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Original Article

# Enhancement of Odor Sensitivity Following Repeated Odor and Visual Fear Conditioning

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## Abstract

Odor detection sensitivity can be rapidly altered by fear conditioning; whether this effect is augmented over time is not known. The present study aimed to test whether repeated conditioning sessions induce changes in odor detection threshold as well as in conditioned responses and whether olfactory stimuli evoke stronger conditioned responses than visual stimuli. The repeated conditioning group participated in repeated sessions over 2 weeks whereas the single conditioning group participated in 1 conditioning session; both groups were presented with visual and olfactory stimuli, were paired with an electric shock (CS+) and 2 matched control stimuli not paired with shock (CS–) while olfactory detection threshold and skin conductance responses (SCRs) were measured before and after the last session. We found increased sensitivity for the CS+ odor in the repeated but not in the single conditioning group, consistent with changes in olfactory sensitivity following repeated aversive learning and of a similar magnitude to what has previously been demonstrated in the periphery. SCR to the visual and olfactory CS+ were similar between groups, indicating that sensory thresholds can change without corresponding change in conditioned responses. In conclusion, repeated conditioning increases detection sensitivity and reduces conditioned responses, suggesting that segregated processes influence perception and conditioned responses.

Key words: aversive conditioning, odor conditioning, odor sensitivity, visual conditioning

## Introduction

Fear conditioning in nonhuman animals, where the individual learns to associate an odorant with the presence of an aversive stimulus, demonstrates that odor conditioning alters not only behavioral responses (Fletcher and Wilson 2002) but also neural processing and morphology within the olfactory system (Jones et al. 2008; Chen et al. 2011). Recent data prove an odor conditioning-dependent modulation even at the very first stage of the olfactory system, the olfactory sensory neuron, after repetitive pairings between the odor of interest and an aversive stimulus (Kass et al. 2013).

Consistent with findings in animals, studies in humans have reported that fear conditioning of an odorant increases behavioral discriminatory performance and modulates the neural processing of the conditioned odor as early as in the primary olfactory cortex (Li et al. 2008). Although no study has yet reported a conditioning-dependent modulation of olfactory sensory neurons in humans, we recently demonstrated a rapid augmentation of odor detection sensitivity by fear conditioning (Åhs et al. 2013). Using a differential conditioning paradigm, where 1 odorant

predicts the onset of a weak electrical shock (CS+) and another odorant never predicts shock (CS–), we demonstrated a significant augmentation in the ability to detect the CS+ odor, irrespective of the chemical characteristics of the odor itself. One pairing session resulted in an estimated 67% reduction in the stimulus concentration needed to detect the CS+ odorant compared with the CS– odorant post conditioning. This reveals that the olfactory system is able to rapidly modulate its sensitivity to better detect relevant threats in the environment. However, 8 weeks post conditioning this effect disappeared, thus indicating the involvement of transient modulatory mechanisms. This is in conflict with findings within nonhuman animals where long-term sensory potentiation has been demonstrated (Jones et al. 2008; Kass et al. 2013). Most of the nonhuman literature is, however, based on long-term aversive conditioning involving multiple sessions of pairing over days, or even weeks. Thus far, no empirical evidence of such long-term conditioning-enhancement of odor sensitivity in humans exists.

Olfactory CSs pass through vastly different anatomical routes than visual CSs before converging in the amygdala, where information about the pairing with an unconditioned stimulus (US) is initially processed. However, in contrast to the architecture of the visual processing stream, the olfactory receptor body is situated merely 1 synapse away from the amygdala, thus suggesting that olfactory information has a prioritized access (Carmichael et al. 1994; Lundström et al. 2011). This anatomical feature is often highlighted as an indication that odors are naturally more emotional in their character than visual stimuli. In line with this notion, Adolph and Pause (2012) recently demonstrated that odors elicited stronger emotional responses than comparable visual stimuli and proposed that perceptually triggered emotional responses are modality-dependent. Whether this mood-induction disparity between the senses also influences fear learning has yet to be determined.

The present study had 2 primary goals. First, we aimed to determine whether repetitive odor-dependent aversive conditioning induces long-lasting changes in olfactory detection thresholds. We predicted that repeated fear-conditioning sessions would increase detection of the reinforced odor. Second, we investigated the effect of repetitive conditioning on conditioned responses to olfactory and visual CSs. Based on the disynaptic connections between the olfactory bulb and the medial and cortical nuclei of the amygdala and the hippocampus (Carmichael et al. 1994), 2 areas associated with fear condition, as well as the above presented findings of odor-superiority in respect to mood induction, we hypothesized that repeated conditioning would facilitate greater conditioned responses to odor CSs relative to visual CSs.

## Methods

### Participants

A total of 47 individuals provided written informed consent to participate in the study. Twenty-three of them were included in the repeated conditioning group and 24 were included in the single conditioning group. Due to the long and time-demanding nature of the study with multiple visits, 4 individuals in the repeated conditioning group and 2 individuals in the single conditioning group elected to discontinue the study past the initial session. After additional data exclusions ($N$ = 4), as detailed in Data reduction and statistical analyses, the final repeated conditioning group consisted of a total of 16 individuals (9 women) and the final single conditioning group of a total of 19 individuals (10 women). See Table 1 for demographic information. All participants were in good general physical and mental health. None were currently taking any medication, suffering from any form of hormonal, neurological, or autoimmune diseases, none had in the past suffered a head trauma leading to unconsciousness, and no participants smoked, minimizing the possibility that participants presented to various degrees an affected olfactory processing (Boesveldt et al. 2011). Participants were instructed to not eat or drink anything but water, to not chew gum 1 h prior to testing, and to not wear any scented products on the day of testing. All aspects of the study were approved by the local Institutional Review Board.

