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ORIGINAL PAPER

Neural correlates of aversive conditioning: development of a functional imaging paradigm for the investigation of anxiety disorders

Isabelle Reinhardt · Andreas Jansen · Thilo Kellermann · André Schüppen · Nils Kohn · Alexander L. Gerlach · Tilo Kircher

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Abstract The purpose of the present study was to establish a short paradigm for the examination of classical aversive conditioning processes for application in patients with anxiety disorders. We measured behavioral, autonomic and neural correlates of the paradigm in healthy subjects, applying functional magnetic resonance imaging (fMRI) and measurement of skin conductance. Therefore, neutral visual stimuli were paired with an unpleasant white noise as unconditioned stimulus. Twenty healthy subjects performed three experimental phases of learning: familiarization, acquisition and extinction. Subjective ratings of valence and arousal after each phase of conditioning as well as skin conductance measurement indicated successful conditioning. During acquisition, fMRI results showed increased activation for the conditioned stimulus ($CS+_{unpaired}$) when compared with the non-conditioned stimulus (CS−) in the right amygdala, the insulae, the anterior cingulate cortex and the parahippocampal gyrus, all regions known to be involved in emotional processing. In addition, a linearly decreasing activation in the right amygdala/hippocampus for the CS− across the acquisition phase was found. There were no significant differences between CS+ and CS− during extinction. In conclusion, the applicability of this paradigm for the evaluation of neural correlates in conditioning and extinction processes has been proven. Thus, we present a promising paradigm for the examination of the fear-circuit in patients with anxiety disorders and additionally effects of cognitive-behavioral interventions.

Keywords Classical aversive conditioning · Amygdala · Anxiety disorders · Extinction · Panic disorder

I. Reinhardt (✉) · T. Kellermann · N. Kohn
Department of Psychiatry and Psychotherapy,
RWTH Aachen University, Pauwelsstr. 30,
52074 Aachen, Germany
e-mail: ireinhardt@ukaachen.de

I. Reinhardt · T. Kellermann · N. Kohn
JARA Translational Brain Medicine, Aachen, Germany

A. Jansen
Department of Psychiatry and Psychotherapy,
Philipps-University Marburg,
Marburg, Germany

A. Schüppen
Central Service Facility "Functional Imaging"
at the ICCR-Biomat, RWTH Aachen University,
Aachen, Germany

N. Kohn
Virtual Project House Gender and Technology,
RWTH Aachen University, Aachen, Germany

A. L. Gerlach
Department of Clinical Psychology, University of Münster,
Munster, Germany

T. Kircher
Department of Psychiatry and Psychotherapy,
Philipps-University Marburg, Marburg, Germany

Introduction

Classical aversive conditioning is a form of associative learning. Its general principles were first demonstrated by Ivan Pavlov in 1927. Typically, a neutral stimulus is repeatedly associated with an aversive stimulus (unconditioned stimulus, US), which provokes a reaction until the neutral stimulus becomes the so-called conditioned stimulus (CS) and elicits the conditioned reaction by itself. When the CS is then presented without being paired with the US, the conditioned reaction becomes weaker

with time (extinction). In a differential conditioning paradigm two similar, initially neutral stimuli are used; one is paired with the aversive stimulus (conditioned stimulus, CS+), whereas the other is presented without pairing (non-conditioned stimulus, CS−). Fear conditioning is considered as one central pathogenic pathway in the development of anxiety disorders [34, 36, 37]. Important hypotheses postulated, e.g. a preparedness of aversive associations [30], a generally enhanced conditionability in anxiety disorders [5, 33] or associative learning deficits in these patients [8, 18, 26], it has also been proposed that anxiety disorders are associated with elevated stimulus generalization [28].

In the last decade, several functional magnetic resonance imaging (fMRI) studies investigated the neural network underlying classical aversive conditioning in healthy subjects. Heterogeneous stimuli were used in these studies, e.g. tones, forms or pictures were used as CS. Furthermore, various US were applied, such as electrical shocks, odors or sounds [39]. Despite the great variety of the stimuli and specific experimental conditions employed in these studies, a characteristic fear network could be identified, which was consistently activated across all studies and consisted of the amygdala, the insulae and the anterior cingulate cortex (ACC) which showed stronger activations during the presentation of the CS+ when compared with the CS− [6, 12, 19, 35]. Most studies that reported increased amygdala activation for the CS+ also found a typical decrease in activation over time, e.g. [6, 22]; other studies however failed to show amygdala activation at all [13, 20, 21]. Extinction processes have also been investigated in several fMRI studies in healthy subjects demonstrating a special role for the amygdala and the medial prefrontal cortex [21, 22, 35]. However, these findings do not seem as clear and concise as in conditioning experiments [3, 29], especially concerning amygdala activation [35].

The brain regions activated in classical aversive conditioning paradigms in healthy subjects show a substantial overlap with the so-called "fear network" which is disturbed in patients suffering from anxiety disorders [12]. Therefore, classical aversive conditioning paradigms provide a pragmatic model to investigate the neural correlates of anxiety disorders [7, 16].

