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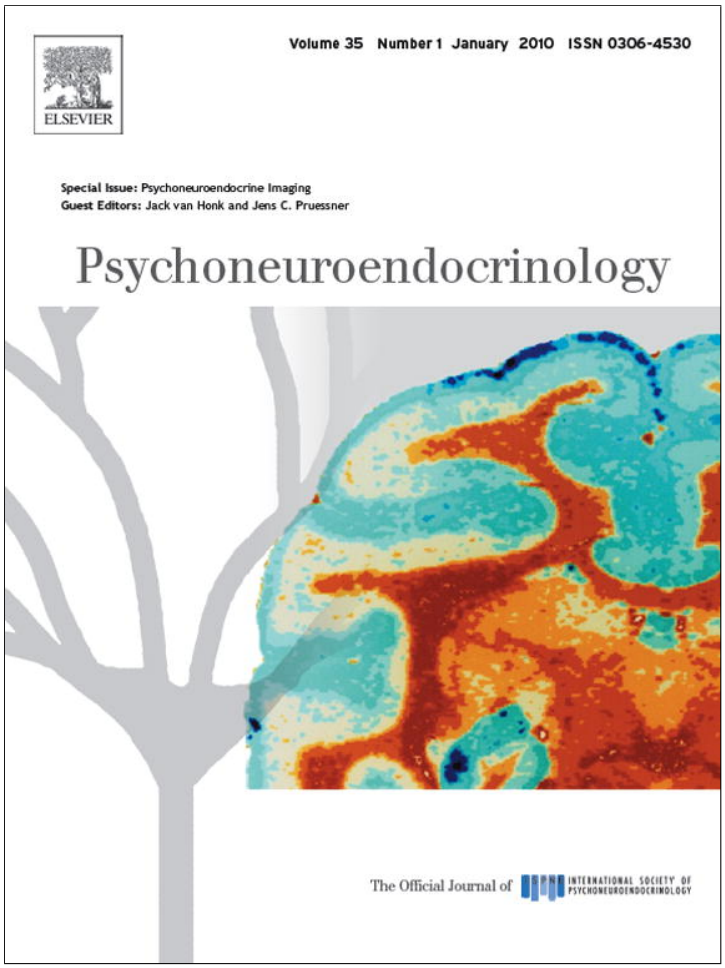

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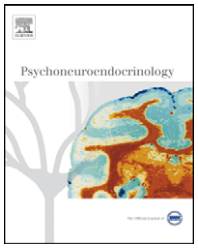

Investigating the impact of sex and cortisol on implicit fear conditioning with fMRI

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Summary Fear conditioning is influenced by stress but opposing effects in males and females have often been reported. In a previous human functional magnetic resonance imaging (fMRI) study, we observed acute effects of the stress hormone cortisol on prefrontal structures. Men showed evidence for impaired fear conditioning after cortisol treatment, while the opposite pattern was found for women. In the current experiment, we tested whether similar sex-dependent effects would occur on the neural level if contingency awareness was prevented experimentally to investigate implicit learning processes. A differential fear conditioning experiment with transcutaneous electrical stimulation as unconditioned stimulus and geometric figures as conditioned stimuli (CS) was conducted. One figure was always paired (CS+), whereas the other (CS−) was never paired with the UCS. Thirty-nine (19 female) subjects participated in this fMRI study, receiving either placebo or 30 mg cortisol (hydrocortisone) before conditioning. Dependent variables were skin conductance responses (SCRs) and neural activity (BOLD signal). In line with prior findings in unaware participants, no differential learning could be observed for the SCRs. However, a sex × cortisol interaction was detected with a reduced mean response to the CS after cortisol treatment in men, while the opposite pattern was observed in women (enhanced mean SCR under cortisol). In the contrast CS+ minus CS−, neural activity showed a sex × cortisol interaction in the insula and further trends in the hippocampus and the thalamus. In these regions, cortisol reduced the CS+/CS− differentiation in men but enhanced it in women. In contrast to these sex specific effects, differential amygdala activation was found in the placebo group but not in the cortisol group, irrespective of sex. Further, differential neural activity in the amygdala and thalamus were positively correlated with the SCRs in the placebo group only. The present study in contingency unaware participants illustrates that cortisol has in some brain regions sex specific effects on neural correlates of emotional learning. These effects might translate into a different vulnerability of the two sexes for anxiety disorders.
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1. Introduction

The acquisition of stress-associated anxiety disorders has been subject to vast research efforts for several decades. Among other factors, this research has identified sex and implicit learning processes as important modulators for these disorders (Armony and LeDoux, 1997; Elzinga and Bremner, 2002; Nemeroff et al., 2006). Despite this fact, the interaction between stress and sex on emotional learning processes has not sufficiently been studied in humans so far. A well-established paradigm to investigate fear acquisition is classical conditioning. As implicit fear learning has been shown to be of specific interest in the acquisition of anxiety disorders, e.g., posttraumatic stress disorder (Armony and LeDoux, 1997; Mineka and Zinbarg, 2006), implicit fear conditioning is a fruitful approach to add to this line of research.

In general, acute stress or cortisol treatment can influence learning and memory processes (Het et al., 2005; Wolf, 2008; van Stegeren, 2009). The underlying mechanism is the stress-induced activation of the hypothalamus-pituitary-adrenal (HPA) axis with a resulting glucocorticoid (GC; cortisol in humans, corticosterone in rats) release and several other hormones such as corticotropin-releasing factor (CRF) or (nor)epinephrine (Joëls and Baram, 2009). The subsequent modulation of memory is due to the released GCs occupying central nuclear mineralocorticoid receptors (MRs) as well as glucocorticoid receptors (GRs). Recently, the membrane bound MR was linked to rapid nongenomic effects in the initial stress reaction possibly accounting for stress-related memory modulation (Diamond et al., 2007; Joëls et al., 2008; Roozendaal et al., 2009; Wolf, 2009).

Further, concerning stress effects on learning and memory, two distinct memory systems can be distinguished: first, declarative or explicit memory with the conscious recall of events and facts and second, nondeclarative or implicit memory mediating, e.g., sensitization, habituation, and conditioning without conscious thought (Milner et al., 1998). Within both systems the strength of the memory trace is influenced by the emotionality of the material and by acute stress (van Stegeren, 2008; Wolf, 2008). An enhanced learning of emotional compared to neutral material (Cahill and McGaugh, 1995, 1996; LaBar and Cabeza, 2006) is related to the activation of the amygdala resulting from a noradrenergic input from the brain stem into the basolateral nucleus (Cahill et al., 1994; McGaugh et al., 1996; Canli et al., 2000; Southwick et al., 2002). Acute stress can further potentiate the enhancing effect on consolidation and the impairment of retrieval of emotional material (Buchanan and Lovallo, 2001; Abercrombie et al., 2003; Kuhlmann et al., 2005; Buchanan et al., 2006; van Stegeren et al., 2006). This effect is due to activity of GCs on GRs and MRs in the neurons of the limbic system (Reul and de Kloet, 1985; Herman et al., 2003), particularly in the hippocampus or in the amygdala (de Leon et al., 1997; Roozendaal, 2002; de Quervain et al., 2003; Oei et al., 2007). To sum, stress is a potent modulator of several aspects of memory, especially regarding the processing of emotional material (Roozendaal et al., 2006b; Wolf, 2008; van Stegeren, 2009).

