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Received: 17 July 2020 | Revised: 11 May 2021 | Accepted: 21 July 2021

DOI: 10.1111/psyp.13917

ORIGINAL ARTICLE

PSYCHOPHYSIOLOGY SPR WILEY

Neural signature of delayed fear generalization under stress

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Funding information
This work was supported by a grant from the German Research Foundation (DFG), in the context of the collaborative research center SFB/TRR58 "Fear, Anxiety, Anxiety Disorder"

Abstract

Although the generalization of fear to stimuli resembling a threatening stimulus is an adaptive mechanism, fear overgeneralization is maladaptive and thought to play a key role in anxiety-related disorders. Since there is typically a delay between an initial fear experience and a situation in which fear (over)generalization may occur, we assessed delayed fear generalization and its neural signature. Moreover, as stress is known to affect fear learning, we further tested whether acute stress modulates fear generalization. Therefore, we conducted a two-day fear generalization study, with initial fear acquisition on Day 1 and a fear generalization test after a 24-hr delay in the MRI scanner. Prior to fear generalization testing, participants were exposed to a stressor or a control manipulation. Our behavioral data showed the expected generalization of fear. At a neural level, fear generalization was accompanied by increased fear-signaling for stimuli that resembled the conditioned stimulus in the bilateral insula and frontal operculum, whereas activity declined in frontal, hippocampal, and temporal regions, including the ventromedial prefrontal cortex, as stimuli became more similar to the conditioned stimulus. Importantly, stress did not modulate fear generalization, neither on a behavioral nor on a neural level. Interestingly, in an exploratory comparison to two other studies that used the same paradigm but tested generalization immediately after acquisition, we observed increased fear generalization in the delayed relative to the immediate generalization test. In sum, our results suggest that stress leaves fear generalization and its neural signature unaffected but that a temporal delay might increase the extent to which fear responses are generalized to stimuli resembling the threatening stimulus.

KEYWORDS

acute stress, fMRI, fear tuning, delay, fear generalization, insula, vmPFC

1 | INTRODUCTION

Fear triggers adaptive behaviors to avoid future threat. Because threatening stimuli rarely occur in the exact same form across situations, the generalization of fear to stimuli resembling the stimulus initially associated with danger promotes the effective avoidance of threat. Research over the past decade suggested that this process of fear generalization

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is implemented by an intricate balance of excitatory and inhibitory mechanisms. In particular, whereas areas such as the insula or amygdala showed declining activity as a stimulus differentiated from the threat-related conditioned stimulus (CS+), the ventromedial prefrontal cortex (vmPFC) and hippocampus showed inclining activity as a stimulus deviated from the CS+ (Greenberg et al., 2013; Lissek et al., 2014; Lopresto et al., 2016; Onat & Büchel, 2015). Although fear generalization is generally adaptive from a survival perspective, an exaggerated generalization of fear to harmless stimuli, that is, fear overgeneralization, is maladaptive and a common characteristic of anxiety disorders or post-traumatic stress disorder (PTSD; Dunsmoor & Paz, 2015; Lissek, 2012).

Stress is known to play a key role in fear-related disorders (Pitman et al., 2012; de Quervain et al., 2017). Moreover, stress impacts fear-learning processes in general (Merz et al., 2016; Raio & Phelps, 2015) and major stress mediators, such as glucocorticoids, act on medial-temporal and prefrontal areas involved in fear generalization (Kim & Diamond, 2002; Krugers et al., 2012; Roozendaal et al., 2006; Schwabe et al., 2012). These findings suggest that stress may induce an overgeneralization of fear. In line with this idea, rodent studies showed that stress or glucocorticoids may result in increased fear generalization (Bender et al., 2018; de Quervain et al., 2017; Dunsmoor & Paz, 2015; Kaouane et al., 2012). Initial evidence from one behavioral study in humans suggests that stress increased fear generalization specifically at a 24 hr-delayed test (Dunsmoor, Otto, et al., 2017). Yet, to date, the neural underpinnings of putative stress effects on fear generalization are unknown.

Beyond potential stress-dependent changes, another factor that may modulate fear generalization is time. In fear-related disorders, there is usually a considerable delay between an initial threatening encounter and situations in which fear (over)generalization may occur. This time interval between fear acquisition and later generalization may be highly relevant because memories undergo a change from detailed to more gist-like representations over time (Dandolo & Schwabe, 2018; Jasnow et al., 2012; Winocur et al., 2007). In rodents, several studies assessed fear generalization at different delays (Asok et al., 2019) and recently enhanced cued fear memory generalization has been reported in humans as time after acquisition proceeded (Pollack et al., 2018). In contrast to the rodent literature, most human studies tested fear generalization shortly after fear acquisition (Dunsmoor, Kroes, et al., 2017; Holt et al., 2014; Lissek et al., 2008, 2014; Onat & Büchel, 2015). To the best of our knowledge, there are only two behavioral studies that explored fear generalization processes after a delay of 24 hr in humans (Andreatta et al., 2020; Dunsmoor, Otto, et al., 2017). Whereas one study focused on the influence of contextual information on fear generalization (Andreatta et al., 2020), another study suggested an increased level of fear generalization due to stress for older memories but not for recent memories, that is, a test of fear generalization after a 24-hr delay compared to an immediate test (Dunsmoor, Otto, et al., 2017). However, to what extent the neural underpinnings of immediate and delayed fear generalization differ is completely unknown.

To date, different fear generalization paradigms exist (Dymond et al., 2015), some of which focus on perceptual similarity (Lissek et al., 2008; Onat & Büchel, 2015), whereas others focus on the influence of conceptual similarity (Dunsmoor & Murphy, 2015). Although using perceptually similar stimuli, Onat and Büchel (2015) were able to show that fear generalization is not just passively driven by perceptual failure because they also found object-sensitive visual areas that rather responded to uncertainty. Here, we aimed to determine the neural signature of fear generalization 24 hr after fear acquisition and to explore its potential modulation by acute stress. In addition, we aimed to explore whether this was different from an immediate test of fear generalization, which is why we used the same fear generalization paradigm of Onat and Büchel (2015) including socially relevant stimuli. On a first experimental day, participants completed a fear conditioning procedure. Twenty-four hours later, participants underwent either a stress or a control procedure before they completed a test of fear generalization in the MRI scanner. Although our study was mainly designed to assess stress effects on (delayed) fear generalization, we also aimed to investigate time-dependent changes in fear generalization and its neural basis. To this end, we contrasted our findings with those of two previous studies that used the same experimental paradigm but without a delay between fear acquisition and generalization test (Kampermann et al., 2019; Onat & Büchel, 2015). We hypothesized that fear generalization would be increased after a 24-hr delay, relative to when tested immediately after acquisition. Furthermore, we expected that stressed participants would show an even wider fear generalization.

2 | METHOD

2.1 | Participants and experimental design

Seventy-three healthy, right-handed volunteers (34 men, 39 women) participated in this experiment. In addition to any contraindications for MRI, exclusion criteria comprised any current medication intake or physical illness, a history of any mental or neurological disorder and drug or tobacco use. Moreover, women were not tested during their menses and those taking hormonal contraceptives were excluded. All participants provided written informed consent before participation and received a monetary compensation of 60€. The study protocol was approved by the ethics committee of the Medical Association Hamburg and in accordance with the Declaration of Helsinki.

In a 2-day, between-subjects design, participants were pseudorandomly assigned to a stress group or a control group, ensuring an equal number of men and women per group. Nine participants had to be excluded from the analyses because they did not show successful (explicit) fear acquisition on Day 1 (i.e., they had a lower US-expectancy rating for the CS+ than for the CS-), which was a requirement for testing fear generalization processes 24 hr later. This left a final sample of 64 participants for behavioral data analysis (age [mean ± *SD*]: 25.5 ± 4.1 years: stress group: $n = 33$ (16 women), control group: $n = 31$ [18 women]). For fMRI analyses, 2 additional participants (both stress group) had to be excluded, due to excessive head movement (>4 mm of maximal translation (in any direction of x, y, or z) and >4.0° of maximal rotation).

The previous studies that tested fear generalization immediately after acquisition and to which we compare the present findings, included 29 participants (Onat & Büchel, 2015) and 74 participants (Kampermann et al., 2019), respectively. In these studies, participants were also young, healthy individuals and largely the same inclusion and exclusion criteria were applied.

