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Reinstatement of Second-Order Fear

Matthew Nelson
University of South Carolina - Aiken

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Matthew Nelson

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Abstract

The purpose of this study was to determine if, after conditioning and extinction of a second-order stimulus, reinstatement of extinguished fear could be produced. The findings of Holmes et al. (2014) do not support reinstatement of second-order fear following post-extinction presentation of the unconditioned stimulus (US). The present study provides a second attempt at uncovering reinstatement following extinction of second-order conditioning. Rat subjects were randomly assigned to 5 groups (n=12), which received first- and second-order conditioning with Light and Tone counterbalanced. Responding to the second-order stimulus (S2) was extinguished through S2-alone presentations for all but the control subjects (Group NE-NR). This group did not receive extinction or reinstatement and served as the baseline of fear conditioned to S2. Comparisons made between Group NE-NR and subjects presented the US (Group US-alone) or additional first-order pairings (Group S1-US) indicated similar levels of freezing and thus reinstatement. Lower levels of freezing were noticed in Group S2-S1 and Group S1-alone and indicated effective extinction through presentation of S2 alone, and a lack of reinstatement for these groups. The clinical applications of these results are discussed.

Reinstatement of Second Order Fear

Fear is generally defined as a phasic, apprehensive arousal to explicit threat of an aversive stimulus (e.g., Davis, 1998). Although fear is generally adaptive, it may become a source of pathology when experienced in the absence of direct threat (i.e., anxiety). Anxiety disorders are among the most prevalent mental health diagnoses, and reported to affect 18.1% of the adult population and 25.1% of 13 to 18 year olds (Kessler, Chiu, Demler, & Walters, 2005). The estimated annual direct cost of these disorders is said to be between $42-47 billion, which jumps to over $100 billion when long-term unemployment and co-morbidity are taken into account (Greenberg, et. al, 1999).

Due to the prominence of anxiety disorders among the population, it is vital to understand successful interventions for individualized treatment. Exposure therapy involves presentation of the feared stimulus in absence of the feared response, and has been proven effective in treating anxiety (e.g., Hofmann and Smits, 2008; Norton and Price, 2007). While exposure is effective in reducing fear, relapse following treatment is common. Craske (1999) reports relapse rates between 19-62% depending on variables such as: population, interval, and evidence criteria indicating a return of fear. These relapse rates parallel findings examining animal behavior (e.g., Bouton & King, 1983; Pavlov, 1927; Rescorla and Heth, 1975), prompting researchers to utilize existing learning principles to help inform treatment.

Classical Conditioning of Fear and Extinction

For years theorists have used classical conditioning as a model to explain the development and maintenance of fear and anxiety (e.g., Watson & Rayner, 1920). Pavlov (1927) was the first to identify the four components of classical conditioning: the unconditioned stimulus (US), the unconditioned response (UR), the conditioned stimulus (CS), and the

conditioned response (CR). Through his work with dogs, Pavlov observed natural saliva production (UR) in response to food presentation (US). He then repeatedly paired a neutral stimulus (tone, CS) with food (US), until presentation of the tone alone resulted in a new excitatory response of salivation (CR). Similar to this excitatory conditioning, fear may be presumed to be classically conditioned by pairing a neutral stimulus with an aversive stimulus, for instance by pairing a simple tone (CS) with electrical shock (US). Through these repeated pairings, fear of the tone may develop as it reliably signals shock. Miller (1948) was one of the first to demonstrate fear conditioning with an animal model. Rat subjects were administered electric shock in the white compartment of a black and white shuttle box. Following this conditioning treatment subjects were returned to the white compartment, where they learned to turn a wheel to escape from the white context. Presumably, pairing the white context (CS) with electrical shock (US) resulted in conditioned fear of that context, along with motivating escape behavior, with successful escape reinforced by fear reduction (negative reinforcement).

Classical conditioning principles have also been extended to include treatment of fear. Pavlov (1927) found that repeated CS-noUS (CS-) presentations produce a decrease in CR – a process known as extinction. A more recent demonstration of extinction comes from the research of McAllister and McAllister (1994). Using an escape-from-fear task, they presented rats the US (footshock) in one side of a two-compartment box. Fear of the context, where shock had been administered, was measured by the subject's speed to escape into the other compartment. Following conditioning, subjects were returned to the conditioning chamber and given 0, 1, 3, or 5 hr of context exposure (i.e., no US was presented) to extinguish fear. Subjects given 1 or 3 hr of exposure displayed high levels of fear, suggesting extinction was not successful. However,

jump times of the 5 hr extinction group were similar to a non-conditioned control group, indicating elimination of fear.

Over the years researchers have used extinction of conditioned fear as a laboratory correspondent to the study of exposure treatment for anxiety disorder (e.g., Hermans, Craske, Mineka and Lovibond, 2006). Hermans, Dirikx, Vansteenwegenin, Baeyens and Eelen (2005) tested the utility of extinction in reducing human fear. A differential conditioning procedure was used in which one CS was always followed by mild unpleasant shock (CS+), whereas the other CS was always presented alone (CS-). Following acquisition, participants were given 72 extinction trials in which neither CS was followed by shock (US). A US-expectancy rating was obtained at both stages of this experiment in which participants were asked to indicate to what extent they expected a US following CS+ and CS- presentation. Results showed significantly lower US-expectancy ratings (i.e., less fear) for the CS+ following extinction compared to ratings made after acquisition. These results support the use of extinction (CS-no US) in the treatment of fear.

Although research has demonstrated that extinction can be effective in reducing fear, several well-documented phenomena exist which highlight the persistence of fear following extinction. These phenomena include spontaneous recovery, renewal, rapid reconditioning and reinstatement.

Spontaneous Recovery

Spontaneous recovery refers to the reappearance of the CR after the response has been extinguished, seen after an interval of time passes without further CS-US pairings. This phenomenon was first observed by Pavlov (1927) when he noticed increased salivation for subjects tested shortly after extinction training compared to those tested immediately.

Haberlandt, Hamsher and Kennedy (1978) reported a positive correlation between post-extinction interval and conditioned responding, with maximal responding 12-24 hours following extinction. Spontaneous recovery is generally short lasting, and further non-reinforced presentations of the CS reduce the effect (e.g., Wagner, Siegel, Thomas and Ellison, 1964).

Researchers have worked to identify factors that attenuate spontaneous recovery. Urcelay, Wheeler and Miller (2009) highlight the importance of extinction trial spacing in the maintenance of fear reduction. Their results show less recovery of fear (i.e., spontaneous recovery) in groups given spaced extinction trials compared to control and mass extinction-trial subjects. Not only is the interval between treatment sessions important, but so too is the delay between fear conditioning and extinction treatment. Results from Huff, Hernandez, Blanding and Labar (2009) demonstrate greater spontaneous recovery when fear is immediately extinguished after conditioning than when there is a delay between acquisition and extinction.

One attempt to explain phenomena such as spontaneous recovery has been made by Bouton (1993, 1994). His memory retrieval model suggests two distinct memories are formed during the acquisition and extinction processes. During acquisition subjects learn that the CS is followed by reinforcement (US), whereas, during extinction a CS-noUS association is formed. Following extinction, these memories compete for retrieval. Bouton argues the CS-noUS memory is context dependent and can only be recalled in the presence of extinction cues (i.e., the extinction context). Bouton suggests a delay between extinction and testing causes a temporal contextual change, which explains a failure to retrieve the CS-noUS memory, and thus recovered responding is observed. This explanation of spontaneous recovery has been supported through research by Bouton and Brooks, (1993) which demonstrated that presentation of an extinction

cue prior to the CS during extinction weakened spontaneous recovery, presumably because the cue "reminds" subjects of the association formed during extinction.

Renewal

A second example of the persistence of fear after extinction is the renewal effect. Fear renewal refers to the reemergence of fear after extinction when the conditioned stimulus is presented in a context different than extinction. Bouton and King (1983) used an ABA design in which subjects received fear conditioning to a CS in one context (Context A), and then fear was extinguished in a separate context (Context B), followed by CS testing in A. Renewal (reappearance) of extinguished fear was observed in Context A, but not if tested in Context B.

