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Enhanced Startle Responsivity 24 Hours After Acute Stress Exposure

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Cortisol release in a stressful situation can be beneficial for memory encoding and memory consolidation. Stimuli, such as odors, related to the stressful episode may successfully cue memory contents of the stress experience. The current investigation aimed at testing the potency of stress to influence startle responsivity 24 hr later and to implicitly reactivate emotional memory traces triggered by an odor involved. Participants were assigned to either a stress (Trier Social Stress Test [TSST]) or control (friendly TSST [f-TSST]) condition featuring an ambient odor. On the next day, participants underwent an auditory startle paradigm while their eyeblink reflex was recorded by an electrooculogram. Three different olfactory stimuli were delivered, one being the target odor presented the day before. Additionally, negative, positive, and pictures of the committee members were included for comparing general startle responsivity and fear-potentiated startle. Participants of the stress group demonstrated an enhanced startle response across all stimuli compared to participants of the control group. There were no specific effects with regard to the target odor. The typical fear-potentiated startle response occurred. Stressed participants tended to rate the target odor more aversive than control participants. Odor recognition memory did not differ between the groups, suggesting an implicit effect on odor valence. Our results show that acute stress exposure enhances startle responsivity 24 hr later. This effect might be caused by a shift of amygdala function causing heightened sensitivity, but lower levels of specificity.

Keywords: fear-potentiated startle, odor, Trier Social Stress Test (TSST), cortisol, eyeblink

Stressful situations activate the sympathetic nervous system and hypothalamus-pituitary-adrenal (HPA) axis. As a consequence, the adrenal medulla releases (nor)adrenalin and the adrenal cortex releases cortisol. These reactions cause increased vigilance during a stressful situation (Hermans, Henckens, Joëls, & Fernández, 2014). Besides, cortisol release during memory encoding promotes consolidation processes, especially of stimuli related to or relevant for the stressful episode itself (Joëls, Pu, Wiegert, Oitzl, & Krugers, 2006; Roozendaal & McGaugh, 2011; Wiemers, Sauvage, Schoofs, Hamacher-Dang, & Wolf, 2013). While most of the studies explored this effect using visual stimuli, odors also have the potential of triggering recognition memory of a stressful episode and may function as retrieval cues to long-term memory contents (Wiemers, Sauvage, & Wolf, 2014). As the olfactory system has a direct access (without thalamic gating) to the amygdala (Doty, 2001; Shepherd, 2007), which is responsible for emotional processing, olfactory stimuli are predisposed to have a strong emotional component. Studies demonstrated the potency of odors to cue emotional memories (Adolph & Pause, 2012; Herz, 1998; Herz & Cupchik, 1995; Herz & Schooler, 2002; Willander & Larsson, 2007). Furthermore, odors led to rather personal and affective associations in comparison to word and visual cues (Hinton & Henley, 1993). Moreover, regional cerebral blood flow in the bilateral amygdala was increased upon emotional olfactory in contrast to visual and auditory stimulus presentation (Royet et al., 2000), illustrating the special involvement of

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olfactory stimuli in emotional processes. A functional magnetic resonance imaging (fMRI) study comparing activations during recall triggered by emotional and control olfactory and visual cues showed stronger amygdala activation for the emotional odor compared to the other cue types (Herz, Eliassen, Beland, & Souza, 2004).

Psychosocial stress influences the affective state of a person. This can be assessed explicitly using questionnaires, but may also be assessed implicitly using the startle paradigm. A startle stimulus, such as a loud (≈100 dB) white noise, is applied via headphones to elicit an eyeblink response which can be measured by electrodes attached to the eye muscle. This reflex is potentiated by presentation of an aversive stimulus while applying the startle stimulus (fear-potentiated startle; Hamm, Cuthbert, Globisch, & Vaitl, 1997; Lang, Bradley, & Cuthbert, 1990). In the same way as visual stimuli, olfactory stimuli can also modulate the human startle response, with negative odors augmenting the startle amplitude (Miltner, Matjak, Braun, Diekmann, & Brody, 1994; Pause, Adolph, Prehn-Kristensen, & Ferstl, 2009). A study revealed that startle responses to anxiety sweat were significantly stronger compared to exercise sweat, even though only 15.8% of the participants were able to consciously distinguish the sweat samples from room air (Prehn, Ohrt, Sojka, Ferstl, & Pause, 2006). Olfactory processing often seems to have an unconscious component, probably due to olfactory stimuli being processed without thalamic gating.

In a previous study using the cold pressor test (CPT), the impact of acute stress on the startle response has been examined to investigate the response during the physiological stress experience as well as 45 min afterward. Startle eyeblink responses during stress did not differ from those during the baseline period, but were diminished after the stress experience (Deuter et al., 2012). The authors suggested that acute stress led to a diminished startle response in favor of focused attention. A pharmacological study, in contrast, suggested that the influence of cortisol on the startle response seems to follow an inverted U-shaped pattern, such that a low dose of cortisol (5 mg) enhances, while a high dose (20 mg) reduces the startle response magnitude (Buchanan, Brechtel, Sollers, & Lovallo, 2001). It remains an open research question whether the emotional aspects of a single stressful experience are maintained as memory traces, which may be triggered by reexperiencing stimuli present during the episode 1 day later, when the physiological stress response has vanished.

