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Human fear extinction and return of fear using reconsolidation update mechanisms: The contribution of on-line expectancy ratings

Victor Taylor Warren^{a,b}, Kemp M. Anderson^{a,b}, Cliffe Kwon^{a,b}, Lauren Bosshardt^{a,b}, Tanja Jovanovic^b, Bekh Bradley^{a,b}, and Seth Davin Norrholm^{a,b,*}

^a Atlanta Veterans Affairs Medical Center, Decatur, GA, United States

^b Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA, United States

Abstract

Disruption of the reconsolidation of conditioned fear memories has been suggested as a non-pharmacological means of preventing the return of learned fear in human populations. A reconsolidation update paradigm was developed in which a reconsolidation window is opened by a single isolated retrieval trial of a previously reinforced CS+ which is then followed by Extinction Training within that window. However, follow-up studies in humans using multi-methods fear conditioning indices (e.g., fear-potentiated startle, skin conductance, US-expectancy) have failed to replicate the retrieval + extinction effects. In the present study, we further investigated the retrieval + extinction reconsolidation update paradigm by directly comparing the acquisition, extinction, and return of fear-potentiated startle in the absence or presence of US-expectancy measures (using a trial-by-trial response keypad) with and without retrieval of a previously acquired CS-US association. Participants were fear conditioned to two visual cue CS+'s, one of which was presented as a single, isolated retrieval trial before Extinction Training and one that was extinguished as usual. The results show that the inclusion of US-expectancy measures strengthens the CS-US association to provide enhanced fear conditioning and maintenance of fear memories over the experimental sessions. In addition, in the groups that used on-line US-expectancy measures, the retrieval + extinction procedure reduced reinstatement of fear-potentiated startle to both previously reinforced CS+'s, as compared to the extinction as usual group.

Keywords

Fear extinction; Reconsolidation; Spontaneous recovery; Reinstatement; Fear-potentiated startle; US expectancy

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* Corresponding author. Address: Atlanta VAMC, MHSL 116A, 1670 Clairmont Road, Decatur, GA 30033-4098, United States. Fax: +1 404 417 2911. snorrho@emory.edu (S.D. Norrholm)..

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1. Introduction

The fear-related symptoms of anxiety disorders such as panic disorder, specific phobia, and posttraumatic stress disorder (PTSD) have been conceptualized within the framework of fear conditioning (Bouton, Mineka, & Barlow, 2001; Friedman, 2000; Mineka & Zinbarg, 2006; Norrholm & Jovanovic, 2010; Wolpe & Rowan, 1988). Empirical evidence suggests that these symptoms can arise as a result of impaired fear inhibition (Jovanovic, Kazama, Bachevalier, & Davis, 2011; Jovanovic & Norrholm, 2011; Jovanovic et al., 2009, 2010), over-generalization of fear responses (Lissek, 2012), and/or dysregulation of fear extinction learning (Norrholm & Jovanovic, 2011; Norrholm, Anderson, et al., 2011; Norrholm, Jovanovic, et al., 2011) and extinction recall (Milad et al., 2008, 2009). According to the principles of fear conditioning, the traumatic event serves as an unconditioned stimulus (e.g., improvised explosive device detonation; termed the US) and previously neutral ambient cues (e.g., smoke, palm trees) in the trauma environment become associated with this “US” as a result of trauma exposure. As a result, intense fear reactions can develop to these cues such that the original fear memory is maintained over time even upon return to safety. From a clinical perspective, the most effective interventions for treating anxiety disorders are extinction-based exposure therapies in which the anxiety disorder patient is exposed to traumatic stimuli in the absence of noxious consequences (Rothbaum & Schwartz, 2002). Despite the success of these interventions, anxiety disorder symptoms can relapse following the completion of treatment (e.g., the clinical homolog of the laboratory observation of the return of fear; (Craske, 1999).

The return of conditioned fear can occur as a result of a change in context (termed renewal; (Bouton, 2004), through the passage of time (termed spontaneous recovery; (Bouton, 1993; Pavlov, 1927), or through re-exposure to the US (termed reinstatement; (Bouton & Bolles, 1979; Rescorla & Heth, 1975). Each of the latter phenomena can be observed by accessing the original fear memory that remains intact after new extinction learning has taken place. Current data in the literature strongly suggest that extinction is a form of new learning in which the organism learns that the CS is no longer associated with the US and that this new memory trace competes with the original fear memory trace (Bouton, 1993; Myers & Davis, 2002).

While a large number of studies suggest that the original fear memory (a consolidated CS–US association) remains intact following extinction learning, it is possible to return this memory into a labile state through the process of reconsolidation. Retrieval of the original fear memory during reconsolidation renders the memory vulnerable to disruption and its return to stability requires *de novo* protein and RNA synthesis (Dudai, 2004; Lee, 2009; Nader, Schafe, & LeDoux, 2000; Sara, 2000; Tronson & Taylor, 2007); this period of lability has been termed the reconsolidation window and has been suggested to last for several hours (Duvarci & Nader, 2004). Previous studies have demonstrated disruption of reconsolidation of the fear memory through pharmacological manipulation in both animals (e.g., inhibition of protein synthesis; as in (Nader, Schafe, & LeDoux, 2000) and, more recently, humans (e.g., b-adrenergic receptor antagonism; as in (Soeter & Kindt, 2011a). It is of great clinical interest to develop potential non-pharmacological mechanisms for

disrupting reconsolidation of fear and several recent investigations have explored this possibility.

One potential non-pharmacological avenue for disrupting the reconsolidation of fear memories was initially investigated in rodents by Monfils, Cowansage, Klann, and LeDoux (2009) and involved the presentation of an isolated retrieval trial (to render the fear memory labile) followed by Extinction Training (to foster new learning of the now nonthreatening nature of the previously reinforced CS; (Monfils et al., 2009) during the reconsolidation time window. The rationale behind this model was that the isolated retrieval trial (reactivation of a previously reinforced CS) would result in a persistent reevaluation of the CS as safe and, as such, weaken the previously acquired CS-US association. This weakening of the fear memory would then in turn prevent the return of fear through renewal, reinstatement, or spontaneous recovery. In their study, the Monfils group, in fact, showed that the reactivation of an isolated CS followed by Extinction Training during the reconsolidation window prevented the return of fear.

