



Fear extinction in humans: Effects of acquisition–extinction delay and masked stimulus presentations

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ABSTRACT

Fear extinction can be viewed as an inhibitory learning process. This is supported by post-extinction phenomena demonstrating the return of fear, such as reinstatement. Recent work has questioned this account, claiming that extinction initiated immediately after fear acquisition can abolish the return of fear. In the current study, participants were fear conditioned to four different conditioned stimuli (CS) and underwent extinction either immediately or after a 24 h delay. During extinction, we manipulated CS contingency awareness by presenting two of the CSs (one CS+, one CS–) under non-masked conditions and the other two CSs under masked conditions. Compared to delayed extinction, immediate extinction of non-masked CSs promoted less extinction of fear-potentiated startle and shock expectancy ratings and less reinstatement of fear-potentiated startle without affecting shock expectancy ratings. Critically, future research should clarify how the differences between immediate and delayed extinction in within-session extinction modulate the recovery of fear.

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1. Introduction

Conditioned fear reflects learning to predict danger, and it is regarded as one of the primary behavioral mechanisms underlying fear-related anxiety disorders (Mineka and Zinbarg, 2006). The principles governing how fears are acquired, stored and suppressed originate from experimental work using Pavlovian conditioning paradigms in which initially neutral stimuli (conditioned stimuli; CSs) acquire behavioral relevance through repeated presentations in a predictive relationship to aversive events (unconditioned stimuli; USs) such as electric shocks (Davis, 1992). The underlying neuroanatomical circuitry has been described in some detail (Davis, 1992; LeDoux, 2000). Briefly, it centers on the amygdala, which houses the basic machinery for forming an association between CSs and USs, and which projects to subcortical structures including the periaqueductal gray (PAG) and other motor control systems in the brainstem commanding overt manifestations of fear. Studies using fear conditioning protocols in human subjects have replicated many of the basic findings in other animals and there is good evidence for common underlying fear circuits across species (Delgado et al., 2006).

Fear extinction is defined as the process whereby the behavioral expression of a previously acquired fear memory is weakened

through repeated presentations of a CS in the absence of its associated US. This topic is currently the target of considerable interest because it promises to reveal the mechanisms of action for effective exposure-based treatments of anxiety disorders (Barlow, 2002). The contemporary view of fear extinction is that it represents an inhibitory learning process involving learning of a new association (CS–no US) that competes with the originally learned CS–US association, as opposed to a process of simple erasure of the original memory trace (e.g., Bouton, 1993). This inhibitory-learning theory of extinction is supported mainly by three post-extinction phenomena in which conditioned responses (CR) return: *spontaneous recovery*, which develops with the passage of time (Rescorla, 2004), *reinstatement* following exposure to the US (Bouton and Bolles, 1979b; Rescorla and Heth, 1975; Westbrook et al., 2002), and *renewal* by change of context between extinction and test (Bouton and Bolles, 1979a; Bouton and King, 1983).

Recent work (Myers et al., 2006) has revived interest in the idea of erasure mechanisms by suggesting that different mechanisms mediate extinction depending on the temporal delay between fear acquisition and extinction. Thus, erasure mechanisms might preferentially be invoked when extinction training is initiated shortly after fear acquisition, whereas inhibitory learning accounts for the mediation of extinction once the fear memory has been consolidated (Myers and Davis, 2007). In a series of studies in rodents, Myers et al. (2006) reported that extinction conducted shortly (10 min) after fear acquisition resulted in resistance to reinstatement, renewal, and spontaneous recovery, as measured by the

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fear-potentiated startle reflex, but that these manifestations of the return of fear were present when extinction training was initiated 72 h after acquisition. The apparent difference between immediate and delayed extinction can be understood in the context of consolidation theory, which suggests that memory is more liable to disruption within an hour of encoding (McGaugh, 2000; Schafe et al., 2001).

Initial findings suggesting consolidation-dependent modulation of the behavioral and psychophysiological manifestations of the return of fear have received mixed support. Partial support for a modulatory role of timing comes from other rodent studies using freezing as primary measure (Maren and Chang, 2006) (but see Archbold et al., 2010). Interestingly, Maren and Chang (2006) showed that within-session decrease in CR in rats receiving a standard extinction procedure did not differ from those in a control condition involving the same context as the standard extinction groups, but in which freezing was assessed during sham trials in the absence of CSs. Thus, it was doubtful whether the suppression of CRs in extinguished rats could be attributed to extinction learning per se. In a series of follow-up studies, Chang and Maren (2009) argued that immediate as opposed to delayed extinction yielded a short-lived and context-independent suppression of conditioned freezing, suggesting that immediate extinction might be mediated by habituation mechanisms rather than to rely on learning of a CS–no US contingency during extinction.

In humans, previous studies have reported significant return of fear after immediate extinction. For instance, there are reports of reinstatement following immediate extinction, evidenced by a stronger return of CR to the CS that was previously paired with the US (CS+) than to a control stimulus (CS–), using verbal measures (Hermans et al., 2005) and indirect behavioral (Dirikx et al., 2004), and psychophysiological indices of fear such as reinstatement of skin conductance responses (LaBar and Phelps, 2005; Schiller et al., 2008) and renewal of fear-potentiated startle (Alvarez et al., 2007). However, none of these studies allow inferences regarding quantitative differences in the degree of fear recovery due to the lack of a comparison group receiving delayed extinction training. Such quantitative differences between immediate and delayed extinction may be of both theoretical and clinical interest as they may help to unravel mechanisms affecting the rate of extinction and the return of conditioned fears.

Previous studies explicitly manipulating acquisition-to-extinction timing in humans have however yielded contradictory results. One study, measuring differential skin conductance responses, reported that delayed compared to immediate extinction attenuated conditioned fear renewal and spontaneous recovery (Huff et al., 2009). Another study measuring fear-potentiated startle, however, reported larger spontaneous recovery in the delayed extinction group compared to the immediate group in a differential conditioning paradigm but not in a single-cue paradigm (Norrholm et al., 2008). Interpretation of the latter results are complicated by the fact that the original rodent studies reporting recovery effects that were specific to the delayed extinction procedure were based on a single-cue conditioning paradigm (Myers et al., 2006).

In summary, although it has previously been shown that CR can recover after immediate extinction in humans (i.e., Alvarez et al., 2007; Dirikx et al., 2004; Hermans et al., 2005; LaBar and Phelps, 2005; Schiller et al., 2008), there is mixed support for whether there are quantitative differences in fear recovery between immediate and delayed extinction (Norrholm et al., 2008; Huff et al., 2009). Also, it remains unclear whether the effects of immediate extinction are mediated by habituation-like processes rather than to rely on learning of a CS–no US association (Chang and Maren, 2009).

