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Tracking Short-Term Auditory Cortical Plasticity during Classical Conditioning Using Frequency-Tagged Stimuli

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Animal studies indicate that short-term plasticity during classical conditioning is a fast process. The temporal details of this process in humans are unknown. We employed amplitude-modulated tones in order to elicit the steady-state field (SSF). Conditioned stimulus (CS+) and CS− had a common low carrier frequency, however, differed in their high-frequency component. Low and high frequencies within one tone were modulated at 29 and 45 Hz, respectively. Mean fast Fourier transformation analysis of each single trial allowed extraction of the cortical response to these modulation frequencies, allowing to track cortical responses trial by trial. Mutilation pictures were used as unconditioned stimulus. Furthermore, heart rate and contingency awareness were assessed. Our main findings are the following: 1) A rapid (within 5 trials) enhancement of the amplitude of the high frequencies in contrast to the low frequency, while the high frequencies differentiated later (toward end of acquisition). This partially replicates rapid plasticity as shown before in animals. 2) Those participants who were less aware of the stimulus contingencies showed a relative heart rate acceleration and greater SSF increase to the CS+. This could possibly imply a stronger early amygdala activation in these participants, which then mediates the development of conditioning-related reorganization in auditory cortical areas.

Keywords: auditory steady-state fields, classical conditioning, contingency awareness, frequency tagging, magnetoencephalography

Introduction

Aversive classical conditioning is a common method to achieve changes in the relevance of a previously neutral stimulus (conditioned stimulus, CS+), by pairing it with an intrinsically significant stimulus (unconditioned stimulus, US). This leads to behavioral changes following CS+ presentation. Even though the exact neural foci of CS–US pairing has been a matter of debate, the amygdala has been ascribed a major role in inducing long-term plasticity within the primary auditory cortex via cholinergic projections from the nucleus basalis (LeDoux 1996; Suga and Ma 2003; Weinberger 2004). However, short-term neuronal changes have also been reported for sensory representational areas.

Electrophysiological studies in the auditory domain of animals show that aversive conditioning shifts the characteristic frequency of neurons in A1 toward the CS tone. The conditioned frequency takes up more space of the tonotopic map than prior to conditioning. In guinea pigs, Edeline and colleagues (Edeline et al. 1993) reported shifts of tuning curves toward the conditioned tone frequency after 5–15 CS–US pairings. The rapidity with which neuronal response properties change in the primary auditory cortex within a behaviorally relevant context has also been shown by Fritz et al. (2003, 2005). Changes in receptive field properties were seen in 70% of all neurons within minutes. These results preclude the possibility of mechanisms associated with long-term changes, such as the formation of new synapses. It is more likely that excitatory and inhibitory influences between neurons can be modulated via top–down mechanisms.

The main aim of the present study was to elucidate the time course of aversive conditioning in humans, possibly building a bridge between previous electrophysiological studies in animals and neuroimaging works in humans. In these latter studies, CS+ related neuronal response increases have not been clear-cut and decreases have even been reported (Morris et al. 1998; Jancke et al. 2001). We adopted measures of the auditory neuromagnetic steady-state response (SSR) for this study, which give an excellent signal-to-noise ratio (Pantev et al. 1996; Ross et al. 2000; Weisz et al. 2004). The SSR can be measured following the presentation of auditory stimuli that are modulated (e.g., amplitude modulated) at a certain frequency, usually around 40 Hz. This evokes a brain response that oscillates at the modulation frequency of the stimulus. The great advantage of the SSR over classical-evoked responses is that one can focus on signals at the modulation frequency (which is set by the investigator) and can choose to ignore the rest. Using different modulation frequencies for different tones presented within the same stimulus (here: CS+ or CS–), it is possible to track auditory cortical responses for each tone separately. This method is termed "frequency tagging" and has been employed with success by other authors (Fujiki et al. 2002). In the context of research on conditioning, this technique has been employed as far back as the late 1950s (John and Killam 1959), but to the best of our knowledge, we are not aware of other conditioning studies employing compound stimuli.

