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Influence of contingency awareness on neural, electrodermal and evaluative responses during fear conditioning

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In an fMRI study, effects of contingency awareness on conditioned responses were assessed in three groups comprising 118 subjects. A differential fear-conditioning paradigm with visual conditioned stimuli, an electrical unconditioned stimulus and two distractors was applied. The instructed aware group was informed about the contingencies, whereas the distractors prevented contingency detection in the unaware group. The third group (learned aware) was not informed about the contingencies, but learned them despite the distractors. Main effects of contingency awareness on conditioned responses emerged in several brain structures. *Post hoc* tests revealed differential dorsal anterior cingulate, insula and ventral striatum responses in aware conditioning only, whereas the amygdala was activated independent of contingency awareness. Differential responses of the hippocampus were specifically observed in learned aware subjects, indicating a role in the development of contingency awareness. The orbitofrontal cortex showed varying response patterns: lateral structures showed higher responses in instructed aware than unaware subjects, the opposite was true for medial parts. Conditioned subjective and electrodermal responses emerged only in the two aware groups. These results confirm the independence of conditioned amygdala responses from contingency awareness and indicate specific neural circuits for different aspects of fear acquisition in unaware, learned aware and instructed aware subjects.

Keywords: fMRI; classical conditioning; evaluative conditioning; skin conductance responses

INTRODUCTION

Differential fear conditioning paradigms, in which the paired (CS^+) but not the unpaired (CS^-) conditioned stimulus predicts an aversive unconditioned stimulus (UCS), are often employed when studying the acquisition of fear responses. Thereby, an ongoing debate exits whether conditioned responses can be expected in the absence of contingency awareness and to which extend contingency awareness influences emotional processing (Pessoa, 2005).

Generally, conditioned skin conductance responses (SCRs) and changes in subjective ratings have reliably been observed in contingency aware subjects only (Hamm and Vaitl, 1996; Lovibond and Shanks, 2002; Tabbert *et al.*, 2006; Dawson *et al.*, 2007; Klucken *et al.*, 2009a; Mitchell *et al.*, 2009). An exception may however exist for certain fear relevant CS presented below the threshold for conscious perception (sub-liminal fear conditioning) (Öhman and Soares, 1993, 1998; Esteves *et al.*, 1994; Olsson and Phelps, 2004; Knight *et al.*, 2009).

One central question concerns the neural circuits supporting awareness-related and -unrelated aspects of fear acquisition (LeDoux, 2000; Öhman and Mineka, 2001; Lovibond and Shanks, 2002; Hamm and Weike, 2005; Öhman, 2005; Mitchell *et al.*, 2009). Previous research identified key neural structures involved in fear conditioning and has begun to define their specific roles (Sehlmeyer *et al.*, 2009; Mechias *et al.*, 2010). Whereas amygdala activation has been observed in the absence of contingency awareness (LeDoux, 2000; Tabbert *et al.*, 2006; Öhman *et al.*, 2007), the anterior cingulate cortex (ACC) and the insula have been associated with aware fear conditioning and the conscious anticipation of threat stimuli (Büchel *et al.*, 1998; Öhman, 2005; Straube *et al.*, 2007; Mechias *et al.*, 2010). The middle prefrontal gyrus (mPFG), the hippocampus and more recently, the ventral striatum have been related to the development of contingency awareness (Carter *et al.*, 2006; Klucken *et al.*, 2009a, b). The orbitofrontal cortex (OFC) has been associated more generally with the evaluation of the reinforcement value of a stimulus and the learning of stimulus–reinforcement associations (Rolls, 1999, 2008; O'Doherty, 2007), while putatively different roles of medial

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and lateral OFC sub-territories in emotion processing exist (Öngür *et al.*, 2003; Milad and Rauch, 2007).

For the research on the effects of contingency awareness, it is fundamentally important how contingency awareness is conceptualized and experimentally manipulated. In the past, contingency awareness has often been conceptualized as the conscious perception of the CS. Thereby, contingency awareness was manipulated by presenting the CS below conscious perception in order to prevent awareness of the CS/UCS contingencies as done for example in backward masking studies (Esteves *et al.*, 1994; Öhman and Soares, 1993; 1998; Knight *et al.*, 2009). An immanent problem of this method concerns the determination of the conscious perception threshold necessary to prevent the development of contingency awareness (Lovibond and Shanks, 2002; Wong *et al.*, 2004). Another methodological approach is to hide the CS/UCS contingencies despite a conscious stimulus perception, for example, via distraction (Carter *et al.*, 2003; Tabbert *et al.*, 2006). There, contingency awareness is conceptualized as the ability of subjects to verbalize the CS-UCS contingencies directly following the acquisition phase. The advantage of this approach is that it allows the investigation of the effects of contingency awareness disentangled from conscious stimulus perception. It may also possess a higher ecological validity because it better resembles real life conditions: In everyday life, sub-liminal stimulus presentations rarely occur. However, despite a conscious stimulus perception, fear responses towards stimuli can occur in, e.g. post-traumatic stress disorder or phobias without the patients being able to associate the eliciting stimuli (CS) with an initial threat (UCS) (LeDoux, 1998; Ehlers and Clark, 2000; Brewin, 2001; Öhman and Mineka, 2001).

Notably, in a recent study, in which contingency awareness was manipulated by distraction, we found conditioning related neural responses in the amygdala, the OFC and the occipital cortex (OCC) in unaware subjects, whereas conditioned SCRs were observed only in aware subjects (Tabbert *et al.*, 2006). In this previous study, medial and lateral parts of the OFC were not explicitly distinguished; yet, *post hoc* inspection revealed that the reported OFC peak voxel was situated in the lateral OFC.

The aim of the present study was to replicate these findings in a larger sample and, additionally, to extend our findings to subjective ratings of the CS. Further, we were interested in the effects of the way contingency awareness is achieved—i.e. via instruction prior to or via learning during the fear acquisition process. We used distraction (2-back task and an additional visual distracting stimulus) in order to hamper the spontaneous detection of CS/UCS contingencies. One group was informed about the contingencies *a priori* (instructed aware group), whereas a second group was not informed about the contingencies and did not detect them spontaneously (unaware group). A third group of participants, not informed *a priori*, learned the contingencies despite distraction (learned aware group). Comparing these three groups allows the in depth investigation of the neural structures involved in aware and unaware fear conditioning. The following structures were expected to show conditioned responses irrespective of contingency awareness: amygdala, OFC and OCC in response to the CS^+ as compared to the CS^-. Only for the instructed and learned aware subjects, differential activity was expected in the ACC, the insula and the mPFG (Öhman, 2005; Carter *et al.*, 2006). Conditioned ventral striatum and hippocampus activity was expected to be particularly pronounced in learned aware subjects (Klucken *et al.*, 2009a, b).