### Stimuli

#### Odor stimuli and delivery mechanisms

Two odors of perceptually neutral character were used as odor stimuli: peanut odor (Takasago Inc.) and *n*-butanol (butanol;

**Table 1.** Means and SD for demographic information, selected shock intensity, and perceptual odor ratings separated by group

| | Repeated conditioning group | | Single conditioning group | | Group differences | | |
|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | *t* | df | *P* |
| Age | 24.68 | 4.58 | 25.01 | 4.88 | –0.210 | 35 | 0.835 |
| Shock level [mA] | 3.82 | 2.30 | 2.59 | 1.64 | 1.882 | 35 | 0.068 |
| Stimulus intensity | | | | | | | |
| Butanol | 7.89 | 1.37 | 7.79 | 2.04 | .173 | 35 | 0.864 |
| Peanut | 8.11 | 0.96 | 8.58 | 1.50 | –1.120 | 35 | 0.270 |
| Stimulus pleasantness | | | | | | | |
| Odor | | | | | | | |
| Butanol | 4.61 | 1.65 | 5.05 | 1.93 | –0.746 | 35 | 0.460 |
| Peanut | 6.44 | 2.20 | 6.32 | 2.11 | 0.182 | 35 | 0.857 |
| Image | | | | | | | |
| Abstract | 6.39 | 1.29 | 6.89 | 1.41 | –1.137 | 35 | 0.263 |
| Orange | 9.14 | 1.19 | 9.68 | 1.06 | –1.479 | 35 | 0.148 |
| Stimulus familiarity | | | | | | | |
| Odor | | | | | | | |
| Butanol | 5.63 | 2.36 | 6.16 | 2.71 | –0.613 | 33 | 0.544 |
| Peanut | 6.42 | 3.06 | 7.50 | 3.03 | –1.067 | 34 | 0.294 |
| Image | | | | | | | |
| Abstract | 2.97 | 2.08 | 3.21 | 2.59 | –0.304 | 34 | 0.763 |
| Orange | 11.00 | 0.00 | 10.84 | 0.50 | 1.335 | 35 | 0.191 |

Independent Student's *t*-test is reported to assess differences among groups for each of the variables reported. df, degrees of freedom; *P*, exact *P* value.

Sigma–Aldrich). Throughout the study, 1,2-propanediol (Sigma–Aldrich) was used as diluting agent and all concentrations below are given as volume to volume (v/v) in liquid phase. The *n*-butanol odor is a mono-molecular odor whereas the peanut odor is a natural mixture (Figure 1); gas chromatography/mass spectrometry analyses of its content demonstrated that it contained about 8 main chemical components. During odor conditioning, a 35% v/v concentration of the peanut odor and a 5% v/v concentration of the butanol odor were used based on pilot data ($N = 15$) indicating that at these concentrations the odors were clearly perceivable and rated as isointense. For the odor detection thresholds, dilution series were prepared for each odor separately using 17% v/v of the peanut odor and 1% v/v of butanol concentrations as dilution starting points. From there, both odors were diluted in 16 consecutive dilution steps using a 1.785 volume dilution series for the peanut odor (end concentration 0.0029% v/v) and a 1.667 volume dilution series for the butanol odor (end concentration 0.00047% v/v). These dilution series were selected based on an independent pilot study ($N = 50$; 25 women; mean age 24.6 SD ± 3.85) demonstrating that these concentrations were able to capture the detection threshold in 95% of healthy individuals in a sample representative for our normal testing population while still maintaining a sensitive separation between each dilution step. Two series of each odor were prepared and used to allow sufficient headspace saturation between potential repetitions of the same dilution step. Detection thresholds were determined using a 3-alternative forced-choice (3AFC) ascending staircase paradigm with 7 reversals (Doty 1991), described in detailed elsewhere (Lundström et al. 2006, 2008). In short, each dilution step and its 2 paired diluent only (lure) bottles were presented in consecutive and random order to the blindfolded participant with the question, "Which 1 of the 3 has an odor?" Either 2 correct or 1 incorrect response triggered a reversal of the "staircase." Detection threshold was defined as the geometric mean of the last 4 reversals. Each dilution step and the 2 matching lure stimuli containing diluent only were delivered using amber 2 oz. glass bottles, all visually identical and containing 10 mL of liquid each.

During the acquisition sessions, odors were presented birhinally using a computer-automated olfactometer capable of delivering odors in a temporally-precise, square-shaped manner (Lundström et al. 2010). Odor onset timing was regulated by the stimulus presentation program E-Prime Professional 2.0 (Psychological Software Tools). To prevent irritation of the nasal mucosa over time (Lotsch et al. 1998; Lundström et al. 2010), we used a low birhinal flow rate of 3.0 L/m (a total of 1.5 L/m per nostril) for a total duration of 3 s per stimuli. Odorous air was directed to the nose when the odor was delivered and clean air was presented subsequently for another 2 s to minimize odor residuals (Seubert et al. 2014). Even though the olfactometer setup used has been demonstrated to not induce any pain sensations due to mucosa dryness even for long presentations (Lundström et al. 2010), we implemented an interstimulus interval (ISI) of 9s of no air (giving a total of 11s of minimum odor ISI) to allow rehumidification of the nasal mucosa. Please refer to Table 1 for details about odor ratings.

### Visual stimuli

Color images of an orange and an abstract picture were selected and presented centrally on the screen on a white background for a total duration of 3 s (Figure 1). We used the visual object orange rather than peanut to avoid potential transfer of learning between the 2 modalities. These images were selected to match the odor stimuli along the food versus nonfood dimension meaning that the orange image and peanut odor are associated with edible objects and the abstract image and the butanol odor are not known to be associated with edible objects. To avoid color-dependent effects, the abstract image was of an orange color. Please refer to Table 1 for details about image ratings. These visual stimuli were selected in order to test the hypothesis that olfactory stimuli are more emotional than visual stimuli (as previously suggested, Adolph and Pause 2012) in the context of aversive conditioning. Therefore, the choice of images was secondary to the choice of odors. Indeed, the very nature of the images used as well as the crossmodal fear conditioning task allowed us to study the physiological responses to images, but not to administer visual sensitivity tests comparable to those used to test olfactory behavior.

