Oral contraceptive usage alters the effects of cortisol on implicit fear learning ☆

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ABSTRACT

An important feature of the human defense system comprises fear learning, which stress hormones can crucially modulate. However, stress hormones might influence men and women differently, in part because of interactions with sex hormones. In women, distinct stages of the menstrual cycle or the intake of oral contraceptives (OC) affect sex hormone levels. In this study, we used a differential fear conditioning paradigm with electrical stimulation as unconditioned stimulus (UCS) following one neutral stimulus (conditioned stimulus, CS+), but not another (CS−). To investigate implicit fear learning, participants were distracted from detecting the contingencies between CS and UCS. To address interaction effects of sex and stress hormones, 32 men, 30 women in the early follicular phase of the menstrual cycle (FO), 30 women in the luteal phase (LU), and 30 OC women received either 30 mg cortisol or a placebo. In the contrast CS+ minus CS−, an interaction between cortisol administration and sex hormone status emerged in the anterior parahippocampal gyrus and the hippocampus. Cortisol reduced fear learning in men, FO, and LU women, but enhanced it in OC women. Additionally, cortisol attenuated differential amygdala activation in the entire group. These results demonstrate that OC usage substantially modifies cortisol effects on emotional learning in women, particularly in memory-related medial temporal lobe regions. Further, a high dose of cortisol reduces amygdala differentiation pointing to a lowered learning ability of the defense system under high cortisol concentrations, irrespective of current sex hormone availability.

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Introduction

Essential features of the human defense system include detecting threats and initiating adequate responses to cope with them. Even if one does not consciously perceive a potential danger, a subcortical fear circuit centered around the amygdala might be activated automatically (LeDoux, 2003). Fear learning is highly adaptive, because it supports recollection of potential dangers and promotes adequate future behavior.

In humans, fear learning can be investigated in the laboratory using differential fear conditioning designs. They typically reveal fear conditioned responses (CRs) at the electrodermal level or in the neuronal fear circuit including the amygdala, the anterior parahippocampal gyrus, the hippocampus, the insula, and the orbitofrontal cortex (Knight et al., 2004a,b; LeDoux, 2000; Mechias et al., 2010; Rolls, 1999). However, a dissociation between electrodermal and neuronal fear responses can be found using neutral, supraliminally presented conditioned stimuli (CS; e.g. Tabbert et al., 2006, 2011). In particular, persons that cannot explicitly report any association between CS and the unconditioned stimulus (UCS) did not exhibit CRs at the electrodermal level, but in the neuronal fear circuit (e.g. in the amygdala). A prolonged or exaggerated activation of the fear module during initial conditioning, even if not accessible to one's awareness, might be associated with the development of pathologic fears (for reviews: Etkin and Wager, 2007; Öhman and Mineka, 2001; Shin and Liberzon, 2010).

An environmental threat triggers this fear module, which initiates a stress response resulting in the release of (nor)epinephrine and cortisol, the major stress hormone in humans, from the adrenal glands. Then, cortisol influences several cortical and subcortical structures such as the amygdala or the hippocampus (for reviews: Rodrigues et al., 2009; Wolf, 2008). Stress and stress hormones have been implicated in the pathogenesis of several psychiatric disorders, in particular of anxiety disorders (for reviews: Korte, 2001; Wolf, 2008). Besides, prominent sex differences in the prevalence of anxiety disorders exist with a more frequent occurrence in women (Kessler et al., 2005). Neurobiological explanations of these discrepant prevalence

rates encompass the involvement of sex hormones (Solomon and Herman, 2009; Toufexis et al., 2006).

Sex hormones such as estradiol and progesterone affect the brain and the periphery through activational and organizational effects. Organizational effects refer to long-term influences of sex hormones on physiology and morphology during development, whereas activational effects relate to circulating sex hormones inducing physiological and morphological changes through the whole lifespan (Gillies and McArthur, 2010). Activational effects can be explored in women during different stages of the menstrual cycle. Low sex hormone levels characterize the early follicular phase (FO), whereas these concentrations increase in the luteal phase (LU), peak levels can be observed during ovulation. Several studies implicate that sex hormones alter fear processing, e.g. using estrogen administration or comparing different cycle stages in female rodents (Gupta et al., 2001; Morgan and Pfaff, 2001; for a review: Morgan et al., 2004) and humans (Milad et al., 2006; Zeidan et al., 2011) or investigating women with low or high estradiol levels (Milad et al., 2010).

However, a considerable percentage of women are using oral contraceptives (OCs). Despite this crucial relevance, reports on specific OC effects in emotional learning tasks are sparse. OCs contain exogenous sex hormones such as ethinylestradiol, which acts centrally and peripherally and continuously suppresses endogenous sex hormones. Therefore, the combined investigation of OC women with free-cycling women exhibiting low (FO) or high (LU) endogenous sex hormone concentrations ignores the evidence concerning activational effects.

