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Memories in context: On the role of cortisol

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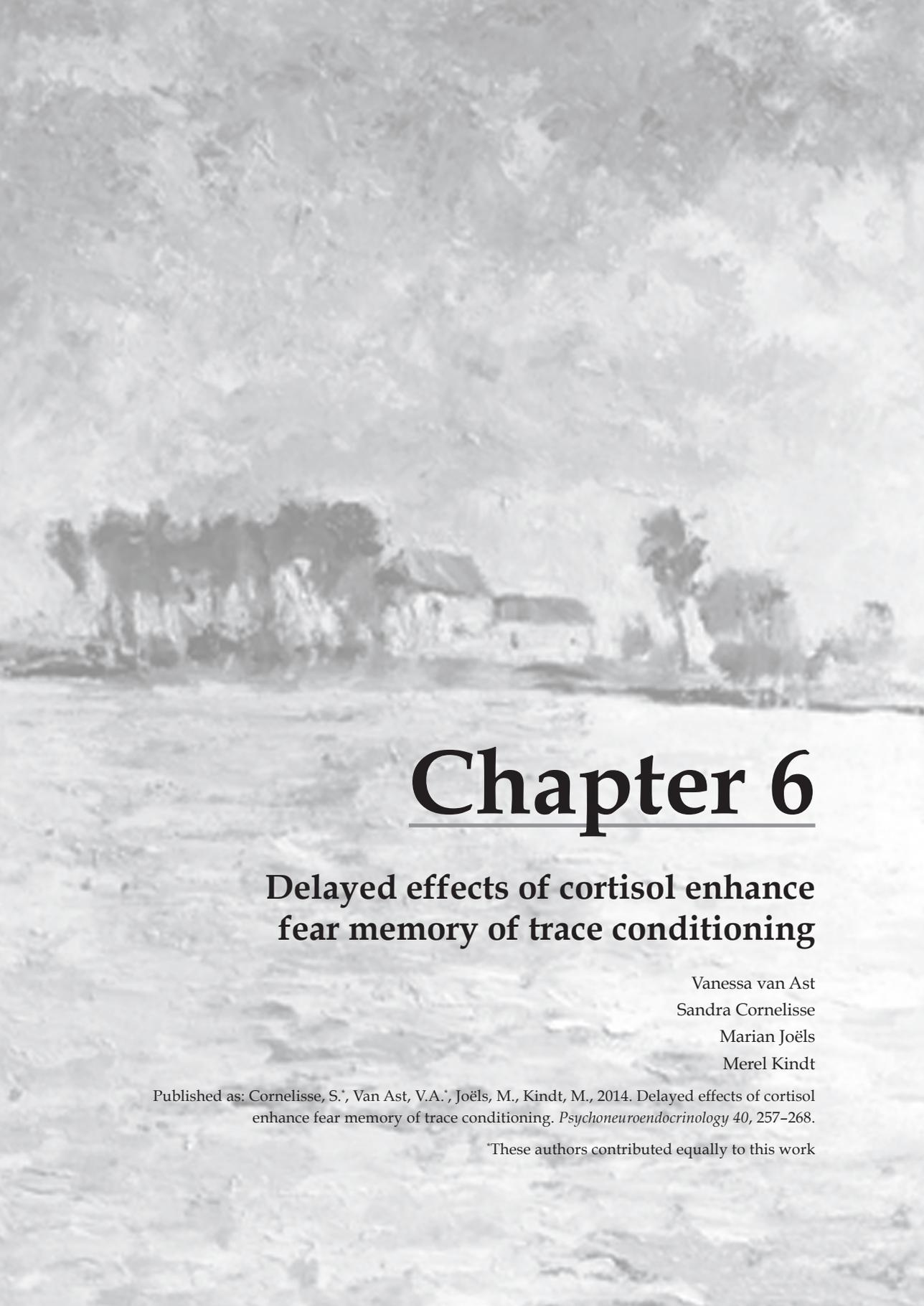
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Chapter 6

Delayed effects of cortisol enhance fear memory of trace conditioning

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SUMMARY

Corticosteroids induce rapid non-genomic effects followed by slower genomic effects that are thought to modulate cognitive function in opposite and complementary ways. It is presently unknown how these time-dependent effects of cortisol affect fear memory of delay and trace conditioning. This distinction is of special interest because the neural substrates underlying these types of conditioning may be differently affected by time-dependent cortisol effects. Delay conditioning is predominantly amygdala-dependent, while trace conditioning additionally requires the hippocampus. Here, we manipulated the timing of cortisol action during acquisition of delay and trace fear conditioning, by randomly assigning 63 men to one of three possible groups: 1) receiving 10mg hydrocortisone 240 minutes (slow cort) or 2) 60 minutes (rapid cort) before delay and trace acquisition, or 3) placebo at both times, in a double-blind design. The next day, we tested memory for trace and delay conditioning. Fear potentiated startle responses, skin conductance responses and unconditioned stimulus expectancy scores were measured throughout the experiment. The fear potentiated startle data show that cortisol intake *240 minutes before* actual fear acquisition (slow cort) uniquely strengthened subsequent trace conditioned memory. No effects of cortisol delivery 60 minutes prior to fear acquisition were found on any measure of fear memory. Our findings emphasize that slow, presumably genomic, but not more rapid effects of corticosteroids enhance hippocampal-dependent fear memories. On a broader level, our findings underline that basic experimental research and clinically relevant pharmacological treatments employing corticosteroids should acknowledge the timing of corticosteroid administration relative to the learning phase, or therapeutic intervention.

1. INTRODUCTION

Corticosteroids are released after stress and modulate learning and memory processes via mineralocorticoid and glucocorticoid receptors. These receptors are abundantly expressed in limbic brain areas, like the hippocampus (Reul and de Kloet, 1985). In humans, effects of cortisol on memory have predominantly been investigated for non-associative and distinct emotional stimuli (de Quervain et al., 2009).

Stress is considered to be an important vulnerability factor for anxiety disorders (Korte, 2001). It is generally assumed that anxiety disorders originate from a learned association between a previously neutral or ambiguous event (conditioned stimulus; CS) and an anticipated aversive event (unconditioned stimulus; US) (Mineka and Oehlberg, 2008). Thus, associative fear learning (i.e., discriminative fear conditioning) seems a suitable experimental model to delineate the mechanism by which corticosteroids contribute to the development of anxiety disorders.

Earlier animal and human studies have shown that stress and/or corticosteroids can indeed alter associative fear learning (Rodrigues et al., 2009; Wolf, 2009). However, the effects of stress hormones on fear acquisition seem equivocal, even when differences in the employed paradigms, dependent variables, and sex are taken into account: Studies investigating relationships between cortisol and *delay* fear conditioning as measured by skin conductance reported enhanced (Jackson et al., 2006; Zorawski et al., 2006; 2005), impaired (Stark et al., 2006; Van Ast et al., 2012; Wolf et al., 2009), or unaltered acquisition (Merz et al., 2013; 2012b) in men. In women, both impairing effects (Merz et al., 2013; Wolf et al., 2009) and no effect of cortisol on fear acquisition (Merz et al., 2012b; Stark et al., 2006; Tabbert et al., 2010; Van Ast et al., 2012) have been reported. Only two studies investigated the relationship between post acquisition cortisol and fear retention, but these studies did not reveal any relationships between cortisol and retention in either men or women (Zorawski et al., 2006; 2005). On the neural level, cortisol seemed not to influence instructed fear conditioning (Merz et al., 2013; 2012b) neither in men nor in women, but when fear learning was involved (either in a learned aware or an unaware sample), men displayed reduced neuronal fear responses after cortisol application, whereas women taking oral contraceptives exhibit enhanced fear responses on the neuronal level (Merz et al., 2013; 2012a; 2010; Stark et al., 2006; Tabbert et al., 2010).

Studies investigating the relationship between cortisol and *trace* fear conditioning (or occasion setting) in men reported impairing (Wolf et al., 2012) or enhancing (Kuehl et al., 2010) effects on fear acquisition as measured by % eyeblink responses, enhancing effects as measured by fear-potentiated startle (Van Ast et al., 2012), or no effects as measured by skin conductance (Van Ast et al., 2012). One study sug-

gested impairing effects of cortisol on trace conditioning, but here sex effects were not investigated (Nees et al., 2008). Taken together, the high variety of paradigms (e.g., delay- or trace conditioning), dependent measurements (e.g., skin conductance responses vs. startle responses), and participants (men vs. women) used across studies, precludes valid comparisons. In addition, the majority of these studies only focused on fear acquisition, that is, they did not examine long-term memory aspects that are exquisitely sensitive to corticosteroids. Revealing the effects of cortisol on retention of fear might be very relevant to understanding maintenance of fear in anxiety disorders, as opposed to cortisol effects on fear learning or mere fear expression (i.e., in instructed conditioning paradigms). For these reasons, we aimed to target the expression of fear memory as assessed one day after fear acquisition.