So far, there are only two fMRI studies that investigated conditioning and extinction processes in patients with anxiety disorders. In both studies, neutral faces were used as CS for patients with social phobia (SP). The authors showed evidence for a differential amygdala involvement in patients when compared with matched healthy controls [38, 43]: one study reported an increase in amygdala and hippocampal activation in the course of time during the acquisition phase in patients with SP, whereas a deactivation was shown in healthy control subjects [38], the other study found differential activation in the limbic–prefrontal circuitry in SP during conditioning [43]. Nevertheless, both clinical studies on conditioning processes in SP have some limitations: In the first study [38], only 16 slices were measured to shorten the scanning time (which of course is advantageous for anxious patients). Moreover, they used a 100% reinforcement strategy. A 50% reinforcement strategy, however, is more appropriate for the examination of extinction processes because this intermittent strategy helps in slowing down extinction processes [35]. In the second study [43], skin conductance was measured during scanning, which prolonged the paradigm due to the necessary inter-stimulus time interval needed in skin conductance measurements. Another limitation of both studies is the use of biologically salient and disorder relevant stimuli (faces were used as conditioned stimuli for social phobics) that may facilitate conditioning for these patients [27], but is limited to the specific disorder which is examined. Furthermore, in both conditioning studies, extinction processes have not been reported despite their relevance for treatment in anxiety disorders [3]. There are no other fMRI studies examining conditioning or extinction processes in anxiety disorders until now. Although several behavioral studies have already been conducted [e.g. 26], the neural basis of conditioning in anxiety disorders, for example in panic disorder, remains elusive.

In the present study, we, therefore, set up a differential conditioning paradigm looking at all three classical phases (familiarization, acquisition and extinction). The US was presented unpaired in the familiarization phase to avoid novelty effects which may interfere with the conditioning process during the acquisition phase. All three phases of conditioning offer important insights into the development of anxiety disorders: The familiarization phase helps in examining habituation processes to the US without any conditioning taking place. Moreover, initial arousal of the patients may be reduced by introducing a longer familiarization. The avoidance of novelty effects concerning the US in the acquisition phase and an additional control for the neutrality of the stimuli are other advantages in this context. This helps to examine the conditioning processes themselves which take place in the acquisition phase. Lastly, the examination of extinction processes leads to a better understanding on how fears can be diminished and is, therefore, an essential part of conditioning studies in anxiety disorders. We used neutral, not biologically salient or disorder-specific stimuli.

The aim of this study was to establish a new paradigm which is suitable for different kinds of anxiety disorders (e.g. panic disorder) without any disorder-specific relevance for the patients. We, therefore, renounced to use

stimuli which could be "biased" by enhancing conditioning processes in certain kinds of disorders, such as e.g. faces in social anxiety disorder or in patients with comorbidities [27]. The use of neutral stimuli (like geometrical forms) has the same impact on all patients and may also help to disentangle the effects of "selective sensitivity to social-related conditioning experiences" from a "general proclivity toward associative fear conditioning" [27]. For the examination of conditioning processes in patients with anxiety disorders, especially for patients with panic disorder with agoraphobia, paradigms have to be as short as possible. Therefore, we aimed to reduce scan time by leaving out skin conductance measurements during scanning which prolong measurements due to longer interstimulus intervals needed for recovery of skin conductance. Subjective ratings of emotional valence and arousal were obtained after each phase of conditioning, whereas autonomic arousal as a physiological index for associative learning was measured in a separate experiment.

Materials and methods

Subjects

Twenty healthy subjects participated in the fMRI study. To have a homogenous sample, all participants were male and right handed (laterality index >80, as measured by the Edinburgh Handedness Inventory) [32] and between 22 and 44 years (mean age 28.8 ± 6.1 years). Written informed consent was obtained prior to the participation according to the declaration of Helsinki. The study was approved by the local ethics committee. Participants were excluded when they had been diagnosed with a past or present psychiatric, neurological, or medical disease. None of the participants showed abnormalities in brain morphology as assessed with T1-weighted MR images. A short test battery comprising the German estimated verbal intelligence test (MWT-B) [24], Beck's Depression Inventory [1] and the State Trait Anxiety Inventory [41] was administered. None of the participants showed abnormalities concerning any of the questionnaires; 14 out of the 20 subjects participated twice in the experiment. In this second experiment, we measured the skin conductance response (SCR) outside the scanner with a parallel version of the same classical conditioning paradigm. Six subjects were not available for the second experiment. The SCR measurements took place either at least 3 weeks before or after the fMRI scan.

Paradigm

The fMRI paradigm consisted of three phases: familiarization, acquisition and extinction (Fig. 1). As neutral stimuli simple geometric forms were chosen, a yellow and a blue sphere.[1] Each sphere was presented visually for 2,000 ms, the inter-stimulus interval (ISI) between the presentation of the spheres varied between 4.785 and 7.250 s. A fixation cross was presented during these intervals and participants were instructed to fixate the cross. As US an unpleasant white noise was used which was presented for 100 ms with a loudness of about 100 dB (adjustment between 95 and 105 dB, depending on individual judgment of aversiveness outside of the scanner before starting of the measurement). After each learning phase, we obtained self-report ratings of emotional valence and arousal (SAM self assessment manikin) [23] for both spheres using a 5-point Likert Scale (1 = 'very unpleasant' to 5 = 'very pleasant' and 1 = 'not arousing' to 5 = 'very arousing'). During the familiarization phase, both neutral stimuli (CS+ and CS−) were presented 16 times each. To eliminate novelty effects for the unpleasant noise during conditioning in the acquisition phase, the US was also presented in the familiarization phase for 16 times; however, without being paired to any of the spheres. The ISI between the US and the preceding as well as the succeeding stimulus, therefore, varied also between 4.785 and 7.250 s. Presentation of all stimuli was randomized. During the acquisition phase, one sphere was pseudo-randomly paired with the US (thus becoming CS+), while the other sphere was not (thus becoming CS−). The presentation of the US occurred 1.9 s after the presentation of the conditioned sphere, both stimuli terminated simultaneously. Each sphere was presented 32 times. A partial reinforcement strategy in which only 50% of the CS+ stimuli were paired with the US ($CS+_{paired}$) was used, while the other 16 events were presented without US pairing ($CS+_{unpaired}$). This strategy makes comparison of brain activity related to the conditioned sphere possible without including potential confounding effects of the associated aversive noise [6]. During the extinction phase, both spheres (CS+ and CS−) were again presented 16 times; however, without pairing with the US, the US was not presented.

