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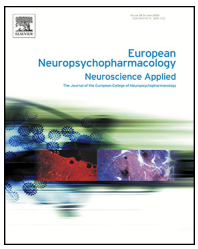

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Noradrenergic stimulation increases fear memory expression

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Abstract
Fear responses are typically not limited to the actual threatening stimulus but generalize to other stimuli resembling the threatening stimulus. Although this fear generalization is generally adaptive, fear overgeneralization is maladaptive and assumed to contribute to anxiety disorders. Despite the clinical relevance of fear (over)generalization, how the extent of fear generalization is modulated remains not well understood. Based on the known effects of stress on learning and memory, we tested here the impact of major stress mediators, glucocorticoids and noradrenergic arousal, on fear generalization. In a laboratory-based, placebo-controlled, double-blind, between-subject design, 125 healthy participants first underwent a fear conditioning procedure. About 24 h later, participants received orally either a placebo, hydrocortisone, the α2-adrenoceptor antagonist yohimbine, leading to increased noradrenergic stimulation, or both drugs before a test of fear generalization. Skin conductance responses as well as explicit rating data revealed that yohimbine intake led to enhanced fear memory expression, i.e. an enhanced responding to the CS+ but not to stimuli resembling the CS+. Moreover, neither enhanced safety learning nor a mere enhancement of perceptual discrimination ability could explain this result. In contrast to yohimbine, hydrocortisone had no significant effect on fear memory. These findings suggest that noradrenergic arousal strengthens fear memory expression and have important implications for mental disorders in which the overgeneralization of conditioned fear is prominent.
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1. Introduction

Learning to fear potentially dangerous stimuli is highly adaptive as it helps to prevent future harm to the organism. Because threatening stimuli rarely occur in the exact same form across experiences, the generalization of fear is an important mechanism that helps us to deal with complexity (Shepard, 1987). However, an exaggerated generalization of fear to stimuli not predicting danger, i.e. fear overgeneralization, is maladaptive and may contribute to anxiety disorders or post-traumatic stress disorder (PTSD) (Dunsmoor and Paz, 2015; Lissek, 2012).

Although fear generalization is a fundamental process with important clinical implications, it is largely unclear how the extent of fear generalization is modulated. Stressful events are known to be a major modulator of learning and memory (Diamond et al., 2007; Joels et al., 2006; Quaedflieg and Schwabe, 2018; Sandi and Pinelo-Nava, 2007; Schwabe et al., 2010), including fear learning processes (Merz et al., 2016; Raio and Phelps, 2015; Simon-Kutscher et al., 2019). For instance, there is evidence that acute stress may alter fear acquisition and extinction (Jackson et al., 2006; Raio et al., 2014). Moreover, stress hormones are known to act on prefrontal and medial-temporal areas, including the amygdala, the hippocampus and the ventromedial prefrontal cortex (Joels and Baram, 2009), which are critically involved in fear generalization (Dunsmoor et al., 2011; Lissek et al., 2014; Onat and Büchel, 2015). There is initial evidence suggesting that stress may affect fear generalization processes, both in animals and in humans (Bender et al., 2018; Dunsmoor et al., 2017; Kaouane et al., 2012). However, the mechanisms involved in the impact of stress on fear generalization remain poorly understood.

The exposure to stressful events initiates a cascade of physiological changes, including the release of numerous hormones, neurotransmitters and peptides (Joels and Baram, 2009). In particular, noradrenaline and glucocorticoids are known to play key roles in the modulation of learning and memory processes (Joels and Baram, 2009; Roozendaal et al., 2006). Several studies revealed that noradrenergic arousal and glucocorticoids may act synergistically to influence learning and memory (Joels et al., 2011; Krugers et al., 2012; Roozendaal et al., 2006; Schwabe et al., 2012). In addition, there is evidence that suggests that glucocorticoids - acting in concert with noradrenergic arousal - may strengthen the noradrenergic effects (Buchanan and Lovallo, 2001; Roozendaal, 2002). On the contrary, however, there is also evidence for distinct roles of noradrenaline and glucocorticoids. For instance, glucocorticoids are known to impair memory retrieval (Cai et al., 2006; de Quervain et al., 1998, 2000) and the sensory reinstatement during a memory test (Gagnon et al., 2019) which may thus result in a less specific, more generalized fear memory. At the same time noradrenergic arousal may even facilitate certain retrieval processes (Murchison et al., 2004; Schönfeld et al., 2014), thereby preventing generalization processes. Furthermore, glucocorticoids after encoding enhance memory strength, while noradrenergic stimulation facilitates the long-term specificity of memory (Atucha et al., 2017).

This experiment aimed to investigate the impact of glucocorticoids and noradrenergic arousal on fear generalization in humans. Therefore, healthy participants underwent a differential fear-conditioning procedure on Day 1, in which one stimulus was followed by a shock (CS+), while another stimulus was never followed by a shock (CS-). Twenty-four hours later, participants received either a placebo, 20 mg hydrocortisone, 20 mg of the α2-adrenoceptor antagonist yohimbine, leading to increased noradrenergic stimulation, or both drugs before a test of fear generalization (Onat and Büchel, 2015). The distribution of fear acquisition and generalization over two days allowed us to isolate drug effects on fear memory generalization, while ruling out influences on fear acquisition and early consolidation processes.

We hypothesized that glucocorticoids and noradrenergic stimulation would exert opposite effects, resulting in enhanced fear generalization after hydrocortisone intake, represented by a wider fear-tuning function, but enhanced fear memory specificity after yohimbine intake, mirrored by an increased amplitude of the Gaussian function. Regarding the concurrent administration of hydrocortisone and yohimbine, it was hypothesized that both drugs might lead to an even further reduction in fear generalization than yohimbine alone. However, given the differential effects we expected after the administration of either drug alone, this hypothesis was more speculative.

2. Experimental procedures

2.1. Participants and experimental design

One-hundred-thirty-six healthy volunteers (68 women, age: $M = 25.41$ years, *SEM*=0.36 years) without a history of any mental or neurological disorder, current medication intake, drug or tobacco use participated in this experiment. This sample size was based on an a-priori power analysis using G*Power 3.1 [28] showing that 136 participants are sufficient to detect a medium-sized effect of $f = 0.25$ with a power of 0.95. Women were not tested during their menses and those taking hormonal contraceptives were excluded from participation. All participants provided written informed consent before taking part in the experiment and received a compensation of 60€ for study participation. The study protocol was approved by the ethics committee of the State Chamber of Physicians Hamburg and in accordance with the Declaration of Helsinki.