The combined examination of the effects of stress and sex hormones (especially concerning free-cycling and OC taking women) on fear learning is important to elucidate basal modulatory influences on the human defense system. Previous experiments from our group observed that cortisol had opposing effects on the neuronal correlates of fear learning in men and women (Merz et al., 2010; Stark et al., 2006; Tabbert et al., 2010). In these studies, the mediating role of sex hormones on this effect could not be investigated due to small groups of women and/or mixed sex hormone status. Thus, in this experiment, men, FO, LU, and OC women were tested receiving either cortisol or placebo prior to a classical fear conditioning paradigm. Because we were particularly interested in implicit fear learning, distractors were introduced to prevent participants from detecting the relationship between the CS and UCS (cf. Merz et al., 2010; Tabbert et al., 2006, 2010, 2011). Inferring from these prior studies, we hypothesized no conditioning signs on the electrodermal, but on the neuronal level in structures involved in fear learning (e.g. anterior parahippocampal gyrus, hippocampus). Further, our prior results concerning implicit fear learning predict that cortisol should reduce fear CRs especially in the amygdala (cf. Merz et al., 2010). Based on previous conditioning studies using functional magnetic resonance imaging (fMRI; Merz et al., 2010; Stark et al., 2006; Tabbert et al., 2010), we hypothesized that cortisol would lead to higher CRs in OC women, but to reduced CRs in men. Notably, the additional investigation of FO and LU women is highly interesting for the interpretation of the obtained results. This approach will reveal if cortisol effects in OC women are due to OC intake or due to lowered endogenous sex hormones. So, for the first time, we examined activational and organizational effects of sex hormones on cortisol-modulated subcortical fear learning.

Material and methods

General background

The present experiment is part of a larger project investigating different groups, which were either instructed about the CS-UCS contingencies in advance or not (i.e. instructed vs. unaware fear conditioning; see Tabbert et al., 2011). In this report, we focus on the latter participants; they were not informed about a relationship between CS and UCS in advance. Adding distractors into the experimental design (a numerical

two-back task and a distractor stimulus; cf. Merz et al., 2010) hampered contingency learning in the course of the experiment. Those subjects, who nevertheless noticed the correct CS-UCS contingencies, were excluded from the present analyses because of the impact of contingency awareness on various correlates of fear conditioning (e.g. Hamm and Vaitl, 1996; Klucken et al., 2009; Tabbert et al., 2006; see Tabbert et al., 2010, 2011 for the exact results).

An analysis of a subsample ($n = 42$ from the placebo group) has been published previously together with two additional groups (learned and instructed aware participants; Tabbert et al., 2011). This prior data analysis was concerned with the differential effect of contingency awareness on fear acquisition, not with the influence of cortisol or sex hormone status. A first report on the effects of cortisol and sex has also been published based on an overlapping small subsample ($n = 39$; Merz et al., 2010). The detailed impact of sex hormone status could not be analyzed there because of low cell frequency in the women groups ($n \leq 5$) leading to a joint examination of LU and OC women. Now, in the available complete large sample, we are able to investigate cortisol effects in men as well as in three different groups of women.

Participants

In total, 122 participants (117 undergraduate and five graduate students) completed the study. To assess different sex hormone statuses, we invited 32 men, 60 free-cycling women, and 30 OC taking women. Free-cycling women did not take any kind of contraceptives. They reported to have a regular menstrual cycle; one half was invited in the early follicular phase (FO; 3rd to 8th day after the onset of their last menstruation) and the other half in the luteal phase (LU; 3rd to 9th day before the onset of their next menstruation) of the individual menstrual cycle. OC women were required to have been taking their birth control pill (only monophasic preparations with an ethinylestradiol (0.02–0.035 mg) and a gestagenic component) for at least the last three months and we tested them during the pill intake phase.

None of the participants was taking regular medication except OCs or had a history of psychiatric or neurological treatment. Exclusion criteria covered somatic diseases, in particular endocrine diseases, which can influence hormone concentrations, as well as standard fMRI exclusion criteria (e.g. implants, previous brain surgery or intrauterine devices). Inclusion criteria comprised an age between 18 and 35 and a body mass index (BMI) between 18 and 28 kg/m^2. The mean age for the eight groups ranged from 21.3 to 24.8 years and the mean BMI from 21.1 to 22.8 kg/m^2. Further, only right-handed persons were included as assessed by the Edinburgh Inventory of Handedness (Oldfield, 1971).

All participants had normal or corrected vision. We instructed them to refrain from smoking, food intake, and drinking anything but water for at least two hours before the start of the experiment. Each session began between 2 and 5 p.m. to guarantee low and relatively stable endogenous cortisol levels. At first, participants got a detailed explanation of the general procedure (naturally, the conditioning schedule was not explained until the end). The cover story concealing the conditioning procedure included the investigation of the impact of cortisol and several distractors (including an electrical stimulation and visual stimuli) on memory performance. Participants were instructed to pay close attention to all stimuli and to complete the implemented two-back task. All participants gave written informed consent and received at least 25 Euros for their attendance. The ethics committee of the German Psychological Society approved this study.

Conditioned stimuli (CS), unconditioned stimulus (UCS), and experimental procedure

Three pictures of geometric figures (a rhomb, a square, and a triangle) served as CS+, CS−, and as distractor stimulus (non-CS; always the triangle). All figures had identical luminance, were gray-colored, and

were presented against a black background for 8 s. Through a mirror mounted on the head coil, participants viewed the stimuli, which were projected onto a screen at the end of the scanner (visual field = 18°) using an LCD projector (EPSON EMP-7250).