The colors of the stimuli serving as CS+ and CS− were counterbalanced for all participants. The stimuli were presented by MR-compatible LCD goggles and a head set (VisuaStim XGA, Resonance Technology Inc., LA, USA). Stimulus presentation was controlled by the software package presentation 10.1 (Neurobehavioral Systems, http://www.neurobs.com).

[1] In order to avoid influences on conditioning processes, we changed the colour of the stimuli during the skin conductance measurements. Instead of yellow and blue spheres, we used green and violet stimuli.

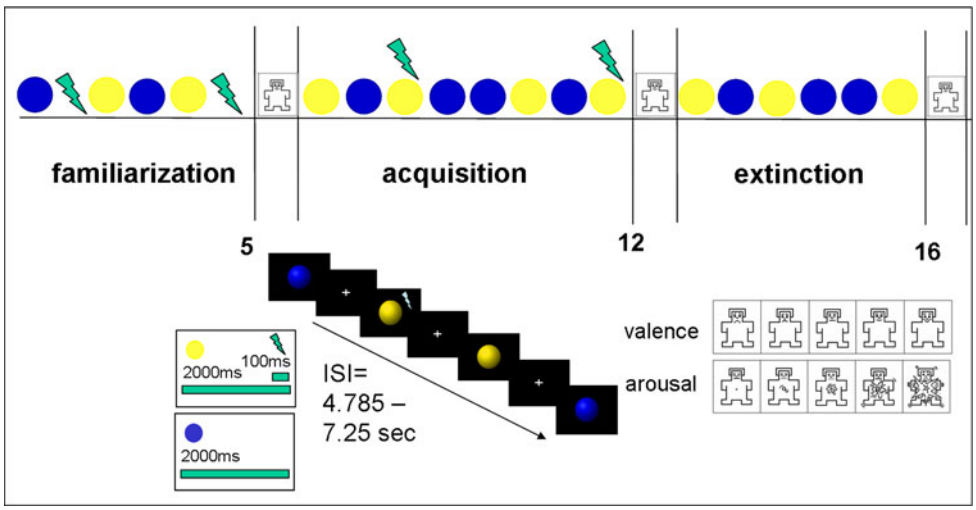

Fig. 1 Description of the paradigm: in the familiarization phase each stimulus was presented randomly 16 times. In the acquisition phase, a 50% reinforcement strategy was employed. One stimulus was presented with the aversive noise (CS+), whereas the other remained without consequence (CS−). During extinction, the stimuli were presented randomly without pairing with the US. After each phase of learning, subjective ratings of valence and arousal were obtained for both spheres

MRI data acquisition

All MRI data were acquired at the University Hospital of Aachen, Germany, on a 3T whole body scanner (Gyroscan Achieva, Philips Medical Systems, Best, The Netherlands) equipped with a standard head coil. Functional images were acquired using a T2*-weighted EPI sequence (TE = 30 ms, TR = 2 s, flip angle 90°, slice thickness 3.75 mm, inter-slice gap 0.4 mm, matrix 64 × 64, FOV = 240 mm, 32 axial slices oriented parallel to the AC-PC line covering the whole brain). In total, 505 volumes were collected (including 5 dummy scans to minimize T1 effects). Head motion was minimized using foam padding. In addition, high resolution structural images were acquired (three-dimensional T1*-weighted MPRAGE sequence, TE = 4 ms, TR = 2.2 s, TI = 1.2 s, flip angle 15°, slice thickness 1 mm, matrix 256 × 256, FOV 256 mm, 160 sagittal slices). Total scanning time for the conditioning paradigm was 16 min 50, the structural scan lasted 11 min. We employed a rapid event-related design.

Analysis of fMRI data

SPM5 (http://www.fil.ion.ucl.ac.uk/spm) standard routines and templates were used for analysis of fMRI data. The functional images were temporally and spatially aligned, normalized (resulting voxel size $2 \times 2 \times 2$ mm^3), smoothed (10-mm isotropic Gaussian filter) and high-pass filtered (cut-off period 120 s). None of the subjects had movement artifacts exceeding a critical threshold of 3 mm as assessed by the realignment parameters. Statistical analysis was performed in a two-level, mixed-effects procedure. In total, we build three different statistical models. The first model was used to analyze brain activation differences related to the onset of the different stimuli (model 1), the other two models were used to investigate time-dependent activation changes in a linear and exponential fashion, respectively (models 2 and 3).