A well established paradigm to investigate emotional learning and memory processes is the above-mentioned classical fear conditioning. Thereby, the presentation of a conditioned stimulus (CS+; e.g., a geometric figure, a former neutral stimulus) is paired with an aversive event (e.g., an electrical stimulation; unconditioned stimulus, UCS), whereas another stimulus (CS−) is not paired with the UCS. Differential responses towards the CS+ and the CS− can then be used as an index of learning. Several brain regions were shown to be involved in fear acquisition, expression and emotional stimulus evaluation such as subcortical (amygdala, thalamus, hippocampus), cortical (anterior cingulate, medial prefrontal cortex, orbitofrontal cortex), and sensory processing structures as the insula, and the sensory cortex (LeDoux, 1995, 2000; Knight et al., 1999, 2004a,b; Rolls, 1999; Büchel and Dolan, 2000; Öhman, 2005; Tabbert et al., 2005). However, the involvement of these neural structures in classical fear conditioning has been shown to be modulated by contingency awareness, i.e., the cognitive representation of the contingency between UCS and CS (e.g., Tabbert et al., 2006; Klucken et al., 2009a,b). Furthermore, backward masking studies that prevent conscious stimulus processing demonstrate enhanced amygdala activation to masked emotional or fear conditioned stimuli (Whalen, 1998; Morris et al., 1998, 2001). Amygdala activation was also found in a group that was experimentally prevented from detecting the contingencies between supraliminal CS and UCS (Tabbert et al., 2006). On the other hand, conditioned skin conductance responses (SCRs) have been reliably shown in contingency aware subjects only (Hamm and Vaitl, 1996; Hamm and Weike, 2005; Tabbert et al., 2006; Weike et al., 2007; Klucken et al., 2009a). However, some studies found conditioned SCRs even in contingency unaware participants (Esteves et al., 1994; Morris et al., 2001; Öhman and Mineka, 2001; Knight et al., 2009).

The basal ganglia (especially the striatum) are also supposed to be involved in implicit learning and memory, e.g., skill and habit learning or instrumental and classical conditioning (Gabrieli, 1998; Packard and Knowlton, 2002; Doyon et al., 2003; Pessiglione et al., 2008; Belin et al., 2009). However, this has to be differentiated from fear conditioning without contingency awareness and using supraliminal CS (in contrast to subliminal CS presentations). In the ventral striatum, we previously observed differential neural activity only in those participants who actually learned the contingency between CS and UCS in the context of fear conditioning (Klucken et al., 2009c). Thus, the ventral striatum may be involved in contingency learning rather than in implicit learning or memory per se.

One further factor that has been shown to influence such learning and memory processes is sex and its resulting functional and structural dimorphisms (Cahill, 2003, 2006; Dalla and Shors, 2009). Studies in rodents investigating male rats showed that stress or GC application enhanced conditioned responses (Beylin and Shors, 2003; Conrad et al., 2004; Shors, 2004; Thompson et al., 2004), whereas diminished conditioned responses were observed in females (Wood and Shors, 1998; Shors, 2004). To date, only few human studies have been conducted investigating the interaction between cortisol and sex on conditioned responses. Zorawski et al. (2005, 2006) report enhanced fear acquisition or a positive correlation between basal cortisol concentrations and acquisition in men only. The same result pattern was found by Jackson et al. (2006) after psychosocial stress. In the first fMRI experiment on this topic, however, we observed the opposite picture (Stark et al., 2006). In a differential fear conditioning paradigm with a previous pharmacological cortisol treatment,

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women showed an enhanced fear acquisition after GC application. In contrast, conditioned reactions were impaired in men after the intake of cortisol in comparison to placebo. This was reflected in a differential response of several prefrontal structures. Furthermore, a modulation by cortisol and sex was observed in SCRs providing enhanced differentiation between CS+ and CS− in men under placebo that was blocked after GC treatment. However, in this experiment almost 60% of the participants became aware of the relationship between CS and UCS, whereas the rest did not, which could have influenced the results.

To investigate the impact of acute GC effects and sex on implicit fear acquisition, we conducted a differential conditioning experiment with an electrical stimulation (UCS) that always followed the CS+, whereas the CS− was presented alone. Additionally, a distractor working memory task and a distractor figure were introduced to prevent participants from detecting the contingencies between the CS and UCS (cf. Tabbert et al., 2006). Prior to the conditioning procedure, half of the participants received an oral dose of hydrocortisone to induce elevated cortisol concentrations. As dependent variables, we measured SCRs and blood oxygenation level dependent (BOLD) responses via fMRI.

Concerning the SCRs we hypothesized that, in line with prior studies from this laboratory (Tabbert et al., 2006; Klucken et al., 2009a), no differentiation between CS+ and CS− would occur, since all participants were contingency unaware. Nevertheless, the influences of sex and cortisol are explored in the SCRs. We expected differences in neural activity (BOLD signal), in particular an interaction between sex and cortisol for the contrast CS+ minus CS− primarily in the insula and in subcortical structures like the amygdala, the hippocampus, and the thalamus. Based on our previous findings (Stark et al., 2006), we predicted that cortisol would reduce the CS+/CS− differentiation in men, while enhancing them in women in one of the mentioned structures and additionally in the frontal cortex. These activations can be independent of each other.

2. Materials and methods

2.1. Subjects

A total of 48 subjects completed the study, which was approved by the ethics committee of the German Psychological Society. All participants were university students (one already finished his diploma) who had been recruited via announcements at bulletin boards at the campus. None of them was taking regular medication or had a history of any psychiatric or neurological treatment. Exclusion criteria were somatic and in particular endocrine diseases that can have an impact on hormonal concentrations (e.g., acute asthma, hypo- or hyperthyroidism). All participants were right-handed as assessed by the Edinburgh Inventory of Handedness (Oldfield, 1971) and had normal or corrected-to-normal vision. Inclusion criteria were age between 19 and 35 and a body mass index (BMI = kg/m^2) between 18 and 26. Free-cycling women should report having a regular menstrual cycle and were invited in the luteal phase of their individual menstrual cycle (days 3—9 before the onset of their next menstruation; Buffet et al., 1998). Women taking oral contraceptives were required to have been taking their birth control pill (only monophasic preparations) at least during the last 3 months and were tested during the ''on phase'' of pill intake. All subjects were instructed to refrain from any caffeine, food intake, and smoking 2 h before the experiment.

At the beginning, participants received a detailed explanation of the procedure in general (the conditioning schedule was of course not explained until the experiment was finished). Written informed consent was obtained. The cover story to conceal the conditioning procedure was the investigation of the impact of cortisol and several distractors (including an electrical stimulation) on memory performance. After finishing the experiment, participants were debriefed about the purpose of the study and received 25 Euros for their participation.