The current study therefore aimed at investigating whether stress effects do have an influence on startle responsivity 24 hr after stress exposure using a stress paradigm which causes stronger activation of the HPA axis and thus higher cortisol release than the CPT. We further aimed at investigating whether lending an emotional component to a previously neutral and unknown odor by presenting it during stress induction (Wiemers et al., 2014) has effects on the startle response when participants are reexposed to it the following day. We hypothesized that exposure to a stressor leads to implicitly acquired negative associations in connection with the odor present and thus to a potentiated startle response. In addition, visual stimuli were included, among which were pictures of the committee members, to see whether this would enhance the response due to stress induction in direct comparison to the odors. Negative and positive pictures were included to test for the presence of the typical fear-potentiated startle response (Greenwald, Bradley, Cuthbert, & Lang, 1998) and its potential modulation by stress. Recognition performance for the odor ambient in the testing room was hypothesized to be better in the stress group in comparison to the control group. Furthermore, participants stressed were hypothesized to rate the target odor as more negative.

Method

Participants

Participants were 70 nonsmoking male (n = 36) and female students from the Ruhr-University Bochum, who reported neither mental and physiological diseases nor regular medication use. Female participants reported having a regular menstrual cycle and were not tested during their time of menses. Pregnant women and those taking hormonal contraceptives were excluded (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999).

The participants' age ranged from 18 to 34 years (M = 24, SD = 3.65), their body mass index ranged from 18.04 to 29 (M = 22.65, SD = 2.74). They were either paid an expense allowance of 25 € or received course credits. The study was approved by the local Ethics Committee of the Faculty of Psychology and the Declaration of Helsinki was followed.

Experimental Session

Participants were randomly assigned to either a stress or a control condition. Stress was elicited by the TSST (Kirschbaum, Pirke, & Hellhammer, 1993), whereas the control condition involved a comparable situation not eliciting stress, the f-TSST (Wiemers, Schoofs, & Wolf, 2013). After arrival, participants signed informed consent. Afterward, participants were to fill out the Social Interaction Anxiety Scale (SIAS; Mattick & Clarke, 1998) and the experimenter handed over the first Salivette (Sarstedt, Nümbrecht, Germany). While collecting the first saliva sample, the participants filled out the Positive and Negative Affect Scale (PANAS; Watson, Clark, & Tellegen, 1988). The experiment started after the participants were brought to a room where either an evaluation or a friendly committee was waiting. Until then, participants did not know about the condition to which they were assigned. In the room, a fixed amount and concentration of an odor, methyl benzoate—in the following referred to as the target odor—was dispersed using a ventilator while the participants held their speech (Wiemers et al., 2014). Afterward, the participants were brought back to the testing room where they again gave affect ratings using the PANAS and delivered another saliva sample (+1 min). Subsequently, the participants filled out the NEO Five Factor Inventory (NEO-FFI; Costa & McCrae, 1992) and delivered two more saliva samples (+10 min and +30 min). By the end of the first experimental session, the participants of the stress group were debriefed about the nature of the TSST being a standardized procedure, including fixed response patterns of the committee.

The second day's testing session again started with the PANAS before the startle procedure was initiated, including two blocks: one visual and one olfactory startle block, having the same temporal sequence and presentation times. Participants pseudorandomly started with either of the two blocks. The duration was approximately 17 min each, during which the startle stimulus was applied 24 times, on average, in the presence and 42 times in the

absence of a stimulus. Afterward, participants rated seven odors for their pleasantness on a 4-point scale (1 = *very pleasant*, 2 = *pleasant*, 3 = *unpleasant*, 4 = *very unpleasant*), including the target odor and two distractor odors (see the Odors section), and had to give a forced-choice reply to which they thought was the odor present the day before. Besides the two distractor odors, which were delivered via the olfactometer for comparing the startle responses to those of the target odor, four other odors were included. Those were the unknown odor damascenone and the known odors lemon, lavender, and vanilla (Sulmont, Issanchou, & Köster, 2002). Their purpose was to mask the target odor, test for general odor rating and ability to name the known odors.

Materials

Trait questionnaires. Participants filled out the SIAS (Mattick & Clarke, 1998) including 20 items to be rated on a 5-point scale for their expression, ranging from 1 = *not at all* to 5 = *extremely*. The German version of the SIAS exhibits a good reliability, quantified by a Cronbach's alpha of .90 (Heinrichs et al., 2002).

The NEO-FFI (Costa & McCrae, 1992) assesses the value of the personality traits Neuroticism, Extraversion, Openness to Experience, Agreeableness, and Conscientiousness. Each scale consists of 12 items to be answered on a 5-point scale ranging from *strongly agree* to *strongly disagree*. The scales of the German version (Borkenau & Ostendorf, 1985) exhibit a good internal consistency with a Cronbach's alpha up to .85 (Neuroticism, Conscientiousness). Our main interest focused on Agreeableness to validate the answers given in the SIAS, and Neuroticism to compare the groups with regard to a reliable personality parameter.

