone ret+ext first showed a significant reduction in freezing to the first four cues of the light extinction session when compared to the rats that only received extinction of the tone. This is consistent with the idea that the within-compound association of the tone and light is better retrieved and targeted through this reconsolidation paradigm as the ret+ext is targeting the compound more successfully than extinction alone.

Our results are consistent with Thompson and Van Hoesen's (1967) prior experiments on compound conditioning in a two-way shuttle avoidance task in rats. In this set of experiments, the authors performed a series of tests demonstrating that if one component of T+L compound CS is a high-intensity stimulus and the other is a low-intensity stimulus, the high-intensity stimulus will show poor extinction if extinguished alone (Thompson and Van Hoesen 1967). This is consistent with our data that show that ext-only of the tone (in this paradigm, the high-intensity stimulus as evidenced by increased freezing compared to the light) after T+L fear conditioning shows spontaneous recovery of fear during long-term memory tests just 24 h after extinction. However, in this experiment, we found that ret+ext of the same tone component prevents the recovery of the fear response to the tone at long-term memory. Thompson and Van Hoesen (1967) went on to examine the effects of a second extinction of the opposite component of the compound and concluded

that both stimuli contribute to responding in a differential manner as determined by the stimulus intensity. We also find that sequential extinction of the two individual components results in decreased freezing, as measured at LTM; however, we also see that ret+ext with the higher intensity cue retrieved and extinguished first results in a further decrease of the fear response when compared to ext-only.

The sequential extinction technique is similar to paradigms that find deepened extinction after acquisition to a single CS through multiple extinction sessions that include a compound of the original CSs (Rescorla 2006; Janak and Corbit 2011) and research that finds enhanced extinction when single cues are conditioned separately and then extinguished in compound (Rescorla 2000; Witnauer and Miller 2012). In these experiments, researchers have found that extinction consisting of a compound of single cues that had been extinguished previously results in a deepened extinction that prevents the return of fear (Rescorla 2006), appetitive responding (Janak and Corbit 2011), and drug seeking (Kearns et al. 2012). However, in the present experiment, the rats were initially conditioned to a compound cue based on the possible translational relevance of fear-inducing cues consisting of cues of more than one sensory modality. The individual components were chosen for extinction because there may be clinical situations in which recreating the entire compound may not be possible, thus there may be a benefit to attenuating memories without ever presenting the entire compound.

When intensities of the cues were matched, there was only a benefit of sequential ret+ext over ext-only when the tone extinction preceded the light extinction. This result is similar to the pattern of fear reduction seen when the tone cue is stronger than the light cue with one important distinction. When the blinking light is extinguished first, both the ret+ext and ext-only groups show significant fear reduction compared to ext-only with the low-intensity tone first and the no ext rats. One possibility is that the blinking light is not interpreted as a single discrete cue but rather as several rapid cues with an intertribal interval (ITI) of 500 msec. It is possible, then, that in experiment 5, instead of simply altering the saliency of the cues, we also introduced a new variable that includes varied spacing between cue presentations. Either way, after fear conditioning to a blinking light and low-intensity tone compound, sequential extinction or sequential retrieval+extinction of the components results in a reduction of freezing to the compound both 24 h after extinction (or retrieval+extinction) and after an unreinforced footshock.

Our data show that after fear conditioning to a compound cue, ret+ext of the auditory cue (when the auditory component is either of higher or equal intensity to the visual component) reduces freezing to the visual cue (both when tested 3 h later with an additional extinction session and when tested the next day) but ret+ext of the visual cue does not reduce freezing to the auditory cue. It is possible that, even though the freezing levels were matched between the auditory and visual cues in experiment 5, the subjects, who in this experiment were Sprague–Dawley rats and are known to have poor visual acuity (Prusky et al. 2002), still interpret the auditory signal as the most salient. This was further evidenced by a stronger reduction in freezing to the visual cue after extinction or retrieval+extinction than that seen after a single extinction or retrieval+extinction of the auditory cue, despite equivalent levels of freezing 24 h after fear conditioning.