MATERIALS AND METHODS

General background

The data presented are part of a larger ongoing study investigating the effects of various variables (cortisol, sex, contingency awareness) on fear conditioning. Participants received either a single oral dose of 30 mg hydrocortisone or placebo ~25 min before the acquisition. To control for the effects of cortisol treatment, salivary samples were taken at baseline (before tablet intake), immediately before the acquisition and after the extinction. It is important to note that in the present study, only participants receiving placebo were included.

Participants gave a written consent, which included all aspects of the experiment (the conditioning schedule was not explained to the unaware and learned aware group until the experiment was finished). Participants also filled out a short questionnaire on mental health, somatic health and demographic data as well as the Edinburgh Inventory of Handedness (Oldfield, 1971). They were debriefed about the purpose of the study after the experiment. The study was approved by the ethics committee of the German Psychological Society.

Subjects

There were 50 subjects in the instructed aware group (20 male, 30 female) and 42 subjects in the unaware group (15 male, 27 female). The third group (learned aware) consisted of 26 subjects (11 male, 15 female), who were originally designated for the unaware group but developed contingency awareness despite the distractors (see also the Manipulation of contingency awareness section), leading to a total sample size of 118 subjects. All 118 subjects were included in the analyses of neural and subjective responses. Concerning SCR analyses, data of three subjects in the learned aware group, one subject in the unaware and one in the instructed aware group were lost due to technical problems, leaving 41 subjects in the unaware, 23 subjects in the learned aware and 49 subjects in the instructed aware group.

Groups did not differ in socio-demographic background. Most of the participants were university students. Mean age of the participants was 24.32 years in the instructed aware (s.d. = 3.05 years, range: 20–34 years), 22.57 years in the

unaware (s.d. = 2.956 years, range: 18–31 years) and 23.73 years in the learned aware group (s.d. = 2.63 years, range: 19–29 years). None of the subjects was taking regular medication, nobody had a previous history of any psychiatric or neurological treatment, and all of them were right-handed.

Conditioned visual stimuli

Two simple geometric figures, a rhombus and a square, served as CS^+ and CS^- in a differential conditioning paradigm. An additional geometrical figure (referred to as non-CS in the following text), a triangle, occurred less often than the CS^+ and the CS^- and served as distractor stimulus. All three stimuli were grey in colour and had identical luminance. All figures were presented with the duration of 8 s. For visual stimulation inside the scanner, an LCD-projector (model EPSON EMP-7250) was used, which projected pictures onto a screen at the end of the scanner (visual field = 18°). Pictures were viewed by means of a mirror mounted to the head coil.

UCS

A custom-made impulse-generator (833 Hz) provided transcutaneous electrical stimulation to the left shin through two Ag/AgCl electrodes (0.1 cm, two surfaces each). The electrical stimulation served as the UCS. The electric stimulation voltage ranged from 50 to 400 V at a resistance of 100 kΩ. Stimulus intensity was set for each subject individually to an 'unpleasant but not painful' level using a gradually increasing rating procedure; i.e. the investigator manually triggered short electrical stimulations, starting with a low intensity below the perception level until the stimulation was perceived as 'unpleasant but not painful' by the respective participant. During the conditioning procedure, each electrical stimulus was applied for 100 ms. The onset and the duration of the electrical stimulation were set by a computer program and the impulse-generator inside the scanning chamber was triggered via an optic fibre cable.

2-back-task

Interspersed in the presentation of the geometric figures, numbers ranging from one to five were presented sequentially for 1 s. By pressing one of two buttons, participants had to indicate after each stimulus whether it was the same or a different number to the number before the last. There were 50 numbers in total, of which 12 were identical and 38 different to the number before the last one. Potential differences in the number of correct responses were calculated in SPSS for Windows (Release 17.0; SPSS Inc. Illinois) via one-way analysis of variance (ANOVA) with the factor awareness (unaware, learned aware, instructed aware).

Conditioning procedure

The conditioning experiment consisted of one acquisition and one extinction session. Only data from the acquisition are reported here. In between the two sessions, subjects remained in the scanner. Directly after the acquisition session, subjects indicated their estimation of CS-UCS-contingencies and CS evaluation for all CS (see below for a detailed description of the contingency awareness check).

For each participant, there was one acquisition run with 20 trials of CS^+ and CS^-, respectively, and 10 trials of non-CS. A 100% reinforcement schedule was used, i.e. each CS^+ was followed by a UCS. Inter-trial intervals between the geometrical figures and the numbers ranged from 5 to 7.5 s. Accordingly, the inter-trial intervals between the CS ranged from 11 to 16 s. The variable inter-trial intervals, ranging from 0 to 2.5 s (i.e. one TR), were introduced to enhance signal quality due to signal sampling at variable peri-stimulus times. The onset of the UCS-presentation was delayed 7.9 s after CS^+ onset and co-terminated with CS^+ presentation (delay-conditioning). One of two pseudo randomized stimulus orders was used comprising the following restrictions: no more than two consecutive presentations of the CS, no more than three consecutive identical numbers, an equal distribution for any number before or after CS^+ trials to avoid conditioning to any of the numbers, and an equal quantity of CS^+ and CS^- trials within every 10th trial (five each). The acquisition procedure started with a CS^+ for half of the subjects, with a CS^- for the other half, and either the rhombus or the square served as CS^+.

Manipulation of contingency awareness

All subjects were informed by a written instruction that they would take part in a study examining the influence of cortisol and several distractor stimuli (including an aversive electrical stimulation and visual stimuli) on a memory task. Instructions for the instructed aware group deviated in one sentence indicating the geometrical figure that would precede the electrical stimulation. After reading the instructions, the participants were invited to ask questions about the procedure. When subjects from the instructed aware group asked for confirmation that the electrical stimulation would only occur at certain times (i.e. after the CS^+), this relation was confirmed by the experimenter. Again, directly before the acquisition, the contingencies were verbally repeated for the instructed aware subjects.