In our study, the CS+ and CS– shared a common low-frequency component modulated at 29 Hz and differed in the high carrier frequency that was modulated at 45 Hz. Thus, the low frequency was not predictive as to whether an aversive US would follow or not and could be used as reference for the informative high-frequency response within one and the same trial. The rationale was that stronger brain responses should be seen specifically for the tone that was paired with an aversive stimulus but not for another tone that was presented within the CS+ and CS–. The primary interest was directed at the temporal development of the SSRs for the different tones. A further question that could be addressed by our study was the relationship between heart rate changes (reflecting fear response), contingency awareness, and auditory cortical changes. In a previous study by group coworkers investigating conditioning of the visual SSR (Moratti and Keil 2005), increased CS+ associated brain responses were only seen in subjects exhibiting heart rate acceleration to the

CS+. This conforms with other studies pointing to a positive association between heart rate acceleration and acquisition of a fear response (Hodes et al. 1985; Hamm and Vaitl 1996). On the other hand, some authors (Chan and Lovibond 1996; Grillon 2002) have shown that lower contingency awareness is related to enhanced anxiety. These separate findings could be unified by showing that participants who are less aware of the contingency show stronger CS+ enhancements in auditory cortex. Our design enables us to tackle this question directly.

Materials and Methods

Participants

Sixteen right-handed subjects (8 females; mean age ± standard error [SE]: 24.63 ± 0.91 years) were recruited from the University of Konstanz. After familiarizing the participant with the magnetoencephalographic (MEG) recording room, both the general procedures were explained and an example of an aversive picture was presented to the participant (Do you think you could bear looking at such pictures?). Written informed consent was then collected from each participant. The experiment was approved by the Ethics Committee of the University of Konstanz. The subjects received €15 for their participation. Vision was corrected to normal with MEG-compatible lenses, if necessary.

Stimuli and Procedure

Two stimuli presented to the left ear served as CS+ and CS–, respectively. Both sounds were compound stimuli (10 s duration; 15 ms rise and fall time) consisting of simultaneously presented tones at 2 different carrier and modulation frequencies (100% modulation depth). The CS– consisted of a 500-Hz tone modulated at 29 Hz and a 1200-Hz tone modulated at 45 Hz. The CS+ was identical to the CS– except that the high-frequency component was a 2850-Hz tone. Each stimulus was presented 40 times in a pseudorandom manner leading to sequences in which CS+ and CS– alternated, although one stimulus could be presented twice in a row (e.g., A-B-A-B-B-A-etc.). The stimuli were separated by inter-stimulus intervals (ISIs) varying between 7100 and 8100 ms. The set up of the trial and spectrograms of the stimuli are depicted in Figure 1*a*,*b*. During the ISI, an image of an eye was presented in order to let the subject know that she/he was allowed to blink. The first 20 trials (10 CS+, 10 CS–) were presented without reinforcement (baseline). These were followed by 40 trials (20 CS+, 20 CS–) with reinforcement, starting 9 s after tone onset and terminating 1.5 s after tone offset. The CS+ was paired with the US, which were randomly chosen from a list consisting of 40 pictures from the International Affective Picture System (IAPS) rated as highly negatively valent and arousing (mostly mutilation pictures). For the CS–, we chose the same quantity of neutral pictures consisting of public places displaying people. No pictorial stimuli were paired with the sounds during the extinction block (10 CS+, 10 CS–). To ensure that participants paid attention to the visual display (and to prevent participants from looking away, e.g., due to disgust), they were instructed to count yellow crosses that could appear during the acquisition phase. Stimulus presentation was carried out using Psyscope X (http://psy.ck.sissa.it/). Data were recorded from subjects lying in a supine position. Pictures were presented via a video projector to the ceiling of the MEG chamber approximately in 1.5 m distance to the subject.