### Shock

Electric pulses (11 Hz) for a total duration of 200 ms were presented using a stimulating bar electrode (ADInstruments) placed on the right forearm 50 mm from the wrist served as US. Shock level was determined individually by presenting the participant with successively increasing stimulus levels starting from 0.5 mA. Each increase was set to 0.5 mA using an interstimulus interval of 20 s and the stimulus intensity was not allowed to go above a predetermined max level of 10 mA. Stimulations increased until the participant reported an irritation rating of 7 on a 0–10 graded scale where 0 was anchored as "no irritation at all" and 10 was anchored as "very irritating." This procedure created a baseline equal to the perceived intensity of the aversive stimulus rather than its absolute mA value. At the beginning of each new session (both intermediate sessions and Session 6), each participant was subjected to electric stimulation to confirm that the

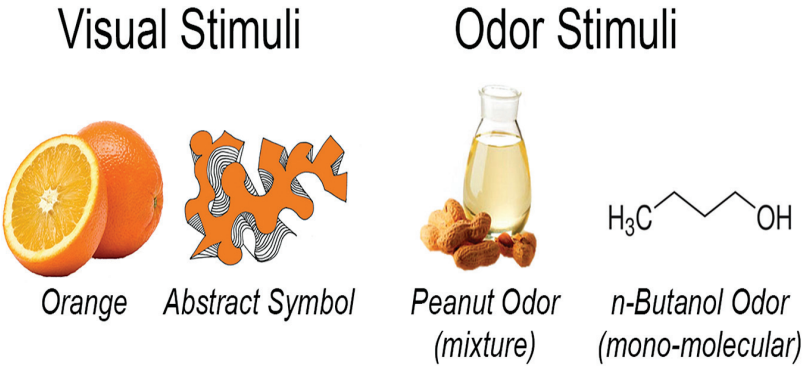


**Figure 1.** Four stimuli were used: 2 visual (left panel) and 2 odor stimuli (right panel), with the 2 left most in both panels associated with edible objects whereas the 2 rightmost in both panels are perceived as abstract and not commonly associated with edible objects.

subjective level of irritation was not changed since last measurement (Figure 1B). If the subjective irritation level was changed, the new subjective intensity corresponding to an irritation level of 7/10 was used.

## Psychophysiological assessments

Skin conductance responses (SCR) were continuously measured throughout the experiment by means of the PowerLab system (ADInstruments) and assessed offline using LabChart Pro, Version 7 (ADInstruments). This measure was acquired as an objective measure of aversive learning (Flykt et al. 2007). Responses were acquired from the palmar surface on the medial phalanges of the fore and middle fingers of the nondominant hand using 10 mm Ag/AgCl round electrodes with a sampling rate of 100 Hz and a high-pass filter of 0.1 Hz (Andreassi 2000).

## Procedures

Both the repeated conditioning and single conditioning groups participated in 2 sessions (initial and final session) with approximately 16 days in-between, and the repeated conditioning group also participated in 4 additional, intermediate, aversive conditioning sessions. Please refer to Figure 2A and 2B for a graphical representation of the experimental design.

### Preacquisition and acquisition phase (Session 1)

Detection threshold tests for the 2 odors were administered with order of the odors tested counterbalanced between individuals. After these initial tests, participants were equipped with the bar electrode used to deliver the aversive stimulus, electrodes on their middle and ring fingers of their nondominant hand to measure SCR, and headphones presenting low continuous levels of white noise to mask potential valve-related auditory cues from the olfactometer. In the post-experiment debriefing, no participants reported that they detected any external sounds. After providing 3 ratings for each of the perceptual parameters (odor intensity, pleasantness, and familiarity) using visual analogue scales implemented in the aforementioned E-Prime program, the preacquisition session was performed. In this phase, stimuli are presented unreinforced, primarily to reduce the orienting response to stimuli before conditioning. During preacquisition, each odor and each image was presented a total of 10 times with no pairing with the aversive stimulus. The (aversive conditioning) acquisition section of the session was initiated by setting the shock level (see Shock) after which 20 presentations of each odor and 20 of each image were presented. One of the odors and one of the visual images had been designated, in a counterbalanced fashion to avoid odor- or image-specific effects, to be paired with the aversive stimulus (CS+) and 1 odor and 1 image to never appear with the aversive stimulus (CS–). Odors and images were presented in a randomized order using an average ISI of 14s (ISI lengths were randomized ±2s to prevent onset anticipatory responses) and a stimulus length of 3s. For 50% of the CS+ designated odor/visual presentations in the acquisition phase, during the last 200 ms, the odor was paired with the aversive stimulus, which coterminated with the odor/visual presentation. To maintain the acquired threat value of the CS+ across the extinction phase, a partial reinforcement schedule—known to slow