Due to the potential impact on the dogma of fear learning and the translation to clinical practice, the retrieval + extinction paradigm introduced by Monfils et al. (2009) has been further examined by other groups in several preclinical human studies using validated psychophysiological measures of fear, namely skin conductance responding and fear-potentiated startle. For example, using skin conductance, Schiller et al. (2010) showed that Extinction Training administered 10 min after a reactivated cue (during the reconsolidation window) prevented spontaneous recovery as compared to groups that underwent extinction 6 h after reactivation (outside the reconsolidation window) and to groups that did not receive a reactivated CS (Schiller et al., 2010). In addition, the Schiller et al. (2010) group demonstrated that the blockade of fear return was cue specific, extended to reinstatement as well as recovery, and persisted for up to a year. Oyarzun et al. (2012) replicated this finding by demonstrating that reinstatement of fear, as measured by skin conductance, to visual stimuli previously paired to an aversive auditory US could be attenuated by presenting an isolated retrieval trial 10 min prior to Extinction Training (Oyarzun et al., 2012). Using fear-potentiated startle measures coupled with skin conductance and on-line ratings of distress (2011) or US-expectancy (2013), Kindt and Soeter (Kindt & Soeter, 2013; Soeter & Kindt, 2011a) did not replicate the finding that return of fear is prevented when Extinction Training occurs within the reconsolidation window. Recently, Golkar, Bellander, Olsson, and Ohman (2012), using both skin conductance and fear-potentiated startle methods, presented both fear-relevant and fear-irrelevant cues in the Schiller et al. (2010) experimental design and did not replicate the disruption of reconsolidation effect either (Golkar et al., 2012).

As described by Schiller and Phelps (2011) and Auber, Tedesco, Jones, Monfils, and Chiamulera (2013), there are many contributing factors to consider with regard to the conditions under which the retrieval + extinction-induced disruption of reconsolidation can be observed (Auber et al., 2013; Schiller & Phelps, 2011). For example, differing methodological approaches have been employed to index fear conditioning (skin conductance only (Oyarzun et al., 2012; Schiller et al., 2010) vs. concurrent startle, skin conductance, and on-line ratings (Kindt & Soeter, 2013; Soeter & Kindt, 2011b) and in the selection of CS type (geometric shapes (Oyarzun et al., 2012; Schiller et al., 2010) vs. fear-

relevant spider images (Soeter & Kindt, 2011b). In addition, there is evidence to suggest that individual differences among participants, such as genetic polymorphisms, may influence the effect of disruption of reconsolidation (Agren, Furmark, Eriksson, & Fredrikson, 2012). Further investigations of reconsolidation update mechanisms are certainly warranted given the inconclusive data reported to date, the potential boundary conditions that may explain reported discrepancies, and the compelling clinical potential of this type of paradigm.

The translation of the Monfils et al. (2009) study into the human preclinical arena (e.g., Kindt & Soeter, 2013) requires certain methodological adjustments in order to account for obvious species-specific differences in the expression of fear behavior as well as the means of effectively capturing these data. For example, human studies often include the use of verbal instructions and on-line ratings of participant US-expectancy, threat, or distress (Norrholm et al., 2006; Vervliet, Kindt, Vansteenwegen, & Hermans, 2010) to enhance learning and foster attention to the experimental contingencies. The inconclusive results observed to date in the study of reconsolidation update mechanisms may be explained, in part, by these procedural differences and the underlying neurobiological learning mechanisms accessed by these divergent procedures. Kindt and Soeter (2013) employed a multi-method approach that included the use of an on-line measure of US-expectancy; a behavior that recruits higher cortical brain regions involved in declarative knowledge (Weike, Schupp, & Hamm, 2007). The results reported by the Monfils et al. (2009) coupled with prior animal work on fear memory formation and reconsolidation (e.g., (Davis, 1997; Han et al., 2009; LeDoux, 2000; Nader et al., 2000)) implicate the amygdala as a potential anatomical substrate for previously observed retrieval + extinction effects. This possibility was directly investigated in humans by Agren, Furmark, et al. (2012) through the integration of brain imaging and skin conductance measures. Agren and others reported that retrieval + extinction with a 10-min, but not 6-h, interval between retrieval and extinction: (1) attenuated reinstatement of fear, (2) significantly reduced amygdala activity during fear memory retrieval, and (3) weakened coupling between the amygdala and other brain regions important for fear memory recall and return of fear (Agren, Engman, et al., 2012; Agren, Furmark, et al., 2012). Thus, it remains possible that the use of on-line ratings and subsequent recruitment of cortical areas disrupts the retrieval + extinction effect based in the amygdala.

The current study expands on recent investigations of reconsolidation update mechanisms by directly comparing the absence or presence of on-line US-expectancy measures during administration of the retrieval + extinction paradigm introduced by Monfils et al. (2009) translated to humans by Schiller et al. (2010) and subsequently investigated by several groups. The results will be discussed in terms of the effects of expectancy ratings on (a) fear acquisition and extinction and (b) extinction during reconsolidation.

2. Materials and methods

2.1. Participants

55 Subjects (20 males/35 females) with a mean age of 20.8 + 1.7 years old participated in the study after signing an informed consent form approved by the Emory University Institutional Review Board, the Atlanta VAMC Research and Development Committee, and

the US Army Medical Research and Materiel Command (USAMRMC)/Office of Research Protections (ORP)/Human Research Protection Office (HRPO). The psychiatrically healthy volunteers included in this study were recruited as part of a larger investigation of fear inhibition and generalization in combat veterans at the Atlanta VAMC. Requirements for participation included no significant visual impairment (corrected 20/20 vision) and tone detection at 30 dB of frequencies ranging from 250 to 4000 Hz (assessed with a Grason-Stadler Model GS1710 pure threshold audiometer). Participants were screened for current or past psychiatric illness through self-report measures and administration of the Structured Clinical Interview for DSM-IV Axis I Disorders, SCID-1. Participants were also screened for illicit drug use via urine toxicology analysis and excluded for current drug or alcohol abuse or dependency. The participants were assigned to one of four age- and sex-matched experimental groups: No Retrieval/No Keypad ($n = 10$), No Retrieval/Keypad ($n = 13$), Retrieval/No Keypad ($n = 20$), or Retrieval/Keypad ($n = 12$).