At the end of the experiment, the Self Assessment Manikin was used to evaluate the arousal and valence of the stimuli (Bradley and Lang 1994). A contingency awareness test was also administered, consisting of 17 questions. The first question asked whether subjects believed that all tones heard during the experiment were the same. This was to ensure that the subjects could discriminate between the different tones, which was found to be the case for all participants. Then they listened to both sounds again, and they were instructed to classify the CS+ as "high tone" and the CS– as "low tone." After this, 16 questions regarding details of the relationship between sounds and picture were posed (e.g., "I think the aversive picture always came after the termination of the high tone," "I think that the high tone predicted the aversive picture"). Answers were Correct, Incorrect, or Don't Know. Correct responses (1 point each) were added to yield a total score of contingency awareness.

Data Acquisition and Analysis

Data (678.17 Hz sampling rate; 0.1–200 Hz online filter) were collected using a 148-channel whole head magnetometer (MAGNES 2500 WH, 4D Neuroimage, San Diego, CA). Prior to each recording, each individual's head shape was digitized. Coils were attached at 5 positions (left and right periauricular points, nasion, a pseudo-vertex, and a pseudo-inion) that were used to measure the position of the head relative to the sensors. Two Ag/Cl electrodes were attached to the forearms for measurement of the electrocardiogram (ECG). Additional electrodes were applied to both outer canthi and above and below the right eye in order to monitor eye movements (electrooculogram [EOG]). Acquisition of EOG and ECG was carried out using a Synamps amplifier (Neuroscan, E1 Paso, TX).

For each of the 80 trials, 8.5-s epochs were extracted. The first 0.5 s were discarded in order to avoid the influence of the transient response. The concatenated epochs were subjected to an independent component analysis using the infomax algorithm implemented in the "runica" function of the "EEGLAB" (http://www.sccn.ucsd.edu/eeglab/) toolbox (Delorme and Makeig 2004). This procedure separates the signal measured at N sensors—assumed to be a linear mixture of various neurophysiological and also artifactual sources—into an equivalent amount of temporally independent and spatially fixed components. From visual inspection, components representing blink, horizontal eye movement and heart beat activity were rejected and the cleansed data reconstructed for the 148 sensors (multiplication of the inverse of the weighting matrix with the component activations). A mean fast Fourier transformation was calculated for each trial by shifting a 512-point hamming-tapered window (1/2 window length overlap) across the time series and the power (square of the complex modulus) extracted. This approach is represented for one sample trial in Figure 1*c*. In order to obtain sensor groups of interest, the 40 amplitude values of the CS+ frequency (2850 Hz) were contrasted with the average values of the residual frequencies at each sensor:

$$\mathrm{AmpContrast}_{i,s} = (\mathrm{AmpResiduals}_{i,s})/3 - \mathrm{Amp2850Hz}_{i,s},$$

with i corresponding to trials and s denoting channels. This vector of 40 values per channel was entered into a principal component analysis using singular-value decomposition yielding 3 matrices: the principal components, the eigenvalues, and the eigenvectors (weight matrix). The screen plot of the component eigenvalues reveals a single dominant component (Fig. 2*a*), its time course over trials showing a steady change (Fig. 2*b*). The activation of the component was backprojected onto the sensors by multiplying the component activation with the eigenvector. This is depicted in Figure 2*c*, which shows increased amplitudes at left temporal sensor (LTS) and right temporal sensor (RTS). Seven sensors were selected from each cluster (left: 54, 55, 76, 77, 78, 97, 98; right: 65, 66, 88, 89, 90, 109, 110) and will be noted as LTS and RTS subsequently.

Heart Rate (HR) (beats per minute)—derived from the interbeat interval—was calculated in 500-ms bins for the first 7.5 s following stimulus onset. A 2-s prestimulus baseline was subtracted from the HR waveform to yield the stimulus-evoked heart rate changes.

