Biological Psychology 92 (2013) 82–89

Contents lists available at SciVerse ScienceDirect

Biological Psychology

journal homepage: www.elsevier.com/locate/biopsycho

Stress hormones are associated with the neuronal correlates of instructed fear conditioning☆

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ARTICLE INFO

Article history:
Received 28 February 2011
Accepted 22 February 2012
Available online 2 March 2012

Keywords:
Amygdala
Cortisol
fMRI
Oral contraceptives
Sex differences

ABSTRACT

The effects of sex and stress hormones on classical fear conditioning have been subject of recent experimental studies. A correlation approach between basal cortisol concentrations and neuronal activation in fear-related structures seems to be a promising alternative approach in order to foster our understanding of how cortisol influences emotional learning. In this functional magnetic resonance imaging study, participants with varying sex hormone status (20 men, 15 women taking oral contraceptives, 15 women tested in the luteal phase) underwent an instructed fear conditioning protocol with geometrical figures as conditioned stimuli and an electrical stimulation as unconditioned stimulus. Salivary cortisol concentrations were measured and afterwards correlated with fear conditioned brain responses. Results revealed a positive correlation between basal cortisol levels and differential activation in the amygdala in men and OC women only. These results suggest that elevated endogenous cortisol levels are associated with enhanced fear anticipation depending on current sex hormone availability.

1. Introduction

Classical conditioning is thought to represent a central mechanism in the development of anxiety disorders (Bangasser and Shors, 2010; Hofmann, 2008; Mineka and Oehlberg, 2008). Stress influences emotional learning and is a potent modulator of psychiatric diseases, in particular concerning anxiety disorders such as post-traumatic stress disorder (de Quervain et al., 2009; Holsboer and Ising, 2010; Wolf, 2008). More detailed knowledge of the neuroendocrine modulation of emotional learning might have valuable implications for the prevention and treatment of anxiety disorders (Bentz et al., 2010). In particular, women are more likely to develop an anxiety disorder (Kessler et al., 2005; McLean et al., 2011), which could suggest an enhanced susceptibility to stress. However, the precise influence of sex and stress hormones on fear conditioning is still not fully understood.

Amongst others, stress induces an activation of the hypothalamus-pituitary-adrenal (HPA) axis (release of glucocorticoids (GCs): corticosterone in rodents; cortisol in humans). Elevated GCs in turn reduce HPA activity via negative feedback. The HPA axis can be inhibited by the orbitofrontal cortex (OFC) and the hippocampus (including the parahippocampal gyrus), but can be activated by the amygdala (Dedovic et al., 2009; Diorio et al., 1993; Herman et al., 2003, 2005; Liberzon et al., 2007; Oei et al., 2007; Prüssner et al., 2008).

These critical brain structures overlap with the neuronal correlates of emotional learning studied in classical fear conditioning paradigms (Cheng et al., 2006; Knight et al., 1999; LeDoux, 2000; Mechias et al., 2010; Rolls, 1999; Sehlmeyer et al., 2009). Differential fear conditioning includes a stimulus paired with an aversive event (unconditioned stimulus, UCS), which becomes a conditioned stimulus (CS+), whereas another stimulus is never paired (CS−). The fear conditioning protocol can be assumed as successful if higher responses, e.g. skin conductance responses (SCRs) or neuronal activation, towards the CS+ compared to the CS− are observed.

As the most prominent structure in the fear circuit, the amygdala is crucial for fear learning and expression (LeDoux, 2000; Maren, 2005). Importantly, research on fear extinction and emotion regulation revealed that amygdala activation is modulated by the medial prefrontal cortex (PFC), largely by inhibitory projections; however, also excitatory projections exist (Kalisch et al., 2006; Milad et al., 2007; Paré et al., 2004; Phelps et al., 2004; for reviews see Delgado et al., 2006; Ochsner and Gross, 2005).

Stress and the accompanying occupation of glucocorticoid receptors in the PFC might change this top–down control. More

☆ The study was carried out at the Bender Institute of Neuroimaging, Justus Liebig University Giessen.

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doi:10.1016/j.biopsycho.2012.02.017

precisely, rodent studies revealed that elevated GC concentrations impair prefrontal function and the PFC is hence no longer able to inhibit the amygdala (e.g. Akirav and Maroun, 2007; Izquierdo et al., 2006). In human studies, the modulating role of stress hormones on the top–down control of the amygdala by the medial PFC is still rather unclear (e.g. Ahs et al., 2006; Kern et al., 2008; Urry et al., 2006; Wang et al., 2005). Besides, the influence of sex and stress hormones on this interplay has been widely neglected; further research on this topic is thus highly relevant.

Rodent studies using eye-blink conditioning (Dalla and Shors, 2009; Shors, 2004) have revealed that stress hormones have sex-dependent effects on conditioned responses (CRs). Stress led to higher CRs in males, but impaired CRs in females. In human fear conditioning, high endogenous or stress-induced cortisol levels are associated with enhanced fear conditioned SCRs in men, but not in women (Jackson et al., 2006; Zorawski et al., 2005, 2006). Neuroimaging studies using a high cortisol dosage (30 mg; Merz et al., 2010; Stark et al., 2006; Tabbert et al., 2010), however, revealed reduced CRs in men after GC application, but enhanced CRs in women in several brain structures.