The conditioning experiment consisted of one acquisition, one extinction phase, and an implemented continuing two-back task. Nine subjects became aware of the contingencies and were excluded from further analyses. They were selectively replaced in the respective groups. Thus, the final sample consisted of 39 participants subdivided into four groups: cortisol women (n = 10), placebo women (n = 9), cortisol men (n = 10), and placebo men (n = 10). The mean age was 23.2 years (SD = 2.5) with no significant differences between the four groups with respect to age. The same was true for BMI, with an entire mean BMI of 22.3 (SD = 2.0). Half of the women in the respective groups were taking oral contraceptives, whereas the other half were free-cycling.

This experiment is part of a larger (and still ongoing) study investigating the effects of cortisol on fear acquisition and extinction with respect to contingency awareness and sex differences. The current manuscript only reports the findings for acquisition of participants from the unaware group, which was tested during the first recruitment wave.

2.2. Conditioned visual stimuli

Two simple geometric figures (a square and a rhombus) served as CS+ and CS−. A triangle was further used to serve as distractor stimulus (non-CS), occurring half as often as the CS− only. The three stimuli had identical luminescence, were grey in color and presented with a duration of 8 s, respectively. Visual stimulation inside the scanner was realized with an LCD projector (model EPSON EMP-7250), which projected pictures onto a screen at the end of the scanner (visual field = 18°). A mirror mounted to the head coil allowed the subjects to look at the screen.

2.3. Unconditioned stimulus (UCS)

A custom-made impulse-generator (833 Hz) provided transcutaneous electrical stimulation to the middle of the left shin through two Ag/AgCl electrodes (1 mm surface each), that was applied as the UCS triggered via an optic fibre cable.

Stimulus intensity was set for each participant individually using a gradually increasing rating procedure to attain an ''unpleasant but not painful'' level of sensation. The electrical stimulation was applied for 100 ms during the conditioning procedure, the duration as well as the onset of the UCS was set by a computer program.

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2.4. Two-back task

This task was included to prevent subjects from detecting the relationship between CS and UCS as has been done before (Carter et al., 2003; Tabbert et al., 2006). Numbers ranging from 1 to 5 were presented sequentially on the screen for 1 s, interspersed in the presentation of the geometric figures. After each number, participants had to indicate whether it was the same or a different number as the number before the last one by pressing one of two buttons. In the acquisition phase, there were 50 numbers in total, 12 were identical (targets) and 38 different (non-targets) to the number before the last one. During extinction, 6 targets and 21 non-targets had to be rated. The order was the same as in the first half of the acquisition excluding the first two numbers (always non-targets).

Performance on the two-back task (percentage of correct responses) with possible differences between the four groups was tested with SPSS for Windows (Release 17.0, SPSS Inc., Illinois) via analysis of variance (ANOVA) with the between subjects factor group (placebo women, cortisol women, placebo men, cortisol men). One placebo women deviated from the two-back instruction and considered every second number only, so she was excluded from this analysis.

2.5. Conditioning procedure

The conditioning procedure was adapted from prior studies in our laboratory (Tabbert et al., 2005, 2006; Stark et al., 2006) with an additional extinction phase. There were 20 trials of CS+ as well as CS− and 10 trials of non-CS presentations throughout the acquisition phase. During extinction, 11 trials of CS+ and CS− were presented and five trials of non-CS. The first CS+ and CS− trial (as well as the first two numbers) in the extinction phase were excluded from analyses, since learning could not have yet occurred (cf. Phelps et al., 2004). Inter-trial intervals between the numbers and the geometrical figures ranged from 5 to 7.5 s (random jitter between 0 and 2.5 s). Correspondingly, the inter-trial intervals between the CS ranged from 11 to 16 s. The onset of the UCS presentation started 7.9 s after CS+ onset and co-terminated with CS+ offset (delay conditioning; 100% reinforcement). Non-UCS was defined as the UCS omission after the CS− in a time window corresponding to UCS application after the CS+ (i.e., 7.9 s after CS− onset). The CS− and the non-CS were never paired with the UCS and no further CS+ pairing occurred in the extinction phase. The conditioning procedure started with a CS+ for half of the subjects and a CS− for the other half and either square or rhombus served as CS+ or CS−.

For each participant, pseudo-randomized stimulus orders were used comprising the following restrictions: no more than two consecutive presentations of the same CS, no more than three consecutive identical numbers, an equal distribution for any number before or after CS+ trials to avoid conditioning to any of the numbers, and an equal quantity of CS+ and CS− trials within the first and the second half of the experiment (10 each).

2.6. Contingency awareness

After the acquisition phase, participants had to rate the contingencies for the CS+, CS− and non-CS, which were presented in random order. Next to the picture of the respective CS, the question was always: "Please estimate how often the electrical stimulation succeeded the following geometrical figure" with the answer possibilities: "I do not know", "never", "sometimes" and "always". A short recognition questionnaire like this has been shown to be the most sensitive and valid method to assess contingency awareness (Dawson and Reardon, 1973). At the end of the experiment, a questionnaire and a short interview were used to further validate subjects' awareness. Participants were classified as (at least partially) contingency aware if they stated that the CS− was never followed by the electrical stimulation, whereas the CS+ always (or sometimes) precedes the UCS. Subjects who recognized the correct relationship between the CS and UCS ($n = 9$) were not further analyzed because of their known effects on several response levels (Hamm and Vaitl, 1996; Tabbert et al., 2006; Klucken et al., 2009a,b). They were selectively replaced in the respective groups, so the remaining sample fully consisted of 39 contingency unaware participants.

2.7. Treatment and cortisol analyses

This study was conducted as a double-blind, randomized and placebo-controlled experiment. Ten women and 10 men received three 10 mg tablets of cortisol (30 mg hydrocortisone; Hoechst) 45 min before the start of the functional scans for conditioning. Visually identical placebos (tablettose and magnesium) were given to the other participants. In previous studies, it was found that this cortisol dose could influence explicit memory (Wolf et al., 2001; Kuhlmann et al., 2005) and fear conditioning (Stark et al., 2006). Each experiment started between 14:00 and 17:00 h to control for the circadian cortisol rhythm with its different occupation of MRs and GRs (Lupien et al., 2002, 2007).

Saliva samples for the analysis of free cortisol were collected from the participants by use of glass tubes. Samples were taken directly before, 25 min (before the fMRI run), and 90 min after the treatment (after the fMRI run). Immediately after sampling, the saliva was stored at −20 °C until assayed. Saliva cortisol was determined by use of a commercial enzyme immunoassay (IBL, Hamburg, Germany). All samples were analyzed within one lot and in duplicates. Inter-assay coefficients of variations (CV) were below 6% with an inter-assay CV below 10%.

Statistical analyses were conducted in SPSS for Windows via ANOVA with the repeated measurement factor time and the between subjects factors sex (women vs. men) and treatment (cortisol vs. placebo). In the case of violation of the sphericity assumption, Greenhouse-Geisser correction was applied.

After the experiment, participants were asked to give a treatment guess with the possible answers "placebo", "hydrocortisone" or "no idea". The McNemar test using binomial distribution including the answers "placebo" and "cortisol" only was performed to check if subjects were somehow aware of their treatment.