The main objective of the current study was to directly assess the effect of varying the acquisition-to-extinction-interval on

reinstatement of fear-potentiated startle. More specifically, we hypothesized that immediate compared to delayed extinction would result in less reinstatement of fear-potentiated startle. Also, to explore whether varying the acquisition-to-extinction interval would have an effect on reinstatement in the absence of CS–US contingency learning during extinction, we manipulated explicit CS–US contingency learning by including masked CS trials. Backward masking is a procedure in which a brief presentation of a target picture is followed by a masking picture, resulting in participants reporting that they only see the masking picture but not the preceding target (Enns and Di Lollo, 2000; Wiens and Öhman, 2007). Previous research has shown that conditioned fear to fear-relevant stimuli can survive masking (Morris et al., 1998; Öhman and Soares, 1993), implying that, under some circumstances (i.e., when stimuli are fear-relevant), explicit awareness of the CS–US contingencies is not necessary for the expression of conditioned fear (Esteves et al., 1994). Thus, we reasoned that a differential decrease in CR during masked extinction conditions could be attributed to habituation of the CR rather than to explicit CS–no US contingency learning.

To address these issues, participants were randomly assigned to an immediate or a delayed extinction group and fear conditioned to four different fear-relevant CSs (two CS+ and two CS–). During extinction, we manipulated contingency awareness within-subjects by repeatedly presenting one CS+ and one CS– under non-masked conditions that allowed for explicit CS–US contingency learning while the other two CSs (one CS+ and one CS–) were presented under masking conditions that precluded CS–US contingency learning during extinction. Immediately after extinction training, all participants received three unsignaled US presentations followed by a reinstatement test. Based on previous results (Myers et al., 2006; Norrholm et al., 2008), we hypothesized that immediate extinction following non-masked CSs would elicit less reinstatement of fear-potentiated startle than delayed extinction without altering reinstatement of shock expectancy ratings. Moreover, we predicted that in the absence of CS–US contingency learning during extinction, reinstatement would be unaffected by the acquisition-to-extinction interval, i.e., we expected that both the immediate and the delayed group would show significant reinstatement of fear-potentiated startle and shock expectancy ratings to the previously masked CSs.

2. Materials and methods

2.1. Participants

Thirty-three students at Karolinska Institutet participated in the study after signing an informed consent form (approved by the ethics committees at Karolinska Institutet) and donating saliva sample for DNA extraction and genotyping (data not reported). All participants were screened for lifetime psychiatric disease and medication. Four participants were excluded from the final analysis due to technical problems, and two participants were excluded because of voluntary interruption, leaving a final sample of 27 (8 men) healthy participants with a mean age of 24.9 years ($SD = 5.3$). Participants were randomly assigned to two groups; immediate extinction ($N = 13$) or delayed extinction ($N = 14$). All participants were given two cinema vouchers for their participation.

2.2. Stimulus material

Four different pictures depicting fearful male faces from the Karolinska Directed Emotional Faces (Lundqvist et al., 1998) served as CSs (model nr: AM14AFS, AM23AFS, AM34AFS, AM35AFS) and two additional neutral faces served as masks (model nr: AM04NES, AM29NES). For each picture, the background was removed and color was converted to grey-scale. A white fixation cross was shown on a black background during the inter-trial intervals (ITI), the duration of which varied between 12 s and 15 s throughout all experimental sessions (acquisition, extinction and reinstatement test). The experiment was run in a sound-attenuated chamber on a desktop PC with a standard 21-in. cathode ray tube (CRT) monitor. Screen resolution was 800×600 pixels and the refresh rate was set to 60 Hz. The experiment was programmed in Presentation 13.1 (Neurobehavioral Systems, www.neurobs.com). Participants viewed pictures at a distance of about 1 m. The US

was a monopolar 100 ms DC-pulse electric stimulation (STM200; Biopac Systems Inc., www.biopac.com) applied to the participant's right wrist. Startle probes were 50 ms bursts of 95-dB[A] white noise with a near instantaneous rise time (<1 ms) based on previous protocols (Weike et al., 2007). Startle probes were presented binaurally through headphones (Sennheiser HD202) four or five seconds after stimulus (CS+, CS–, ITI) onset throughout all experimental sessions.

2.3. Measurements and recordings

2.3.1. Startle

The eyeblink component of the startle response was measured through electromyographic (EMG) recordings of the left orbicularis oculi muscle using two miniature Ag/AgCl electrodes filled with electrolyte gel. A third ground electrode was placed behind the left ear over the mastoid. The raw EMG signal was amplified and filtered through a 28–500 Hz bandpass, and rectified and integrated with a time constant of 20 ms.

2.3.2. Behavioral ratings

To collect trial-by-trial ratings of US expectancy during each presentation of a CS, subjects indicated their response on a visual analog scale (TSD115, Biopac Systems Inc., www.biopac.com). Subjects were instructed to move a lever to the right side of the scale if they expected the CS to be followed by a shock (max = 100% certain of receiving a shock) or to the left side if they did not expect the CS to be followed by a shock (max = 100% certain of not receiving a shock), and remain in the middle when they did not know what to expect. Subjects were instructed to look at the monitor throughout the experiment. Given that subjects were not informed about the masking manipulation, during extinction, participants rated their shock expectancy to the CS that they were aware of being exposed to (i.e., the neutral mask). This was confirmed by a post-experimental interview (data not shown).

2.4. Procedure

2.4.1. Experimental groups

Prior to starting the experiment, participants were randomly assigned to one of two experimental groups. For the immediate extinction group, the extinction session immediately followed the conditioning session without any delay. For the delayed extinction group, the acquisition and extinction sessions were separated by approximately 24 h to allow for consolidation of the acquired fear memory. At the beginning of the extinction session, participants in the delayed group were told that the experiment would be resumed and that all settings would be identical to the previous day. No other instructions were given.

2.4.2. Fear acquisition

For both groups, the experimental procedure on day 1 included individual adjustment of the US to a level described by the subject as “highly unpleasant but not painful”, presentation of three startle habituation trials and a preconditioning phase during which two non-reinforced presentations of each CS and a total of five startle probes were presented. To simplify the discrimination between the different CSs, participants were instructed that the four faces could be categorized as belonging to two pairs; one pair would always be presented with a blue background and the other pair with a yellow background. Furthermore, they were instructed that only one face of each pair (i.e., one face with blue background and one with yellow background) could be coupled to a shock. During the conditioning phase, each picture was presented for six seconds. Two of the faces (one CS from each pair), designated CS+, co-terminated with the presentation of the US (nine reinforced presentations/CS), while the other two faces, designated CS–, were never coupled to the US (nine non-reinforced presentations/CS). The coupling between face and US within a CS pair as well as the pairing of a CS pair and background color was counterbalanced between participants. Acoustic startle probes were presented on six presentations of each CS and during six ITIs based on previously published protocols (Weike et al., 2007).