In a double-blind, placebo-controlled, fully crossed, between-subject design with the factors hydrocortisone (yes/no) and yohimbine (yes/no) administration, participants were pseudo-randomly assigned to one of four experimental groups: placebo (20 mg; PLAC), hydrocortisone (20 mg; CORT), yohimbine (20 mg; YOH), and hydrocortisone+yohimbine (20 mg each; CORT+YOH). To ensure full blindness, every participant received four pills that were not distinguishable. Two participants had to be excluded because of data loss during acquisition on experimental Day 1. In addition, nine participants had to be excluded from the analyses because they did not show successful (explicit) fear acquisition on Day 1 (i.e. their US-expectancy rating was not higher for the CS+ than for the CS-), leaving a final sample of 125 participants (PLAC: $n = 31$, 16 women; age: $M = 25.29$ years, SEM=0.87 years; CORT: $n = 31$, 15 women; age: $M = 24.84$ years, SEM=0.69 years; YOH: $n = 34$, 17 women; age: $M = 25.15$ years, SEM=0.71 years; CORT+YOH: $n = 29$, 15 women; age: $M = 25.62$ years, SEM=0.72 years).

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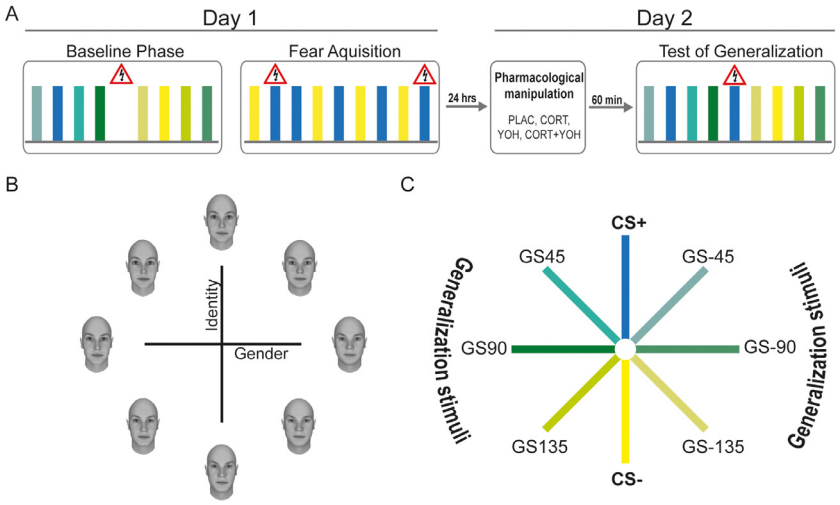

Fig. 1 Fear generalization paradigm and stimulus organization. (A) Fear generalization paradigm with three phases. The baseline and fear acquisition phases took place on Day 1, the test phase on Day 2, after the pharmacological manipulation. During the baseline phase, the complete set of stimuli (represented by colored bars) was shown to the participants and US were signaled by a shock symbol. During the fear acquisition phase, just two stimuli from opposite sides of the circular similarity continuum were shown to the participants. These stimuli represented one pair of the most dissimilar faces and were used as CS+ and CS-, respectively. During fear acquisition, the CS+ was followed by the US in ~23% of the trials. During the test phase, the complete set of faces was shown to the participants again. To avoid extinction, there was a reinforcement rate of ~23% for the CS+. (B and C) There were 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).

2.2. General procedure and measurements

All testing took place between 1:00pm and 7:00pm on two consecutive days. On both experimental days, saliva samples were collected repeatedly using Salivette® collection devices (Sarstedt, Germany) and stored immediately after testing at −18 °C (−0.4°F). At the end of data collection, free cortisol and alpha-amylase concentrations were analyzed from saliva with a luminescence immunoassay and enzyme assay, respectively (IBL-International, Hamburg, Germany). In addition, systolic and diastolic blood pressure were obtained using a Critikon Dinamap system (Tampa, Fl, USA), with a cuff placed on the right upper arm. Potential changes in subjective mood were tracked on both testing days with a German version of the Positive and Negative Affect Schedule (PANAS; Krohne et al., 1996).

2.2.1. Day 1 - baseline phase and fear acquisition

Upon participants' arrival at the lab, baseline measurements of vital signs (i.e. systolic and diastolic blood pressure) and saliva samples were taken. Afterwards an electrode for the electrical stimulation, serving as unconditioned stimulus (US), was placed on participants' back of the right hand. Further, two electrodes for skin conductance recordings were attached to the left hand. Then, the individual pain threshold was determined using the QUEST procedure (Watson and Pelli, 1983). On a scale from 1 (no pain) to 10 (worst pain possible), participants were asked to indicate the shock intensity and we aimed to obtain a shock intensity that was unpleasant but not painful, represented by a score of 5 on the scale.

Next, the baseline phase of the fear generalization paradigm started (Fig. 1A). The paradigm contained eight face stimuli (500 × 500 pixels) arranged on a circular similarity continuum with two axes (x-axis: gender; y-axis: identity; Fig. 1B; (Onat and Büchel, 2015). The stimuli were always presented for 1.5 s. The face stimulus chosen as CS+ was counterbalanced across subjects and groups. All other faces were quantified in their distance to the CS+ on the circular similarity continuum. By having eight stimuli in a circular arrangement, this resulted in a quantification of 45° between each stimulus. The two faces opposite to each other (i.e. 180°) represented the most dissimilar faces and were used as CS+ and CS-. The faces in between represented the generalization stimuli (GS), whereby the most similar GS to the CS+ were positioned 45° next to the CS+ and the most dissimilar GS were positioned 135° away from the CS+ (Fig. 1C). During the baseline phase, the complete set of faces was shown to the participants, to control for any a priori differences between the faces. The same number of electric shocks (i.e. 10 shock trials) was administered as in the other phases to maintain a comparable arousal due to electrical stimulation throughout the task. Participants were informed that the US was always signalized by a shock symbol. This was done to ensure full predictability and prevent any association of the shock with any of the faces. Additionally, participants were asked to respond to 10 trials of oddball targets, i.e. faces with artificially added freckles. These oddball trials occurred without prior notice and served to control for attention. In total, there were 293 trials (~29 min).