2.8. Skin conductance responses

Skin conductance responses (SCRs) were sampled simultaneously with fMRI scans using Ag/AgCl electrodes filled with

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isotonic (0.05 M NaCl) electrolyte medium, placed hypothenar at the non-dominant hand. SCRs were defined in three analysis windows (cf. Prokasy and Ebel, 1967): the maximum response within a window of 1—5 s after the CS onset was counted as the first interval response (FIR), within the time window of 5—8.5 s as the second interval response (SIR), and within the time window of 8.5—13 s as the unconditioned response (UCR). Conditioned responses were defined as larger response magnitudes in reaction to the CS+ than to the CS− in the FIR and SIR.

The data were transformed with the natural logarithm in order to render the distribution more towards normal. Statistical comparisons were performed via ANOVA in a 2 (CS-type: CS+ and CS− for the FIR and SIR; UCS and non-UCS for the UCR) × 20 (trial) factorial design within the general linear model as it is implemented in SPSS for Windows. Sex (women vs. men) and treatment (cortisol vs. placebo) were introduced as between subjects factors. Greenhouse-Geisser correction was applied when sphericity assumption was not met. Post hoc ANOVA were performed for significant interactions. Electrodermal data of one woman in the cortisol group and one man in the placebo group had to be discarded because of technical problems.

2.9. Magnetic resonance imaging

Brain images were acquired using a 1.5 T whole-body tomograph (Siemens Symphony with a quantum gradient system) with a standard head coil. Structural image acquisition consisted of 160 T1-weighted sagittal images (magnetization-prepared, rapid acquisition gradient echo sequence, 1 mm slice thickness). For functional imaging, a total of 750 volumes (480 for the acquisition and 270 for the extinction phase) were registered using a T2*-weighted gradient echo-planar imaging sequence with 25 slices covering the whole brain (slice thickness = 5 mm; 1 mm gap; descending slice order; TA = 100 ms; TE = 55 ms; TR = 2.5 s; flip angle = 90°; field of view = 192 mm × 192 mm; matrix size = 64 × 64). The first three volumes were discarded due to an incomplete steady state of magnetisation. The orientation of the axial slices was parallel to the orbitofrontal cortex—bone transition in order to minimise susceptibility artifacts in prefrontal areas. A gradient echo field map sequence was measured before the functional run to get information for unwarping B_0 distortions.

Data were analyzed using Statistical Parametric Mapping (SPM5, Wellcome Department of Cognitive Neurology, London, UK, 2005) implemented in MatLab R2007b (Mathworks Inc., Sherborn, MA). Realignment (2nd degree b-spline interpolation to the first image) and unwarping, slice time correction (reference slice: 13), coregistration of functional data to each participant's anatomic image, and normalisation to the standard space of the Montreal Neurological Institute (MNI) brain were performed. Spatial smoothing was executed with an isotropic three-dimensional Gaussian filter with a full width at half maximum of 9 mm to allow for corrected statistical inference. The acquisition and extinction were integrated as separate sessions in one model, including the following experimental conditions in each case: CS+, CS−, non-CS, UCS, non-UCS, target, and non-target (excluding UCS and non-UCS for the extinction). The linear temporal trend of the CS+, CS−, non-CS, UCS, and non-UCS were added as regressors in our statistical design to account for possible habituation or sensitization effects. An additional regressor was introduced containing the first two numbers and the first two geometrical figures of the extinction. These regressors were modeled by a stick function convolved with the canonical hemodynamic response function in the general linear model, without specifically modeling the durations of the different events. The six movement parameters of the rigid body transformation, applied by the realignment procedure, were introduced as covariates in the model separately for the acquisition and extinction phase. The voxel-based time series were filtered with a high pass filter (time constant = 128 s).

For the statistical analyses, we used explorative whole brain as well as region of interest (RoI) analyses to enhance the statistical power. As we investigated implicit fear conditioning, the following subcortical structures were included as RoIs: the amygdala, the hippocampus, and the thalamus. Based on prior reports in unaware participants and concerning the sex × cortisol interaction, we added the insula and the extended frontal cortex[1] as further RoI (Stark et al., 2006; Klucken et al., 2009a). The required masks for these analyses were designed using the software-program MARINA (Walter, 2002), besides, significant peak voxels within the frontal RoI were labeled with MARINA. Regressors of interest were CS+, CS−, CS+ by time, CS− by time, UCS and non-UCS. Statistical analyses were done in a random effects design and focused on the contrasts CS+ minus CS−, CS+ by time minus CS− by time, and UCS minus non-UCS. Since the time-modulated BOLD responses towards the CS and UCS were added as regressors, the reported contrast effects for CS+ minus CS− and UCS minus non-UCS are independent of linear increases or decreases. ANOVA was conducted with the group factors treatment (placebo vs. cortisol) and sex (women vs. men) in the flexible factorial model implemented in SPM5. The CS analyses (i.e., CS+ minus CS−, CS+ by time minus CS− by time) were conducted for the separate groups and the respective group comparison (women vs. men, placebo vs. cortisol) as well as the interaction between sex and treatment. Reactions towards the UCS minus non-UCS were analyzed only in the entire group as well as with respect to differences between the groups without the RoI in the frontal cortex.

In order to link BOLD responses to SCRs, we correlated (simple regression) the mean differential SCRs for the FIR and the SIR with neural activations in the contrast CS+ minus CS− for each subject. This analysis was focused on the significant hemodynamic results within the placebo and cortisol group.

For the explorative whole brain analyses, the significance threshold was set to $\alpha = 0.05$ on voxel-level, corrected for multiple testing (family-wise error (FWE) correction), and a minimum cluster size of 5 voxels. RoI analyses were performed using the small volume correction options of SPM5 ($p < .05$). Additionally, trends up to a threshold of $p_{corr} < .10$ are reported.

[1] The frontal RoI was created with all frontal structures of MARINA as well as the anterior and medial cingulate cortex.

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Table 1 Mean (*SE*) cortisol concentrations (in nmol/l) before the administration of cortisol (30 mg) or placebo, 25 min, and 90 min after administration; separated for both sexes and the treatment condition.

Group	Before treatment	25 min after treatment	90 min after treatment
Placebo			
Women	8.85 (2.29)	5.67 (1.06)	7.00 (1.84)
Men	7.86 (1.17)	5.72 (0.53)	6.87 (1.15)
Cortisol			
Women	4.59 (1.54)	116.80 (34.10)	136.34 (34.15)
Men	3.65 (0.71)	119.05 (23.63)	80.18 (20.60)

Unrealistic high cortisol concentrations (larger than 1000 nmol/l) were excluded from the descriptive statistics in this table.

3. Results

3.1. Cortisol concentrations

Some participants (four women and four men) displayed extremely high cortisol levels (larger than 1000 nmol/l) 25 min after cortisol intake. These subjects were excluded from hormonal analyses because these concentrations most likely reflect some micro hydrocortisone residue of the uncoated tablet in the mouth of the participants. Elevated cortisol concentrations were not observed in the placebo group. The ANOVA with the within subjects factor time and the between subjects factors sex and treatment revealed a significant main effect of time ($F(1.886, 49.035) = 31.543$; $p < .001$) and a significant time $\times$ treatment interaction ($F(1.886, 49.035) = 34.088$; $p < .001$). Thus, treatment was successful by elevating cortisol levels in the cortisol group, whereas cortisol concentrations in the placebo group remained unchanged (see Table 1). The main effect or interactions for the factor sex did not reach statistical significance.