Corticosteroids are known to affect neurobiological processes in a time-dependent manner (Diamond et al., 2007; Joëls et al., 2012); this may have added to seemingly inconsistent cortisol effects across studies. Shortly after stress, corticosteroids interact with noradrenaline to synergistically promote rapid increases in neuronal activity (Karst et al., 2010; 2005). This effect is most pronouncedly sustained in the basolateral amygdala (BLA) through a nongenomic pathway (Karst et al., 2010; 2005). In humans, such rapid corticosteroid effects have indeed been described for emotion- and arousal-related brain areas, such as the amygdala (van Marle et al., 2010). They promote habitual, reflex-like behaviour (Schwabe et al., 2010a) and attention (Vedhara et al., 2000), at the expense of goal-directed behaviour (Schwabe et al., 2010a) and higher cognitive functioning (Elzinga and Roelofs, 2005). Indeed, shortly after stress a vigilance network including the amygdala is activated, as demonstrated with fMRI in humans (see Hermans et al., 2011). During memory encoding after stress, hippocampal and prefrontal cortex activity is generally suppressed (Qin et al., 2009a; van Stegeren et al., 2010). All in all, most observations in animals and humans agree that rapid cortisol effects -presumably through nongenomic pathways and in interaction with arousal-evoked central adrenergic release- enhance amygdala activity while reducing hippocampal and PFC activity. This may help the organism to focus and subsequently remember the most significant aspects of an event (Roosendaal et al., 2006b), at the cost of the more complex, cognitive aspects (Joëls et al., 2012; Karst et al., 2010).

By contrast, some hours after stress, slower long-lasting genomic corticosteroid actions develop (de Kloet et al., 2005; Wiegert et al., 2005). Such delayed effects of cortisol on the brain are thought to restore homeostasis following stressful periods (Diamond et al., 2007; Joëls et al., 2006). Although slower genomic effects have not been investigated in fear conditioning studies, other studies indicated that genomic effects promote consolidation (Barsegyan et al., 2010), cognitive self-control (Oitzl

et al., 2001), enhance working memory (Henckens et al., 2011), promote sustained attentional processing (Henckens et al., 2012b), and strengthen connectivity between the PFC and amygdala (Henckens et al., 2010). As such, slower genomic corticosteroid effects may facilitate remembering a certain event in a more cognitively controlled manner.

Here, we examined the potential role of these time-domains in corticosteroid effects on discriminative human fear conditioning, with special interest in the slow effects since these have not been addressed so far. Hereto, we designed a within-subjects delay- and trace-conditioning paradigm, concurrently measuring fear potentiated startle responses (FPS), skin conductance responses (SCR) and subjective shock expectancies (EXP) during fear acquisition and, importantly, a fear memory retention phase. In delay conditioning, the US and the CS co-terminate, whereas in trace conditioning a stimulus free period (the trace interval) passes between the offset of the CS and the US delivery. This distinction is of special interest because different neural substrates are believed to underlie delay and trace conditioning. Both animal and human research has identified the amygdala as predominantly involved in delay conditioning, while trace conditioning requires the dorsal hippocampus and the prefrontal cortex in addition to the amygdala (Burman et al., 2006; Knight et al., 2004). By including delay and trace conditioning in a within-subjects paradigm, we modelled relatively simple, amygdala-dependent features and more complex, hippocampal- and prefrontal cortex-dependent features of learning and memory, respectively.

We targeted time-dependent cortisol effects by randomizing participants into one of three experimental groups: 1) receiving 10 mg hydrocortisone either 240 or 2) 60 minutes before delay- and trace acquisition, or 3) placebo at both times. Approximately 24 hours later, fear memory expression (extinction and reinstatement) was tested. Trace and delay conditioning were assessed by a within-subjects design. During acquisition, the delay conditioned stimulus (CSdel+) was directly followed by the US (a shock), whereas for the trace conditioned stimulus (CStr+) a time gap was introduced between the end of the CS and the start of the US. A control stimulus (CS-) was never followed by the US. During extinction and reinstatement, these stimuli were presented unreinforced (CSdel-, CStr-, CS-). Concerning time-dependent effects of cortisol on subsequent fear memory expression (i.e., retention), we hypothesized that cortisol administration 240 minutes before acquisition (slow cort) would enhance memory of trace conditioning, in line with the idea that slow genomic corticosteroid actions promote consolidation of more complex, hippocampal- and prefrontal cortex-dependent stress-related memories for future use. By contrast, cortisol administration 60 minutes before acquisition (rapid cort) would enhance

more simple, amygdala-dependent memory of delay conditioning (Zorawski et al., 2006; 2005), whereas trace conditioning memory would be impaired, in line with the idea that the most significant, aspects of an event will be remembered, at the cost of the more complex aspects.

2. METHODS

2.1. Participants

Sixty-three male participants (mean age 21.4 years (SD=2.9 years); mean body mass index 22.2 (SD=2.9)) gave written informed consent. The local ethical committee of the University of Amsterdam approved the study. Inclusion was conditional on having no past or present psychiatric or neurological condition, assessed by self-report. In addition, participants having any somatic or endocrine disease (e.g. acute asthma), or taking medication known to influence central nervous system or endocrine systems were excluded from participation. Participants were asked not to eat, drink or smoke two hours before participation. Participants were rewarded for participation with either course credits or a monetary reward of €65,-. Due to technical errors, extinction and reinstatement data from one participant were missing, as well as reinstatement data from another.

2.2. Physiological Measures

2.2.1. Drug administration and assessment

A single dose of 10mg hydrocortisone was employed to elevate cortisol to a level equivalent to acute stress. Hydrocortisone and placebo (albochin) pills looked identical. To assess salivary free cortisol concentrations for each participant, Salivette collection devices (Sarstedt, Nümbrecht, Germany) were employed. After testing, salivettes were stored at -25°C. Upon completion of the study, samples were sent to Dresden (Technische Universität, Dresden, Germany) for biochemical analysis.

2.2.2. Fear-potentiated startle

Startle probes to induce fear-potentiated startle reflexes were 104dB, 40ms bursts of white noise with a near instant rise time and delivered binaurally through headphones (Sennheiser, model HD25-1II). Sound pressure and dB level were calibrated using a sound level meter (Rion, NA-27, Japan). Startle reflexes were measured through electromyography (EMG) of the left orbicularis oculi muscle. Hereto, two 6mm Ag/AgCl electrodes filled with a conductive gel (Signa, Parker) were placed approximately 1cm under the pupil and 1cm below the lateral canthus (Fridlund

and Cacioppo, 1986). A ground electrode was placed on the forehead, 1cm below the hairline. The EMG amplifier consisted of two stages. The input stage or pre-amplifier had an input resistance of 10.000Ω . The EMG signal was set at a frequency response of DC-1500Hz and was then amplified by 200. A 50Hz notch filter was used to reduce interference from the mains noise. For the second stage, the signal was amplified with a variable amplification factor of 0-100 times. Finally, the EMG signal was digitized at a rate of 1000S/s.

2.2.3. Skin Conductance Response

Electrodermal activity was measured by two curved Ag/AgCl electrodes of 20 by 16mm that were attached with adhesive tape to the medial phalanges of the first and third fingers of the left hand. The in-house built amplifier applied a sine-shaped excitation voltage (1V peak-peak) of 50Hz derived from the mains frequency to the electrodes in order to detect changes in the electrodermal activity. The signal from the input device was led through a signal-conditioning amplifier. The analogue output was digitized at 1000 S/s by a 16-bit AD-converter (National Instruments, NI-6224).

2.2.4. US-expectancy ratings

US-expectancies were measured continuously throughout acquisition, extinction, and reinstatement, thus enabling us to collect ratings during the CS presentation and CS traces. Ratings were given by sliding a lever on a box (custom made from a joystick) that in turn operated a cursor on a scale that showed at the bottom of the computer screen. The scale was continuous, ranging from 'certainly no electrical stimulus' through 'certainly an electrical stimulus'. Expectancy data were sampled at 1000 S/s. Startle responses, electrodermal activity and US-expectancy ratings were recorded with the software program VSSRP98 v6.0 (Versatile Stimulus Response Registration Program, 1998; Technical Support Group of the Department of Psychology, University of Amsterdam).

2.3. Subjective measures

Participants filled out the Trait and State Anxiety Inventory (Spielberger et al., 1970) and the Positive Affect and Negative Affect Schedule (Watson et al., 1988). In a post experimental questionnaire Unpleasantness ratings of the US and startle probes were assessed on 9-point scales ranging from "not unpleasant" (1) to "extremely unpleasant" (9). We also probed knowledge of which substance (placebo or hydrocortisone) was administered at the two time points. To assess contingency awareness, participants indicated which CSs were followed by the US. Participants were classified as

“aware” if they correctly recognized that the CSdel+ and the CStr+ were followed by a shock, while the CS- was not followed by a shock.