2.4.3. Extinction and reinstatement testing

During extinction, all participants were presented with twelve non-reinforced presentations of each of the four CSs. For each participant, one previously reinforced CS+ and one CS– was immediately masked by a neutral face (33 ms CS presentation immediately followed by a six second presentation of a neutral mask) while the other two CSs (one previously reinforced CS+ and one CS–) were presented non-masked under the reversed timing parameters (33 ms neutral mask immediately followed by a six second CS presentation). Hence the overall exposure time to the CSs during extinction training differed between the masked and the non-masked CS presentations. However, the critical effect of backward masking, in contrast to merely very short stimulus presentations, is that it allows controlled exposure to a short stimulus but precludes its access to conscious processing, thus preventing association of the CS with US omission (Enns and Di Lollo, 2000). Masking parameters were selected based on previous research (Esteves and Öhman, 1993) and the combination of mask and CS pair was preselected based on pilot testing that assured efficient masking of the CS (data not shown). The masked CS pair presented during extinction was counterbalanced across participants so that masking of a particular CS pair differed between participants. For each participant, the background color of the CS pair was held constant throughout the experiment and the background color

of the mask varied according to the background color of the masked CS pair. During extinction, startle probes were presented on eight of the twelve presentations of each CS and during eight ITIs. At the end of the extinction session, all participants received three unsignaled presentations of the US followed by four non-reinforced and non-masked six-second presentations of each of the four CSs. During reinstatement testing, startle probes were presented on three of the four presentations of each CS and during three ITIs.

2.4.4. Forced choice recognition task

To verify that the parameters used during the extinction session efficiently masked the CSs, at the end of the experiment, both groups completed a forced choice recognition task involving presentations of the previously masked CS pair. Participants were informed that they would be shown brief presentations of the faces previously seen during the experiment, which would be immediately followed by the neutral face. Their task was to indicate which face the first picture depicted. After each face-mask presentation, the two target faces were shown on opposite sides of the screen and participants had to indicate with a keyboard press (1 = face on the left side of screen; 2 = face on the right side screen) which face was presented. There was no time limit for responding and participants were told to respond even if they felt that they were guessing. For each participant, trial order of the CS was randomized and the location of the target faces on the screen (left, right) was balanced against the correct button press (1, 2). The task consisted of 30 trials (10 trials/CS and 10 blank trials in which the mask served as target) shown under the same masking parameters as during the previous extinction session except that the duration of the mask was shortened from six to three seconds.

2.5. Data reduction and response definition

Startle eyeblink magnitude (microvolts) was measured as the amplitude from onset to peak and T-standardized, resulting in a distribution with an overall mean of 50 and a standard deviation of 10 for each participant.¹ To avoid confounding effects of Group (immediate, delayed), the raw startle magnitude during acquisition was normalized separately from extinction and reinstatement testing. Behavioral ratings of shock expectancy were scaled from –10 (100% certain of not receiving a shock) to +10 (100% certain of receiving a shock) and scored as the mean shock expectancy during the presentation of each CS. Note that scoring expectancy ratings at CS+ immediately before the US and at the corresponding point of the CS– did not alter the reported results.

2.6. Data analysis

Data were analyzed with SPSS 17.0 for Windows. Mean startle difference scores were calculated as [mean startle magnitude to startle probe in the presence of the CS] – [mean startle magnitude to startle probe during ITI for each session] as has been described previously in similar work (Norrholm et al., 2008). To decrease the influence of trial-by-trial variability, startle data were analyzed in blocks (Norrholm et al., 2006, 2008; Weike et al., 2007) and each startle block consisted of two startle probes throughout all experimental sessions (acquisition, extinction, reinstatement test). Each session of the experiment was analyzed separately with repeated-measures analysis of variance (ANOVA). We adopted a significance level of .05, only reporting significant effects not qualified by a significant higher-order interaction (see Supplementary data for all significant ANOVA effects). We report partial η^2 as the estimate of effect size and Greenhouse–Geisser adjustments of degrees of freedom were used when appropriate. Given our a priori hypothesis regarding the differences between immediate and delayed extinction following non-masked extinction specifically, reinstatement data were analyzed separately for the non-masked and masked CSs (see Supplementary data for exploratory full factorial analysis, i.e., Stimulus \times Mask \times Block \times Group of the reinstatement data).

3. Results

To confirm that the obtained results were not confounded by differences in baseline startle, we first ran one-way ANOVAs with raw and T-standardized ITI startle scores for each session separately (conditioning, extinction and reinstatement testing) with Group as a between subject variable. None of these analyses yielded any significant differences ($p > .05$ for all sessions) between the immediate and delayed extinction groups in baseline startle (see Table S1 for means and standard deviations for each session).

¹ Although we present results based on normalized T-scores, analysis using the raw startle scores yielded essentially the same results, as has been reported previously (Grillon and Ameli, 2001).

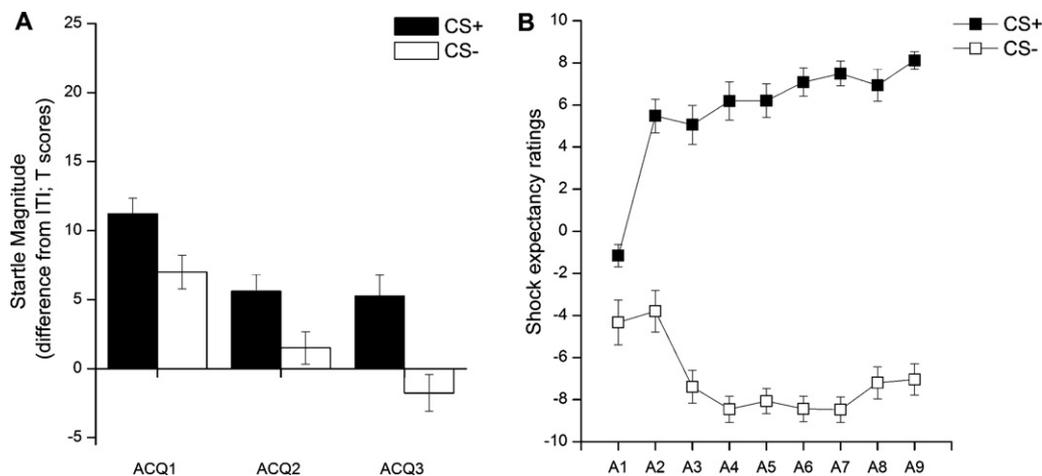


Fig. 1. Fear acquisition. (A) Magnitude of the fear-potentiated startle response elicited during the presentation of the conditioned stimuli (CS+; CS-) expressed as differences scores to the startle response elicited during the inter-trial interval (ITI). ACQ refers to the separate acquisition blocks. (B) Trial-by-trial shock expectancy ratings to the conditioned stimuli (CS+; CS-) during fear acquisition. Note that the CS+ and CS- scores represent data collapsed across the two separate CS+s and CS-s. Error bars represent standard errors.