2.2 | Fear generalization paradigm

In order to assess fear generalization processes, we used a recently introduced paradigm (Onat & Büchel, 2015). If not specified otherwise, the procedure was exactly the same as in the previous studies (Kampermann et al., 2019; Onat & Büchel, 2015). This paradigm included eight face stimuli arranged on a circular similarity continuum with two axes (x-axis: identity; y-axis: gender; Figure 1a). The two faces opposite to each other represented the most dissimilar faces and were later used as CS+ and CS-, respectively. The face stimulus chosen as CS+ was counterbalanced across participants and groups. The faces in between the CS+ and CS- represented the generalization stimuli (GS), which were quantified in their distance to the CS+ (Figure 1b). The paradigm comprised three phases: a *baseline phase*, a *fear acquisition phase*, and a *test of generalization* (Figure 1c). A moderate electric shock served as US. Face stimuli were shown for 1.5 s and, in shocked trials, the US was presented after 1.4 s and co-terminated with face offset. The mean inter-trial interval (ITI) was 3.5 s, ranging between 1.5 and 5.5 s. The ITI was slightly different (3.5 s vs. ~4 s) to the previous studies (Kampermann et al., 2019; Onat & Büchel, 2015). During the *baseline phase*, the complete set of faces was shown, to control for any a priori differences between the faces. During the *fear acquisition phase,* only two faces, that is, the most dissimilar faces, were shown. One face was followed by the US in ~30% of the trials and served as CS+, whereas the other face was never paired with the US and served as CS-. During the *test of fear generalization*, again the complete set of faces was presented. A detailed description of these phases is provided in the supplement.

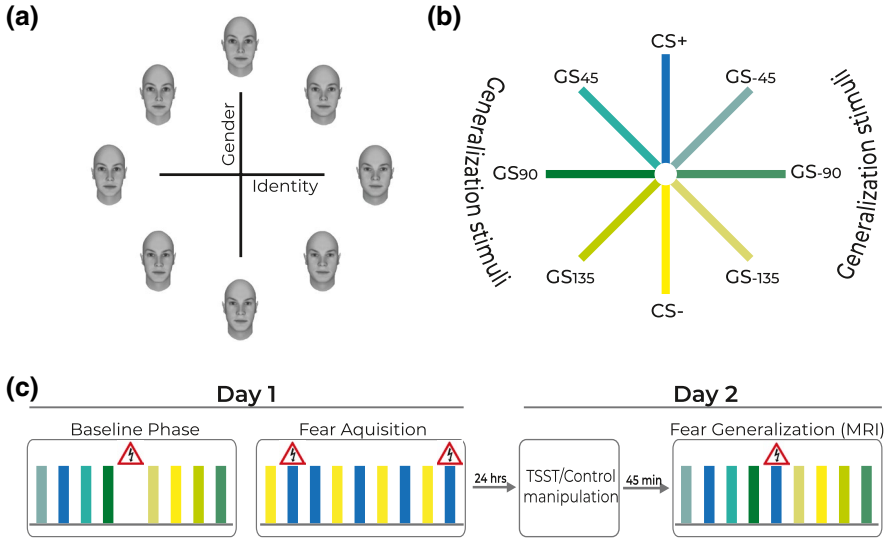

FIGURE 1 Fear generalization paradigm and stimulus organization. (a and b) There are eight different face stimuli in total, arranged on a circular similarity continuum with the axes gender and identity. The stimuli in between the CS+ and CS- represent the generalization stimuli (GS). (c) Fear generalization paradigm with three phases. On Day 1, the baseline and fear acquisition phases take place. On Day 2, the test of fear generalization follows after the stress manipulation or control condition. During the baseline phase, the complete set of stimuli (represented by colored bars) is shown to the participants and US are signaled by a shock symbol. During the fear acquisition phase, the two most dissimilar stimuli from opposite sides of the circular similarity continuum are shown to the participants, representing the CS+ and CS-. During fear acquisition, the CS+ is followed by the US in ~30% of the trials. During the test phase, again the complete set of faces is shown to the participants. To avoid extinction, there is a reinforcement rate of ~30% for the CS+ in the test phase

After each phase, each face was presented two times in randomized order and US-expectancy ratings were assessed using a visual analogue scale (VAS; anchors: "1" = certain, no shock; "10" = certain, shock) to measure explicit fear learning.

2.3 | Experimental procedure

Testing took place on two consecutive days, between 12:30 p.m. and 7:30 p.m., with fear acquisition on Day 1 and the stress manipulation and the test of fear generalization in the MRI scanner on Day 2. To induce stress, we used the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), a standardized protocol for experimental stress-induction in humans that reliably increases subjective stress levels and activates both the autonomic nervous system and the hypothalamus-pituitary-adrenal axis (Kirschbaum et al., 1993). In brief, participants were asked to give a free speech and to perform a mental arithmetic task while being videotaped and evaluated by a panel of two cold, non-reinforcing experimenters. In the control condition, participants talked about a topic of their choice and performed a simple arithmetic task, while being alone in the room, without video recordings. To validate the successful stress induction, we obtained subjective ratings and physiological stress indicators, that is, blood pressure, pulse, and salivary cortisol at several time points across the experiment. For a detailed description of the task and timings of measurements, see Supporting Information.

2.3.1 | Day 1—Baseline phase and fear acquisition

Upon participants' arrival at the lab, they completed several questionnaires assessing control variables of interest (depression, Beck Depression Inventory [BDI-II; Beck et al., 1996]; anxiety, State-Trait-Anxiety-Inventory [STAI; Spielberger & Syndeman, 1994]; and chronic stress, Trier Inventory for the Assessment of Chronic Stress [TICS; Schulz & Schlotz, 1999]). After completing an unrelated, non-arousing task, the electrodes for US application and for recordings of electrodermal activity (EDA) were attached. For a detailed description of electrical stimulation and EDA analysis, see Supporting Information. Then, the individual pain threshold was determined using the QUEST procedure (Watson & Pelli, 1983), aiming at a shock intensity that was unpleasant but not painful. Next, the *baseline phase* of the fear generalization paradigm started which was immediately followed by the *fear acquisition phase*. At the end of Day 1, the pain strength rating was measured again.

2.3.2 | Day 2—Stress manipulation and test of fear generalization

About 24 hr later (range: 30 min to 3 hr), participants returned to the lab, the individual pain threshold was determined and depending on the experimental group, participants either underwent the TSST or the control manipulation. Immediately thereafter, participants were placed in the MRI scanner, completed again an unrelated, non-arousing task, before the critical *fear generalization phase* started. After the generalization test, all of the eight face stimuli were shown to the participants in a randomized circular arrangement and participants had to indicate which of the faces was followed by the shock. Outside of the scanner, participants performed a perceptual discrimination task, to check for participants' general discrimination ability (Supporting Information). At the end of Day 2, participants were debriefed and compensated for participation.

2.4 | Analysis of fear-tuning profiles

To characterize individual fear-tuning, we followed the approach of Onat and Büchel (2015) and set up a Gaussian model with two parameters (α, amplitude; σ, width), using MATLAB (Release 2016b, Natick, MA). We restricted our Gaussian model to be centered on the CS+-face. Fear-tuning profiles were calculated for z-scored skin-conductance response (zSCR) and rating data separately. For further statistical analyses, we extracted the two parameters (amplitude, width) of each profile.

2.5 | Behavioral and physiological data analysis

Statistical analyses were performed with SPSS 25.0 (IBM). Subjective and physiological data were analyzed by mixed-design ANOVAs with time and stimulus as within-subject factors and group (stress vs. control) as between-subjects factor. For simple group comparisons, independent sample *t* tests were used and for repeated measurements analyses we applied rmANOVAs. To investigate fear-tuning over time, we calculated a sharpening index (SI) by subtracting the width of the fear-tuning profile obtained for the test phase from the width of the fear-tuning profile obtained for the acquisition phase, that is, $\sigma_{Rating(Acqui)} - \sigma_{Rating(Test)}$. To analyze the perceptual discrimination ability, we calculated a discrimination score by subtracting the mean false alarm rate from the mean hit rate. Frequency of distribution was analyzed by means of Chi^2-tests and Cramer's V was used for group comparisons.

To investigate how time influenced the responding to the stimuli, we additionally calculated the mean response for the

stimuli most similar to the CS+ (|GS45|) and created a difference variable by subtracting this mean response from the CS+. In addition, we re-analyzed the behavioral results of two previous studies using the exact same paradigm in which the test phase was presented immediately after the fear acquisition phase (Kampermann et al., 2019; Onat & Büchel, 2015) and compared those results to ours.

All reported *p*-values are two-tailed, using a α-error threshold of $p = .05$. Significant main or interaction effects were pursued using the post hoc test, which were corrected for multiple comparisons. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied.