2.2. Trial definitions

The eyeblink component of the acoustic startle response was measured according to previously published methods (Norrholm et al., 2006; Norrholm, Anderson, et al., 2011; Norrholm, Jovanovic, et al., 2011). The startle probe was a 108-dB [A], 40 ms burst of white noise with near instantaneous rise time delivered binaurally with headphones. Acoustic startle response magnitude was recorded via electromyography (EMG) readings of the right *orbicularis oculi* muscle. Two 5 mm Ag/AgCl electrodes filled with electrolyte gel were placed 1 cm below the pupil and 1 cm below the lateral canthus. EMG signals were amplified and digitalized with the BIOPAC MP150 monitoring system (Biopac Systems, Inc., Aero Camino, CA). Impedances through these electrodes were less than 6 kX. Startle magnitude was determined as the peak amplitude of the EMG contraction 20–250 ms following the acoustic stimulus. Similar to several of our previous studies (e.g., (Jovanovic et al., 2005; Norrholm et al., 2008), the aversive stimulus (US) was a 250 ms, 140 p.s.i. airblast directed at the larynx. The CSs were geometric shapes presented on a computer monitor approximately 1 m in front of the participant. On CS+ trials (Fear Acquisition), the shape was presented for 6s total, with the 40 ms startle probe presented 5210 ms after CS onset followed 500 ms later by the 250 ms, 140 p.s.i. airblast that co-terminated with CS presentation. On CS- trials (Fear Acquisition) and nonreinforced CS+ trials (Extinction, Test, Re-extinction, Reinstatement), the shape was presented for 6 s total, with the startle probe occurring 5960 ms after CS onset. On noise alone (NA) trials, the 40 ms startle probe was presented alone without the CS's. Startle trials were averaged across blocks (4 trials per block) in order to reduce variability as in our previous work (e.g., Norrholm et al., 2008).

2.3. Session definitions

The experimental sessions occurred over three consecutive days (see Fig. 1). The Fear Acquisition session occurred on Day 1, the Retrieval trial (or equivalent passage of time) and Extinction Training sessions were administered on Day 2, and the Extinction Test (to assess extinction retention or spontaneous recovery), Re-extinction, and the Reinstatement Tests occurred on Day 3. All test sessions occurred in the same context. The Fear Acquisition session began with a 1-min acclimation period followed by a habituation phase consisting of three noise alone (NA) presentations. Next, a CS habituation phase was

presented consisting of four presentations of each CS without the airblast US. After habituation to the CSs, the Fear Acquisition session continued with three blocks of four trials of each trial type (CS+a, CS+b, CS-, NA). Shapes were counterbalanced across participants for each CS type. The Fear Acquisition session used a 100% schedule of reinforcement for the CS+'s such that each presentation of the CS+'s was reinforced with the airblast US. The inter-trial interval (ITI) was randomized between 9 and 22 s.

Twenty-four hours after Fear Acquisition, participants were either presented with a single non-reinforced presentation of CS+a and the CS- (Retrieval groups) or an equivalent period of time (No Retrieval groups). The CS+b cue was not reactivated. Ten minutes after the Retrieval trial, participants were administered the Extinction Training session. Extinction Training consisted of six blocks of four presentations each of the CS's and NA. Twenty-four hours after Extinction Training, an Extinction Test of extinction retention or spontaneous recovery was presented. The Extinction Test consisted of one block of four trials of each CS and NA. Ten minutes after the Extinction Test, Re-extinction occurred using the same protocol as that used for Extinction Training. Finally, a Reinstatement Test was presented at the conclusion of Re-extinction. The Reinstatement Test consisted of four unsignaled presentations of the airblast US followed by one block of four presentations of each CS and NA. In order to remove any sensitization effects of the US on NA trials, 6 NA startle probe trials were delivered prior to the block with the CS and NA trials.

2.4. US-expectancy

A three-button response keypad (SuperLab, Cedrus Corporation, San Pedro, CA) was used during each acoustic startle session to record the expectancy of the participants of the US on each CS presentation. Participants received verbal instructions prior to each session on how to respond with the keypad. Participants were instructed to press a button marked "+" if they expected the shape to be followed by the US, a button marked "-" if they did not expect the airblast US, or a button marked "0" if they were uncertain. Instructions were to press the button within three seconds of conditioned stimulus onset. Any responses occurring after the airblast US were discarded.

2.5. Data analysis

US-expectancy ratings (scored as -1 for the "-" button, 0 for the "0" button, and 1 for the "+" button) during fear acquisition were analyzed using repeated measures analysis of variance (RM-ANOVA) with Trial (16 levels: 4 trials during CS Habituation and 12 trials during Acquisition) and Trial Type (3 levels: CS+a, CS+b, CS-) as within-subject factors and Retrieval group (2 levels: Yes, No) as the between-groups factor. Startle response potentiation to CS+ trials was tested with a RM-ANOVA with Block (4 levels: 1 block of CS Habituation, 3 blocks of Acquisition) and Trial Type (3 levels: NA, CS+a, CS+b) as within-subjects factors and Retrieval group (2 levels) and Keypad group (2 levels: Yes, No) as between-groups variables. The degree of potentiation to the CS's was compared by calculating a fear-potentiated startle score. Fear-potentiated startle was expressed as a Difference score using the formula: Difference score = [Mean startle magnitude to probe in presence of CS] - [Mean startle magnitude to startle probe alone (NA) for each session]. The Difference score was then used as the dependent variable in further analyses of startle

data. During fear acquisition, a RM-ANOVA comparing Block (4 levels) and Trial Type (3 levels: CS+a, CS+b, CS-) as within-subjects factors and the same between-groups factors as in the previous analysis.