One possible explanation of these divergent results could be the exact cortisol concentration during fear conditioning. Basal endogenous or stress-induced cortisol levels might exert effects quite different to those induced by exogenous GC application often leading to supraphysiological hormone concentrations. An inverted U-shaped curve concerning cortisol and memory processes, but also a more linear relationship has been proposed (de Kloet et al., 1999; Lupien et al., 2007; Sandi and Pinelo-Nava, 2007). In males but not in females, linear associations between stress hormones and CRs have been reported (Jackson et al., 2006; Wood et al., 2001; Zorawski et al., 2005, 2006).

The differing fear learning patterns in men and women in response to elevated cortisol levels could be due to the influence of circulating sex hormones on brain activation. The impact of menstrual cycle phase and stress hormones on emotional learning has already been studied in rodents (Shors et al., 1998; Wood and Shors, 1998; Wood et al., 2001). These studies indicate enhanced conditioning performance in females when estradiol levels are high; heightened stress hormones abolished this enhancement. No experiment on this topic exists in humans so far, in particular concerning a correlation approach between basal cortisol concentrations and fear conditioned neuronal activation.

In the present study, we conducted a differential conditioning experiment with an electrical stimulation as UCS. All participants were instructed about the CS-UCS-contingencies before the experiment. Thus, we most likely measured fear expression rather than fear learning. The present sample has already been compared with a group receiving 30 mg cortisol prior to fear conditioning (Merz et al., in press) revealing no effects of exogenous cortisol on CRs. In the present report, we were interested in endogenous cortisol and its correlation with differential neuronal activation.

Based on previous human studies investigating endogenous (basal or stress-induced) cortisol levels (Jackson et al., 2006; Zorawski et al., 2005, 2006), we expected positive correlations between cortisol concentrations and differential amygdala activation, in particular in men. The CS+/CS− differentiation in the PFC should also be associated with endogenous cortisol either positively or negatively depending on the particular subregion (medial PFC vs. OFC). We had additional specific hypotheses concerning the (para)hippocampal complex and the insula. Heightened cortisol levels consistently influence these structures sex-dependently, as has been shown before (Merz et al., 2010; Tabbert et al., 2010). All these hypotheses are independent of each other. Because of the inconsistent results in the literature regarding women, we exploratively investigated two groups of women with different hormonal statuses. We were particularly interested in two groups of women, which are most different from each other in terms of sex hormone status. More specifically, we tested women in the luteal phase of the menstrual cycle (LU; high endogenous estradiol and progesterone levels) and women taking oral contraceptives (OC; low endogenous estradiol and progesterone levels because of pill intake; cf. Buffet et al., 1998; Kirschbaum et al., 1999).

2. Materials and methods

2.1. General background

The data presented are part of a larger ongoing project investigating the effects of contingency awareness, stress, and sex hormones on fear conditioning. Participants received either 30 mg cortisol (hydrocortisone; Hoechst) or placebo (tablettose and magnesium) orally about 45 min before the fear conditioning protocol. In the present data analysis, only participants, who were informed about the relationship between CS and UCS in advance of the experiment (i.e. instructed fear conditioning; see Tabbert et al., 2011), were included. Further, only participants receiving placebo were included to explore the impact of endogenous cortisol levels on fear CRs. The effects of the exogenous cortisol administration resulting in supraphysiological cortisol levels as well as results of the extinction phase will be reported elsewhere (Merz et al., in press). A group analysis of the same sample has been published previously together with two additional groups (unaware and learned aware participants; cf. Tabbert et al., 2011). This prior analysis was concerned with the differential impact of contingency awareness on fear acquisition, not with sex hormone status or the relation between cortisol concentrations and fear responses.

2.2. Subjects

In total, 50 participants completed the study; 44 were undergraduate students, the remaining six had already graduated. To assess different sex hormone statuses in women, we invited 15 free-cycling women and 15 OC taking women. We also investigated 20 men. Free-cycling women reported to have a regular menstrual cycle and were invited in the luteal phase (LU) of their individual menstrual cycle (3rd to 9th day before the onset of their next menstruation; Buffet et al., 1998). OC women were required to have been taking their birth control pill (only monophasic preparations with an ethinylestradiol component) for at least the last three months. They were tested 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 were, in addition to somatic diseases, in particular endocrine diseases, which can influence hormone concentrations. Inclusion criteria were an age between 18 and 35 and a body mass index (BMI) between 18 and 28 kg/m^2. The mean age for the three sex hormone status groups (men: 24.15 ± 3.08 (standard deviation); LU women: 25.27 ± 3.69; OC women: 23.60 ± 2.13) as well as the mean BMI (men: 23.04 ± 1.94; LU women: 22.79 ± 1.59; OC women: 21.57 ± 1.85) were comparable (both $ps > .05$).

All participants were right-handed as assessed by the Edinburgh Inventory of Handedness (Oldfield, 1971) and had normal or corrected vision. They were instructed to refrain from smoking, food intake, and drinking anything but water for at least two hours before the experiment. Each experimental session was scheduled to begin between 2 and 5 p.m. to guarantee low and relatively stable endogenous cortisol concentrations. At first, participants received a detailed explanation of the procedure in general. All participants gave written informed consent and received at least 25 Euros for their attendance. The study was approved by the ethics committee of the German Psychological Society.