The McNemar test showed that subjects were not able to indicate whether they had received hydrocortisone or placebo ($n = 25$; exact $p > .10$). Four participants correctly indicated having received hydrocortisone. However, three placebo subjects also guessed that they were in the cortisol group. The remaining participants assumed the intake of placebo ($n = 18$) or had no treatment guess ($n = 14$).

3.2. Two-back task performance

There were no significant group differences in the percentage of correct responses in the two-back task during acquisition ($F(3,37) = 0.972$; $p > .10$). Percentage of correct responses was 86.75% ($SE = 4.36$) for the placebo women, 86.80% ($SE = 3.44$) for the cortisol women, 88.60% ($SE = 2.44$) for the placebo men, and 81.40% ($SE = 2.80$) for the cortisol men.

3.3. Skin conductance responses (SCRs)

The ANOVA with the repeated measurement factors CS-type and trial and the between subjects factors sex and treatment demonstrated a main effect of trial for the FIR ($F(7.843, 258.829) = 6.617$; $p < .001$), reflecting a general habituation of SCRs. Besides, a sex $\times$ treatment interaction occurred ($F(1,33) = 7.693$; $p < .01$) irrespective of CS-type. In women, post hoc *t*-tests indicate a significant reduction in general SCRs after cortisol intake in comparison to placebo ($t(16) = 2.397$; $p < .05$). In men, cortisol slightly enhanced the SCR reaction to both CS compared to placebo ($t(17) = 1.638$; $p = .120$; see Fig. 1).

Similarly in the SIR, the sex $\times$ treatment interaction ($F(1,33) = 7.378$; $p = .01$) reached statistical significance. Post hoc *t*-tests could determine the same pattern as in the FIR with cortisol women displaying significant lowered mean SCRs than placebo women ($t(16) = 2.311$; $p < .05$). Men's mean SCRs were marginally heightened after cortisol in comparison to placebo ($t(17) = 1.470$; $p = .160$; see Fig. 1). Thus, while cortisol treatment had no effect on peripheral (SCR) correlates of learning (CS+/CS− differentiation), the hormone influenced the overall responsivity of the sympathetic nervous system (SNS) in a sex specific manner.

Concerning the UCR, a main effect of CS-type ($F(1,33) = 118.415$; $p < .001$) and a main effect of trial ($F(8.526, 281.367) = 7.142$; $p < .001$) occurred as well as the interaction CS-type $\times$ trial ($F(8.632, 284.840) = 2.697$; $p < .01$). The interaction reflected larger SCRs and a faster

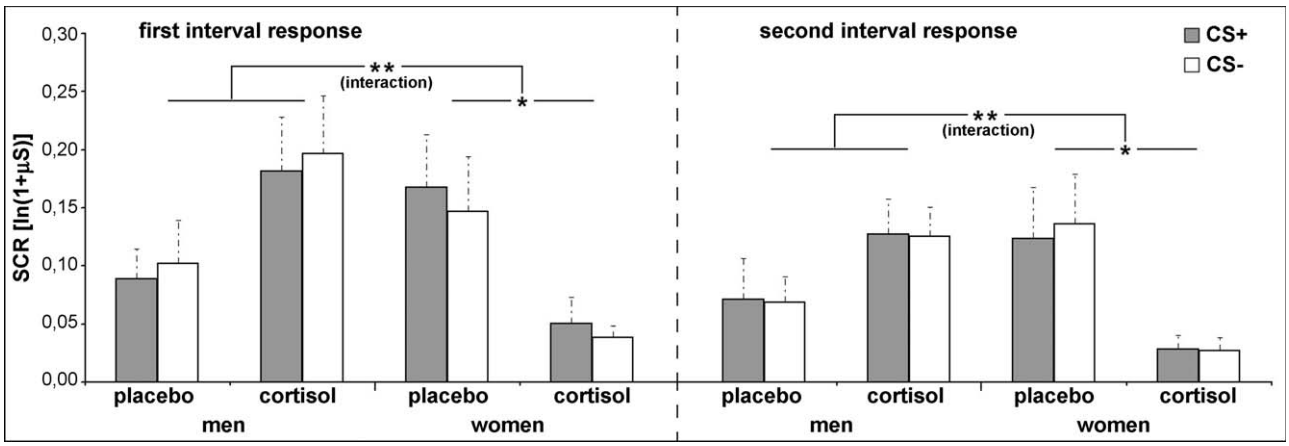

Figure 1 Mean (*SE*) logarithmic skin conductance responses (SCRs) towards the CS+ and CS− for the first interval response (left side) and second interval response (right side), separated for women and men in the cortisol and placebo condition. $^{**}p \leq .01$, $^{*}p < .05$.

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decline of the UCR to the UCS than to the non-UCS. No other main effects or interactions reached statistical significance.

3.4. Hemodynamic responses

3.4.1. CS+ minus CS−

The ANOVA with the group factors treatment and sex revealed that, irrespective of treatment, men had a significant differentiation of CS+ and CS− in the right frontal cortex (namely the frontal superior gyrus), the right insula and the thalamus (bilaterally). Women showed significant neural activity in the left frontal cortex (namely the frontal superior gyrus), the right insula, and the right thalamus in the contrast CS+ minus CS− (see Table 2). The group analyses men minus women and vice versa revealed no significant differences.

With respect to the effect of cortisol treatment the following results were observed. In the placebo group, the CS+ as compared to the CS− (irrespective of sex) provoked significant neural activity in the left amygdala, the right hippocampus, the right insula as well as bilaterally in the thalamus. In the cortisol group, only the right frontal cortex (namely the frontal superior gyrus) resulted in stronger hemodynamic responses in the CS+ compared to the CS−, a further trend was found in the right thalamus. Group analyses comparing the cortisol with the placebo group did not show significant differential neural activity except for a trend ($p = .054$) in the left amygdala for placebo minus cortisol. This was due to a higher differentiation of CS+ and CS− in the placebo group in comparison to the cortisol group (see Fig. 2).

A significant interaction between cortisol and sex could be detected in the right insula and trends in the right hippocampus and the left thalamus. In women, the differentiation between CS+ and CS− within these brain structures was enhanced after cortisol administration, while it was basically absent under placebo. Men displayed the opposite activation pattern with a robust differentiation between CS+ and CS− in all these regions, which was abolished after cortisol application (see Fig. 3 for the relevant brain slices and descriptive illustration of the CS+ minus CS− in the respective peak voxels). Post hoc ANOVA in these brain regions could not trace back the interaction effect to a single significant difference within or between the groups.

3.4.2. CS+ by time minus CS− by time

In the placebo group, but not in the cortisol group, a significant CS+/CS− differentiation that is built up linearly could be observed in the right hippocampus ($x = 39$, $y = -21$, $z = -12$, $T_{max} = 4.62$, cluster size = 74, $p_{corr} = .007$). No further group analysis revealed statistical significant effects.