2.6 | fMRI acquisition and analysis

fMRI data were acquired using a 3T MRI Scanner (Prisma, Siemens, Germany) with a 64-channel head coil. Sixty transversal slices were sequentially acquired using a T2-weighted echo-planar imaging sequence (2 s TR, 30 ms TE, 30° slice tilt, voxel size = 2 × 2 × 2 mm, 905 volumes). In addition, a high-resolution T1-weighted anatomical image was acquired (256 coronal slices, 2.5 s TR, 2.12 ms, voxel size = 0.8 × 0.8 × 0.8 mm).

Preprocessing and analysis of the fMRI data was performed using SPM12 (Wellcome Trust Centre for Neuroimaging, London). The first five functional scans were discarded, to allow for T1 equilibration. All functional volumes were motion-corrected and co-registered to anatomic images using rigid-body transformations. Both functional and structural images were normalized to the MNI standard brain. Finally, the normalized functional images were smoothed using a 4 mm FWHM Gaussian kernel.

To investigate neural fear generalization we followed the procedure described by Onat and Büchel (2015) and set up two different models. With the first model, we aimed to identify brain areas that mirrored a Gaussian shaped fear-tuning response. Therefore, we set up a linear regression model with the primary regressor representing the face onsets and two regressors of no interest (onsets of oddball trials and US trials), all of which were convolved with a canonical hemodynamic response function. In addition, we included two parametric modulators on responses evoked by our primary regressor, that is, the face stimuli, and the six realignment parameters as movement regressors. The parametric modulators were the same as in Onat and Büchel (2015) and represented (i) a Gaussian basis function and (ii) a numerical approximation of the derivative of the Gaussian function with respect to its standard deviation parameter ($dG/d\sigma$) to model a large variety of Gaussian-tuning profiles. On the individual first level, data were filtered in the temporal domain using a nonlinear high-pass filter with 128 s cut-off and we tested different combinations of the contrasts for the two parametric modulators, using a *t*-test. In line with common recommendations, we first conducted an exploratory whole-brain analysis, followed by a theory-driven analysis of a-priori defined regions of interest (ROIs; Poldrack, 2007). Those areas that exceeded a family-wise error (FWE) corrected statistical threshold of 0.05 (whole-brain) were defined as our ROIs. FWE-correction was performed without a cluster-extent threshold. Given their importance in fear generalization, we predefined the vmPFC, the insula and the amygdala as ROI and if not found on whole-brain level, we would investigate those areas with small-volume correction (SVC). In a second step, we aimed to precisely determine the activity in our ROIs to each individual face and to explicitly compare responding to CS+ versus CS-. Therefore, we set up a second linear model on the first level, that contained eight primary regressors, one for each face as well as the two regressors of no interest, again using the canonical hemodynamic response function and added the six realignment parameters as movement regressors. We extracted the eight beta-weights representing the activation levels for every individual face stimulus for each participant. Then, those beta-weights were used for the final parameterization of the fear-tuning profiles using the Gaussian-fitting procedure.

Anatomical locations were determined based on Automated Anatomical Labeling atlas (Tzourio-Mazoyer et al., 2002). At the group level, contrast images were analyzed using one-sample *t* tests and two-sample *t* tests for group comparisons. Correlations with the different stress parameters and brain regions were Bonferroni-corrected (critical *p*-value: $p/9 = .05/9 = .006$).

3 | RESULTS

3.1 | Control variables

Groups did not differ regarding their trait or state anxiety and subjective level of chronic stress (all $t \leq 1.194$; all $p \geq .237$; all $d \leq 0.301$, Table 1). However, there was a trend for a group difference in depressive mood ($t(61) = -1.958$, $p = .054$, $d = 0.494$), indicating a slightly higher degree of depressive mood in the stress group. To rule out a possible influence of depressive mood on our results, we included the BDI score as a covariate in all our analyses. Because this covariate left our results largely unaffected, we decided to report the analyses results without the covariate. In addition, we ran explorative analyses of our control variables with fear-tuning parameters for the behavioral and neural data, of which the results can be found in the Table S2. Due to the exploratory nature of this analysis, these results should be interpreted with caution.

TABLE 1 Control variables, psychophysiological and subjective measures on Day 1

Variable	Control	Stress
Control variables		
STAI-T	35.39 (1.47)	36.72 (1.57)
STAI-S	36.23 (1.31)	35.25 (1.00)
TICS	12.71 (1.31)	15.28 (1.70)
BDI-II	3.65 (0.59)	5.97 (1.02)
Experimental variables		
Salivary cortisol (nmol/L)	4.99 (0.68)	4.87 (0.53)
Systolic BP (mmHg)	122.37 (2.80)	120.74 (2.23)
Diastolic BP (mmHg)	80.82 (1.70)	81.92 (1.34)
Pulse (bpm)	81.68 (2.45)	78.88 (2.15)
Positive affect	2.91 (0.15)	2.69 (0.09)
Negative affect	1.28 (0.07)	1.21 (0.06)
Pain threshold (V)	52.75 (2.44)	53.47 (2.40)
Pain strength start	5.29 (0.36)	5.27 (0.34)
Pain strength end	5.35 (0.34)	5.09 (0.40)

Note: Data represent mean (standard error of the mean).

3.2 | Day 1: Baseline phase and fear acquisition

Before the beginning of testing on Day 1, groups did not differ in their subjective mood, salivary cortisol, blood pressure, heart rate, estimated pain threshold or pain strength rating (all $t \leq 1.292$; all $p \geq .201$; all $d \leq 0.326$, Table 1). In addition, participants experienced the US as uncomfortable from beginning until the end of testing, without differences between groups (all $F \leq 0.217$; all $p \geq .750$; all $\eta^2 \leq 0.003$).

3.2.1 | Baseline responses to face stimuli

As displayed in Figure 2a, both groups rated the faces comparably after the baseline phase (both $F \leq 0.974$; both $p \geq .328$; both $\eta^2 \leq 0.015$). There was a face stimulus main effect (F (3.4049, 211.356) = 3.197, $p = .019$, $\eta^2 = 0.049$). However, after correcting for multiple testing, no post hoc comparison approached statistical significance (all $p \geq .130$). Regarding the zSCR data, the stress group showed a slightly higher SCR than the control group during the *baseline phase* (F (1, 62) = 4.042, $p = .049$, $\eta^2 = 0.061$; Figure 2b). More importantly, however, there was no main effect of face stimulus and no group × face stimulus interaction (both $F \leq 1.725$; both $p \geq .120$; both $\eta^2 \leq 0.027$).

3.2.2 | Successful fear acquisition

Participants showed successful fear acquisition, as indicated by higher responding to the CS+ compared to the CS-, in both the subjective rating data (F (1, 62) = 507.982, $p < .001$, $\eta^2 = 0.891$; Figure 2c) and the zSCR data (F (1, 62) = 21.272, $p < .001$, $\eta^2 = 0.255$; Figure 2d). Importantly, there were no group differences in fear acquisition, neither in the rating nor in the zSCR data (all $F \leq 1.597$; all $p \geq .211$; all $\eta^2 \leq 0.025$).

3.3 | Day 2: Stress exposure and delayed test of fear generalization

Upon their arrival on Day 2, groups did not differ in subjective mood, salivary cortisol, blood pressure, heart rate or pain strength rating (all $t \leq 1.649$; all $p \geq .104$; all $d \leq 0.412$; see Table S1). With respect to the pain strength rating, participants rated the US as more painful after compared to before the *fear generalization phase* (F (1, 62) = 17.726, $p < .001$, $\eta^2 = 0.222$), independent of experimental group (both $F \leq 1.667$; both $p \geq .201$; both $\eta^2 \leq 0.026$).

3.3.1 | Successful stress-induction by the TSST

Significant changes in subjective and physiological parameters verified the successful stress induction by the TSST (Figure 3). On the subjective level, participants of the stress group felt more challenged, uncomfortable and stressed after the task than participants of the control group (all $t \geq -4.948$; all $p \leq .001$; all $d \geq 1.238$). Salivary cortisol, blood pressure, and heart rate increased from before to after the manipulation in the stress group (all $F \geq 6.251$; all $p < .001$; all $\eta^2 \geq 0.168$) but not in the control group. For the control group, there was even a significant decrease in salivary cortisol and pulse over time (both $F \geq 7.229$; both $p < .001$; both $\eta^2 \geq 0.194$). Importantly, post hoc t tests showed that groups significantly differed in their cortisol concentrations 20 min, 60 min and 110 min after the treatment, implicating significantly elevated cortisol concentrations in the stress group throughout the critical fear generalization test (all $t \geq -3.035$; all $p \leq .004$; all $d \geq 0.759$). Regarding the autonomic measurements, the stress group showed increased systolic and diastolic blood pressure compared to the control group during and 20 min after the TSST (all $t \geq -2.980$; all $p \leq .004$; all $d \geq 0.751$). The pulse was only significantly different during the stress/control manipulation ($t = -2.054$, $p = .045$, $d = 0.508$).