Model 1

At the first level, the BOLD response for each event type (CS+, CS−, US) and each phase (familiarization, acquisition, extinction) was modeled by the canonical hemodynamic response function employed by SPM5 within the framework of the general linear model. In total, ten regressors were used: In the familiarization phase, the BOLD responses for both spheres (CS+, CS−) and the US were modeled. In the acquisition phase, regressors for the non-conditioned sphere (CS−), the US, the conditioned sphere presented with the US ($CS+_{paired}$) and the conditioned sphere presented without US ($CS+_{unpaired}$) were modeled. In the extinction phase, the BOLD responses for both spheres (CS+, CS−) were modeled. In addition, a regressor modeling the BOLD response during the behavioral assessments was created to reduce nuisance in baseline activation. Parameter estimates (β-) and t statistic images were calculated for each subject. At the second level, the individual β-contrast images were used to determine activations at the group level. Group activations were assessed by a flexible factorial model within the framework of SPM5 with ten different conditions (familiarization CS+, CS−, US, acquisition $CS+_{sphere}$, $CS+_{noise}$, $CS+_{unpaired}$, CS−, extinction CS+, CS−, SAM).

Models 2 and 3

We additionally investigated time-dependent activation changes within each phase, specifically to test the hypothesis that the amygdala shows a time-specific activation decrease in the acquisition phase. Therefore, we included two further first-level statistical models nine

additional regressors for each condition (expect for the behavioral assessment) that modeled linear and exponential activation changes, respectively. At the second level, group activations were assessed by one-sample *t* tests. We were specifically interested in the following contrasts:

1. Basic contrasts (model 1): activity related to the onset of the US and activity related to the onset of CS+ and CS−, separately for each phase as well as summed over all phases. For these contrasts, strong activation in the auditory and the visual cortex, respectively, were expected. These contrasts were used on a group as well as on an individual level to test that technical equipment and data analysis processes were correct.
2. Differential contrasts (model 1): the fear-circuit was investigated by the comparison of brain activity related to the CS+ and the CS−. Activation differences were assessed in two ways: First, the direct contrast of $CS+_{unpaired}$ and CS− within each phase ("direct comparison") were examined. Second, the interaction effects of stimulus type and conditioning phase were assessed by the differential contrast between the CS+ and the CS− which was subtracted from the same differential contrast in the other phases ('interaction analysis').
3. Time-dependent contrasts (models 2 and 3): time-dependent activation changes were assessed by the appropriate phase-specific contrasts as described before.

Because we assumed that the differences between CS+ and CS− might be small, we chose to employ Monte-Carlo simulation of the brain volume to establish an appropriate voxel contiguity threshold [40]. This correction has the advantage of higher sensitivity to smaller effect sizes, while still correcting for multiple comparisons across the whole brain volume. Assuming an individual voxel type-I error of $P < 0.001$, a cluster extent of 81 contiguous resampled voxels was indicated as necessary to correct for multiple voxel comparisons at $P < 0.05$. In addition, we performed a region of interest analysis for the amygdala and the insulae, both regions being key regions for conditioning. Here, we threshold the activations patterns at $P < 0.01$ (uncorrected) and applied a small volume correction (as implemented in SPM5) using a statistical threshold of $P < 0.05$ corrected for multiple comparisons. Coordinates are listed in Talairach and Tournoux atlas space [42]. In addition, amygdala activations were confirmed by the anatomy toolbox as implemented in SPM [10].

Analysis of behavioral data

Behavioral data was analyzed using SPSS 15 (SPSS Inc., Chicago, USA). SAM ratings were obtained after each phase of conditioning concerning both spheres. They were analyzed separately for valence and arousal with a two-factor analysis of variance (ANOVA) for repeated measures with the factors phase (familiarization; acquisition; extinction) and stimulus (CS+; CS−); *t* tests were performed afterwards.

Analysis of skin conductance data

The measurement of skin conductance was performed in a separate session outside of the scanner. We employed the same paradigm as in the fMRI part, but extended the inter-ISI to 12–14 s after each stimulus onset. This was necessary because SCRs need more time for recovery [4]. SCRs were measured from two electrodes on the hypothenar of the left hand by a BIOSEMI skin conductance system (Biosemi Systems, The Netherlands) using AgAgCl electrodes and electrode gel. The electrode cable was grounded and we used an auto-offset of 3 s. The signal was amplified and sampled at 2,048 Hz; further offline processing was performed with self-written MATLAB routines (The Matworks, Natick, MA). Data were re-sampled at 16 Hz and subsequently filtered (2 Hz). We were specifically interested in skin conductance differences related to the presentation of $CS+_{unpaired}$ and CS−, respectively, during the acquisition phase. This difference is a widely accepted index for learning in Pavlovian conditioning [4]. For quantitative analysis of the evoked signal, responses were characterized by the maximum of the SCR signal between 1 and 5 s after stimulus onset. To account for baseline fluctuations, this value was subtracted from a baseline, defined by the mean of the SCR in a 1-s time interval before the onset of the stimulus. SCR in the acquisition phase was only analyzed on trials that did not co-terminate with the presentation of the US. After the data were *z* standardized, all values exceeding more than two standard deviations from the mean of the values of each subject were considered as artifacts and eliminated from the evaluation. The mean responses towards $CS+_{unpaired}$ and CS− were compared by a Wilcoxon test for non-parametric comparisons.