2.4. Experimental task

Three pictures (Langner et al., 2010) of male neutral faces served as conditioned stimuli. Assignment of the three faces as CSdel+, CStr+ and CS- was counterbalanced across the three experimental groups. During the preconditioning phase, participants were presented a habituation phase consisting of 8 startle probes (“noise alone” trials, NA) with an inter-probe interval of 20 ± 5 s. Then, the CSdel+, CStr+ and CS-, were presented unreinforced once in random order, along with one NA trial. The acquisition phase commenced with a habituation phase, that consisted of 8 NA trial presentations. During acquisition, the four stimulus types (CSdel+, CStr+, CS-, NA) were randomized within blocks. These blocks were presented 10 times in total. During acquisition, the CSdel+ and the CStr+ were followed by the US at all times, except for the first trial. We did so in order to obtain a measure of possible time-dependent effects of cortisol on responses to the stimuli themselves (i.e., from pre-conditioning to first presentations of each trial during acquisition). CSs were presented for 10 seconds. Startle probes were presented during CSs (9 s after CS onset) and during traces (9 s after CS offset). In case of the CSdel+, the CS probe was followed by the US after 0.5 s. In case of the CStr+, the trace probe was followed by the US after 0.5 s. Inter trial intervals (ITI) were $30 (\pm 5)$ s. In case a noise alone (NA) probe was presented during an ITI, it followed the trace probe after $10 (\pm 5)$ s (see **Figure 1**).

During extinction, all stimulus types were again presented 10 times, but CSs were never followed by the US. Fifteen seconds after the last extinction ITI, three unsignaled USs (respectively intermitted by 40 and 30s time lag) were presented. The reinstatement test phase started 30s after the last reinstating US, during which each stimulus type was again presented four times. Trial timing was similar for all experimental phases.

2.5. Design and procedure

In a between-subjects, placebo-controlled, double blind study design, participants were randomly assigned to either the ‘slow cort’ (hydrocortisone 240 and placebo 60min prior to acquisition), ‘rapid cort’ group (placebo 240 and hydrocortisone 60min prior to acquisition) or placebo group (placebo at both 240 and 60min prior to acquisition), resulting in 21 participants per group. Testing took place in between 12am and 8pm, when endogenous cortisol levels are stable and relatively low (Pruessner et al., 1997). A schematic overview of the experiment is depicted

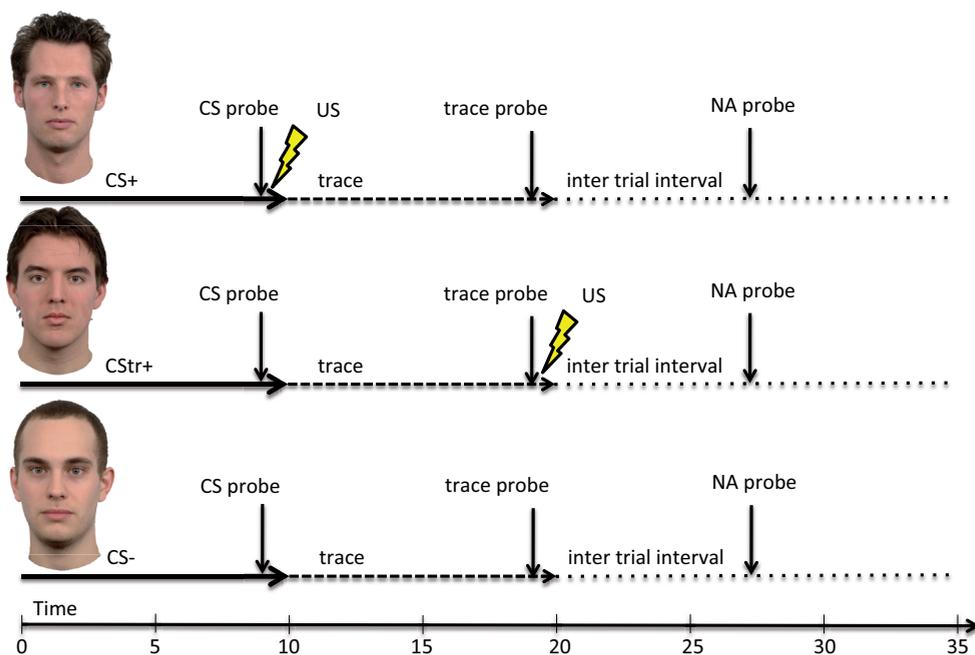


Figure 1. Trace and delay conditioning procedure. CSs were presented for 10 seconds. Startle probes during CSs (denoted with: ‘CS probe’) were presented 9 s after CS onset. In case of the CSdel+, the CS probe was followed by the US after 0.5 s. Startle probes during traces (denoted with: ‘trace probe’) were presented 9 s after CS offset. In case of the CStr+, the trace probe was followed by the US after 0.5 s. Inter trial intervals (ITI) were 30 (± 5) s. In case a noise alone (NA) probe was presented during an ITI, it followed the trace probe after 10 (± 5) s.

in **Figure 2.** Participants filled out the STAI-T, PANAS and STAI-S to assess baseline self-reported mood states and a first saliva sample (S1) was taken. To acquire physiological baseline responding to the startle probes and the different CS stimuli, a preconditioning phase followed. A shock workup procedure was completed to establish a shock level that was “unpleasant, but not painful”. Participants were told that this phase involved baseline response assessment to the stimuli, and no shocks would be administered. Then, directly following sample S2, participants received their first pill (hydrocortisone or placebo). The second pill (hydrocortisone or placebo) was given 180min later. While waiting, participants read or studied, and they were allowed to eat lunch. During this period, samples S3-S6 were taken. After Pill 2, participants gave sample S7 and again filled out the PANAS and STAI-S. Prior to conditioning, participants were told they would see three faces, one of which would never be followed by the US, while the other two could be followed by the US at different time points. They were told to learn to predict whether and when they

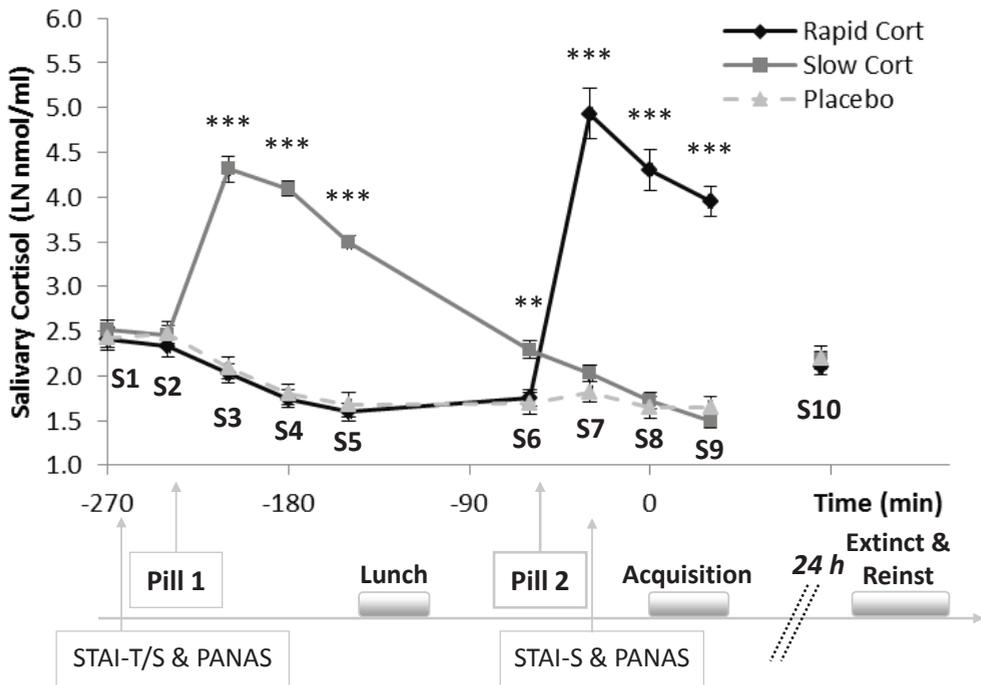


Figure 2. Overview of the experimental design and salivary cortisol levels during the experiment. Participants received a pill 240 and 60 minutes prior to delay- and trace fear acquisition on day 1 (Pill 1 and Pill 2). The pills contained either hydrocortisone (10 mg) or placebo (albochin). Two baseline saliva samples were taken at the beginning of the experiment ($t = -270$ and $t = -240$), six more before acquisition ($t = -210, -180, -150, -60, -30, 0$), one directly after acquisition ($t = 30$) and a last sample was taken before the extinction procedure on day 2. In the slow cort condition cortisol levels were increased from 30 minutes after pill intake until 180 minutes later and in the rapid cort condition cortisol levels were increased from 30 minutes after pill intake until the end of the first session. Error bars represent standard error of the mean (S.E.M.). Significant Bonferroni corrected differences with placebo are depicted by ** = $p < 0.01$; *** = $p < 0.001$.

would receive an electrical stimulus. These explicit instructions were given because awareness is a necessary condition to acquire conditioned responses in hippocampus dependent tasks such as trace conditioning (Weike et al., 2007). In addition, paradigms using explicit instructions concerning the CS-US relationships are best suited to investigate subsequent memory effects (Kindt et al., 2009; Merz et al., 2012b), as they reduce variability on day 1 that could perhaps explain subsequent variability in memory tests on day 2. Sixty minutes after second pill intake, sample S8 was taken and conditioning started. At the end of the first experimental day, participants gave a final saliva sample (S9). Time between the two testing sessions was kept at 24h, in order to substantiate consolidation of fear memories. The following day, participants

were told that they would see the same faces again and tested for memory of what was learned the day before. A final set of mood questionnaires (STAI-S, PANAS) was filled out and sample S10 was taken, followed by extinction and reinstatement phases. The experiment was completed by the post-experimental questionnaire. The experiment described here was part of a larger study into time-dependent cortisol effects on cognitive function. The other task (i.e., a 5-minute delay discounting task (Berns et al., 2007)) took place approximately 20 minutes prior to fear acquisition. Results on this task will be reported elsewhere.