Two additional procedures have been shown effective in producing renewal following extinction. In the ABC paradigm, conditioning occurs in context A, followed by extinction in context B. Subjects are then transferred to a third different context (C), unlike context A or B, where the CS is tested and renewed fear is observed. Similar renewal of fear to the CS is seen in an AAB design (Bouton & Ricker, 1994), where conditioning and extinction occur within the same context (A) and testing in a different context (B). These data suggest that the process of extinction does not erase original conditioning, but rather masks its expression. Similar to spontaneous recovery, renewal has also been explained using Bouton's (1993, 1994) memory retrieval model as both highlight the importance of context in conditioning and post-conditioning procedures.

Manipulation of certain contextual cues has proven effective in reducing the renewal of fear. A study by Havermans, Keuker, Lataster and Jansen (2005) demonstrates renewal in humans. During acquisition, participants were instructed to respond to stimuli on a computer based on the background color of the screen. Incorrect responses were "punished" by delaying

the participants' ability to produce the required response. No punishment was administered during extinction to help eliminate fear. It is also important to note that researchers manipulated screen and stimulus color during extinction. These conditions mimic an ABA design, in which extinction occurred in a context different than conditioning and testing. Results demonstrated that simply changing the background color between the extinction and acquisition context was not sufficient to support renewal, whereas the combination of change in both background and stimulus color was enough to allow distinction between these two contexts, and thus renewal was observed.

Thomas, Larsen and Ayers (2003) demonstrate a similar result with rats. They were able to successfully demonstrate ABA renewal following a change in odor and location of lighting between Context A and B. Their results show that removal of the odor cue attenuated renewal, as it was presumed subjects were unable to discriminate between the extinction and acquisition context, thus allowing for recall of the CS-no US association. The results of these two studies suggest the more similar the extinction and conditioning context the more effective extinction will be. Extinction in multiple contexts has also been shown to alleviate the effects of renewal as learning is thought to generalize to a multitude of settings (i.e., Chelonis, Calton, Hart and Schachtman, 1999; Neumann, 2006). Bandarian, Balooch, and Neumann (2011) combined these factors (i.e., number and similarity of contexts) to show that extinction in multiple similar contexts was most effective in reducing ABA renewal.

Rapid Reacquisition

It is well documented that following extinction, reconditioning often occurs quicker than original conditioning (e.g., Macrae & Kehoe, 1999; Smith & Gormezano, 1965), a phenomenon known as rapid reconditioning (reacquisition). Rapid reconditioning provides further evidence of

extinction's failure to erase fear. Napier, Macrae, and Kehoe (1992) conditioned an eye-blink response to a tone in rabbits. Following conditioning, this response was extinguished for all subjects. Results from a subsequent reacquisition phase demonstrated a faster rate of learning (reconditioning) for subjects with conditioning experience compared to controls (i.e., no previous CS-US pairings). McAllister, McAllister, Scoles and Hampton (1986) conditioned fear of context to one side of a two-compartment box. Upon return to the conditioning chamber, subjects learned to hurdle jump to escape the context CS. No shocks were administered during this portion of the experiment, thus serving as extinction trials. Following extinction, some subjects received an additional CS-US pairing while others were given no such pairing. Results demonstrated that subjects given an extra CS-US pairing required significantly more trials to reach a second, subsequent extinction than controls (i.e., rapid reacquisition), indicating some fear remained after extinction.

Reinstatement

Research by Rescorla and Heth (1975) provides further evidence of the inability of extinction to completely erase original conditioning. They classically conditioned rats to fear a tone (CS) and then presented the tone alone to extinguish fear. Half the subjects then received a single presentation of the US (shock) alone (no CS), while the other half received no shock. Testing with the CS showed significantly lower suppression ratios (higher fear) in those subjects presented the US following extinction than those who received no stimulus presentation between extinction and test. This increase in CR after extinction, with an additional US presentation, is known as reinstatement. To explain these results Rescorla and Heth (1975) argued that two separate processes occur during acquisition. Individual representation of the CS and US enter into memory during original conditioning and an association is formed between these two

memories. Presentation of the CS alone during extinction decreases the memorial representation of the US in addition to the CS-US association, producing a decrease in responding. They argue that presentation of the US following extinction helps to inflate and restore memory of the US, reinstating the CS-US association and CS fear, and thus new responding. This is a non-associative interpretation of reinstatement as the increase in US representation is said to increase CS fear, and presumably no additional CS-US pairings are necessary.

An alternative explanation of reinstatement suggests that presentation of the US alone following extinction results in greater fear of the context where the US was administered (e.g., Bouton and Bolles, 1979). This contextual conditioning along with the small amount of remaining fear of the CS may therefore combine to reinstate responding (cf. Reberg, 1972). This is considered to be an associative view of reinstatement as no increase in actual CS fear is thought to occur; instead the combination of CS plus context fear results in increased CR. According to this interpretation, reinstatement should only be seen when testing and post-extinction shock occurs in the same context.

Bouton and Bolles (1979) suggested that the "different" contexts used for reinstatement in the Rescorla and Heth (1975) study were similar enough that the contextual fear produced through US-alone presentation may have generalized to the test context; they therefore examined reinstatement using two distinctly different contexts to reduce any influence of generalization. Following conditioning and extinction, US-alone shocks were given in one of the two different contexts; subsequent fear testing revealed reinstated fear only when shock and test occurred in the same context. These results highlight the importance of context in reinstatement and shed doubt on the explanation provided by Rescorla and Heth (1975), whose theory would suggest reinstatement should occur for both groups if presentation of the US serves to inflate US

representation. In a follow-up study, Bouton and Bolles (1979) exposed subjects to the reinstatement context following post-extinction shock in an attempt to extinguish contextual fear. This exposure, which presumably decreased any influence of context fear, was sufficient to prevent reinstatement, providing further support for the associative hypothesis.

Callen, McAllister and McAllister (1984) pointed out that the study by Bouton and Bolles (1979) may not provide definitive support for an associative view of reinstatement. They highlight the research of Reberg (1972) and Hendry (1982), which demonstrates a CS insufficient by itself to demonstrate fear may, in combination with an additional weak CS, produce demonstrable fear effects. Callen et al. (1984) note the possibility of the US inflation effect going unnoticed in the different context group of the Bouton and Bolles experiment, due to the absence of contextual cues at test. In order to rule out this possibility, Callen et al. (1984) used only context cues to condition fear (no discrete CS). Subjects were conditioned to fear one side of a two-compartment box, and then, fear was extinguished through non-reinforced exposure to the fear compartment. Post-extinction shock was delivered in the conditioning context for some subjects, while others were presented the same US in a distinctly different context. Using an escape from fear measure, significantly more fear was reported for the group reinstated in the original compartment compared to the different context reinstatement condition, which showed no reinstated fear. Even nine additional shocks (to maximize any potential US inflation effect) in the different context did not produce any evidence of reinstated fear. These results are consistent with an associative account of reinstatement, and do not support non-associative explanations of reinstatement.

Westbrook, Iordanova, McNally, Richardson, and Harris (2002) note that US presentation in the extinction context, might also partially restore the CS-US association contributing to the

reinstating effects observed (e.g., Callen et al., 1984). To test this, conditioning and extinction was conducted in the same context. Following extinction, some subjects received post-extinction shock in the extinction context, whereas some received shock in a distinctly different context. Half the rats in each of these groups were tested in the reinstatement context, while the other half were tested outside this context. Subjects presented the US and test in the same context displayed significantly higher levels of fear compared to the different context group. To differentiate between renewal and reinstatement an additional study was conducted, in which three distinct contexts were used. Conditioning, extinction, and post-extinction shock procedures were identical to the previous study; however testing was conducted in a novel context for all subjects. Results showed subjects who received re-exposure to shock in the original context, froze significantly more than rats given shock outside the conditioning context and controls. This study demonstrates that presenting the US in the extinction context partially renews the CS-US association learned during acquisition training. These results taken together indicate reinstatement is most pronounced when extinction, US re-exposure, and testing all occur in the same context.