For statistical analysis, the neuromagnetic data for LTS and RTS of the acquisition and extinction trials were clustered into 6 blocks with 5 trials: acquisition 1 (A1; trials 11–15), acquisition 2 (A2; trials 16–20), acquisition 3 (A3; trials 21–25), acquisition 4 (A4; trials 26–30), extinction 1 (Ext1; trials 31–35), and extinction 2 (Ext2; trials 36–40). For each subject and each frequency, the 10 baseline trials were averaged and the values of each block were divided by the baseline value to yield relative changes for each frequency. Because there were only sizeable amplitude enhancements for the high frequencies, as was predicted, the amplitudes of the 500-Hz blocks were subtracted from the corresponding high-frequency value, which returns the net high frequency–related enhancements. A linear mixed-effect model was performed for each sensor group with frequency (condition) and time (block) as fixed factors. Because our prediction was that the CS+ frequency should yield stronger amplitudes than the CS– frequency, this analysis was followed up by planned comparisons using paired t-tests. HR responses were divided in 2 time windows: 0.5–4 and 4.5–7 s relative to tone onset. The HR values were grouped into blocks of 10 trials: baseline (B; trials 1–10), acquisition 1 (A1; trials 11–20), acquisition 2 (A2; trials 11–30), and extinction (Ext; trials 31–40). Paired t-tests were