A custom-made impulse-generator (833 Hz) provided transcutaneous electrical stimulation (UCS; 100 ms) through two Ag/AgCl electrodes (1 mm² surface each) fixed to the middle of the left shin. Intensity was set individually using a gradually increasing rating procedure to attain an "unpleasant but not painful" level of sensation. The UCS onset started 7.9 s after CS+ onset (100% reinforcement; delay conditioning). The CS− and the non-CS were never paired with the UCS. The UCS omission 7.9 s after the onset of the CS− defined the non-UCS. The non-CS is a further CS−, which only occurred half as often as the CS− and which served as an additional distractor stimulus to hamper contingency learning.

The conditioning experiment consisted of an acquisition phase, an extinction phase, and an implemented two-back task (cf. Merz et al., 2010 for further details). In short, 20 trials of CS+ as well as CS− and ten trials of non-CS were presented in the acquisition phase. The extinction phase consisted of eleven trials of CS+ and CS− as well as five trials of non-CS. Inter-trial intervals (ITI) between the numbers of the two-back task and the geometrical figures were randomly jittered (ITI duration: 5 to 7.5 s). Each participant received pseudo-randomized stimulus orders (cf. Merz et al., 2010).

Ratings concerning contingency awareness

Immediately after the acquisition phase, participants had to rate the contingencies between UCS and CS+, CS−, and non-CS, presented in random order inside the scanner. Next to the picture of the respective CS, the question read always: "Please estimate how often the electrical stimulation succeeded the following geometrical figure"; with the answer to be chosen between "I do not know", "never", "sometimes", or "always". At the end of the experiment, a questionnaire and a short interview further validated subjects' contingency awareness.

We classified participants as (at least partially) contingency aware if they stated higher probabilities for the UCS occurrence after the CS+ than after the CS− (i.e. possible combinations for CS+ and CS− were: always–sometimes; sometimes–never; always–never). Subjects who recognized the correct relationship between the CS and UCS (n = 58) were excluded and selectively replaced in the respective groups. Thus, the remaining sample consisted of 122 contingency unaware participants.

Cortisol administration, hormone analyses, and treatment guess

Description

In a double-blind, randomized, and placebo-controlled experiment, 17 men, 15 FO, 15 LU, and 15 OC women received three 10 mg tablets of cortisol (hydrocortisone; Hoechst) 45 min before the start of the functional scans for conditioning. Visually identical placebos (magnesium and tablettose) were given to the remaining participants (15 in each sex hormone status group).

We used glass tubes for the collection of saliva samples of cortisol, estradiol, progesterone, and testosterone. Samples were taken directly before tablet intake as well as 25 min (immediately before the fMRI run) and 90 min (immediately after the fMRI run) after tablet intake, and stored at −20 °C until assayed. Commercially available enzyme immunoassays (IBL International, Hamburg, Germany) subserved to measure free hormone concentrations. Samples of one participant were run on the same kit and analyzed within one lot and in duplicates. Inter-assay coefficients of variations (CV) for all analyses were below 8% with an inter-assay CV below 11%. Sensitivity was 0.83 nmol/l for cortisol, 3.30 pmol/l for estradiol, 25.36 pmol/l for progesterone, and 26.372 pmol/l for testosterone.

At the end of the experiment, participants had to give a treatment guess with the possible answers "cortisol", "placebo", or "no idea".

Statistics

All statistical analyses were performed in IBM SPSS Statistics for Windows 19.0 with Greenhouse–Geisser correction and the statistical significance level set to $\alpha = .05$. We conducted analyses of variance (ANOVA) for cortisol including the repeated measurement factor time (first vs. second vs. third sample) as well as the between subjects factors treatment (cortisol vs. placebo) and sex hormone status (men vs. FO women vs. LU women vs. OC women). Estradiol, progesterone, and testosterone were analyzed via ANOVA with the between subjects factors treatment and sex hormone status, without the repeated measurement factor time. Sex hormones were only determined in the first and the third saliva sample. The average of these two concentrations subserved to check for expected differences between men, FO, LU, and OC women (cf. Merz et al., in press). Effect sizes were calculated using partial η^2 for overall ANOVA effects and using Cohen's d for pair-wise comparisons.

To check if subjects possibly became aware of their treatment, Fisher's exact test including the answers "cortisol" and "placebo" was performed in IBM SPSS Statistics for Windows.

Image acquisition and analyses

Description

Brain images were acquired using a 1.5 T whole-body tomograph (Siemens Symphony with a quantum gradient system) with a standard head coil (cf. Merz et al., 2010 for details concerning structural and functional image acquisition). Data were preprocessed using Statistical Parametric Mapping (SPM5, Wellcome Department of Cognitive Neurology, London, UK, 2005) implemented in MatLab R2007b (Mathworks Inc., Sherborn, MA). Unwarping and realignment (2nd degree b-spline interpolation to the first volume), slice time correction (reference slice: 13), co-registration of functional data to each participant's anatomical image, segmentation into gray and white matter, and normalization to the standard space of the Montreal Neurological Institute (MNI) brain were performed. To allow for corrected statistical inference, spatial smoothing was executed with an isotropic 3D Gaussian filter with a full width at half maximum of 9 mm.

