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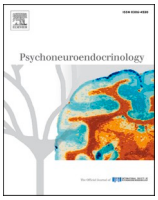

Cortisol before extinction generalization alters its neural correlates during retrieval

Bianca Hagedorn, Oliver T. Wolf, Christian J. Merz *

Department of Cognitive Psychology, Institute of Cognitive Neuroscience, Faculty of Psychology, Ruhr University Bochum, Germany

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ABSTRACT

While generalization of fear seems to be naturally acquired as frequently observed in fear-related disorders, extinction learning appears to be stimulus-specific. Thus, treatments aiming to generalize extinction learning comprise the chance to overcome stimulus-specificity and consequently reduce relapse. One suggested candidate is the timing-dependent administration of the stress hormone cortisol.

In the present pre-registered, three-day fear conditioning study, we aimed to create a generalized extinction memory trace in 60 healthy men and women using multiple sizes of one conditioned stimulus (CS+G; generalized) during extinction training, whereas the other CS (CS+N; non-generalized) and the CS- were solely presented in their original sizes. Extinction training took place either after pharmacological administration of 20 mg cortisol or placebo. Following successful fear acquisition on day one, generalization effects during extinction training and retrieval were investigated in the comparison of CS+G and CS+N. Insula and dorsal anterior cingulate cortex (dACC) activation for CS+G as compared to CS+N extending to the second half of extinction training indicated prolonged fear processing during extinction training for the CS+G on day two. During retrieval on day three, an activation of the anterior hippocampus occurred for CS+N minus CS+G in the cortisol but not in the placebo group. Additionally, a more posterior hippocampal activation (compared to the other hippocampal activation) was observed for the contrast CS+G minus CS+N. In accordance with our hypotheses, amygdala and dACC responding during reinstatement test was reduced for the CS+G as compared to CS+N. However, cortisol did not modulate amygdala responding, but abolished the CS+G/CS+N differentiation in the dACC relative to placebo. Generalization and cortisol effects were not mirrored in skin conductance responses. In conclusion, extinction generalization processes appear to rely on prolonged fear processing still present in the second half of extinction training that in turn leads to reduced fear-related processing after reinstatement. Cortisol administration prior to extinction training, however, selectively reduced fear-related activation for standard extinction but did not further reduce fear-related activation for extinction generalization.

1. Introduction

Relapse after initially successful exposure therapy constitute one of the major limitations of today's first line treatment for anxiety disorders (Arch and Craske, 2009; Craske et al., 2014; Lipp et al., 2020) with estimations ranging between 19% and 62% (Craske and Mystkowski, 2006). As extinction learning represents the crucial model for exposure therapy (Forcadell et al., 2017; Lange et al., 2020), enhancing extinction learning could contribute to increasing the success of exposure therapy. One major source of relapse encompasses return of fear evoked by perceptually (Struyf et al., 2017, 2015; Zaman et al., 2019) and conceptually (Dunsmoor et al., 2012; Starita et al., 2019; Vervoort et al., 2014) related stimuli to the original fear-related stimulus. Thus, extinction is required to generalize across features of the original fear-related stimulus (Vervliet et al., 2006) to counteract the generalization of fear (Dunsmoor and Murphy, 2015; Lissek et al., 2014). The stress hormone cortisol is able to enhance extinction learning when administered prior to extinction training (Meir Drexler et al., 2019) and ultimately promote exposure therapy (de Quervain et al., 2019, 2017). We aimed to combine the strategies of stimulus-based extinction generalization (Hagedorn et al., 2021; Waters et al., 2018) and pre-extinction cortisol administration (Meir Drexler et al., 2019) to

* Correspondence to: Department of Cognitive Psychology, Faculty of Psychology, Institute of Cognitive Neuroscience, Ruhr University Bochum, Universitätsstr. 150, 44780 Bochum, Germany.
E-mail address: Christian.J.Merz@rub.de (C.J. Merz).

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enhance extinction learning compared to standard extinction.

Fear generalization appears to rely on schematic matching processes via the hippocampus, which do not initiate pattern separation mechanisms leading to discrimination between stimuli, but rather pattern completion mechanisms (Lissek et al., 2014). Consequently, this process is assumed to mediate the downregulation of the ventromedial prefrontal cortex (vmPFC) and in turn disinhibition of the fear network with its main structures (Fullana et al., 2016): insula, amygdala and dorsal anterior cingulate cortex (dACC).

Importantly, activation of areas involved in fear expression and inhibition were also observed during extinction generalization: During extinction training, amygdala and insula activation were upregulated and the vmPFC downregulated, indicating enhanced fear responding (Hagedorn et al., 2021). In contrast, during retrieval, amygdala and hippocampal activation were reduced to both, the original and a formerly unpresented stimulus size, arguing for overall reduced fear responding (Hagedorn et al., 2021). However, enhanced responding during extinction training and decreased responding during retrieval were not reflected in skin conductance responses (SCRs; Hagedorn et al., 2021). Nevertheless, this neuroimaging pattern (but not the SCR result) is in line with a previous study that observed increased SCRs during extinction learning, but decreased SCRs in a later generalization test in a multiple extinction stimuli compared to a single extinction stimulus group (Waters et al., 2018). In addition, the integration of multiple stimuli during exposure therapy was observed to prolong fear expression but ultimately reduce the return of fear (Rowe and Craske, 1998). Of note, the highest fear short- and long-term reduction in phobic patients was reported after exposure involving multiple stimuli in contrast to multiple contexts or a combination of both (Shiban et al., 2015).

As a first hypothesis for the current extinction generalization study, we expect increased SCRs and activation of the fear network and decreased activation of the inhibitory vmPFC during the first and second half of extinction training for a generalized extinguished conditioned stimulus (CS+G) compared to a standardly extinguished conditioned stimulus (non-generalized; CS+N). As a second hypothesis, successful extinction generalization (based on enhanced extinction learning) is expected to reduce SCRs, amygdala, insula and dACC activation and increase vmPFC activation to the CS+G as compared to the CS+N during retrieval processes on the next day (retrieval and reinstatement test).

Although extinction generalization appears to enhance the success of exposure therapy by strengthening the extinction memory trace and reducing the return of fear, additional manipulations are required to further stabilize these effects. Administration of the stress hormone cortisol before retrieval was shown to block the beneficial effects of extinction generalization on the neural level as observed for the amygdala and insula (Hagedorn et al., 2021). Hence, extinction generalization alone appears to be vulnerable to detrimental influences on extinction memory retrieval like increased stress hormones (Kinner et al., 2018, 2016; for a review: Meir Drexler et al., 2019).

However, cortisol in itself also comprises the potential to enhance the extinction memory trace: If cortisol concentrations are high prior to extinction training rather than prior to retrieval, they strengthen and reduce the context-specificity of the extinction memory as illustrated in the Stress Timing affects Relapse (STaR) model (Meir Drexler et al., 2019). Cortisol administration prior to extinction training reduced amygdalar and hippocampal activation, increased the functional connectivity to the vmPFC and attenuated SCRs during early extinction training (Merz et al., 2018a). Importantly from a translational perspective, cortisol was also already successfully established as an add-on treatment to enhance exposure therapy (Bentz et al., 2010; de Quervain et al., 2019, 2017; Soravia et al., 2014).