During the fear acquisition phase, only two faces i.e. the most dissimilar faces, were presented. In ~23% of the trials, one face (CS+) was followed by a shock (US), resulting in 45 unreinforced CS+ trials, that later entered our analyses, whereas the other face (CS-; 44 trials) was never paired with the US. In contrast to the baseline phase, participants were informed that during this phase the US will always follow a certain face. Same as in the baseline phase, 10 oddball trials were presented to keep participants attentive. In total, there were 123 trials (~15 min). After both phases, US-expectancy ratings were assessed to measure explicit fear learning. For this purpose, we presented each face stimulus two times in randomized order and asked participants to rate for each stimulus their subjective shock expectancy using a visual analogue scale ranging from 1 (certain, no shock) to 10 (certain, shock).

At the end of Day 1 testing, pain strength rating as well as vital signs were measured again and another saliva sample was taken.

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2.2.2. Day 2 - pharmacological manipulation and test phase

At the beginning of Day 2, participants completed the State-Trait Anxiety Inventory (STAI-S; Spielberger and Syndeman, 1994) to check for differences in subjective state anxiety. Depending on the experimental condition, participants then received orally either a placebo, 20 mg of hydrocortisone, 20 mg of yohimbine or 20 mg of both drugs. Timing as well as dosage of the drug administration were chosen in accordance with previous studies (Kluen et al., 2017; Schwabe et al., 2012). Saliva samples and vital signs were collected at several time points: before drug administration, 45 min after drug intake, 60 min after drug intake, i.e. immediately before the test phase of the fear generalization paradigm, 90 min after drug intake, i.e. after the test phase, and 120 min after drug intake at the end of testing.

During a waiting period of 60 min, participants completed several questionnaires assessing control variables of interest (depression, Beck Depression Inventory (BDI-II; Beck et al., 1996); trait anxiety, STAI-T (Spielberger and Syndeman, 1994); and chronic stress, Trier Inventory for the Assessment of Chronic Stress (TICS; Schulz and Schlotz, 1999)) as well as an unrelated, non-arousing task (Zerbes et al., 2019). Then, the individual pain threshold was determined again, using the same procedure as on Day 1, before the critical fear generalization test started. In the fear generalization phase, the complete set of faces was shown to the participants again. Every face was shown ~34 times, except for the CS+ which was shown ~44 times. This was realized because only unreinforced CS+ trials later entered analysis and the US followed the presentation of the CS+ in ~23% of the trials to avoid extinction learning to the CS+. Again, 10 oddball trials were presented. Same as the baseline phase, this phase contained 293 trials (~29 min). At the end of the fear generalization phase, US-expectancy ratings were collected using the same procedure as on Day 1.

After these ratings, all of the eight face stimuli were presented to the participants as shown in Fig. 1B but in a randomized circular arrangement and participants had to indicate which of the faces was followed by the shock. Participants had to use the arrow keys to navigate around the circle and confirm their selection with the space bar. This task was self-paced.

Finally, a perceptual discrimination task was presented to assess participants' perceptual discrimination ability. In this task, participants were presented two faces one after another, each for 1.5 s and were asked to rate the faces as being the same or different. There was no time limit for the response but participants were instructed to decide quickly. Participants could use the arrow keys to select the "same" or "different" button and had to confirm their choice using the space bar. In total, the discrimination task consisted of 192 trials (each of the eight face stimuli was shown 24 times) and lasted for about 30 min.

At the end of the experiment, participants were asked to indicate which treatment they thought they received (i.e. placebo, hydrocortisone, yohimbine, both drugs or any drug) to check for successful blinding. They were then debriefed and compensated for participation.

2.3. Electrodermal stimulation and SCR analysis

The US consisted of trains of 5-ms electrical pulses at 66 Hz lasting in total 100 ms, co-terminating with the shock symbol or the face stimulus and applied via a constant voltage stimulator (STM200, BIOPAC Systems, Goleta USA) with a surface bar electrode. Electrodermal activity was recorded from the distal phalanx of the index and middle fingers of the left hand, using two 8 mm Ag/AgCl electrodes, connected to the MP-150 BIOPAC System (BIOPAC Systems, Goleta USA), assessed according to common guidelines (Boucsein et al., 2012). A deconvolution technique as implemented in Ledalab version 3.4.9 (Benedek and Kaernbach, 2010) was used to divide raw skin conductance recordings into the slowly varying tonic activity, i.e. skin conductance level, and a rather fast varying phasic activity, i.e. skin conductance responses (SCRs). As part of the procedure, skin conductance data were downsampled to a resolution of 20 Hz and optimized using four sets of initial values. The optimization procedure was used to find the best starting point for the deconvolution. To obtain the anticipatory SCRs, we derived the average phasic driver within a response window from 1 s to 4 s after stimulus onset. By setting the minimum amplitude threshold to 0.01μS, we controlled for non-responding on a trial-by-trial level. As such, trials with an amplitude smaller than 0.01μS were set to 0 and were not included when averaging the SCR. To correct for inter-individual differences, SCRs were z-transformed (zSCRs) separately for the three different phases (Ben-Shakhar, 1985). Because US- trials and CS+ trials in which a shock was presented did not enter further analyses, we excluded these trials before z-transformation. We then calculated the responses associated with the onset of individual faces at a single subject level. Finally, responses to the different stimuli were averaged and single subject fear-tuning profiles for each phase were derived (Onat and Büchel, 2015).

2.4. Analysis of fear-tuning profiles

Individual fear-tuning profiles were analyzed using MATLAB (Release 2016b, Natick, MA). To characterize the fear-tuning, a Gaussian model with two parameters (α, amplitude, i.e. the strength of fear memory specificity or expression; σ, tuning width (full width at half maximum), i.e. the strength of fear generalization) was used. We restricted our Gaussian model to be centered on the CS+-face. Fear-tuning profiles were calculated for zSCR and rating data separately. For further statistical analyses, we extracted the two parameters of each profile (Onat and Büchel, 2015).