2.3. Conditioned visual stimuli, 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 were gray-colored, had identical luminance, and were presented against a black background for 8 s. Using an LCD projector (EPSON EMP-7250), stimuli were projected onto a screen at the end of the scanner (visual field = 18°) and were viewed through a mirror mounted on the head coil. A custom-made impulse-generator (833 Hz) provided transcutaneous electrical stimulation (UCS) for 100 ms through two Ag/AgCl electrodes (1 mm^2 surface each). Electrodes were fixed to the middle of the left shin and stimulus intensity was set individually using a gradually increasing rating procedure to achieve a level of sensation, which was “unpleasant but not painful”. The onset of the UCS presentation started 7.9 s after CS+ onset (100% reinforcement; delay conditioning). The CS− and the non-CS were never paired with the UCS. Non-UCS was defined as the UCS omission 7.9 s after the onset of the CS−.

The conditioning experiment consisted of an instructed fear phase, an extinction phase, and an implemented two-back task (cf. Merz et al., 2010 and Tabbert et al., 2010 for further details). Twenty trials of CS+ as well as CS− and ten trials of non-CS were presented in the instructed fear phase. Inter-trial intervals (ITI) between the numbers of the two-back task and the geometrical figures lasted 5 s and were randomly jittered between 0 and 2.5 s (i.e. ITI of 5–7.5 s). For each participant, pseudo-randomized stimulus orders were used (cf. Merz et al., 2010).

Participants were told precisely, which geometrical figure (the rhomb or the square, randomized over all subjects) would precede the electrical stimulation (instructed fear conditioning). Immediately after the instructed fear phase, participants had to rate the contingencies between UCS and CS+, CS−, and non-CS, presented in random order. 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 possible answers: "I do not know", "never", "sometimes", and "always". In all participants, contingency awareness was confirmed by indication that the CS+ "always" and the CS− "never" preceded the UCS.

2.4. Hormone analyses and skin conductance responses (SCRs)

Saliva samples for the analyses of free cortisol, estradiol, progesterone, and testosterone were collected by use of glass tubes. Samples were taken directly before placebo tablet intake (see Section 2.1) as well as 25 min (immediately before the fMRI run) and 90 min (immediately after the fMRI run) after placebo tablet intake. Saliva was stored at −20 °C until assayed by use of commercial enzyme immunoassays (IBL International, Hamburg, Germany). All samples were 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%.

SCRs were sampled concurrently with fMRI scans using Ag/AgCl electrodes filled with isotonic (0.05 M NaCl) electrolyte medium placed hypothenar at the non-dominant hand. SCRs were defined in two analysis windows: the maximum amplitude within a window of 1–8.5 s after the onset of the CS was counted as the CR and within the time window of 8.5–13 s as the unconditioned response (UCR). The baseline was the skin conductance level immediately preceding the inflexion point. Electrodermal data were transformed with the natural logarithm in order to attain a normal distribution.

All statistical analyses were conducted in IBM SPSS Statistics for Windows 19.0. Greenhouse-Geisser correction was applied when the sphericity assumption was not met and the statistical significance level was set to $p < .05$. Additionally, trends will be reported up to a threshold of $p < .10$. Analyses of variance (ANOVA) were performed for cortisol including the repeated measurement factor time (first vs. second vs. third sample) and the between subjects factor sex hormone status (men vs. LU women vs. OC women). Estradiol, progesterone, and testosterone were analyzed with the between subjects factor sex hormone status only, without the repeated measurement factor time. Sex hormones were only determined in the first and the third saliva sample. Their concentrations were averaged to check for expected differences between men, LU, and OC women.

Statistical comparisons of SCRs were performed with the within subjects factor stimulus-type (CS+ and CS− for the CR; UCS and non-UCS for the UCR) and the between subjects factor sex hormone status. Only main effects or interactions with the factor stimulus-type will be reported to emphasize conditioning-related modulations. Pearson product-moment correlations were computed between mean differential conditioned SCRs and cortisol concentrations right before fear conditioning (second saliva sample) reflecting cortisol levels during instructed fear most appropriately.

2.5. Image acquisition and analyses

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; Tabbert et al., 2010, 2011 for details concerning structural and functional image acquisition). Data were analyzed 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 FWHM of 9 mm.

The instructed fear phase was integrated in a statistical model including the following experimental conditions: CS+, CS−, non-CS, UCS, non-UCS, targets, and non-targets. A rapid initial fear response is assumed as before (cf. Merz et al., 2010; Stark et al., 2006; Tabbert et al., 2010, 2011) in contrast to more long lasting processes, which might be obscured by emotion regulation processes (e.g. Hermann et al., 2009). So, all regressors were modelled by a stick function convolved with the canonical hemodynamic response function in the general linear model, without specifically modelling the durations of the different events (i.e. event-related approach). The six movement parameters of the rigid body transformation obtained by the realignment procedure were introduced as covariates in the model. 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− and UCS minus non-UCS. ANOVA was conducted with the group factor sex hormone status in the full factorial model implemented in SPM8. Further, we correlated cortisol concentrations with the contrast CS+ minus CS− in order to investigate the specific relationship between endogenous cortisol levels and fear conditioned neuronal activation (measured by blood oxygenation level dependent (BOLD) responses). Therefore, cortisol concentrations before conditioning (second sample) were included as regressor in a simple regression model on second level (i.e. group level). Significant t-values indicate brain regions, in which the functional differential activation significantly correlates with cortisol levels. To gain a quantitative measure for the magnitude of these correlations, the t-values of the peak voxels of the respective analysis were transformed into the correlation coefficient r (Rosenthal, 1994).