In order to test effects of Extinction Training on Day 2, we used RM-ANOVA with US-expectancy as the dependent variable with Trial (2 levels: 1st Extinction trial, last Extinction trial) and Trial Type (2 levels: CS+a, CS+b) as within-subjects factors and Retrieval group (2 levels) and as the between-groups factors. Given that the US-expectancy ratings were derived from the keypad, these analyses were limited to the Keypad groups and included only Retrieval group as a between-group factor. Analyses of fear-potentiated startle used the Difference score as the dependent variable and Block (2 levels: 1st and last block of Extinction) and Trial Type (2 levels) as within-subject factors and Retrieval and Keypad groups as between-group factors. Analyses of extinction focused on comparing the two CS+'s: CS+a was the reactivated cue and the CS+b was the non-reactivated cue.

For Day 3, we tested spontaneous recovery of fear by comparing the last trial of Extinction Training on the previous day with the first trial of the Extinction Test session for US-expectancy. Fear-potentiated startle was analyzed by comparing the last block of Extinction with the first Test block. Trial Type, Retrieval and Keypad groups were defined the same as above. Re-extinction used RM-ANOVA to compare US-expectancy on the first and last trial of Re-extinction and fear-potentiated startle on the first and last block of Re-extinction, with all other factors the same as above. Finally, Reinstatement tested the increase in US-expectancy after the delivery of 4 unpaired airblasts by comparing the last trial of Re-extinction and the first trial of the Reinstatement Test. As in the analyses above, fear-potentiated startle compared the last block of Re-extinction to the Reinstatement Test block. Fear-potentiated startle was calculated using the Difference score of CS minus NA, as described above. Because unpaired airblasts increase baseline startle responses (i.e. NA), 6 startle probes were delivered in order to normalize the NA level. The NA used as the reference in the calculation of fear-potentiated startle during Reinstatement did not include those trials, but only the 4 in the Reinstatement Test.

Interaction effects of Retrieval and Keypad groups were followed-up by comparisons within the four groups defined above: No Retrieval/No Keypad, No Retrieval/Keypad, Retrieval/No Keypad, or Retrieval/Keypad. Given the differences in cell size, we report effect sizes for these analyses using partial Eta squared. All analyses were performed using SPSS 20.0 for Windows, with alpha level set at .05.

3. Results

3.1. Day 1: fear acquisition

We examined US-expectancy ratings on each trial during conditioning using the response keypad comparing the two CS+'s and the CS-. Participants responded with "1" if they expected the air-blast on the trial, "0" if they were uncertain, and "-1" if they did not expect an airblast. Instructions were to press the button within three seconds of CS appearance. All participants were compliant with this timeframe, in fact, most responses occurred within the first 1500 ms of CS onset. Fig. 2A shows the US-expectancy across Trials and Trial Type. A

RM-ANOVA revealed a significant Trial by Trial Type interaction, $F(30, 690) = 35.00, p < .001$, with US-expectancy increasing to both CS+'s and decreasing to CS- across trials during Fear Acquisition. In the groups that included the Keypad, the No Retrieval and Retrieval groups did not differ on US-expectancy during Acquisition. Although the Retrieval trial occurred after the Acquisition session, we compared the two groups in order to ensure that there were no prior differences in the groups.

Startle magnitude was significantly potentiated during acquisition to both CS+'s compared to NA trials (see Fig. 2B), as seen by a significant Block by Trial Type interaction, $F(6, 318) = 14.20, p < .001$. Contrasts showed that startle magnitude to both CS+s was significantly higher than NA (both p 's $< .001$). While there was no effect of Retrieval group during Acquisition, there was a significant interaction of Trial Type and Keypad group, $F(2, 106) = 7.41, p < .001$. We examined the effect of Trial Type in each group separately: while those without the Keypad still showed significant conditioning effects to CS+a ($p = .02$) and CS+b ($p = .0003$), the increase in startle magnitude was more robust in those who used a Keypad to both the CS+a ($p = .00002$), and CS+b ($p = .000006$). Fear-potentiated startle calculated as a Difference Score between the CS+'s and NA examined during the last block of Acquisition was significantly higher for the two CS+ trials compared to the CS-, $F(2, 106) = 13.26, p < .001$, with no interaction effects with either Retrieval group or Keypad group (Fig. 2C). Contrast analyses showed that CS+a and CS+b had significantly greater fear-potentiated startle than the CS- ($F(1, 53) = 19.20, p < .001$ and $F(1, 53) = 11.32, p < .001$, respectively).

3.2. Day 2: Extinction Training

For the Extinction Training session, we examined US-expectancy to the CS+ trials during the first and last block of extinction with Retrieval group as the between-groups factor. We found a significant main effect of Trial, $F(1, 23) = 58.90, p < .001$, and no main or interaction effects of Retrieval group. There was also no main or interaction effect of Trial Type, indicating that US-expectancy ratings to both CS+s were equally diminished during Extinction Training (see Fig. 3).

Fig. 4 shows startle data across study Days 2 and 3. Comparing fear-potentiated startle on the first and last block of Extinction Training across the above groups revealed a significant main effect of extinction Block, $F(1, 51) = 31.64, p < .001$ and an interaction of Block and Trial Type, $F(1, 51) = 5.69, p = .02$. However, analyses of each Trial Type separately showed that there was significant extinction to CS+a trials, $F(1, 54) = 35.20, p < .001$, and to CS+b trials, $F(1, 54) = 32.36, p < .001$. In addition, there was a significant 3-way interaction of Trial Type \times Retrieval group \times Keypad group, $F(1, 51) = 3.98, p = .05$. The interaction was followed up by comparing Trial Type (CS+a vs CS+b) within each of four groups: (a) No Retrieval and No Keypad; (b) Retrieval and No Keypad; (c) No Retrieval and Keypad, and (d) Retrieval and Keypad. This analysis revealed that only the Retrieval and No Keypad group showed lower fear-potentiated startle to the reactivated CS+ compared to the non-reactivated CS+ during extinction, $F(1, 19) = 5.75, p = .03, \eta^2 = 0.23$ (Fig. 4B). The two CS+ trials did not differ from each other in any of the other groups (No Retrieval/No Keypad: $p = 0.75, \eta^2 = 0.01$; No Retrieval/Keypad: $p = 0.10, \eta^2 = 0.21$; Retrieval/Keypad: p

= 0.55, $\eta^2 = 0.03$). Finally, there was a significant main effect of Keypad, with higher fear-potentiated startle to both Trial Types in the groups that used the Keypad, $F(1, 51) = 4.25$, $p = .04$ (Fig. 4C and D).