3.3.2 | No influence of stress on behavioral fear generalization

Twenty-four hours after fear acquisition, participants still showed intact fear memory, indicated by a higher response to the CS+ compared to the CS- in both rating and zSCR data (both $F \geq 42.465$; both $p < .001$; both $\eta^2 \geq 0.410$), without

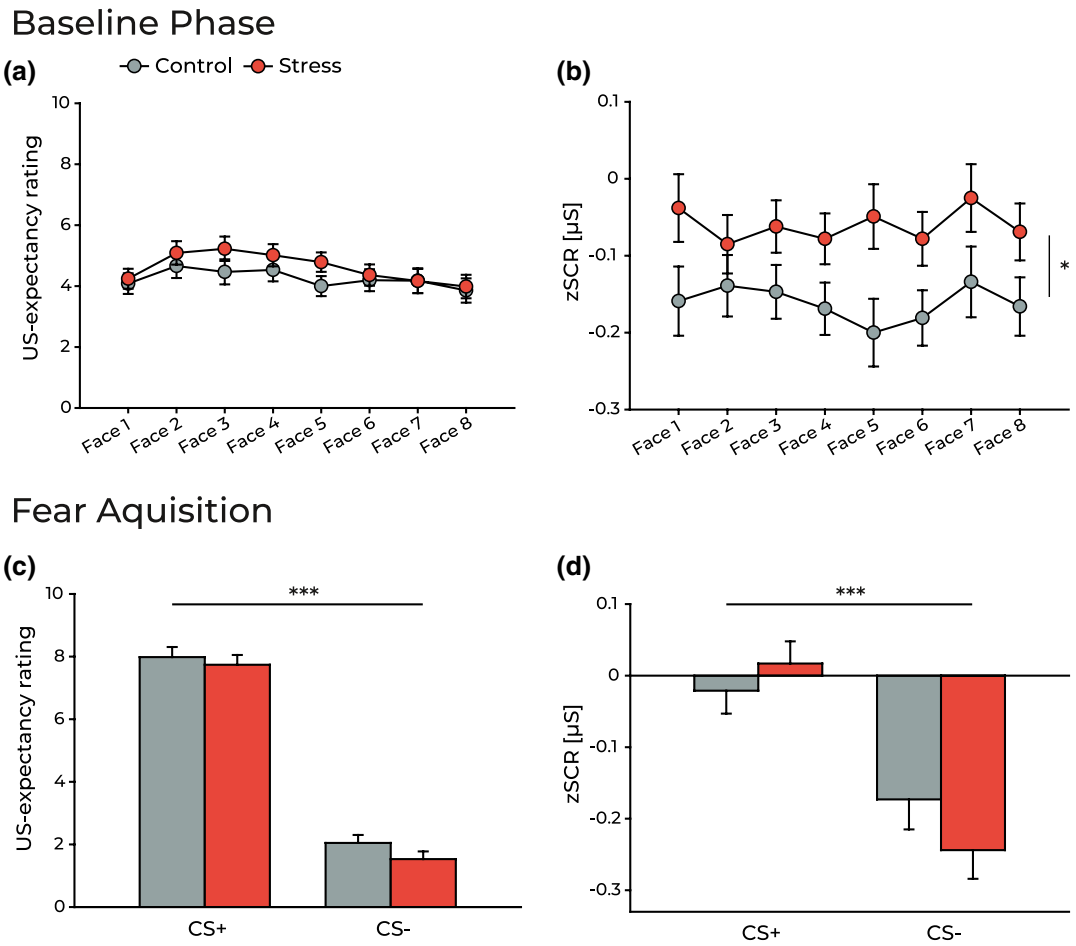

FIGURE 2 Day 1: Physiological and subjective responses to the face stimuli during the baseline and fear acquisition phases. (a) Explicit rating data as well as (b) zSCR data show no systematic a priori differences between faces and no group differences during or after the baseline phase. During and after fear acquisition, both (c) explicit US-expectancy rating data as well as (d) zSCR data show successful fear learning reflected in higher responses to the CS+ than to the CS-. Error bars represent standard errors of the mean. Asterisks denote differences between stimuli (*$p < .05$, ***$p < .001$)

any influence of group (all $F \leq 0.662$; all $p \geq .419$; all $\eta^2 \leq 0.011$). In addition, we obtained clear evidence for fear generalization, shown by fear-tuning profiles, resembling a Gaussian function, for both explicit and implicit fear data (Figure 4a,b, respectively). Statistically, an rmANOVA for the eight stimuli showed a main effect of stimulus in both measures (both $F \geq 24.794$; both $p \leq .001$; both $\eta^2 \geq 0.289$) with larger quadratic within-subject contrasts compared to linear ones (both $F \geq 45.884$; both $p \leq .001$; both $\eta^2 \geq 0.429$). Importantly, there was no main effect of group or stimulus × group interaction effect (all $F \leq 1.445$; all $p \geq .234$; all $\eta^2 \leq 0.023$), that is, stress did not modulate fear generalization. To investigate the influence of stress on delayed fear generalization in more detail, we compared the parameters of our Gaussian fear-tuning profiles obtained on Day 2 between groups. Results again showed that groups did not differ regarding their amplitude or width across measurements on Day 2 (all $t \leq 1.052$; all $p \geq .297$; all $d \leq 0.265$). Moreover, stress did not influence the change of amplitude or width of the rating data from fear acquisition to the test of fear generalization (all $F \leq 1.163$; all $p \geq .285$; all $\eta^2 \leq 0.019$).

Exploratively, we also conducted correlational analyses between the different stress mediators, that is, systolic and diastolic blood pressure, pulse and cortisol, and different fear-tuning widths. In general, there were only few significant correlations, which were not constant. These results can be found in the Supporting Information.

3.3.3 | Fear generalization requires fear reactivation

To investigate if the reminder US in the test phase had an impact on fear generalization, we calculated fear-tuning curves for reinforcement bins, representing different distances to the last US and subjected these data to a Gaussian fear-tuning analysis (Figure 5). As shown in Figure 5, a first US was necessary for a reinstatement of fear in general and consequently for fear generalization, in both groups. We hypothesized that a reminder US is necessary for the development of a Gaussian fear-tuning curve. This assumption was confirmed by rmANOVAs, showing a significant stimulus × proximity

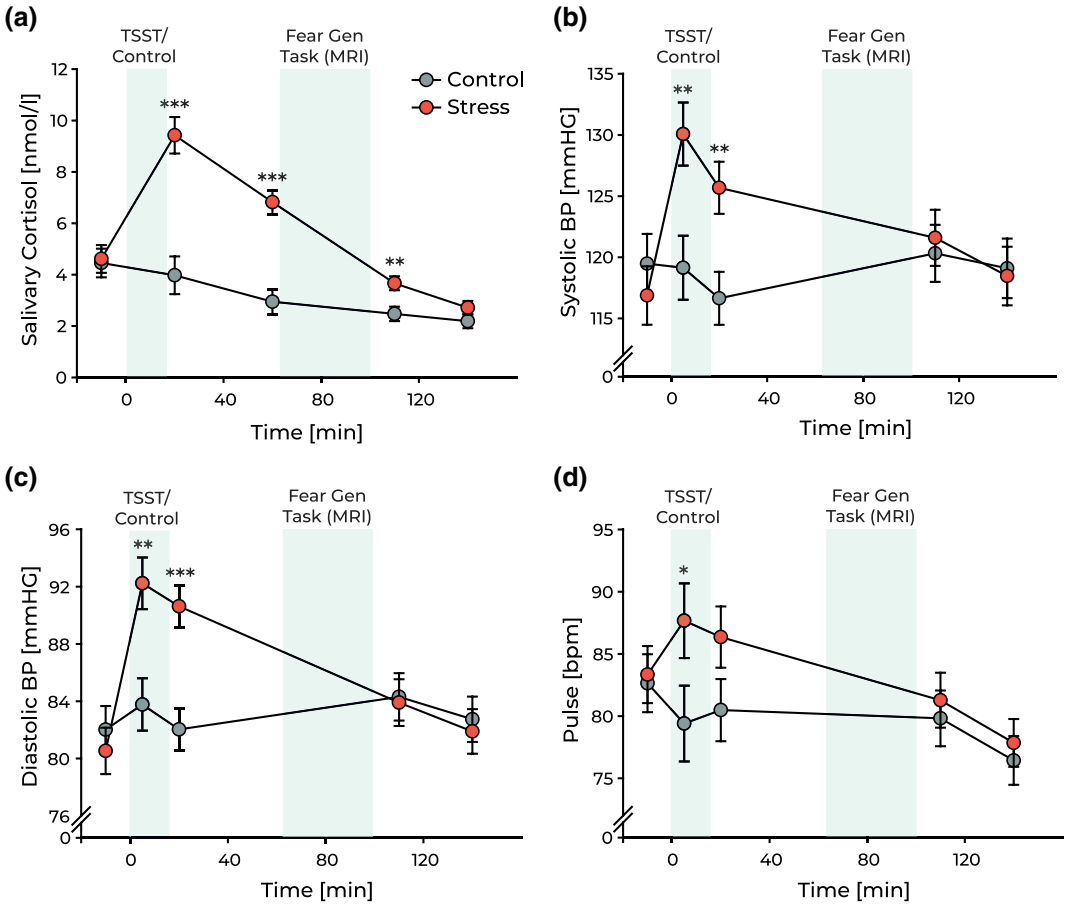

FIGURE 3 Stress manipulation check. (a) Salivary cortisol increase. (b) Systolic blood pressure increase. (c) Diastolic blood pressure increase. (d) Pulse increase. Error bars represent standard errors of the mean. Asterisks denote difference between groups. (***$p < .001$, **$p < .01$, *$p < .05$)