3.1. Fear conditioning

3.1.1. Startle

To assess fear acquisition, we measured startle differentiation between the two CS+s and the two CS-s (differential conditioning) by collapsing data across stimulus type in a repeated-measures analysis of variance (ANOVA) with Stimulus (CS+, CS-) and Block (Block1, Block2, Block3) as within-subjects variables and Group (immediate, delayed) as a between-subject variable. This analysis revealed robust startle CS+/CS- differentiation as indicated by a significant main effect of Stimulus on startle blink responses during fear conditioning, $F(1,25)=20.04$, $p<.001$, $\eta^2=.45$, and a significant main effect of Block $F(2,50)=32.09$, $p<.001$, $\eta^2=.56$, Greenhouse-Geisser $\epsilon=.98$ (Fig. 1A). There were no significant differences between the immediate and delayed extinction groups during fear acquisition (largest $F=2.20$).

3.1.2. Ratings

A 2 (Stimulus) \times 9 (Trial) \times 2 (Group) repeated-measures ANOVA revealed increased CS+/CS- differentiation over time as supported by a significant Stimulus \times Trial interaction $F(8,200)=24.93$, $p<.001$, $\eta^2=.50$, Greenhouse-Geisser $\epsilon=.56$ (Fig. 1B). There were no significant effects involving Group (largest $F=1.27$).

3.2. Extinction and reinstatement testing

3.2.1. Startle

To examine whether there was a significant decrease in CR across extinction training, we ran a 2 (Stimulus) \times 2 (Mask) \times 4 (Block) \times 2 (Group) repeated-measures ANOVA. This analysis revealed overall better CS+/CS- differentiation during non-masked CSs in the immediate compared to the delayed extinction group. This was supported by a significant Stimulus \times Mask \times Group interaction, $F(1,25)=4.08$, $p=.05$, $\eta^2=0.14$ (see Fig. 2). To further disentangle the effect of Group on CS+/CS- differentiation, follow-up analysis confirmed overall better CS+/CS- differentiation in the immediate group compared to the delayed group during non-masked CSs (Stimulus \times Group interaction: $F(1,25)=8.40$, $p=.01$, $\eta^2=.25$), whereas there were no differences between the groups during masked CSs (largest $F=.52$). The latter analysis did however reveal a main effect of Stimulus $F(1,25)=19.76$, $p<.001$, $\eta^2=.44$, indicating overall higher startle responses to the CS+ compared to the CS- also for the masked CS pair.

Given our a priori hypothesis regarding the differences between immediate and delayed extinction following non-masked extinction specifically, we analyzed reinstatement data separately for the non-masked and masked CS pair.² First, for the non-masked CSs, we assessed end extinction performance and CS+/CS- differentiation during the reinstatement test. There was a significant difference between the immediate and delayed extinction groups in end extinction performance (Stimulus \times Group interaction: $F(1,25)=6.53$; $p=.02$; $\eta^2=.21$). Follow-up t -test revealed that in contrast to the delayed group, the immediate group maintained significant CS+/CS- differentiation at the last extinction block, immediate: $t(12)=2.96$; $p=.01$; delayed $t(13)=.53$, $p=.61$. This group difference in CS+/CS- differentiation at end extinction did not remain significant at the reinstatement test block (Stimulus \times Group: $F(1,25)=.15$) but there was a significant main effect of Stimulus ($F(1,25)=16.08$, $p<.001$, $\eta^2=.39$), indicating overall higher responses to the CS+ than to the CS- in both extinction groups.

In a second step, we analyzed reinstatement of fear-potentiated startle, evidenced as an increase in fear-potentiated startle from the last block of extinction training to the first block of reinstatement testing (Kindt et al., 2009; Norrholm et al., 2006, 2008; Soeter and Kindt, 2011) in a Stimulus (2) \times Block (2) \times Group (2) repeated-measures ANOVA. Importantly, this analysis allowed us to address whether a change in fear-potentiated startle response from end extinction to reinstatement testing was the result of an increase in CS+/CS- differentiation (as opposed to an absolute increase in CS+ response which is more susceptible to differences in baseline response levels) and whether this increase was driven by the CS+ response specifically (as compared to a stimulus independent increase that might reflect shock sensitization). As predicted, this analysis revealed that reinstatement of fear-potentiated startle differed between the immediate and delayed extinction groups, which was supported by a significant Stimulus \times Block \times Group interaction, $F(1,25)=6.23$, $p=.02$, $\eta^2=.20$. Only the delayed group displayed an increase in CS+/CS- differentiation from end

² Analyzing reinstatement data in a Stimulus (2) \times Mask (2) \times Block (2) \times Group (2) repeated measures ANOVA revealed two significant three-way interaction. There was a significant Stimulus \times Mask \times Block interaction ($F(1,25)=4.24$, $p=.05$, $\eta^2=.15$, Greenhouse-Geisser $\epsilon=1.00$) and a significant Stimulus \times Block \times Group interaction ($F(1,25)=4.97$, $p=.04$, $\eta^2=.17$, Greenhouse-Geisser $\epsilon=1.00$). See Supplementary data for further details.