3.4.3. UCS minus non-UCS

The entire sample demonstrated an extended activation cluster to the UCS as compared to the non-UCS in the parietal lobe (supramarginal gyrus, $x = 60$, $y = -18$, $z = 24$, $T_{max} = 12.94$, cluster size = 17,732, $p_{corr\ for\ the\ whole\ brain} < .001$) and bilaterally in all RoIs with a $p_{corr} < .001$. In the contrast UCS minus non-UCS, the ANOVA revealed that men had higher neural activity than women in the left hippocampus ($x = -36$, $y = -24$, $z = -15$, $T_{max} = 3.81$, cluster size = 170, $p_{corr} = .036$), the left ($x = -9$, $y = -27$, $z = 9$, $T_{max} = 3.89$, cluster size = 237, $p_{corr} = .020$), and the right thalamus ($x = 6$, $y = -18$, $z = 6$,

Table 2 Significant RoI activations for the contrast CS+ minus CS− for the main effects, and group comparisons of sex, cortisol, and the interaction sex × cortisol.

Group	Brain structure	Cluster	x	y	z	T_{max}	p_{corr}
Men	R frontal cortex (fr. superior gyrus)	1497	15	−12	60	5.19	.030
	R insula	120	45	6	12	4.04	.043
	L thalamus	191	−3	−9	12	3.61	.041
	R thalamus	165	6	−9	12	3.69	.035
Women	L frontal cortex (fr. superior gyrus)	990	−21	0	45	5.08	.040
	R thalamus	148	15	−15	18	3.62	.040
	R insula	*99*	*33*	*15*	*12*	*3.82*	*.069*
Placebo	L amygdala	54	−27	3	−18	3.59	.016
	R hippocampus	121	36	−27	−9	3.92	.032
	R insula	254	45	6	12	4.37	.020
	L thalamus	228	−12	−15	15	3.71	.033
	R thalamus	180	15	−15	18	4.06	.015
Cortisol	R frontal cortex (fr. superior gyrus)	2008	15	−12	60	6.85	<.001
	R thalamus	*115*	*12*	*−21*	*15*	*3.17*	*.097*
Placebo > cortisol	*L amygdala*	*32*	*−27*	*3*	*−18*	*3.01*	*.054*
Sex × cortisol	R insula	192	45	−3	12	4.30	.024
	R hippocampus	*70*	*39*	*−12*	*−21*	*3.59*	*.066*
	L thalamus	*84*	*−18*	*−24*	*6*	*3.38*	*.067*

The threshold was $p_{corr} < .05$ (FWE-corrected according to SPM5; for RoIs: small volume correction). All coordinates (x, y, z) are given in MNI space. Trends up to a threshold of $p_{corr} < .10$ are written in italics. L = left, R = right.

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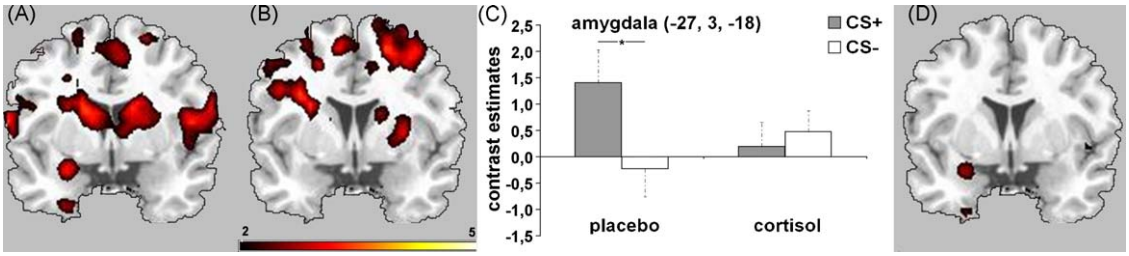

Figure 2 Neural activations for the contrast CS+ minus CS− in (A) the placebo group, (B) the cortisol group, and (D) the comparison placebo minus cortisol. For illustration, data were thresholded with a $T \geq 2.0$ (see color bar for exact t values) and the depicted slice was selected according to the reported activation in the left amygdala ($y = 3$). Additionally, mean (*SE*) contrast estimates to CS+ and CS− for the cortisol and placebo group in the respective peak voxel are illustrated in the bar graph (C). $^{*}p < .05$.

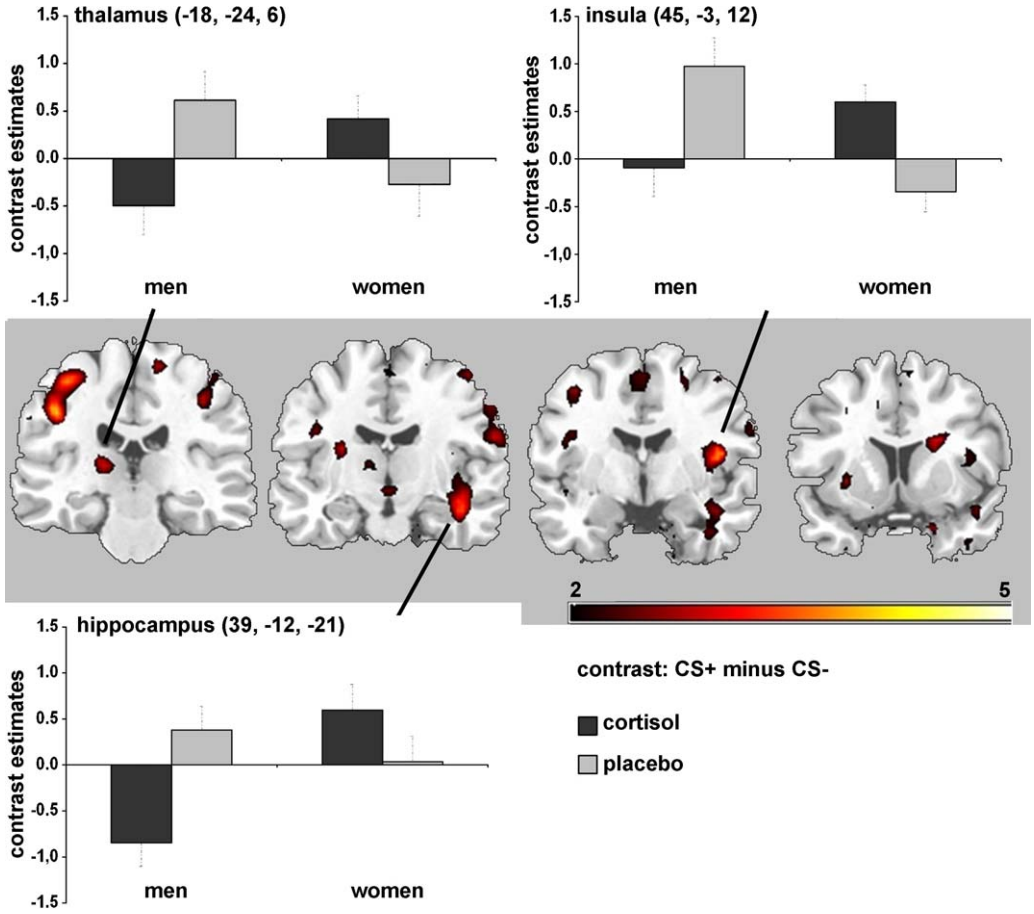

Figure 3 Neural activations for the sex × cortisol interaction in the contrast CS+ minus CS− in a series of brain slices. For illustration reasons, the data were thresholded with a $T \geq 2.0$ (see color bar for exact t values) and the depicted slices were selected according to the reported activations. Mean (*SE*) contrast estimates of the contrast CS+ minus CS− for women and men in the cortisol and placebo group in the insula, hippocampus, and thalamus in the respective peak voxels are illustrated in the bar graphs.