Persistence of freezing to the T+L compound stimulus may be considered adaptive when only one of the elements has been extinguished. Where one element of the compound may lose associative value with the fear-inducing stimulus after extinction, the elements presented together may still predict danger. This persistence of conditioned responding to multimodal cues is reliably found in occasion-setting experiments and depends on temporal properties of cue presentation (Ross and Holland 1981; Holland 1984) as well as the saliency of the cues combined (Rescorla 1986; Holland 1989). When a light and tone CS are presented in compound, and the tone is more salient than the light, the light will acquire occasion-setting properties where it serves to modulate the associative value the tone has with the US (Holland 1989). This is in support of the idea that the saliency of the cues presented in compound will determine how extinction of one component affects conditioned responding to the other and to the compound. Extinguishing the Pavlovian response to the occasion setter does not extinguish its ability to set the occasion (rats may not freeze to the light anymore, but will when presented in compound with the tone [Ross 1983; Rescorla 1986]). Although requiring additional research, one possible explanation for the results seen in experiment 4 is that sequential ret+ext with the more salient cue preceding the less salient cue targets both the Pavlovian responding to the cues and any occasion-setting properties of the less salient cue therefore reducing the conditioned response to the compound stimulus.

Our sequential extinction paradigm shows promise in translational research given the practicality of exposing a patient to single aspects of a complex memory instead of attempting to recreate all of the cues present at the time of learning. Additional research in pathological conditions would need to be performed, but if complex traumatic situations can be targeted through the retrieval and then exposure therapy of the individual elements instead of the entire event, this approach may have potential in a clinical setting.

Materials and Methods

Subjects

Male Sprague–Dawley rats (250–300 g; Harlan Lab Animals Inc.) were used in all experiments. Twenty-two rats were used in experiment 1, 24 rats were used in experiment 2, 324 rats were used for experiment 3, 72 rats for experiment 4, and 60 rats for experiment 5. Procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the University of Texas at Austin Animal Care and Use Committee. Rats were housed in pairs in clear plastic cages and maintained on a 12-h light–dark cycle with food and water provided ad libitum. Rats were handled for several minutes each 24 h prior to the start of each experiment.

Apparatus and stimuli

All behavioral procedures took place in standard conditioning chambers equipped with two metal walls and two clear walls and stainless-steel rod floors connected to a shock generator (Coulbourn Instruments). Each conditioning chamber was enclosed in an acoustic isolation box (Coulbourn Instruments) and lit with a red light. Behavior was recorded with infrared digital cameras mounted on the top of each unit. The chambers were cleaned with Windex between each session.

Stimulus delivery was controlled using Freeze Frame software (Coulbourn Instruments). The conditioned stimuli (CS) used were a tone (5 kHz, 80 dB), light (white LED), or tone+light (T+L), all cues were 20 sec in duration. In experiment 5 only, the amplitude of the tone CS was reduced to 70 dB and the 20-sec continuous LED light was changed to 20 sec of a flashing LED light (500 msec on, 500 msec off). The unconditioned stimulus (US) used was a 0.7-mA footshock 500 msec in duration.

Behavioral procedures

Habituation

Rats were habituated to the chambers used for fear conditioning for 12 min 24 h prior to fear conditioning. This was done to reduce association of the US to the context during fear conditioning.

Fear conditioning

On the fear conditioning day, after a 5-min habituation period, all rats received three 20-sec simultaneous presentations of T+L (ITI = 180 sec), each coterminating with a 500-msec, 0.7-mA footshock. After fear conditioning, all rats were returned to their home cage. In experiment 1 only, the rats were fear conditioned to the light CS alone. In experiment 5, the T+L CS consisted of a flashing light (500 msec on, 500 msec off) and a 70-dB, 5-kHz tone.

Retrieval+extinction/extinction only

Experiment 1

Rats were fear conditioned, as described above, to the light CS and divided into a retrieval+extinction (ret+ext) group ($n = 12$) and extinction-only (ext-only) group ($n = 10$). The next day, rats were returned to the same context used in fear conditioning and either were exposed to a single presentation of the light (ret) or received no CS and were only exposed to the context (no ret). The rats were immediately returned to their home cages in the colony for 10 min. After 10 min, the rats underwent an extinction of either 18 light CSs (ret+ext) or 19 light CSs (ext-only).