For all these statistical analyses, we used exploratory whole brain as well as region of interest (ROI) analyses: the amygdala and the insula were included as ROI for the UCS and CS analyses. Additionally, the frontal medial cortex, the hippocampus, the anterior parahippocampal gyrus, and the OFC were included for CS analyses. All ROI were tested separately for the left and the right hemisphere except the frontal medial cortex. The required masks for these analyses were 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) with the probability threshold set to .5.

For 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. Regarding 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). Trends in the amygdala will also be reported at a more liberal level ($p < .10$ on voxel-level, FWE-corrected) due to its prominent role in fear conditioning.

3. Results

3.1. Endocrinological data and SCRs

ANOVA for cortisol revealed a significant main effect of time ($F(1.4, 65.4) = 13.7$; $p < .001$) and a time x sex hormone status interaction ($F(2.8, 65.4) = 3.2$; $p = .032$). Post hoc ANOVA and t-tests showed different variations over time for the three sex hormone status groups, but no significant differences at baseline, in the second and third sample (see Table 1A). All sex hormone status groups showed a decline in cortisol concentrations from the first to the second sample (all $p = .001$). The second and third sample did not differ in LU and OC women (both $p > .10$); in men, higher cortisol levels were found after the conditioning procedure in comparison to the concentrations before the fMRI run ($p = .005$).

Analyses of sex hormones revealed implausibly high levels for some participants, which could point to sample contamination. Explorative data analyses displayed six outliers (three men, two LU women, and one OC woman) for estradiol and four outliers (two men and two OC women) for testosterone. These subjects were excluded from sex hormone analyses. ANOVA showed a significant main effect of sex hormone status for estradiol ($F(2, 41) = 6.7$; $p = .003$), progesterone ($F(2, 47) = 26.0$; $p < .001$), and testosterone ($F(2, 43) = 16.7$; $p < .001$; see Table 1B). Compared to OC women and men, LU women had significantly higher estradiol (both $p < .030$) and progesterone levels (both $p < .001$). Men had higher testosterone concentrations than LU and OC women (both $p \leq .001$), and LU women had higher levels than OC women ($p = .018$).

Concerning SCRs, ANOVA demonstrated a main effect of stimulus-type for the CR ($F(1, 47) = 56.8$; $p < .001$) and the UCR ($F(1, 47) = 136.0$; $p < .001$). These effects were based on higher SCRs towards the CS+ (UCS respectively) than to the CS− (non-UCS respectively). By trend, a stimulus-type x sex hormone status interaction occurred in the CR ($F(2, 47) = 2.9$; $p = .068$), but not in the UCR ($p > .20$). Post hoc t-tests revealed that the CS+/CS− differentiation was higher in men compared to OC women ($T(33) = 2.6$; $p = .013$; see Fig. 1), whereas LU women did not significantly differ from men ($p > .65$) or OC women ($p > .10$).

Correlation analyses showed a negative relationship between cortisol concentrations and differential conditioned SCRs in OC women only ($r = -.70$; $p = .004$), but no significant correlation in men ($r = -.24$; $p > .30$), LU women ($r = .04$; $p > .85$), or the whole sample ($r = -.16$; $p > .25$). Tests of the correlation coefficients between

Table 1
(A) Mean (*SE*) salivary cortisol levels (in nmol/l) at baseline, before, and after fear conditioning for men, LU, and OC women. *P* values for the main effect time (separate for men, LU, and OC women) and the main effect sex hormone status group (separate for the first, second, and third sample) are also included. (B) Mean (*SE*) salivary estradiol, progesterone, and testosterone concentrations (in pmol/l) for men, LU, and OC women. *P* values for the main effect sex hormone status group are included.

(A) Cortisol (nmol/l)	Baseline	Before conditioning	After conditioning	Main effect: time
Men	5.94 (.67)	4.26 (.62)	6.16 (.80)	$p = .010$
LU women	7.27 (1.56)	4.68 (1.08)	4.13 (.68)	$p = .019$
OC women	6.62 (.79)	4.59 (.56)	5.40 (.77)	$p = .005$
Main effect: group	$p = .643$	$p = .917$	$p = .177$	

(B) Sex hormone (pmol/l)	Estradiol	Progesterone	Testosterone
Men	5.56 (.79)	137.48 (17.82)	274.41 (40.81)
LU women	1.95 (1.55)	567.38 (82.28)	97.35 (15.41)
OC women	6.48 (.95)	164.63 (24.48)	52.08 (8.85)
Main effect: group	$p = .003$	$p < .001$	$p < .001$

Unrealistically high sex hormone levels were excluded from the analyses and the descriptives of this table.

the sex hormone status groups revealed a significantly stronger (negative) correlation in OC women compared to men ($Z_{diff} = 1.65$; $p = .049$) and compared to LU women ($Z_{diff} = 2.22$; $p = .013$); men and LU women did not differ from each other ($p > .20$).