interaction (F (8.513, 510.783) = 1.752, $p < .001$, $\eta^2 = 0.101$). After the first US-administration during the test phase, participants showed in all proximity bins successful fear-tuning, that is, the strongest reaction toward the CS+, that decreased with increasing dissimilarity. This was confirmed by post hoc rmANOVAs for each proximity bin separately. Those showed that the reaction to the stimuli followed a quadratic trend (all $F \geq 31.579$; all $p < .001$; all $\eta^2 \geq 0.345$) compared to a linear trend (all $F \leq 5.595$; all $p \geq .021$; all $\eta^2 \leq 0.085$). In contrast, before any US was administered, the stimulus reaction rather followed a linear trend ($F = 8.388$; $p = .005$; $\eta^2 = 0.123$) instead of a quadratic one ($F = 4.073$; $p = .048$; $\eta^2 = 0.064$), suggesting that a precise fear memory was missing. Furthermore, the analyses of the Gaussian model parameters revealed a significant proximity effect for the amplitude (F (1.796, 107.779) = 12.138, $p < .001$, $\eta^2 = 0.168$) and post hoc comparisons revealed a significant lower fear reaction from before compared to after US-administration (all $p \leq .042$). These results further underpin the need for a reminder to reactivate fear-memory. Regarding the width of fear generalization, results revealed no significant effect of proximity (F (3, 180) = 0.768, $p = .513$, $\eta^2 = 0.013$). This pattern did not differ between the stress and control groups, suggesting that stress had no modulatory effects on the need of a reminder US for the development of fear-tuning (all main or interaction effects: all $F \leq 0.323$; all $p \geq .702$; all $\eta^2 \leq 0.005$).

3.3.4 | Hyper-sharp fear-tuning in brain regions beyond the insula after a 24-hr delay, irrespective of stress

To investigate the neural underpinnings of delayed fear generalization, we analyzed in a first step which brain areas showed a fear-tuning comparable to our behavioral data (i.e., following a Gaussian function). At the whole-brain level (FWE-corrected $p < .05$), several areas showed the predicted fear-tuning (Table 2), many of them overlapping with previous reports on the neural underpinnings of fear generalization (Dunsmoor et al., 2011; Greenberg et al., 2013; Lissek et al., 2014; Onat & Büchel, 2015). Most importantly, two of these regions, the bilateral insula and the right frontal operculum, showed increased activity in response to the CS+ and declining activity as the face stimuli became more dissimilar

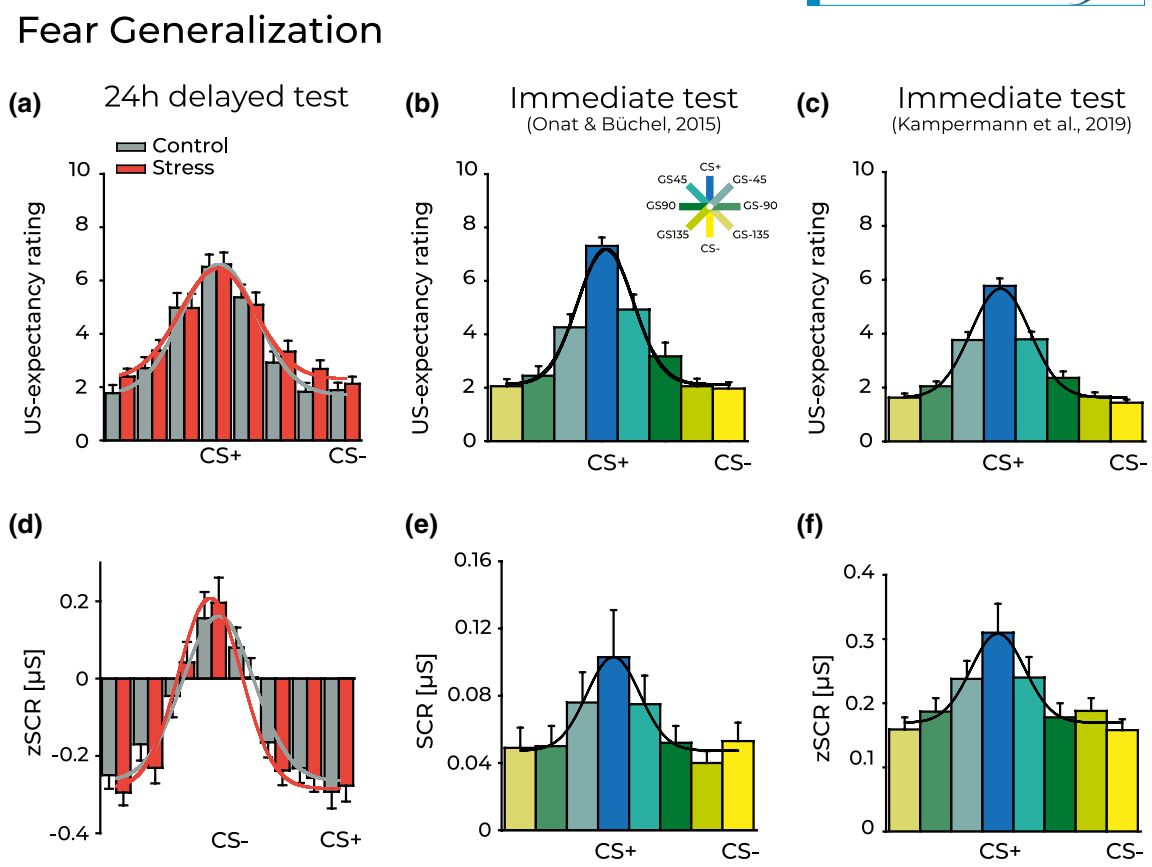

FIGURE 4 Day 2: Fear generalization phase of the different studies. Figures depict the responses to the different stimuli. Across all studies, fear-tuning is observed in (a-c) explicit fear learning, represented by US-expectancy ratings as well as (d-f) implicit fear learning, represented in electrodermal activity. For the current study (a+d) responses are depicted for the stress group and the control group separately. Error bars represent standard errors of the mean

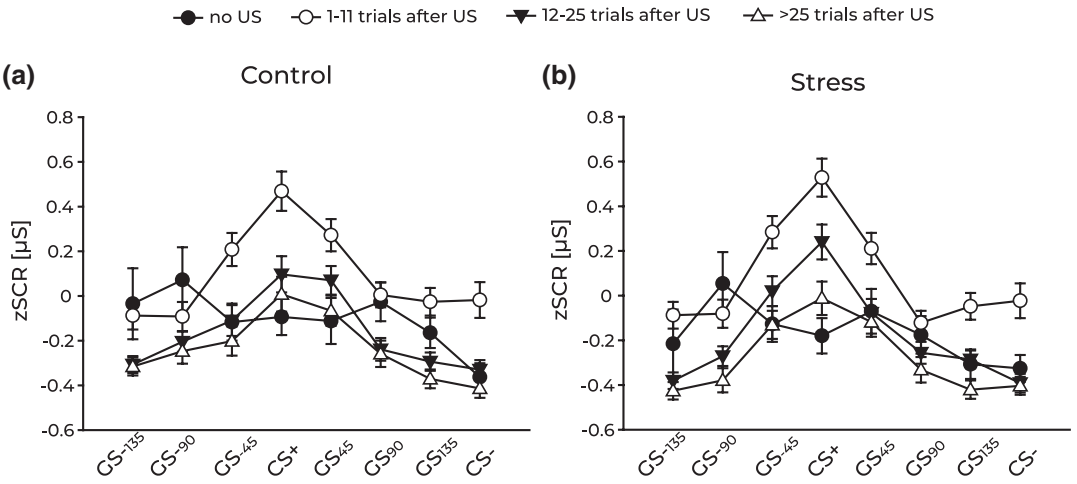

FIGURE 5 Fear-tuning dependent on US-proximity. For both the stress group and the control group, a reminder US during the test phase is necessary for the evolvement of an actual quadratic fear-tuning

to the CS+. All other regions (frontal, temporal, and hippocampal regions, angular gyrus and left precuneus) showed an inverted gauss function, that is, reduced activation to the CS+ (Figure 6a,b). Interestingly, in line with previous results (Onat & Büchel, 2015), we found a hyper-sharp tuning of the bilateral insula (i.e., smaller width compared to the behavioral data) even after a 24-hr delay. This is depicted by a significant difference of the fear-tuning width in the left insula compared to the width of rating as well as zSCR data, being narrower on the neural level (both $p \leq .042$). For the right insula, the