Statistics

Acquisition and extinction were integrated as separate sessions in one statistical model in SPM5 including the following experimental conditions: CS+, CS−, non-CS, UCS, non-UCS, targets, and non-targets. An additional regressor was introduced containing the first two numbers and the first two geometrical figures of the extinction. The general linear model uses only the orthogonal parts of the regressors, so the variance that accounts for CS+ as well as UCS responses alike is not taken into account neither for the CS+ nor for the UCS regressor. All 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 from the realignment step constituted covariates in the model separately for acquisition and extinction. A high pass filter (time constant = 128 s) was implemented by using cosine functions in the design matrix.

The individual contrasts were analyzed in random effects group analyses in SPM8 (Wellcome Department of Cognitive Neurology, London, UK, 2009) and focused on the contrasts CS+ minus CS− during fear acquisition. Results of the contrast UCS minus non-UCS can be found in Table S1 (see supplementary material). ANOVA was conducted with the group factors treatment and sex hormone status in the full factorial model implemented in SPM8. In particular, we were interested in the interaction between treatment and sex hormone status as well as the main effect of cortisol. For all statistical analyses, we used exploratory whole brain as well as region of interest (ROI) analyses including the

following ROI: amygdala, anterior parahippocampal gyrus, hippocampus, insula, and orbitofrontal cortex. All ROI were tested separately for the left and the right hemisphere. The required masks for these analyses were maximum probability masks with the probability threshold set to .25, taken from the probabilistic Harvard-Oxford Cortical and Subcortical Structural Atlas provided by the Harvard Center for Morphometric Analysis (http://www.cma.mgh.harvard.edu/fsl_atlas.html).

Regarding the exploratory whole brain analyses, the intensity threshold was set to $p < .05$ corrected for multiple testing (family-wise error (FWE) correction), the minimal cluster size (k) was 10 voxels, and the significance threshold was set to $p < .05$ on voxel-level, FWE-corrected. For the ROI analyses, the intensity threshold was set to $p < .05$ uncorrected, $k = 0$, and the significance threshold was set to $p < .05$ on voxel-level, FWE-corrected (using the small volume correction options of SPM8).

Skin conductance responses (SCRs)

Description

SCRs were sampled with an in-house built optical fiber SCR coupler concurrently with fMRI scans using Ag/AgCl electrodes filled with isotonic (.05 M NaCl) electrolyte medium attached hypothenar at the non-dominant (left) hand. Raw SCR data were low pass filtered with a cutoff frequency of 10 Hz. We defined SCRs in three analysis windows (cf. Prokasy and Ebel, 1967): the maximum amplitude within a window of 1 to 5 s after the CS onset was counted as the first interval response (FIR), within the time window of 5 to 8.5 s as the second interval response (SIR), and within the time window of 8.5 to 13 s as the unconditioned response (UCR). The baseline comprised the skin conductance level immediately preceding the inflexion point. Electrodermal data of six participants had to be discarded because of malfunction of the SCR coupler, fallen off electrodes, or random noise in the dataset.

Statistics

Data were transformed with the natural logarithm in order to attain a normal distribution. Statistical comparisons of SCRs were conducted in IBM SPSS Statistics for Windows via ANOVA with the between subjects factors treatment and sex hormone status; the mean differential SCR (CS + minus CS − for the FIR and the SIR; UCS minus non-UCS for the UCR) was entered as dependent variable.

Results

Cortisol and treatment guess

Salivary cortisol levels increased after hydrocortisone compared to placebo administration (main effect time ($F_{(1.11;100.57)} = 65.39$; $p < .001$; $\eta^2 = .42$); main effect treatment ($F_{(1;90)} = 150.99$; $p < .001$; $\eta^2 = .63$);

time × treatment interaction: $F_{(1.11;100.57)} = 67.87$; $p < .001$; $\eta^2 = .43$) pointing to a successful treatment. No significant main or interaction effect with sex hormone status was found. In particular, no significant differences between the four sex hormone status groups emerged in the placebo group in the first, second or third sample (all $p > .16$). Further, we found no significant baseline differences in the cortisol group ($p > .69$). As shown in Table 1, each sex hormone status group displayed higher cortisol concentrations 25 min (all $p < .02$) and 90 min (all $p < .001$) after *cortisol* intake compared to baseline. Reduced cortisol levels occurred in men, LU, and OC women 25 min after *placebo* intake compared to baseline (all $p < .05$), but not in FO women or 90 min after pill administration.

Results from the treatment guess revealed that participants could not correctly indicate whether they had received cortisol or placebo (Fisher's exact test: $p > .40$). In the placebo group, 27 subjects correctly indicated to have received placebo and eight were mistaken in guessing cortisol. In the cortisol group, 26 participants assumed the intake of placebo and 13 correctly indicated cortisol. The remaining 48 subjects had no treatment guess at all.

Sex hormones

For estradiol, the difference between the sex hormone status groups missed the statistical significance threshold ($F(3;112) = 2.54$; $p = .060$; $\eta^2 = .06$). For progesterone and testosterone, the expected differences between the sex hormone status groups emerged (main effect sex hormone status in progesterone ($F_{(3;113)} = 40.24$; $p < .001$; $\eta^2 = .52$) and in testosterone: ($F_{(3;111)} = 33.88$; $p < .001$; $\eta^2 = .48$)). As can be seen in Table 2, LU women had higher progesterone levels compared to men, FO, and OC women (all $p < .001$), whereas these three groups did not differ significantly among each other. Men displayed lowered estradiol and elevated testosterone concentrations than the three groups of women (estradiol: all $p < .05$; testosterone: all $p < .001$), which were comparable in their estradiol and testosterone levels. Cortisol administration had no impact on sex hormones, neither a statistically significant main effect (all $p > .085$) nor an interaction with sex hormone status (all $p > .50$) occurred.