3.3. Day 3: Extinction Test

Next, we administered an Extinction Test to assess the degree of extinction retention or spontaneous recovery 24 h after Extinction Training by comparing US-expectancy ratings on the last trial of extinction and the first test trial for both CS+'s. We again found that both Trial Types showed a significant increase in US-expectancy, $F(1, 23) = 19.21$, $p < .001$, but no interaction or main effects of Trial Type or Retrieval group.

With respect to fear-potentiated startle, we also found a significant increase to the Test block compared to the last block of Extinction Training, $F(1, 50) = 20.23$, $p < .001$, with no interaction effects with Retrieval or Keypad group. There was also no interaction effect with Trial Type, indicating that the CS+a and CS+b showed similar levels of spontaneous recovery. However, there was a significant main effect of Keypad group, $F(1, 50) = 11.44$, $p = .001$, with the Keypad group showing higher levels of fear-potentiated startle than the No Keypad group (Fig. 4C and D).

3.4. Day 3: Re-extinction

Analysis of US-expectancy on the first and last trial during Re-extinction revealed a significant decrease across Trials, $F(1, 23) = 11.31$, $p = .003$, but again no interaction effects of Retrieval group, Keypad group, or Trial Type. On the other hand, the startle data showed a significant 3-way interaction of Block (1st vs 6th Block of Re-extinction) \times Retrieval group \times Keypad group, $F(1, 50) = 4.55$, $p = .04$. We subsequently compared the Re-extinction blocks within each of the four experimental groups. These results showed significant re-extinction only in the two groups that used a Keypad: the No Retrieval/Keypad group, $F(1, 12) = 31.34$, $p < .001$, $\eta^2 = 0.72$, and the Retrieval/Keypad group, $F(1, 11) = 7.08$, $p = .02$, $\eta^2 = 0.39$. The groups that did not use a Keypad did not demonstrate significant re-extinction (No Retrieval/No Keypad: $p = 0.97$, $\eta^2 = 0.00$; Retrieval/No Keypad: $p = 0.21$, $\eta^2 = 0.08$). None of the groups showed any main or interaction effects of Trial Type, i.e. both CS+'s were re-extinguished in the Keypad groups, and neither in the No Keypad groups. There were no effects of Retrieval group.

3.5. Day 3: Reinstatement Test

After Re-extinction, we tested for reinstatement effects on US-expectancy by comparing the last trial of Re-extinction to the first trial of Reinstatement after the delivery of 4 unsignaled airblast USs. Trial Type was included as a within-subjects factor, and the Retrieval group as a between-groups factor. This analysis revealed a significant main effect of Trial, $F(1, 19) = 10.19$, $p = .005$, as well as an interaction effect of Trial by Retrieval group, $F(1, 19) = 4.89$, $p = .04$. The group that received the reactivation cue showed less of an increase in US-expectancy during Reinstatement compared to the group who did not get a reactivation cue, see Fig. 3.

The same analysis of Trial Type and Block (last block of Re-extinction compared to Reinstatement Test block) as within-subjects factors and Retrieval group and Keypad group as between-groups factors with fear-potentiated startle as the dependent variable revealed a main effect of Trial Type, $F(1, 44) = 7.50, p = .009$, with higher startle during the Reinstatement Test to the reactivated cue (CS+a) compared to the non-reactivated cue (CS +b); this should not to be confused with reinstatement of fear which is based on the within-subject change from the end of re-extinction to the Reinstatement Test (see next set of analyses). There was also a significant 3-way interaction of Block \times Retrieval group \times Keypad group, $F(1, 44) = 5.68, p = .02$. Analyses of Block within each of the four groups revealed significant reinstatement in the No Retrieval/Keypad group, $F(1, 8) = 6.56, p = .03, \eta^2 = 0.45$, and no effect of Block in the Retrieval/Keypad ($p = 0.47, \eta^2 = 0.05$), Retrieval/No Keypad ($p = 0.06, \eta^2 = 0.18$), or No Retrieval/No Keypad groups ($p = 0.89, \eta^2 = 0.003$), see Fig. 4.

4. Discussion

The purpose of the present study was to further examine the putative reconsolidation update mechanism reported in rodents by Monfils et al. (2009) that has since been translated to human studies (e.g., Schiller et al., 2010). Previous results from the Monfils group and others (e.g., Oyarzun et al., 2012; Schiller, Raio, & Phelps, 2012; Schiller et al., 2010) demonstrated a blockade of the return of fear when the reconsolidation window was opened by an isolated single exposure to a previously reinforced CS+ followed by Extinction Training. Recent skin conductance and fear-potentiated startle data from other groups (e.g., Soeter & Kindt, 2011a, Kindt and Soeter (2013); Golkar et al., 2012) failed to replicate these findings. One of the significant discrepancies between the Schiller human study and the procedures used by other groups was the inclusion of on-line US-expectancy measures. In the current study, we performed a direct examination of the effects of retrieval + extinction in the presence or absence of a response keypad to report participant US-expectancy.

4.1. Summary of Findings

The primary findings of the present study were as follows: (1) all participants acquired robust fear-potentiated startle responses to the reinforced CS+a and CS+b as compared to NA trials and significant discrimination between the reinforced CS+'s and the CS , (2) the inclusion of the Keypad for US-expectancy enhanced the degree of fear-potentiated startle as compared to groups without the Keypad during Fear Acquisition, (3) the inclusion of the Keypad increased participant retention of fear-potentiated startle to the CS+'s at the beginning of Extinction Training as compared to those groups without the Keypad, (4) in the No Keypad/Retrieval group, there was significantly less fear-potentiated startle to the reactivated CS+a as compared to the control CS+b during Extinction Training, (5) all groups displayed some level of spontaneous recovery of fear-potentiated startle to both previously reinforced CS+'s during the Extinction Test with the Keypad groups showing a greater degree of fear recovery as compared to the No Keypad groups, (6) only the groups with a Keypad showed significant re-extinction to both previously reinforced CS+'s, (7) only the No Retrieval/Keypad group showed significant reinstatement of fear-potentiated startle to the previously reinforced CS+'s.