2.5. Statistical data analysis

Statistical analyses were performed with SPSS 25.0 (IBM). To ensure that groups had not baseline differences, Day 1 data were subjected to ANOVAs with the between-subjects factor group with four levels (PLAC, CORT, YOH, CORT+YOH). As within-subject factor, we used time (start vs. end), face-number (eight levels) and stimulus (CS+ vs. CS-). Day 2 data were analyzed by means of mixed-design ANOVAs with time (five levels) and stimulus (eight levels) as a within-subject factor and hydrocortisone (yes/no) and yohimbine (yes/no) administration as between-subject factor, in order to analyze the main effect of each drug separately as well as a drug interaction effect. To explicitly compare responding to the CS+ and the most similar GS, we averaged responding of these GS and calculated the variable |GS45|. To analyze the perceptual discrimination ability, we calculated a discrimination score by subtracting the mean false alarm rate from the mean hit rate. To avoid extinction learning during fear generalization test, participants still received the US in ~23% of the trials. To investigate, if this reinforcement had an impact on fear generalization on Day 2, we calculated reinforcement bins by counting the number of trials between the US and the different GSs and CS-. We calculated the mean and grouped the time after US occurrence in three percentiles, whereby we obtained four bins: before any US had occurred, 1-11 trials after US occurrence, 12-25 trials after US occurrence, and >25 trials after US occurrence. We then performed a fear-tuning analysis for the stimuli dependent on the time of US occurrence and extracted the same parameter as for the general fear-tuning. As such, our independent variables were the between-factors group (Day 1 and baseline Day 2 analyses), hydrocortisone and yohimbine (Day 2 analyses) and the within-subject factors time, face-number and stimulus. Our

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Table 1 Physiological, endocrine, and subjective response to the pharmacological manipulation.

Variable	PLAC	CORT	YOH	CORT+YOH
Day 1				
Salivary cortisol (nmol/L)	4.00 (0.77)	4.94 (0.77)	4.55 (0.74)	5.09 (0.80)
Alpha-Amylase (U/ml)	99.48 (17.68)	108.18 (17.77)	132.39 (16.60)	106.51 (17.98)
Systolic BP (mmHG)	124.08 (2.57)	125.24 (2.57)	126.15 (2.45)	123.98 (2.65)
Diastolic BP (mmHG)	69.95 (1.82)	69.37 (1.82)	70.69 (1.74)	72.78 (1.89)
Negative affect				
Day 1	1.23 (0.05)	1.28 (0.05)	1.26 (0.05)	1.25 (0.06)
Day 2 baseline	1.20 (0.06)	1.16 (0.06)	1.20 (0.06)	1.26 (0.06)
Day 2 45 min post drug	1.14 (0.04)	1.11 (0.04)	1.15 (0.04)	1.15 (0.05)
Day 2 60 min post drug	1.14 (0.04)	1.11 (0.04)	1.14 (0.04)	1.17 (0.05)
Day 2 90 min post drug	1.17 (0.07)	1.27 (0.07)	1.24 (0.07)	1.26 (0.08)
Day 2 120 min post drug	1.06 (0.05)*	1.14 (0.05)	1.14 (0.04)	1.12 (0.05)•
Positive affect				
Day 1	2.70 (0.10)	2.71 (0.10)	2.80 (0.09)	2.72 (0.10)
Day 2 baseline	2.67 (0.11)	2.81 (0.11)	2.84 (0.11)	2.66 (0.12)
Day 2 45 min post drug	2.43 (0.12)**	2.51 (0.11)**	2.64 (0.11)*	2.41 (0.12)*
Day 2 60 min post drug	2.38 (0.12)***	2.49 (0.12)**	2.63 (0.12)	2.36 (0.13)**
Day 2 90 min post drug	2.03 (0.12)***	2.13 (0.12)***	2.38 (0.11)***	2.31 (0.13)**
Day 2 120 min post drug	2.19 (0.12)***	2.26 (0.11)***	2.31 (0.11)***	2.30 (0.12)**
Pain Threshold				
Day 1	38.53 (2.23)	46.00 (2.23)	43.73 (2.13)	42.06 (2.30)
Day 2	41.40 (1.99)	45.68 (1.99)	45.93 (1.90)	45.40 (2.06)

The table presents physiological, endocrine, and subjective responses before testing on Day 1 as well as the change over time in response to the pharmacological manipulation on Day 2. Groups did not differ in any of the measurements on Day 1 or before pill intake on Day 2. However, there were significant changes in all of the measurements in response to the pharmacological manipulation, thus confirming the action of the drugs. Data represent mean (standard error). Asterisks denote difference to Day 2 baseline: •$p < .1$, *$p < .05$, **$p < .01$, ***$p < .001$.

dependent variables were zSCR and US-expectancy rating data and the fear-tuning parameters of these data.

All reported p-values are two-tailed, using an α-error threshold of p=.05. Significant main or interaction effects were pursued using post-hoc planned comparisons, with Sidak correction if indicated. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied.

3. Results

3.1. Day 1

Before the beginning of the baseline phase, the four groups differed neither in their subjective mood, physiological markers such as cortisol, alpha-amylase, systolic or diastolic blood pressure, nor in their estimated pain threshold (all $Fs \leq .749$; all $ps \geq .544$; all $\eta^2 s \leq .018$; Table 1). With respect to the pain strength rating, there was a significant time effect ($F(1113)=22.23$, $p<.001$, $\eta^2=0.164$), indicating that participants rated the US as less painful at the end of testing compared to before testing, without differences between groups (main effect of group and time × group interaction: both $Fs \leq 1.834$; both $ps \geq .145$; both $\eta^2 s \leq .046$).

3.1.1. Baseline phase

During the baseline phase, there were no main effects of group or group × face-number interaction effects, neither for the zSCR data nor for the rating data. However, for both measurements there was a face-number main effect (zSCR: $F(7847)=5.340$, $p<.001$, $\eta^2=0.042$; rating data: $F(3.097,374.724)=3.831$, $p=.009$, $\eta^2=0.031$; Fig. 2A and B). Regarding the rating data, no post-hoc comparison for individual faces reached statistical significance (all $ps>0.067$), for zSCR data face 3 elicited higher SCR compared to face 4 and face 6 (both $ps<0.002$) and face 7 elicited higher SCR compared to face 8 ($p=.017$). However, since we counterbalanced CS+ and CS- assignment across subjects and groups, the influence of this difference on our conditioning data should be negligible.

3.1.2. Successful fear acquisition

As expected, the results of the fear acquisition phase revealed a significantly higher responding to the CS+ compared to the CS-, indicated by a higher zSCR ($F(1120)=14.583$, $p<.001$, $\eta^2=0.108$) as well as a higher US-expectancy rating for the CS+ ($F(1121)=719.459$, $p<.001$, $\eta^2=0.856$). There were no differences between groups (all $Fs \leq 1.466$; all $ps \geq .227$; all $\eta^2 s \leq .035$; Fig. 2C and D).