Immediately following the acquisition and the CS evaluation, participants completed a rating of contingencies for each CS (CS^+, CS^-, non-CS) inside the scanner. The rating contained one multiple-choice question for each CS (CS^+, CS^-, non-CS) regarding its relation to the UCS. The participants were asked to estimate 'how often the electrical stimulation followed this geometric figure' with the following possible answers: 'always', 'sometimes', 'never', 'I don't know'. The respective geometrical figure was placed above these answer alternatives. The awareness checks were complemented by additional assessments including (i) an open statement given by the participants describing their assumption of the study aim and (ii) an indication of the participants whether they noticed a relation between the CS and the

UCS and if so, which relation was perceived and when (via index on a timeline). Participants were only included if the initial multiple-choice questions were confirmed by the additional assessments.

All subjects of the instructed aware group indicated that the UCS 'always' followed the CS^+ and 'never' the CS^-. Subjects originally designated for the unaware group remained in this group if they did not state higher probabilities for the UCS delivery after the CS^+ than after the CS^-. Subjects originally designated for the unaware group were included in a third group (learned aware) if they stated higher probabilities for the UCS application after the CS^+ than after the CS^- (i.e. possible combinations for CS^+ and CS^-, respectively, were: always–sometimes; sometimes–never, always–never).

Evaluative conditioning

Before and after the acquisition (i.e. directly before the contingency ratings), participants rated their subjective valence and arousal for each CS (CS^+, CS^-, non-CS) on a 9-point Likert scale, which ranged from 1 ('very unpleasant' or 'calm and relaxed') to 9 ('very pleasant' or 'very arousing'). The non-CS was rated together with the CS^+ and the CS^-, yet it was not included in the analyses as it only served as a distractor stimulus. Statistical analyses of evaluative conditioning (EC) were performed via ANOVA in a 2 (CS-type: CS^+ or CS^-) $\times$ 2 (rating time) $\times$ 3 (between subjects factor group: unaware, learned aware, instructed aware) factorial design within the general linear model as implemented in SPSS. For significant interactions, appropriate follow-up-tests were conducted (ANOVA or *t*-tests). We expected conditioned subjective ratings in learned and instructed aware, but not in unaware subjects.

Responses to the non-CS were also analysed analogous to the described analyses of CS^+ and CS^- (see Supplementary Data; 'Methods and Results' section for details).

SCRs

SCRs were sampled simultaneously with fMRI scans using Ag/AgCl electrodes filled with isotonic (0.05 M NaCl) electrolyte medium, placed hypothenar at the non-dominant hand. SCRs were defined in three analysis windows (Prokasy and Ebel, 1967): The maximum response within a time window of 1–5 s after the CS onset was counted as a first interval response (FIR), within the time window of 5–8.5 s as a second interval response (SIR) and within the time window of 8.5–13 s as the unconditioned response (UCR). The FIR may reflect orienting to the CS and the SIR the anticipation of the UCS, with both responses indicating fear-conditioning related changes (for a more detailed discussion see Pineles *et al.*, 2009). To estimate SCRs, inflexion points were detected automatically but manually controlled using the software EDR_Para (3.71; Schäfer F., unpublished data). The immediately preceding SCR level to the inflexion point served as the baseline. Conditioned responses were defined as larger response magnitudes in reaction to the CS^+ than to the CS^- in the FIR and SIR. Data were logarithmically transformed (natural logarithm) in order to attain statistical normality. Statistical comparisons were performed via ANOVA in a 2 (CS-type: CS^+ and CS^- or UCS and non-UCS for the UCR) $\times$ 20 (trial) $\times$ 3 (between subjects factor group: unaware, learned aware, instructed aware) factorial design within the general linear model as it is implemented in SPSS. As for the EC measures, appropriate *post hoc* tests (ANOVA or *t*-tests) were performed for significant interactions. Due to data loss, SCR analyses were performed with 41 subjects in the unaware, 23 subjects in the learned aware and 49 subjects in the instructed aware group ('Subjects' section).

Similar to the subjective ratings, we expected conditioned SCRs in learned and instructed aware, but not in unaware subjects.

Additional analyses were performed also with the non-CS (Supplementary Data).

Magnetic resonance imaging

Brain images were acquired using a 1.5 Tesla whole-body tomograph (Siemens Symphony with a quantum gradient system) with a standard head coil. For functional imaging, a total of 750 volumes (480 for the acquisition and 270 for the extinction phase) were registered using a T2*-weighted gradient echo-planar imaging sequence (EPI) with 25 slices covering the whole brain (slice thickness = 5 mm; 1 mm gap; descending slice order; TA = 100 ms; TE = 55 ms; TR = 2.5 s; flip angle = 90°; field of view = 192 mm $\times$ 192 mm; matrix size = 64 $\times$ 64). The first three volumes were discarded due to an incomplete steady state of magnetization. The orientation of the axial slices was parallel to the OFC tissue—bone transition to keep susceptibility artefacts in the OFC and the amygdala to a minimum. A gradient echo-field map sequence was measured before the functional run to get information for unwarping B_0 distortions. Data were analyzed using Statistical Parametric Mapping (for preprocessing and first-level analyses: SPM5, Wellcome Department of Cognitive Neurology, London, UK; 2005; for group analyses: SPM8, Wellcome Department of Cognitive Neurology, London, UK; 2009) implemented in MatLab R2007b (Mathworks Inc., Sherborn, MA, USA). Unwarping and realignment (b-spline interpolation), slice-time correction and normalization to the standard space of the Montreal Neurological Institute brain (MNI-brain) were performed. Smoothing was executed with an isotropic 3D Gaussian filter with a full width at half maximum (FWHM) of 9 mm.

For subject-level analyses, acquisition and extinction were integrated as separate sessions in one model, each including the following experimental conditions: 'CS^+', 'CS^-', 'non-CS', 'UCS', 'non-UCS', numbers of the 2-back-task (excluding 'UCS' and 'non-UCS' for the extinction). A further regressor contained the first two numbers and two geometrical figures of the extinction, because at this time (re-)learning of the CS meaning had not yet been possible

(Phelps *et al.*, 2004). These 11 regressors were modelled by a stick function convolved with a haemodynamic response function (hrf) in the general linear model, without specifically modelling the durations of the different events. 'Non-UCS' was defined as the time window after CS^- presentation corresponding to the time window of UCS presentation after the CS^+ (7.9 s after CS^- onset). The six-movement parameters of the rigid body transformation, applied by the realignment procedure, were introduced as covariates in the model for each session (acquisition and extinction) separately. The voxel-based time series were filtered with a high pass filter (time constant = 128 s). For the statistical analyses, we employed an ANOVA with one factor (awareness) to analyze its potential effect on conditioned responses (CS^+ minus CS^-). *Post hoc* two-sample *t*-tests were done only for structures that showed a significant main effect ($P_{corr} < 0.05$) or a tendency ($P_{corr} < 0.10$) of awareness to further clarify the direction of group differences. Additionally, main effects of awareness on UCS (UCS minus non-UCS) and on number processing were analyzed. Conditioned responses within the three groups were tested via separate one-sample *t*-tests for descriptive purposes. Results of the two- and one-sample *t*-tests are reported until a threshold of $P_{corr} \leq 0.05$. We used explorative whole brain as well as regions of interest (ROI) analyses to enhance the statistical power. For the present analyses, only the following five regressors of the acquisition session were relevant: 'CS^+', 'CS^-', 'UCS', 'non-UCS' and 'numbers'.