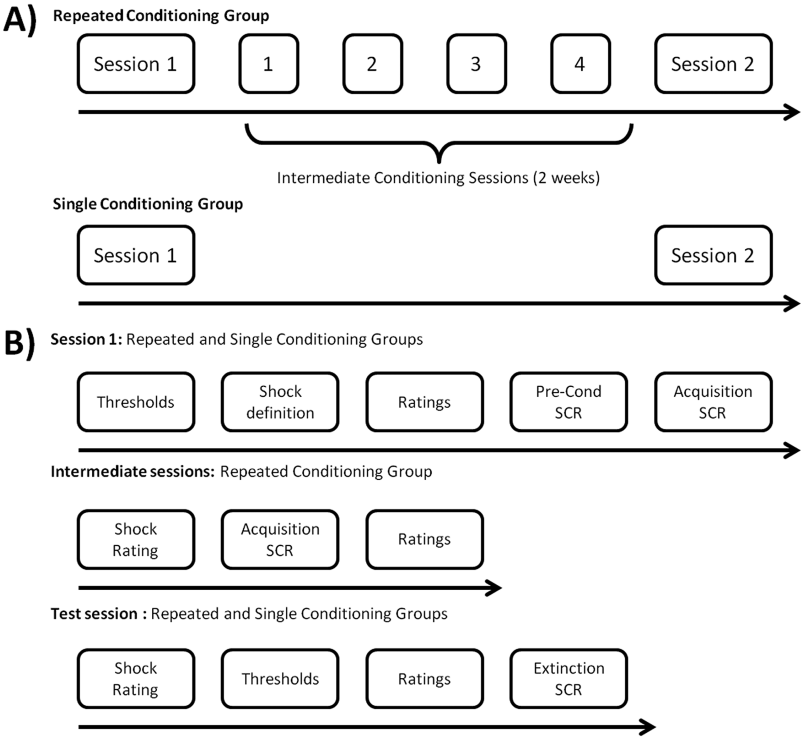


**Figure 2.** (**A**) Experimental design. Both groups participated in an initial conditioning session (Session 1). Additionally, the repeated conditioning group only participated in 4 conditioning sessions (Sessions 2–5), each separated by 2–3 days. Both groups then returned for a final test session (Session 6), 3 days following the last conditioning session for the repeated conditioning group. (**B**) The initial conditioning session (Session 1) consisted of baseline measurements of detection thresholds to the olfactory CSs. A shock work-up procedure followed. Subsequently, participants rated the pleasantness and intensity of all CSs. Baseline SCRs to olfactory and visual CSs were then measured before the conditioning session started. All intermediate sessions were identical and consisted of an initial shock presentation that was rated on pleasantness and intensity before the conditioning block started. CSs were subsequently rated on pleasantness and intensity at the end of the session. In Session 6, the shock stimulus was rated before measurement of detection thresholds to olfactory CSs. Ratings of all CSs were then collected before SCRs to the CSs were registered.

extinction processes—has been used (partial reinforcement extinction effect; LaBar et al. 1998; Phelps et al. 2004).

**Intermediate sessions (Sessions 2–5)**

Twice a week during the 2-week period between the initial and the final sessions, the repeated conditioning group participated in reinforcement sessions that were identical to the previously mentioned aversive conditioning portion of the initial session (40 trial repetition, 10 per odor and 10 per image). Between Session 2 and 3, there was a 3-day break for the weekend.

**Test phase (Session 6)**

Both groups subsequently participated in the final session that was seemingly identical to the first session but with the important difference that at no time was any aversive stimulus delivered. In other words, each odor and each image was presented a total of 20 times each with no pairing with the aversive stimulus for either odor and either image in the final session.

### Data reduction and statistical analyses

SCRs were computed for each condition separately. For each condition, the average electrodermal activity 0.5 s prestimulus was subtracted from the peak response within 10 s post-stimulus onset (Boucsein 2012). Responses greater than 2.5 standard deviations (SDs) away from the individual mean were excluded on an individual level (max. 2 responses per condition/participant) and average evoked SCR for each condition and individual were calculated (Boucsein 2012). Two participants (1 in each group) had averaged SCR more than 2.5 SDs away from the mean and were excluded from analyses. Note that only the 50% of the trials where the odor/visual stimuli were not followed by the shock stimulus are included in the SCR analyses, except for Supplementary Figure S1 where the response to the US (the shock) itself is displayed separately.

To align and equate the 2 odor detection threshold tests independent of their unique distribution, we initially *Z*-transformed the threshold tests for each odor separately. One individual in the Repeated Conditioning group expressed a *Z*-score greater than 3.0 SDs from the mean for the threshold of 1 odor. This participant was designated as an outlier and subsequently removed from further analyses. The effect of repeated conditioning on detection threshold was statistically assessed in 2 ways. First, to assess main and interaction effects, we performed repeated-measures analyses of variance (rm-ANOVA) with session (initial vs. final) and conditioned stimulus (CS) type (CS+ and CS–) as within-subjects repeated variables, separately for both groups, using the *Z*-transformed detection threshold scores as independent measures. Second, to directly assess whether aversive pairing altered detection threshold independent of initial values, we subtracted initial from final session values on an individual level for each odor condition and submitted these to 1-sample Student's *t*-tests against the expected value indicating no change due to aversive pairing, that is, 0. The comparison between modality-dependent conditioning effects was explored by means of rm-ANOVAs with modality (vision vs. olfaction), session (initial vs. final) and CS-type (CS+ and CS–) as within-subjects repeated variables, separately for both groups. For all ANOVAs, simultaneous multiple comparisons were adjusted with the Bonferroni's method and whenever the sphericity assumption was violated, Greenhouse-Geisser corrections were applied. Spearman's correlations were calculated to assess the relationships between arousal and olfactory threshold detection as well as the intensity of the aversive electrical stimulus.

## Results

Refer to Table 1 for demographic information, psychometric, and perceptual characteristics as well as for the assessment of statistical differences between the 2 groups. Demographic, psychometric, and perceptual rating measures were similar across groups and across time (Supplementary Figure S1).