An alternative explanation of reinstatement comes from Bouton (1991, 1993), which suggests the contextual conditioning produced by post-extinction US presentation returns organisms to acquisition conditions, where contextual conditioning also occurred. This return to acquisition conditions allows for recall of the CS-US memory, and therefore reinstated responding to the CS. Richardson, Duffield, Bailey and Westbrook (1999) utilized a procedure similar to that used by Callen et al. (1984), in which fear of contextual stimuli was conditioned and extinguished in one side of a two-compartment box. Post-extinction shock was administered in the reinstatement context, which varied markedly from the conditioning context. The interval

at which shock was delivered varied between groups. Previous research suggests immediate shock does not allow formation of an association between context and US; whereas delayed shock is conducive to contextual conditioning (e.g., Fanselow, 1990; Kiernan & Westbrook, 1993). According to the non-associative account both groups should exhibit a similar amount of reinstatement; an associative model would predict reinstatement for only the delay condition, because it leads to greater context conditioning. Using an escape from fear measure revealed reinstatement of fear (i.e., faster jump times) for the group given delayed shock in comparison to the group given immediate shock. This finding is consistent with Bouton's prediction, however; contextual conditioning seems to only partially explain the results obtained. According to Bouton's model reinstatement should only occur when reinstatement shock and test occur in the same context. However, results from the current study demonstrate reinstatement despite a contextual change between post-extinction shock and test. This can be taken as further support for an associative account of reinstatement, as fear of the acquisition context (CS) appeared to summate with the contextual fear produced from post-extinction shock.

Research by McAllister and McAllister (2006) provides an alternative interpretation of the results obtained by Richardson et al. (1999). McAllister and McAllister used a passive avoidance procedure to measure fear. For this experiment, the floors of two distinct compartments were dissimilar in addition to visual color differences. All subjects were conditioned to fear a black wall, grid floor compartment. Following conditioning, fear was extinguished through non-reinforced exposure to the conditioning chamber. After extinction, all subjects received a single post-extinction shock. One group received shock after a short delay (24 hours following extinction), while the other group was presented the US following a long delay (168 hours later). This shock was given either in a distinctly different context consisting of a solid floor, or in the

original context. Results showed significantly longer passive avoidance latencies (i.e., more reinstated fear) for rats given post-extinction shock in the grid floor environment after a short delay compared to those given shock in the solid floor (different) environment. These results are in agreement with previous studies which demonstrate higher levels of fear in rats given post-extinction shock in the extinction context, or as this study illustrates a context highly similar. Analyses also indicated significantly more fear for subjects in the long-delay condition compared to their respective short-delay counterparts, regardless of reinstatement context. McAllister and McAllister explain these results based on the broadening of the stimulus generalization gradient over time. They suggest that the short-delay group was able to easily distinguish the reinstatement context from the training context, and that only fear reconditioned to the grid-floor would generalize back to the test context. However, with a long delay between extinction and reinstatement the contexts would be more difficult to discriminate, thus allowing for generalization of fear across the reinstatement and conditioning contexts, and therefore higher levels of reinstated responding.

Recent research provides evidence that reinstatement may occur in the absence of US presentation. Halladay, Zelikowsky, Blair, and Fanselow (2012) successfully demonstrated a return of fear following presentation of only an unextinguished CS, without a US, a phenomenon referred to as conditional reinstatement. First, all subjects were trained to fear a tone and light CS. Responding was then extinguished to one of the two CSs. Then subjects in the conditional reinstatement group were given presentations of the unextinguished CS, while another group received eight presentations of a novel stimulus. The results indicated that conditional reinstatement produced more freezing during extinguished CS presentation than control groups. The researchers suggested that presentation of an unextinguished CS produces a fear reaction

similar to the one experienced during acquisition, thus allowing for recall of the original CS-US association. This suggests that return to the internal state that was present during acquisition, through experience of any fear-related sensation, may be enough to reinstate responding.

Although most research on reinstatement has utilized animal models, Dirikx, Vansteenwegen, Eelen and Hermans (2009) successfully demonstrated reinstatement of fear in humans. They used a differential conditioning paradigm in which one CS was paired with an aversive US (CS+), while a different CS was not (CS-). Following acquisition, each CS was presented alone, without the US, to extinguish fear. One group of participants was presented the US (shock) alone prior to test, while the other received no shock between extinction and test. After extinction, results showed a significant increase in fear of both the CS+ and CS- in the reinstatement but not control group. Researchers explain these results through contextual conditioning. They suggest the reinstatement context is made excitatory due to unpredictable US presentation. This fear of the reinstatement context is thought to generalize, leading to increased fear of stimuli not initially followed by the US, a finding similar to that of McAllister and McAllister (2006).

Understanding the context specificity of extinction Dunsmoor, Ahs, Zielinski and LaBar (2014) tested the impact of extinction in multiple contexts in a human population. A differential conditioning procedure was used in which fear was conditioned to one of two different virtual reality characters. Fear was extinguished in the conditioning context for one group of participants, a second group received extinction training in multiple contexts, while the final group received non-reinforced presentations of the CS+ in a distinct context. All contexts used varied in both color and texture. After extinction, all participants were exposed to a novel context where they received three unsignaled shocks (US) prior to test. Results showed that participants

who received extinction in only one context responded significantly more to the CS+ than the CS-, thus indicating reinstatement of fear. However, no difference in fear between CS+ and CS- was noticed in the group receiving extinction in multiple contexts. These results demonstrate that extinction in multiple contexts can reduce reinstatement effects.

Second-Order Conditioning

The studies mentioned thus far have examined fear following first-order CS-US pairings (i.e., first-order conditioning). Pavlov (1927) identified that a first-order conditioned stimulus could serve as the basis for conditioning of a different CS, a process known as second-order conditioning or higher-order conditioning. First, fear was established through CS-US pairings. Pavlov took this a step further and demonstrated fear conditioning by pairing the newly conditioned CS (S1) with a second CS (S2). More recently, Rizley and Rescorla (1972) administered first-order CS-US pairings, and after a reliable CR to the CS was produced, they paired a second neutral stimulus with the first (e.g., S2-S1), without any US. Conditioned responding was observed to S2 even though it had not been paired with a US, presumably because of its association to S1.

An explanation of these results provided by Rizley and Rescorla (1972) suggest learned associations during both stages of conditioning. They suggest an association forms between S1 and the US during first-order training, which is carried over and recalled during second-order conditioning when an association develops between S2 and S1. According to this theory, extinction of first-order conditioning, and thus a decrease in S1-US associative strength, should result in extinction of second-order responding. Rizley and Rescorla (1972) tested this assumption. Following second-order conditioning, fear of S1 was extinguished for one group of subjects and not a second group. Results showed little difference in fear across the extinction and

non-extinction groups, thus suggesting different extinction processes may be operating in second compared to first-order conditioning.

Rescorla (1982) provides an explanation of these results based on the type of association formed across first- and second-order conditioning. He suggests that during first-order pairings an association is created between the stimulus properties of S1 and the US, whereas during second-order pairings an association forms between the properties of S2 and the emotional response evoked by S1. Extinction of the stimulus-stimulus association (i.e., S1-US) does little to effect the stimulus-response relationship formed during second-order conditioning. Rescorla tested his assumption through manipulation of the temporal relationship between stimuli by varying the type of presentation used during second-order conditioning. Subjects received either simultaneous presentation of S1 and S2 or sequential pairings (e.g., S2 offset co-occurring with S1 onset). It was assumed that simultaneous presentation promotes S-S learning more than sequential presentation (e.g., Rescorla, 1980; 1981). Responding to S1 was then extinguished and the results showed less fear and thus successful extinction of second-order responding in the simultaneous group compared to sequential group. These findings support Rescorla's hypotheses and highlight the importance of the temporal relationship between stimuli during second-order conditioning procedures.