For the first time, this study aims to investigate whether extinction generalization can be further enhanced by pre-extinction cortisol administration. As third hypothesis, we propose that cortisol compared to placebo should reduce SCRs as well as activation of the fear network (insula, amygdala and dACC) and enhance activation of the vmPFC during extinction training, especially for the CS+G as compared to CS+N. As fourth hypothesis, we assume the same patterns to emerge for retrieval processes (retrieval and reinstatement test): cortisol compared to placebo should diminish SCRs as well as the activation of the fear network and increase activation of the fear-inhibitory vmPFC for the CS+G as compared to CS+N. In addition to our four hypotheses, the hippocampus and its functional connectivities to fear-related and extinction-related areas will be investigated to further elucidate the mediating role of the hippocampus for extinction generalization processes. Connectivity analyses will be conducted to investigate the (interconnected) network underlying extinction generalization and the mediating role of the hippocampus in this network. Additionally, sex-dependent cortisol effects modulating extinction generalization processes will be explored in view of previously reported potential sex differences (Merz et al., 2018b; Stockhorst and Antov, 2016).

2. Methods

2.1. Participants

This study was pre-registered on the Open Science Framework (https://osf.io/8e9wm/?view_only=efc38b7efd2343aa9aea0b499c6bed83). Out of 63 participants recruited via advertisements at the Ruhr University Bochum, three had to be excluded due to artifacts, missing contingency awareness and technical issues, leaving a final sample of 60 participants (thirty women; mean age: 23.9 years, SD: 4.1; mean BMI: 22.9 kg/m^2, SD: 2.3). According to our performed power analysis in G*Power 3.1 (Faul et al., 2007), 60 participants would be needed to attain a power of 1-$\beta = 0.80$ with an assumed effect size of $f = 0.105$, a repeated measures correlation of 0.8 and the significance level set to $p = .05$ to detect a significant interaction of CS x treatment. The effect size was calculated from a meta-analysis on the effects of stress on memory (Shields et al., 2017).

Exclusion criteria encompassed standard fMRI exclusion criteria, history of mental or neurological diseases and acute or regular intake of medication. All participants exhibited corrected-to-normal or normal vision and reported right-handedness as assessed with the Edinburgh Inventory of Handedness (Oldfield, 1971). Women reporting current pregnancy or current menstruation were excluded from our study. In addition, women taking oral contraceptives were excluded due to differences in fear learning after cortisol administration (Merz et al., 2018b; Merz and Wolf, 2017). Thus, only free-cycling women with sex hormones varying over the menstrual cycle were included.

To achieve relatively stable endogenous cortisol levels, appointments were arranged between 1 p.m. and 8 p.m. on three consecutive days (with 24 h ± 2 h between the sessions). Participants were instructed to refrain from eating, drinking anything but water and exercising two hours before each testing session. All participants signed informed consent prior to the beginning of the experiment and were screened for fMRI eligibility on each of the testing days. After the last day of the experiment, participants were debriefed and received a compensation of 45€. Procedures were conducted in accordance with the Declaration of Helsinki and approved by the Medical Faculty ethics committee at Ruhr University Bochum (registration number: 16–5789).

2.2. Fear conditioning procedure

Conditioned stimuli (CS) consisted of white geometrical shapes (rhomb, parallelogram and square) with similar luminescence on a black background (Fig. 1). To ensure similar luminescence, all white geometrical shapes were designed to match in surface area between stimuli. Each trial had a total duration of 20 s and consisted of a black screen presented for 0–2.5 s (jittered) at the beginning followed by an 8 s presentation of the CS and a jittered 9.5–12 s inter-trial interval (see Hagedorn et al., 2021). The three shapes were randomly assigned to the three CS and balanced between groups. The UCS consisted of a 100 ms

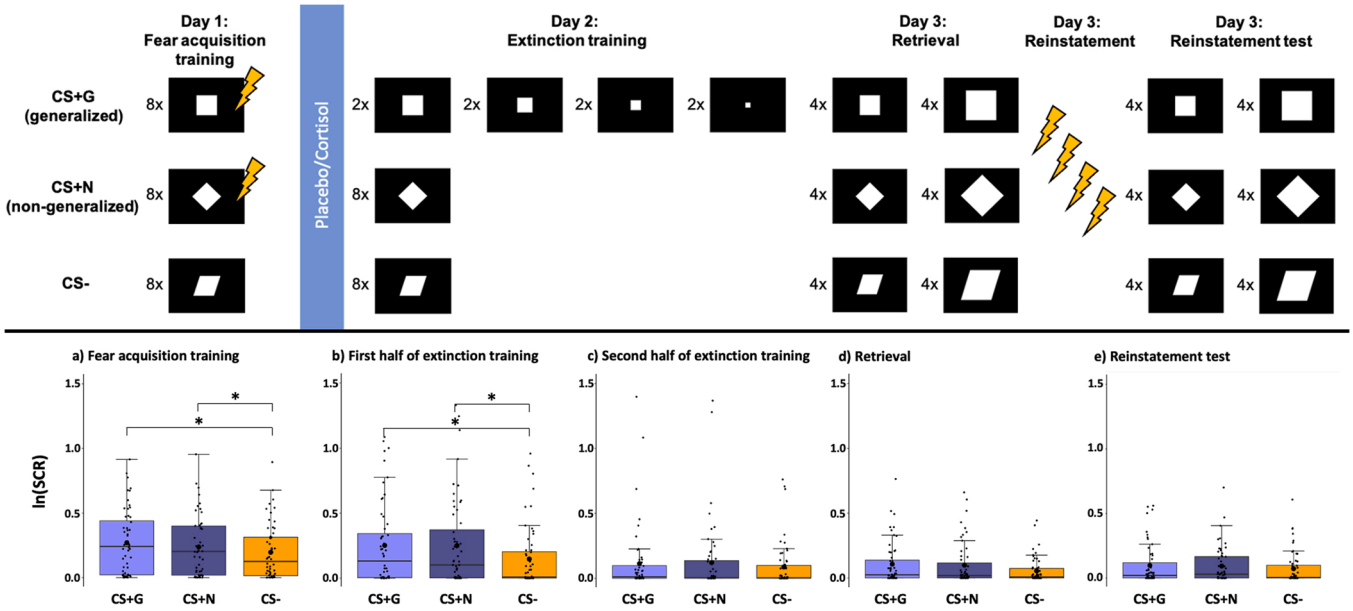

Fig. 1. Extinction generalization paradigm. Fear acquisition training, extinction training, retrieval, reinstatement and reinstatement test were performed on three consecutive days with a time lag of 24 (± 2) hours. During fear acquisition training on day 1, each of the three geometrical shapes was presented eight times. Lightning bolts during fear acquisition training represent a 100 ms electrical stimulation as unconditioned stimulus (62.5% of CS+G and CS+N trials). Prior to extinction training on day 2, 20 mg cortisol or placebo was pharmacologically administered. During extinction training, the CS- and CS+N (non-generalized) were presented eight times, each of the four sizes of the CS+G (generalized) was presented two times, thus, in total eight presentations of the CS+G were realized. During retrieval and reinstatement test on day 3, each of the two sizes of each CS was presented four times to account for a total of eight trials for each CS. Retrieval and reinstatement test were separated by four UCS presentations serving for reinstatement.
Log-transformed skin conductance responses (SCRs; boxplots with thick black dots representing the mean) for a) fear acquisition training, b) first half of extinction training, c) second half of extinction training, d) retrieval and e) reinstatement test.
Asterisks indicate statistical significance ($p \leq .05$): SCRs to CS+ as compared to CS- revealed a significant difference during fear acquisition training (* $p < .001$). During the first half of extinction training, there was a significant difference between CS+G (* $p < .001$) and CS+N (* $p = .001$) as compared to CS-. During retrieval and reinstatement test, there were no significant differences between the three CS.

electrical stimulation applied to the fingertips of the participant's right index- and middle-finger via two 1 cm^2 electrodes using a constant voltage stimulator (STM200; BIOPAC systems, CA, USA). The stimulation level was adjusted individually to be unpleasant but not painful (Hagedorn et al., 2021). The paradigm was presented via MR suitable LCD-goggles (Visuastim Digital, Resonance Technology Inc., Northridge, CA, USA) and realized in Matlab 2017a (Mathworks Inc., Sherborn, MA, USA).