TABLE 2 Brain areas showing Gaussian fear-tuning

Brain region	T-value	$P_{FWE\text{-}corr}$	MNI coordinates		
			X	*Y*	*Z*
Whole-brain					
L. middle temporal gyrus	−7.56	0.000	−60	−6	−20
L. angular gyrus	−7.28	0.000	−42	−72	36
R. parahippocampal gyrus	−7.02	0.000	24	−16	−20
L. middle orbital gyrus (vmPFC)	−6.91	0.000	−6	60	2
R. angular gyrus	−6.78	0.000	48	−68	30
L. precuneus	−6.73	0.000	−6	−56	16
L. insula	6.63	0.000	−34	22	6
R. insula	6.62	0.000	34	30	6
L. middle frontal gyrus	−6.53	0.000	−26	22	50
L. parahippocampal gyrus	−6.50	0.000	0	20	−16
R. middle temporal gyrus	−5.92	0.003	62	−14	−18
R. frontal operculum	5.41	0.017	34	10	26
R. middle frontal gyrus	−5.36	0.019	26	32	46
Small-volume corrected					
R. amygdala	−3.77	0.007	20	8	−18
L. amygdala	−3.70	0.007	−18	−6	−22

differences were non-significant (both $p \geq .163$). The reversed fear-tuning pattern of the vmPFC showed a significantly narrower width compared to the zSCR data ($p = .037$), suggesting an increased neural inhibition of fear-tuning after 24 hr. Compared to the rating data, fear-tuning in the vmPFC was also narrower but this difference was not significant ($p = .190$). While all of the aforementioned results are based on a whole-brain analysis, we also performed a pre-defined ROI analysis that focused on the amygdala, an area known to play a key role in fear processing (Büchel & Dolan, 2000; Phelps et al., 2001). In line with the result of the previous study on immediate fear generalization testing (Onat & Büchel, 2015), the amygdala displayed an inversed fear-tuning curve (Figure S1).

Next, we contrasted the fear-tuning related contrast images between the stress and control groups, to investigate a possible influence of stress on the neural signature of fear generalization. On a whole-brain level with a FWE-corrected threshold, we did not observe any differences. Using SVC, we could show that participants of the stress group showed a stronger fear-tuning in the left insula ($T = 3.34$, $p_{SVC} = 0.019$ (FWE)). There was no influence of stress on any other of our ROIs (all $T \leq 2.05$, $p_{SVC} = 0.276$ (FWE)).

3.3.5 | Increased fear generalization after a 24-hr delay

Because we used the same paradigm as in two previous studies, which investigated immediate fear generalization (Kampermann et al., 2019; Onat & Büchel, 2015), we aimed to exploratively compare our results of delayed fear generalization with those of generalization tested immediately after fear acquisition. For this comparison, we only included the control group of the present study. In general, there was a wider fear generalization after a delay of 24 hr (Table 3). To investigate how fear responding changed from fear acquisition to the test of fear generalization, we first compared the sharpening index (SI) between studies (Figure 7a). Interestingly, results indicated that fear-tuning of subjective data decreased from fear acquisition to the test of fear generalization when tested on the same day but increased when fear generalization is tested 24 hr later (F (2, 130) = 5.256, $p = .006$, $\eta^2 = 0.075$). Post hoc comparisons revealed a significant difference between the current study and the non-fMRI study ($p = .002$), but only a non-significant trend in the same direction between the current study and the previous fMRI study ($p = .113$). There was no statistically significant difference between the two studies with an immediate test of fear generalization ($p = .201$).

Next, we compared the parameters of the fear-tuning profiles on Day 2 across studies. Results of the rating data for the fear-tuning width, mirrored results of the SI, showing a trend for a wider fear-tuning after a 24-hr delay (F (2, 130) = 2.791, $p = .065$, $\eta^2 = 0.041$; Figure 7b). Post hoc tests corrected for multiple comparisons revealed a significant difference between the current study and the non-fMRI study ($p = .026$) and a trend for a difference between the two fMRI studies ($p = .059$), without any statistically significant difference

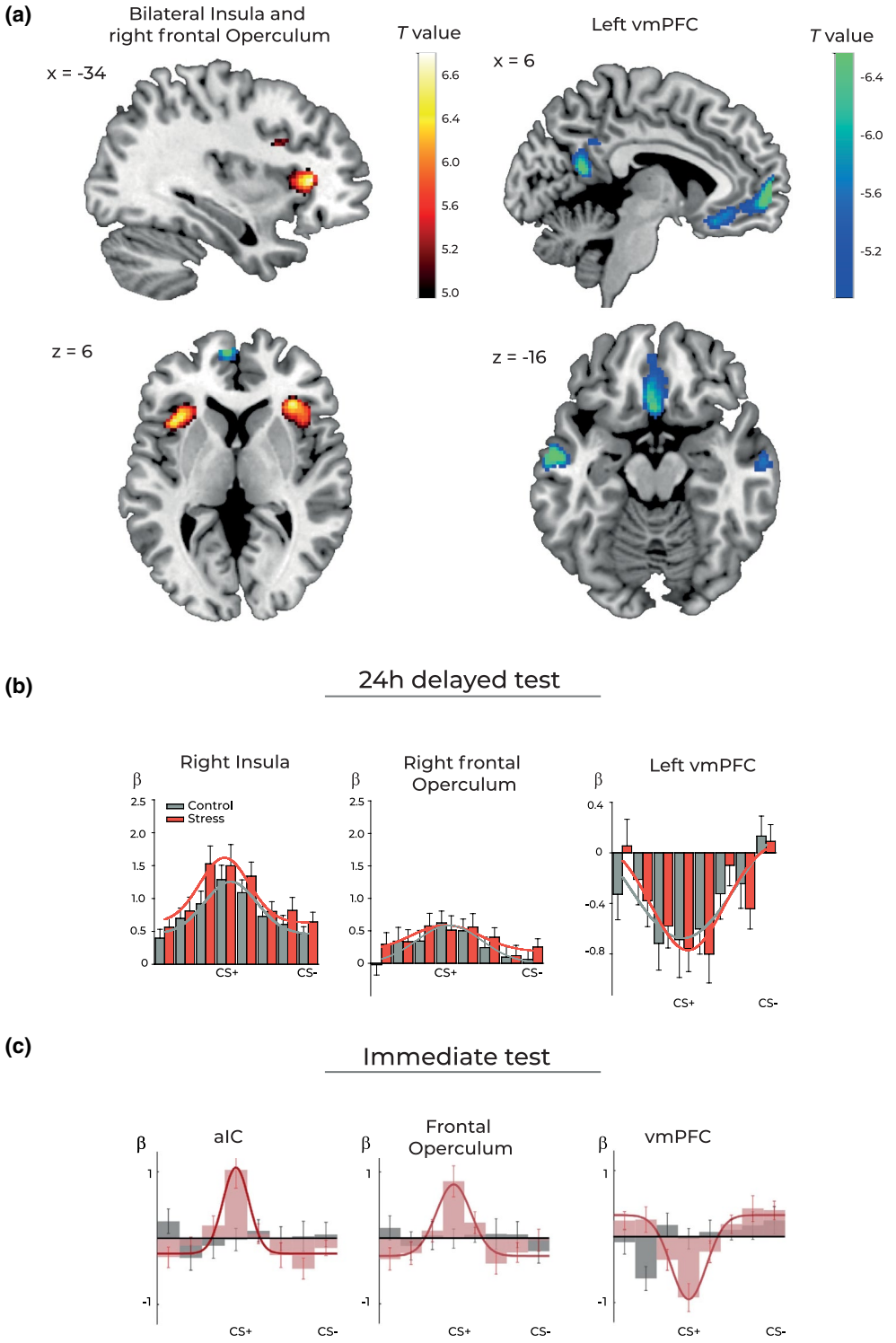

FIGURE 6 Brain areas showing Gaussian fear-tuning at the whole-brain level. (a) The bilateral insula as well as the right frontal operculum show a positive association with fear-tuning, whereas the left vmPFC is found to be negatively related to fear-tuning, thus reflecting safety tuning. Thresholded statistical maps ($p < .05$, FWE-corrected) depict fear-tuning clusters and functional maps are normalized to MNI space. (b) Fear-tuning profiles of the peak-voxel of the clusters depicted in (a) during the test of fear generalization. Bars represent the averaged neural responses across participants for each stimulus separately. The fourth bar represents the CS+, the eighth bar, the CS-. Error bars represent standard errors of the mean

between the two previous studies ($p = .966$). Regarding the amplitude, there was a significant study effect (F (2, 130) = 3.422, $p = .036$, $\eta^2 = 0.050$; Figure 7c). However, post hoc tests suggested that this effect was mainly driven by the environment of testing. Participants that were tested outside of the scanner showed a lower amplitude compared to those