2.6. Data reduction

Raw EMG data were conditioned to a band-pass between 28-500Hz. Relative to startle probe onset the latency window for the blink reflex was 0-120ms and maximum peak amplitude was determined within a window of 20-150ms. Electrodermal responses *during* the CSs were obtained by subtracting the baseline (1s average before CS onset) from the maximum absolute SCR score obtained from a window of 1-9s following CS onset. Electrodermal responses for the *traces* were obtained by subtracting the baseline (1s average before CS onset) from the maximum absolute SCR score obtained from a window of 1-9s following trace onset. Raw SCR and FPS scores were standardized across all experimental phases and converted to T-scores ($T=(z \times 10) + 50$). To obtain US-expectancy ratings for the CSs and the traces, 1s averages were calculated immediately before each startle probe onset.

2.7. Data analysis

In order to assess group differences in sample characteristics, univariate ANOVAs with the between subject factor Condition (slow cort, rapid cort, placebo) were employed.

Cortisol levels showed a skewed distribution with the Shapiro-Wilk test of normality and were log transformed. The effect of hydrocortisone administration on salivary cortisol during the first session was assessed by means of a mixed ANOVA with the within subject factor Time (S1-S9) and between subjects factor Condition. Bonferroni-corrected post-hoc comparisons were used to detect significant group differences at all sample points.

Analyses to assess whether the trace and delay conditioning paradigm was successful are described in the **Supplementary Methods**.

To check for cortisol effects on baseline FPS responses, FPS on baseline habituation trials and habituation trials before acquisition were entered into a mixed ANOVA with Pre-Post (before vs. after cort manipulation) and Trial as within subject factors and Condition as between subject factor. Further, to check for possible

time-dependent effects of cortisol on responses to the stimuli themselves, FPS and SCR data from the pre-conditioning phase and the first (non-reinforced) trials of acquisition were entered into mixed ANOVAs with Conditioning type (trace vs. delay conditioning), CS type (CSdel+, CStr+, CS-) and Pre-Post as within subject factors and Condition as between subject factor. To assess slow and rapid corticosteroid effects on acquisition, extinction and reinstatement, FPS, SCR and EXP data were entered into mixed ANOVAs with Conditioning type (trace vs. delay conditioning), CS type (CSdel+, CStr+, CS-) and Trial as within subject factors and Condition as between subject factor. If this overall analysis revealed an interaction effect between Conditioning type, CS type and Condition, trace and delay conditioning were further analyzed separately. Thus, for analysis of *delay* conditioning, data obtained *during presentation* of the CSs were entered into mixed ANOVAs with CS type and Trial as within subject factors and Condition as between subject factor. To assess time-dependent corticosteroid effects on *trace* conditioning, data obtained from the *traces* of the CSs were entered in a similar analysis. Planned comparisons to assess group differences were performed on difference scores of the relevant CSs (i.e., traces of CStr vs. CS- for trace conditioning and CS responses of CSdel vs. CS- and possibly CStr vs. CS- for delay conditioning). A Greenhouse-Geisser procedure was used in case of violation of the sphericity assumption in ANOVAs. Alpha level was set at .05 for all statistical analyses.

3. RESULTS

3.1. Participant characteristics

The slow cort, rapid cort and placebo groups did not differ in terms of age, body mass index, trait anxiety and anxiety sensitivity (all $F_s < 1.98$, *n.s.*). Shock intensity ranged from 6 to 56mA ($M=22.42$, $SD=11.74$), and did not differ between the three experimental groups ($F=1.11$, *n.s.*). The subjective evaluation of the US and the startle probe did not differ either (all $F_s < 1.89$, *n.s.*). Furthermore, groups did not differ on subjective mood (STAI-S, PANAS) (all $F_s < 2.34$, *n.s.*). Three participants were classified as unaware of the CS-US contingencies (one participant in each group). Those three participants were excluded from analyses. Inclusion of all subjects led to similar results.

3.2. Manipulation check

Figure 2 displays salivary cortisol levels for all groups (see **supplementary table 1** for the raw cortisol data). As expected, the ANOVA for salivary cortisol levels showed a significant Time \times Condition interaction ($F_{16,456} = 116.79$, $p < 0.001$, $\eta^2 = 0.804$). In addi-

tion, a significant main effect of Time ($F_{8,456}=24.20, p<0.001, \eta^2=0.302$) and Condition ($F_{2,57}=25.41, p<0.001, \eta^2=0.471$) emerged. Bonferroni-corrected post-hoc comparisons showed that in the slow cort condition cortisol levels were increased from 30min after pill intake until 180min later (S3-S6; $ps<0.002$) but had returned back to baseline before and during the acquisition phase (S7-S9). In the rapid cort condition, cortisol levels were increased from 30min after pill intake until the end of the first session (S7-S9; $ps<0.001$). On day 2, cortisol levels did not differ between the three groups (S10; $F=0.102, n.s.$). Participants were unable to identify the substance received during the exit interview ($X^2(1)=0.185, n.s.$; in total 8 subjects correctly identified both pills they received; 1 subject in the rapid cortisol group, 4 subjects in the slow cortisol group and 3 subjects in the placebo group).

3.3. Delay and trace conditioning paradigm

As described in the **Supplementary Results** and illustrated in **Supplementary Figure 1**, successful acquisition and extinction was demonstrated for EXP, FPS and SCR.

3.4. Rapid and slow corticosteroid effects

3.4.1. Fear potentiated startle

Figure 3 displays the fear potentiated startle responses during the acquisition, extinction and reinstatement phases in delay- and trace conditioning for the three experimental groups. Cortisol did not affect baseline FPS responses to habituation NA trials before vs. after the cortisol manipulation (Pre-Post), as evidenced by the absence of a Condition \times Pre-Post \times Trial interaction effect ($F=1.11; n.s.$) or a Condition \times Pre-Post interaction effect ($F=0.130; n.s.$). Cortisol did also not affect FPS responses during the CSs when pre-conditioning trials before pill intake were compared with the first (un-reinforced) trials of acquisition, as evidenced by the absence of a Condition \times Pre-Post \times CS Type \times Conditioning type interaction ($F=2.00; n.s.$), nor were there any other significant interaction effects involving Condition and Pre-Post ($Fs<2.38, n.s.$).

Acquisition: Analysis of acquisition data did not show any interaction effects involving Condition, CS type and Conditioning type ($Fs<1.23, n.s.$), nor a main ef-

Table 1. Salivary cortisol levels over the course of the study (mean \pm SE).

Time	Placebo (nmol/L)	Hydrocortisone (20 mg) (nmol/L)
Baseline	7.81 \pm 0.72	12.05 \pm 1.60
Pre-conditioning	6.10 \pm 0.83	170.35 \pm 38.35
Post-conditioning	5.5832 \pm 0.52	158.86 \pm 30.43