4.2. Relationship between on-line US-expectancy measures and psychophysiological indices

As in several of our previous studies (e.g., Norrholm et al., 2008), a three-button response keypad was used as a measure of US-expectancy on a trial-by-trial basis during each of the test sessions previously described. Traditionally, these data provide the experimenter with validation that the CS-US association was successfully learned during fear acquisition in that there is often a high correlation between physiological indices of fear (e.g., startle, skin conductance) and US-expectancy responses (e.g., Norrholm, Anderson, et al., 2011; Norrholm, Jovanovic, et al., 2011). As the study of conditioned fear is extended into fear extinction and return of fear paradigms (which often include multiple test sessions with varying temporal and contextual factors), dissociations begins to emerge between physiological measures and US-expectancy responses (see (Kindt & Soeter, 2013; Norrholm et al., 2008; Soeter & Kindt, 2010, 2011b; Weike et al., 2007). For example, in the current study, participant keypad responses increase from a level of “safety” at the end of the Extinction Test to that of “uncertainty” at the beginning of the re-extinction session (see Fig. 3). This is in contrast to the level of fear-potentiated startle observed at the beginning of re-extinction in the keypad groups (see Fig. 4). This dissociation is consistent with previous reports using on-line US-expectancy measures during extended fear extinction paradigms (Kindt & Soeter, 2013; Norrholm et al., 2008). As discussed by Boddez et al. (2013), this dissociation is not surprising and may be due, in part, to (1) the subjective nature of US-expectancy, (2) the failure of US-expectancy measures to capture arousal, and (3) the sensitivity of this measure to experimental demand, or the experimental artifact created when a participant changes his or her behavior based on their interpretation of the experiment's purpose (Grillon, 2008; Lipp, 2006).

Based on a large body of evidence accumulated over the past two decades, it is clear that fear-potentiated startle, skin conductance response, and US-expectancy measures can be used as complementary research tools in the area of human fear conditioning (Jovanovic et al., 2013; Kindt & Soeter, 2013). However, each methodology has its own inherent strengths and limitations. Fear-potentiated startle provides a non-zero baseline, is a proxy measure of amygdala activity, and shows sensitivity to the valence of experimental stimuli (for review see Davis, Falls, Campeau, & Kim, 1993; Glover et al., 2011). A potential limitation of the use of fear-potentiated startle is the potential for the acoustic startle probes to be perceived as aversive and an additional unconditioned stimulus (e.g., Golkar et al., 2012). Skin conductance response is generally viewed as a measure of arousal and a less specific measure of valence and/or fear (e.g., Glover et al., 2011; Kindt & Soeter, 2013). SCR is also preferentially used in conjunction with neuroimaging studies (Agren, Engman, et al., 2012; Milad et al., 2008) since startle introduces movement artifact in the scanner. As previously described, US-expectancy measures tend to show greatest sensitivity at the peak of fear acquisition (i.e., at the end of fear conditioning and at the outset of Extinction Training) and much less sensitivity once the initial fear memory has been extinguished; this pattern of US-expectancy responding has been observed in response to prepared stimuli, such as fear-relevant images of spiders (Kindt & Soeter, 2013) and non-prepared stimuli, such as neutral shapes (Norrholm et al., 2008, current results). In addition, these measures have been employed concurrently and there is little evidence to suggest that either the administration of

acoustic startle probes or the active recording of one's US-expectancy disrupts skin conductance responses (for discussion see Soeter & Kindt, 2011a, 2011b).

4.3. The effect of US-expectancy measures on fear acquisition and extinction

The results of the present study indicated that the presence of a keypad for on-line assessment of US-expectancy enhanced the acquisition, maintenance, spontaneous recovery, and reinstatement of fear-potentiated startle as compared to test groups without the US-expectancy measure. Critics of the use of on-line US-expectancy measures have argued that the inclusion of these procedures intentionally draws participant attention to the experimental contingencies and, as such, potentially influences pure conditioning (Boddez et al., 2013; Lipp, 2006). This criticism has its roots in early work by Razran (1955) who believed that the effect of a participant's conscious awareness of the CS–US association was an “unfortunate error” and not reflective of true conditioning (Razran, 1955). More recently, it has been argued that active awareness of experimental contingencies is essential to fear conditioning and provides greater ecological validity (De Houwer & Beckers, 2002; Grillon, 2002). While initial fear conditioning was evident in the groups that did not have on-line US-expectancy measures in the present study, it appears as though active detection of the experimental contingencies via US-expectancy responses produced a stronger CS–US association that was more readily retrieved following Extinction Training. The observed enhancement of fear conditioning associated with on-line US-expectancy ratings may also reflect recruitment of two mechanisms of learning described by Ohman and Mineka (2001): (1) contingency learning and (2) fear module-based emotional learning (Ohman & Mineka, 2001).

Additionally, the more robust fear memories observed in the Keypad groups in the present study may also be the result of rehearsal. Although this study was not a controlled study of rehearsal *per se*, the act of reporting one's US-expectancy on each trial is similar to mentally rehearsing the CS–US contingencies (e.g., Joos, Vansteenwegen, Vervliet, & Hermans, 2013). Rehearsal has been shown to strengthen and sustain conditioned fear responses to a rehearsed CS+ as compared to a non-rehearsed CS+. According to this interpretation, the groups without a Keypad did not have the opportunity to mentally rehearse the CS–US associations as they anticipated and executed their expectancy ratings (whereas the Keypad group did) and thereby did not show sustained fear memory expression across the experimental sessions.