Like first-order conditioning, second order fear can be conditioned to contextual stimuli. In an experiment by Helmstetter and Fanselow (1989) a differential first-order conditioning procedure was used in which one stimulus was followed by aversive footshock (S1+), while the other was not (S1-). Following first-order conditioning, animals were moved to two distinct contexts where they received presentation of S1+ in one context and S1- in the other. Researchers hypothesized that presentation of an excitatory CS, much like a US, would result in

contextual conditioning. At test, subjects were returned to the contexts used during second-order training and freezing behavior was measured. Results showed that the rats were able to discriminate between the two contexts as more freezing was observed in the context associated with the reinforced first-order stimulus (S1+). Therefore, it can be said that second-order conditioning was successful as fear of the context (S2) developed based on its pairings with S1+. In a related study, Marlin (1983) was able to successfully demonstrate second-order conditioning using contextual stimuli as S1. One group of rats were shocked in one context (S1+), and a second group exposed to a different context without shock (S1-). Half the subjects were then presented a tone (S2) in the context paired with shock (e.g., S2-S1+ pairings), whereas the other half were presented the tone in the non-excitatory context (e.g., S2-S1- presentation). Testing in a third context revealed significantly more fear during CS presentation for rats given S2-S1+ pairings compared to the S2-S1- group. These results highlight a similarity between first-and second-order conditioning, as both are sensitive to contextual manipulations.

Over the years, researchers have identified factors to attenuate second-order conditioning. Since trial spacing has been identified as a factor that affects spontaneous recovery and reinstatement, Miller and Whitnauer (2011) examined its impact on contextually mediated and conventional second-order conditioning. One group received massed conditioning trials with shorter CS-US intertrial latencies, while a second group received spaced conditioning with a long intertrial interval. Subjects received S1-US pairings to produce first order fear, interspersed with S2-S1 pairings to establish fear of S2. Rats in the context condition received US-alone presentations (i.e., first-order contextual fear) interspersed with S2-alone presentations (i.e., second-order fear conditioned to contextual S1). All subjects were tested in a different context for conditioning to S2. Results showed successful second-order conditioning for subjects

receiving massed conditioning with contextual cues and for those receiving spaced trials with a discrete stimulus. This experiment demonstrates that optimal trial spacing is influenced by the stimulus used during first-order training.

Stout, Escobar and Miller (2004) identified two other factors that impact strength of second-order conditioning: number of second order trials and temporal relationship between stimuli. After first-order conditioning, one group of rats received 100 S2-S1 pairings, a second group received 20 pairings, while a third group received 4 parings. Half the subjects in each group received simultaneous presentation of S1 and S2, whereas the other half received serial presentation of these stimuli (i.e., S2 followed by S1). All subjects received separate conditioning to a tone (i.e., tone-US), which researchers used for comparison during summation testing. Results from the summation test show negative summation (i.e., inhibition) with increased S2-S1 pairings for both simultaneous and serial presentation methods. These results demonstrate increased second-order pairings may actually inhibit development of fear as subjects learn S2 signals non-reinforcement over many trials. Results also showed less fear with simultaneous compared to serial presentation. From this study it is clear increased S2-S1 pairings attenuate conditioning, especially when S1 and S2 are presented in a simultaneous fashion.

There is not a lot of research examining reinstatement and other return of fear processes as they apply to second-order conditioning. A recent study by Holmes, Cai, Lay, Watts, and Westbrook (2014) examined renewal and reinstatement following extinction of S2 fear. Subjects received first- and second-order conditioning in two different contexts. Groups were then given S2-alone presentations to extinguish fear of S2. One group received S2-alone presentations in the first-order conditioning context, while the other received S2 extinction in the second-order conditioning context. Half of the subjects in each group were tested with S2 in the context where

extinction had occurred, whereas the other half received testing outside the extinction context. Results showed renewal of fear for rats tested in a context different than extinction compared to those tested in the presence of extinction context cues. These results demonstrate renewal does apply to extinguished second-order conditioned stimuli, much like first-order conditioned stimuli. They conducted a second experiment to test whether reinstatement might also be observed following extinction of a second-order CS. First- and second-order conditioning were conducted in the same context, followed by extinction of S2 with S2-alone presentations. Then one group of subjects was presented the US alone, a second was presented S1 alone, while a third group was given no stimulus. Testing revealed no difference in responding among the three groups, suggesting reinstatement does not occur following extinction of a second-order CS. This study was the first to examine return of fear phenomena, specifically reinstatement, as they apply to second-order conditioned stimuli. The findings were interesting, yet incomplete, as the observation of renewal suggests that first and second-order CS tended to behave similarly, yet no reinstatement was found.

The aim of the current study is to provide a second attempt at uncovering reinstatement of second-order fear. The current study will replicate the procedures used by Holmes et al. (2014), and will include two additional groups to test for reinstatement effects. These two groups will each receive presentation of the first- and second-order stimulus compounds (i.e., S2-S1; S1-US). Presentation of these compounds in addition to S1 alone and US alone presentations should help to uncover the associations underlying first and second-order conditioning processes. Freezing during CS presentation will serve as the dependent measure of fear. The present study should also provide additional support for the use of S2-alone presentations as an effective method of extinguishing second-order fear. Several hypotheses will be tested:

Hypothesis 1. Observed fear will decrease across S2-alone presentations, indicating successful extinction.

Hypothesis 2. Following extinction, presentation of the US or S1 (the US analogous in S2-S1 pairings) will produce contextual conditioning. This context fear, in addition to the small amount of fear remaining towards S2 will reinstate responding.

Hypothesis 3. Following extinction, presentation of the stimulus compound S1-US or S2-S1 will evoke an emotional reaction, similar to conditional reinstatement, thus allowing for recall of the S2-S1 memory and increased S2 freezing.

Method

Subjects and Design

The subjects were 60 naive albino Sprague Dawley rats, (30 male, 30 female) approximately 120 days old, supplied by the USC Aiken Psychology Department animal vivarium. All subjects were individually housed and allowed free access to food and water for the duration of the experiment. Subjects were randomly assigned to 1 of 5 groups: S1-US, S2-S1, S1-alone and US-alone, and NE-NR. The experimental design is presented in Table 1. All subjects received S1-US pairings (first order conditioning) followed by S2-S1 pairings (second-order conditioning). For all groups except NE-NR responding to S2 was extinguished with S2-alone presentations, followed by one of four different reinstatement treatments. Group NE-NR did not receive extinction or reinstatement treatment and served as the baseline for second-order conditioned fear. All fear testing was then conducted with the second-order stimulus (S2) and freezing behavior to that S2 stimulus was measured. The stimuli used as S1 and S2 (flashing light and tone) were counterbalanced. All sessions were conducted in the same apparatus and approximately 24 hours separated treatment sessions.

Apparatus

Treatments were administered in four identical Med Associates chambers (Env-022MD). Background illumination was provided by an incandescent light bulb (28-V, 170mA) centered on the left wall and mounted 16.67 cm from the floor, along with two stimulus lights centered and located on the right wall. The stimulus lights (28-V bulbs) were programmed to flash on and off two times per second and provided the source for one of the CS stimuli. A speaker, located on the rear wall, provided the 80db auditory stimuli (3000 Hz pure tone) and was the other CS. Foot shock (1 mA, 0.7-s) was delivered through the grid floor and served as the unconditioned stimulus (US). All chambers were housed in sound and light attenuating boxes. The operant chambers were connected to a computer via a Med Associates Interface (version 4.0), through which all programming and data collection was monitored. A Fujinon Fish Eye camera, mounted on the left front door 30 cm from the floor, recorded subject behavior during test trials. Med Associates Video Monitor Software (version 1.4.0) was used to record and analyze freezing behavior.

Procedure

Exploration: On Days 1 and 2, all subjects received exploration of the context. During these sessions subjects were placed in the operant chamber for 30 min and allowed to explore. No stimuli were presented during this phase of the experiment. This procedure allowed for contextual familiarization and thus the elimination of any unconditioned contextual fear prior to conditioning.