$T_{max} = 3.77$, cluster size = 217, $p_{corr} = .025$). No other main effects or interactions were observed.

3.4.4. Correlation of BOLD responses with SCRs in the contrast CS+ minus CS−

Concerning the group analysis placebo minus cortisol, neural responses to CS+ minus CS− positively correlated with enhanced differential FIRs in the placebo group only but not in the cortisol group. Looking at the significant neural structures that differentiated between CS+ and CS− in the placebo group, there was a positive correlation with differential FIRs in the left amygdala, bilaterally in the thalamus as well as a trend in the right hippocampus (see Table 3). Correlations with the SIR did not reach statistical significance.

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Table 3 Correlations of conditioned brain activity (CS+ minus CS−) with differential SCRs (logarithmic FIR; CS+ minus CS−) in the placebo and the cortisol group.

Group	Brain structure	Cluster	*x*	*y*	*z*	T_{max}	p_{corr}
Placebo	L amygdala	73	−30	−6	−15	3.83	.031
	R hippocampus	*134*	*39*	*−24*	*−6*	*4.34*	*.061*
	L thalamus	130	−6	−27	12	4.21	.048
	R thalamus	172	15	−18	−3	4.06	.006
Cortisol	No significant activations						

The threshold was $p_{corr} < .05$ (FWE-corrected according to SPM5; for RoIs: small volume correction). All coordinates (*x*, *y*, *z*) are given in MNI space. Trends up to a threshold of $p_{corr} < .10$ are written in italics. L = left, R = right.

4. Discussion

In this experiment, we investigated the influence of acute cortisol treatment on human fear conditioning in contingency unaware subjects and its possible modulation by sex. First, the impact of cortisol as well as the cortisol × sex interaction on learning-related neural activity (i.e., CS+ evoked higher responses than the CS−) and their implications will be highlighted. Second, we will discuss the results concerning the SCRs before describing some limitations and drawing a general conclusion.

4.1. Cortisol influences neural activity

A main effect of cortisol treatment could be observed in the amygdala. Whereas the placebo group showed enhanced learning-related neural activity towards the CS+ compared to the CS− in line with previous studies (Knight et al., 2004a,b; Tabbert et al., 2006; Klucken et al., 2009a), this effect was abolished by cortisol. Reduced blood flow in limbic regions was observed when cortisol concentrations are elevated during a psychosocial stressor (Prüssner et al., 2008). Behaviorally, cortisol treatment has been shown to reduce fear in phobic patients (Soravia et al., 2006). Similarly in healthy subjects, pretreatment with cortisol was able to reduce negative mood after a stressful movie or psychosocial stress suggesting a protective effect of GCs on mood during stress (Reuter, 2002; Het and Wolf, 2007). The present finding of reduced amygdala reactivity to the CS+ might be a neuronal correlate of these anxiolytic properties of the stress hormones. Surprisingly, the amygdala effect was not modulated by sex as could have been suggested from the relevant literature (Sergerie et al., 2008; van Stegeren, 2009). The amygdala expresses MRs and GRs, so this brain structure is susceptible to influences of circulating GCs besides other regions in the limbic system and the prefrontal cortex (Reul and de Kloet, 1985; Joëls and Baram, 2009). However, only the amygdala showed an almost significant group effect. This could be due to the used experimental paradigm, i.e., fear conditioning without contingency awareness. Here, the amygdala is critically involved in contrast to other MR and GR rich structures mentioned above.

Having said this, other studies reported a rather enhancing effect of GCs on amygdala activity in animals as well as humans (Quirarte et al., 1997; van Stegeren et al., 2007). Other hormones as norepinephrine (NE) are involved in this effect and may explain part of the heterogeneous results (van Stegeren et al., 2005, 2008; Roozendaal et al., 2006c; Kukolja et al., 2008). It has been suggested that central NE is a prerequisite for GC actions to occur (Roozendaal et al., 2006c). Animal studies indicate that this interaction appears to be of relevance for fear conditioning as well (Roozendaal et al., 2006a). We cannot rule out the possibility that the results in our present experiment are modulated by NE influences on amygdala activity. Markers of noradrenergic activation, such as alpha amylase (Chatterton et al., 1996; van Stegeren et al., 2006), should be considered in future studies to account for this fact. Finally, acute cortisol treatment could have led to an inhibition of the HPA axis through the negative feedback mechanism and subsequent lower central CRF concentrations. Within this framework, our reported activations could be possibly due to a modulating effect of CRF concentrations on emotional behavior (Croiset et al., 2000).

Concerning the linear temporal trends, we found significant differences between CS+ and CS− in the hippocampus for the placebo group only. So, it could be suggested that the hippocampus is involved in the linear learning-related CS+/CS− differentiation that gradually increases over time possibly reflecting synaptic plasticity (cf. Maren, 2001). Linear increases in the hippocampus were already demonstrated in response to fearful faces (Surguladze et al., 2003). However, the remaining analyzed regions did not reveal such an effect, which could be accounted for by higher order (i.e., non-linear) temporal trends that may underlie the differential neural responses in these brain regions (cf. Surguladze et al., 2003).

Correlation analyses of differential SCRs (CS+ minus CS−) with differential brain activation (CS+ minus CS−) revealed that although unaware participants did not yield significant SCR differentiation, they displayed a correlation of SCRs and amygdala, hippocampus, and thalamus activity. SCRs have before been linked to subcortical activity in fear conditioning studies (Critchley, 2002; Knight et al., 2005; Petrovic et al., 2008), so our results confirm these findings. The correlations implicate that these brain structures can modulate electrodermal responding, even in the absence of differential SCRs.

4.2. Sex modulates cortisol effects on neural activity

The most remarkable result was the facilitation of the CS+/CS− differentiation in the insula for women after cortisol administration, whereas it was basically absent in women from the placebo group. In contrast, fear acquisition was superior in placebo men but impaired by cortisol treatment.

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The insula is associated with emotional processing not only regarding fear but also disgust (Augustine, 1996; Phillips et al., 1997; Schienle et al., 2002; Stark et al., 2005). Besides, this area plays a major role in fear conditioning (Büchel et al., 1998; Phelps et al., 2001; Klucken et al., 2009a) and the anticipation and evaluation of future emotional states (Nitschke et al., 2006; Paulus and Stein, 2006; Simmons et al., 2008). So, the insula can be regarded as a central structure that integrates internal somatic cues of danger with emotional experience (Reiman et al., 1997; Damasio et al., 2000). Integrating the present results into this view, we assume that the insula can differentiate between CS+ and CS− in men more easily under normal (resting) conditions, but acute cortisol elevations can disrupt this function. Women were more prone to a facilitation of this structure during enhanced GC levels possibly revealing an unconscious shift towards the acquisition of potential danger cues in a stressful situation. A high density of estrogen and androgen receptors in the insula, which are differentially activated in men and women in the presence of circulating sex steroids, could account for these effects as well as organisational sex differences in brain structure and functioning (Sibug et al., 1991; Sisk and Zehr, 2005; Dalla and Shors, 2009).