4.4. The effect of US-expectancy measures on fear extinction during reconsolidation

The putative reconsolidation update mechanism has been proposed as a means of disrupting the reconsolidation of an acquired CS–US association (fear memory) by reactivating a previously reinforced CS+ through the presentation of a single, isolated, unreinforced CS+ trial followed by Extinction Training. Monfils et al. (2009) suggested the lateral amygdala as a potential anatomical locus for the retrieval + extinction effects on fear return. Schiller et al. (2010) and Oyarzun et al. (2012) employed a single method for assessing fear responses (skin conductance) in their studies that produced very similar effects to those reported by Monfils et al. (2009). A possible explanation for the failure of subsequent groups to replicate the retrieval + extinction blockade of fear return may be the recruitment of neural systems

that extend beyond the amygdala, which has been suggested as a principal neurobiological substrate for this phenomenon (Agren, Engman, et al., 2012), to involve areas such as hippocampus and frontal cortices (Weike et al., 2007) as a result of including on-line US-expectancy measures. In other words, cortical representations of the CS-US association may have elicited amygdala activation and, as such, strengthened the original fear memory (Olsson & Phelps, 2007).

The results of the present study in the groups that did not have on-line US-expectancy measures indicate some effects of reactivation of a previously reinforced CS+ during an isolated retrieval trial. Fear-potentiated startle to the reactivated CS+a was lower than that which was observed to the non-reactivated CS+b at the outset of Extinction Training. This reduction was not observed in the Keypad/Retrieval group and interpretation of this finding is masked by the decreased fear learning, and loss of fear expression signal throughout the experimental sessions, that was seen in the No Keypad groups. An additional effect of reactivation observed in the current study was the lack of reinstatement of fear-potentiated startle in the Keypad/Retrieval group. In fact, the only group to show reinstatement in our study was the Keypad/No Retrieval group. While the No Keypad/Retrieval group also did not show reinstatement, it is unclear whether this effect is due to the absence of the Keypad rather than the presence of the reactivation cue, given that the No Keypad/No Retrieval group also did not show reinstatement. Notably, this lack of reinstatement was not specific to the reactivated CS+a, but extended to the CS+b. The potential to reduce the reinstatement of learned fear to similar (potentially generalized) cues has compelling clinical implications and certainly warrants further study.

It has become clear to investigators as the retrieval + extinction paradigm is increasingly studied that there are boundary conditions that exist under which reconsolidation does not occur (Nader & Hardt, 2009). As described by Golkar et al. (2012), the inability to replicate the disruption of reconsolidation by Extinction Training within the reconsolidation time window has been most often discussed in terms of boundary conditions such as acquisition memory strength.

With regard to acquisition memory strength, one issue to consider with regard to the discrepancies reported using the retrieval + extinction paradigm is the schedule of reinforcement utilized during fear acquisition (as discussed by Auber et al., 2013). Our current study used 100% reinforcement on the CS+ trials during fear acquisition similar to Soeter and Kindt (2011a, 2011b). The studies by Schiller et al. (2010) and Oyarzun et al. (2012), however, employed a partial reinforcement schedule (37.5%). It is possible that the lack of a disruption of reconsolidation (as evidenced by spontaneous recovery observed in all groups of the present study) may be due to stronger conditioning and subsequent resistance to reconsolidation update. This possibility is supported by animal work showing that an increased number of reinforced trials down-regulates cellular and molecular mechanisms in the amygdala that are necessary to open a reconsolidation window thereby preventing the potential for disruption (Wang, de Oliveira, & Nader, 2009). However, recent findings by Agren, Engman, et al. (2012) and Agren, Furmark, et al. (2012) showed that reconsolidation could be disrupted using retrieval + extinction methods and 100%

reinforcement during fear acquisition. Thus, it remains unclear whether acquisition strength contributes to the discrepant results reported regarding the disruption of reconsolidation.

4.5. Limitations

There are a number of limitations in the current study that should be addressed in future investigations. First, in the No Keypad groups, it is difficult to assess the potential contribution of decreased attention to the reduced fear expression signal. Incorporation of other measures such as skin conductance response or eye-tracking in the study might assess attention and increase consistency with other studies in the literature. Next, we based the experimental design on our previous studies on extinction (Norrholm et al., 2008; Norrholm, Anderson, et al., 2011; Norrholm, Jovanovic, et al., 2011) that used 6 blocks, with 4 trials of each type per block. However, it is possible that this large number of trials (i.e., 96 trials), coupled with the absence of active responding on a keypad, results in a “floor effect” in the No Keypad groups. A shorter extinction session may provide a stronger fear expression signal across Days 2 and 3 of testing. Finally, the four groups had relatively small sample sizes (ranging from 10 to 20 per group) which may have been underpowered to detect significant effects. However, some of the smaller groups did show significant effects, indicating that there was sufficient power. In addition, the small effect sizes in the groups with non-significant effects suggested that these differences would not be significant even with larger sample sizes.

5. Conclusions: an update to the disruption of reconsolidation through update mechanisms

In conclusion, the present study was aimed at extending the reconsolidation update mechanism (retrieval + extinction) that has putatively been shown to prevent the return of fear in some studies while having no reported effect in others. The data we now report suggest that even with the inclusion of US-expectancy measures (and the associated recruitment of higher brain circuits) fear-potentiated startle paradigms that capture the acquisition, maintenance, extinction, recovery, re-extinction, and reinstatement of conditioned fear (see Fig. 4, panel C) are well-suited to advance the study of the retrieval + extinction mechanism and may shed further light on its neurobiological mechanism of action and, ultimately, its potential utility as a non-pharmacological intervention for the treatment of anxiety disorders.

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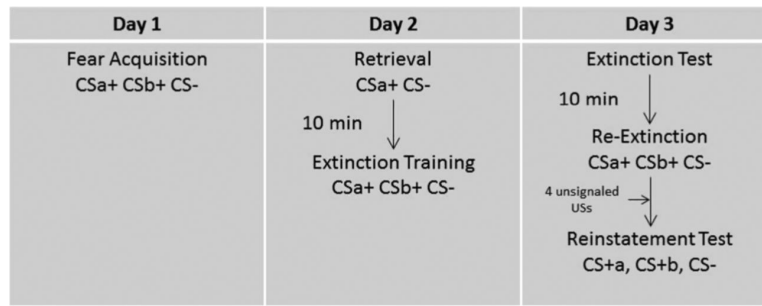


Fig. 1.
Representative schematic of the experimental design.