Fear acquisition training on day one served to establish a CS-UCS association: two of the three CS (CS+G and CS+N) were immediately followed by the UCS in 5/8 trials (62.5% partial reinforcement rate), whereas the CS- was never followed by the UCS (0% reinforcement rate, 8 trials). Importantly, there was no difference in the fear acquisition protocol for CS+G and CS+N (Fig. 1). Prior to fear acquisition training, participants were instructed to pay attention towards possible associations between the presentation of a geometrical shape and the electrical stimulation because they would be asked about them using a questionnaire afterwards. Participants were classified as contingency aware in case they were able to correctly identify the two CS+ (sometimes followed by an electrical stimulation) and the CS- (never followed by an electrical stimulation) after fear acquisition training (Hagedorn et al., 2021; Tabbert et al., 2011). Before extinction training and retrieval, the participants were informed that the associations learned on day one would not change over the course of the entire experiment to avoid expectancy of contingency reversal. Importantly, participants were not informed about the actual contingencies on any of the three experimental days.

During extinction training on day two, the CS+N and the CS- were presented solely in their original size testing the effects of standard extinction training. The CS+G, however, was additionally to its original size presented in three smaller sizes (75%, 50% and 25% of the original size) to test for the effects of generalized extinction training. The three CS were presented eight times each during extinction training. Each size of the CS+G was presented two times to reach a total number of eight extinction trials; learning effects based on a higher number of presentations (e.g., eight for each size of the CS+G) were avoided using this approach.

During retrieval on day three, all stimuli were presented in their original size for four trials intermixed with four presentations of a formerly unpresented size (175% of the original size) to test for generalization effects to this new size. Four electrical stimulations, each separated by 5 s (after 2 s, 7 s, 12 s and 17 s) were applied during the 20 s presentation of a grey background for reinstatement after retrieval followed by a reinstatement test (identical to retrieval). After retrieval and before the reinstatement test, a black background was presented for 15 s each.

2.3. Cortisol administration and concentrations

On day two, 40 min before extinction training, half of the participants (15 women and 15 men) received 20 mg hydrocortisone tablets (Hoechst) in a randomized double-blind design, whereas the other half received visually identical placebos. 20 mg of hydrocortisone appeared to induce beneficial effects in previous fear conditioning and exposure therapy studies (de Quervain et al., 2011; Hagedorn et al., 2021; Soravia et al., 2014). Saliva samples using Salivettes (Sarstedt, Nümbrecht, Germany) were taken on each day to measure circulating cortisol levels: on day one and three, saliva samples were taken before and after scanning. On day two, saliva samples were taken before tablet intake (baseline) as well as 30 and 60 min after tablet intake (before and after extinction training). Saliva samples were stored at − 20 °C until analyzed using a commercial enzyme-linked immunosorbent assay (IBL

International, Hamburg, Germany). Inter- and intra-assay coefficients of variations were below 10%.

For the performed analysis, values transformed with the natural logarithm were used to attain a normal distribution. In addition to the three participants excluded from all analyses due to missing contingency awareness, cortisol data of one participant had to be excluded due to undetectable cortisol levels in the placebo group, while cortisol data of three participants in the cortisol group had to be excluded due to undefinably high cortisol concentrations (>1738.80 nmol/l, maximum value at 20-fold dilution). These four additional exclusions were only applied to the cortisol analysis leading to a reduced sample size of n = 29 (15 women) in the placebo and n = 27 (14 women) in the cortisol group.

2.4. Skin conductance responses

Ag/AgCl electrodes filled with an isotonic (0.05 NaCl) electrolyte medium fixed at the hypothenar of the left hand served for the measurement of SCRs. Data was acquired at 5000 Hz using the Brain Vision Recorder software, resampled at 10 Hz and filtered at 4.5 Hz in the Brain Vision Analyzer (Brain Products GmbH, Munich, Germany). Ledalab 3.4.9 (Benedek and Kaernbach, 2010) served for the analysis of conditioned responses, which were defined as trough-to-peak maximum amplitudes in a time window of 1–8 s after CS onset. Zero and missing responses were entered as 0 in our analyses, since non-responding also reflects parts of the learning process. Raw SCRs were transformed with the natural logarithm to attain a normal distribution for the performed analyses. In addition to the three participants excluded from all analyses, SCR data of four participants (one woman) in the placebo group had to be excluded due to technical issues (broken connection to recording computer) at least on one of the three days. These additional exclusions, however, were only applied to the SCR analysis finally performed on a reduced sample size of n = 26 (14 women) in the placebo and n = 30 (15 women) in the cortisol group.

2.5. Statistical analyses

Statistical analyses of SCRs and cortisol concentrations were performed in IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). Mixed ANOVAs served for the analyses with the significance threshold set to $p \leq .05$ (Bonferroni-corrected for multiple comparisons). If the assumption of sphericity was violated, Greenhouse-Geisser corrected values were reported. Partial eta square (η^2_p) was used as a measure of effect size. For all analyses, treatment (placebo vs. cortisol) and sex (women vs. men) were entered as between-subjects factors.

The analyses of cortisol concentrations encompassed the within-subjects factor time (baseline, 30 and 60 min after tablet intake). Prior to the analyses, responses were averaged across trials for each CS for fear acquisition training, halves of extinction training, retrieval and reinstatement test. For the analyses of SCRs during fear acquisition training, both CS + (mean of all acquisition-trial responses to the CS+G and CS+N) were compared to the CS-. For extinction training, the analysis encompassed the within-subjects factors half (first vs. second half) and CS (CS+G vs. CS+N vs. CS-). Although hypotheses apply for both halves of extinction training, analyses encompassed the factor half to capture time-dependent changes. For the analyses of the retrieval and reinstatement test, the within-subjects factors CS and size (original vs. modified) were entered.

2.6. Functional magnetic resonance imaging

Whole- brain images were acquired with a 3 T whole-body scanner and a 32 channel head coil (Philips Achieva 3.0 T X-Series, Philips, the Netherlands). Structural images obtained with a T1 weighted FTE sequence encompassed 220 transversally oriented slices (FOV: 240 mm x 240 mm, voxel size: 1 mm x 1 mm x 1 mm). Functional images obtained with a T2 weighted gradient echoplanar imaging sequence (TR: 2.5 s, TE: 30 ms, flip angle: 67°, slice gap: 0.75 mm) encompassed 40 slices measured parallel to the orbitofrontal-bone transition in ascending order (FOV: 192 mm x 192 mm, voxel size: 2 mm x 2 mm x 3 mm). 201 volumes were recorded during fear acquisition training and extinction training, while 411 volumes were recorded during retrieval (201 volumes), reinstatement (9 volumes) and reinstatement test (201 volumes). To reach stable magnetization, the three dummy scans preceding each functional scan and the first three functional volumes were discarded.

Functional data was preprocessed and analyzed with the software Statistical Parametric Mapping (SPM12, Wellcome Department of Cognitive Neurology, London, UK) applied in Matlab 2017a (Mathworks Inc., Sherborn, MA, USA). The preprocessing encompassed realignment, slice time correction, co-registration, normalization to MNI standard space and smoothing with an 8 mm Gaussian kernel.