Results

Behavioral data (SAM)

A two-factor repeated measures ANOVA for the ratings of valence of the CS during the three experimental conditions revealed a significant main effect for the factor stimulus (CS+; CS−) ($F = 8.00$; $df = 1$; $P < 0.01$, partial η squared (h_p^2) $= 0.30$) and a significant interaction between phase and stimulus ($F = 9.48$; $df = 2$; $P < 0.01$, $h_p^2 = 0.51$). Concerning the arousal, a two-factor repeated measures

ANOVA for the ratings of the CS during the three experimental conditions revealed a significant main effects for the phase ($F = 5.50$; $df = 2$; $P < 0.01$, $h_p^2 = 0.38$) and the stimulus ($F = 21.92$; $df = 1$; $P < 0.01$, $h_p^2 = 0.54$) as well as a significant interaction between phase and stimulus ($F = 8.91$; $df = 2$; $P < 0.01$, $h_p^2 = 50$). Post hoc t test revealed that both visual stimuli were judged equal in valence with a tendency to a positive rating after the familiarization phase [CS+ = 3.79 ± 0.79; CS− = 3.63 ± 1.07; $t(20) = 0.59$; $P = 0.56$]. In addition, there were no differences in the judgments concerning the arousal after the familiarization phase [CS+= 1.68 ± 1.00, CS− = 1.84 ± 0.90; $t(20) = -0.77$; $P = 0.45$]. After the acquisition phase, a conditioning effect was observed concerning in the evaluation of valence [$t(20) = -5.08$; $P < 0.01$] as well as the judgment of arousal [$t(20) = 5.64$; $P < 0.01$]: the CS+ was given a significant more negative evaluation of valence (CS+ = 2.84 ± 1.17; CS− = 4.05 ± 0.71) and a significantly higher evaluation of arousal (CS+ = 2.42 ± 0.1.07; CS− = 1.11 ± 0.32) after his association with the aversive sound, while the CS− became even more agreeable and retained its non-arousing attributes. In the extinction phase, no significant differences for subjective ratings were observed concerning the valence of both stimuli [CS+ = 3.37 ± 1.11; CS− = 3.63 ± 1.47; $t(20) = -1.55$; $P = 0.14$], whereas regarding the ratings of arousal, a slight difference between both stimuli remained (CS+ = 1.47 ± 0.84; CS− = 1.16 ± 0.38) [$t(20) = 2.33$; $P < 0.03$] indicating that the formerly CS+ was still evaluated as slightly more arousing when compared with the CS− after the extinction phase. In summary, behavioral data indicated a conditioning effect that mostly disappeared after the extinction phase (Fig. 2). Subjective ratings that were obtained during the measurement of the skin conductance in a separate session outside of the scanner were very similar to those acquired inside the scanner and are, therefore, not reported in detail here.

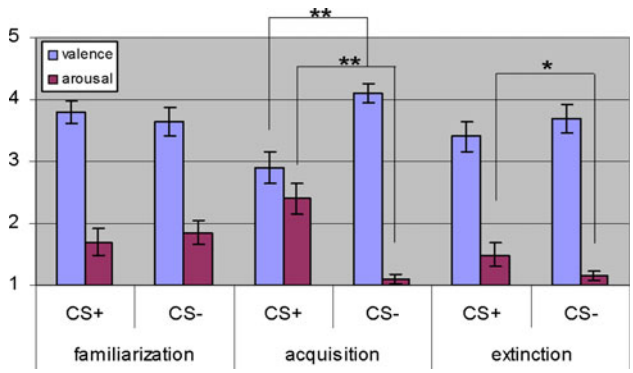

Fig. 2 Subjective evaluations of valence and arousal were obtained after each phase of conditioning: after familiarization, there were no differences concerning the ratings of valence and arousal towards both spheres. After the acquisition phase, the CS+ was given a significantly lower rating concerning its valence and a significantly higher rating in arousal as compared to the CS−. After extinction, there was no difference in the ratings of valence in both spheres, but the CS+ was rated slightly higher concerning its arousing attributes compared to the CS− (range 1–5) (*$P < 0.05$, **$P < 0.01$)

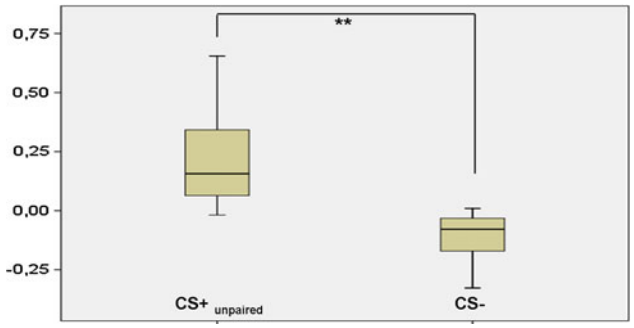

Fig. 3 Differential skin conductance responses between CS+$_{unpaired}$ and CS− during the acquisition phase were obtained in a separate session outside of the scanner ($N = 12$)

Skin conductance

We compared SCRs associated with the presentation of CS+$_{unpaired}$ and CS− across the acquisition phase. One subject was considered as a non-responder, another subject had to be excluded from the data analysis due to technical problems during the measurement. Across all subjects, a Wilcoxon test revealed differential SCRs between CS+$_{unpaired}$ and CS− ($W = -2.98$, $P < 0.003$). Subjects showed a significant increase in skin conductance during the presentation of CS+$_{unpaired}$ as compared to CS− across the acquisition phase (Fig. 3).