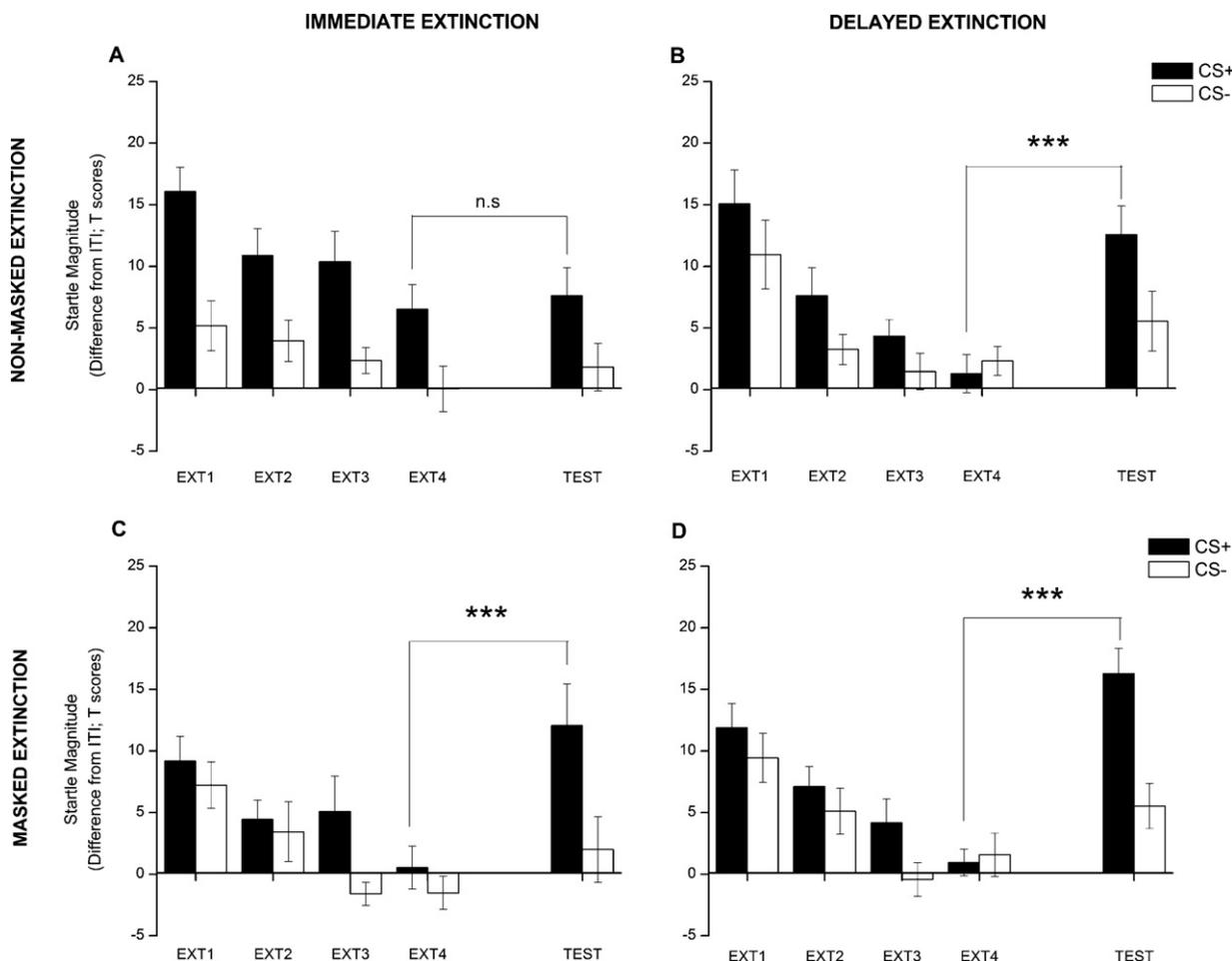


Fig. 2. Fear-potentiated startle during fear extinction and reinstatement testing. Magnitude of the fear-potentiated startle response elicited during the presentation of the conditioned stimuli (CS+; CS-) expressed as differences scores to the startle response elicited during the inter-trial interval (ITI) in the immediate (left side) and delayed (right side) extinction groups. Results are displayed for the non-masked (A and B) and masked CSs (C and D) separately. EXT refers to the separate extinction blocks and TEST refers to the first reinstatement test block. Error bars represent standard errors. Asterisks indicate significant differences, * $p < .05$, ** $p < .01$, *** $p < .001$; ns, non-significant.

extinction to reinstatement (Stimulus \times Block: $F(1,13) = 11.20$, $p = .01$, $\eta^2 = .48$) whereas this differential increase in response to the CS+ vs. the CS- from the last extinction block to the reinstatement test block was not significant in the immediate group ($F(1,12) = 1.74$, $p = .21$). Follow-up paired samples t -tests confirmed that there was a CS+ specific increase from extinction to reinstatement testing only in the delayed extinction group (delayed: CS+ $t(13) = 3.65$, $p = .01$, CS- $t(13) = 1.20$, $p = .25$; immediate: CS+ $t(12) = .42$, $p = .68$, CS- $t(12) = .71$, $p = .49$). Data for the non-masked CS pair are presented in Fig. 2A and B.

For the masked CS pair, there was no significant CS+/CS- differentiation (main effect of Stimulus: $F(1,25) = .32$, $p = .58$) or between-group difference at the last block of extinction training (largest $F = 1.10$). During the reinstatement test block, fear-potentiated startle responses were overall higher to the CS+ than to the CS- (main effect of Stimulus: $F(1,25) = 15.76$, $p = .001$; $\eta^2 = .39$) in the absence of any between-group differences (largest $F = .03$). Furthermore, both groups showed successful reinstatement, assessed as a differential increase in fear-potentiated startle to the CS+ vs. the CS- from the last extinction block to the first reinstatement block (Stimulus \times Trial: $F(1,25) = 18.20$, $p < .001$, $\eta^2 = .42$), again in the absence of a difference between the immediate and delayed extinction groups (largest $F = .55$). Planned follow-up paired samples t -tests confirmed that there was a CS+ specific increase from extinction to reinstatement testing in both extinction groups (immediate: CS+ $t(12) = 3.56$, $p = .01$, CS- $t(12) = 1.05$,

$p = .32$; delayed: CS+ $t(13) = 7.77$, $p < .001$, CS- $t(13) = 1.56$, $p = .14$). Data for the masked CS pair are presented in Fig. 2C and D.

Critically though, in contrast to extinction with masked CSs, the immediate and delayed extinction groups differed at the last block of extinction training with non-masked CSs. Thus, this difference in end extinction performance with non-masked CSs might confound the observed differences between the immediate and delayed extinction groups in reinstatement of fear-potentiated startle. Therefore, we ran an exploratory analysis separately for the non-masked CSs in which we only included participants who met a 50% extinction performance criterion (difference score to CS+ in Block1 vs. Block4 of extinction training) that has previously been adopted by Davis and colleagues (Norrholm et al., 2006, 2008) in similar work. Including extinguishers only (immediate group $N = 8$; delayed group $N = 13$), we first assessed performance at the last block of extinction training in a 2 (Stimulus) \times 2 (Group) analysis that neither revealed a main effect of Stimulus ($F(1,19) = .09$, $p = .77$), nor a significant Stimulus \times Group interaction ($F(1,19) = 1.95$, $p = .18$), confirming that CS+/CS- differentiation was absent at the end of extinction in both groups in this subsample of extinguishers. We then analyzed fear reinstatement data in this subsample of extinguishers (see Fig. 3). This exploratory analysis confirmed that the change in CS+/CS- differentiation from extinction to reinstatement testing was restricted to the group that received delayed extinction training. This was supported by a significant Stimulus \times Block \times Group interaction, $F(1,19) = 7.37$,