First-order Fear Conditioning: On Days 3 and 4, subjects were returned to the operant chamber and received two trials of first-order conditioning (S1-US pairings). Following a 6 min adaptation period, S1 (tone or flashing light, counterbalanced) was presented for 10 s, co-

terminating with the .7 s, 1 mA US. After a 6 min intertrial interval (ITI), a second S1-US presentation was administered, and subjects were then returned to their home cages. Thus, a total of 4 S1-US pairings were received by all subjects.

Second-order Fear Conditioning: On Day 5, all subjects received second-order conditioning, with S2 being a different stimulus than that used as S1 (tone or light). Following a 6 min adaptation period, all groups received eight paired presentations of a 30 s S2 followed by a 10 s presentation of S1, with an ITI of 6 min. No shock was given on this day.

Extinction: On Day 6, all Groups except NE-NR were given a S2 extinction treatment. After a 3 min adaption period, eight S2-alone trials were presented for 30 s each with a 3 min ITI (no S1 presentations were given). Subjects in Group NE-NR were placed in the conditioning context for an equal amount of time but received no stimulus presentation.

Reinstatement: On Day 7 two trials of different reinstatement treatments were administered, depending on group designation (see Table 1). After 3 min in the conditioning apparatus, Group S1-US received a first-order conditioning treatment consisting of two paired presentations of the 10 s S1 co-terminating with a .7 s, 1 mA US (ITI=3 min). Group S2-S1 received a second-order conditioning treatment of two paired presentations of a 30 s S2 followed by a 10 s S1 (ITI=3 min). Subjects in Group S1-alone received two 10 s S1-alone presentations (ITI=3 min), whereas subjects in Group US-alone were given two .7 s US-alone presentations (ITI=3 min) and neither S1 nor S2. The remaining group (NE-NR) spent an equal amount of time in the conditioning context without either S1, S2, or US presentations.

Fear Testing: On Day 8, all subjects were tested for fear of the second-order stimulus, S2. Eight 10 s S2-alone presentations were administered in the conditioning context (ITI=2 min) and

behavior was videotaped during stimulus presentations. Freezing behavior during each trial was subsequently coded and analyzed.

Freezing was defined as an absence of all movements, except those related to breathing (Fanselow, 1980). Freezing was measured using a time-sampling procedure for each test trial. During each S2 stimulus presentation, each subject was observed every 3 s and scored as either *freezing* or *not freezing*, by two independent observers blind to the experimental conditions. Mean percentage of freezing was calculated for each trail, based on the three scores obtained during sampling. For example, if the subject was noticed freezing once during the 10s S2 presentation (i.e., one score of 1), the mean percentage of freezing score for that trial would be 33% since freezing only occurred at one of the three times sampled. Interater coding agreement for freezing behavior was 94%.

Results

The results were analyzed to determine the effectiveness of the independent variable manipulations in the current study. First, effective second-order conditioning would be indicated by fear of S2 being present in Group NE-NR, the group that received first- and second-order conditioning but no extinction or reinstatement. Second, the effectiveness of the various reinstatement treatments would be measured by the fear of S2 present at test in the reinstatement groups compared to that of Group NE-NR. Fear levels lower than NE-NR would reflect the successful extinction of S2 without reinstatement, whereas fear levels equal to or higher than that of NE-NR would reflect reinstated S2 fear. Finally, since each Test trial presentation of S2 is in effect an extinction trial, fear (i.e., freezing) should be observed to eventually decrease over trials. Because two originally neutral stimuli were used as S1 and S2, there was no a priori prediction that their effectiveness as stimuli would be different.

To test these hypotheses, freezing behavior over trials was examined. Analysis of Sex (M vs. F) indicated no significant effects or interactions (Fs < 1.41), and therefore that variable was not included in any further analysis. A series of mixed design repeated measures analyses of variance (ANOVAs) were performed, with Group (S1-US, S2-S1, S1-alone, US-alone and NE-NR) and S2 Stimulus (Tone or Light) as the between-subjects factors, and Trials as the within-subjects factor. In general, the results supported the hypotheses and demonstrated conditioning, extinction, and reinstatement of second-order conditioning, the latter being dependent on the stimuli presented during the second-order reinstatement treatment.

Figure 1 presents the freezing behavior of all Groups over all Trials. This figure presents several interesting results, consistent with the present hypotheses. The performance of Group NE-NR, which received first-order conditioning followed by second-order conditioning, but received neither extinction nor reinstatement, is an indication of the baseline amount of fear of S2. The high steady level of freezing in this group demonstrates that successful second order conditioning took place in the current study. Comparison of the other four groups to NE-NR allows a determination of the effectiveness of the extinction and reinstatement manipulations. The generally lower freezing levels of Groups S2-S1 and S1-alone, compared to NE-NR, suggest that the S2 extinction manipulation was effective in reducing S2 fear. However, those two reinstatement manipulations did not appear to be effective in reinstating any S2 fear.

As Figure 1 suggests, the reinstatement procedures administered in Groups S1-US and US-alone did appear to be effective in producing reinstatement of S2 fear, as their fear levels approached or exceeded that of Group NE-NR. One other impression from Figure 1 is that overall fear levels appeared to be stable, as little change occurred across the eight trials.

An overall 5 (Group) x 2 (Stimulus) x 8 (Trials) repeated measures ANOVA supported these impressions. The results indicated a significant effect of Group, F (4, 50) = 5.122, p = .002, and Stimulus, F (1, 50) = 7.609, p = .008. There was also a significant Stimulus x Trials interaction, F (7, 350) = 2.411, p = .020, as well as a significant Group x Stimulus x Trials triple interaction, F (28, 350) = 1.561, p = .037. No other effects or interactions were significant (F's < 1.240).

Given the significant Group x Stimulus x Trials interaction, follow-up Group x Trials repeated measures ANOVAs were performed separately for the Light S2 and the Tone S2. An initial analysis conducted with Group NE-NR comparing differences in freezing across stimuli, revealed no significant effect nor interactions (F's < 1.533). This finding shows that fear was not conditioned differentially to the stimuli used. Therefore, results of the separate analyses conducted represent group differences based on reinstatement manipulations regardless of the stimulus used as S2. Figure 2 displays the freezing behavior for subjects receiving Tone as S1 and Light as S2. Although this figure indicates a similar pattern of results as Figure 1, the groups that received the Light as S2 seemed to produced more variability over trials, and group differences appeared later in testing. In addition, overall fear levels appeared somewhat higher under this condition for most groups.

Results from the Group x Trials ANOVA for the subjects that had Tone as S1 and Light as S2 indicated a significant effect of Group, F (4, 25) = 2.854, p = .045, and Trials, F (7, 175) = 2.912, p = .007. A significant Group x Trials interaction, F (28, 175) = 1.710, p = .020, was also observed. Because of the significant interaction, One-Way ANOVAs were conducted at each Trial. These analyses indicated significant Group effects at Trial 2, F (4, 25) = 2.790, p = .048, Trail 6, F (4, 25) = 3.996, p = .012, and Trial 7, F (4, 25) = 4.406, p = .008.

Multiple comparisons were conducted using Fisher's least significant difference method (LSD) and are presented in the top half of Table 2. On Trials 2 and 6 Group S2-S1 froze significantly less than Group US-alone and NE-NR, which did not differ. The lack of significant difference between Group NE-NR and US-alone suggests that presentation of the US following extinction reinstates fear to pre-extinction levels. Comparisons at Trial 6 also reveal less fear for Group S1-alone in contrast to Group US-alone and Group NE-NR, which did not differ. This shows that presentation of S1, the US analogous in S2-S1 pairings, did little to reinstate responding. On Trial 7 there was significantly higher freezing for subjects in Group S1-US and US-alone compared to Group S2-S1, Group S1-alone, and Group NE-NR, which did not differ. These results appear to indicate extinguished responding towards S2 in Group NE-NR, consistent with test trials also serving as extinction trials. In addition, paired presentation of S1 and the US successfully reinstated responding, as indicated by the significantly higher level of fear in S1-US compared to NE-NR, S1-alone, and S2-S1 on Trial 7.