FMRI

Basic activation

During the presentation of the auditory stimulation (analysis of all auditory stimuli in the familiarization and the acquisition phase) increased activation in the auditory cortex A1 and associated areas were found (Fig. 4 left). During the presentation of the visual stimulation (analysis of all visual stimuli including the behavioral ratings), significant activation in the visual cortex could be shown (Fig. 4 right).

Differential activation of CS+ and CS−

In the direct comparison between CS+ and CS−, no differential activations could be found in the familiarization phase. Contrasting CS+$_{unpaired}$ and CS− during acquisition revealed increased activation in the CS+$_{unpaired}$ in areas which are well known to be part of the 'fear-circuit' in the

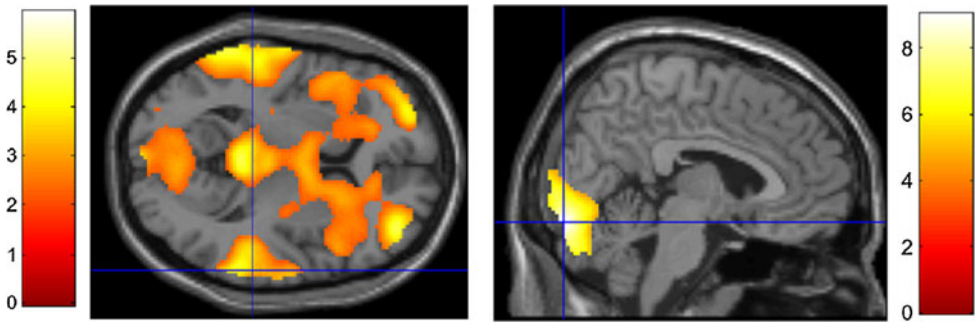

Fig. 4 The results indicate increased activation in the primary auditory area (A1) and associated areas ($P < 0.01$ FDR corr.) during the presentation of the auditory stimulation as well as increased activation in areas associated with primary visual processes (V1; $P < 0.05$ FWE corr.) during the presentation of the visual stimulation over all phases of the experiment

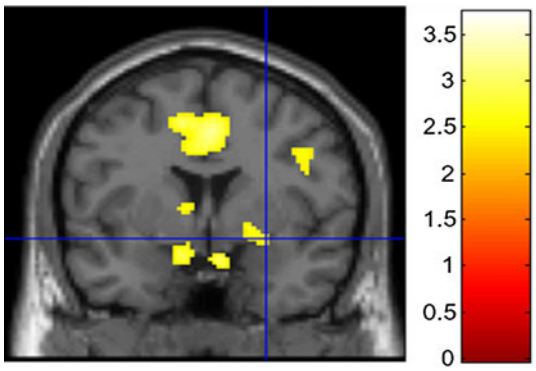

Fig. 5 Differential activation between $CS+_{unpaired}$ related to CS− during acquisition in the right amygdala, the insulae, the sub-genual ACC and the parahippocampal gyrus (the picture centered on the amygdala is shown, for illustrational purposes, at a statistical threshold of $P < 0.01$ uncorr.)

brain, such as the right amygdala, the insula bilaterally, the sub-genual ACC (BA 25) and the right parahippocampal gyrus (Fig. 5; Table 1). On the contrary, there were no increased activations in the CS− relative to the $CS+_{unpaired}$. In the extinction phase, there were no differential activations comparing CS+ and CS−.

Differential effects between CS+ and CS− were not only assessed within the different phases of conditioning, but also across different phases ('interaction analysis'). Although contrasting the acquisition and the familiarization phase, increased activations in the right amygdala and the right insula during the acquisition phase could be shown (Table 2). The inverse contrast did not reveal any differences in brain activation.

When comparing the differential contrasts in the acquisition and the extinction phase showed increased differential activation the right amygdala during acquisition. The opposite contrast revealed increased bilateral insula activation (Table 3).

There were no differences in fMRI activations between those subjects that differed concerning the time point of the fMRI measurement (before or after skin conductance measurement).

Temporal changes in the amygdala

Across the acquisition phase, a significant linear decrease in activation in the CS− was found in the right amygdala/hippocampus (see Fig. 6), but this activation did neither become significant for the $CS+_{unpaired}$ nor for the differential contrast between both conditions. There were no significant decreases in amygdala activation during familiarization or extinction. There were no significant increases in activation in the amygdala across all three phases. No significant exponential activation changes in the amygdala were detected in either condition.

Discussion

The aim of the study was to establish a new and very short paradigm with all three phases of conditioning for the examination of classical aversive conditioning processes which is suitable for an application in patients with anxiety disorders. Therefore, we examined behavioral, autonomic and neural correlates of the paradigm in healthy subjects. The main results can be summarized as follows:

Behavioral data and skin conductance measurements

Behavioral ratings of valence and arousal after each phase of the experiment indicated a clear conditioning effect: after the familiarization phase, there were no significant differences in the judgments of valence and arousal of both spheres. On the contrary, after the acquisition phase, the CS+ was rated significantly more negative in valence and significantly higher in arousal than the CS−. After the extinction phase, the CS+ was rated equally in valence, but still slightly more arousing than the CS−, indicating a (albeit not absolute) extinction in subjective evaluation.