ROI for the analyses of the CS and the UCS were the ACC, the amygdala, the insula, the mPFG and the OFC (lateral and medial) and additionally for the CS-analyses the extended OCC, the ventral striatum and the hippocampus. ROI for the analyses of the 2-back-task were frontal structures (mPFG, medial OFC, lateral OFC, ACC) and the hippocampus as central structures for explicit memory processes. For the explorative whole brain analyses, the significance threshold was set to $\alpha = 0.05$ on voxel-level, corrected for multiple testing [family wise error (FWE) correction]. ROI analyses were performed using the small volume correction options of SPM8 ($P_{corr} < 0.05$). The required masks for the ROI analyses were designed from predefined anatomical regions using the software-program MARINA (Walter *et al.*, 2003). The ventral striatum mask was taken from the Human Brain Project Repository database (THOR Center for Neuroinformatics; http://hendrix.ei.d-tu.dk/; labelled as ventral-striatum region and prefrontal medial and superior region) with the threshold at 0.5, because for this structure no predefined region is provided by MARINA. The original data for the ventral striatum mask was based on the BrainMap database (Fox and Lancaster, 1994; Nielsen and Hansen, 2002).

RESULTS

EC

ANOVA of the valence and arousal ratings of all subjects revealed main effects of CS-type and rating time as well as interactions of CS-type × rating time, CS-type × contingency awareness, and rating time × contingency awareness (all $P < 0.05$; Figure 1). All main effects and interactions merged into 3-fold interactions of CS-type × rating time × contingency awareness (all $P < 0.001$).

Subsequent separate ANOVAs of the valence and arousal ratings revealed significant learning effects in learned and instructed aware subjects only. Here, interactions of CS-type × rating time emerged, indicating a decrease in valence and an increase in arousal for the CS^+ but not for the CS^- from the first (before acquisition: baseline) to the second rating (post acquisition; all $P < 0.001$; Figure 1).

For unaware subjects, there was a significant increase in arousal rating from the first to the second rating ($P < 0.05$; Figure 1), irrespective of CS-type.

Analyses of the non-CS revealed similar results (Supplementary Data).

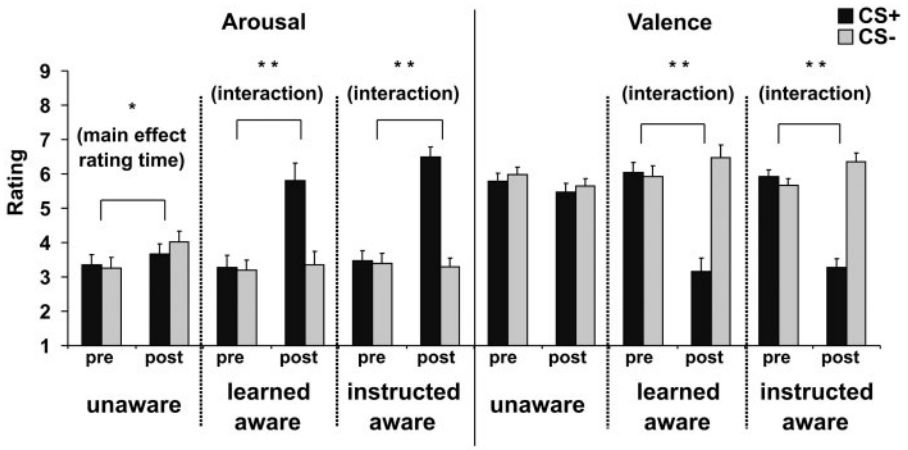

Fig. 1 Mean subjective arousal [1 (calm and relaxed) to 9 (very arousing)] and valence [1 (very unpleasant) to 9 (very pleasant)] ratings in unaware, learned aware and instructed aware subjects before (pre) and after (post) the acquisition phase. Error bars are standard errors of the mean. *$P < 0.05$; **$P < 0.001$.

SCRs

In the FIR, there were main effects of CS-type and trial as well as interactions of trial × contingency awareness and of CS-type × contingency awareness (all $P < 0.001$). Further, there was a 3-fold interaction of CS-type × trial × contingency awareness [$F_{(25.4,1399.7)} = 2.13$; $P = 0.001$; Figure 1].

For the SIR, there was a main effect of CS-type and trial as well as an interaction of CS-type × trial and of CS-type × contingency awareness (all $P < 0.05$). The main effects and interactions merged in a significant 3-fold interaction of CS-type × trial × contingency awareness [$F_{(23.5,1293)} = 1.68$; $P = 0.023$].

Regarding the UCR, enhanced responses to the UCS compared to UCS-omission and habituation of SCRs were revealed in a main effect of UCS and trial; differential time courses for responses to UCS and UCS-omission were reflected in an interaction of UCS × trial (all $P < 0.001$). There were no main effects of or interactions with contingency awareness in the UCR.

Follow-up analyses showed that the interactions of FIR and SIR responses were due to main effects of CS-type in instructed and learned aware (all $P \leq 0.001$) but not in unaware subjects (all $P > 0.4$), with higher responses to the CS^+ as compared to the CS^-. For the FIR and the SIR in instructed aware subjects, there was also a significant interaction of CS-type × trial (all $P \leq 0.007$).

Results concerning the non-CS were comparable (Supplementary Data).

2-back-task: number of correct responses

There were no significant group differences in the number of correct responses in the 2-back-task [$F_{(2,115)} = 0.57$; $P = 0.57$]. Mean correct responses in the unaware group were 43.67 (s.d. = 6.0) out of 50, in the instructed aware group 42.32 (s.d. = 7.0) and in the learned aware group 42.31 (s.d. = 6.9).

fMRI data

Main effects of awareness on conditioning effects (CS^+ minus CS^-)

Main effects of awareness emerged in the superior parietal, supra-marginal, post-central and superior temporal cortex, as well as in the supplementary-motor area (SMA), the rolandic operculum, bilateral ACC, bilateral insula, the right ventral striatum and the left lateral OFC (Table 1, Figure 2). Concerning the ACC, activation peaks were located in dorsal rather than ventral parts. Trends for a main effect could be observed in the right medial OFC and bilateral hippocampus (Table 1, Figure 2).