In the first-level model, the three scan sessions were entered separately for each participant. CS+G, CS+N and CS- were used as regressors in each session. For fear acquisition training, the parameters half (first and second half), UCS transmission and UCS omission were entered separately for each CS. Extinction parameters also included half, whereas retrieval/reinstatement/reinstatement test parameters encompassed size (original and modified), the UCS and grey screen applied during reinstatement. Six realignment parameters were added as covariates and a 128 s high-pass filter was applied. All parameters were modeled using the general linear model with a stick function convolved with the hemodynamic response function.

The second-level model encompassed full-factorial models with the between-subjects factors treatment and sex. The contrasts for each phase were chosen in accordance with the SCR analyses: successful fear learning should be captured in the contrast CS+G and CS+N minus CS-. The contrast CS+G minus CS+N for each half of extinction training was realized to examine time-dependent changes during extinction learning: the first half of extinction is expected to reflect rather fear retrieval processes while the second half more likely captures the formation of the extinction memory trace (Lonsdorf et al., 2017). For retrieval, the contrast CS+G minus CS+N for both sizes taken together was analyzed to investigate the success of the extinction generalization procedure. The contrast CS+G minus CS+N appeared to be the most important one to directly test the hypotheses as it compares the generalized extinction to the standard extinction protocol (Hagedorn et al., 2021). In addition, avoiding the comparison to the CS- circumvents the problem of involving a learned safety stimulus (Lissek et al., 2005) that might mask critical effects (Fullana et al., 2019). The contrast CS+G minus CS+N is supposed to capture extinction generalization effects beyond standard extinction, while the contrast CS+N minus CS+G should reflect more pronounced effects of standard extinction as compared to extinction generalization.

Region of interest (ROI) analyses included areas previously identified to be involved in fear and extinction generalization processes (Hagedorn et al., 2021; Lissek et al., 2014): insula, amygdala and dACC as part of the fear network (Fullana et al., 2016), the vmPFC for fear inhibition processes (Fullana et al., 2018) and the hippocampus and parahippocampal gyrus (PHG) as possible areas mediating the generalization effect via pattern separation or completion (Lissek et al., 2014). Maximum probability masks (1 mm, threshold = 0.25) were taken from the Harvard-Oxford Cortical- and Subcortical-Atlases (http://www.cma.mgh.harvard.edu/fsl_atlas.html). The dACC and vmPFC masks comprised a 5 mm sphere around the peak voxels taken from meta-analyses concerning fear acquisition for the dACC (MNI: x = 0 y = 16 z = 36; Mechias et al., 2010) and extinction learning for the vmPFC ((MNI: x = 0 y = 40 z = −3; Schiller and Delgado, 2010).

Family-wise error (FWE) correction for small volumes (Penny et al., 2007) was applied with the significance threshold of $p \leq .05$ for the defined ROIs. For exploratory whole brain analyses, the significance threshold was set to $p \leq .05$ (FWE-corrected) and a minimal cluster size

of 10 voxels had to be exceeded (results are reported in Table A.1 in the supplement). ROIs significantly activated during extinction training, retrieval and reinstatement test or in interaction with cortisol were entered as seed regions (volume of interest with 5 mm sphere around the peak voxel) within the frame of psychophysiological interaction (PPI) analyses to test the related functional connectivity.

Results of the factor sex were only reported in case of interactions with the factor treatment to focus on possible sex-dependent cortisol effects modulating extinction generalization (for fMRI data: CS+G vs. CS+N) and not on sex differences per se.

3. Results

3.1. Day 1: fear acquisition training

Enhanced activation of the bilateral insula as well as dACC (Table 1a) and higher SCRs ($F_{(1,52)}$= 13.84, $p < .001$, η^2_P= .210; Fig. 1a) were present for both CS+ as compared to CS-, indicating successful fear acquisition. In addition, the enhanced activation of the right insula for CS+ in comparison to CS- was confirmed in exploratory whole brain analyses (see supplement: Table A.1a). Importantly, there were no other significant interactions with the factors CS or cortisol ($p > .05$).

3.2. Day 2: cortisol administration and extinction training

Analyses of salivary cortisol indicated significant main effects of time ($F_{(1.69,87.72)} = 252.11$, $p < .001$, $\eta^2_P = .829$), sex ($F_{(1,52)} = 4.30$, $p = .043$, $\eta^2_P = .076$; overall higher cortisol concentrations in men compared to women) and treatment ($F_{(1,52)} = 184.19$, $p < .001$, $\eta^2_P = .780$) as well as a significant time*treatment interaction ($F_{(1.69,87.72)}$= 330.67, $p < .001$, η^2_P= .864). Post-hoc comparisons indicated no differences during baseline ($F_{(1,52)} = 1.61$, $p = .211$, $\eta^2_P = .030$), but higher cortisol levels in the cortisol as compared to the placebo group 30 min ($F_{(1,52)} = 271.02$, $p < .001$, $\eta^2_P = .839$) and 60 min after cortisol administration ($F_{(1,52)} = 358.91$, $p < .001$, $\eta^2_P = .873$), demonstrating a successful cortisol manipulation (see supplement: Table B.1).

The comparison of CS+G and CS+N during the first half of extinction training revealed no significant activations (Table 1b). However, in (partial) support of our first hypothesis, an increased activation of the bilateral insula and dACC occurred for CS+G as compared to CS+N (Fig. 2a-c; Table 1c) during the second half of extinction training. In addition, PPI analyses revealed that the activation of the right insula was functionally connected to the right amygdala (Fig. 2d; Table 1c; see supplement: Table C.1-C.3 for exploratory separate analyses of the CS+G and CS+N relative to the CS-). Since no interactions with cortisol were observed, there was no evidence for our third hypothesis stating a cortisol-related modulation of the neural correlates of extinction training.

SCRs revealed a significant main effect of CS ($F_{(1.42,73.60)} = 9.40$, $p = .001$, $\eta^2_P = .153$) during extinction training: SCRs to the CS+G ($F_{(1,52)} = 12.31$, $p = .003$, η^2_P= .191) as well as CS+N ($F_{(1,52)} = 9.82$, $p = .008$, $\eta^2_P = .159$) were increased in comparison to the CS-, but there was no difference between CS+G and CS+N ($F_{(1,52)}$= 0.10, $p > .999$, η^2_P= .002). In addition, a main effect of half ($F_{(1,52)} = 12.35$, $p = .001$, $\eta^2_P = .192$) indicated decreased responding from the first to the second half of extinction training. The significant CS*half interaction ($F_{(1.49,77.65)}$= 3.94, $p = .035$, η^2_P= .070) revealed higher SCRs for CS+G ($F_{(1,52)}$= 17.13, $p < .001$, $\eta^2_P = .248$) and CS+N ($F_{(1,52)} = 14.83$, $p = .001$, $\eta^2_P = .222$) as compared to CS- in the first half of extinction training (Fig. 1b). There were no significant differences between the three CS during the second half of extinction ($F_{(1.35,70.13)}$= 1.60, $p = .213$, $\eta^2_P = .030$), indicating successful extinction learning (Fig. 1c). Importantly, no differences between CS+G and CS+N or any interactions with cortisol were observed, neither for the first nor the second half of extinction training. Thus, enhanced responding to CS+G as compared to CS+N were not reflected in SCRs during extinction training, providing no additional evidence for our first hypothesis. In

Table 1
Peak-voxel statistics and localizations for the contrast CS+ vs. CS- for a) fear acquisition training and the contrast CS+G vs. CS+N (directions of the contrasts are marked) during b) early, c) late extinction training, d) retrieval and e) reinstatement test. Due to our aim to investigate differences between CS+G and CS+N during early and late extinction training, retrieval and reinstatement test, additional psychophysiological interaction (PPI) analyses were not performed for fear acquisition training. Early extinction training contrasts revealed no significant activations and, thus, no PPI analyses were performed. Respective seed regions and underlying contrasts are depicted directly above each PPI result.