3.2. Day 2

We obtained no group differences regarding depressive mood, chronic stress, or state anxiety (all $Fs \leq 1.134$; all $ps \geq .338$; all $\eta^2 s \leq .027$; Table 2). There was a trending group difference in trait anxiety scores ($F(3121)=2.560$, $p=.058$,

Table 2 Subjective assessments of depressive mood, chronic stress and anxiety.

Variable	PLAC	CORT	YOH	CORT+YOH
Depressive score (BDI-II)	5.07 (0.94)	5.52 (0.94)	7.12 (0.89)	5.45 (0.97)
State anxiety (STAI-S)	2.39 (0.03)	2.42 (0.03)	2.42 (0.03)	2.46 (0.03)
Trait anxiety (STAI-T)	2.02 (0.05)	2.03 (0.05)	2.19 (0.05)	2.06 (0.05)
Subjective chronic stress (TICS)	63.03 (5.72)	71.36 (5.72)	76.88 (5.47)	66.83 (5.92)

Subjective assessments of depressive mood, chronic stress and anxiety through various questionnaires reveal low levels in all of the measures and no differences between the four groups. Data represent mean (standard error).

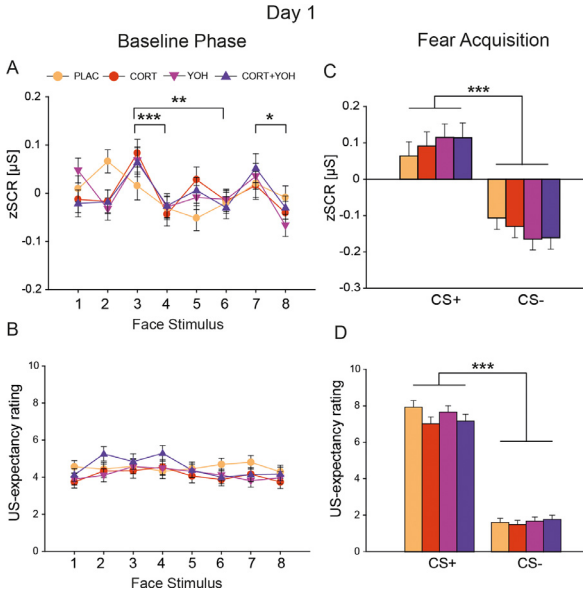

Fig. 2 Day 1: Physiological and subjective responses to the face stimuli during the baseline and fear acquisition phases. (A) zSCR data as well as (B) explicit rating data showed no systematic a priori differences between faces and no group differences during or after baseline phase. During and after fear acquisition, both (C) zSCR as well as (D) explicit US-expectancy rating data showed successful fear learning reflected in higher response to the CS+ than to the CS-. Error bars represent standard errors off the mean. Asterisks denote differences between stimuli ($^{*}p < .05$, $^{**}p < .005$, $^{***}p < .001$).

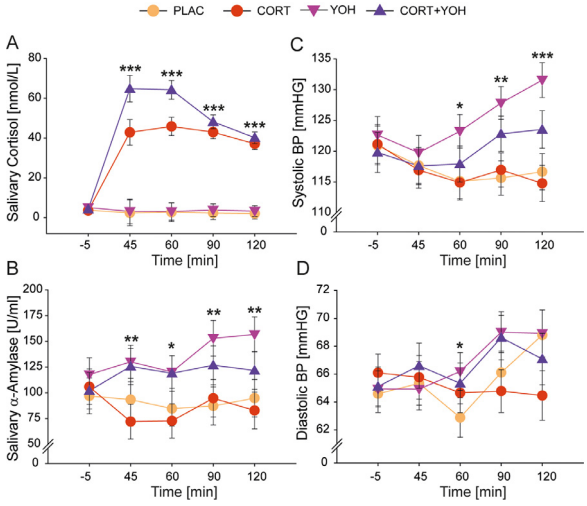

Fig. 3 Pharmacological manipulation check. (A) Salivary cortisol increase. (B) Salivary alpha-amylase increase. (C) Systolic blood pressure increase. (D) Diastolic blood pressure increase. Error bars represent standard errors or the mean. Asterisks denote difference between factors either hydrocortisone (yes/no) for salivary cortisol or yohimbine (yes/no) for alpha-amylase, systolic and diastolic blood pressure. ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$).

η^2=0.060). Sidak post-hoc tests indicated no significant differences (all *ps*≥.104). To ensure that trait anxiety did not modulate our results, we re-analyzed our data including the STAI-T score as a covariate, which had, however, no significant influence on our main findings. Furthermore, groups did not differ in subjective mood, cortisol, alpha-amylase, systolic or diastolic blood pressure at the beginning of Day 2, i.e. before pill intake (all *Fs*≤1.473; all *ps*≥.225; all η^2*s*≤.035; Table 1 and Fig. 3). Regarding their pain threshold, participants had in general a slightly higher pain threshold on Day 2 compared to Day 1 ($F(1121)=6.672$, $p=.011$, $\eta^2=0.052$), but rated the US as less painful ($F(1113)=12.585$, $p=.001$, $\eta^2=0.100$). Importantly however, this change from Day 1 to Day 2 was not influenced by group (all $F \leq 1.726$; all $p \leq .165$; all $\eta^2 \leq .041$). With respect to the pain strength rating only on Day 2, there was a trend for a main effect of time ($F(1114)=2.929$, $p=.090$, $\eta^2=0.025$), suggesting that groups rated the US as slightly more painful at the end of testing compared to before testing. This was not affected by our manipulation (all interactions including hydrocortisone and yohimbine: all *Fs*≤.183; all *ps*≥.670; all η^2*s*≤.001).