Follow-up two-sample t-tests

Follow-up tests in the structures that showed at least a trend for a main effect of awareness revealed higher differential responses in the learned aware compared with instructed aware subjects in the right hippocampus (Table 2).

The learned aware compared with unaware subjects showed enhanced conditioning-related responses in the left ACC, bilateral insula, the right ventral striatum and the bilateral hippocampus (Table 2).

The instructed aware subjects showed higher responses than the unaware subjects in the superior parietal, supra-marginal and superior temporal cortex, the rolandic operculum, SMA, bilateral ACC, bilateral insula, right ventral striatum and left lateral OFC (Table 2).

The unaware subjects showed higher differential responses than the instructed aware subjects in the left post-central cortex and the right medial OFC (Table 2).

The sample size of the learned aware group was smaller than the sample size of the other two groups. Therefore, effect sizes for the group comparison are depicted in Table 2 as these are more independent of sample size.

Table 1 Main effect of awareness on differential responses to CS^+ minus CS^- during fear acquisition

Brain structure (correction)	Side	*x*	*y*	*z*	F_{max}	P_{corr}
Superior parietal cortex (whole brain)	Right	21	−45	66	22.23	<0.001
Supra-marginal cortex (whole brain)	Left	−60	−33	27	19.74	0.002
Post-central cortex (whole brain)	Left	−39	−30	48	19.09	0.004
Supplementary motor area (whole brain)	Right	12	−6	66	17.40	0.011
Superior temporal cortex	Right	63	−39	21	16.55	0.019
ACC (whole brain)	Left	−3	21	24	16.22	0.024
ACC (whole brain)	Right	0	6	36	16.34	0.023
Rolandic operculum (whole brain)	Left	−51	3	6	15.91	0.030
Insula (ROI)	Left	−48	3	6	15.58	0.001
Insula (ROI)	Right	30	18	−12	10.57	0.020
Lateral OFC (ROI)	Left	−45	15	−6	10.61	0.016
Ventral striatum	Right	15	0	−3	8.15	0.020
Medial OFC (ROI)	*Right*	*6*	*36*	*−27*	*7.99*	*0.078*
Hippocampus (ROI)	*Left*	*−30*	*−18*	*−9*	*7.52*	*0.094*
Hippocampus (ROI)	*Right*	*39*	*−6*	*−21*	*8.46*	*0.050*

The threshold was $P_{corr} < 0.05$ (FWE-corrected according to SPM8; for ROI: small-volume correction). Additionally, trends are reported in italic letters up to a threshold of $P_{corr} < 0.1$. All coordinates (*x*, *y*, *z*) are given in MNI space.

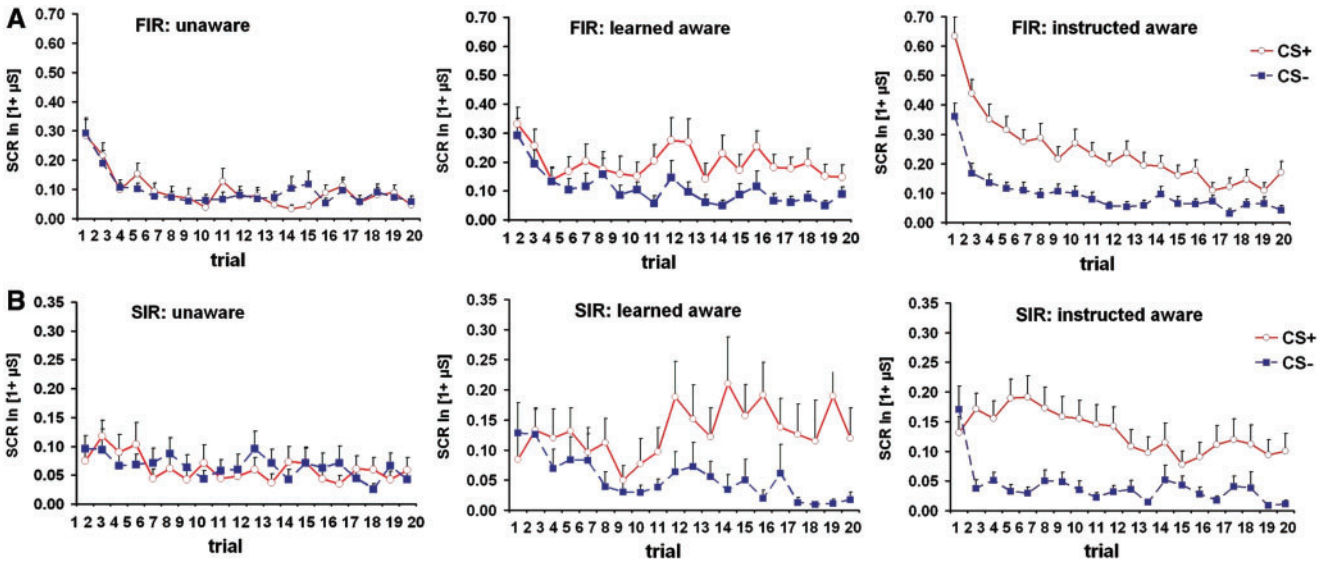

Fig. 2 SCRs for the unaware, the learned aware and the instructed aware group. (**A**) FIR. (**B**) SIR. Please note the different scaling of the *y*-axis in (A) and (B). Error bars are standard errors of the mean.