Skin conductance data could only be obtained by 14 out of the 20 subjects in a separate session outside of the scanner which of course constrains its validity. Nevertheless, a significantly higher autonomic arousal after the presentation of the $CS+_{unpaired}$ as compared to the CS− during the acquisition phase could be shown. These results

Table 1 Differential activations of $CS+_{unpaired}$ and CS− during the acquisition phase

Anatomical region	BA	MNI coordinates			*z* Value	No. of voxels
		x	*y*	*z*		
$CS+_{unp} > CS-$						
Right amygdala[a]		28	0	−12	2.56	36
Right insula[a]		52	−40	16	2.65	21
Left insula/superior temporal gyrus[a]		−56	−32	12	3.34	81
Left subgenual anterior cingulate cortex (ACC)	25	8	10	−20	3.67	87
Right parahippocampal gyrus	37	22	−48	−14	3.39	96
Left superior temporal gyrus (STG)	42	−56	−32	12	3.34	92

Significance level and size of the respective activation cluster (number of voxels) at $P < 0.05$ corr

Coordinates are listed in Talairach and Tournoux [42] atlas space

BA Brodmann area nearest to the coordinate and should be considered approximate

[a] Corrected for small volume

Table 2 Differential activations of $CS+_{unpaired}$ and CS− in acquisition and familiarization

Anatomical region	BA	MNI coordinates			*z* Value	No. of voxels
		x	*y*	*z*		
Acq (CS+ > CS−) > Fam (CS+ > CS−)						
Right amygdala[a]		20	−2	−12	2.94	48
Right insula[a]	13	50	−40	20	2.92	60
Right middle occipital gyrus	19	36	−72	10	3.90	237

Significance level and size of the respective activation cluster (number of voxels) at $P < 0.05$ corr

Coordinates are listed in Talairach and Tournoux [42] atlas space

BA Brodmann area nearest to the coordinate and should be considered approximate

[a] Corrected for small volume

Table 3 Differential activations of $CS+_{unpaired}$ and CS− in acquisition and extinction

Anatomical region	BA	MNI coordinates			*z* Value	No. of voxels
		x	*y*	*z*		
Acq (CS+ > CS−) > Ext (CS+ > CS−)						
Amygdala[a]		22	4	−10	2.53	28
Ext (CS+ > CS−) > Acq (CS+ > CS−)						
Right insula[a]	13	38	−18	20	2.67	44
Left insula[a]	13	−40	−12	24	2.77	42

Significance level and size of the respective activation cluster (number of voxels) at $P < 0.05$ corr

Coordinates are listed in Talairach and Tournoux [42] atlas space

BA Brodmann area nearest to the coordinate and should be considered approximate

[a] Corrected for small volume

can be evaluated as a further evidence for successful conditioning processes which have taken place in acquisition phase. Our results nicely replicate prior conditioning experiments, lending further support to the notion of applicability in a patient group.

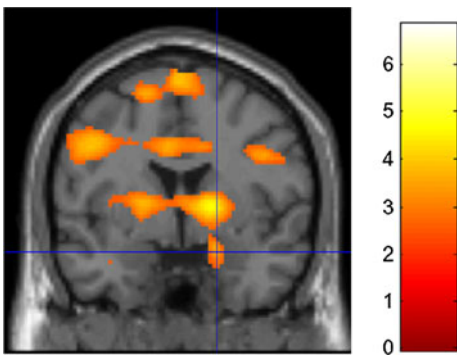

Fig. 6 The picture shows increased activation in the amygdala/hippocampus after modeling of a linear decrease in the CS− across the acquisition phase (MNI coordinates $x(=18)$, $y(=0)$, $z(=-16)$; $P < 0.05$ FWE corr. (ROI); $Z = 2.99$; cluster size = 62 voxels)

FMRI data

During the familiarization phase, there was no activation differences between CS+ and CS−which indicated that both stimuli were perceived similarly before one of the spheres was paired with the aversive noise. In the

acquisition phase, brain activation differences between CS+$_{unpaired}$ and CS− were found in areas associated with the so-called 'fear-circuit' such as the right amygdala, the insulae, the left sub-genual ACC and the right parahippocampal gyrus. These activations are in line with the previous studies on classical aversive conditioning [6, 12, 19, 22]. In addition, activation differences between both spheres were investigated by an analysis of the interaction effect between phase and stimulus type. This analysis supported our findings. Increased activation in limbic areas comparing the acquisition and the familiarization phase indicated that results were not only a time-dependent artifact, but that there was a real effect in the transition of familiarization to acquisition phase. During the extinction phase, the differential contrast between CS+ and CS− within the phase did not become significant. Recent studies showed that the prefrontal cortex and ACC seem to play an important role in interaction with amygdala in the regulation of extinction processes [2, 17, 35, for review see also 3]. Some studies were able to demonstrate a special role for the amygdala and the medial prefrontal cortex in extinction [21, 22, 35], however, extinction processes do not seem as clear as the mechanisms of fear acquisition yet [3, 29]. A number of studies could show increased activation in the amygdala in CS+ related to CS−, especially during the early period of extinction [17, 19, 22]. Phelps et al. [35] found, contrary to the main finding, elevated amygdala activation for the CS− relative to the CS+ in the extinction phase. Lowering the threshold for the contrast between CS+ and CS− during the extinction phase in the present study revealed increased bilateral hippocampal, parahippocampal and inferior frontal activations in the CS+ related to the CS− However, no amygdala activations could be detected, not even in the linear modulation. The results suggest that special experimental conditions (i.e. neutral stimuli and reinforcement strategy used) may have contributed to generally smaller effect sizes during acquisition may also attribute for the lack of amygdala activation during extinction in this paradigm. Moreover, there was discontinuance between acquisition and extinction due to the administration of the self-assessment manikin (which of cause lasted only 30 s). An important point may also be that we did not separate the extinction phase into an early and a late part.