3.2. Hemodynamic responses

In the contrast UCS minus non-UCS for the whole group, significant BOLD responses were found bilaterally in the amygdala and the insula (all $p_{corr.} < .001$; see Supplementary Table 1A). In addition to these ROI, the whole brain analyses revealed significant BOLD responses in the frontal pole, the right middle frontal gyrus, and the right insula (in a different voxel than in the ROI analysis). No group differences emerged in the UCR in the ANOVA with the group factor sex hormone status.

In the contrast CS+ minus CS−, significant results were found bilaterally in the insula and the OFC as well as the right amygdala and the left anterior parahippocampal gyrus (see Supplementary Table 1B). Additionally, the exploratory whole-brain analyses showed significant BOLD responses in the right angular gyrus, the left anterior supramarginal gyrus, the left central opercular cortex, the right inferior frontal gyrus, the precentral gyrus, and bilaterally in the superior parietal lobule. Further, ANOVA revealed a significant group difference in the right amygdala ($x = 21$, $y = 0$, $z = -21$; $F = 7.24$; $p_{corr} = .041$; see Fig. 2). Post hoc *t*-tests indicated a significantly higher CS+/CS− differentiation in LU women compared to OC women ($x = 21$, $y = -6$, $z = -21$; $T = 4.06$; $p_{corr} = .006$) and trendwise compared to men ($x = 21$, $y = 0$, $z = -18$; $T = 2.59$; $p_{corr} = .090$), whereas men and OC women did not differ significantly from each other.

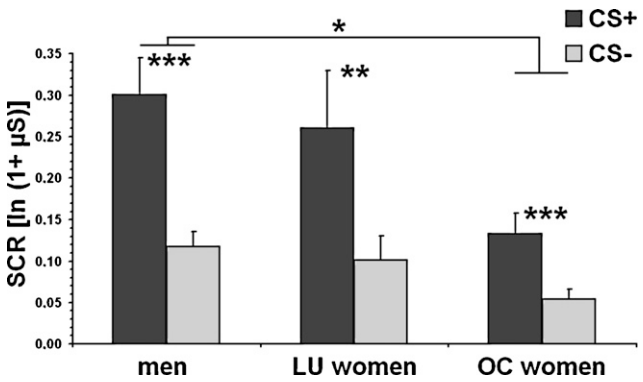

Fig. 1. Mean (SE) skin conductance responses (SCRs; transformed with the natural logarithm) in response to CS+ and CS−. Each sex hormone group exhibited significantly conditioned SCRs. The almost significant interaction stimulus-type x sex hormone status is illustrated with OC women demonstrating lesser CS+/CS− differentiation compared to men. ** $p \leq .005$, *** $p \leq .001$, resulted from post hoc *t*-tests in each sex hormone status group; * $p \leq .05$, resulted from post hoc *t*-tests between the sex hormone status groups.

In the correlation analyses in men, a significant positive correlation between endogenous cortisol levels and the contrast CS+ minus CS− emerged in the left amygdala (see Table 2 and Fig. 3). Further correlations in the respective peak voxel separately for CS+ and CS− revealed that the differential correlation can be traced back to responses towards the CS+ only ($r = .59$; CS−: $r = -.06$). In OC women, a positive correlation between cortisol and the CS+/CS− differentiation occurred in the right amygdala and the right anterior parahippocampal gyrus (see Table 2 and Fig. 3). For the amygdala, the correlation is driven by a combination of responses to the CS+ ($r = .33$) and CS− ($r = -.41$). For the anterior parahippocampal gyrus, the correlation can be traced back to responses to the CS+ only ($r = .68$; CS−: $r = -.01$).

In men and OC women, we further tested if the correlation coefficients in the peak voxels of the respective analyses were significantly different from the two other sex hormone status groups. In men, the correlation coefficient of the left amygdala was not significantly larger than in LU or OC women. However, the correlation coefficient in the right amygdala in OC women significantly differed from men ($Z_{diff} = 2.58$; $p = .005$) and LU women ($Z_{diff} = 2.24$; $p = .013$). The same pattern was found for the right anterior parahippocampal gyrus (OC women compared to men: $Z_{diff} = 2.81$; $p = .002$; and compared to LU women: $Z_{diff} = 3.00$; $p = .001$).

Besides the correlations in men and OC women, we also found a positive correlation in the right OFC ($x = 27$, $y = 12$, $z = -21$; $T = 3.36$; $p_{corr} = .039$; $r = .436$) in all participants. Similarly in LU women, positive correlations emerged in the left anterior parahippocampal gyrus ($x = -24$, $y = 0$, $z = -33$; $T = 3.49$; $p_{corr} = .046$; $r = .695$) and the right OFC ($x = 27$, $y = 12$, $z = -21$; $T = 5.11$; $p_{corr} = .011$; $r = .817$) as well as a negative correlation in a different voxel in the right OFC ($x = 12$, $y = 15$, $z = -21$; $F = 4.27$; $p_{corr} = .035$; $r = -.764$). However, inspection of the scatter plots (see Supplementary Fig. 1) revealed that the results in the whole group as well as in LU women were mainly driven by two outliers. After removing these participants, the respective correlations in the whole group and in LU women did no longer reach the significance threshold.

Supplementary material related to this article can be found in the online version, at doi:10.1016/j.biopsycho.2012.02.017.