Taken together, the administration of cortisol as well as the prior assignment according to sex hormone status was successful.

Differential neuronal activation

In the placebo group as a whole, significant BOLD responses emerged in the contrast CS + minus CS − bilaterally in the amygdala, the anterior parahippocampal gyrus, and the hippocampus as well as in the right insula, the right orbitofrontal cortex, and the right lateral occipital cortex (see Table 3). Thus, we successfully detected fear CRs at the neuronal level. In the cortisol group, the CS +/CS − differentiation was only

Table 1

Mean (*SE*) salivary cortisol levels (in nmol/l) before, 25 min after, and 90 min after the administration of placebo or cortisol (30 mg) in men, FO, LU, and OC women. A minority of participants in the cortisol group (five men, six FO, eight LU, and three OC women) showed extremely high cortisol levels (>700 nmol/l) 25 min or even 90 min after hydrocortisone intake. We excluded these subjects from cortisol analyses and the descriptive statistics of this table, because these high concentrations most likely reflect micro hydrocortisone residues of the uncoated tablet in the mouth of the participants. For comparisons between samples within each cell, Cohen's d is given as an estimate of effect size (< or > indicating the direction of the significant difference; vs. indicating non-significant differences).

Cortisol (nmol/l)	Before treatment (sample 1)	25 min after treatment (sample 2)	90 min after treatment (sample 3)	Comparisons between samples
Men: placebo	6.78 (0.91)	4.94 (0.65)	6.55 (1.00)	1 > 2 ($d = 0.71$); 1 vs. 3 ($d = 0.05$)
Men: cortisol	4.95 (1.26)	214.68 (58.31)	96.77 (17.26)	1 < 2 ($d = 1.85$); 1 < 3 ($d = 3.63$)
FO women: placebo	8.17 (1.47)	7.34 (1.15)	8.04 (1.50)	1 vs. 2 ($d = 0.19$); 1 vs. 3 ($d = 0.02$)
FO women: cortisol	6.34 (0.79)	268.28 (60.58)	166.30 (18.25)	1 < 2 ($d = 2.23$); 1 < 3 ($d = 4.02$)
LU women: placebo	7.53 (1.37)	4.79 (0.82)	5.23 (0.71)	1 > 2 ($d = 1.45$); 1 vs. 3 ($d = 0.56$)
LU women: cortisol	6.03 (1.07)	162.96 (47.77)	104.96 (12.02)	1 < 2 ($d = 1.94$); 1 < 3 ($d = 4.97$)
OC women: placebo	8.41 (1.85)	5.81 (0.80)	7.68 (1.09)	1 > 2 ($d = 1.35$); 3 vs. 1 ($d = 0.20$)
OC women: cortisol	5.04 (0.84)	225.55 (56.90)	134.38 (14.98)	1 < 2 ($d = 1.69$); 1 < 3 ($d = 3.52$)

Table 2

Mean (SE) salivary estradiol, progesterone, and testosterone concentrations (in pmol/l) for men, FO, LU, and OC women. Analyses of sex hormones revealed implausibly high levels for some participants (>3 SD above the mean of the respective sex hormone status group), which could point to sample contamination. For estradiol, we excluded two men from sex hormone analyses and the descriptive statistics of this table as well as one FO woman for progesterone, and one man, one FO, and one OC woman for testosterone. For between group comparisons, Cohen's d is given as an estimate of effect size (< or > indicating the direction of a significant difference; vs. indicating non-significant differences).

Sex hormone (pmol/l)	Estradiol	Progesterone	Testosterone
Men	5.94 (0.46)	193.62 (23.21)	318.39 (28.84)
FO women	8.74 (1.07)	144.99 (14.85)	115.06 (25.13)
LU women	8.85 (0.95)	525.53 (45.92)	84.49 (7.85)
OC women	8.21 (0.83)	154.16 (17.78)	69.01 (9.80)
Group comparisons	M<FO ($d=0.67$)	LU>M ($d=1.01$)	M>FO ($d=1.37$)
	M<LU ($d=0.76$)	LU>FO ($d=2.30$)	M>LU ($d=2.30$)
	M<OC ($d=0.64$)	LU>OC ($d=2.13$)	M>OC ($d=2.34$)
	FO vs. LU ($d=0.02$)	M vs. FO ($d=0.46$)	FO vs. LU ($d=0.28$)
	FO vs. OC ($d=0.10$)	M vs. OC ($d=0.35$)	FO vs. OC ($d=0.49$)
	LU vs. OC ($d=0.13$)	FO vs. OC ($d=0.10$)	LU vs. OC ($d=0.23$)

evident in the left hippocampus and the left orbitofrontal cortex. The direct comparison between both groups showed that the cortisol group displayed reduced neuronal activation in the left amygdala (see Fig. 1) and the left anterior parahippocampal gyrus (which is included in the interaction treatment×sex hormones status; see below).