contrast	structure	cluster size	x	y	z	Tmax	pcorr
(a) fear acquisition training: CS+ vs. CS-							
CS+ > CS-	dACC	65	2	20	34	2.96	.026
CS+ > CS-	L insula	674	-28	24	-4	4.05	.022
CS+ > CS-	R insula	243	36	22	4	5.54	< .001
(b) early extinction training: CS+G vs. CS+N							
no significant activations							
(c) late extinction training: CS+G vs. CS+N							
CS+G > CS+N	dACC	29	-2	18	40	3.41	.010
CS+G > CS+N	L insula	237	-34	24	-2	4.20	.017
CS+G > CS+N	R insula	193	30	24	-4	4.29	.013
→PPI CS+G > CS+N	R amygdala	42	30	-2	-16	3.36	.044
(d) retrieval: CS+G vs. CS+N							
CS+G > CS+N	L hippocampus	25	-24	-40	-4	3.66	.040
→PPI CS+G > CS+N	L amygdala	52	-20	-10	-12	3.50	.029
→PPI placebo > cortisol	L insula	639	-44	2	-4	3.82	.046
→PPI placebo > cortisol	R hippocampus	19	14	-38	4	3.64	.045
→PPI placebo > cortisol	vmPFC	81	4	42	0	3.41	.010
CS+G < CS+N	L hippocampus	76	-34	-32	-12	3.80	.028
placebo < cortisol	L hippocampus	95	-34	-24	-12	4.03	.015
→PPI placebo < cortisol	dACC	27	-4	18	38	3.01	.026
(e) reinstatement test: CS+G vs. CS+N							
CS+G < CS+N	L amygdala	82	-32	-4	-20	3.72	.016
→PPI CS+G > CS+N	R insula	109	36	4	-6	3.77	.048
CS+G < CS+N	dACC	51	0	14	40	2.96	.028
placebo > cortisol	dACC	72	0	12	34	3.79	.004
→PPI CS+G < CS+N	L hippocampus	56	-24	-30	-12	3.71	.034

The significance threshold was set to $p \leq 0.05$ (FWE-corrected for small volume correction). All coordinates (x, y, z) are given in MNI space. L = left, R = right.

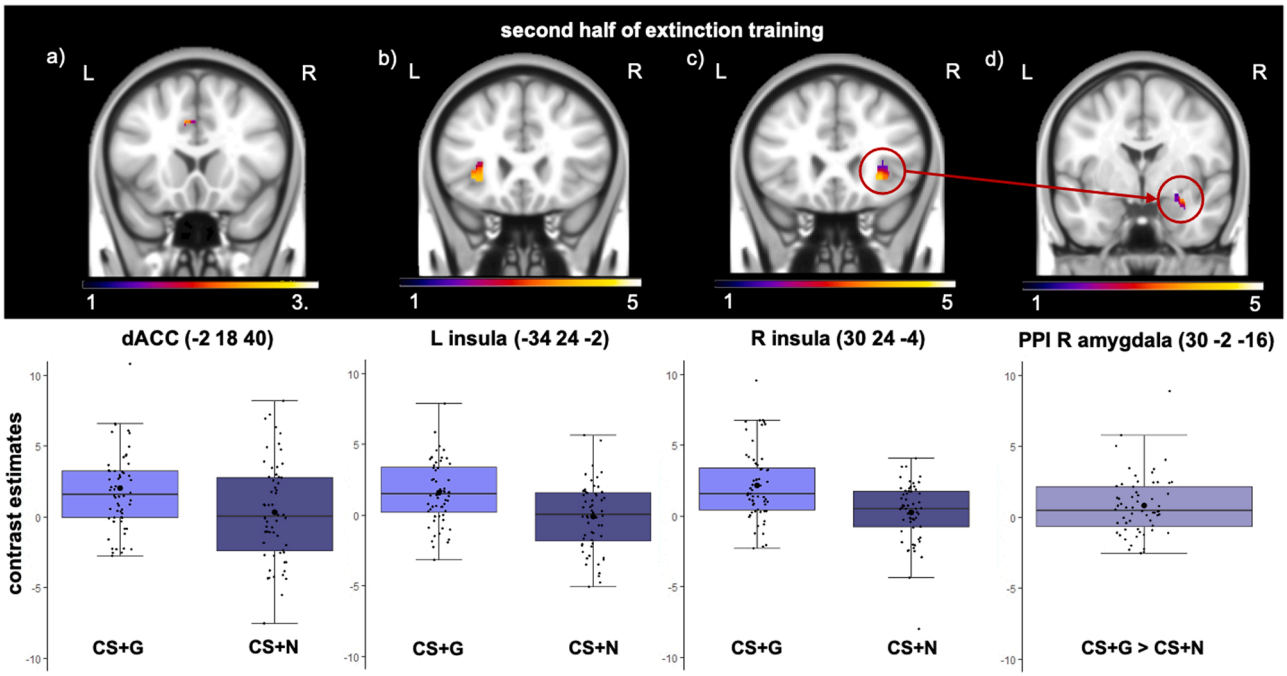

Fig. 2. Differential neural responding for the contrast CS+G minus CS+N during the second half of extinction training. The slices were selected according to peak voxels of the activated ROI: a) dorsal anterior cingulate cortex (dACC), b) left insula, c) right insula and d) the functional connectivity from the right insula to the right amygdala. Data is presented on the standard MNI brain template and thresholded to T ≥ 1 (see color bar for exact T-values). Boxplots represent contrast estimates of the respective peak voxel (with thick black dots representing the mean). L = left, R = right.
Whereas no differential responding for the contrast CS+G minus CS+N was observed for the first half of extinction training, during the second half of extinction training, a) dACC as well as b) left and c) right insula activation were increased for CS+G as compared to CS+N. In addition, the right insula showed d) stronger functional connectivity to the right amygdala for the contrast CS+G minus CS+N. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

addition, cortisol effects on extinction training were neither observed in neural activations nor in the SCRs, providing no evidence for our third hypothesis.

3.3. Day 3: retrieval

An activation of the left anterior hippocampus was decreased for CS+G as compared to CS+N (Table 1d). Notably, this region was modulated by cortisol as well: while no CS+G/CS+N differentiation occurred in the placebo group, cortisol increased hippocampal responding to the CS+N relative to CS+G (Fig. 3d; Table 1d). The region of the left hippocampus modulated by cortisol also showed stronger functional connectivity to the dACC in the cortisol as compared to the placebo group in the contrast CS+N minus CS+G (Fig. 3e; Table 1d).

In contrast, a more posterior activation of the left anterior hippocampus (compared to the activation in the reverse contrast) was found for the contrast CS+G minus CS+N (Fig. 3a; Table 1d) during retrieval. In addition, this hippocampal region was functionally connected to the left amygdala (Fig. 3b; Table 1d). The hippocampal functional connectivities to the left insula, right hippocampus and vmPFC for the contrast CS+G minus CS+N were reduced in the cortisol as compared to the placebo group (Fig. 3c; Table 1d).

SCRs revealed a main effect of CS ($F_{(1.60,83.32)} = 7.02$, $p = .003$, $\eta^2_P = .119$; Fig. 1d) during retrieval. Post-hoc tests indicated significantly enhanced SCRs for CS+G ($F_{(1,52)} = 9.47$, $p = .010$, $\eta^2_P = .154$) and CS+N ($F_{(1,52)} = 7.14$, $p = .030$, $\eta^2_P = .121$) as compared to CS-, whereas no difference emerged between CS+G and CS+N ($F_{(1,52)} = 0.55$, $p > .999$, $\eta^2_P = .010$). No differences between CS+G and CS+N or any interactions of CS with size or treatment occurred during retrieval.