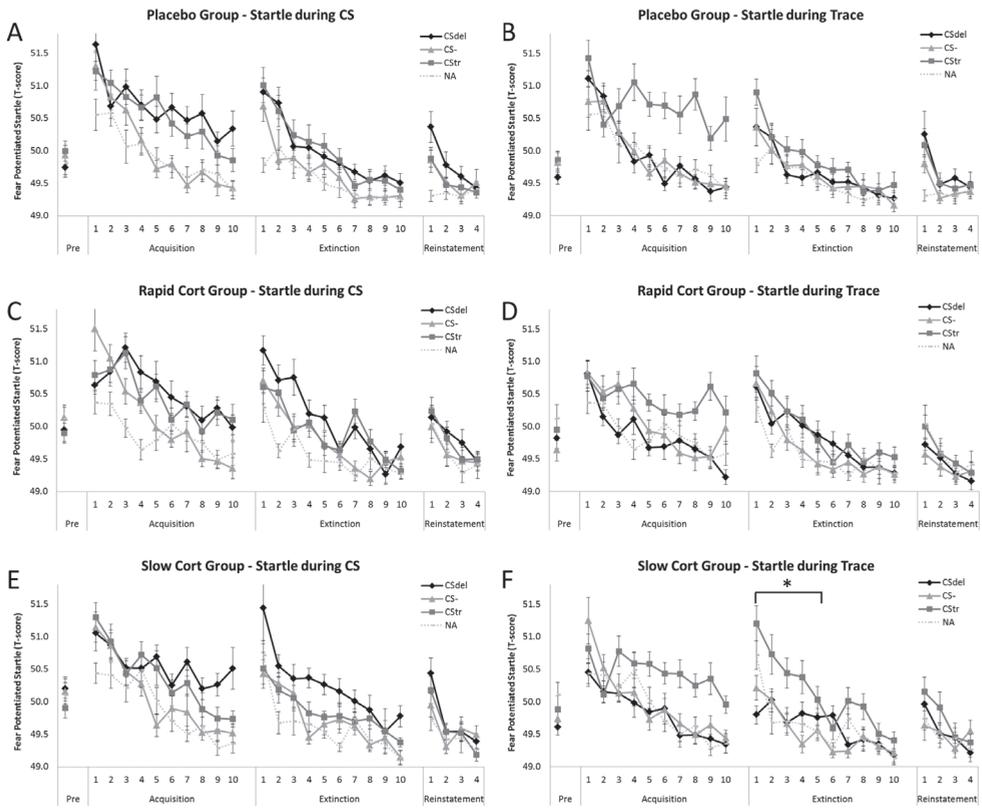


Figure 3. Fear potentiated startle data acquired during the CS pictures (A, C, E) and during the CS traces (B, D, F) of all three trial types (i.e., CSdel+, CStr+ and CS-) for the placebo (A, B), rapid cort (C, D) and slow cort (E, F) groups. Note that Participants in the slow cort group uniquely demonstrate enhanced startle responses during early extinction, suggesting that cortisol, given 4 hours before acquisition, enhances fear memory of the trace stimulus 24 hours later. Error bars represent standard error of the mean (S.E.M.). Significant post-hoc differences with placebo are depicted by * = $p < 0.05$. Error bars represent standard error of the mean (S.E.M.).

fect of Condition ($F=0.644$, *n.s.*). This suggests that the cortisol manipulation did not affect delay- or trace acquisition.

Extinction: For extinction, we did observe a significant interaction effect between Conditioning type, Condition and CS type ($F_{4,112}=4.32$, $p=0.003$, $\eta^2=0.134$). To further disentangle this effect, we analyzed delay and trace conditioning separately. For delay conditioning, the cortisol manipulation did not affect extinction ($F_s < 1.98$, *n.s.*). For trace conditioning however, we did observe a significant CS type \times Condition interaction ($F_{4,112}=3.04$, $p=0.020$, $\eta^2=0.026$). If this reflected a memory retention effect, we expected it to originate mainly from the first part of extinction. Indeed, we found a

significant CS Type \times Condition interaction during the first part ($F_{4,112}=3.42, p=0.011, \eta^2=0.109$), but not the second part of extinction ($F=0.83, n.s.$). Planned comparisons revealed that the slow cort group showed more differentiation of the CStr- vs. the CS- compared to the placebo group ($p=0.027$) and marginally more differentiation of the CStr- vs. the CS- compared to the rapid cort group ($p=0.058$). Differentiation of the CStr- vs. the CS- did not differ between the placebo group and the rapid cort group ($n.s.$). Also, differentiation between the CSdel- and the CS- did not differ between any of the groups ($n.s.$). Analysis of the last half of acquisition (i.e., the last 5 trials) vs. the first half (i.e., the first 5 trials) of extinction also revealed a Phase (last half acquisition versus first half of extinction) \times CS type \times Condition effect ($F_{2,56}=5.519, p=0.003, \eta^2=0.187$). Follow-up analyses showed that the difference between the differential responding from the end of acquisition to the beginning of extinction was enhanced in the slow cortisol group as compared to the placebo group ($F_{1,37}=10.81, p=0.002, \eta^2=0.226$), while the rapid and placebo group did not differ ($F=3.76, n.s.$). This effect was not caused by altered baseline (NA) FPS responses during extinction ($F=0.56, n.s.$). These same effects were significant when the raw (untransformed) data were used in analyses. Notably, although the slow cort group showed more differentiation of the CStr- vs. the CS-, both the placebo and the rapid cort group showed significantly higher startle responses on the CStr- vs. the CS- during the first half of extinction (Placebo: $F_{1,19}=6.55, p=0.019, \eta^2=0.256$; Rapid Cort: $F_{1,19}=5.388, p=0.031, \eta^2=0.212$). Together, this suggests that cortisol, given 4 hours before acquisition, enhanced fear memory of the trace stimulus 24 hours later.

Reinstatement: There were neither any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.27, n.s.$), nor a main effect of Condition ($F=0.10, n.s.$) suggesting that the cortisol manipulation did not affect delay- and trace reinstatement.

3.4.2. Skin conductance responses and expectancy ratings

Analysis of the EXP and SCR data did not show any effects of the experimental manipulation (see **Supplementary Results**).

4. DISCUSSION

A first aim of the present study was to develop a paradigm in which delay and trace conditioning can be tested within subjects, concurrently measuring FPS, SCR and EXP. We show that participants are able to acquire delay and trace conditioning simultaneously, and retain the same pattern of conditioned responses the following day on all dependent measures. Although delay and trace conditioning were tested

within-subjects before (Cheng et al., 2008; Knight et al., 2004), the present paradigm adds in important ways to current test models of associative fear memory, as this is the first human trace conditioning study including FPS, *and* a memory retention test one day later. On a broader level, the inclusion of FPS and a retention test renders this paradigm more relevant to understand the root and maintenance of fear-related disorders in humans (Mineka and Oehlberg, 2008).

Further, we tested time-domain effects of cortisol action during acquisition on subsequent memory. FPS data showed that cortisol intake 240 minutes before fear acquisition - focusing on genomic actions - uniquely bolstered trace, but not delay, fear memory. The hippocampus plays an additional role in trace versus delay conditioning (Burman et al., 2006; Knight et al., 2004) and successful trace conditioning relies to a large extent on working memory (Carter et al., 2003) and selective attention (Han et al., 2003). These processes were recently found to be enhanced by slow corticosteroid actions (Henckens et al., 2012b; 2011). Thus, perhaps by means of boosted executive processing after acquisition, we here provide the first evidence that slow (presumably gene-mediated) corticosteroid effects during fear acquisition can strengthen subsequent fear memory in humans. Most likely though, this effect on trace conditioning is mediated by genomic effects during fear conditioning and (early) consolidation (Joëls et al., 2012) of trace memories that were not yet present in the rapid cort group. Because cortisol levels were too low for non-genomic actions to occur, and there were sufficient hours in between cortisol administration and encoding, likely genomic effects were at play. Such a slow effect on consolidation fits with earlier findings in humans and rodents on the role of gene-mediated corticosteroid actions in consolidation (Oitzl et al., 2001; Van Ast et al., 2013). The most explicit example is provided by Oitzl et al. (2001) who showed that a mutant mouse in which GR cannot bind to the DNA (which prohibits any genomic actions) is impaired with respect to its consolidation of spatial information. We are not aware of any other studies in humans investigating exogenous cortisol effects on the retention of fear memory (as opposed to fear acquisition). Our findings suggest that slow effects of cortisol do not merely restore baseline functioning, but may actually lead to a redistribution of neural resources towards superior executive functioning (Henckens et al., 2012b; 2011) that can enhance subsequent fear memory as well.

Since cortisol levels during fear acquisition in the slow cort group already had gone back to baseline, not only the absolute levels of cortisol per se may play an important role in modulating certain memory processes, but also the time lag that exists between cortisol level enhancement and encoding. This is in line with the temporal dynamics model (Diamond et al., 2007), the emotional tagging hypothesis (Richter-Levin and Akirav, 2003), and theories by Joëls and colleagues (Joëls et al.,

2006), which all predict differential effects by corticosteroids in the time-domain shortly after stress as opposed to several hours later. At the cellular level, rapid activation, followed by inhibition of neuroplasticity has been observed in the hippocampus in response to several types of stress manipulations (Richter-Levin and Akirav, 2003), threat (Diamond et al., 2007), or cortisol administration (Joëls et al., 2011). How exactly these cellular effects relate to the overall efficiency of network function and concomitant behavioral performance is not easy to predict.