The interaction treatment×sex hormone status revealed that cortisol significantly influenced neuronal activation in the left anterior parahippocampal gyrus and the left hippocampus only dependent on sex hormone status (see Table 3). As can be seen in Fig. 2, in both structures, cortisol reduced the CS+/CS− differentiation in men, FO, and LU women, but enhanced it in OC women (for an illustration of the separate CS+ and CS− responses, please see Fig. S1 the supplementary material).

The exploratory whole brain analyses did not reveal any significant effects, neither did the main effect sex hormone status.

Table 3

Localization and statistics of the peak voxels for the placebo and the cortisol groups as well as their direct comparisons in the contrast CS+ minus CS−. Further, the interaction effect treatment (cortisol vs. placebo)×sex hormone status (men vs. FO women vs. LU women vs. OC women) is shown in this contrast.

Analysis	Brain structure	x	y	z	T_{max}	p_{corr}
Placebo	L amygdala	−30	−9	−21	4.16	.003
	R amygdala	27	−3	−27	3.88	.006
	L anterior parahipp. gyrus	−27	−6	−30	4.10	.005
	R anterior parahipp. gyrus	24	−3	−30	3.98	.007
	L hippocampus	−30	−12	−21	4.70	.001
	R hippocampus	24	−9	−27	3.87	.012
	R insula	33	24	0	3.59	.036
	R orbitofrontal cortex	36	30	−3	4.24	.007
	R lateral occipital cortex, superior division (WB)	15	−63	48	5.54	.020
Cortisol	L hippocampus	−12	−39	3	3.53	.030
	L orbitofrontal cortex	−21	36	−12	3.83	.025
Placebo–cortisol	L amygdala	−30	−9	−21	2.97	.043
	L anterior parahipp. gyrus	−21	−9	−33	3.66	.011
Cortisol–placebo	No significant activations					

Analysis	Brain structure	x	y	z	F_{max}	p_{corr}
Treatment×sex hormone status	L anterior parahipp. gyrus	−21	−9	−33	9.14	.002
	L hippocampus	−24	−9	−30	6.70	.031

The significance threshold was $p_{corr}<.05$ (FWE-corrected; small volume correction or corrected for the whole brain (WB)). The peak voxel from the WB analysis was labeled based on the Harvard-Oxford Cortical and Subcortical Structural Atlas. All coordinates (x, y, z) are given in MNI space (L = left, R = right).

Differential skin conductance responses (SCRs)

ANOVA demonstrated a significant difference in the UCR ($F_{(1;108)}=327.75$; $p<.001$) revealing higher SCRs to the UCS compared to the non-UCS. No differences between CS+ and CS− in the FIR or SIR occurred. Neither treatment nor sex hormone status influenced differential SCRs in the FIR, SIR, or UCR.

Discussion

Our results demonstrate that the stress hormone cortisol influences neuronal correlates of fear conditioning depending on the current sex hormone availability. Most importantly, cortisol reduced fear learning in the anterior parahippocampal gyrus and the hippocampus in men, FO, and LU women, whereas it enhanced the neuronal differentiation in OC women. To the best of our knowledge, this is the first time that an influence of sex hormones and OC usage on the basic modulation of emotional learning processes and their modulation by cortisol have been observed in humans.

In fear conditioning designs, OC women obviously constitute a particular group of participants exhibiting contrary results in neuronal activation compared to men or free-cycling women. In detail, cortisol administration led to enhanced fear acquisition (Stark et al., 2006; Tabbert et al., 2010; in part: Merz et al., 2010) as well as increased fear extinction learning in OC women (Tabbert et al., 2010). Moreover, an independent effect of sex hormone status on extinction learning was recently found pointing to altered neuronal processing in OC women compared to men and LU women (Merz et al., in press). The described effects might be attributable to the direct intake of OCs and not to low endogenous sex hormones as demonstrated for the first time in the present experiment. FO women have low endogenous sex hormone levels comparable to OC women and should thus have displayed the same response pattern as OC women, if sex hormones were responsible. Yet, FO women exhibited the same results as men and LU women. So, OC intake per se induced a completely changed fear learning pattern.

Sparse hints for the biological mechanisms of OC effects can be derived from the animal literature. The main estrogenic compound in OCs, ethinylestradiol, binds to estrogen receptors (ERs), whereas the gestagenic component binds to progestin receptors (PRs). Various brain areas express ERs and PRs including subcortical structures such as the hippocampal complex (Mitterling et al., 2010; Shughrue et al., 1997). A higher estradiol and/or progesterone binding after continuous OC intake might lead to a subsequent downregulation and/or to a desensitization of ER or PR. Hence, hippocampal activation could be reduced, in consequence slightly impairing learning processes. Possibly both receptors act in concert in reducing learning and memory processes during OC intake rather than one receptor alone.