In our prior fear conditioning study without the distractor task (Stark et al., 2006), we observed a similar opposing response pattern in men and women, but it was restricted to prefrontal structures. In the current experiment, an overall highly similar response pattern was found but for different brain regions prominent in fear conditioning (hippocampus, insula, thalamus). Several facts could be responsible for this sexual dimorphic effect after GC treatment in these additional areas: different hippocampal receptor affinity for GCs in women and men, sex steroid-induced changes in neuronal excitability or alterations in dendritic structures caused by sex steroid fluctuations (Madeira and Lieberman, 1995; Cahill, 2006). The thalamus has several connections to the insula, prefrontal and medial temporal brain structures (Öngür and Price, 2000; Craig, 2003). Therefore, it can subserve as mediator in this sexual dimorphic functional network of implicit fear conditioning during different GC levels. But the results of our prior experiment (Stark et al., 2006) cannot be directly compared to the present one for the following reasons. First, several distractors should prevent participants from detecting the contingencies between CS and UCS in order to investigate fear acquisition in unaware participants only. Almost 60% of the participants in the previous study were able to correctly identify the contingencies, whereas the occurrence of awareness was intentionally prevented in the current experiment. Second, participants were informed that the purpose of the study was to examine the impact of cortisol on memory processes. This may have lead participants to shift their attention more to the two-back task and away from the CS, the latter possibly obtaining a slightly different meaning.

Regarding sex differences in learning and memory, animal studies reported general stronger conditioned freezing behavior in males (Pryce et al., 1999). But in eyeblink conditioning females outperform males under unstressed conditions (Shors, 2004). After stress, a facilitation effect occurred in males but a blockade of conditioned responses in females (Shors, 2004). In humans, some studies could replicate this response pattern in men, albeit discrepant findings are reported in women (Zorawski et al., 2005, 2006; Jackson et al., 2006). There are several methodological differences that could explain discrepancies between the previous human studies and the present one. First, the three prior studies used emotional CS in contrast to neutral CS in the current experiment, so heterogeneous results could rely on the partly changed fear acquisition process. Second, basal or stress-induced cortisol concentrations were investigated leading to less saturated MRs and GRs in comparison to our acute hydrocortisone treatment. Cortisol can influence memory processes dependent on its action on GRs or the balance of MR/GR activation resulting in the inverse U-shaped cortisol response curve (Lupien and McEwen, 1997; Conrad et al., 1999; de Kloet et al., 1999; Lupien et al., 2007). Thus, differential saturations could partially explain the discrepancies between our current and earlier findings (Stark et al., 2006) compared to observations obtained with basal cortisol concentrations or laboratory stressors (Zorawski et al., 2005, 2006; Jackson et al., 2006). In addition, it has to be emphasized that stress induction, in contrast to cortisol application, causes a multitude of additional neuroendocrine responses (e.g., activation of the SNS as well as CRF and adrenocorticotropic hormone release as part of the HPA activation). Since there is good evidence that the SNS and the HPA system interact at multiple levels (e.g., Roozendaal et al., 2006b), it would be of interest to investigate the effects of exposure to a laboratory stressor on fear conditioning with fMRI.

4.3. Skin conductance responses

We found no conditioned SCRs in contingency unaware participants, which might at first sight be interpreted as a failure to successfully induce fear learning. But a closer look at prior studies indicates that our findings are well in line with the literature. Fear conditioning without contingency awareness could be demonstrated only with particular CS-types, i.e., subliminal CS or biological salient CS as snakes, spiders or angry faces (Esteves et al., 1994; Morris et al., 2001; Öhman and Mineka, 2001; Knight et al., 2009). In contrast to that, our CS were neutral geometric figures presented supraliminally. Previous studies with contingency unaware participants and the same CS reported no conditioned SCRs but differentiation on the neural level (Tabbert et al., 2006; Klucken et al., 2009a). Our current findings nicely replicate these results. Therefore, we suggest that conditioning can occur without contingency awareness at the neural but not at the electrodermal level. A better way to measure peripheral conditioned responses in unaware participants may be the startle reflex, as it does not depend on contingency awareness as SCRs (Hamm and Vaitl, 1996; Hamm and Weike, 2005; Weike et al., 2007).

Surprisingly, there was a sex × cortisol interaction reflecting attenuated mean SCRs in cortisol women compared to the respective placebo group. In men, the opposite direction was observed with marginally higher SCR in the cortisol in comparison to the placebo group. So, while cortisol had no effect on peripheral correlates of emotional learning (differential SCR to the CS+ compared to the CS−), it enhanced the overall responsivity of the SNS to the presented stimuli in women, while reducing it in men. This was not a simple effect on SCRs in principle, since only the FIR and SIR were differentially

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modulated. The UCR did not show this response pattern, even though a ceiling effect might have occurred. A direct sex specific effect of cortisol on SNS reactivity could be a plausible explanation.

Thus, the present study suggests that in unaware participants peripheral and central indices of emotional learning and emotional arousal seem to be modulated by cortisol and sex in an opposing direction. In our prior article with mostly aware participants (Stark et al., 2006), the response pattern for the differential SCRs and for several prefrontal brain regions was in the same direction (reduction after cortisol in men, enhancement after cortisol in women). So, the current study highlights the fruitfulness of measuring peripheral and central correlates of fear conditioning in parallel, when investigating the effects of stress hormones.

4.4. Limitations

Our current sample is slightly different from the previous one (Stark et al., 2006), as free-cycling women in their luteal phase were included at equal parts in the placebo and cortisol women group. This resulted in an increase in variance since sex steroid milieus are different between these two samples. Different effects of the stage of the menstrual cycle on the BOLD signal and SCRs have to be taken into account in further examinations (cf. Fernández et al., 2003; Goldstein et al., 2005; Protopopescu et al., 2005; Milad et al., 2006). The present sample cannot statistically account for this fact because of undersized cell frequency. A correlation approach examining the relation between sex steroids and neural activity in a fear conditioning paradigm would be promising in future studies.

Furthermore, we only used a dose of 30 mg of hydrocortisone. An auspicious approach would be to test multiple cortisol doses (Lupien et al., 1999). At present, we were interested in the fear acquisition process only. GC effects on the several stages of extinction (i.e., acquisition, consolidation, retrieval of the extinction; see Quirk and Müller, 2008) should be considered as far as stress-related disorders and their maintenance are concerned (de Quervain and Margraf, 2008).

5. Conclusion

In sum, this experiment provides initial evidence that cortisol effects on implicit fear conditioning are partially modulated by sex at the central (hemodynamic responses) and the peripheral autonomic level (SCRs). Amygdala activation in contrast was blocked after cortisol administration but no modulation of sex was found suggesting a general inhibitory effect on learning in this particular structure. In addition, sexually dimorphic effects were observed treatment dependent for specific regions, namely the insula, the hippocampus, and the thalamus. Understanding how unconscious fear learning is modulated by stress hormones and sex is a first step towards translating basic research into an enhanced understanding of the origin of anxiety disorders.

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Conflict of interest statement

None declared.

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