4. Discussion

In the present fear conditioning study, we observed a higher CS+/CS− differentiation in the amygdala occurring in women with higher levels of female sex hormones (women in the luteal phase of their menstrual cycle). Using a correlation approach, we were further able to identify neuronal structures showing associations between endogenous cortisol concentrations and instructed fear

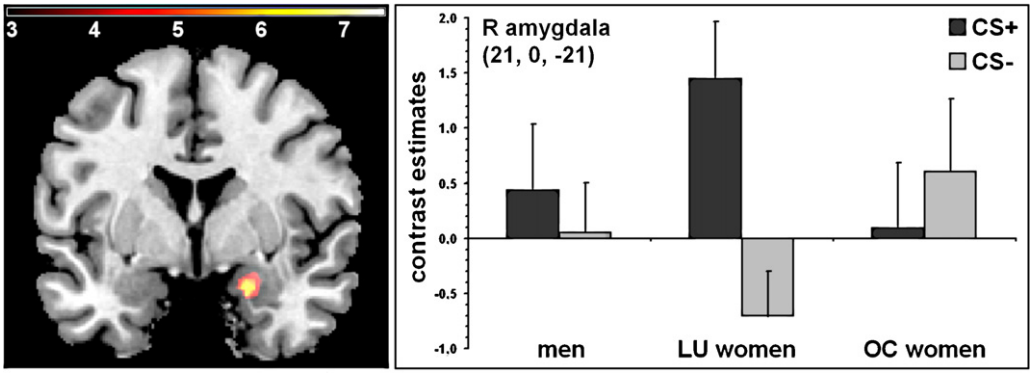

Fig. 2. Differences between the sex hormone status groups for neuronal activation in the contrast CS+ minus CS−. LU women exhibited significantly higher CRs in the right amygdala compared to OC women and trend-wise compared to men.
Data are masked with the respective ROI (see color bar for exact *F* values). The depicted slice was selected according to the reported activation, MNI coordinates are given (L = left; R = right).
The bar graph depicts the group mean of peak voxel activation in the right amygdala, error bars are standard errors of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Localization and statistics of the peak voxels for the correlation analyses between cortisol concentrations and BOLD responses in the contrast CS+ minus CS−. Results are shown for all participants as well as separately for men, LU, and OC women within the respective ROI. Correlation coefficients (*r*) are inserted at the right side. After removal of two outliers (see also Supplementary Fig. 1), the correlations in all participants as well as in LU women did no longer reach the statistical significance threshold.

Group	Brain region	*x*	*y*	*z*	T_{max}	$p_{corr.}$	*r*
All	no significant activations						
Men	L amygdala	−24	−12	−12	3.13	.045	.594
LU women	no significant activations						
OC women	R amygdala	18	−3	−24	3.64	.042	.710
	R anterior parahipp. gyrus	15	−3	−27	4.20	.023	.759

The significance threshold was $p_{corr} < .05$ (FWE-corrected; small volume correction). All coordinates (*x*, *y*, *z*) are given in MNI space (L = left, R = right).

conditioning (which probably more closely reflects fear expression rather than fear learning). Further, the correlation patterns were influenced by the current sex hormone status. Before discussing these main results, a brief summary of the important prerequisites for the interpretation of our findings will be given.

First, recruiting of the present sample was successful, because sex hormone concentrations of the three sex hormone status groups were in the expected range compared with values reported by the producer of the enzyme immunoassays as well as a previous study in LU women (Schoofs and Wolf, 2009). Second, the electrical stimulation was effective in evoking UCRs in all preselected ROI and in additional structures obtained by the whole brain analyses (see Supplementary Table 1), which is also mirrored in SCRs. Third, in the contrast CS+ minus CS−, we observed significant activations in the amygdala, the insula, the OFC, and the anterior parahippocampal gyrus. Besides these predefined ROI, the exploratory whole brain