Another possible explanation for the observed OC effects involves an effect of low sex hormone availability on emotional learning. OCs disrupt the function of the gonadotropin-releasing hormone (GnRH) pulse generator as part of the hypothalamus-pituitary-gonadal axis via negative feedback processes resulting in low endogenous sex hormone release (cf. Buffet et al., 1998). Importantly, men as well as free-cycling women exhibit high testosterone concentrations, which can be aromatized to estrogens (cf. Milad et al., 2010). However, OC women displayed the lowest estradiol and testosterone concentrations, at least descriptively (see Table 2). Thus, in OC women, direct effects of estrogens as well as indirect effects of aromatized testosterone into estrogens are reduced compared to the other groups. This explanation would not exclude low sex hormone (in particular estrogen) concentrations as an important mechanism contributing to the current results. In sum, this argumentation would add OC effects on emotional learning to recent observations proposing that low estradiol levels can alter fear conditioning, which constitutes a vulnerability factor for the development of PTSD (Glover et al., 2012; Lebron-Milad et al., 2012). Future studies
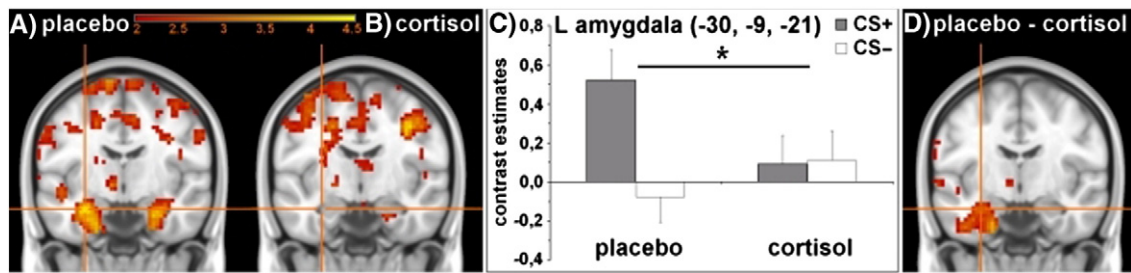
Fig. 1. Neuronal activation in the contrast CS + minus CS − for the placebo group (A), the cortisol group (B), and the comparison placebo minus cortisol (D). The depicted coronal slice was selected according to the reported activation in the left amygdala ($y = −9$). For illustration reasons, data were thresholded with $T ≥ 2.0$ (see color bar for exact T values) and displayed on the standard MNI brain template. In the bar graph (C), mean contrast estimates to CS + and CS − are additionally given for the placebo and the cortisol group in the respective peak voxel. Error bars are standard errors of the mean. Cortisol significantly attenuated the CS +/CS − differentiation in the amygdala. *$p < .05$ for the main effect of treatment.

have to delineate the molecular mechanism of OC usage and sex hormones on emotional learning.

Our results eminently emphasize the importance of activational effects of sex hormones on the acquisition of emotional relevant material (e.g. Andreano and Cahill, 2010; Dalla and Shors, 2009). If only organizational effects were related to the modulation by cortisol, the three groups of women should have displayed the same response pattern. Putatively, OC women are more susceptible to information carrying negative connotations in extremely stressful situations than under normal conditions. On the contrary, men and free-cycling women are less susceptible potentially due to an evolutionary-based survival mechanism. This adaptive response might be disrupted by the interference of the hormonal milieu because of continuous intake of exogenous sex hormones.

Apart from the present results from implicit fear learning, the parahippocampal gyrus and the hippocampus account for encoding, consolidation, and retrieval of explicit memories. In men, exogenously heightened cortisol levels reduce activation in the hippocampus and the parahippocampal gyrus during memory retrieval (de Quervain et al., 2003; Oei et al., 2007). Thus, cortisol effects emerge not only during implicit, but also during explicit memory formation. Translating these important results of basic research into pathological fear, it has been

shown that cortisol impairs disorder-related fear memories (Aerni et al., 2004; de Quervain et al., 2011; Soravia et al., 2006), most likely due to the involvement of the hippocampus. In line, it would be extremely interesting to examine if women taking OCs have a higher prevalence in developing psychiatric disorders, in which conditioning processes and stress are critically involved in the acquisition and maintenance of the disorder.

In the present design, we successfully distracted participants from detecting the contingencies between CS and UCS by use of a distractor task and an additional distractor stimulus. On the one hand, this approach prevented a CS +/CS − differentiation at the electrodermal level (cf. Merz et al., 2010; Tabbert et al., 2006, 2011). But on the other hand, it induced reliable activation of an automatic fear network centered around the amygdala as can also be seen in studies using subliminal presented, auditory, or emotional CS (Hamm and Weike, 2005; Knight et al., 2009; Öhman and Mineka, 2001; Tabbert et al., 2006, 2011). The fact that no conditioning signs emerged in SCRs should not lead to the assumption that the unaware group was not conditioned at all. At the neuronal level, clear evidence occurred for a heightened activation in many fear-related structures to the CS + compared to the CS − in the placebo group. In the cortisol group, the CS +/CS − differentiation was
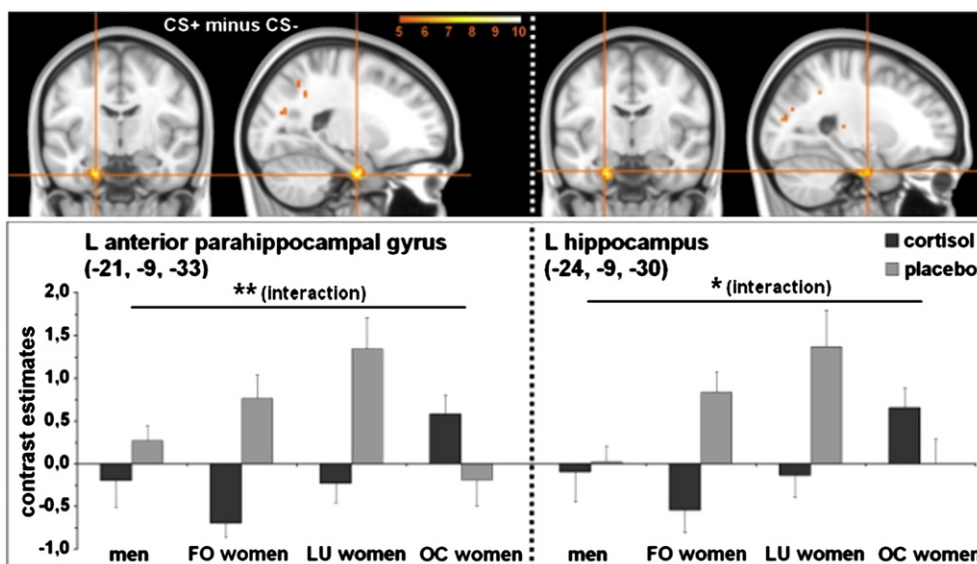
Fig. 2. Neuronal activation in the contrast CS + minus CS − for the treatment × sex hormone status interaction. The depicted coronal and sagittal slices were selected according to the reported activation in the left anterior parahippocampal gyrus ($x = −21$: $y = −9$) and the left hippocampus ($x = −24$; $y = −9$). For illustration reasons, data were thresholded with $F ≥ 5.0$ (see color bar for exact F values) and displayed on the standard MNI brain template. In the bar graphs, mean contrast estimates to CS + minus CS − are given for men, FO, LU, and OC women separately for treatment (cortisol vs. placebo) in the respective peak voxel. Error bars are standard errors of the mean. In both brain regions, cortisol reduced the CS +/CS − differentiation in men, FO, and LU women, but enhanced it in OC women. * $p < .05$; ** $p < .005$ for the treatment × sex interaction.