We did not observe effects of cortisol administered 60 minutes before task onset on either delay or trace memory. This is in line with two other studies that did not find a relationship between post-acquisition endogenous cortisol and fear retention (Zorawski et al., 2005, 2006). The delay between the onset in rise of cortisol level (certainly in the brain) and the start of the behavioral task (<45 minutes) was too short to allow gene-mediated actions to develop. However, fear acquisition took on average 25 minutes, so that by the time subjects had completed the task cortisol possibly no longer exclusively exerted non-genomic corticosteroid actions. Because cortisol levels were still high at the beginning of consolidation, non-genomic effects may have been at work at the same time as genomic effects. Perhaps, these processes cancel each other out, but we can only speculate about this. Alternatively, we may not have had enough power to find an effect, or the hydrocortisone dose that we utilized could have been too low (i.e. 10 mg). Also, cortisol by itself may not be sufficient to strengthen cued fear memory as this process may require strong noradrenergic activation; e.g. manipulations targeting the adrenergic system affect cued fear memory to a great extent (Soeter and Kindt, 2011a). Finally, the fact that the rapid group was only marginally different from the slow group (as measured during the traces) may reflect that similar effects of cortisol occurred in the slow and rapid groups, but that these were just more clearly expressed in the slow group. This would fit with the putative partial onset of genomic actions in the rapid group during the consolidation phase. Taken together, we found no support for the expected rapid effects on delay conditioned memory and in light with the raised issues mentioned before it is hard to make any claims concerning changes in consolidation in this group.

As described in the introduction, earlier studies into effects of cortisol on acquisition of delay or trace acquisition have been equivocal. It is important to note that the nature of the instructions concerning the conditioning contingencies prevent us from drawing strong conclusions on fear *learning*. Rather, the acquisition data reflect fear *expression* (Merz et al., 2013; Tabbert et al., 2010). Such paradigms are best suited to investigate subsequent memory effects (e.g. Kindt et al., 2009; Merz et al., 2013), in which we were foremost interested. Here we did not observe altered fear expression on day 1 in either delay or trace conditioning, irrespective of timing of corticosteroid

treatment. The absence of cortisol effects on psychophysiological measures due to cortisol is in line with other delay conditioning studies using explicit instructions that found no effects of cortisol either on psychophysiological expression of fear, or cortisol induced alterations of fear learning on the neural level (Merz et al., 2012, 2013). Taken together, we conclude that cortisol does not exert time-dependent effects on the expression of fear in delay and trace conditioning. In order for cortisol to exert strong effects on psychophysiological expression of fear, less explicit prior conditioning instructions may be required.

The absence of an effect in the slow cort group at day 1 seems in apparent contrast with the *enhancing* memory effects on trace conditioning the next day. This corroborates the idea of a dissociation between fear expression during acquisition learning and subsequent long term fear memory. Such a dissociation has been convincingly illustrated by studies in which pharmacological manipulations, administered before acquisition, affected the extinction process several days later while leaving the acquisition process intact (Soeter and Kindt, 2011a; 2011b). Post-learning processes may account for this dissociation, as they induce the structural changes underlying the stabilization of a memory trace after its acquisition (McGaugh, 1966). Thus, the implementation of a test phase of associative fear memory after consolidation is most appropriate to assess long-term fear memory.

Some considerations regarding our results should be mentioned. First, effects of the experimental manipulation were only apparent on the startle measure. This corroborates the idea that SCR and FPS variables reflect rather distinct aspects of conditioned responses, and can display opposite effects after certain experimental manipulations (Hamm and Weike, 2005; Soeter and Kindt, 2010). At the same time, the absence of strong (time-dependent) effects of cortisol on any other dependent variable also suggests that the present findings are preliminary and require replication. Second, this study used a within-subjects discrimination procedure that likely recruited abilities over and beyond those that are needed for delay or trace conditioning alone (Knight et al., 2004). We cannot exclude the possibility that delay and trace conditioning may influence each other. Further, we only tested men, to exclude unwanted effects of the menstrual cycle; for instance, women are known to display different HPA axis reactivity than men (Kajantie and Phillips, 2006). Further, interactions between sex and stress hormone levels can have important consequences for fear learning and its later expression (Merz et al., 2010; Milad et al., 2010). Indeed, women in the follicle or luteal phase seem to respond to cortisol manipulations in comparable ways as men, while oral contraceptive use enhances differential fear learning on the neural level, in an implicit fear-conditioning paradigm (Merz et al., 2012b). It is important to realize that within the context of etiological models of

anxiety disorders sex specific sensitivity to stressful events has been repeatedly associated with the higher prevalence of mood and anxiety disorders in women (Cahill and McGaugh, 1995; Kessler et al., 1993). Consequently, the omission of women in this study is an important limitation to how these data may be translated to women, or the clinic. Also, this is the first human study to include an extinction and reinstatement phase in a within-subjects trace-delay (or trace alone) conditioning paradigm. Therefore, it is not known what a trace extinction curve typically looks like under basal (e.g., placebo) circumstances. However, analyses of the difference between the CStr and the CS- startle data do show fear retention on startle responses during the first half of extinction in the placebo as well as both cortisol groups. Further, we must note that a full reinforcement schedule during acquisition was used (after one initial unreinforced trial) during acquisition, which may have fastened extinction processes. However, as pilot testing showed that including a partial reinforcement rate hampered fear acquisition itself, we utilized a full reinforcement acquisition schedule. Concerning reinstatement, the present study showed differential reinstatement for both trace and delay conditioning as measured by the US-expectancies, but non-differential reinstatement as measured by FPS or SCR. Non-differential reinstatement has earlier been found in even simpler paradigms (e.g. Dirikx et al., 2009). Thus, most likely, due to the complicated paradigm, the reinstatement procedure was not sensitive enough to detect effects of our subtle manipulation. Finally, a dose of 10 mg hydrocortisone was used, while in this field of research a typical dose of 30 mg is being used (Merz et al., 2012b; Stark et al., 2006; Tabbert et al., 2010). Several studies have shown that experimental effects can alter (or even flip over to the opposite side) depending on the dose (Abercrombie et al., 2003). Hence, dose-response studies may be necessary to get a more complete view on the effects of cortisol on fear memory.

In summary, we show that slow, presumably genomic, effects of cortisol enhance memory for more complex, hippocampus-mediated fear learning, but not for simple amygdala-mediated fear learning. The present findings emphasize that corticosteroids can affect associative fear memory in a time-dependent manner, adding to earlier findings showing time-dependent cortisol effects on other neurobiological processes (Henckens et al., 2011; 2010; 2012a). The insight that cortisol exerts time-dependent effects on associative fear memory has implications for experimental human research into corticosteroid effects on associative fear memory, and perhaps more generally, also on declarative memory research, fields that are often characterized by disparate findings. The majority of studies have typically tested memory performance 30-120 minutes after cortisol administration. Since gene-mediated transcriptional changes are discernible already one hour after cortisol exposure (Morsink

et al., 2006), the majority of current human experimental research has tested in a time window where both genomic and non-genomic processes are active, complicating a straightforward interpretation of results. Clinically, this is very relevant for the development of pharmacological treatments, especially since cortisol has been suggested as a pharmacological add on to cognitive-behavioral intervention in anxiety disorders (de Quervain and Margraf, 2008).

SUPPLEMENTARY MATERIAL

1. SUPPLEMENTARY METHODS

1.1. Data analysis

To assess whether the trace and delay conditioning paradigm was successful, US-expectancy, FPS, and GSR data of the acquisition, extinction and reinstatement phases were subjected to repeated measures ANOVAs with the within-subjects factors Conditioning type (trace vs. delay), CS type (CSdel+, CStr+, CS-) and Trial. If this overall analysis revealed an interaction between those three within-subject factors or an interaction between Conditioning type and CS type, we further disentangled this effect by performing separate analyses for data obtained during CS presentation and trace presentation. In these analyses, within-subject factors are the CS type (CSdel+, CStr+, CS-) and Trial. Only for analysis of GSR data the trace of the CStr+ was omitted, since this point of measurement was still high due to occurrence of the US. A repeated measures ANOVA with the factor Block (average first two trials, versus average last two trials) and CS type assessed successful acquisition or extinction learning. Further, specific planned contrasts were used to assess whether conditioned responses to each individual CS significantly evolved from the first to the last block. Finally, reinstatement effects were tested by running an ANOVA with Block (last block of extinction, first block of reinstatement) and CS type as within-subject factors. Planned contrasts revealed which individual CSs significantly increased from the last extinction block after the reinstating shocks.