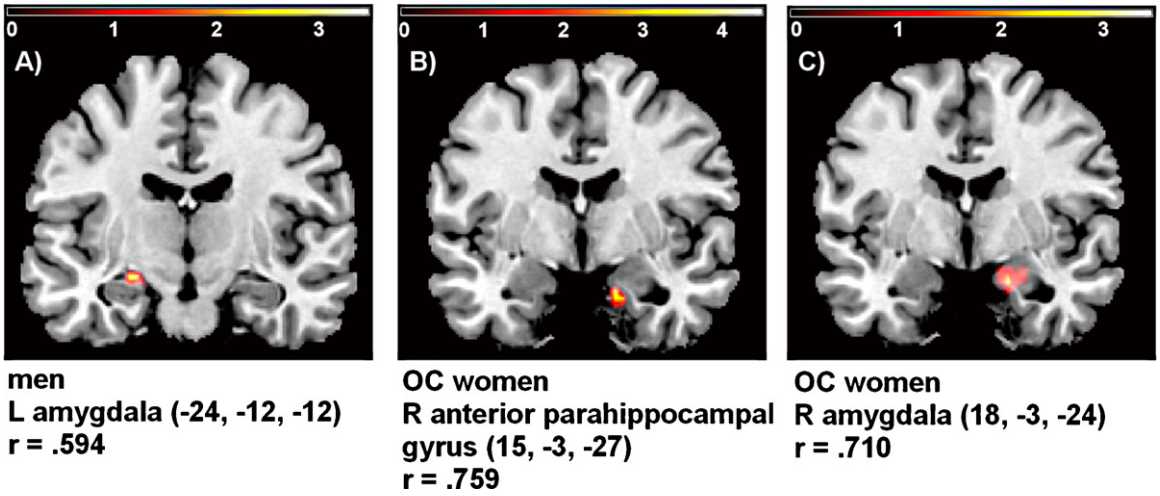

Fig. 3. Neuronal activation for the correlation analyses between differential brain activation (contrast CS+ minus CS−) and endogenous salivary cortisol levels. Data are masked with the respective ROI (see color bar for exact *T* values). The depicted slices were selected according to the reported activations (see Table 2), MNI coordinates and correlation coefficients are given (L = left; R = right). Scatter plots of these correlations can be found in the supplementary Fig. 1.
(A) Analyses for *men only*, significant *positive* correlation between cortisol and differential activation in the *left amygdala*.
(B) Analyses for *OC women only*, significant *positive* correlation between cortisol and differential activation in the *right anterior parahippocampal gyrus*.
(C) Analyses for *OC women only*, significant *positive* correlation between cortisol and differential activation in the *right amygdala*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

analyses revealed significant results in the angular, the anterior supramarginal, the inferior frontal, and the precentral gyrus as well as in the central opercular cortex and the superior parietal lobule (see Supplementary Table 1). Thus, the fear conditioning protocol was successful in eliciting CRs in fear- and anticipation-related structures, which can also be seen in conditioned SCRs.

In the contrast CS+ minus CS−, a group difference emerged in the right amygdala (see Fig. 2), where LU women had a significantly higher CS+/CS− differentiation than OC women and also trendwise compared to men. This result is in line with studies in female rats, in which high estradiol and progesterone levels (as present in LU women) led to enhanced eye-blink conditioning compared to other stages of the menstrual cycle (Shors et al., 1998; Wood et al., 2001). Thus, heightened concentrations of female sex hormones might sensitize amygdala neurons resulting in enhanced CRs. A dose-dependent sex hormone effect has also been reported with high doses of estradiol leading to enhanced fear learning while low doses impair it (Diaz-Veliz et al., 1991). Because women with elevated female sex hormones exhibit higher differential responses in the amygdala, our finding is highly relevant for future conditioning studies investigating, and in particular contrasting, men and women.

Previous studies in men and male rodents have shown that basal cortisol levels as well as either acute or chronic stress and GC treatment have facilitating effects on fear CRs (Conrad et al., 1999; Hui et al., 2004; Jackson et al., 2006; Zorawski and Killcross, 2002; Zorawski et al., 2005, 2006). In women or female animals, the picture is less clear; they were either not investigated or differences in sex hormone levels (because of OC usage or particular stage of menstrual cycle) were not controlled for or reported. The present fMRI study addressed this problem by examining men as well as two groups of women, either free-cycling tested during the luteal phase or taking OC.

Correlation analyses revealed a positive relationship between endogenous cortisol levels and the CS+/CS− differentiation in the amygdala. This was the case for men on the left side and for OC women on the right. Results also pointed to a positive correlation in the right anterior parahippocampal gyrus in OC women (see Table 2 and Fig. 3). Recent studies showed increased activation in the amygdala during the learning of emotional pictures (van Stegeren et al., 2010), depending on cortisol levels and on the activation of the autonomous nervous system (van Stegeren et al., 2005, 2007). Further, the amygdala is strongly connected to anterior hippocampal regions (cf. Goosens, 2011; van Strien et al., 2009). Stress hormones can strengthen emotional memories via their influence on the amygdala and hippocampal regions (Roozendaal et al., 2006; for a recent review see Schwabe et al., 2011).

Taken together, our data support the notion that endogenous stress hormones are positively associated with instructed fear conditioning – in particular the resulting amygdala activation – in men and women with low endogenous female sex hormones (OC women). Applying supraphysiological cortisol levels (obtained by the administration of 30 mg hydrocortisone) in unaware and learned aware participants, we found enhanced CRs in several brain regions in OC women, yet reduced CRs in men (Stark et al., 2006; Merz et al., 2010; Tabbert et al., 2010). Lower doses (10 mg) also reduced amygdala responsivity to emotional faces in men (Henckens et al., 2010). From the present data, a linear relationship between stress hormones and memory processes (cf. Sandi and Pinelo-Nava, 2007) might be proposed at least in OC women. In men, the available data argue more for an inverted U-shaped relationship (cf. de Kloet et al., 1999; Lupien et al., 2007). Thus, the exact relation between stress hormone levels and conditioned responding seems to be different in men and OC women. Contrary, we did not observe any association between cortisol and differential neuronal activation in LU women. This could be due to ceiling effects and/or low variance in neuronal activation in this group. Therefore, it remains to be shown how exactly stress hormones influence fear conditioning in free-cycling women.

Regarding differential electrodermal activity, we found a significant but negative correlation between cortisol and differential SCRs in OC women. No associations between cortisol and SCRs were observed in the other two groups (men and LU women). OC women also displayed a slightly changed overall conditioning effect in the SCRs, as can be inferred from the almost significant stimulus-type x sex interaction. Descriptively, OC women had the least expressed CRs compared to men and LU women (see Fig. 1, only the difference between men and OC women reached statistical significance).