Table 2 Follow-up two-sample *t*-tests for the main effects of contingency awareness on conditioned responses (CS^+ minus CS^-)

Comparison	Brain structure (correction)	Side	*x*	*y*	*z*	T_{max}	P_{corr}	*r*
Instructed > learned aware	No significant differences							
Learned > instructed aware	Hippocampus (ROI)	Right	39	−9	−24	3.55	0.036	0.38
Learned aware > unaware	ACC (ROI)	Left	−3	9	30	3.98	0.019	0.44
	Insula (ROI)	Left	−48	3	6	3.86	0.035	0.43
	Insula (ROI)	Right	36	15	−12	3.73	0.048	0.42
	Ventral striatum (ROI)	Right	18	3	−3	3.70	0.009	0.41
	Hippocampus (ROI)	Left	−30	−18	−9	3.75	0.026	0.42
	Hippocampus (ROI)	Right	39	−6	−18	3.92	0.016	0.43
Unaware > learned aware	No significant differences							
Instructed aware > unaware	Superior parietal cortex (whole brain)	Right	21	−45	66	6.85	<0.001	0.59
	Supra-marginal cortex (whole brain)	Left	−60	−33	27	6.07	0.001	0.54
	SMA (whole brain)	Right	12	−3	69	6.09	0.001	0.54
	ACC (whole brain)	Left	−6	21	24	5.66	0.004	0.51
	ACC (whole brain)	Right	9	9	39	5.74	0.003	0.52
	Superior temporal cortex (whole brain)	Right	63	−42	21	5.32	0.014	0.49
	Rolandic operculum (whole brain)	Left	−51	3	6	5.26	0.017	0.48
	Insula (ROI)	Left	−48	3	6	5.15	<0.001	0.48
	Insula (ROI)	Right	39	9	6	4.45	0.004	0.42
	lateral OFC (ROI)	Left	−45	15	−6	4.58	0.002	0.43
	Ventral striatum (ROI)	Right	15	0	−3	3.46	0.014	0.34
Unaware > instructed aware	Postcentral cortex left	Left	−42	−30	51	5.95	0.001	0.53
	medial OFC (ROI)	Right	6	36	−27	3.69	0.027	0.36

The threshold was $P_{corr} < 0.05$ (FWE-corrected according to SPM8; for ROI: small-volume correction). All coordinates (*x*, *y*, *z*) are given in MNI space; *r* indicates the effect size (point biserial correlation) of the respective *t*-value.

Conditioning effects (CS^+ minus CS^-) in the three subgroups

In the instructed aware group, conditioned responses were found in all ROI (all $P_{corr} < 0.05$) except the left amygdala, the left hippocampus, the right OCC and the right medial OFC. Additionally, significant differential responses emerged from whole-brain analysis in the following regions [all $P_{corr\ (whole\ brain)} < 0.05$]: bilateral superior parietal cortex, precuneus, SMA, bilateral supramarginal cortex, bilateral medial and superior temporal cortex, left superior and right inferior frontal cortex, medial cingulate cortex (outside our mask comprising ventral and dorsal ACC regions) and rolandic operculum.

In the learned aware group, conditioned responses were found in all ROI [all $P_{corr} < 0.05$] except the right medial OFC [right: $P_{corr} = 0.051$] and the left lateral OFC. No additional structures emerged from whole-brain analyses.

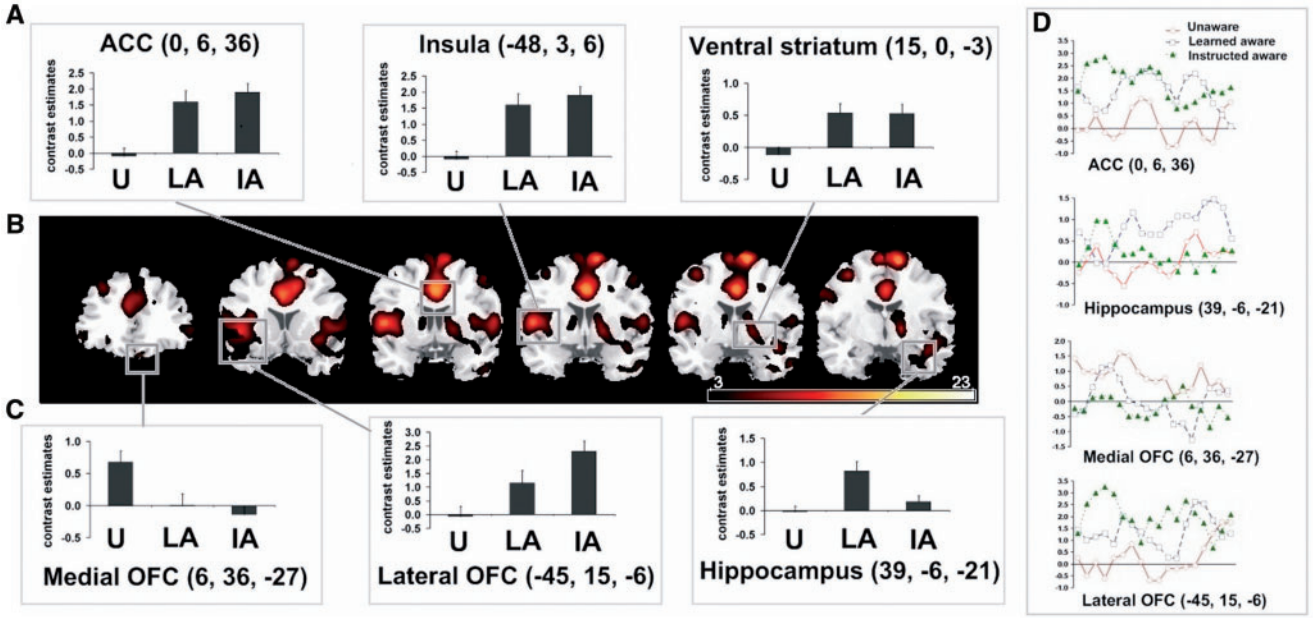

Fig. 3 (**B**) Main effect of awareness on conditioned responses (CS^+ minus CS^-). The colour bar depicts *F*-values for the main effect. The bar graphs show group mean contrast estimates for the respective peak voxel (*U* = unaware group, LA = learned aware group, IA = instructed aware group) for (**A**) structures that showed higher differential responses in both aware compared to the unaware group and (**C**) structures, for which one of the three groups showed higher responses than either one or both of the other groups (Table 2). Error bars are standard errors of the mean. (**D**) Representative learning curves for the three groups are illustrated for selected peak voxel. To enhance temporal smoothness, a moving average over three trials is depicted.

In the unaware group, significant conditioned responses were found in the left amygdala, the right medial OFC and the bilateral hippocampus (all $P_{corr} < 0.05$; Figure 3). The right amygdala showed a trend for differential activation ($P_{corr} = 0.053$). No additional structures emerged from whole brain analyses.

Main effect of awareness on control conditions (2-back-task; UCS minus non-UCS)

No main effects of awareness on the 2-back-task were observed in the relevant ROIs (mPFG, medial OFC, lateral OFC, ACC, hippocampus) or in other structures from the whole-brain-corrected analysis.

There were no significant main effects of awareness on reactions to the UCS. Trends were observed in the left amygdala ($P_{corr} = 0.095$), with the highest responses in the learned aware group and in the left insula ($P_{corr} = 0.058$), with higher responses in the instructed and learned aware subjects.