Stress Procedure and Control Condition

TSST. The participants of the stress group underwent a modified version of the TSST (Kirschbaum et al., 1993) in order to elicit psychosocial stress in the laboratory. The TSST has proven to reliably activate the HPA axis, leading to a strong cortisol release (Dickerson & Kemeny, 2004). In the paradigm, stress is elicited by socioevaluative threat combined with uncontrollability. Participants have to give a free speech in front of an evaluation committee, during which they are videotaped. In line with Wiemers, Sauvage, et al. (2013) the mental arithmetic task was replaced by an additional 5 min of the speech. Thus, the speech is extended to 10 min. The committee, consisting of one female and one male, is introduced as trained behavioral psychologists analyzing the participants' behavior. The participant is instructed to apply for a desired job position in a simulated job interview by only referring to character traits. The committee behaves neutral and reserved, with interaction reduced to a minimum. Before the speech, the participant is allowed a 5-min preparation time.

f-TSST. The recently introduced friendly version of the TSST has proven to be an adequate control condition not activating the HPA axis. The participants' affect ratings show that also subjectively the control condition is experienced as pleasant, not enhancing negative affect (Wiemers, Sauvage, et al., 2013). The procedure is identical to the TSST except for the following. The committee members are introduced by their names and interact in a friendly manner with the participant. The participant is allowed to choose from a set of topics comparable to the contents of a job interview. This situation is not videotaped.

Physiological Stress Measures

Salivary cortisol. For the salivary cortisol assessment, participants were instructed in advance to their appointed time to refrain from taking medication or other drugs, drinking alcohol, or engaging in excessive sports for 24 hr, as well as drinking anything except water and brushing their teeth 1 hr before testing. After collecting four saliva samples on the first and two on the second day of testing using Salivettes, samples were deep-frozen at −18° C and analyzed at our local biochemical laboratory using the Demeditecs cortisol free in saliva enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's manual. A coefficient of variation (CV%), expressed as the percentage deviation from the mean of ≤15% to retain any given duplicate sample, was used. Intra- and interassay CVs were below 10%. Since cortisol release follows a circadian rhythm, testing took place in the afternoon, between 12:30 and 4:00 p.m.

Salivary alpha-amylase. For assessing the response of the sympathetic nervous system, the enzyme alpha-amylase (sAA) was additionally analyzed from the saliva samples (Rohleder & Nater, 2009; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004). The measurement is based on an enzymatic action of the sAA with CNP-G3 used as the substrate. The enzymatic action of sAA can be spectrophotometrically measured at 405 nm. The amount of sAA activity present in the sample is directly proportional to the increase in absorbance at 405 nm. Intra- and interassay variability were both less than 10%.

Affect Measurements

Participants rated their affect using the PANAS (Watson et al., 1988), which includes 20 items divided into 10 positive and 10 negative emotions, rated on a 5-point scale for their intensity, ranging from 1 = *very slightly or not at all* to 5 = *extremely*. The ratings result in a positive (PA) and a negative affect (NA) score.

Startle Procedure

Startle evocation. The startle stimulus consisted of a 100 dB white noise with 50 ms duration and an instantaneous rise time. It was randomly presented via 80 Ω headphones (DT770M, beyerdynamic GmbH & Co. KG, Heilbronn, Germany), between 5 and 7 s after odor delivery or picture presentation onset, respectively. At a 50% rate the white noise was combined with a stimulus, the same for the interstimulus intervals (ISIs). Startle stimuli were delivered in two blocks, one with startle stimuli accompanied by pictures, the other by odors. Both blocks started with a 30 s habituation phase including six startle stimuli.

Data recording. The electrooculography recordings were executed with two bio potential electrodes (EASYCAP GmbH, Herrsching, Germany) attached to the orbicularis oculi muscle of the left eye as described elsewhere (Blumenthal et al., 2005; Fridlund & Cacioppo, 1986; Lang et al., 1990). Further, a disposable ground electrode (GOLMED GmbH, Weddel, Germany) was attached to the forehead. The signal of the electrodes was ampli-

fied and transmitted by means of the MP150 data acquisition device (BIOPAC Systems Inc., Essen, Germany) with filter settings of 10 to 500 Hz. MatLab (version R2012a, MathWorks Inc., Ismaning, Germany) was used to control for operation of the process sequence.

Startle Stimuli

Odors. Three substances (methyl benzoate, bornyl acetate, linalool; Sigma-Aldrich Co., Munich, Germany), previously rated as unfamiliar and neutral (Sulmont et al., 2002), served as olfactory stimuli. The odor essences were dissolved in 50 ml scentless paraffinum liquidum, in concentrations of 60 μl, 850 μl, and 100 μl, respectively, to achieve comparable odor intensity (Wiemers et al., 2014). The mixtures were delivered by means of an in-house-built 6-channel constant-flow (50 ml/s) olfactometer as described elsewhere (Lorig, Elmes, Zald, & Pardo, 1999) via oxygen masks covering nose and mouth. The olfactometer's mean latency is 447.5 ms for onset of the odor and 608.5 ms for offset. Channel activation was adjusted to the latency of the olfactometer to ensure maximum and constant intensity of odor delivery.

Previous studies demonstrated that participants comply well with breathing instructions and produce reliable inspiration patterns (Adolph & Pause, 2012; Prehn et al., 2006). Thus, we abstained from establishing a breathing belt. To make sure the odors were inhaled, each was delivered for 7 s (average respiratory frequency of an adult ≈ 12/min; Silverthorn, 2009) with an ISI of 20 s between offset of the previous and onset of the next odor. Furthermore, a countdown appeared on the screen, counting backward from 3 to 0, featuring the instruction to inhale when 0 is displayed. The odor was delivered 500 ms before to overcome the latency of the olfactometer's odor delivery to the oxygen mask. Each odor was presented seven times in a pseudorandomized manner, so that no odor was presented twice in a row.