2. SUPPLEMENTARY RESULTS

2.1. Manipulation check hydrocortisone

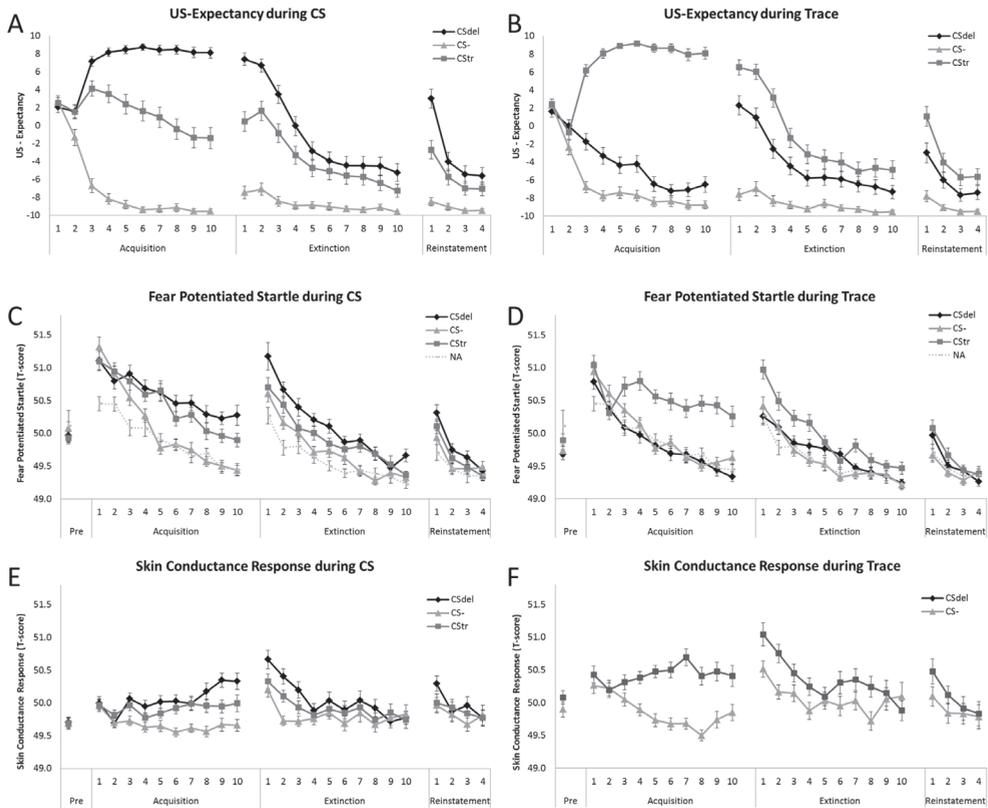
See **Supplementary Table 1** for the manipulation check of hydrocortisone.

2.2. Delay and trace conditioning paradigm

2.2.1. US-expectancy ratings

In **Supplementary Figure 1A** and **1B** US-expectancies during the acquisition, extinction and reinstatement phases of conditioned responses during the CS presentation (1A) and traces (1B) are shown for all subjects.

Acquisition: The overall analysis for acquisition of US-expectancies revealed a Conditioning type \times CS type \times Trial interaction ($F_{18,1026}=46.05$, $p<0.001$, $\eta^2=0.447$). For conditioned responses during the CSs, we observed differential evolvement of the



Supplementary Figure 1. EXP, FPS and SCR data for the delay and trace conditioning paradigm. EXP scores showed successful acquisition, extinction and reinstatement for the CSdel+ during the CS (delay conditioning; A) and for the CStr+ during the trace (trace conditioning; B). On FPS responses, differential acquisition and extinction, but no differential reinstatement was found for both the CSdel- during the CS (delay conditioning; C) and for the CStr- during the trace (trace conditioning; D). Also on SCR successful acquisition and extinction, but no differential reinstatement were observed for the CSdel- during the CS (delay conditioning; E) and for the CStr- during the trace (trace conditioning; F). Error bars represent standard error of the mean (S.E.M.).

three CS types over the course of acquisition (CS type \times Trial interaction, $F_{18,1062}=40.98$, $p<0.001$, $\eta^2=0.410$). Follow-up analyses confirmed differential involvement of the three CS types from the first 2 trials to the last 2 trials of acquisition (CS type \times Block interaction, $F_{2,118}=92.55$, $p<0.001$, $\eta^2=0.611$). Planned contrasts revealed significant acquisition for both the CSdel+ (increases vs. CS- from first to last block; $p<0.001$) and the CStr+ (increases vs. CS- from first to last block; $p<0.001$). Notably, acquisition was stronger for the CSdel+ compared to the CStr+ (increases from first to last block; $p<0.001$).

We also observed differential evolvment of the three CS types over the course of acquisition during the traces (CS type \times Trial interaction, $F_{18,1062}=49.17$, $p<0.001$, $\eta^2=0.455$). Follow-up analyses confirmed differential evolvment of the three CS types from the first 2 trials to the last 2 trials of acquisition (CS type \times Block interaction, $F_{2,118}=180.90$, $p<0.001$, $\eta^2=0.721$). Planned contrasts revealed significant acquisition only for the CStr+ (increases vs. CS- from first to last block; $p<0.001$), but not for the CSdel+ (increases vs. CS- from first to last block; *n.s.*).

Extinction: The overall analysis for extinction of US-expectancies revealed a Conditioning type \times CS type \times Trial interaction ($F_{18,1008}=7.54$, $p<0.001$, $\eta^2=0.119$). For conditioned responses during the CSs, we observed a differential evolvment of the three CS types over the course of extinction (CS type \times Trial interaction, $F_{18,1044}=18.62$, $p<0.001$, $\eta^2=0.243$). Follow-up analyses confirmed differential evolvment of the three CS types from the first 2 trials to the last 2 trials of extinction (CS type \times Block interaction, $F_{2,116}=42.68$, $p<0.001$, $\eta^2=0.424$). Planned contrasts revealed significant extinction for both the CSdel+ (decreases vs. CS- from first to last block; $p<0.001$) and the CStr- (decreases vs. CS- from first to last block; $p<0.001$). Notably, extinction was stronger for the CSdel- compared to the CStr- (decreases from first to last block; $p=0.002$).

Furthermore, for conditioned responses during the traces we observed a differential evolvment of the three CS types over the course of extinction (CS type \times Trial interaction, $F_{18,1044}=17.28$, $p<0.001$, $\eta^2=0.230$). Follow-up analyses confirmed differential evolvment of the three CS types from the first 2 trials to the last 2 trials of extinction (CS type \times Block interaction, $F_{2,116}=43.38$, $p<0.001$, $\eta^2=0.428$). Planned contrasts revealed significant extinction for both the CStr- (decreases vs. CS- from first to last block; $p<0.001$) and the CSdel- (decreases vs. CS- from first to last block; $p<0.001$). Notably, extinction was stronger for the CStr- compared to the CSdel- (decreases from first to last block; $p=0.018$).

Reinstatement: The overall analysis for reinstatement of US-expectancies revealed a Conditioning type \times CS type \times Trial interaction ($F_{6,330}=9.60$, $p<0.001$, $\eta^2=0.149$).

Supplementary Table 1. Descriptive statistics (mean and standard error) of the raw salivary cortisol data (nmol/l) at the different time points throughout the experiment.

		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Rapid Cort	mean	12.83	11.61	8.29	6.21	5.19	6.53	259.41	112.65	68.84	9.38
	S.E.	1.29	1.15	0.68	0.47	0.38	0.51	77.30	77.29	16.31	0.96
Slow Cort	mean	13.68	13.25	102.86	68.03	35.41	9.86	7.97	5.83	4.51	9.61
	S.E.	1.11	1.16	15.72	6.25	2.53	0.86	0.61	0.45	0.30	0.75
Placebo	mean	13.52	13.70	9.09	6.59	6.30	6.43	7.15	5.94	5.80	9.49
	S.E.	1.51	1.58	0.93	0.77	1.23	0.80	0.82	0.71	0.62	1.24

Reinstatement for conditioned responses during the CSs was successful, as indicated by differential increases in US-expectancies from the last 2 trials of extinction to the first 2 trials of testing after reinstatement (CS type \times Block interaction, $F_{2,114} = 12.18$, $p < 0.001$, $\eta^2 = 0.176$). Planned contrasts revealed significant reinstatement for both the CSdel+ (increases vs. CS- from first to last block; $p < 0.001$) and the CStr- (increases vs. CS- from first to last block; $p = 0.001$). Notably, there was a trend for stronger reinstatement for the CSdel- compared to the CStr- (decreases from first to last block; $p = 0.064$).

Reinstatement for conditioned responses during the traces was successful as well, indicated by differential increases in US-expectancies from the last 2 trials of extinction to the first 2 trials of testing after reinstatement (CS type \times Block interaction, $F_{2,114} = 7.41$, $p = 0.001$, $\eta^2 = 0.115$). Planned contrasts revealed significant reinstatement for both the CStr- (increases vs. CS- from first to last block; $p = 0.002$) and the CSdel- (increases vs. CS- from first to last block; $p < 0.001$). However, there was no difference in reinstatement for the CStr- and the CSdel- (*n.s.*).

2.2.2. Fear potentiated startle

In **Supplementary Figure 1C** and **1D** fear potentiated startle responses during the acquisition, extinction and reinstatement phases of conditioned responses during the CSs (1C) and traces (1D) are shown for all subjects.