TABLE 3 Comparison of fear-tuning width for behavioral and neuronal data

Variable	Current study (control group)	Onat and Büchel (2015)[1]	Kampermann et al. (2019)
$\sigma_{SCR(Test)}$	1.03 (0.48)	0.72 (0.36)	0.92 (0.61)
$\sigma_{Rating(Test)}$	0.99 (0.37)	0.78 (0.42)	0.78 (0.46)
SI	−0.13 (0.48)	0.06 (0.40)	0.18 (0.44)
$\sigma_{R.\ Insula}$	0.86 (0.55)	0.65	
$\sigma_{L.\ vmPFC}$	0.82 (0.57)	0.46	
$\sigma_{L.\ Insula}$	0.76 (0.49)		

Note: Data represent mean (standard deviation).

Abbreviations: SI = Sharpening index, that is, $\sigma_{Rating(Acqui)}-\sigma_{Rating(Test)}$.

[1]Behavioral data were re-analyzed with inference statistical analysis, why results differ to the results reported in the original paper. Data of neural fear-tuning are taken directly from Onat and Büchel (2015).

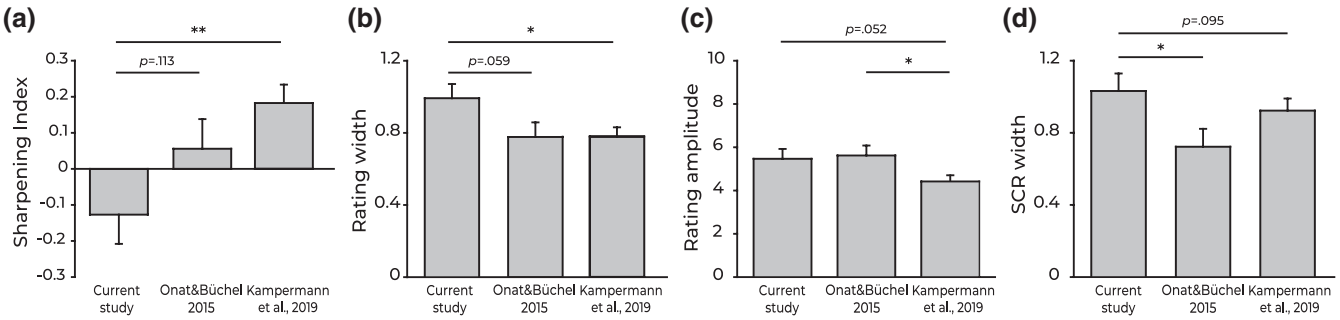

FIGURE 7 Fear-tuning results across studies. The current study tests fear generalization after a 24-hr delay, whereas the two previous studies (Onat & Büchel, 2015; Kampermann et al., 2019) tested fear generalization. (a) When fear generalization is tested after a delay compared to immediately after fear acquisition, the strength to differentiate between the CS+ compared to the stimuli most similar to it, decreases. This is revealed by a negative sharpening index (SI; $\sigma_{Rating(Acqui)}-\sigma_{Rating(Test)}$) for the current study compared to a positive SI for the studies having an immediate test of fear generalization. Furthermore, (b) the width of fear-tuning for the test of fear generalization is wider in the current study than in previous studies. (c) The amplitude of the rating data, however, shows a higher fear-tuning amplitude in both fMRI studies compared to the non-fMRI study. (d) The comparison of the fear-tuning width of the SCR data mirrors the results of the rating data, showing a broader fear generalization of the current study compared to the previous studies. Error bars represent standard errors of the mean. Asterisks denote difference between studies. (**$p < .01$, *$p < .05$)

tested in the MRI, immediately after fear acquisition ($p = .026$) and after a 24-hr delay ($p = .052$).

Because the three studies differed in the preprocessing of SCR data, which precluded a direct comparison of SCR amplitudes, we compared only the width of the fear-tuning profiles. Results again revealed a marginal effect of study (F (2, 119) = 2.612, $p = .078$, $\eta^2 = 0.042$), suggesting a trend for wider fear-tuning after a delay of 24 hr compared to an immediate test (Figure 7d). Post hoc tests revealed a significant difference between the two fMRI studies ($p = .027$) and a trend between the two previous studies ($p = .095$), but no statistically significant difference between the current study and the non-fMRI ($p \geq .355$).

In a next step, we compared the neural representation of fear generalization when tested shortly after fear acquisition versus after a 24-hr delay, by comparing the width parameter of the Gaussian fear-tuning reported after an immediate fear generalization test (Onat & Büchel, 2015) with those obtained by our delayed testing (Table 3). Descriptively, the neural fear-tuning was wider after a delay of 24 hr, mirroring the pattern of the behavioral data.

3.3.6 | Increased responding to similar stimuli accounts for broader fear generalization

To rule out that a wider fear generalization is due to a change in CS+/CS- discrimination from fear acquisition to fear generalization testing, but rather due to altered responding to stimuli most similar to the CS+, that is, the GS45 and GS-45, we compared the difference in responding to the respective stimuli across phases and studies. Because we only showed CS+ and CS- during the fear acquisition phase but obtained US-expectancy ratings for all of the eight faces, only rating data were analyzed.

For the CS+/CS- discrimination, results revealed significant main effects of phase, stimulus and study (all $F \geq 8.833$; all $p < .001$; all $\eta^2 \geq 0.119$). In addition, there was a significant phase × stimulus interaction (F (1, 131) = 8.586, $p = .004$, $\eta^2 = 0.062$), showing a decrease in responding to the CS+ ($p < .001$) but not to the CS- ($p = .085$) across phases, without any difference between studies (all $F \leq 1.961$; all $p \geq .145$; all $\eta^2 \leq 0.029$). Interestingly, the analysis of CS+/|GS45| differentiation revealed, in addition to the main effects of time, stimulus, and study (all $F \geq 7.002$; all $p \leq .001$; all $\eta^2 \geq 0.097$), a time × stimulus × study interaction (F (2, 131) = 3.079, $p = .049$, $\eta^2 = 0.045$; Figure 8). Following up on this interaction revealed that after fear acquisition, there was no statistically significant difference in CS+/|GS45| differentiation between studies (F (2, 131) = 0.978, $p = .379$, $\eta^2 = 0.015$), but a trend for such a difference after the test of fear generalization (F (2, 131) = 2.931, $p = .057$, $\eta^2 = 0.043$). After a 24-hr delay, participants did not differentiate between the CS+ and the most similar stimuli as strongly as participants did when tested immediately after fear acquisition. Statistically, this was supported by a strong trend for a difference between the two fMRI studies ($p = .051$). The other comparisons were non-significant (both $p \geq .421$).

Together, these results show that the broader fear generalization cannot be explained by a changed threat-safety discrimination but rather by a reduction in the discrimination between the threatening stimulus CS+ and the stimuli most similar to the CS+. Importantly, this reduction only occurs after a delay of 24 hr but is not found when the test of fear generalization follows immediately after the phase of fear acquisition.