Thus, on the one hand, OC women exhibited lesser CS+/CS− differentiation in SCRs than men and lesser differential responses in the right amygdala than LU women. On the other hand, *higher cortisol concentrations* in OC women led to reduced differential SCRs but enhanced neuronal activation in the right amygdala and the right anterior parahippocampal gyrus. These results contradict prior human studies, in which no correlations between stress hormones and CRs were found in women (Jackson et al., 2006; Zorawski et al., 2005, 2006). Nevertheless, as noted above, these previous findings can be criticized, because the authors did not control for varying sex hormones over the course of the menstrual cycle and OC intake. Our results concerning OC women suggest a dissociation between the influence of cortisol at the electrodermal and the neuronal level. Having this in mind, contradictory findings obtained in women might not only be due to fluctuating sex hormone levels, but also due to differences in the measured response levels.

In the present study, only women taking OC or in the luteal phase of the menstrual cycle were recruited. These two groups differ from each other concerning the levels of female sex hormones (low in OC women; high in LU women) as well as how these levels are reached (low female sex hormones in OC women as a consequence of OC intake; high female sex hormones because of natural fluctuations across the menstrual cycle). Subsequently, a clear distinction between a direct OC effect and an effect of sex hormone availability cannot be made. For this purpose, women in the follicular phase should be included in future studies (cf. Kuhlmann and Wolf, 2005), because they have low female sex hormone levels comparable to OC women, but due to fluctuations over the menstrual cycle and not due to OC intake. A direct comparison of these two groups would allow to answer the question if there is an underlying effect of low sex hormone availability (if responses of women in the follicular phase resembled those of OC women) or a direct OC effect (if women in the follicular phase resembled LU women).

On first sight, laterality seems to influence the correlation between cortisol and differential responses in the amygdala: In men, an association was observed in the left amygdala, however, in OC women, the correlation was found on the right. This is in contrast to previous reports indicating sex differences in emotional learning, where the left amygdala was recruited in women and the right amygdala in men (Cahill, 2006; but see van Stegeren et al., 2010). Though, as reviewed by Wager et al. (2003), lateralization of emotional processing is quite complex and needs to be explored in future studies.

Furthermore, third variables might account for the results of the current correlation analyses. For example, a mediating effect of corticotropin releasing factor (CRF) cannot be excluded, because high cortisol concentrations lead to reduced CRF levels due to the negative feedback mechanisms of the HPA axis (Aguilera et al., 2007). Yet, in the amygdala, GCs sensitize rather than reduce CRF release (Schulkin et al., 1998). Besides, higher cortisol levels could also reflect higher state anxiety, neuroticism, or depression-related

constructs (Biondi and Picardi, 1999; Portella et al., 2005; van Goozen et al., 1998). Still, since large parts of our results are well in accordance with previous stress research, as noted above, it is very likely that cortisol per se is critically involved in the enhanced fear processing in our experimental design rather than conceivable confounding variables.

Because of the correlational nature of our results, it needs to be elucidated in future studies how exactly cortisol influences CRs in SCRs and neuronal activation and whether OC intake or fluctuating sex hormones are involved in this effect. The precise relationship between stress hormones and CRs in prefrontal areas also remains to be shown in additional experiments. According to the assumed top–down control of the amygdala by structures of the PFC (Amano et al., 2010; Likhtik et al., 2005; Pape and Paré, 2010; Quirk et al., 2003), a correlation between cortisol levels and prefrontal differential activation was hypothesized, yet not verified in the present study.

Lastly, we cannot be sure that the concurrent two-back task has not intervened in fear responding and the association between cortisol and neuronal activation. This distractor task was introduced mainly for two reasons: on the one hand, we were also interested in an additional group, which should not notice the CS-UCS contingencies (cf. Tabbert et al., 2011). These unaware participants were distracted in order to investigate automatic or implicit fear learning (cf. Merz et al., 2010). On the other hand, the two-back task enhanced general attention in order to ensure that participants pay close attention to all visual stimuli. Since all the effects that may be introduced with the two-back task account for CS+ and CS− alike, differential responses should not be affected. Still, a generalization from the current results on a design without a distractor task remains speculative and needs to be addressed by additional studies using a fear conditioning paradigm without a secondary working memory task.

In sum, fear conditioning-related neuronal responses were higher in the amygdala in LU women compared to OC women and men. Positive associations with endogenous cortisol concentrations and neuronal activation were found, but these depended on the specific group tested. Higher cortisol concentrations accompanied higher CS+/CS− differentiation in the amygdala in men and OC women as well as in the anterior parahippocampal gyrus in OC women only. The cortisol-related activation of these subcortical structures might enhance the acquisition and consolidation of fearful events.

Role of the funding source

Funding for this study was provided by the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) to R. Stark (STA 475/7-1) and O.T. Wolf (WO 733/8-1). The DFG had no role in study design, collection, analysis and interpretation of data, writing of the manuscript, or in the decision to submit the paper for publication.

Acknowledgments

We thank Prof. Dr. Dr. Jürgen Hennig, Dr. Yvonne Küpper, and Cornelia Meineke (Justus Liebig University Giessen) for assistance with the hormone analyses as well as Dr. Carlo Blecker (Bender Institute of Neuroimaging) for technical assistance and Dr. Bertram Walter (Bender Institute of Neuroimaging) for statistical support. Further, we thank Lisa Bulganin, Kristina Haase, Klio Hilber, Adriane Icenhour, Agnes Kroczek, and Lisa Koob for subject recruitment and data collection. At last, we would like to thank the anonymous reviewers for their helpful and constructive comments.

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