Figure 3 presents the grand mean freezing data over all trials for groups that received the Light as S1 and the Tone as S2. As can be seen here, the general performance trends observed in Figures 1 and 2 are present. The two groups that received the US during reinstatement, S1-US and US-alone, produced reinstated fear levels approaching that of Group NE-NR, whereas the two groups that did not receive the US in reinstatement demonstrated the lowest fear and no fear reinstatement. In addition, the effectiveness of the S1-US reinstatement treatment was somewhat less than that observed in Figures 1 and 2.

Results of the Group x Trials repeated measures ANOVA indicated a significant effect of Group, F (4, 25) = 3.665, p = .018. No other effects or interactions were significant (F's < 1.089). The lack of a significant Trials effect is interesting as it suggests that unlike Light S2

tested subjects, freezing did not significantly decrease across Trials for subjects tested with Tone (S2). Because there was not a Trials nor interaction effect, the significant Groups effect was examined with post hoc comparisons of the grand mean freezing behavior, and are presented in the bottom half of Table 2. These indicate significantly higher freezing for Group NE-NR compared to Groups S2-S1 and S1-alone, consistent with that found previously indicating the lack of reinstatement under these two conditions. In addition, presenting US-alone resulted in reinstated fear not significantly different than that of the control group, NE-NR. However, under this Tone S2 condition, S1-US reinstatement pairings did not result in any significant increase in fear compared to S1-alone and S2-S1, as it did with the Light S2, and in fact was marginally lower in fear than Group NE-NR. In addition, the amount of reinstated fear with the US-alone was sufficient to only significantly exceed that of S1-alone.

Discussion

The primary goal of the present experiment was to determine if, after conditioning and extinction of a second-order stimulus, reinstatement of extinguished fear could be produced. To test this, subjects were initially presented either a Light or Tone neutral stimulus (S1) paired with shock to produce first-order fear conditioning to that S1 stimulus. Subjects then received forward pairings of the second stimulus (S2) with S1 in order to produce second-order fear conditioning to S2. S2 fear extinction treatments were then administered through S2-alone presentations to produce extinction of second-order conditioned fear. Lastly, following extinction, subjects were administered various reinstatement treatments in an attempt to determine whether extinguished second-order fear can be reinstated. The results of these reinstatement manipulations were compared to the level of freezing observed in Group NE-NR, the control group that did not receive extinction or reinstatement, to measure the presence of reinstated second-order fear.

The results indicated differences in final S2 freezing levels among the different conditions, suggesting that the reinstatement manipulations were differentially effective in producing change in S2 fear. Essential to any interpretation of reinstatement effects is a demonstration of successful second-order conditioning to S2, and that was supported by the finding that Group NE-NR displayed a high level of freezing across trials. Overall, subjects presented with the US following extinction, whether alone or paired with a CS, displayed freezing comparable to that seen in Group NE-NR. These findings suggest that, like first-order conditioning, presentation of the US after extinction increases second-order fear, thus providing evidence for reinstatement of S2 fear, and suggesting the necessity of the US in producing such reinstatement. The lower levels of freezing observed in Group S2-S1 and Group S1-alone in contrast to Group NE-NR indicates two effects. First it demonstrates that, since Group NE-NR reflects the baseline level of S2 fear prior to extinction, the significantly lower fear levels observed in Groups S2-S1 and S1-alone suggest that presentation of S2 alone effectively extinguished S2 fear, and further that these two reinstatement procedures did little to increase S2 fear. This finding supports one of the present hypotheses and is in agreement with findings obtained from Holmes et al. (2014), who also found that S2-alone presentations reduce second-order fear, and subsequent presentations of S1-alone or S2-S1 fail to produce a reinstatement effect.

It is important that the current results be interpreted in light of the reinstatement and second-order conditioning research highlighted above. According to the explanation of reinstatement provided by Rescorla and Heth (1975), fear would be expected to increase for subjects presented with the S1 alone. Their theory explains reinstatement according to associations underlying first-order conditioning, with US presentation following extinction

functioning to inflate and restore memory of the S1-US association. This can be extended to suggest that presentation of S1, the US correspondent in S2-S1 pairings, should result in restoration of the S2-S1 association created across second-order conditioning trials. This inflated representation of the S2-S1 association should increase CS fear, and therefore reinstate responding; however, this did not occur.

The research of Halladay et al. (2012) would also predict reinstatement of fear for Group S1-alone. Conditional reinstatement refers to increased fear at test following presentation of an unextinguished CS. Since responding to S1 was never extinguished, its presentation after extinction would be expected to conditionally reinstate responding. This should be especially true according to the type of association formed during second-order conditioning described by Rescorla (1982). He suggested that the relationship between S2-S1 is stimulus-response (S-R) by nature in that the physical properties of S2 associate with the emotional response elicited by S1. In explanation of their results, Halladay et al. (2012) suggests that presentation of an unextinguished CS produces a fear reaction. These two theories combined suggest that the fear produced through presentation of an unextinguished CS should return subjects to the S-R conditions present during second-order conditioning, thus allowing for recall of the S2-S1 association and reinstatement of S2 fear (e.g., Bouton, 1991; 1993). Additionally, Helmsetter and Fanselow (1989) reported that presentation of an excitatory CS within a neutral context results in fear of that context. This contextual fear along with the small amount of fear remaining towards S2 at test should have combined to produce reinstatement, according to associative explanations of reinstatement (e.g., Bouton and Bolles, 1979; Callen et al., 1984).

The lack of reinstatement seen in Group S1-alone might seem inconsistent with much of the research presented previously in this paper. However, this can be partially explained by the

method utilized in this study, specifically the second-order conditioning procedures. Paired presentation of S2-S1 during second-order conditioning trials serves two purposes. First, it serves to strengthen the relationship between S2-S1, while simultaneously weakening the association created during first-order conditioning between S1 and the US. Therefore, it can be said that each S2-S1 presentation also acts as an S1-alone extinction trial. So while responding towards S1 was never explicitly extinguished, it can be assumed that fear of S1 diminished across second-order conditioning trials. This might explain the absence of contextual fear and conditional reinstatement anticipated for Group S1-alone.

The reinstatement of fear observed in Groups US-alone and S1-US seems most consistent with associative accounts of reinstatement (e.g., Bouton, 1984; Bouton and Bolles, 1979; Callen et al., 1984). According to this theory, presentation of the US following extinction creates contextual conditioning (i.e., fear of the context where the US was administered). Fear of the context is then thought to summate with the small amount of fear remaining towards the CS (S2) to produce reinstatement. While manipulations of context were not used in the present study, the differences observed between Group S2-S1 and Group US-alone can be assumed to represent the amount of fear conditioned to context; as presentation of the stimulus compound (S2-S1) was not expected to produce contextual conditioning, whereas presentation of the US-alone reliably produces contextual fear. As suggested by Hendry (1982) and Callen et al. (1984), any contextual fear after reinstatement with a US-alone may summate with residual stimulus fear, thus providing a foundation for S2 fear to be present here during testing. The absence of reinstatement seen in Group S2-S1-could be explained by the lack of contextual conditioning obtained during reinstatement treatment. The reinstatement effect observed in Group S1-US is consistent with proposals by Callen et al. (1984) that reconditioning plays a critical role in the

reinstatement phenomenon. For subjects in group S1-US, first order fear was presumably increased with the US pairings to that stimulus, thus providing an opportunity for the S2 stimulus in testing to evoke a stronger fear response through its association with a stronger S1 fear response.