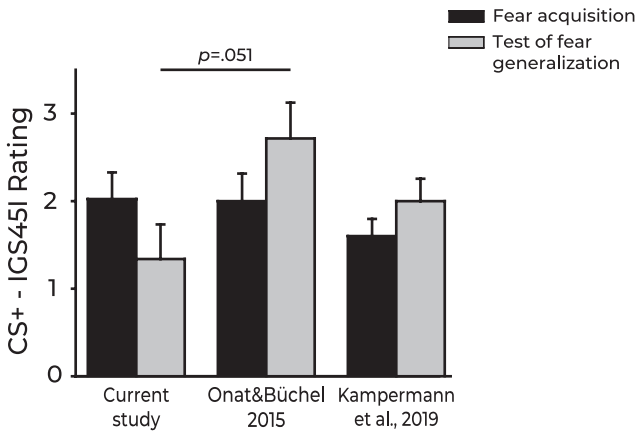

FIGURE 8 Difference in US-expectancy rating between the CS+ and the most similar stimuli, that is, |GS45|, from fear acquisition to test of fear generalization. Whereas there is no difference in CS+/|GS45| differentiation between studies during fear acquisition, the current study, which has a delayed test of fear generalization, shows a decreased differentiation strength during the test of fear generalization compared with studies having fear generalization immediately after fear acquisition

4 | DISCUSSION

Fear generalization is assumed to be a critical process in the development and maintenance of anxiety disorders (Lissek et al., 2005). While virtually all previous studies tested fear generalization shortly after fear acquisition, we investigated fear generalization and its neural underpinnings after a delay of 24 hr. Our findings showed intact fear memory and a pronounced fear generalization—both at the subjective, physiological, and neural level—after 24 hr. In addition, we determined the impact of acute stress on fear generalization. Although subjective and physiological parameters confirmed the successful stress induction, the generalization of fear was left largely unaffected by the stress manipulation. Moreover, a direct comparison of our findings to two previous studies using the same fear generalization paradigm but with the test phase presented immediately after fear acquisition (Kampermann et al., 2019; Onat & Büchel, 2015) revealed that the generalization of fear increased at the longer delay. This was reflected in a stronger responding to the stimuli most similar to the CS+.

Our neural data showed a fear-tuning profile indicative of fear generalization in the same brain regions that have been reported before during immediate fear generalization (Greenberg et al., 2013; Lissek et al., 2014; Onat & Büchel, 2015). In particular, the insula and the frontal operculum were associated with fear-signaling, showing the highest activation for the CS+, which declined for the other GSs with increasing dissimilarity to the CS+. In contrast, the vmPFC, hippocampal, middle frontal, and middle temporal regions rather reflected safety-signaling, that is, activation of these areas was associated with a strong deactivation toward the CS+, which declined with increasing similarity toward the safety-signaling CS-. It is important to note that we did not find fear-tuning in the amygdala in our whole-brain analyses. Given that the amygdala is a key structure in fear-learning processes (Büchel & Dolan, 2000), we also analyzed fear-tuning in the amygdala with an ROI-driven approach, obtaining an inversed fear-tuning curve. This is generally in line with a previous study that also did not observe significant fear-tuning in this region (Onat & Büchel, 2015). The amygdala's strong habituation effects (Breiter et al., 1996), deep location, and the fact that it is a relatively small brain structure that is difficult to image (Zald, 2003) make it particularly difficult to detect amygdala activity in a whole-brain FWE-corrected analysis. Importantly, when comparing the width of the neural fear-tuning to the previous findings observed immediately after fear acquisition (Onat & Büchel, 2015), results revealed wider neural fear-tuning curves.

How can the increased fear generalization after a delay of 24 hr be explained? One possibility might be a diminished fear memory in general. However, the CS+/CS- differentiation was comparable between the two testing days, same as

the threat/safety differentiation between an immediate and a delayed test of fear generalization, suggesting that fear memory was intact after 24 hr. In contrast to the CS+/CS- differentiation, the differentiation between the CS+ and the stimuli most similar to it changed over time. Interestingly, this differentiation also changed in the two studies that tested fear generalization immediately after fear acquisition, but in the direction of an increased differentiation, whereas we observed a diminished differentiation between the previously conditioned CS+ and the stimuli most similar to it. Support for the influence of time on a broader fear memory generalization comes from studies, suggesting that sleep plays an important role regarding a transformation process from a detailed to a more gist-like memory representation (Gais et al., 2007; Menz et al., 2013, 2016).

While the behavioral and neural fear generalization appeared to become broader after a 24-hr delay, it remained largely unaffected by stress. Acute stress shortly before the test of fear memory generalization did not alter fear memory expression or fear generalization, expressed as SCR, nor the neural underpinnings of fear generalization. This is in contrast to the only previous study that focused on the influence of acute stress prior to a test of fear generalization in humans (Dunsmoor, Otto, et al., 2017), which suggested that acute stress led to a heightened fear generalization. Previous rodent studies, however, also yielded inconsistent results. Whereas two studies found an increased fear generalization after corticosterone administration (Bender et al., 2018; Kaouane et al., 2012), another study failed to obtain any impact of corticosterone administration on the extent of fear generalization (Bueno et al., 2017). Importantly, there are some major differences between these studies and our study that may explain the different findings. First of all, the other human study used a different fear generalization paradigm (Dunsmoor, Otto, et al., 2017), using auditory stimuli instead of visual and socially relevant face stimuli. Furthermore, significantly fewer trials were administered during fear acquisition and fear generalization, with a higher reinforcement rate. Thus, fear learning was much more intense in the present study, which may have resulted in a reduced vulnerability to the stress manipulation. Moreover, while fear generalization was tested 15 to 30 min post-stress onset in the previous study, we conducted our test 60 to 95 min post-stress onset in the MRI scanner that could have resulted in a heightened arousal in general in both groups (Muehlhan et al., 2011). Our results support the idea that the environment of testing influences fear memory, specifically the amplitude of fear-tuning was heightened when participants were tested in the MRI scanner compared to outside. Together with the longer delay for testing after stress onset, this could have impeded a possible influence of stress on fear generalization to evolve. When comparing our study to the animal studies, there are crucial differences in timing and type of stress system manipulation. Most of the studies investigated the impact of corticosterone injections (Bueno et al., 2017; Kaouane et al., 2012), which is entirely different from a psychological stress manipulation which targets both the autonomic nervous system and the HPA axis and additionally increases subjective stress levels (Kirschbaum et al., 1993). Moreover, instead of manipulating stress system activity shortly before a test of fear generalization, these studies induced stress either before (Bender et al., 2018) or immediately after fear acquisition (Bueno et al., 2017; Kaouane et al., 2012). Thus, all of these studies affected the process of fear memory consolidation, which prevents a specific analysis of the impact of stress on fear memory generalization.

In line with a published review that highlights the role of conceptual knowledge for fear generalization (Dunsmoor & Murphy, 2015), Onat and Büchel (2015) suggested that fear generalization was not just passively driven by perception but was an active process, in which multiple source of information were integrated. They based their conclusion on the finding that the insula showed less generalization than behavioral responses and that the inferotemporal cortex, known to be implicated in perceptual processing, rather responded to uncertainty. However, the paradigm still included perceptually similar stimuli and we cannot fully rule out the possibility that a paradigm using higher order conditioning might have resulted in a different outcome. Furthermore, it is well known that sleep is highly relevant for the consolidation of memory (Diekelmann & Born, 2010), including fear memory (Pace-Schott et al., 2015). Thus, future studies that aim to investigate time-dependent changes in fear memory generalization should include measures of sleep quality and duration between acquisition and test sessions. Finally, it is to be noted that our explorative analysis of time-dependent changes in the magnitude and neural underpinnings of fear generalization was based on a comparison across separate studies, that is, without a random allocation of participants to experimental conditions (immediate vs. delayed test). Therefore, it cannot be fully ruled out that any differences between studies may have driven the seeming differences in fear generalization. Future studies that include explicit immediate and delayed test conditions are required to determine whether there are time-dependent changes in fear generalization.

In sum, we show that stress leaves 24 hr-delayed fear generalization and its neural signature largely unaffected. Furthermore, we provide first evidence suggesting that a delay of 24 hr results in a broader generalization of conditioned fear. This increase of fear generalization was reflected both in SCRs and the neural substrates of fear generalization. This finding may be highly relevant in the context of anxiety disorders, in which the threatening event typically dates back long in time. Based on our results, one might expect an even broader fear generalization in these long-established fear memories which may well contribute to the maintenance of the disorder. Identifying ways to interfere with old fears and strong fear generalization remains a challenge for future research.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical support by Carlo Hiller and the assistance of Hannah Biel, Beyza Karakaya, Judith Keemss, and Pavlina Lazaridou during data collection. Open access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Franziska Kausche: Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing-original draft; Writing-review & editing. **Gundula Zerbes:** Data curation; Investigation; Writing-review & editing. **Lea Kampermann:** Formal analysis; Resources; Validation; Writing-review & editing. **Christian Büchel:** Conceptualization; Resources; Software; Writing-review & editing. **Lars Schwabe:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing-original draft; Writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Supplementary Material

How to cite this article: Kausche, F. M., Zerbes, G., Kampermann, L., Büchel, C., & Schwabe, L. (2021). Neural signature of delayed fear generalization under stress. *Psychophysiology*, 58, e13917. https://doi.org/10.1111/psyp.13917