Pictures. To have a comparable component, which is known to influence the startle response, a block consisting of picture stimuli was implemented. Pictures included photographs of the committee members present the day before and unfamiliar committee members for a direct comparison of modality effects in relation to stress exposure. The committee pictures were in-house made, showing only the face, with other features like hair and background masked. As only two committee members were involved, the pictures were repeated three times each to ensure valid startle responses to both. Pictures of the International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 2008) were selected to control the sensitivity and validity of the experimental startle setup. They consisted of six positive (mainly landscapes) and six negative (mainly attacks) photographs, matched for arousal within each category. They were adapted from a previous study showing a robust effect on the fear-potentiated startle response (Bradley, Codispoti, Cuthbert, & Lang, 2001). To prevent any conditioning effects to the visual stimuli, each picture was presented twice, only once in combination with a startle stimulus. Pictures were presented on a 15 in. × 12 in. screen with brightness adjusted to 100 and a resolution of 1,280 × 1,024 pixels. In between the stimulus presentations, a 20 × 20 pixels fixation cross was displayed at the center of the screen. The participants were seated in a chair at a 45 cm distance approximately.

Startle Data Processing

Valid data were selected by a semiautomatic mechanism using the software BrainVision Analyzer 2.0 (Brain Products GmbH, Gilching, Germany). The detection mechanism was adjusted to startle responses occurring within a time frame from 50 ms to 225 ms from startle onset. A 50 Hz notch filter was applied. The signal was baseline corrected (0–50 ms) and rectified before applying a peak detection mechanism and further manually verifying the output. Of the overall startle amount during stimulus presentation, 1.33% of the responses were rejected in total due to reactions outside the usual time scope for startle responses (0.48%), artifacts of eyeblinks occurring close to the probable startle response (0–20 ms before or after startle probe onset; 0.43%), or nonresponsiveness (amplitude did not exceed largest baseline amplitude by a factor of 2; 0.43%; Adolph & Pause, 2012).

Data Analysis

Mean values were calculated for each single stimulus (three odors, four picture types) and each sensory modality (olfactory, visual). An analysis of variance (ANOVA) with within-subjects factors modality (odors, pictures) and between-subjects factors condition (stress vs. control) and sex (male, female) was conducted. Further, the ANOVA was repeated for both modalities separately, with Odors (3) × Condition (2) × Sex (2) or Pictures (4) × Condition (2) × Sex (2), respectively. Statistical analyses were performed using SPSS 20.

Results

Participants

Since we were especially interested in the impact of a stress-induced HPA response on the startle response, we used a stringent responder criterion. For cortisol, a difference value was calculated by subtracting the baseline value from that of the saliva sample collected 10 min after termination of the stressor (+10), representing the peak of the hormonal response (Dickerson & Kemeny, 2004; Het, Schoofs, Rohleder, & Wolf, 2012; Kirschbaum & Hellhammer, 1994; Kirschbaum et al., 1993). For the current analysis, a stress response was defined as a difference score of ≥2.5 nmol/L, while a nonresponse was defined as <2.5 nmol/L (Kirschbaum, Wüst, & Strasburger, 1992; Schoofs & Wolf, 2009; Wüst et al., 2000). This cutoff value led to exclusion of nine participants who failed to show a robust cortisol response in the stress condition and seven who expressed a cortisol increase to the control condition. Further exclusions concerned six participants who were nonresponding to the startle stimulus, another seven participants due to technical issues with the electrodes, and one who did not show up on the second day.

The 40 participants remaining are equally distributed over the two groups. According to self-reports, seven of the 21 female participants were in their luteal, 11 in their follicular phase, and three were ovulating. To check for menstrual cycle phase differences between the groups, we compared the distribution of the different cycle phases in female participants using the chi-square test. No significant differences between the groups in menstrual cycle phase were found ($\chi^2(2) = 1.25$, $p = .535$).

Trait Questionnaires

For social interaction anxiety measured by the SIAS, no differences between the stress and control groups were revealed, $t(38) = -.672$, $p = .506$. As for the NEO-FFI, no differences in the measured factors Neuroticism, Extraversion, Openness to Experience, Agreeableness, and Conscientiousness between stress and control group were found (all $p > .10$).

Stress Induction

Affect ratings. As expected, participants of the stress group scored lower in PA and higher in NA compared to the control group, after the experimental manipulation (Table 1). A repeated measures ANOVA with the within-subjects factors time (pre, post) and the between-subjects factors stress (stress vs. control) and sex (female, male) was conducted for the two affect scales (PA, NA) separately. Participants of the stress and the control group did not differ in their affect ratings before the experimental manipulation.

Data for the NA show a significant two-way interaction of Time × Stress, $F(1, 36) = 9.63$, $p = .004$. A follow-up ANOVA for comparing pre- and postassessments of NA within the respective group resulted in both a significant effect of time for the control, $F(1, 18) = 9.53$, $p = .006$ and the stress group, $F(1, 18) = 4.48$, $p = .049$ in opposing directions, with a decline of NA in the control and an increase in the stress group. When comparing pre- and postassessments of NA between the groups in a follow up ANOVA, a significant difference with regard to the post assessment, $F(1, 36) = 5.52$, $p = .024$ could be shown.

For PA, we found a significant within-subjects effect of time, $F(1, 36) = 41.22$, $p < .001$, as both groups had a higher PA score after the experimental manipulation.