3.4. Day 3: reinstatement test

During the reinstatement test, decreased activation of the left amygdala was observed for the contrast CS+G as compared to CS+N (Fig. 4a; Table 1e), in line with our second hypothesis. PPI analyses revealed a functional connectivity of the left amygdala with the right insula in the contrast CS+N minus CS+G (Fig. 4b; Table 1e).

In addition, a decreased activation of the dACC in the contrast CS+G as compared to CS+N (Table 1e) further supported the second hypothesis. The overall dACC activation was further modulated by cortisol: whereas enhanced dACC activation for the CS+N as compared to CS+G emerged in the placebo group, cortisol blocked this differential effect (Fig. 4c; Table 1e). In addition, the cortisol-modulated area of the dACC was functionally connected to the left hippocampus (Fig. 4d; Table 1e). Thus, there was contradicting evidence for our fourth hypothesis predicting a decreased activation of the fear network and increased activation of fear-inhibitory areas in the cortisol as compared to the placebo group especially for the CS+G during retrieval and reinstatement test.

SCRs revealed no significant effects of CS or any interactions with CS or treatment ($p > .05$; Fig. 1e). Thus, observed differences between CS+G and CS+N as well as treatment effects in imaging data were not mirrored in SCRs during the reinstatement test.

4. Discussion

In this pre-registered study, we investigated whether extinction generalization learning, analogous to standard extinction learning, could be boosted by the stress-hormone cortisol. In addition, we aimed to further investigate the neural correlates of extinction generalization processes during extinction training, retrieval, and reinstatement test identified in our previous study (Hagedorn et al., 2021).

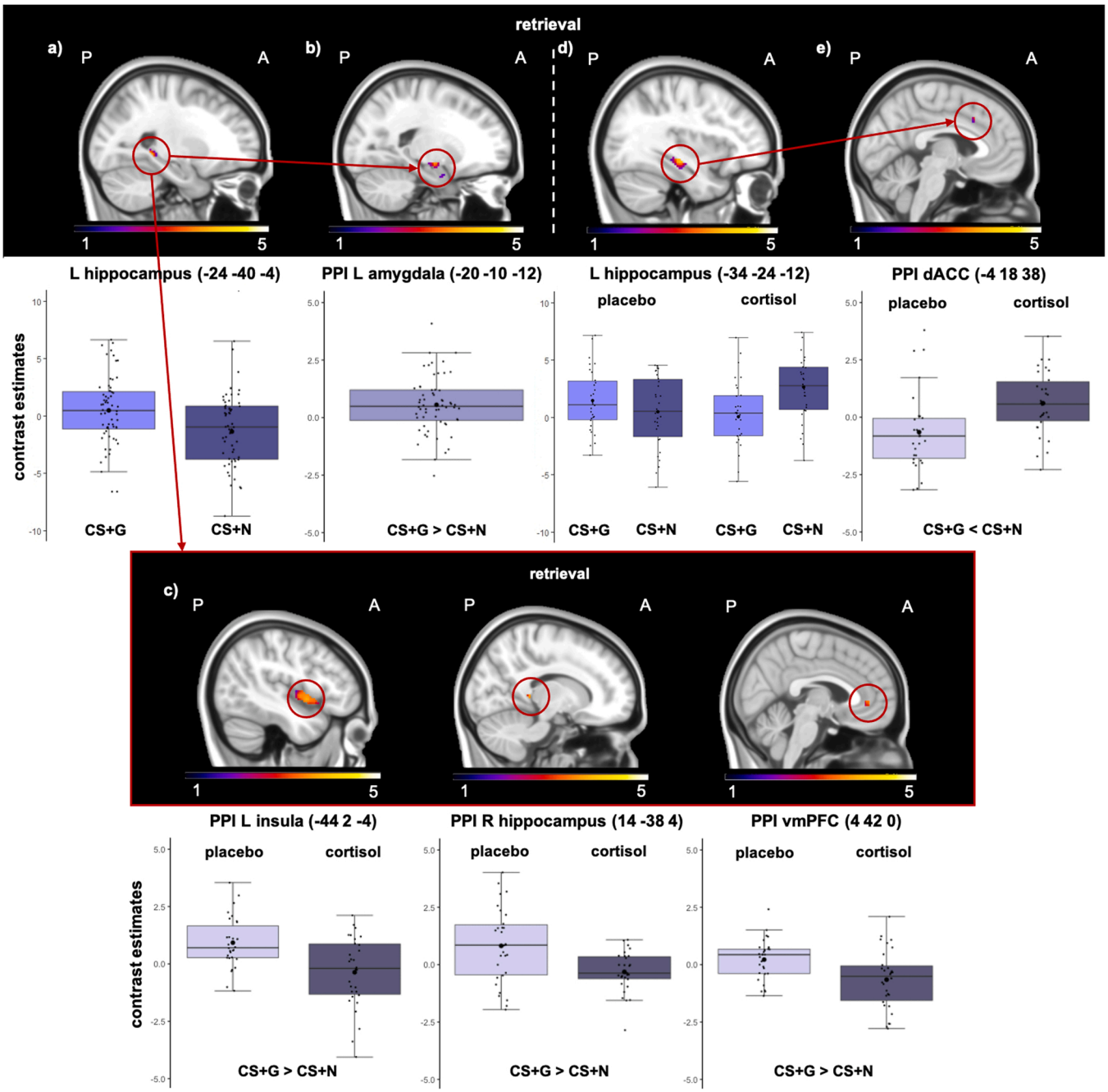

Fig. 3. Differential neural responding for the contrast CS+G minus CS+N during retrieval. The slices were selected according to peak voxels of the activated ROI: a) more posterior part of the hippocampus and its functional connectivities b) to the left amygdala as well as c) to the left insula, right hippocampus and the vmPFC in the placebo relative to the cortisol group. In addition, d) cortisol effects on the more anterior part of the left hippocampus and e) the functional connectivity to the dACC in the cortisol relative to the placebo group are depicted. Data is presented on the standard MNI brain template and thresholded to $T \geq 1$ (see color bar for exact T-values). Boxplots represent contrast estimates of the respective peak voxel (with thick black dots representing the mean). A=anterior, P = posterior.
During retrieval, differential regions of the left hippocampus were activated: a) a posterior region of the left hippocampus for CS+G as compared to CS+N and d) a more anterior and lateral region of the left hippocampus for CS+N as compared to CS+G. The more posterior region of the left hippocampus was functionally connected to b) the left amygdala and revealed stronger functionally connectivities to c) the left insula, right hippocampus and vmPFC in the placebo relative to the cortisol group for the contrast CS+G minus CS+N. The activation of the d) more anterior region of the left hippocampus was modulated by cortisol: Whereas no CS+G/CS+N differentiation occurred in the placebo group, cortisol increased hippocampal responding to the CS+N relative to CS+G. This cortisol-modulated region of the left hippocampus revealed e) a stronger functional connectivity to the dACC in the cortisol relative to the placebo group in the contrast CS+N minus CS+G. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

4.1. Extinction generalization effects

In line with our first hypothesis, enhanced activation of the bilateral insula and dACC was observed for CS+G as compared to CS+N (Fig. 2a-c; Table 1c), arguing for the expected increased fear responding to generalization stimuli during the second half of extinction training. Additionally, the right insula was functionally connected to the right amygdala (Fig. 2d; Table 1c), which might also indicate prolonged activation of fear-related areas for the CS+G during extinction training. Thus, on the neural level, areas assumed to be involved in extinction