### Aversive learning increases sensory detection sensitivity

To determine whether there were differences in the threshold performance toward the 2 odors, we conducted an rm-ANOVA with stimulus (butanol, peanut) and session (1, 6) as within-subjects factors per group (repeated conditioning vs. single conditioning group). As expected, results indicate no significant main or interaction effects ($P_s > 0.05$). Data were subsequently collapsed and analyzed according to their learning value (i.e., stimulus paired [CS+] or not paired [CS–] with shock) to explore effects of training on odor sensitivity. When separately exploring the 2 groups, a significant change in odor detection threshold was evident for the repeated conditioning group [Session: $F(1, 15) = 14.43$, $P = 0.002$, $\eta^2 = 0.11$] but not for the single conditioning group [Session: $F(1, 18) = 0.02$, $P = 0.90$, $\eta^2 = 0.0004$; Figure 3]. To further investigate the source of the CS-type × session interaction, we conducted *post hoc* analyses within groups. The interaction in the repeated conditioning group was due to a significant increase in odor detection threshold sensitivity for the CS+ odors between before and 2 weeks after the initial aversive pairing, that is, an effect of training [$t$ (15) = –2.84, $P = 0.01$, $d = 0.71$]. In addition, a significant decrease in odor detection sensitivity was further observed in this group for the CS– odors [$t$ (15) = 2.10, $P = 0.05$, $d = 0.52$; Figure 3]. Importantly, there were no significant differences in the single conditioning group between the initial and the final session 2 weeks after the first aversive pairing for either the CS+ odors [$t$ (18) = –0.30, $P = 0.80$, $d = 0.06$] or the CS– odors [$t$ (18) = 0.45, $P = 0.66$, $d = 0.10$]. Within the repeated conditioning group, 12 out of 16 individuals demonstrated a nominal increase in detection performance for the CS+ odors, 2 a nominal decrease, and 2 no difference (*Z*-score change ± 0.1). Similarly, 10 out of 16 individuals demonstrated a nominal decrease in detection performance for the CS– odors, 3 a nominal increase, and 4 no difference (*Z*-score change ± 0.1; Figure 4). Although one should note the very limited statistical power, there were no significant differences between the 2 odors in either the repeated conditioning group or the single conditioning group for the CS+ and CS– condition. For a depiction of the data in dilution steps, please refer to Supplementary Figure S2.

**Correlation between intensity of the shock level and detection threshold**

Given that subjective intensity of the shock was similar for all participants (i.e., all determined a subjective intensity of 7/10 in an irritation scale), we assessed whether the corresponding objective intensity of the aversive stimuli was responsible for the magnitude of the above-demonstrated odor learning augmentation. One might suggest that the higher the intensity of the aversive stimulus, the greater the interoceptive signals processed and therefore the greater the learning dependent shift in CS+. Spearman's rank correlations tests were conducted on the average stimulus intensity (in mA) selected by the participant with the magnitude of their learning-dependent shift in sensitivity for the CS+ odor. Although the objective intensity of the shock tends to differ between the repeated and the single conditioning groups [3.82 (2.30) vs. 2.59 (1.64) mA, $t$

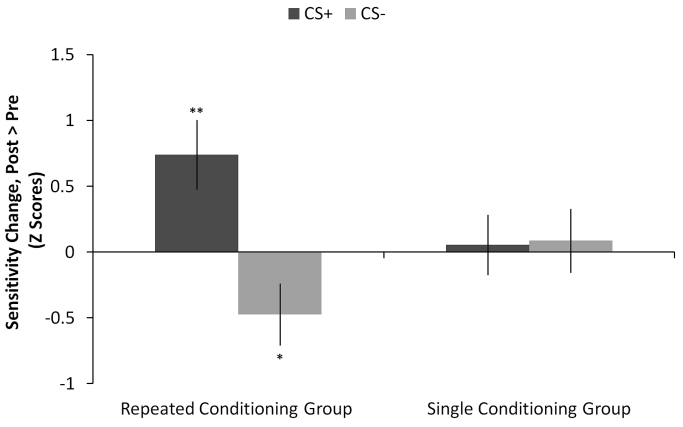


**Figure 3.** Effects of aversive conditioning on odor detection thresholds for each group and odor CS type. Values are displayed in *Z*-transformed dilution steps, to allow merging of the 2 detection thresholds, and are plotted as mean change from Session 1 to the last post-acquisition session 2 weeks later (Session 6). The repeated conditioning group participated in 4 fear conditioning sessions between Session 1 and 6, whereas the single conditioning group did not receive any additional training beyond Session 1. Dark gray bars indicate odor paired with the aversive stimulus (CS+) and light gray bars indicate odor not paired with the aversive stimulus (CS–). Error bars in graphs denote standard error of the mean. $*P < 0.05$ and $**P < 0.01$.

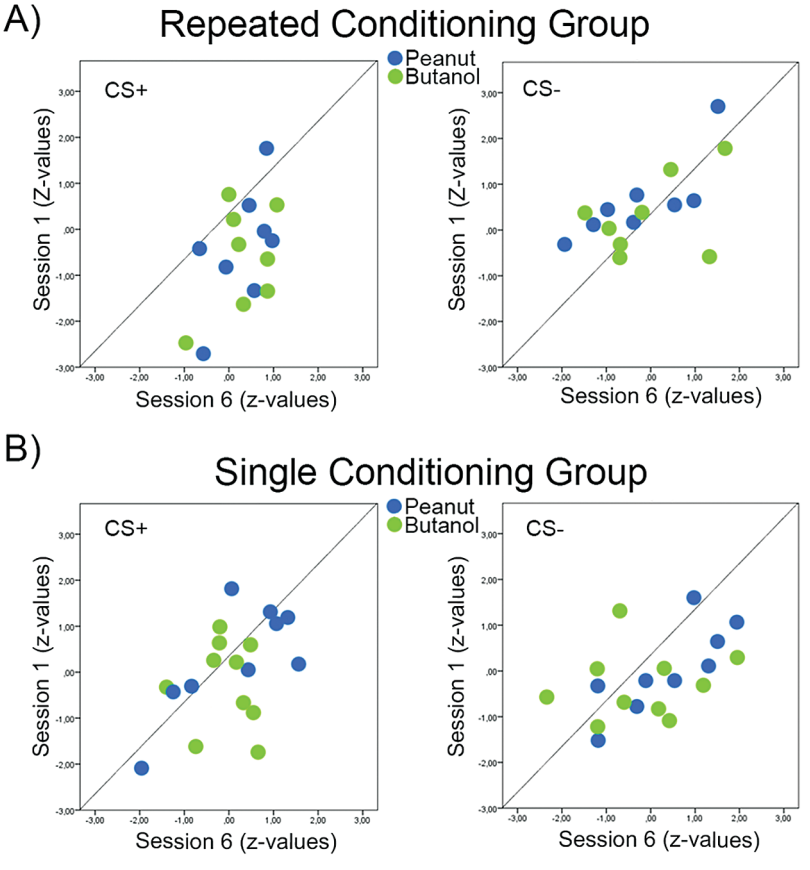


**Figure 4.** Individual changes in detection threshold for each experimental manipulation expressed in *Z*-scores. The diagonal line in each graph indicates no change and blue dots indicate peanut odor as CS +/CS– and green dots indicate butanol odor as CS+/CS–. (**A**) Individuals within the repeated conditioning group change in odor detection threshold from Session 1 to 2 weeks later in Session 6 with repetitive reinforcement of CS+ but not CS–; each shown in separate graphs. (**B**) Individuals within the single conditioning group change in odor detection threshold from Session 1 to 2 weeks later (3 days since last training) in Session 6 with no repetitive reinforcement of either CS+ or CS– in-between; each shown in separate graphs.