Acquisition: The overall analysis for acquisition of fear potentiated startle responses revealed a Conditioning type \times CS type \times Trial interaction ($F_{18,1026} = 2.81$, $p < 0.001$, $\eta^2 = 0.047$). For conditioned responses during the CSs, we observed a differential evolvment of the three CS types over the course of acquisition (CS type \times Trial interaction, $F_{18,1062} = 2.74$, $p < 0.001$, $\eta^2 = 0.055$). Follow-up analyses confirmed differential evolvment of the three CS types from the first 2 trials to the last 2 trials of acquisition (CS type \times Block interaction, $F_{2,118} = 15.00$, $p < 0.001$, $\eta^2 = 0.203$). Planned contrasts revealed significant acquisition for both the CSdel+ (increases vs. CS- from first to last block; $p < 0.001$) and the CStr+ (increases vs. CS- from first to last block; $p = 0.005$). Notably, there was stronger acquisition for the CSdel+ compared to the CStr+ (increases from first to last block; $p = 0.021$).

For conditioned responses during the traces, we also observed a differential evolvment of the three CS types over the course of acquisition (CS type \times Trial interaction, $F_{18,1062} = 2.88$, $p < 0.001$, $\eta^2 = 0.064$). Follow-up analyses confirmed differential evolvment of the three CS types from the first 2 trials to the last 2 trials of acquisition (CS type \times Block interaction, $F_{2,118} = 18.56$, $p < 0.001$, $\eta^2 = 0.239$). Planned contrasts revealed significant acquisition only for the CStr+ (increases vs. CS- from first to last block; $p < 0.001$), but not for the CSdel+ (increases vs. CS- from first to last block; *n.s.*).

Extinction: The overall analysis for extinction of fear potentiated startle responses revealed a Conditioning type \times CS type \times Trial interaction ($F_{18,1008}=1.47$, $p=0.029$, $\eta^2=0.026$). For conditioned responses during the CSs, no CS type \times Trial interaction was found ($F=0.91$, *n.s.*). In analyses on the first 2 trials and last 2 trials however, a marginally significant CS type \times Block interaction was found ($F_{2,116}=2.21$; $p=0.095$). Planned contrasts did reveal significant extinction only for the CSdel- (decreases vs. CS- from first to last block; $p=0.028$), but not for the CStr- (decreases vs. CS- from first to last block; *n.s.*).

For conditioned responses during the traces, differential involvement of the three CS types over the course of extinction was found (CS type \times Trial interaction, $F_{18,1044}=1.61$, $p=0.050$, $\eta^2=0.027$). Follow-up analyses on the first 2 trials and the last 2 trials also showed a CS type \times Block interaction ($F_{2,116}=2.72$, $p=0.044$, $\eta^2=0.051$). Planned contrasts revealed significant extinction only for the CStr- (decreases vs. CS- from first to last block; $p=0.049$), but not for the CSdel- (decreases vs. CS- from first to last block; *n.s.*).

Reinstatement: The overall analysis for reinstatement of fear potentiated startle responses revealed a Conditioning type \times CS type \times Trial interaction ($F_{6,330}=0.27$, $p=0.010$, $\eta^2=0.003$). For conditioned responses during the CSs, analysis of reinstatement (first 2 trials of reinstatement test vs. last 2 trials of extinction) did not show a CS type \times Block interaction ($F=0.47$, *n.s.*), but overall startle responses increased after the reinstatement procedure, as evidenced by a main effect of Block ($F_{1,57}=27.16$, $p<0.001$, $\eta^2=0.323$).

For conditioned responses during the traces, no CS type \times Block interaction ($F=1.70$, *n.s.*) was found, but overall startle responses increased after the reinstatement procedure, as evidenced by a main effect of Block ($F_{1,57}=40.86$, $p<0.001$, $\eta^2=0.418$).

2.2.3. Skin conductance responses

In **Supplementary Figure 1E** and **1F** skin conductance responses during the acquisition, extinction and reinstatement phases of conditioned responses during the CSs (1E) and traces (1F) are shown for all subjects.

Acquisition: The overall analysis for acquisition of skin conductance responses revealed a Conditioning type \times CS type \times Trial interaction ($F_{18,1008}=10.88$, $p<0.001$, $\eta^2=0.150$). For conditioned responses during CSs, we observed a differential involvement of the three CS types over the course of acquisition (CS type \times Trial interaction, $F_{18,1008}=4.14$, $p<0.001$, $\eta^2=0.059$). Follow-up analyses confirmed differential involvement of the three CS types from the first 2 trials to the last 2 trials of acquisition (CS type \times Block interaction, $F_{2,116}=10.45$, $p<0.001$, $\eta^2=0.161$). Planned contrasts revealed

significant acquisition only for the CSdel+ (increases vs. CS- from first to last block; $p < 0.001$), but not for the CStr+ (increases vs. CS- from first to last block; *n.s.*).

For conditioned responses during the traces, we observed a differential involvement of the CStr+ and the CS- over the course of acquisition (CS type \times Trial interaction, $F_{9,508} = 7.47$, $p < 0.001$, $\eta^2 = 0.105$). Follow-up analyses revealed significant acquisition for the CStr+ (increases vs. CS- from first 2 trials to the last 2 trials; CS type \times Block interaction, $F_{1,62} = 9.30$, $p = 0.004$, $\eta^2 = 0.130$).

Extinction: The overall analysis for extinction of skin conductance responses revealed no Conditioning type \times CS type \times Trial interaction ($F_{18,1008} = 1.07$, *n.s.*). However, a Conditioning type \times CS type interaction was found ($F_{2,116} = 10.02$, $p < 0.001$, $\eta^2 = 0.139$). For conditioned responses during the CSs, no CS type \times Trial interaction was found ($F = 1.07$, *n.s.*). In follow-up analyses on the first and last block however, we did find a CS type \times Block interaction ($F_{2,116} = 5.51$, $p = 0.005$, $\eta^2 = 0.082$), indicating differential involvement of the three CS types from the first 2 trials to the last 2 trials of extinction. Planned revealed significant extinction only for the CSdel- (decreases vs. CS- from first to last block; $p = 0.003$), but not for the CStr- (decreases vs. CS- from first to last block; *n.s.*).

For conditioned responses during the traces, no CS type \times Trial interaction was found ($F = 1.39$; *n.s.*). In analyses on the first 2 trials and last 2 trials however, significant extinction of the CStr- was found (decreases vs. CS- from first 2 trials to the last 2 trials; CS type \times Block interaction, $F_{1,57} = 5.46$, $p = 0.026$, $\eta^2 = 0.086$).

Reinstatement: The overall analysis for reinstatement of skin conductance responses revealed no Conditioning type \times CS type \times Trial interaction ($F_{6,330} = 0.57$, *n.s.*). However, a Conditioning type \times CS type interaction was found ($F_{6,330} = 8.27$, $p < 0.001$, $\eta^2 = 0.121$). For conditioned responses during the CSs, analysis of reinstatement (first 2 trials of reinstatement test vs. last 2 trials of extinction) did not show a CS type \times Block interaction ($F = 0.56$, *n.s.*), but overall startle responses increased after the reinstatement procedure, as evidenced by a main effect of Block ($F_{1,57} = 5.76$, $p = 0.024$, $\eta^2 = 0.089$).

For conditioned responses during the traces, a trend was shown for reinstatement of the CStr+ (increases vs. CS- from first 2 trials of reinstatement test vs. last 2 trials of extinction; CS type \times Block interaction, $F_{1,57} = 3.17$, $p = 0.087$, $\eta^2 = 0.057$).

2.3. Rapid and slow corticosteroid effects

2.3.1. US-expectancy ratings

Acquisition: Analysis of delay and trace acquisition data did not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.79$, *n.s.*),

nor a main effect of Condition ($F=0.28$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace acquisition of US-expectancies.

Extinction: Analysis of delay and trace extinction data did not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.94$, *n.s.*), nor a main effect of Condition ($F=0.90$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace extinction of US-expectancies.

Reinstatement: Regarding reinstatement, analysis of delay and trace data did also not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.89$, *n.s.*), nor a main effect of Condition ($F=0.69$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace reinstatement of US-expectancies.

2.3.2. Skin conductance responses

Cortisol did not affect SCR responses during the CSs when pre-conditioning trials before pill intake were compared with the first (un-reinforced) trials of acquisition, as evidenced by the absence of a Condition \times Pre-Post \times CS Type \times Conditioning type interaction ($F=0.74$; *n.s.*), and no other interaction effects involving Condition and Pre-Post ($F_s < 2.36$, *n.s.*).

Acquisition: Analysis of delay and trace acquisition data did not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.98$, *n.s.*), nor a main effect of Condition ($F=0.79$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace acquisition of SCR.

Extinction: Analysis of delay and trace extinction data did not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 1.58$, *n.s.*), nor a main effect of Condition ($F=2.61$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace extinction of SCR.

Reinstatement: Regarding reinstatement, analysis of delay and trace data did also not show any interaction effects involving Condition, CS type and Conditioning type ($F_s < 0.42$, *n.s.*), nor a main effect of Condition ($F=1.96$, *n.s.*). Together, this suggests that the cortisol manipulation did not affect delay- and trace reinstatement of SCR.