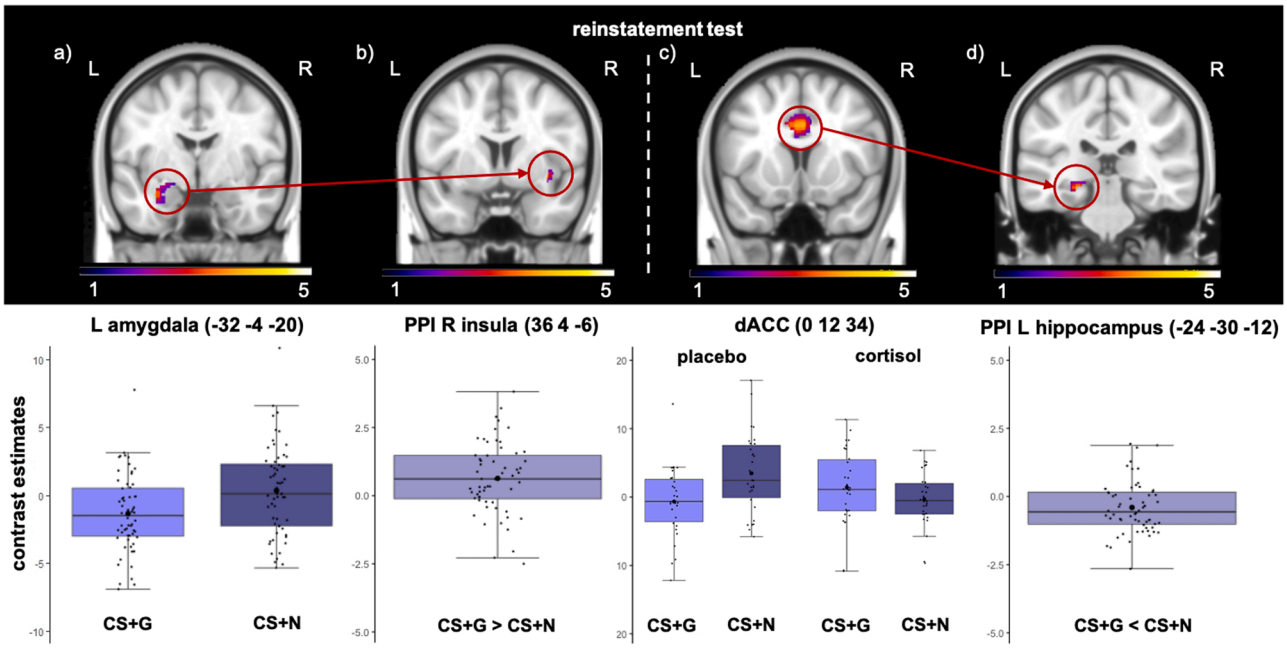

Fig. 4. Differential neural responding for the contrast CS+G minus CS+N during the reinstatement test. The slices were selected according to peak voxels of the activated ROI: a) left amygdala and its b) functional connectivity to the right insula, c) cortisol effects on the dorsal anterior cingulate cortex (dACC) and d) the functional connectivity of the cortisol-modulated dACC to the left hippocampus. Data is presented on the standard MNI brain template and thresholded to $T \geq 1$ (see color bar for exact T-values). Boxplots represent contrast estimates of the respective peak voxel (with thick black dots representing the mean). L=left, R=right. During the reinstatement test, decreased activations of a) the left amygdala and c) the dACC occurred for CS+G as compared to CS+N. The decreased activation of the left amygdala for the CS+G as compared to CS+N revealed b) a negative functional connectivity to the right insula (depicted in the reversed comparison CS+G minus CS+N). Furthermore, c) the differentiation of the dACC for CS+G as compared to CS+N was modulated by cortisol and emerged in the placebo group, but not in the cortisol group. Additionally, d) the cortisol-modulated area of the dACC was functionally connected to the left hippocampus. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

generalization (insula, dACC and amygdala observed in functional connectivity) except for less fear-inhibitory signaling in the vmPFC for the CS+G were confirmed. However, these results were not reflected in enhanced SCRs to the CS+G in comparison to CS+N. Although not all expected activations mentioned in the hypotheses were confirmed, the observed activations are in accordance with the assumed underlying network of extinction generalization.

Enhanced fear-related processing during extinction training, reflected in activation of fear network structures (Hagedorn et al., 2021), increased SCRs (Struyf et al., 2018; Waters et al., 2018) and anticipatory anxiety (Rowe and Craske, 1998), appears to be a key mechanism underlying successful extinction generalization. One possible mechanism underlying extinction generalization processes are stronger prediction errors that might occur due to the presentation of multiple similar stimuli during extinction training (Lipp et al., 2020): in comparison to standard extinction training in which all stimuli were formerly presented during fear acquisition training, generalization stimuli formerly unpresented exert greater uncertainty in the prediction of the UCS. Thus, prediction errors are assumed to be stronger during extinction generalization training as compared to standard extinction training, improving but also prolonging extinction learning. However, the exact underlying mechanisms driving extinction generalization in our present study remain elusive, since fear processing or related processes like increased arousal, novelty or altered attention might also underly these results.

During retrieval, we did not observe the expected downregulation of fear-related areas (insula, dACC and amygdala) and upregulation for fear-inhibitory areas (vmPFC) for CS+G as compared to CS+N, neither in neuroimaging, nor in SCR data. However, differential effects in the left hippocampus were observed: a more posterior part showed enhanced activation, whereas a more anterior part displayed decreased activation to CS+G as compared to CS+N (Table 1d). These activations appear to reflect differential processes: The more posterior part of the anterior hippocampus can be associated with construction (and navigation) and separation processes (Stevenson et al., 2020; Zeidman and Maguire, 2016). Furthermore, this activation might reflect pattern separation processes (Stevenson et al., 2020; Zeidman and Maguire, 2016) occurring during our paradigm and thus one proposed mechanism underlying fear generalization (Lissek et al., 2014). In contrast, the more anterior part is more likely associated with encoding and retrieval processes (Bowen and Kensinger, 2017; Stevenson et al., 2020).

Although we observed no downregulation of fear-related areas during retrieval (Hagedorn et al., 2021), we observed a deactivation of the left amygdala (Fig. 4a; Table 1e) and the dACC (Fig. 4c; Table 1e) for the CS+G in comparison to CS+N during the reinstatement test, in line with our second hypothesis. However, on the neural level we did not observe an upregulation of the fear-inhibitory vmPFC. In addition, the downregulation of fear-related areas was not mirrored in decreased SCRs to CS+G in comparison to CS+N. As additional extinction generalization learning appeared to have taken place over the course of the retrieval trials (also previously observed in functional connectivity between the parahippocampal gyrus and a hippocampal region (Hagedorn et al., 2021)), differential effects for CS+G minus CS+N could occur after re-extinction processes. In addition, unsignaled UCS during reinstatement resulted in a stronger return of fear (Bouton, 2004) enabling differences in CS+G/CS+N activations to emerge, which were possibly weaker during retrieval.

4.2. Cortisol effects on extinction generalization

Cortisol effects were not observed during extinction training for CS+G minus CS+N, contradicting our third hypothesis, but being in line with two studies from our laboratory investigating the influence of stress prior to extinction training on context generalization (Meir Drexler

et al., 2018, 2017). However, cortisol administration prior to extinction training did not decrease the activation of the fear network during extinction training as observed previously for an extinguished CS+ compared to the CS- (Merz et al., 2018a). Importantly, cortisol administration prior to extinction training is assumed to directly influence extinction learning, while fear acquisition learning taking place one day before could not be directly affected by cortisol. Thus, in this design, cortisol should exert effects specifically on the extinction memory trace rather than on the original fear memory trace. Hence, one possible explanation for the current findings might include missing direct cortisol effects on extinction generalization learning due to a required consolidation of the respective memory trace. Cortisol effects might not become visible until retrieval and reinstatement test one day later as also observed for context-related pre-extinction stress hormone effects (Meir Drexler et al., 2018, 2017).