Experiment 3

Rats were fear conditioned as described above and then divided into three groups categorized as retrieval+extinction (ret+ext), extinction only (ext-only), or no retrieval and no extinction (no ext), with three subgroups in each category. The conditioning chambers were altered to a new context (context B) that included black floors and a peppermint scent for retrieval, extinction, and later for long-term memory. The ret+ext category received an isolated retrieval session in order to initiate the reconsolidation period of either the light only, tone only, or the tone and light compound (T+L) in the absence of the US. The ext-only category was placed into the retrieval context for the same amount of time as the ret+ext group but received no CS. All rats were returned immediately to their home cages for 10 min. At the conclusion of the 10-min interval in the colony, ret+ext and ext-only underwent an extinction session (repeated presentation of the CS in the absence of the US) of the same CS that was retrieved (tone, light, or T+L). The extinction session consisted of either 18 CS presentations for the rats that had the retrieval or 19 CS presentations for the rats that had context exposure to ensure equal exposure to the CS between groups. The final category (no ext) remained in their home cages during the extinction day.

Experiment 4

Rats were fear conditioned as described above and divided into five groups again categorized as ret+ext, ext-only, and no ext. However, in this experiment, ret+ext and ext-only groups received two extinction sessions (or two ret+ext sessions, with a 10-min interval between retrieval and extinction) within 1 d, one group received tone extinction (or tone ret+tone ext) followed by light extinction (or light ret+light ext) whereas another one received the light extinction first followed later by tone extinction. The final groups (no ext) remained in their home cages during the extinction day.

Experiment 5

The extinction procedure employed in experiment 5 was identical to the procedure in experiment 4 except that the light component of the compound consisted of a blinking light and the tone component consisted of a 70-dB tone.

Long-term memory tests/spontaneous recovery/reinstatement

Experiment 1

Twenty-four hours after extinction, all rats were tested for long-term memory (four CSs; variable ITI = 180 sec) to the light. Freezing during the four CS presentations was averaged for graphical representation and analyzed as a repeated measure. Spontaneous recovery (SR) was tested 24 d after extinction using four CS presentations of the light. All procedures (extinction, LTM, SR) were performed in the same context. Previous research in our laboratory has indicated that three CS presentations and four CS presentations for LTM and SR tests produce similar measures of freezing behavior and, as such, despite the use of four CS presentations for experiment 1, three CSs were used for the remaining experiments reported here.

Experiment 3

Twenty-four hours after extinction, all rats were tested for long-term memory (three CSs) for either the tone alone, light alone, or tone and light compound in context B. Experimental groups (ret+ext, ext-only, no ext) were divided into three equal cohorts and tested for freezing to three CS presentations of either T, L, or T+L. Each animal was tested to only one CS modality for LTM. The intertrial interval (ITI) was variable, averaging 180 sec. Behavior during the three CS presentations was averaged for graphical representation and analyzed statistically as a repeated measure. For experiment 1, long-term memory was analyzed with separate mixed factor ANOVAs with retrieval group (ret+ext, ext-only, or no ext) and LTM component (T+L, tone, or light) as the factors and LTM cue as a repeated factor.

Experiment 4

Long-term memory tests were performed in a similar manner as above, except that all of the rats in each experimental group (ret+ext, ext-only, no ext) were tested for LTM to all three components of the compound (T+L, T, L) within the same day in context B. All rats were tested to T+L first, followed by tone, and completing with light. *t*-tests comparing the freezing of the no ext rats during each sequential LTM test to the freezing of the no ext rats in experiment 3 to each cue after T+L fear conditioning did not yield any significant differences in freezing (all P's > 0.1). For this reason, in the following experiments, each rat underwent tests to each cue in descending order of intensity (T+L followed by T followed by L).

Experiment 5

Long-term memory was tested 24 h after the sequential extinctions/retrieval+extinctions to the compound T+L. In order to avoid inducing extinction during the long-term memory test, only the compound was tested (three CS presentations). Twenty-four hours after LTM rats were exposed to a single footshock in the absence of any CS. The following day, they were returned to the chambers and tested for reinstatement to each of the cues. This test was performed sequentially, in a manner consistent with the LTM tests performed in experiment 4 (three separate testing sessions: T+L followed by T followed by L). In experiment 5, all procedures were performed in the fear conditioning context (context A).

Scoring—freezing

Freezing was defined as the absence of any movement, excluding breathing and whisker twitching. The total number of seconds spent freezing throughout the CS presentation is expressed as a percentage of CS duration (20 sec).