3.2.1. Manipulation check

Before the beginning of the fear acquisition phase, groups did not differ in subjective mood, salivary cortisol, salivary alpha-amylase and systolic or diastolic blood pressure (Table 1 and Fig. 3). Significant changes in salivary cortisol, alpha-amylase and blood pressure confirmed the action of the drugs (Fig. 3). In an ANOVA with the factors hydrocortisone, yohimbine and time point of measurement, the effectiveness of cortisol was shown by a significant time × hydrocortisone interaction effect ($F(1.542,186.602)=42.320$, $p<.001$, $\eta^2=0.259$). Post-hoc tests verified a significant increase in salivary cortisol from baseline to after hydrocortisone administration for the participants receiving hydrocortisone ($F(1.592,88.707)=36.977$, $p<.001$, $\eta^2=0.389$), whereas participants who had not received hydrocortisone

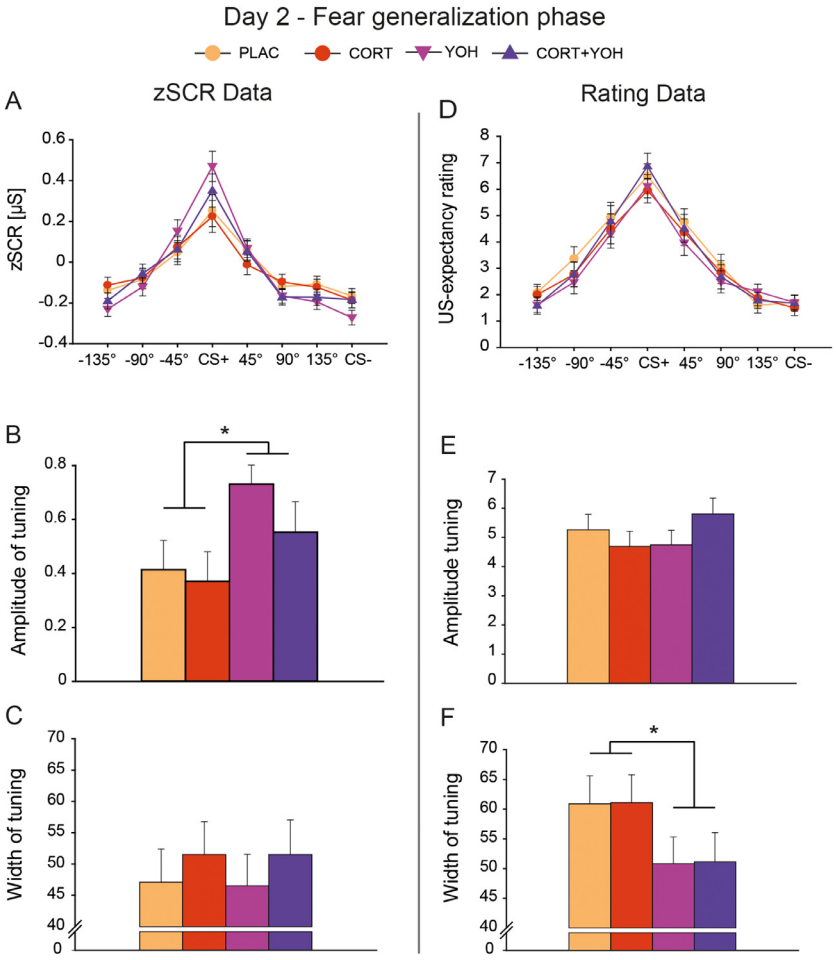

Fig. 4 Day 2: Fear generalization phase. (A) Overview of zSCR to different stimuli during the test phase. Yohimbine administration effects (B) the amplitude of Gaussian model for zSCR data as the amplitude increases. (C) There was no significant influence of yohimbine or hydrocortisone administration for the width of zSCR fear-tuning. (D) Overview of US-expectancy rating to different stimuli after the test phase. (E) There seems to be no effect of yohimbine or hydrocortisone administration on the amplitude of Gaussian model. (F) However, there is a significant influence of yohimbine administration for the width of fear-tuning, as after yohimbine administration the fear-tuning profile gets narrower. Asterisks denote difference between groups ($^*p < .05$).

even showed a decrease ($F(84.565,98.850)=6.884$, $p<.001$, $\eta^2=0.099$) for all time points after drug administration (Fig. 3).

Conversely, yohimbine administration led to significant increases in alpha-amylase as well as in systolic and diastolic blood pressure (time × yohimbine interactions: all $Fs\geq 2.887$; all $ps\leq .042$; all $\eta^2s\geq .023$). This increase was significant for participants receiving yohimbine across all variables (all $Fs\geq 3.341$; all $ps\leq .018$; all $\eta^2s\geq .052$), whereas there was a decrease for participants not receiving yohimbine (all $Fs\geq 2.046$; all $ps\leq .130$; all $\eta^2s\geq .033$; Fig. 3). For diastolic blood pressure, there was additionally a significant time × hydrocortisone interaction ($F(2.639.319.291)=3.989$, $p=.011$, $\eta^2=0.032$) and time × hydrocortisone × yohimbine interaction, which were, however, driven by the PLAC group ($F(1.764,52.917)=3.095$, $p=.060$, $\eta^2=0.094$), without a significant effect for the CORT group ($F(2.988,89.634)=1.025$, $p=.385$, $\eta^2=0.033$). In addition, participants were not aware of the administered drug. The majority (71%) guessed that they had received a placebo, without any difference between the four groups ($\chi^2(3)=3.687$, $p=.297$, Cramer's $V = 0.229$).

3.2.2. Fear generalization phase: noradrenergic arousal boosts fear memory expression

When comparing the fear-tuning parameters for the zSCR data (Fig. 4A), results showed that yohimbine increased the fear memory expression. This was indicated by a higher amplitude of the Gaussian model in both yohimbine groups, i.e. YOH and CORT+YOH compared to the groups that did not receive yohimbine, i.e. PLAC and CORT ($F(1120)=5.677$, $p=.019$, $\eta^2=0.045$; all other main or interaction effects $p\geq .374$; Fig. 4B). The strength of fear generalization, as reflected in the model width parameter, was not significantly altered by yohimbine or hydrocortisone (all main or interaction effects: all $Fs\leq .789$; all $ps\geq .376$; all $\eta^2s\leq .007$; Fig. 4C). To confirm that the yohimbine effect can be explicitly attributed to a higher responding towards the CS+ and not the similar GSs, we compared by means of an rmANOVA the

influence of our manipulation on responding to the CS+ and |GS45|. Besides a main effect of stimulus ($F(1120)=65.872$, $p<.001$, $\eta^2=0.354$) and yohimbine ($F(1120)=4.963$, $p=.028$, $\eta^2=0.040$), results showed a significant yohimbine × stimulus interaction ($F(1120)=4.287$, $p=.041$, $\eta^2=0.034$). Post-hoc t-tests confirmed that the administration of yohimbine resulted in a higher responding towards the threatening CS+ ($t(122)=-2.384$, $p=.019$, $d = 0.428$) but not the similar GSs ($t(122)=-1.427$, $p=.156$, $d = 0.256$).