The interaction analysis between acquisition and extinction revealed increased amygdala activation in the acquisition phase. The decline of amygdala activation in the last phase of the experiment may be interpreted in the way that extinction was successful in the present study. The inverse contrast showed increased insula activation during the extinction phase.

Previous studies suggested that the amygdala has a phaseal reaction pattern [e.g. 6]. Therefore, we explicitly tested for temporal increases as well as decreases in amygdala activation. The results showed a linear decrease in amygdala/hippocampal activation in the CS− across the acquisition phase, but neither a significant decrease during the presentation of the CS+$_{unpaired}$ nor a significant difference in the decrease of activation in the contrast between CS+$_{unpaired}$ and CS− could be found. On the contrary to prior studies that showed an exponential decrease in activation in the amygdala for the presentation of the CS+ during the acquisition phase [6, 22], we did no find such an exponential decrease, neither for CS+$_{unpaired}$ and the CS− nor for the differential contrast between both conditions. No other temporal changes of amygdala activity within any other phase of the experiment were found. This difference may again be a result of the experimental conditions employed, other studies used human faces as CS [6] which may generally have higher effects of amygdala activation, or employed a 100% reinforcement strategy [22]. Although results concerning temporal changes in the amygdala/hippocampus are not in line with the prior results, suggesting a decrease in the CS+$_{unpaired}$, but not in the CS− across the acquisition phase [6, 22], it seems plausible, that both stimuli, the CS+$_{unpaired}$ and the CS−, initially elicit amygdala activation until, after several pairings of the CS+ with the US during the acquisition phase, the CS− stimulus becomes a predictable safety signal and no longer represents a threat. This fact may result in a decrease in amygdala activation. Gläscher and Büchel [14] suggest that, when stable contingencies are given, less amygdala activation is observed the more predictable a CS becomes. This suggests an involvement of this structure in encoding and processing of contingency changes. Amygdala/hippocampal activation, therefore, decreased in the CS− over time, but not in the CS+$_{unpaired}$ which was not predictable due to the intermittent reinforcement strategy. This becomes also clear in Fig. 8 which illustrated the temporal changes of the predicted responses in the right amygdala over time. The plot shows, that the amygdala activation decreases in both stimuli, but the decrease is stronger in the CS−.

Comparing the fMRI results of the present study to other conditioning studies reveals somewhat smaller effect sizes. This may be a result of the specific experimental conditions: the use of neutral, biologically non-prepared stimuli may have led to generally smaller effects during conditioning [11, 31]; in addition, the presentation of the US in the familiarization phase may have caused inhibitory effects of conditioning [28]. We explicitly wanted to already integrate the presentation of the US in the habituation phase to examine the initial arousal which may be caused by the US. This may give us the opportunity to examine the general characteristics of the habituation process and it may help in avoiding novelty effects in the

acquisition phase. Another possible explanation for smaller effects may be the use of a 50% reinforcement strategy that may require a larger number of trials to reach the same strength as a 100% reinforcement strategy. It has been reported that amygdala activity increases parallel to the pairing rate [9], therefore, a partial reinforcement strategy may generally lead to smaller activations. Moreover, in differential conditioning paradigms, increased limbic activation may be shown towards both stimuli in the beginning, until, after several pairings, contingencies have been learned and the activation related to the presentation of the CS− decreases. Another shortcoming of the study may consist of the small number of events during the acquisition phase. Moreover, in the main contrast between $CS+_{unpaired}$ versus CS−, there was an imbalance of statistical power of the two regressors, since $CS+_{unpaired}$ consisted of only 16 events (caused by the 50% reinforcement strategy), whereas CS− contained 32 events. This might also have contributed to the observed lower effect sizes in our sample. Nevertheless, we aimed to keep our paradigm short, and a small number of events were, therefore, necessary due to the time restraints on patients with anxiety disorders in scheduled clinical studies [15].

Outlook: application of the conditioning paradigm to patients with anxiety disorders

We selected the paradigm to suit the following demands: first, it should be sensitive to investigate aversive conditioning processes in all three phases of conditioning: familiarization, acquisition and extinction while meeting strict time restraints. Besides this demand, we wanted to use neutral stimuli with regard to their disorder-specific relevance, to avoid pre-existing differences in stimulus relevance to have an impact on the conditioning process, especially in clinical samples. This confound is additionally controlled by the introduction of the familiarization phase in which differences in stimulus relevance can easily be detected and controlled. Finally, we presented the US in the familiarization phase to control for novelty effects and examine habituation processes to the US. Especially in patients with anxiety disorders we, expect stronger non-conditioning related effects related to the aversive stimulus. In summary, we established a new conditioning paradigm successfully showing conditioning processes in healthy subjects on the behavioral and autonomic level and in characteristic neural correlates ("fear-circuit"). Thus, it can be employed in a multi-site fMRI study investigating patients with anxiety disorders (http://www.paniknetz.de). The aim of future studies will be to examine the fear-circuit and therapeutic effects of a cognitive–behavioral intervention in clinical studies [15, 25].

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