Interpretation of the results was challenged somewhat by a significant interaction of groups and trials with the stimulus used as S1 and S2. This finding was unexpected and required that separate analyses be conducted for each stimulus. These analyses produced generally similar results, although the reinstatement effect appeared to emerge somewhat later in testing for the light as the S2 than for the tone (see Table 2). These findings are consistent with previous research showing differences in salience of Light and Tone CSs (e.g., Rodriguez, Alonso and Hall, 2012). For this study, one group of subjects received separate non-reinforced presentations of Light and Tone prior to conditioning. The remaining subjects were presented no stimulus during this portion of the experiment. It has been shown that non-reinforced presentation of a stimulus prior to conditioning diminishes its effectiveness as an excitatory CS, a process known as latent inhibition. All subjects then received compound conditioning, in which Light and Tone were presented simultaneously and immediately followed by shock. A difference in response was noticed between groups. The group receiving pre-exposure prior to conditioning demonstrated less suppression (i.e., lower level of fear) for Light compared to Tone; whereas those not exposed to the stimuli prior to conditioning demonstrated less suppression to Tone compared to Light. In effect the results were reversed according to pre-exposure treatment. The stronger fear produced towards Light suggests a qualitative difference between the two stimuli currently under investigation, with Light appearing to be more salient. Also, greater inhibition of subsequent conditioning with pre-exposure to Light can be taken to indicate greater salience.

The difference in salience identified in the previous study do not appear to be present in the current study. Recall, results from the analysis conducted for Group NE-NR revealed no significant difference in freezing when comparing stimuli. This finding indicates the effect of stimulus salience did not significantly impact overall results, as conditioning was equally effective regardless of the stimulus used.

The findings of the current study are in stark contrast to the results reported by Holmes et al. (2014), which did not demonstrate successful reinstatement following post-extinction US presentation. Habituation, conditioning and extinction procedures in the current study were similar to the procedures used by Holmes et al. (2014). However, there were differences in the number and types of reinstatement treatments administered across both studies. The current study included additional groups that Holmes did not include to test for reinstatement effects. These groups received presentation of the first- and second-order stimulus compounds (i.e., S2-S1; S1-US). These groups were intended to provide a more sensitive measure of reinstatement as presentation of these pairings should help reestablish the associations formed across first- and second-order conditioning, thus increasing fear. Another difference from the present study was that Holmes et al. (2014) calculated a difference score between CS (S2) and baseline (pre-CS) freezing as the dependent measure of fear. Those results showed increased pre-CS levels of freezing (i.e., more contextual fear) for subjects presented the US after extinction. This increased pre-CS level of freezing lowered the difference between scores, therefore, diminishing the effects observed (e.g., higher freezing) during S2 presentation. Only freezing during S2 presentation in the current study was used as a dependent measure of remaining S2 fear in an attempt to circumvent this issue.

Classical conditioning principles have been used to explain the development, maintenance and treatment of fear and anxiety; therefore, conceptualizing anxiety disorders in classical conditioning terms should prove useful in understanding results and their application outside the laboratory setting (e.g., Hermans et al., 2006; Watson & Raynor, 1920; Pavlov, 1927). Posttraumatic stress disorder (PTSD) is an anxiety disorder, characterized by the re-experiencing of a past traumatic event (American Psychiatric Association, 2013). The original traumatic event represents the US in conditioning paradigms; while any stimulus present prior to trauma can be thought of as S1. Future pairings of these trauma-related cues (S1) with other neutral stimuli (S2) elicits fear towards that previously neutral stimulus, similar to the second-order conditioning procedures utilized and described throughout this study. A study by Wessa and Flor (2007) administered first- and second-order conditioning to subjects diagnosed with PTSD and healthy controls. Results showed higher S2 fear and slower S1-US extinction for subjects with PTSD compared to healthy controls. Researchers concluded that increased second-order fear helped to maintain fear of the original trauma in PTSD subjects; therefore, they suggest clinicians start with extinction of higher order fear when treating this population. The results of the current study only add to the difficulty in treatment of clients with PTSD, as extinction of S2 fear can be reinstated through experience of additional trauma.

Although the general pattern of reinstatement effects were the same, the interaction effects with S1/S2 stimulus modality can be considered a limitation of the current study. In the future, use of a fixed stimulus as S1 and S2 might help simplify results. A second option would be to use stimuli from the same sense modality (i.e., auditory, visual) to help alleviate these effects. Another limitation of the current study was the lack of contextual control, as conditioning, extinction and US re-exposure all occurred within the same context. This lack of

contextual manipulation requires that the role of context be inferred as it relates to second-order reinstatement. Future research should utilize manipulations of context to help highlight its effect in reinstating second-order fear. Exposure to the context following reinstatement procedures might prevent reinstatement, through elimination of contextual fear, much like the results reported by Bouton and Bolles (1979). Also, it would be beneficial for research to be conducted regarding whether other principles applying to first-order conditioning (i.e., rapid reacquisition, spontaneous recovery, renewal) might also apply to second-order stimuli.

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Table 1
Design of Experiment

Group	First Order	Second Order	S2 Extinction	Reinstatement	Test
S1-US	S1-US	S2-S1	S2-	S1-US	S2-
S2-S1	S1-US	S2-S1	S2-	S2-S1	S2-
S1-alone	S1-US	S2-S1	S2-	S1-	S2-
US-alone	S1-US	S2-S1	S2-	US-	S2-
NE-NR	S1-US	S2-S1	NE	NR	S2-

Table 2
LSD Post-hoc multiple comparisons.

	Subjects tested with Light as S2		
Trial 2	Group	Group	Sig.
	S2-S1	US-alone	0.014
	S2-S1	NE-NR	0.007
	US-alone	S2-S1	0.014
	NE-NR	S2-S1	0.007
Trial 6			
	S2-S1	US-alone	0.014
	S2-S1	NE-NR	0.006
	S1-alone	US-alone	0.014
	S1-alone	NE-NR	0.006
	US-alone	S2-S1	0.014
	US-alone	S1-alone	0.014
	NE-NR	S2-S1	0.006
	NE-NR	S1-alone	0.006
Trial 7			
	S1-US	S2-S1	0.007
	S1-US	S1-alone	0.028
	S1-US	NE-NR	0.028
	S2-S1	S1-US	0.007
	S2-S1	US-alone	0.004
	S1-alone	S1-US	0.028
	S1-alone	US-alone	0.015
	US-alone	S2-S1	0.004
	US-alone	S1-alone	0.015
	US-alone	NE-NR	0.015
	NE-NR	S1-US	0.028
	NE-NR	US-alone	0.015
	Subjects tested with Tone as S2		
Trials 1-8	Group	Group	Sig.
	S1-US	NE-NR	0.064
	S2-S1	NE-NR	0.007
	S1-alone	US-alone	0.045
	S1-alone	NE-NR	0.002
	US-alone	S1-alone	0.045
	NE-NR	S1-US	0.064
	NE-NR	S2-S1	0.007
	NE-NR	S1-alone	0.002