Cortisol

Due to a lack of normal distribution, the data were log-transformed. A repeated measures ANOVA was conducted, with time of measurement as a within-subjects factor (baseline, +1, +10, +30) and stress (stress vs. control) and sex (female vs. male) as between-subjects factors. Mauchly's test revealed a violation of sphericity ($\chi^2(5) = 26.77$, $p < .001$), hence Greenhouse-Geisser corrected p values ($\varepsilon = .654$) are reported. The cortisol responses show that stress induction was successful, with participants of the stress group expressing a rise in cortisol concentration, reflected in a significant Time × Stress interaction effect, $F(1.96, 70.60) = 35.73$, $p < .001$, as well as a significant main effect of time, $F(1.96, 70.60) = 12.89$, $p < .001$ and stress, $F(1, 36) = 47.93$, $p < .001$. As expected, salivary cortisol levels at time points +1 ($t(38) = -6.588$), +10 ($t(38) = -8.181$), and +30 ($t(38) = -7.580$) differed significantly between the stress and control groups (all $p < .001$), with maximum difference occurring at time point +10 (Figure 1). Significant salivary cortisol baseline level differences between the stress and the control group were shown, $t(38) = -3.083$, $p = .004$ which are accounted for by conducting an analysis of covariance proving them not to be the cause of any of the resulting group differences (see the Startle Responses section). For the second day, we found a significant within-subjects effect of time, $F(1, 36) = 14.26$, $p = .001$ and a trend toward a Time × Stress interaction, $F(1, 36) = 3.33$, $p = .076$. Further, a main effect of stress was detected, $F(1, 36) = 10.35$, $p = .003$. Significant differences in salivary cortisol concentration between the groups were found before, $t(38) = -3.428$, $p = .001$, and a trend after testing, $t(38) = -1.681$, $p = .101$.

Table 1
Mean and Standard Deviation (SD) of Pre- and Postassessment for Negative Affect (NA) and Positive Affect (PA) of the Positive and Negative Affect Scale for Stress and Control Group

Mean (*SD*)	Stress	Control
NA-pre	13.55 (3.25)	13.70 (2.77)
NA-post	15.10 (7.10)	12.25 (2.80)
PA-pre	27.45 (4.24)	31.00 (4.55)
PA-post	30.15 (9.60)	35.35 (5.72)

Alpha Amylase

As the sAA data lacked normal distribution, they were log-transformed. A repeated measures ANOVA was conducted with factors Time (baseline, +1 min, +10 min, +30 min) × Stress (stress, control) × Sex (male, female). A significant within-subjects effect of time, $F(3, 108) = 22.5$, $p < .001$ for both groups was found, with a peak of sAA release 1 min after the intervention, declining steadily afterward (Figure 2). No significant main effect of stress, $F(1, 36) = .075$, $p = .786$ and no Time × Stress interaction, $F(3, 108) = 1.765$, $p = .158$ were found, indicating a similar time course of sAA release for participants across both groups. Neither the factor sex nor the included two-way interactions resulted in significant effects.

Startle Responses

Since startle data were not normally distributed, they were log-transformed for further analyses. A repeated measures ANOVA for comparing startle responses between the two different groups was conducted with the between-subjects factor stress (stress vs. control) and within-subjects factor startle (3 odors, 4 picture types). Since Mauchly's test resulted in a violation of sphericity ($\chi^2(20) = 168.492$, $p < .001$), Greenhouse-Geisser corrected p values ($\varepsilon = .302$) are reported. The ANOVA resulted in a significant main effect of stress, $F(1, 36) = 4.530$, $p = .040$, indicating enhanced startle responsivity in the stress group across both modalities (Figure 3).[1]

For the within-subjects factor startle, a trend was revealed, $F(1.810,65.158) = 2.723$, $p = .078$. As for the baseline cortisol differences between the two groups, which have been described in the Cortisol section, the ANOVA was repeated, adding baseline cortisol concentrations as a covariate, resulting in an even larger significance of the difference in responsivity between the groups when accounting for the baseline difference on the first, $F(1, 35) = 8.730$, $p = .006$ as well as the second day, $F(1, 35) = 9.199$, $p = .005$.

[1] Stress cortisol nonresponders showed a lower startle response than stressed cortisol responders. Control cortisol responders showed a slightly stronger startle response than the control nonresponders. This pattern is in line with the idea that cortisol is one out of several mediators involved in producing the observed effects on the startle and on the ratings.

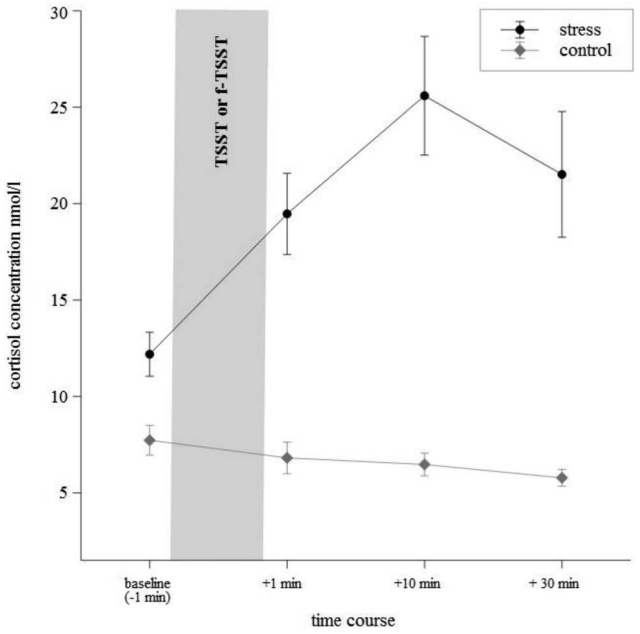

Figure 1. Time course in cortisol concentration of the stress and the control group before experimental manipulation (baseline) and 1, 10, and 30 min after the Trier Social Stress Test (TSST) or friendly TSST (f-TSST), respectively. The statistical analyses were performed with log-transformed data, but the graph displays the raw cortisol data for illustrative purpose. The conducted analysis of variance revealed a Group × Time interaction reflecting an increase in cortisol in the stress group compared to a decrease in the control group.