During retrieval, the observed activations in the left hippocampus for CS+G minus CS+N were directly modulated by cortisol or functionally connected to cortisol-modulated areas. The relatively more posterior hippocampal part was functionally connected to the left amygdala (Fig. 3b; Table 1d) and to areas assumed to be involved in generalization processes (left insula, right hippocampus, vmPFC; Hagedorn et al., 2021; Lissek et al., 2014) for CS+G minus CS+N. These functional connectivities were more pronounced in the placebo as compared to the cortisol group (Fig. 3c; Table 1d). Taken together, cortisol administered prior to extinction training might reduce extinction generalization associated processes during retrieval (or re-extinction). As the hippocampus is assumed to mediate generalization processes (Lissek et al., 2014), its concerted action with areas involved in extinction generalization could argue for enhanced re-extinction learning in the placebo group. Previously, comparable results were observed when cortisol was administered prior to extinction training: increased activation of the hippocampus with other areas of the extinction learning network in the placebo group (Merz et al., 2014, 2018a) during late extinction training argued for the establishment of the extinction memory trace. Cortisol, however, appeared to block neural processes required for successful extinction learning in this study (Merz et al., 2014). While direct downregulation of fear-related areas or upregulation of extinction-related areas as indicators of successful extinction generalization (Hagedorn et al., 2021) were not observed during retrieval, but during the later reinstatement test, additional extinction generalization learning processes have likely taken place during retrieval.

The relatively more anterior part of the left hippocampus was also modulated by cortisol: Whereas no differential activation was observed in the placebo group, increased responding occurred towards the CS+N in comparison to CS+G (Fig. 3d; Table 1d) in the cortisol group. Additionally, this part of the hippocampus was functionally connected to the dACC for CS+N minus CS+G (Fig. 3e; Table 1d). This functional connectivity between the hippocampus and fear-related dACC was stronger in the cortisol in comparison to the placebo group, possibly indicating higher retrieval of the original fear-related memory trace (Mechias et al., 2010; Milad and Quirk, 2012), especially in the cortisol group. As the dACC connects to the fear-excitatory areas of the amygdala rather than fear-inhibitory areas (Milad and Quirk, 2012), the dACC can be regarded as a fear-signaling area which is more functionally connected with the hippocampus in the cortisol group. Thus, fear-related activation observed for the CS+N > CS+G in the cortisol group could indicate higher fear retrieval for the standard extinction stimulus. However, differences between the placebo and cortisol group were not reflected in SCRs and should thus be interpreted with caution. In addition, the expected cortisol-induced enhancement of extinction generalization reflected in less fear-related and more fear-inhibitory signaling and decreased SCRs in the cortisol group for CS+G was not observed.

During the reinstatement test, activations of the left amygdala and dACC were decreased for CS+G in comparison to CS+N (Table 1e). Importantly, this dACC activation was also modulated by cortisol: although there was a significant difference in the placebo group indicating higher dACC activation to the CS+N as compared to the CS+G, no difference was observed in the cortisol group (Fig. 4c; Table 1e). Either increased activation to the CS+G, decreased activation to the CS+N in the cortisol in comparison to the placebo group, or both might be involved in these effects. Activation towards the CS+G might have increased since enhanced fear-related processing as well as elevated cortisol levels were still observed at the end of extinction training as compared to CS+N (Fig. 2; for separate comparisons of the CS+G and CS+N relative to CS- see supplement: Tables C.1-C.3). Thus, not the extinction memory trace but the fear-related processing still present at the end of extinction training might have been preferentially consolidated for the CS+G in comparison to CS+N as previously observed for stress hormone effects on emotional relative to neutral material in episodic memory consolidation (Wolf, 2009). However, the effect appears to mainly rely on the decreased activation to the CS+N in the cortisol in comparison to the placebo group (Fig. 4c). Thus, the decreased fear-related processing to the CS+N in the cortisol group might argue for more retrieval of the extinction memory trace (or less retrieval of the original fear-related memory trace) after cortisol administration for the standard extinction stimulus as predicted by the STaR model (Meir Drexler et al., 2019). Taken together, our fourth hypothesis stating decreased fear-related (insula, dACC and amygdala) and enhanced fear-inhibitory signaling (vmPFC), also reflected in decreased SCRs in the cortisol group for CS+G during retrieval processes was not confirmed. However, we observed less fear-related activation for the standard extinction stimulus rather than for the generalized extinguished stimulus in the cortisol as compared to the placebo group.

4.3. Limitations

One limitation of this study is the missing reflection of the neural results in CS+G/CS+N comparisons in SCRs. In addition to evidence for successful extinction generalization in SCRs (Struyf et al., 2018; Waters et al., 2018), not all studies on stimulus-based fear or extinction generalization investigated (Lissek et al., 2014; Rowe and Craske, 1998) or found (Hagedorn et al., 2021; Shiban et al., 2015) effects in SCRs for CS+G as compared to CS+N. Thus, SCRs might not always be sensitive enough to capture slight differences between CS+G and CS+N, especially in the fMRI environment. Neural correlates (Hagedorn et al., 2021; Lissek et al., 2014) and behavioral data (Rowe and Craske, 1998; Shiban et al., 2015) might be more suitable to reveal differences between standard extinction and extinction generalization. Although decreased fear-related activation was observed for retrieval in our previous study (Hagedorn et al., 2021), this effect emerged only during the reinstatement test in the current study. However, the studies critically differ regarding the timing of cortisol administration and are thus not directly comparable: in the present study, cortisol was given prior to extinction training, whereas cortisol was given before retrieval in our previous study (Hagedorn et al., 2021). This timing difference could have influenced the results as reflected by cortisol modulations on activations and functional connectivities during retrieval. In this experiment, we chose an approach to study extinction generalization on a mechanistical level using geometrical shapes. Although this might state an advantage to generalize results over certain variations of stimuli, more naturalistic stimuli like phobic stimuli (Waters et al., 2018) in virtual reality (Shiban et al., 2015) or in vivo exposure (Rowe and Craske, 1998) might be promising to further investigate clinical implications of extinction generalization and implications of add-on treatment with cortisol. While we observed no enhancing effects of cortisol administration prior to extinction generalization training, the extinction memory trace might nevertheless potentially be strengthened by cortisol: If extinction learning had been successful for the CS+G, cortisol might also have enhanced the consolidation of extinction generalization memory trace. Thus, it remains to be shown whether a prolonged extinction training phase would lead to differential results concerning cortisol effects.

5. Conclusions

Taken together, our results characterize the neural correlates of extinction generalization: activation of fear-related areas was increased during extinction training and reduced during reinstatement test for the generalized extinguished stimulus in comparison to the standard extinction stimulus. However, the expected downregulation of fear-related and upregulation of fear-inhibitory areas was not found during retrieval. Although not confirmed in SCRs, these findings correspond to previously identified neural correlates of successful extinction generalization. Cortisol administration prior to extinction generalization training did not enhance the generalized extinction memory trace but rather improved the extinction memory for the standard extinction stimulus (CS+N) during the reinstatement test. In conclusion, these results hint to future avenues to combine extinction generalization with stress hormones to reduce both, responding to generalization stimuli and the original fear-related stimuli, respectively.

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CRediT authorship contribution statement

Bianca Hagedorn: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Oliver T. Wolf:** Conceptualization, Funding acquisition, Writing – review & editing. **Christian J. Merz:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

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Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105607.

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