DISCUSSION

The question as to how far contingency awareness influences fear conditioning and whether conditioned responses can be acquired without awareness is of high relevance for the understanding of the genesis and the therapy of anxiety and mood disorders. The present study is the first to compare conditioned responses of instructed aware, learned aware and unaware subjects within one experiment. It is also one of few studies assessing the impact of contingency awareness using a supra-threshold CS presentation. It thus provides unique information for the current discussion on contingency awareness.

Replicating previous findings, we observed conditioned SCRs only in contingency aware subjects (learned and instructed aware group) (Hamm and Vaitl, 1996; Lovibond and Shanks, 2002; Hamm and Weike, 2005; Tabbert *et al.*, 2006). Similar results emerged for the subjective ratings: a significant differentiation of CS^+ and CS^- in EC measures was found in aware subjects only, with higher ratings of arousal and negative valence in response to the CS^+. These findings support the assumption that reliable EC can be observed only in combination with contingency awareness (Lovibond and Shanks, 2002; de Houwer *et al.*, 2005; Dawson *et al.*, 2007).

Contrary to SCRs and subjective measures and consistent with our previous study (Tabbert *et al.*, 2006), conditioning related neural responses were observed in the two aware groups but also in the unaware group. Concerning the amygdala, no main effects of contingency awareness emerged. Instead, differential responses were observed in all three groups, also in unaware subjects. This result is in line with previous evidence for the role of the amygdala as a central fear module, which can be activated independent of contingency awareness, potentially via direct projections from the thalamus (LeDoux, 2000; Morris *et al.*, 2001; Öhman and Mineka, 2001; Tabbert *et al.*, 2006; Öhman *et al.*, 2007).

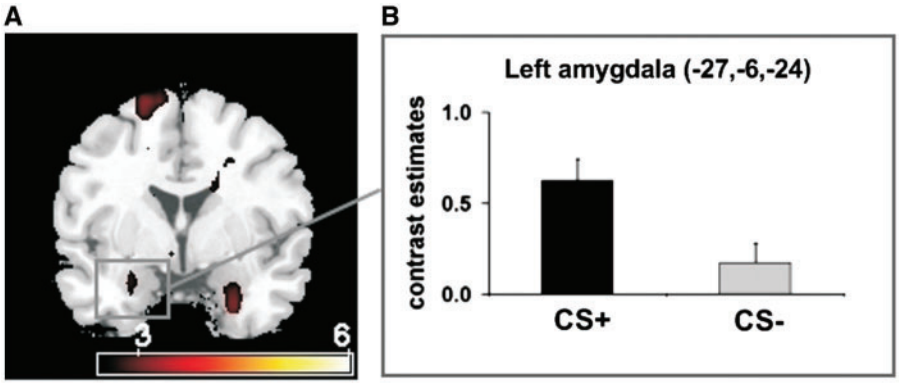

Fig. 4 (**A**) Differential amygdala activation (CS^+ minus CS^-) in the unaware group. (**B**) Mean contrast estimates for the left amygdala peak voxel in the unaware group for the CS^+ and the CS^- separately. Error bars are standard errors of the mean. The colour bar depicts the *t*-values for this contrast.

Yet, for visual stimuli this projection has been evidenced only indirectly so far (for an overview see Öhman *et al.*, 2007). Other than previously (Tabbert *et al.*, 2006), we observed differential amygdala activation also in instructed aware subjects. Although not expected, this is consistent with the assumption of a central fear module and with findings of amygdala activation in response to announced threat cues (Phelps *et al.*, 2001; Ueda *et al.*, 2003).

In the OFC, main effects of awareness emerged. In the lateral OFC higher responses in the instructed aware as compared with the unaware group were observed, while the medial OFC showed the opposite pattern of activation. The latter finding is relatively consistent with previous observations of enhanced OFC activation in unaware as compared to instructed aware subjects (Tabbert *et al.*, 2006). However there, more lateral OFC regions were observed, without an explicit differentiation between lateral and medial regions. Thus, it is unclear whether the medial OFC showed a similar response pattern in the previous study (Tabbert *et al.*, 2006). The OFC has been related to the evaluation of the reinforcement value of a stimulus and the learning of stimulus–reinforcement associations (Rolls, 1999, 2008; O'Doherty, 2007). Previous work has pointed to a functional subdivision of the OFC into lateral and medial parts (Milad and Rauch, 2007), but without explicating the potential influence of contingency awareness. For example, the lateral OFC has been related to behaviour in response to punishment and the processing of negative emotions, but also to reward expectancy; the medial OFC has been ascribed a role in the processing of positive emotions, the avoidance of an aversive outcome, and fear extinction (Kim *et al.*, 2006; Milad and Rauch, 2007). In this context, the higher lateral OFC activation observed in the instructed aware compared with the unaware group may be explained by the anticipated punishment (UCS). Yet, the enhanced medial OFC activation in unaware subjects does not fit the above described function. Altogether, procedural differences between studies (e.g. differences between instrumental learning and classical conditioning) restrict the conclusions, which can be drawn from previous studies, on lateral *vs* medial OFC functions in the present study. Another functional subdivision of the OFC—into a lateral and a medial part—is related to the ambiguity of a situation and the steadiness of stimulus–outcome associations in decision making (O'Doherty *et al.*, 2003; Windmann *et al.*, 2006). It is suggested that the lateral OFC is preferentially activated in situations involving ambiguous conditions or changing stimulus–outcome associations, while medial parts of the OFC monitor relevant reinforcement contingencies, especially if the outcome is steady. This feature of the medial OFC may have been particularly important in our unaware group, who did not recognize the contingencies, leading to the relatively enhanced activation in this group as compared to the instructed aware group. Yet, if contingencies are known, as was the case in the instructed group, the focus of OFC processing may switch to other important features of the situation (e.g. preparing for an upcoming aversive event).

A trend for a main effect emerged in the bilateral hippocampus. *Post hoc* tests confirmed a role of this structure in the development of contingency awareness as suggested also by Carter and colleagues (2006): Enhanced differential responses of the right hippocampus emerged in the learned aware group compared with both other groups (instructed aware, unaware).