Olfactory Startle Paradigm

A repeated measures ANOVA with the within-subjects factor odor (target vs. two distractor odors) and between-subjects factors stress (stress vs. control) and sex (female vs. male) showed no differences in responsivity to the different odors, $F(2, 72) = .326$, $p = .723$. For the between-subjects factors, Stress had a significant influence on the startle response, $F(1, 36) = 4.339$, $p = .044$, but none of the other factors.

Visual Startle Paradigm

The repeated measures ANOVA for the pictures revealed a main effect of picture, $F(2.485,89.462) = 5.056$, $p = .005$, but only a trend toward a main effect of stress, $F(1, 36) = 3.160$, $p = .084$, and no Picture × Stress, $F(2.485,89.462) = .627$, $p = .570$ nor other interactions. A grouped comparison for picture types showed a significantly higher startle responsivity to negative compared to positive stimuli, $t(39) = 2.615$, $p = .013$. Further, startle responsivity to the familiar committee members compared with unfamiliar committee members was higher and close to significance, $t(39) = 1.975$, $p = .055$.

Subjective Odor Ratings and Odor Recognition

In order to test for differences in affective quality of the target odor between the groups, the subjective ratings of the three odors from the participants of the two groups were compared conducting an ANOVA with Odor Rating (3 odors) × Stress (control, stress). A trend toward an Odor Rating × Stress effect was shown, $F(2, 68) = 3.071$, $p = .053$, with participants of the stress group rating the target odor more aversive than participants of the control group ($M = 3.06$, $SD = .539$ vs. $M = 2.55$, $SD = .826$). A paired-samples t-test showed that only participants of the stress group rated the target odor significantly more aversive than the two distractor odors, $t(17) = 3.198$, $p = .005$ and $t(19) = 6.469$, $p < .001$. The odor ratings of the two distractor odors show no group differences (Table 2).

To assure that the startle exposure during odor delivery itself did not influence the odor ratings toward a stronger aversion as found for heart rate measures in a rodent study (Young & Leaton, 1994), we compared the ratings of each of the odors included in the startle block with the rating of another neutral and unknown odor, damascenone. It was included in the odor recognition task and the odor ratings, but not in the startle block. Differences in pleasantness ratings were not influenced by the previous combination with the aversive startle stimulus, as ratings varied independently of that. In the control group, the rating of the target odor did not differ from the ratings of the other distractor odors from the startle session, nor damascenone (all $p \geq .167$). In contrast, the stress group rated the target odor significantly more aversive than any of the other odors, included in the startle block or not (all $p < .019$).

As for odor recognition performance we found no differences between the groups. Only two participants per group identified the target odor in the forced-choice task, whereas the other participants

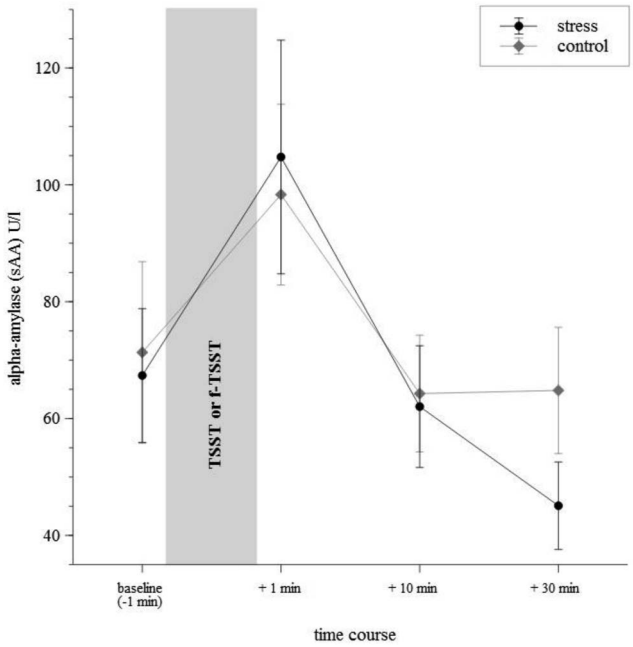

Figure 2. Time course in salivary alpha-amylase (sAA) concentration of the stress and the control group before experimental manipulation (baseline) and 1, 10, and 30 min after the Trier Social Stress Test (TSST) or friendly TSST (f-TSST), respectively. The statistical analyses were performed with log-transformed data, but the graph displays the raw sAA data for illustrative purpose. The conducted analysis of variance detected a main effect of time reflecting a rapid increase followed by a rapid decrease in sAA in both groups.