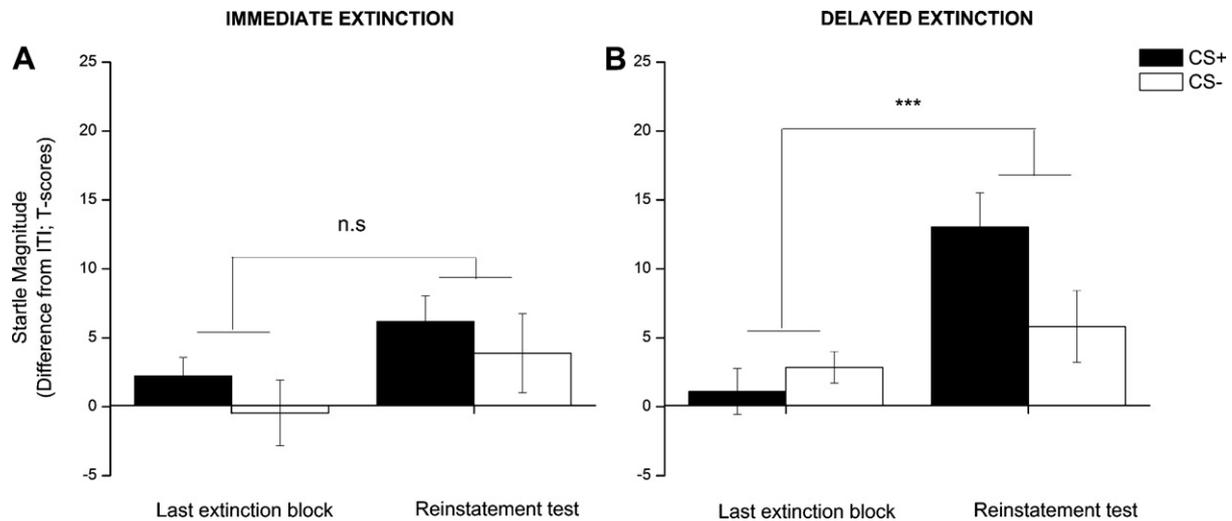


Fig. 3. Reinstatement of fear-potentiated startle following non-masked CSs in extinguishers. Fear-potentiated startle magnitudes elicited during the presentation of the conditioned stimuli (CS+; CS-) expressed as differences scores to the startle response elicited during the inter-trial interval (ITI) at the last block of extinction training and at the first reinstatement test block in the subsample of extinguishers. Results are displayed separately for (A) the immediate extinction group ($N=8$) and (B) the delayed extinction group ($N=13$). Error bars represent standard errors. Asterisks indicate significant differences, $***p < .001$, ns, non-significant.

$p = .01$, $\eta^2 = .28$. Thus, the Stimulus \times Block interaction was significant in the delayed ($F(1,12) = 35.66$, $p < .001$, $\eta^2 = .75$) but not in the immediate extinction group ($F(1,7) = .04$, $p = .85$). Interestingly, the change in CS+/CS- differentiation from the last block of extinction training to reinstatement test was remarkably small in the immediate group even after excluding the non-extinguishers (see Fig. 3).

3.2.2. Ratings

To examine whether there was a significant decrease in shock expectancy ratings during extinction training, we ran a 2 (Stimulus) \times 2 (Mask) \times 12 (Trial) \times 2 (Group) repeated-measures ANOVA that revealed better CS+/CS- discrimination in the immediate than in the delayed group during non-masked extinction trials, as supported by a significant Stimulus \times Mask \times Trial \times Group interaction, $F(11,275) = 2.55$, $p = .04$, $\eta^2 = .09$, Greenhouse-Geisser $\epsilon = .35$ (see Fig. 4). To further disentangle the effect of Group on shock expectancy ratings, follow-up analysis confirmed that shock expectancy ratings to the non-masked CSs were higher to the CS+ than to the CS- during immediate compared to delayed extinction (Stimulus \times Trial \times Group: $F(11,275) = 2.87$, $p = .03$, $\eta^2 = .10$, Greenhouse-Geisser $\epsilon = .36$), but there were no differences between the immediate and delayed groups in shock expectancy ratings to the masked CS pair (largest $F = 1.12$).

During the last trial of extinction training, shock expectancy ratings to the non-masked CS pair were overall higher to the CS+ than to the CS- (main effect of Stimulus: $F(1,25) = 4.69$, $p = .04$; $\eta^2 = .16$) in the absence of any between-group differences (largest $F = 2.33$). Likewise, shock expectancy ratings were overall higher to the CS+ than to the CS- during the first reinstatement test trial (main effect of Stimulus: $F(1,25) = 15.76$, $p = .001$; $\eta^2 = .39$), in the absence of between-group differences (largest $F = 2.11$). Furthermore, reinstatement of shock expectancy ratings, assessed as a differential increase in ratings to the CS+ vs. the CS- from the last extinction trial to the first reinstatement test trial, was successful (Stimulus \times Block: $F(1,25) = 9.52$, $p = .01$; $\eta^2 = .28$), again in the absence of a between-group difference (Stimulus \times Trial \times Group: $F(1,25) = .50$, $p = .49$). Planned follow-up paired samples t -tests confirmed that both groups displayed a CS+ specific increase in shock expectancy ratings from the last trial of extinction to the reinstatement test trial (immediate group: CS+ $t(12) = 3.77$, $p < .001$, CS- $t(12) = .94$, $p = .37$,

delayed group: CS+ $t(13) = 2.84$, $p = .01$, CS- $t(13) = 1.51$, $p = .15$). Data for the non-masked CS pair are presented in Fig. 4A and B.

For the masked CS pair, there were no differential shock expectancy ratings to the CS+ vs. the CS- (main effect of Stimulus: $F(1,25) = 1.59$, $p = .22$) or any between-group differences at the last trial of extinction training (largest $F = .52$), although there was a marginally significant difference between the extinction groups in shock expectancy ratings to the CS+ and the CS- at the first reinstatement test trial (Stimulus \times Group: $F(1,25) = 3.41$, $p = .08$; $\eta^2 = .12$). Thus, although both the immediate and delayed extinction groups showed higher shock expectancy ratings to the previously masked CS+ than to the previously masked CS- (immediate: $t(12) = 7.30$, $p < .001$; delayed: $t(13) = 2.43$, $p = .03$) this CS+/CS- discrimination was more pronounced in the immediate group. Furthermore, assessing reinstatement of shock expectancy ratings, defined as an differential increase in ratings to the CS+ vs. the CS- from end extinction to reinstatement testing, also revealed a marginally significant difference in reinstatement between the extinction groups (Stimulus \times Group: $F(1,25) = 3.60$, $p = .07$, $\eta^2 = .13$). Although both groups showed a significant increase in shock expectancy rating to the previously masked CS+ (immediate: $t(12) = 7.56$, $p < .001$; delayed: $t(13) = 5.53$, $p < .001$) the delayed group also showed a significant increase in shock expectancy ratings to the previously masked CS- (immediate: $t(12) = .77$, $p = .46$; delayed: $t(13) = 3.28$; $p = .01$). Data for the masked CS pair are presented in Fig. 4C and D.

3.3. Forced choice task

Results from the post-experimental forced choice recognition task involving masked CS presentations indicated that subjects did not perform significantly above chance-level. The observed proportion of incorrect responses was .59, which was not statistically different from what is expected by chance ($p = .44$; binomial test two-tailed).