The (dorsal) ACC, the insula and the ventral striatum showed main effects of awareness due to enhanced conditioning in both aware groups as compared to the unaware group. The ventral striatum has been related to the development of contingency awareness (Klucken *et al.*, 2009b). Yet, other than expected, we did not observe significant differences between the learned and the instructed aware group with respect to differential ventral striatum responses. Instead, both aware groups showed higher ventral striatum differentiation than the unaware group. Thus, the present study more closely relates the ventral striatum to the presence of contingency awareness, independently of how it was

gained. However, the smaller sample size in the learned aware group may have concealed existing differences between the learned and instructed aware groups in this structure, weakening the reliability of this conclusion. Concerning the (dorsal) ACC, the present result complements previous suggestions linking this structure, together with the insula, to the conscious processing of fear relevant stimuli (Büchel *et al.,* 1998; Öhman, 2005), but again independent of how awareness was achieved (see also Mechias *et al.*, 2010). Further, among other structures, the ACC has been linked to the production of SCRs (Critchley, 2005; Milad *et al.,* 2007). Thus, the ACC may well have been involved in the production of conditioned SCRs in the instructed aware group.

Several structures of parietal, frontal, temporal and cingulate cortex beyond the predefined ROI showed a whole brain corrected main effect of awareness. These structures also showed significant differential responses in the conditioning analyses of only the instructed aware group and higher differential responses in the instructed aware as compared with the unaware group. These activations potentially reflect enhanced salience of and attention allocation to the CS^{+} due to the instruction and action preparation especially in this group (Downar *et al.,* 2000, 2002). Yet again, the smaller sample size in the learned aware group might have distorted the result pattern, potentially concealing activation characteristic for instructed and learned aware fear conditioning.

Crucially, there are varying experimental approaches by which contingency awareness can be manipulated. These might lead to different neural response patterns. Frequently used methods to prevent or manipulate awareness are backward masking (Öhman and Soares, 1993), distraction (Tabbert *et al.*, 2006), attentional load (Carter *et al.*, 2003; Kalisch *et al.*, 2006) or binocular rivalry (Pasley *et al.*, 2004). For instance, attentional load has been shown to influence neural activation, with decreasing but also enhancing effects on amygdala and dorsal ACC activation (Pessoa *et al.*, 2002; Williams *et al.*, 2005; Kalisch *et al.*, 2006). A recent highly relevant meta-analysis identified common and distinct activation patterns in instructed aware fear conditioning *vs* classical fear conditioning without instruction (Mechias *et al.*, 2010): instructed fear most reliably activated a region centred on rostral parts of the dorsal medial prefrontal cortex (dmPFC) and the dorsal ACC [in an area closely situated to the (dorsal) ACC activation reported in the present study]. Slightly more posterior parts of the dorsal ACC/dmPFC were activated in uninstructed classical fear conditioning, but to a lesser extent (Mechias *et al.*, 2010). It is important to note, however, that the meta-analysis included studies with uninstructed contingency aware and unaware subjects, while they were explicitly separated in the present study. Nevertheless, a joint conclusion of the present study and the meta-analysis could be the involvement of the dorsal ACC in instructed and uninstructed aware fear conditioning (Mechias *et al.*, 2010). A difference between the present study and previous backward masking studies lies in the duration of CS presentation: we presented the CS above a perception threshold (i.e. supra-liminally), whereas CS are presented subliminally during backward masking. One might thus expect less recruitment of cortical brain structures in backward masking studies, while the amygdala may commonly respond to sub- and supra-liminally presented stimuli (LeDoux, 2000; Tabbert *et al.*, 2006; Öhman *et al.,* 2007).

Regarding UCSs, we did not observe a main effect of contingency awareness. There were, however, trends in the left amygdala with the highest responses in learned aware subjects and the left insula with enhanced responses in learned and instructed aware subjects. Both structures are involved in emotion processing and react to aversive sensory stimuli like an electrical stimulation (Öhman and Mineka, 2001; Critchley, 2005; Nitschke *et al.,* 2005; Paulus and Stein, 2006). An altered processing of the UCS, which can be described in terms of diminution or facilitation (i.e. reduced or enhanced UCRs in aware subjects), may be an important outcome of fear conditioning (Domjan, 2005). Thus, we interpret the tendency of enhanced insula and amygdala activation in aware subjects as an UCR facilitation (Domjan, 2005; Tabbert *et al.,* 2006; Dunsmoor *et al.,* 2008; Knight *et al.,* 2010).

We would finally like to address potential shortcomings of the present study. First, sample sizes differed between the groups, with the lowest number of subjects in the learned aware group. Therefore, only medium to large-sized effects could be detected in this group. Failure of detection of potential smaller effects does not preclude their presence. Further, the three groups were not experimentally predetermined to the same extent, because the learned aware group consisted of subjects originally designated for the unaware group. However, as results in the 2-back task as well as the socio-economic and educational background of the three groups were comparable, we assume that the development of contingency awareness in designated unaware subjects occurred by chance. Finally, contingency awareness was not assessed online. However, an online assessment of contingency awareness can influence its development potentially reducing the number of unaware subjects. Further, the use of a short recognition questionnaire has been shown to be the most valid and sensitive measure of awareness among several post-conditioning questionnaires, including short and long recall as well as recognition (Dawson and Reardon, 1973; see also Lovibond and Shanks, 2002). Additionally, in one previous study, online expectancy measures of awareness and post-experimental verbalization were in perfect agreement with respect to awareness classification (Purkis and Lipp, 2001).

To sum it up, main effects of awareness in the dorsal ACC, the insula and the ventral striatum could be traced back to higher neural responses in both aware groups. The OFC

showed opposite findings in lateral and medial structures based on differences between unaware and instructed aware subjects. Activation was specifically enhanced in the hippocampus in the learned aware group. No other structures of interest showed a main effect of contingency awareness. Crucially, there were no main effects of contingency awareness on amygdala activation. Taken together, the activation patterns in the contrast CS^+ minus CS^- in relation to contingency awareness are in line with current models of fear conditioning (Öhman and Mineka, 2001; Hamm and Weike, 2005) proposing that conditioned responses in a fear network centred around the amygdala can be acquired independent of the ability to correctly verbalize the CS/UCS contingencies. Sustained responding of sub-cortical structures may be involved in the development of pathologic fear as for example in anxiety disorders (Öhman and Mineka, 2001; Hamm and Weike, 2005; Etkin and Wager, 2007; Shin and Liberzon, 2010; see also Mineka and Zinbarg, 2006 on the role of conditioning for the development of anxiety disorders). The fact that a fear response can be acquired without explicit knowledge about the association of the conditioned stimuli with the initially fear eliciting event (UCS) explains the clinical observation that pathologic fear reactions sometimes occur without any obvious cause. The results of the present experiment imply that this is not due to a failure to remember but that the explicit knowledge of contingencies was not achieved despite the development of a kind of sub-cortical fear memory.

SUPPLEMENTARY DATA

Supplementary data are available at *SCAN* online.

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