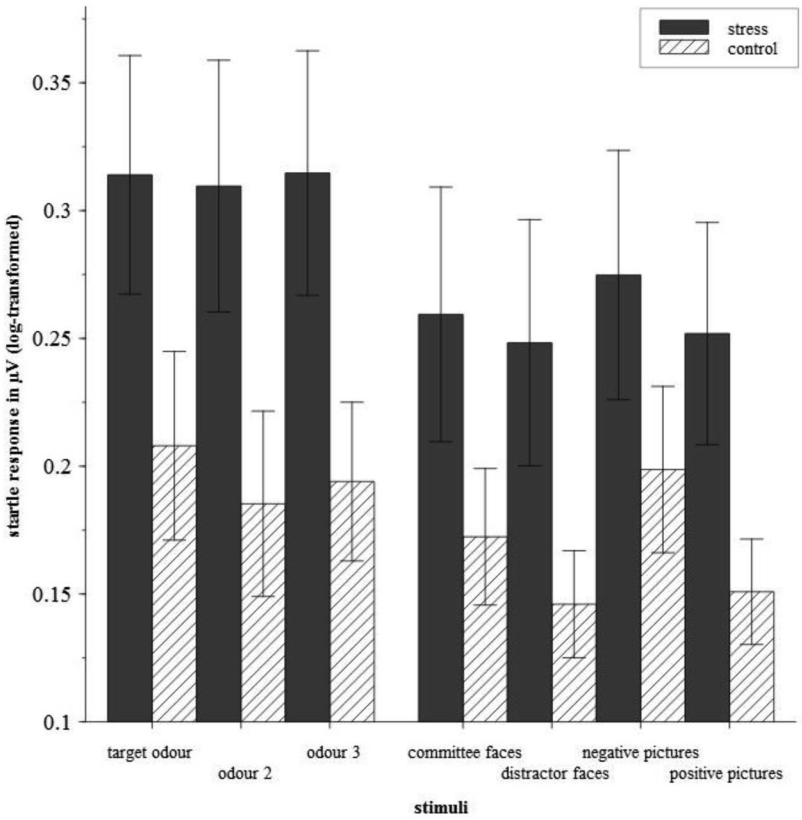

Figure 3. Startle responses to the three odors (1: methyl benzoate (target), 2: bornyl acetate, 3: linalool), negative and positive pictures of the International Affective Picture System, and the pictures of the committee members known and unknown to the participant. The conducted analysis of variance revealed a main effect of stress reflecting an overall enhanced startle responsivity of the stress group.

chose one of the other six odors as having been the odor present in the room the day before.

Discussion

The current study tested the impact of acute stress exposure on the startle response assessed 24 hr afterward. We were interested in global stress effects as well as in specific modulations induced by the odor present during stress exposure. Indeed, we found an enhanced startle response in the stress group 24 hr later in contrast to the control group. Stressed participants expressed stronger startle responsivity across both stimulus modalities for any given stimulus. This extends the previous findings of Deuter et al. (2012), demonstrating a diminished startle response 45 min after the stressor, to a 24 hr period showing that stress can have a long-lasting enhancing effect. Our findings are in line with a previous pharmacological study which reported that a low dose of cortisol (5 mg) enhanced the startle response irrespective of the valence of the pictures used (Buchanan et al., 2001). However, Buchanan and colleagues observed this effect shortly after cortisol administration. Our current findings indicate that a similar pattern can be observed even 24 hr after the termination of the stressor. This lasting effect of stress exposure on the previous day was shown with the startle paradigm in the absence of any conscious alteration of the participant's mood, as the affect ratings did not differ between the stress and the control group on the second day. Similar findings have been reported by Miller McKinney, Kanter, Korte, and Lovallo (2011), suggesting that neuroendocrine factors that influence startle modulation may not be reflected by the subjective sense of fear- and anxiety-related emotions and their verbal expression.

Table 2
Odor Ratings of the Three Odors Delivered During the Startle Block and Damascenone, an Odor Which Was Included in the Odor Rating and Recognition Task

Rating	Condition	Mean	*SD*
Methyl benzoate	Stress	3.10	.553
Target odor	Control	2.55	.826
Bornyl acetate	Stress	2.33	.594
Distractor odor 1	Control	2.55	.826
Linalool	Stress	1.75	.716
Distractor odor 2	Control	2.15	.745
Damascenone	Stress	2.40	.754
Not included in startle block	Control	2.50	.946

Since we were especially interested in the long-term impact of acute stress-induced HPA activation, we employed rather stringent exclusion criteria in order to ensure that all participants in the stress condition and no participant in the control condition showed a robust cortisol increase. The number of stress nonresponders and control responders was slightly higher than in our previous study (Wiemers, Schoofs, et al., 2013), but not higher than in other studies employing the TSST (e.g., Kirschbaum et al., 1993). It has to be acknowledged that, without the exclusion of the nonresponders, the reported effects on the startle response decreased in size and would have turned into a nonsignificant trend. Our findings thus emphasize the relevance of validating the efficacy of the stress paradigm using salivary stress markers in order to assure that potential findings are not masked by stress nonresponders and control responders. Of course, HPA activation is only one out of many psychoneuroendocrine alternations in response to stress. However, it was the focus of the present study and the presence of an HPA response apparently was a prerequisite for the observed effects on startle responsivity. Only a pharmacological cortisol administration study could provide direct mechanistic causal evidence.

As we found small but significant baseline differences in cortisol between the stress and the control group, we included cortisol baseline as a covariate in the ANOVA, leading to an even stronger main effect of stress on startle responsivity. Thus, baseline cortisol differences between the two groups are not responsible for the observed differences in startle reactivity on the next day. In fact, cortisol might not be mediating the stress effect on the startle response at all. Roemer, Nees, Richter, Blumenthal, & Schächinger (2009) could show that metyrapone, suppressing endogenous cortisol production, significantly increased startle eyeblink responses. They suggested that increased release of central corticotropin-releasing hormone might have mediated the observed metyrapone effect on startle responsivity. This hypothesis might explain why the subtle cortisol baseline differences between the groups did not affect their startle outcome in the current study.