(18) = 1.882, $P = 0.068$], correlation tests revealed no significant modulation of shock levels on the demonstrated learning effects [repeated conditioning group: $r_s = 0.08$, $P = 0.77$; single conditioning group: $r_s = 0.22$, $P = 0.37$].

## Skin conductance responses

### Repeated versus single conditioning

Considering that both groups differ in training (repeated vs. single exposure), as well as in time since last training (14 days for the

repeated conditioning group vs. 3 days for the single conditioning group), we did not directly compare the 2 groups in between-subjects analyses. To reveal the presence of differences in the skin conductance responses toward the 2 odors, we conducted an rm-ANOVA with stimulus (butanol, peanut) and session (1, 6) as within-subjects factors. For both groups, only the main effect of session reached the significance level [repeated conditioning: $F(1, 15) = 3.99$, $P = 0.06$, $\eta^2 = 0.21$; single conditioning: $F(1, 18) = 8.95$, $P = 0.001$, $\eta^2 = 0.33$], indicating a stimulus-independent post-conditioning change in SCR. As hypothesized, results indicate no other significant main or interaction effects involving the stimulus ($P_s > 0.05$). The same procedure was applied to determine the differences between visual images. The main effect of session was significant in both groups [repeated conditioning: $F(1, 15) = 6.80$, $P = 0.02$, $\eta^2 = 0.31$; single conditioning: $F(1, 18) = 9.55$, $P = 0.006$, $\eta^2 = 0.35$] whereas the 2-way interaction image × session was significant only for the single conditioning group: $F(1, 18) = 4.44$, $P = 0.05$, $\eta^2 = 0.20$]. However, *post hoc* contrasts indicated that the difference in the interaction was only driven by the session factor. Therefore, data were collapsed and accounted for according to their learning value. Consistent with fear conditioning, we observed greater SCRs to the CS+ than to the CS– in both groups [repeated conditioning: $F(1, 15) = 5.93$, $P = 0.03$, $\eta^2 = 0.28$; single conditioning: $F(1, 18) = 5.48$, $P = 0.03$, $\eta^2 = 0.23$; Figure 5 for mean SCR]. SCR was greater to olfactory as compared with visual CSs in the repeated conditioning group only: $F(1, 15) = 4.82$, $P = 0.04$, $\eta^2 = 0.24$, although following the first acquisition session this pattern was present overall: $F(1, 34) = 5.74$, $P = 0.02$, $\eta^2 = 0.14$ and habituated from the initial Session 1 to the final Session 6, 2 weeks later in both groups [session: repeated conditioning: $F(1, 15) = 5.27$, $P = 0.04$, $\eta^2 = 0.26$; single conditioning: $F(1, 18) = 9.49$, $P = 0.006$, $\eta^2 = 0.35$]. We also found a greater decrease in SCR from the acquisition phase to Session 6 to olfactory CSs than to visual CSs in the single conditioning group (modality × session: $F(1, 18) = 5.01$, $P = 0.04$, $\eta^2 = 0.22$). We did not observe any difference in conditioned responses between visual and olfactory CS+ as compared with the modality controlled CS– in either group [modality × CS-type: repeated conditioning: $F(1, 15) = 0.67$, $P = 0.80$, $\eta^2 = 0.004$; single conditioning: $F(1, 18) = 1.98$, $P = 0.18$, $\eta^2 = 0.10$].

### Correlation between skin conductance responses and detection threshold

There was no significant association between the increased arousal demonstrated toward the target odor in Session 6 and change in detection threshold for either the repeated conditioning group [$r_s(14) = 0.12$, $P = 0.67$] or the single conditioning group [$r_s(17) = -0.29$, $P = 0.22$].

### Visual versus olfactory repeated conditioning

Focusing on the differences between visual and olfactory conditioning, we explored the SCRs of the repeated conditioning group only. The rm-ANOVA with modality × CS-type x session (1 through 6) revealed that odors produced a generally higher SCR amplitude than visual stimuli (0.44 vs. 0.31 µS; $F(1, 15) = 4.64$, $P = 0.05$, $\eta^2 = 0.02$), but this modality effect was not interacting with either CS-type [$F(1, 15) = 1.67$, $P = 0.22$] or session [$F(1, 15) = 1.22$, $P = 0.31$]. As expected, the SCR amplitude associated with the stimulus paired with the shock was greater than that for the stimulus unpaired with the shock [$F(1, 15) = 7.85$, $P = 0.01$, $\eta^2 = 0.02$] and the SCR amplitude recorded from each session was not significantly different; however, a tendency toward significant habituation was detected [$F(1, 15) = 2.73$, $P = 0.07$, $\eta^2 = 0.10$, Greenhouse-Geisser corrected].

### SCR and rating responses over time for repeated conditioning

We finally explored the pattern over time for the paired, unpaired, and US conditions. By merging the SCR data from the visual and olfactory CSs, we documented a significant difference between the CS+ and CS– conditions in Session 2, 3 and 4. This difference became nonsignificant in the subsequent sessions (5–6), indicating that habituation processes have occurred. The SCR responses for both visual and olfactory stimuli presented with the shock stimulus (US; Figure 6) significantly decreased from Session 1–3, from where they plateaued. Contrary to these trends, the subjective ratings of the shock intensity and shock pleasantness remained stable throughout the 5 conditioning sessions.