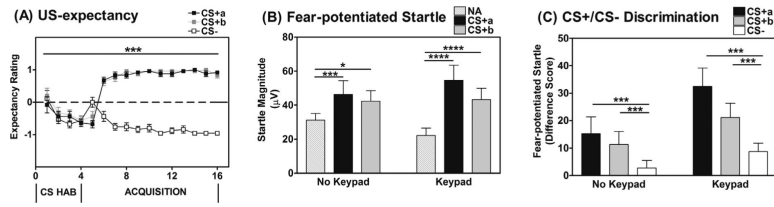


Fig. 2.

Fear Acquisition. (A) For groups with a Keypad, participants displayed significant discrimination between the reinforced CS+'s and the CS-. CS HAB = CS habituation phase. For each trial, participant responses were coded as “+1” if they expected the airblast on the trial, coded “0” if they were uncertain, and coded “-1” if they did not expect an airblast. (B) Participants with and without a Keypad showed robust fear-potentiated startle to the CS+a and CS+b with an enhanced degree of fear-potentiated startle to both the CS+a, and CS+b in those who used a Keypad. (C) Fear-potentiated startle calculated as a Difference Score between the CS+'s and NA examined during the last block of Acquisition was significantly higher for the CS+a compared to the CS-, with no interaction effects with either Retrieval group or Keypad group. **** $p < 0.0001$, *** $p < 0.001$, $p < 0.05$; Difference Score = [startle magnitude in the presence of a CS] – [startle magnitude in response to noise alone (NA)].

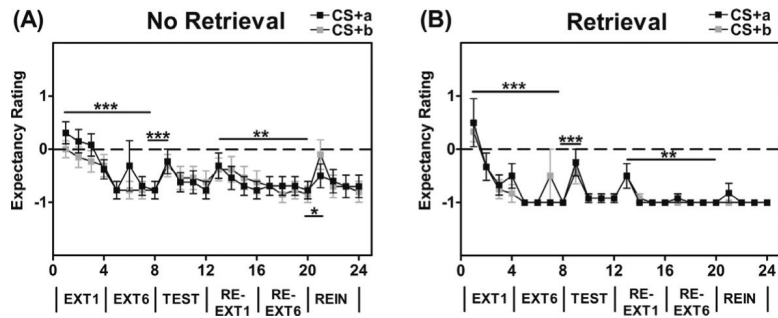
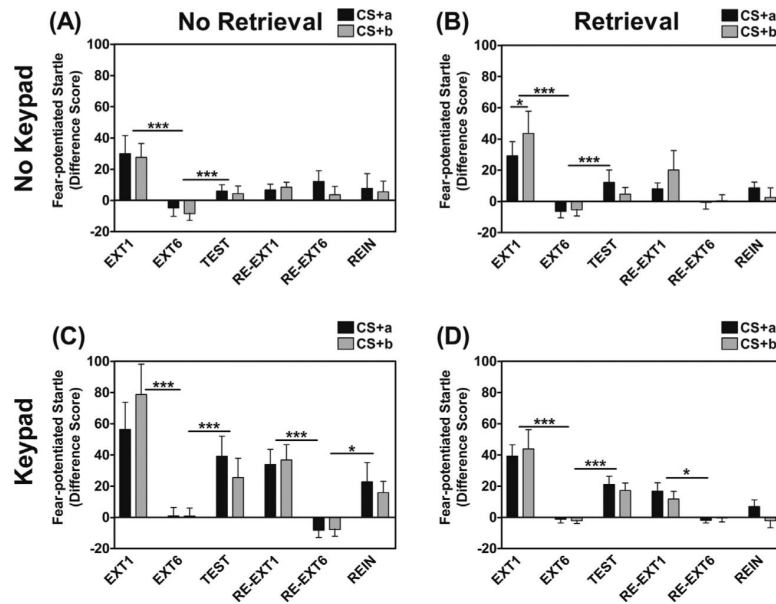


Fig. 3.

US-expectancy ratings for the (A) No Retrieval group and (B) Retrieval group, during Extinction Training (EXT), Extinction Test (TEST), Re-extinction (RE-EXT), and Reinstatement Test (REIN). During Extinction Training, US-expectancy ratings to both CS+s (CS+a = reactivated cue; CS+b = non-reactivated cue) were equally diminished in the Retrieval and No Retrieval groups. During the Extinction Test, both Trial Types showed a significant increase in US-expectancy, when comparing US-expectancy ratings on the last trial of extinction (EXT6) and the first test trial (EXT1) for both CS+s. Next, analysis of US-expectancy on the first and last trial during Re-extinction revealed a significant decrease from RE-EXT1 to RE-EXT6. After Re-extinction, we tested for reinstatement effects on US-expectancy by comparing the last trial of Re-extinction to the first trial of the Reinstatement Test after the delivery of 4 unsignaled airblast USs. This analysis revealed a significant main effect of Reinstatement, as well as an interaction effect of with Retrieval group, in that the group that received the retrieval cue showed less of an increase in US-expectancy during Reinstatement compared to the group who did not get a reactivation cue. *** $p < 0.001$, ** $p < 0.01$.

**Fig. 4.**

Fear-potentiated startle data for the Extinction Training (EXT), Extinction Test (TEST), Re-extinction (RE-EXT), and Reinstatement Test (REIN) sessions for the four experimental groups: (A) No Retrieval/No Keypad; (B) Retrieval/No Keypad; (C) No Retrieval/Keypad, and (D) Retrieval/Keypad. All groups showed significant extinction of fear-potentiated startle from EXT1 to EXT6. However, only the Retrieval/No Keypad group showed lower fear-potentiated startle to the reactivated CS+ (CS+a) compared to the non-reactivated CS+ (CS+b) during Extinction Training. The two CS+ trials did not differ from each other in any of the other groups. There was higher fear-potentiated startle in the groups that used the Keypad compared to the No Keypad groups. All four groups showed a significant increase from the last block of Extinction Training (EXT6) to the Extinction Test (TEST), however there was also a significant main effect of Keypad group. Re-extinction (RE-EXT1 vs RE-EXT6) was only significant in the two groups that used a Keypad. None of the groups showed any effects of Trial Type, i.e. both CS+ were re-extinguished in the Keypad groups, and neither in the No Keypad groups. Significant reinstatement (RE-EXT6 vs REIN) was only observed in the No Retrieval/Keypad group, with no effect of Trial Type.