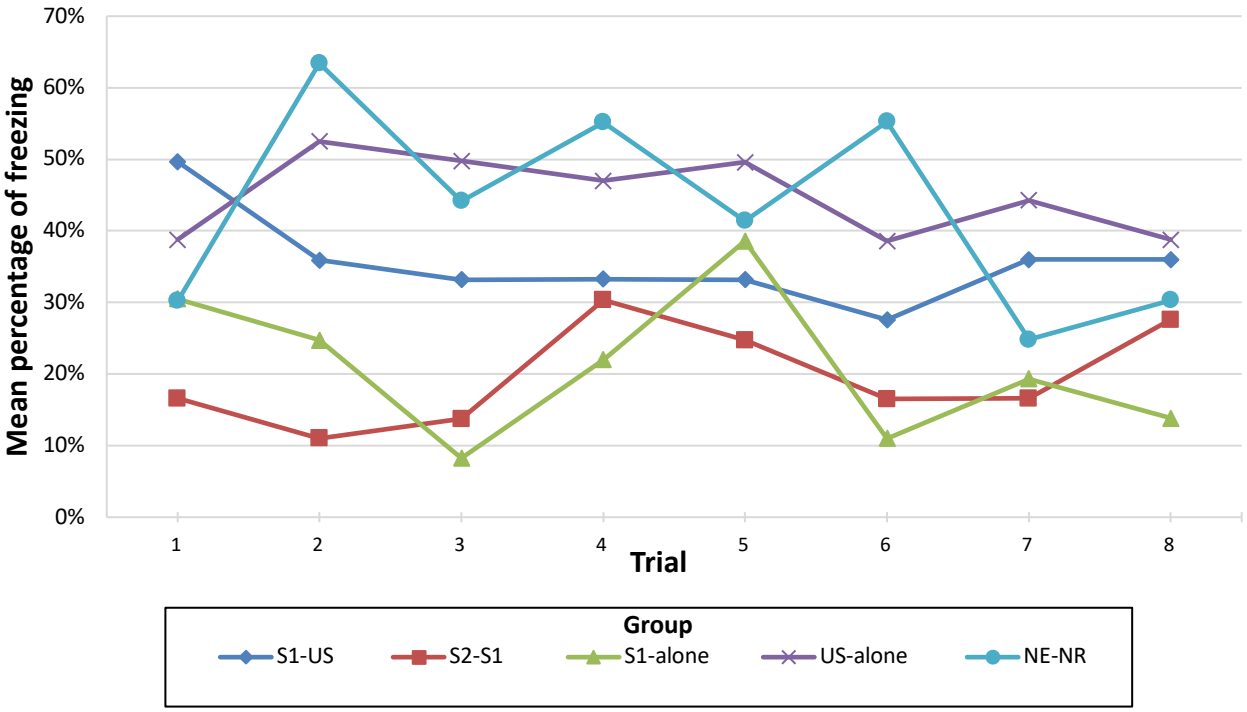

Figure 1. Mean percentage of freezing across Trials 1-8 for all subjects with Tone and Light stimuli counterbalanced as S1 and S2.

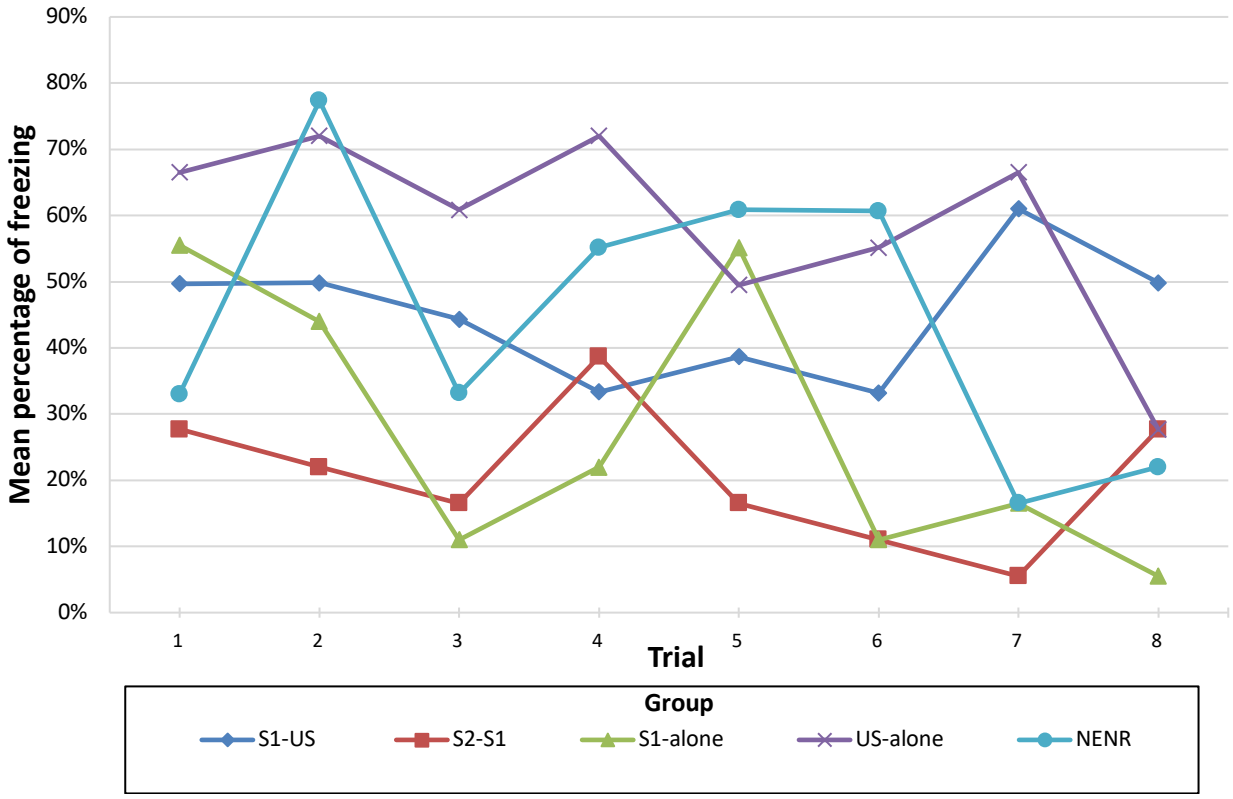

Figure 2. Mean percentage of freezing across Trials 1-8 for subjects receiving Tone as S1 and Light as S2.

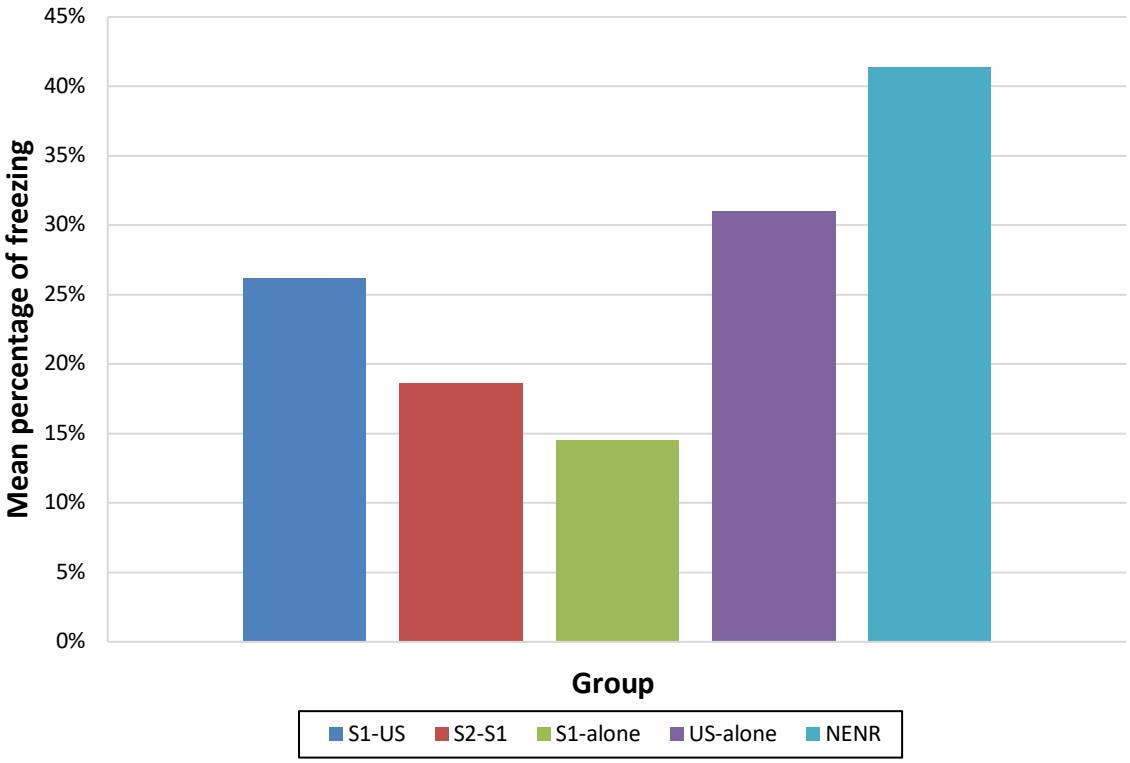

Figure 3. Mean percentage of freezing when combining all eight Trials for subjects conditioned with Light as S1 and Tone as S2.