4. Discussion

Our results provide partial support for the hypothesis that reinstatement of fear-potentiated startle varies with the timing of extinction training relative to acquisition. Thus, for the non-masked CS pair, there was a CS+ specific increase in fear-potentiated startle

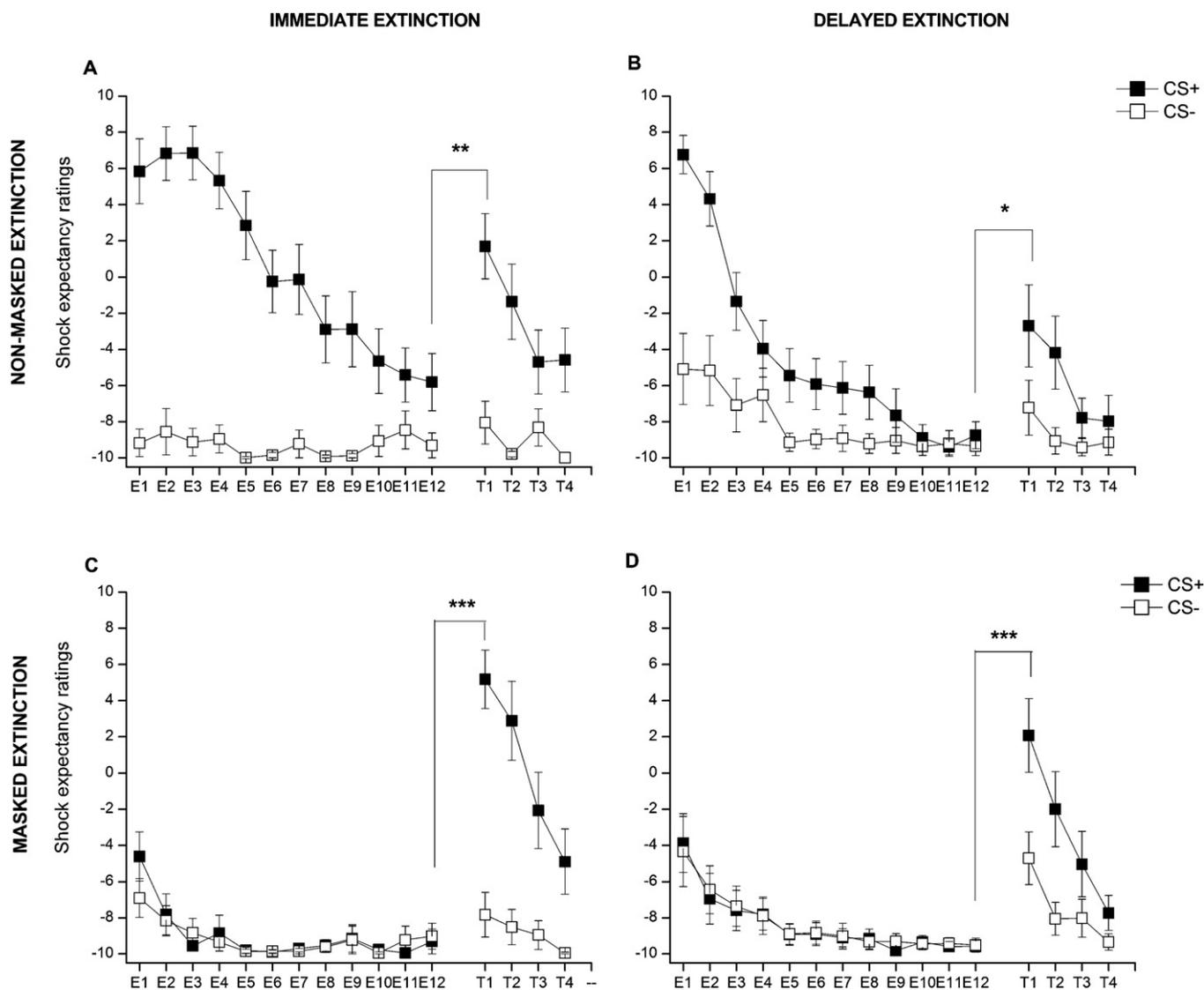


Fig. 4. Shock expectancy ratings during fear extinction and reinstatement testing. Trial-by-trial shock expectancy ratings to the conditioned stimuli (CS+; CS-) in the immediate (left side) and delayed (right side) extinction groups. Results are displayed for the non-masked (A and B) and masked CSs (C and D) separately. Error bars represent standard errors. Asterisks indicate significant differences, * $p < .05$, ** $p < .01$, *** $p < .001$.

following delayed extinction, which was not evident for the immediately extinguished CS+ (see Fig. 2A). Notably though, successful startle differentiation between the CS+ and CS- during the reinstatement test block did not differ between the immediate and delayed extinction groups. Thus, this pattern of data suggests that although reinstatement of fear-potentiated startle, defined as a significant increase in CS+/CS- differentiation from extinction to reinstatement testing, varied with the timing of extinction training relative to acquisition, this did not preclude that both extinction groups differentiated between the CS+ and CS- at test. In contrast, reinstatement of shock expectancy ratings occurred independently of the acquisition-to-extinction delay as both extinction groups showed a significant increase in shock expectancy ratings to the non-masked CS+ from end extinction to reinstatement testing.

Critically however, under non-masked conditions, immediate extinction training produced slower within-session decrement of both shock expectancy ratings and fear-potentiated startle than did delayed extinction. In fact, CS+/CS- differentiation, as measured by fear-potentiated startle, was significantly higher in the immediate than in the delayed extinction group at the last extinction block. This finding is consistent with work in both humans

(Norrholm et al., 2008) and rodents (Myers et al., 2006), which have reported less within-session extinction of fear-potentiated startle during immediate compared to delayed extinction. Nonetheless, this raises the possibility that incomplete extinction in the immediate group could have confounded the observed lack of reinstatement of fear-potentiated startle. To address this issue in our data, we selected a subset of participants, who could be defined as extinguishers, for an exploratory analysis using the same extinction performance criteria as reported previously (Norrholm et al., 2006, 2008). In this subset of extinguishers, there were no significant differences between the immediate and delayed extinction groups at the end of extinction training but the differences at reinstatement testing were still preserved, i.e., only the delayed extinction group showed a significant increase in CS+/CS- differentiation from extinction to reinstatement testing.

This exploratory analysis should however be interpreted with caution given the small sample size (8 vs. 13) and as such requires replication in a larger sample of participants. Also, preselecting participants based on performance criteria introduces the risk of equating differences that might reflect properties that are fundamental to the process of interest. For instance, the timing of fear

extinction relative to acquisition might not only be relevant for the recovery of fear, but might also be relevant for within-session extinction learning per se. In this context, it is noteworthy that such differences in within-session extinction between immediate and delayed extinction have been reported in several prior studies. For instance, [Norrholm et al. \(2008\)](#) reported that immediate extinction resulted in less terminal within-session extinction and less pronounced recovery of fear-potentiated startle than delayed extinction even after participants had been equalized based on extinction performance. Moreover, the original rodent studies reporting abolished reinstatement of fear-potentiated startle after immediate but not delayed extinction training ([Myers et al., 2006](#)) also reported poorer extinction in groups extinguished with a relatively short interval (10 min) than in groups extinguished with longer intervals between acquisition and extinction. In fact, none of the groups given extinction training after a 10 min interval showed complete extinction. Also, three recent reports measuring freezing in rats ([Archbold et al., 2010](#); [Chang and Maren, 2009](#); [Kim et al., 2010](#)) reported significantly less within-session extinction in the immediate extinction groups compared to the delayed extinction groups. Interestingly, extinction strength acquired during initial training did not moderate the effects of fear recovery ([Archbold et al., 2010](#)). Data from our lab (under review) using fear-relevant stimuli as CSs, showed that doubling the number of immediate extinction trials (from 12 trials to 24 trials) still did not promote complete within-session extinction of fear-potentiated startle nor did it result in a differential increase in startle response from end extinction to reinstatement testing. Thus, we cannot rule out that differences in end extinction confound the recovery of fear-potentiated startle in the current study, and it remains to be resolved exactly how differences in within-session performance predict subsequent fear recovery, as the data from both experimental and clinical studies have failed to show a consistent relationship between within-session extinction and subsequent fear recovery ([Craske et al., 2008](#) for a review).