Statistical analysis

Data was analyzed with PASW Statistics software version 18.0 using mixed factor ANOVAs with the CS cue as a repeated measure and retrieval group membership as a between subject factor. Where appropriate, post-hoc tests were performed with Tukey's honestly significant difference mean comparison. Percent freezing during LTM tests is expressed as the mean over three CS presentations to each of the components 24 h after extinction or retrieval+extinction.

Pre–CS freezing

For all experiments, pre-CS freezing (freezing measured during the 20 sec prior to the first CS presentation) was minimal, and was not significantly different between retrieval groups for either of the three extinction cues (separate one-way ANOVAs with retrieval group as a fixed factor: light ext, $F_{(2,103)} = 2.076$, $P = 0.131$; tone ext, $F_{(2,101)} = 0.922$, $P = 0.401$; T+L ext, $F_{(2,105)} = 0.683$, $P = 0.507$) which suggests that freezing to the CS during the test sessions was specific to the CS presentation. This can also be described as freezing to the context and when necessary, contextual freezing is reported by measuring pre-CS freezing.

Immunofluorescence

c-Fos protein expression was analyzed in rats that were fear conditioned to the compound T+L CS as described above after the retrieval of either tone, light, T+L, or context-only. All rats were perfused 1 h after completion of an isolated retrieval of one of the components. All rats were deeply anesthetized with a lethal dose of sodium pentobarbital and then perfused intracardially with 0.01 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The brains were removed and stored in PFA for 24 h at 4°C and then transferred to 30% sucrose for cryoprotection until sectioning. The brains were sectioned coronally at 40 μm on a freezing microtome and stored in 0.01 M PBS at 4°C.

For c-Fos immunofluorescence, every fourth free-floating section was rinsed in 0.01 M PBS and then incubated in anti-c-Fos rabbit polyclonal antibody diluted in .01 M PBS and 0.2% Triton X-100 (PBST, 1:1000; Santa Cruz) for 24 h at 4°C. After three PBS washes, sections were incubated for 1 h at room temperature in Alexa Fluor 488 goat anti-rabbit conjugated antibody in PBST (1:1000; Invitrogen).

Sections were mounted and cover-slipped with ProLong Gold anti-fade reagent (Molecular Probes) for analysis.

Quantification of immunofluorescence

For each brain section, the number of c-Fos positive cells was counted by an observer blind to the experimental group using a fixed counting frame in a given structure as described below. For analysis, a density is expressed by dividing the number of positive cells in the counting frame by the area occupied by the counting frame used. Images were taken using a Zeiss AxioCam MRm digital camera at 10× magnification. Cells were counted using ImageJ software (NIH) for Mac. Care was taken to match sections for each brain and the regions were sampled from both the left and right hemispheres.

Lateral amygdala

Three sections were selected for each brain representing bregma –2.76, –3.00, and –3.24 (Paxinos and Watson 2009). Three identical circular counting frames (total area = 0.072 mm^2) were fitted within the boundaries of the lateral amygdala.

Prefrontal cortex

Cells in the infralimbic cortex were sampled from sections corresponding to bregma 3.72 and 3.24. Three identical circles (total area = 0.159 mm^2) were arranged in a way that one was tangent to the lateral border of the IL as indicated by the lower point of the forceps minor of the corpus callosum, the second was tangent to the midline, and the third circle was placed equidistant between the first two circles to allow for unbiased sampling. The cells in the prelimbic area were sampled in a similar manner except that an additional section was added corresponding to bregma 3.00 (total counting frame area = 0.159 mm^2)

Hippocampus

Cells in the hippocampus were sampled in the CA1, CA3, and dentate gyrus (DG) regions from sections corresponding to bregma –3.24, –3.48, and –3.72. An image was taken from each section by aligning the top left corner of the frame with the apex of the pyramidal layer of CA1. The imaging frame for CA3 and DG was taken in a similar manner by aligning the edge of the image with the apex of the granular layer of the DG. Counting frames consisted of identical circles placed within the visible region of interest and cells were counted from alternating circles to provide an unbiased sampling. The total area sampled for each section was 0.019 mm^2 for the CA1, 0.0495 mm^2 for CA3, and 0.0393 mm^2 for DG.

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