## Discussion

The present study investigated whether repeated fear conditioning over 14 days induced lasting changes in olfactory sensitivity to the odors that served as CSs. We found that repeated fear conditioning, in contrast to a single fear conditioning session that was followed by a long retention interval, increases detection sensitivity of the CS+ at least 3 days following training. The increase was of similar magnitude to previously published data employing a brief learning paradigm (Åhs et al. 2013). Also, for the first time we directly compared visual and olfactory learning effects over time, exploring how different sensory modalities characterize aversive conditioning. We demonstrate that odors are more arousing than visual stimuli, yet aversive learning is similar across stimulus-modalities. These findings reveal that olfactory sensitivity is experience-dependent and can last for at least 3 days (potentially 2 weeks) as well as that olfaction

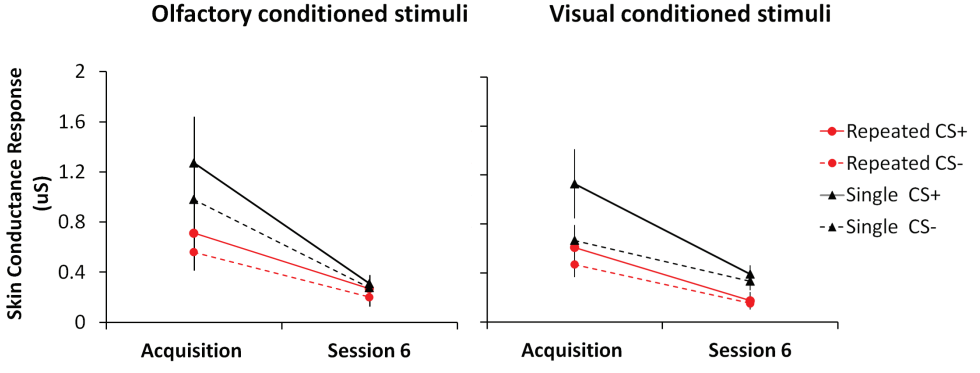


**Figure 5.** Mean skin conductance responses to (**A**) olfactory and (**B**) visual CSs during initial fear conditioning. CS+: reinforced conditioned stimulus; CS–: unreinforced control stimulus.

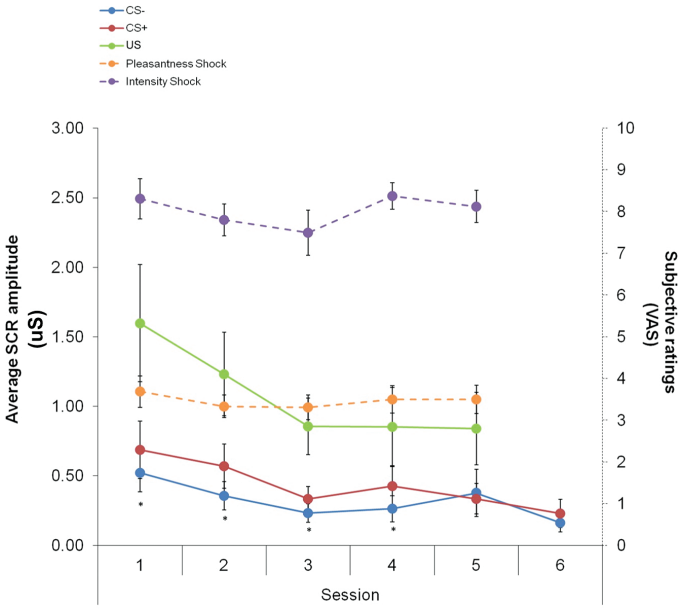


**Figure 6.** Solid lines depict the average skin conductance responses at each of the 6 measuring times for merged visual and olfactory CS paired with shock (CS+) and unpaired with shock (CS–) as well as merged US, that is trials in which the shock was simultaneously present with the visual or the olfactory stimulus. Dotted lines depict subjective ratings of the intensity and unpleasantness of the shock stimulus for each of the 6 measuring time points. Note that scale for the latter is indicated on the right side of the graph. Stars in graph indicate time points of statistical differences between CS+ and CS– ($P < 0.05$) and error bars indicate standard error of the mean (SEM). CS+: reinforced conditioned stimulus; CS–: unreinforced control stimulus.

has a unique role in emotional experiences. The nature of the paradigm, composed around our primary interest, namely olfaction, prevented from the use of threshold visual tests analogous to those used in the olfactory modality. Therefore, whether these differences in cross-modal arousal have a behavioral counterpart has yet to be determined in the visual modality.

It has been demonstrated that aversive conditioning of an odorant not only modulates processing within the olfactory cortex (Li et al. 2008; Chen et al. 2011; Choi et al. 2011) and the olfactory bulb (Fletcher and Wilson 2003; Moreno et al. 2009), but also at the level of the olfactory receptors (Jones et al. 2008; Kass et al. 2013). Based on these findings, it has been speculated that aversive conditioning could modify absolute detection threshold of an odor (Kass et al. 2013). We can here demonstrate that aversive conditioning indeed modulates detection threshold for several days (at least 3) following acquisition. These findings extend our previous discovery of transient sensory augmentation following a single brief fear conditioning session (Åhs et al. 2013). Due to the limitations of human models, we are unable to directly assess whether these effects are mediated by receptor augmentation. It is, however, of interest to note that the participants following repeated fear conditioning on average detected a stimulus that was approximately 69% lower in concentration in comparison to their initial threshold, as estimated from absolute stimulus values (note that these are interpolated estimations from dilution steps and not absolute measures of the active compound). This increase in sensitivity closely matches the increase in the odor-driven synapto-pHluorin optical responses to olfactory stimuli in olfactory sensory neurons post-conditioning (Kass et al. 2013). It is therefore possible that the sensitivity-enhancing mechanisms reported by Kass et al. (2013) extend to humans. Although Kass et al. (2013) suggest that the mechanism can be related to a perceptual increase in suprathreshold odor intensity, our data do not seem to support such claim but rather indicate a specific effect on absolute detection.