In contrast to cortisol and affect, the sAA data showed no differences between the groups, demonstrating that the activity and reactivity of the sympathetic nervous system was similar in both conditions. This is in line with previous studies using the f-TSST (Wiemers, Sauvage, et al., 2013; Wiemers, Schoofs, et al., 2013).

The two groups did not differ in trait aspects, such as social interaction anxiety, assessed by the questionnaires. Thus, with regard to several relevant personality traits, the participants of the two groups were highly comparable.

Our hypothesis that the participants of the stress group would subjectively rate the target odor as more negative than participants of the control group could be confirmed at a trend level. Thus, a negative association with the target odor due to stress experience could be induced, suggesting that our paradigm was successful. Despite the differences between the groups with regard to the odor rating, no memory differences were found for identification of the target odor—only two participants in each group identified the target odor correctly. This finding suggests that it is an implicit effect causing the stressed participants to rate the odor more negatively.

Despite the remarkable finding of a single stress exposure inducing an enhanced startle response 1 day later, the expected differentiation between the target odor and the two distractor odors could not be confirmed. It is conceivable that the stress reaction causing the main effect was that strong, that other differential effects were masked, leading to the lack of specificity regarding the responses to the odors. Stressed participants rather seem to react with generally pronounced startle responsivity. A shift of amygdala function might be responsible for this effect as revealed in an fMRI study using strongly aversive movie clips (van Marle, Hermans, Qin, & Fernández, 2009). Stress caused heightened sensitivity to potential threat, but lower levels of specificity. As the olfactory sense has direct access to the amygdala, the influence of its shift of function might be more pronounced for odors than for pictures. That is, the difference in startle responsivity to negative and positive pictures might be less affected by the shift of amygdala function in comparison to responsivity to the different odors. Hence, startle responses to pictures might show a more distinctive specificity compared with responses to odors.

In line with our findings of an enhanced startle response 24 hr after stress exposure, Beylin and Shors (1998) found in a rodent study that the effects of a one-time stress experience can intensify eyeblink conditioning 24 hr later in male rats. Hence, the stress effect on emotional responding is not only apparent in an immediate response, but can be maintained, dependent on intensity and duration, over days. Obviously, the lateral/basolateral nucleus of the amygdala is involved in facilitated eyeblink conditioning during stress exposure, mediated via *N*-methyl-D-aspartate receptor activation (Shors & Mathew, 1998). Another rodent study (Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005) tested the impact of repeated acute immobilization stress on anxiety and revealed a gradual build-up of avoidance behavior up to 10 days later which was accompanied by increased spinogenesis in the basolateral amygdala. These findings suggest that altered amygdalar processing might underlie our observed findings.

The typical fear-potentiated startle response, as repeatedly reported previously (Ameli, Ip, & Grillon, 2001; Bradley, Cuthbert, & Lang, 1993; Greenwald et al., 1998; Paschall & Davis, 2002), was successfully induced and detected in our experiment. Negative pictures were associated with the most pronounced startle response. This illustrates that our implemented startle paradigm had a high sensitivity and validity. Considering the startle responses in the visual block, the differentiation is descriptively more prominent in the control in comparison to the stress group. This again suggests more pronounced startle responses to be less specific.

Our data show that startle responses to the odors, in general, were more pronounced than those to the pictures. It has to be considered that participants were wearing an oxygen mask for odor presentation, causing a slightly uncomfortable feeling, which could be a confounding variable when comparing the modalities (Adolph & Pause, 2012). Still it can be observed that, for the stress group, also in the visual block, startle responses are not as specific with regard to picture valence as the case in the control group. Differences between responses to negative and positive pictures are less distinct in the stress group. Hence, generally stronger responses go along with a lower specificity. As the testing room and experimenter were familiar to the participants from the previous day, the influence of context effects cannot be ruled out. However, subjective affect ratings did not differ between the groups on the second day. Thus, there were no subjectively perceived affect differences between the groups when returning to the testing room.

It would be interesting for future investigations to extend the current findings of long-term effects of stress exposure on the startle eyeblink response to more immediate effects, using the TSST, especially in combination with an odor. The findings of Deuter et al. (2012) are based on the CPT as a primarily physiological stressor, whereas the TSST is mainly based on a social evaluative threat. Hence, the TSST typically causes a larger HPA response than the CPT (McRae et al., 2006). As the described rodent studies showed an effect of stress on anxietylike behavior up to 10 days later, investigating long-term stress effects 1 week after exposure to the TSST on the human startle response would be of interest. A long-term effect of a stressful experience causing the described amygdala involved shift may occur in people suffering from posttraumatic stress disorder (PTSD). Grillon and Morgan (1999), for example, showed that Gulf War veterans suffering from PTSD demonstrate an enhanced sensitivity to stressful experimental contexts due to generalizing fear across stimuli, expressed by enhanced startle reactivity.

In sum, the present study provides evidence for enhanced human startle responsivity 24 hours after exposure to an acute psychosocial laboratory stressor. This effect is apparently rather broad than specific for olfactory or visual stimuli experienced during the stressor. Our findings are in line with the notion of enhanced sensitivity at the expense of reduced specificity of amygdalar responding in the aftermath of stress.

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