substantially reduced to significant findings in the left hippocampus and left orbitofrontal cortex only.

Comparing the placebo and the cortisol group directly revealed that cortisol significantly diminished the amygdala differentiation compared to placebo intake, thus, confirming our previous observation (Merz et al., 2010) in a larger sample. Congruently, limbic areas including the amygdala are suppressed after stress exposure or cortisol administration (e.g. Henckens et al., 2010; Prüssner et al., 2008). In contrast, stress hormones also enhanced amygdala activation in animals and humans (e.g. Quirarte et al., 1997; van Stegeren et al., 2007). This discrepancy might partly be explained by the additional involvement of (nor)epinephrine, which is also released in the course of the stress response, acting as a prerequisite for glucocorticoid effects (Kukolja et al., 2008; Roozendaal et al., 1999).

Moreover, it has to be noted that stress induction, in contrast to our pharmacological approach, leads to a different picture in rodents (Dalla and Shors, 2009; Wood et al., 2001) and humans (Duncko et al., 2007; Jackson et al., 2006; Zorawski et al., 2006). Stress led to heightened CRs in males, but the same stressor impaired conditioning in females. Our current observations support these prior results in free-cycling women only, but not in men or OC women. However, menstrual cycle and OC usage were previously not controlled for. Investigating women in the ovulatory phase (linked to high estradiol levels) as well as men, psychosocial stress impaired eye-blink conditioning in both groups (Wolf et al., 2009). These results confirm our present findings in men and free-cycling women. This effect in men is also in line with our previous studies using exogenous administration of 30 mg hydrocortisone, in which cortisol consistently reduced conditioned neuronal activation in men, but enhanced it in OC women (Stark et al., 2006; Tabbert et al., 2010; in part: Merz et al., 2010). Overall, the interpretation of the present state of knowledge is complicated by the usage of different methodologies (single cue vs. differential conditioning; eye-blink vs. context vs. fear conditioning; neutral vs. emotional CS) and species. In addition, different concentrations of stress hormones (basal vs. stress-elevated vs. supraphysiological levels), and how increased levels are reached (tail shock vs. swim stress vs. psychosocial stress vs. pharmacological induction) have to be considered.

As mentioned above, men displayed lower estradiol concentrations than the three groups of women, but estradiol levels should be much higher in LU women compared to FO and OC women. One reason for this result could be the fact that we used a between group instead of a within group design. The latter design might be more appropriate to detect slight cycle-associated changes in particular regarding estradiol. The highest estradiol concentrations should be found in the late follicular phase just before ovulation (Buffet et al., 1998), which we did not investigate. Individual differences in each woman's menstrual cycle might account for the not significantly different estradiol levels between FO, LU, and OC women. Nevertheless, progesterone (and testosterone) concentrations showed the expected differences and women were explicitly invited with respect to their menstrual cycle. Thus, we assume a correct classification of the three groups of women.

In summary, heightened stress hormones lower fear learning in subcortical structures. The current sex hormone availability plays a major role in this result: the reducing effect of cortisol concerns men and free-cycling women. However, cortisol causes an enhancement of fear learning in OC women. Thus, OC women constitute an interesting subgroup of participants that future conditioning research needs to explore more closely. In addition, cortisol generally impairs the amygdala differentiation across all participants. Fear learning processes, even not explicitly conscious, and stress are crucial factors in the development and maintenance of psychiatric disorders, in particular anxiety disorders. Sex hormone concentrations modulate the interplay of these factors on the neuronal level, clearly advising to consider activational and organizational effects of sex hormones in modern etiologic models.

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