Similarly, yohimbine intake led to a more specific US-expectancy rating to the CS+ (Fig. 4D). Specifically, the US-expectancy data showed a significant yohimbine effect for the width of rating data with a narrower fear-tuning curve after yohimbine intake (YOH and CORT+YOH groups) compared to groups that received no yohimbine (PLAC and CORT groups; $F(1120)=4.537$, $p=.035$, $\eta^2=0.036$; Fig. 4F). For the amplitude there were no significant effects in the subjective US-expectancy ratings (all $Fs\leq2.476$; all $ps\geq.118$; all $\eta^2s\leq.020$; Fig. 4E).

3.2.3. Noradrenergic arousal supports fear memory expression rather than safety learning

In order to test whether noradrenergic stimulation increased specifically the responding to the CS+ or safety learning, reflected in a reduced responding to the CS-, we analyzed in a next step the CS+/CS- differentiation. The results revealed a significant stimulus × yohimbine interaction ($F(1120)=4.637$, $p=.033$, $\eta^2=0.037$; without any main or interaction effects of cortisol: all $ps\geq.361$). Separate post-hoc analyses for each stimulus showed that yohimbine administration compared to no yohimbine administration specifically increased responding to the CS+ ($F(1)=5.308$, $p=.023$, $\eta^2=0.042$), without influencing the responding to the safety stimulus CS⁻ ($F(1)=2.004$, $p=.159$, $\eta^2=0.016$).

For the subjective rating data, there was a stimulus main effect ($F(1120)=297.925$, $p<.001$, $\eta^2=0.713$), but no interaction effect with yohimbine or hydrocortisone (all $Fs\leq1.867$; all $ps\geq 0.174$; all $\eta^2s\leq.015$).

3.2.4. Noradrenergic arousal boosts fear memory expression independent of the temporal distance to the threatening stimulus

To investigate how US presentation in the test phase affected fear generalization, we calculated fear-tuning curves for reinforcement bins, reflecting the distance to the last US, and applied Gaussian fear-tuning to these data (Fig. 5). The analyses of the Gaussian model parameters revealed again that the administration of yohimbine led to higher amplitudes independent of the number of elapsed trials after a US ($F(1120)=4.937$, $p=.028$, $\eta^2=0.040$), indicating once more increased fear memory expression. In contrast to yohimbine, the administration of hydrocortisone had no effects on amplitude or width (all main or interaction effects $ps\leq.129$).

3.2.5. Increased fear memory expression after noradrenergic stimulation cannot be attributed to enhanced attention or perceptual discrimination of fear-related cues

To ensure that the effect of yohimbine cannot be explained by merely enhanced attention, we analyzed responses to the oddball targets that were presented during all phases.

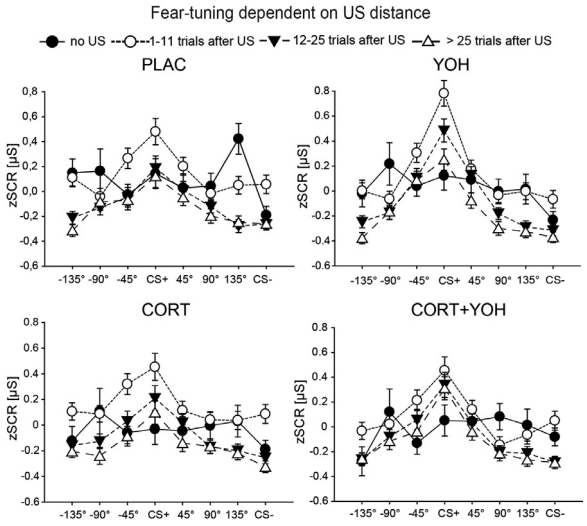

Fig. 5 Fear-tuning dependent on US distance. Descriptively, the fear-tuning curves support the statistical findings, as with the YOH Group, the strongest reaction to the CS+ can be seen, independent of time after US occurrence.

In general, participants were very attentive and reacted correctly in 98.47% of the trials, without any influence of hydrocortisone or yohimbine (all $Fs\leq1.358$; all $ps\geq.246$; all $\eta^2s\leq.011$).

Although groups did not differ in their ability to correctly identify the CS+ as assessed by the presentation of all eight faces at the end of Day 2 (both $Fs\leq1.480$; both $ps\geq.226$; both $\eta^2s\leq.012$), testing the discrimination ability between faces, results revealed an improved perceptual discrimination after yohimbine administration ($F(1121)=4.590$, $p=.032$, $\eta^2=0.037$).

To test whether the effects of noradrenergic stimulation on the expression of fear memory were due to the enhanced perceptual discrimination ability or whether the yohimbine effect goes beyond the mere discrimination ability, we re-run our analyses including the discrimination score as covariate. These analyses showed that taking the discrimination ability into account left our results largely unaffected. In particular, the yohimbine effect on the amplitude remained significant after controlling for differences in perceptual discrimination ability ($F(1119)=4.012$, $p=.049$, $\eta^2=0.034$). When directly comparing the reaction to the CS+ and CS-, there is still a trend for a stimulus × yohimbine interaction ($F(1119)=2.914$, $p=.090$, $\eta^2=0.024$). For the reinforcement bins analysis, the significant yohimbine main effect for the amplitude remains on a trend level ($F(1119)=3.316$, $p=.071$, $\eta^2=0.027$).

4. Discussion

Whereas the generalization of fear is crucial to avoid harm to the organism, fear overgeneralization is maladaptive and may contribute to anxiety disorders and PTSD (Dunsmoor and Paz, 2015; Lissek, 2012). We tested here, to the best of our knowledge, for the first time the impact of major stress mediators, i.e. noradrenergic stimulation

and glucocorticoids, on fear generalization in humans. Our results show that yohimbine intake led to a higher amplitude of fear-tuning in SCR data across a similarity continuum from CS+ to CS-, indicating that noradrenergic stimulation enhanced fear memory expression, mirrored in an increased responding to the CS+, while responding to the similar generalization stimuli and the safety signaling CS- remained unaffected by noradrenergic stimulation. In addition, effects were independent of the distance to the US, and the impact of noradrenergic stimulation on fear memory expression could not be explained by a mere increase in perceptual discrimination ability. Whereas noradrenergic arousal increased fear memory expression, there was a trend suggesting that cortisol may increase fear generalization on an explicit level.