On a neural level, previous research ([Myers et al., 2006](#)) has suggested that extinction learning might rely on partly separate mechanisms depending on the temporal delay between acquisition of fear memory and extinction. Thus, in contrast to delayed extinction, which is thought to reflect inhibitory learning of a new association, immediate extinction has been proposed to be preferentially mediated by erasure mechanisms that abolish the return of fear. This effect has been hypothesized to reflect that immediate extinction interferes with a time-dependent consolidation process that is required to turn previous acquired memories into a stable state ([Myers et al., 2006](#)). In this context, it is important to define exactly which consolidation processes are the target of the interference. By varying the timing between fear acquisition and extinction but holding constant the extinction-to-reinstatement interval, we were able to specifically target the effects of interfering with the consolidation of fear acquisition without confounding these with extinction consolidation. This approach however leaves open long-term effects of immediate and delayed extinction that can only be studied once the extinction memory has been consolidated. Indeed, recent data in rodents suggest that delayed, compared to immediate extinction, results in better suppression of conditioned fear during a subsequent retrieval test ([Kim et al., 2010](#); [Liu et al., 2009](#)), even though the short-term and long-term consequences of immediate and delayed extinction have not yet been studied separately. Also, whereas our approach held constant the extinction-to-test interval between the immediate and delayed extinction groups, it did not control for the acquisition-to-test interval. Thus, this introduces an important limitation to the present findings and emphasizes the need for future research to separately manipulate both the extinction-to-test interval and the acquisition-to-test interval. Two previous studies in rodents ([Maren and Chang, 2006](#);

[Chang and Maren, 2009](#)) have addressed this issue by separately comparing the effects of holding constant the acquisition-to-test interval and the extinction-to-test interval. Neither of these studies suggested that the difference between immediate and delayed extinction was attributable to different test intervals ([Maren and Chang, 2006](#); [Chang and Maren, 2009](#)). Nonetheless, more empirical data are needed to clarify this issue.

With few exceptions ([Lonsdorf et al., 2009](#); [Norrholm et al., 2006](#)), human data on fear extinction almost invariably derive from immediate extinction (e.g., [Knight et al., 2004](#); [LaBar and Phelps, 2005](#); [Phelps et al., 2004](#); [Vervliet et al., 2004](#)). Therefore, the current results, suggesting differential effects with regard to both within-session extinction and the recovery of fear, emphasize the need to further explore the differences between immediate and delayed extinction. Future research should optimally assess if the present findings can be replicated in a larger sample of participants and address the generalizability of these findings by systematically varying procedural properties (e.g., fear relevant vs. non-fear relevant stimuli, shock reinforcement rate during acquisition, the training-to-test interval) and develop research strategies that more closely can target the underlying mechanisms that mediate these differences. Critically, in order to exclude that differences in end extinction performance between immediate and delayed extinction confound differences in the return of fear, future research should directly address how the resistance to extinction that accompanies immediate extinction of fear-relevant stimuli affects the subsequent return of fear.

As expected from theories positing relatively independent levels of cognitive and emotional learning in fear conditioning (e.g., [Hamm and Vaitl, 1996](#); [Öhman and Mineka, 2001](#)) we observed a dissociation between expectancy ratings and startle data. Thus, although reinstatement of fear-potentiated startle differed between the immediate and delayed groups following non-masked extinction, reinstatement of shock expectancy ratings was preserved in both groups. Although we cannot exclude that differences in end extinction performance confound these results, this dissociation between fear-potentiated startle and shock expectancy ratings has been reported in similar work ([Kindt et al., 2009](#); [Norrholm et al., 2008](#)). Interestingly, when the CSs were masked during extinction, we observed differential startle potentiation to the CS+ and the CS- across extinction training in the absence of any differentiation for expectancy ratings (see [Esteves and Öhman, 1993](#) for similar data) in both the immediate and delayed extinction groups. Both measures did however result in reliable reinstatement independently of the acquisition-to-extinction delay. Taken at face value, and giving reinstatement the role of a diagnostic for extinction learning, the masking data would suggest that nonconscious presentation of the CS might result in extinction learning both for the immediate and delayed extinction groups. However, the reinstatement results for the masked condition are hard to interpret. For instance, it is unclear whether reinstatement testing after masked extinction trials reflects reinstatement and/or renewal and given the present within-subject design, these effects are indistinguishable. As such, although masking CS trials successfully blocked explicit CS contingency learning while preserving the recovery of fear independently of the acquisition-to-extinction delay, the masking manipulation gives limited insight into whether the differences between immediate and delayed extinction might be due to a habituation-like process that would be independent of CS-US contingency learning during extinction. Moreover, in operational terms, reinstatement of an extinguished response is close to identical with dishabituation of a habituated response ([Thompson and Spencer, 1966](#)). Both these phenomena involve the sudden return of the response to a stimulus, the effect of which has been attenuated by prior presentations, following unexpected presentation of a different,

intense (usually aversive) stimulus. As consciously perceived, the masked extinction condition incorporated a novel face (the six second neutral mask) that was repeatedly presented, eventually to be followed by three unanticipated presentations of the aversive US, and then non-masked presentations of the CS+ and the CS-. Dishabituation reflects a sensitization process that augments responding to any subsequently presented stimulus (Groves and Thompson, 1970), which would promote enhanced responses to the following CSs indicative of reinstatement. This effect, furthermore, would be enhanced by the recovery of the habituated response by the change in stimulus to the CS+ and CS- previously seen during acquisition. Thus, it remains unclear whether extinction learning is needed to explain our masking findings and further research elucidating the relationship between dishabituation and reinstatement and other measures of fear recovery (spontaneous recovery and renewal) is necessary.

In summary, the main findings from this study are that, compared to delayed extinction, immediate extinction of non-masked CSs (a) promoted less extinction of fear-potentiated startle and shock expectancy ratings and (b) elicited less reinstatement of fear-potentiated startle without affecting reinstatement of shock expectancy ratings. Notably, both groups showed successful startle differentiation between the CS+ and CS- during the reinstatement test block. Critically, the differences between immediate and delayed extinction in fear-potentiated startle at the end of extinction warrants further research to clarify the relationship between within-session extinction and the recovery of fear, on the one hand, and the effects of varying the acquisition-to-extinction interval on the other.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biopsycho.2012.07.007>.

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