Besides the increase in sensitivity to the CS+ odor, there was a significant decrease in sensitivity to the control odor (CS–). This is in line with the decrease in sensitivity to the CS– odorant reported by Åhs et al. (2013). The differential conditioning paradigm used here has been demonstrated to result in a reduction in odor receptor field size within the olfactory cortex (Chen et al. 2011), potentially leading to an increased odor specificity by presynaptic modulation of the olfactory sensory neuronal output (McGann 2013). There is an obvious evolutionary advantage for a learning-induced decrease in sensitivity, as it would reduce competition with dangerous stimuli and allow for swift and accurate response to these. The differential fear learning protocol may reduce the fear generalization observed when no control stimulus is present during fear learning (Resnik et al. 2011).

As outlined above, we can demonstrate that conditioning of an odorant augments participants' absolute sensitivity to an odor. Detection could, however, potentially be mediated by a heightened arousal felt towards the target odor (CS+) which would aid in detecting, consciously or nonconsciously, the presence of the odor in the detection threshold test (Johnston and Dark 1982). In other words, when exposed to the odor, the visceral information about an increase in arousal could alarm the individual and inform about the presence of the target odor even in the absence of a perceptual detection in line with the somatic marker hypothesis (Damasio 1996). The change in detection threshold of the target odor was, however, not correlated with the change in neither the objective intensity of the shock, nor in arousal, as measured *via* SCR in both the repeated conditioning and the single conditioning group, thus suggesting that visceral signals did not drive the increased sensitivity to the reinforced odor.

The differences in olfactory sensitivity to the CSs, as a function of repeated or single conditioning, were not reflected in SCRs. Although we noted robust acquisition and extinction of SCRs to the CS+ relative to the CS–, SCRs were similar in participants exposed to repeated or single fear conditioning in the final session 14 days later. This suggests that sensory reorganization following repeated conditioning, which

enhances olfactory sensitivity, can occur independently of changes in fear memory. The increased sensitivity for the CS+ does hence not directly trigger more intense conditioned responses. Enhanced sensory sensitivity following conditioning could serve as a bottom-up mechanism that explains previous reports of increased attentional bias for fear cues predicting shock (Pischek-Simpson et al. 2009). It is interesting to note that a distinct habituation occurred in the repeated conditioning group over time. Whereas there were clear differences between the reinforced and nonreinforced stimuli after Sessions 2, 3, and 4, after the 3-day delay between Sessions 5 and 6 (the weekend), the significant difference disappeared. Likewise, a similar reduction in SCR to the US stimuli was observed even though the perceptual intensity and unpleasantness of the shock stayed constant throughout the experiment. To the best of our knowledge, this is the first study exploring the effect on conditioned responses during aversive conditioning over an extended period of time (2 weeks) in humans and the mechanisms thereof are not well known. The origin of this dissociation between the stable subjective perception of the aversive stimulus and the marked reduction in orienting response should be addressed in future studies where potential mediating mechanisms can be more thoroughly explored.

This extensive characterization of the perceptual effects of olfactory aversive learning is well complemented by the demonstration that odors overall elicit greater physiological arousal as compared with similarly treated visual stimuli, suggesting a baseline difference between the SCR responses elicited by the 2 sensory modalities. However, the fact that differential conditioning (CS+/CS−) was not significantly affected seems to suggest that aversive learning does not have modality-specific correlates within this temporal window. Although this is the first direct comparison of aversive learning effects in the visual and olfactory domains, the present findings are in line with recent research showing a differential temporal course in emotional olfactory and visual information (Adolph and Pause 2012). These effects were not limited to 1 single odor or 1 image. It should be noted, however, that because we did not use odorants or visual stimuli spanning the entire stimulus space available to us, further studies is needed to determine whether odors demonstrate a superior aversive learning effect relative to visual images across the full with of both the odor and the visual stimulus space. Nonetheless, disgusting (aversive) odors produced greater withdrawal reactions as compared with pictures, suggesting that odors might be more potent emotional stimuli as compared with their visual counterparts (Adolph and Pause 2012). This would be no news for patients suffering from post-traumatic stress disorder (PTSD) or panic attacks, for whom odors constitute potent triggers (Hinton et al. 2004) and serve as traumatic reminders (Vermetten and Bremner 2003). Future research is left to determine how different modalities impact on the neural routes responsible for aversive conditioning in healthy participants as well as in patients with anxiety disorders.

Although the present study contributes significantly to the underresearched topic of olfactory emotional elicitation and long-term effects of fear-learning on detection sensitivity, the longitudinal design needed to assess this comes with limitations. By necessity, 1 possible confound in our study was that the time that elapsed between the last conditioning session and the test session differed between the single conditioning group (14 days) and the multiple conditioning group (3 days), meaning that we were not able to directly replicate our previous studies using an identical design and we were not able to completely trace the odor learning curve. To guarantee the feasibility of the present study, only the most relevant control conditions were included. However, to fully disentangle the effects of "time since last training" from the effects of "repeated training," and therefore account for the extinction learning that intermittent testing introduces, the inclusion of multiple additional groups would be necessary. Ideally, the to-be-added controls would include: (i) a group having Session 1 and 6 separated only by 3 days (single conditioning group—3 days); (ii) a no-odor control group; (iii) a no-visual info control group; (iv) a group experiencing noncontingent associations between the aversive stimulus and odors and images. Furthermore, a prepost threshold test for an odor not present in the learning procedure could be included to verify the effects of mere exposure on repeated olfactory sensitivity. These are questions that future studies will explore.

In conclusion, we demonstrate that the previously reported aversive conditioning-dependent effects on olfactory sensory neurons in rodents (Kass et al. 2013) may translate to humans. These results suggest that the sensitivity of the human olfactory sensory system is dynamically regulated by aversive learning and that olfaction is a sense forging a unique emotional experience whose features require further exploration.

## Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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