The observed increase in fear memory expression is generally in line with previous findings suggesting that noradrenergic arousal can have enhancing effects on memory accuracy (Atucha et al., 2017; McGaugh, 2013) and is further necessary for the retrieval of recent contextual memories (Murchison et al., 2004). Previous neuroimaging studies on fear generalization showed specific responses to the CS+ and declining activity as stimuli differentiated from the CS+ in the amygdala, insula, thalamus, and striatum (Dunsmoor et al., 2011; Lissek et al., 2014; Onat and Büchel, 2015). Conversely, activity in the hippocampus and vmPFC inclined as stimuli differentiated from the CS+, suggesting an inhibition of responses to stimuli similar to the CS+ (Greenberg et al., 2013; Lissek et al., 2014; Onat and Büchel, 2015). Noradrenergic arousal has been shown to increase the activity of areas implicated in the enhanced responding to the CS+ as well as in areas involved in the inhibitory control of fear responses (Arnsten, 2009; Atucha et al., 2017; Tully and Bolshakov, 2010). However, our finding that yohimbine enhanced specifically the responding to the CS+, while it led CS- responses unaffected, might be taken as evidence that the increased fear memory expression after yohimbine intake was primarily due to enhanced activity in areas involved in CS+ responding, such as the amygdala or insula.

Traditionally, it has been argued that fear generalization is due to the perceptual similarity of the CS+ and graded versions of CS+ and CS-, assuming that the neural fear generalization is directly linked to the perceptual similarity of the stimuli (Lissek, 2012; Lissek et al., 2014). More recently, an alternative model has been proposed according to which fear generalization is an active process that may even occur when individuals are able to perceptually discriminate the CS+ (Dunsmoor and Paz, 2015; Onat and Büchel, 2015). In the present experiment, yohimbine increased participants' perceptual discrimination capacity. Critically, however, the impact of yohimbine on fear memory expression remained, at least at trend-level, when we statistically controlled for the increase in perceptual discrimination ability, thus providing further evidence that there is a fear generalization process that is at least partly independent of the perceptual discrimination capacity. Based on our results, we suggest that this active process, which maintains fear memory expression, may be shaped by noradrenergic stimulation. Although the processes of fear memory generalization and expression seem to be related (Rozeske et al., 2015), there is evidence that these are two distinct processes (Xu and Sudhof, 2013). Based on our results, we propose that the individual stress mediators have distinct effects on these two processes and disentangling glucocorticoid and noradrenaline effects on either fear generalization and fear memory expression remains a challenge for future research.

In contrast to yohimbine, hydrocortisone did not enhance fear memory expression but tended to increase fear generalization. Although this trend needs to be interpreted with caution, this finding is generally in line with the disruptive effects of cortisol on memory retrieval (Buchanan et al., 2006; Roozendaal, 2002) and more specifically, with rodent data, suggesting enhanced fear generalization after glucocorticoid administration (Kaouane et al., 2012). A cortisol-driven increase of fear memory generalization would further be in line with a previous study reporting enhanced fear memory after stress because this study tested fear generalization when stress-induced cortisol had reached peak levels while stress-induced arousal had already vanished (Dunsmoor et al., 2017).

While the present data provide initial evidence for opposite roles of noradrenaline and glucocorticoids in fear memory expression and generalization, it is important to note that there is compelling evidence for synergistic interactions of glucocorticoid and noradrenergic activity in the modulation of learning and memory (Krugers et al., 2012; Roozendaal et al., 2006). We explicitly tested for such interactions by including the CORT+YOH group. Participants in this group showed a very similar pattern as those in the YOH group, suggesting that the impact of noradrenergic impact superimposed the glucocorticoid effect. Evidence suggests that there are time-dependent effects of glucocorticoids, related to non-genomic and genomic modes of action, with rapid effects resembling those of catecholamines but opposite delayed effects (Joels et al., 2011; Schwabe and Wolf, 2014). As such, it would be interesting to test the influence of glucocorticoids at different time-intervals before the generalization test, to assess whether glucocorticoids active at longer time intervals before test would result in more pronounced effects on fear generalization or to altered interactions with noradrenergic activity.

While there was a specific increase in the response to the CS+ in our SCR data, reflected in the amplitude of tuning, our US-expectancy rating data appeared to represent fear generalization processes, reflected in the width of tuning. Previous studies suggested that SCR and expectancy ratings may reflect different types of fear memory, i.e. implicit and explicit memory systems, respectively (Manassero et al., 2019; Schultz et al., 2013). In line with our results, a recently published study found that implicit reactions, represented by the SCR, were selectively triggered by the CS+, but not by a similar stimulus. In contrast, participants were more susceptible to misidentify the same similar stimulus as the CS+ on an explicit level (Manassero et al., 2019). The authors interpret their results as a support for the two-system framework (LeDoux and Pine, 2016), that proposes a defensive survival circuit which may be mainly depending on behavioral and physiological reactions, hence fast implicit fear learning and a rather cognitive circuit, reflected in subjective experience of fear.

Finally, it should be noted that increased noradrenergic arousal after yohimbine intake might be assumed to induce

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an overall increase in SCR. However, even if there is such an effect of yohimbine on SCR per se, this should have led to an overall increase in SCR to all stimuli, which was not observed here, rather than to the stimulus-specific responses that are suggested by our analyses based on parameters of Gaussian fits.

To conclude, fear generalization is a fundamental process that allows us to deal with complexity in our environment, yet overgeneralization of fear to non-threatening stimuli may be maladaptive. We show here for the first time that noradrenergic stimulation may increase the expression of 24hrs-old fear memories and that this effect could not be explained by increases in safety learning or a mere increase in perceptual discrimination ability. These findings provide novel insights into the regulation of fear memory by major stress systems and might point to novel treatment approaches for mental disorders in which the overgeneralization of fear is a hallmark feature.

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Contributors

L.S. conceived and planned the experiment. F.M.K. and G.Z. collected the data. L.K. and C.B. contributed analysis tools. F.M.K. performed the analyses. F.M.K., L.K., C.B., J.C.M., K.W., and L.S. contributed to the interpretation of the results. F.M.K. drafted the manuscript, L.S. provided critical revisions. All authors contributed to the final version of the manuscript.

Conflict of interest

The authors declare no competing interests.

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