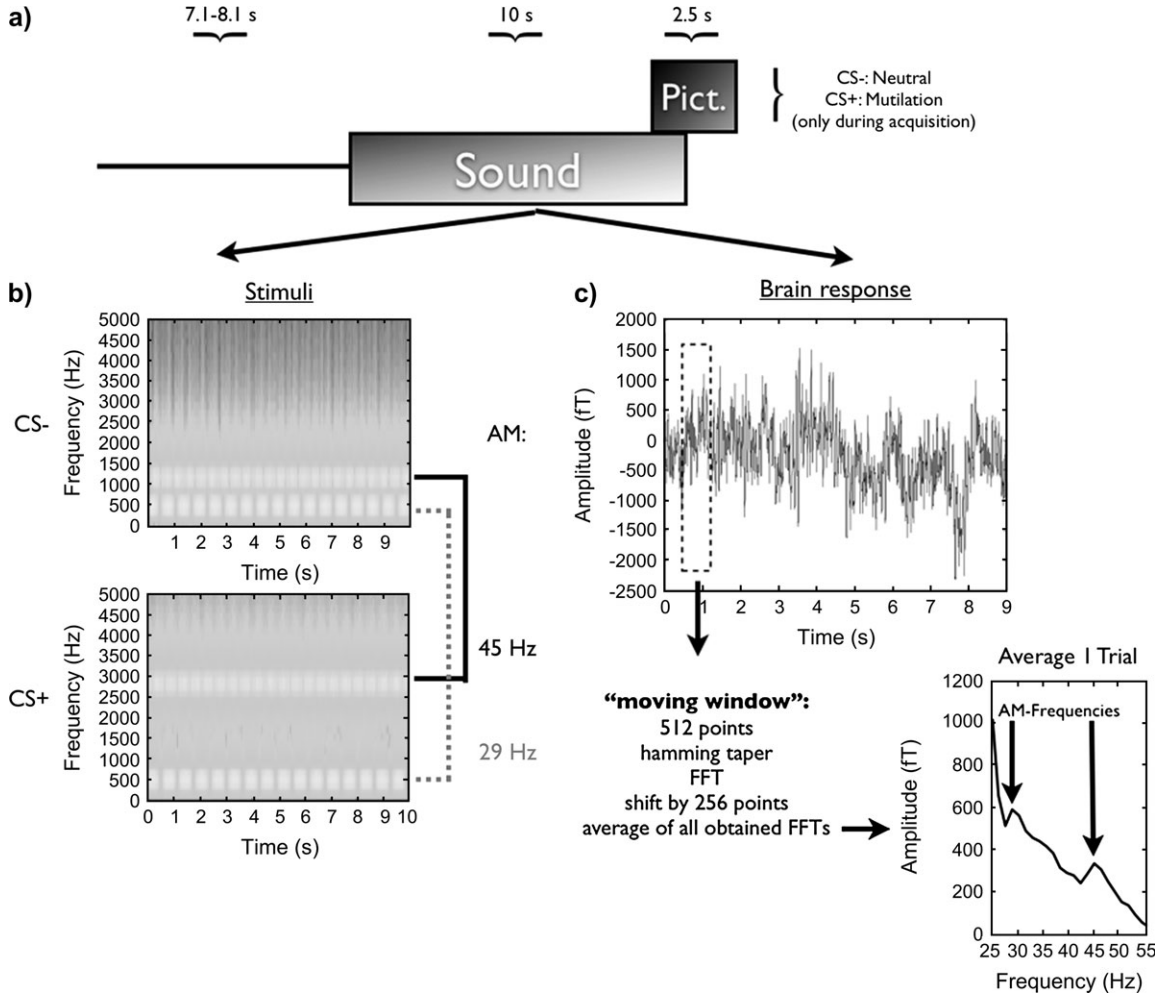

Figure 1. (*a*) Set up of a single trial. Pictures (presented only during acquisition) commenced 9 s after sound onset and terminated 1.5 s after sound offset. (*b*) Stimuli were compound sounds consisting of 2 amplitude-modulated tones. A common carrier frequency (500 Hz) was modulated at 29 Hz, whereas the high carrier frequencies (different for CS+ and CS−) were modulated at 45 Hz. (*c*) Depiction of basic signal analysis for one trial of a single subject. A moving window was applied across the raw data (top panel) moving at half window length, in which data were hamming tapered to avoid spectral leakage. For each window, a fast Fourier transformation (FFT) was calculated and finally averaged to yield the "mean FFT." Note the peaks at the AM frequencies.

calculated between B and A1, A2, or Ext, respectively. Furthermore, to elucidate the association between HR changes and steady-state field (SSF) changes that signified differential processing of CS+ and CS– (trials 26–35; see Results), product moment correlations were calculated.

Results

Stimulus Ratings

The aversive properties of our USs (mutilation pictures) were validated by ratings given by the participants. In comparison to neutral stimuli, they were rated as more arousing (means ± SE: 7.30 ± 0.47 vs. 2.24 ± 0.30; t_{14} = 11.01, P < 0.001) and more negative regarding valence (means ± SE: 1.64 ± 0.27 vs. 6.02 ± 0.22; t_{14} = –10.60, P < 0.001).

Amplitude

For each participant, we obtained one amplitude value for each trial (i.e., 40 values) and 2 for each condition (CS+: 500 and 2850 Hz; CS–: 500 and 1200 Hz), yielding overall 4 time series vectors. A singular-value decomposition (see Materials and Methods for details) indicating changes over trials for the 2850-Hz (informative) tone relative to the other tones resulted in one dominating component with relatively high eigenvalues (see Fig. 2*a*). The component activation shows an increasing change following the end of the baseline trials (first 10 trials; Fig. 2*b*). Backprojection of the component activation onto our 148 sensors shows the topographic distribution of the first component as can be seen in Figure 2*c* (blue–red corresponding to 0–0.3). From this analysis, 2 spatial clusters over temporal areas are evident, showing amplitude increases for the 2850-Hz tone. These clusters, consisting of 7 sensors each, were subsequently regarded as regions of interest. To obtain one amplitude time series each for LTS and RTS, values were averaged across the respective sensors. Because we were interested in conditioning-related changes over time, the amplitude values were divided by the mean baseline amplitude (10 trials). This is depicted in Figure 3*a* (acquisition and extinction runs blocked with 5 trials; baseline collapsed across all 10 trials). A striking feature is that whereas the amplitudes for both 500-Hz tones show a slight decrease over the course of the experiment, the amplitudes for the high-frequency tones increase. This difference can be observed very early on within the first 5 trials ($F_{1,30}$ = 24.66, P < 0.001) and increases over the course of the experiment,

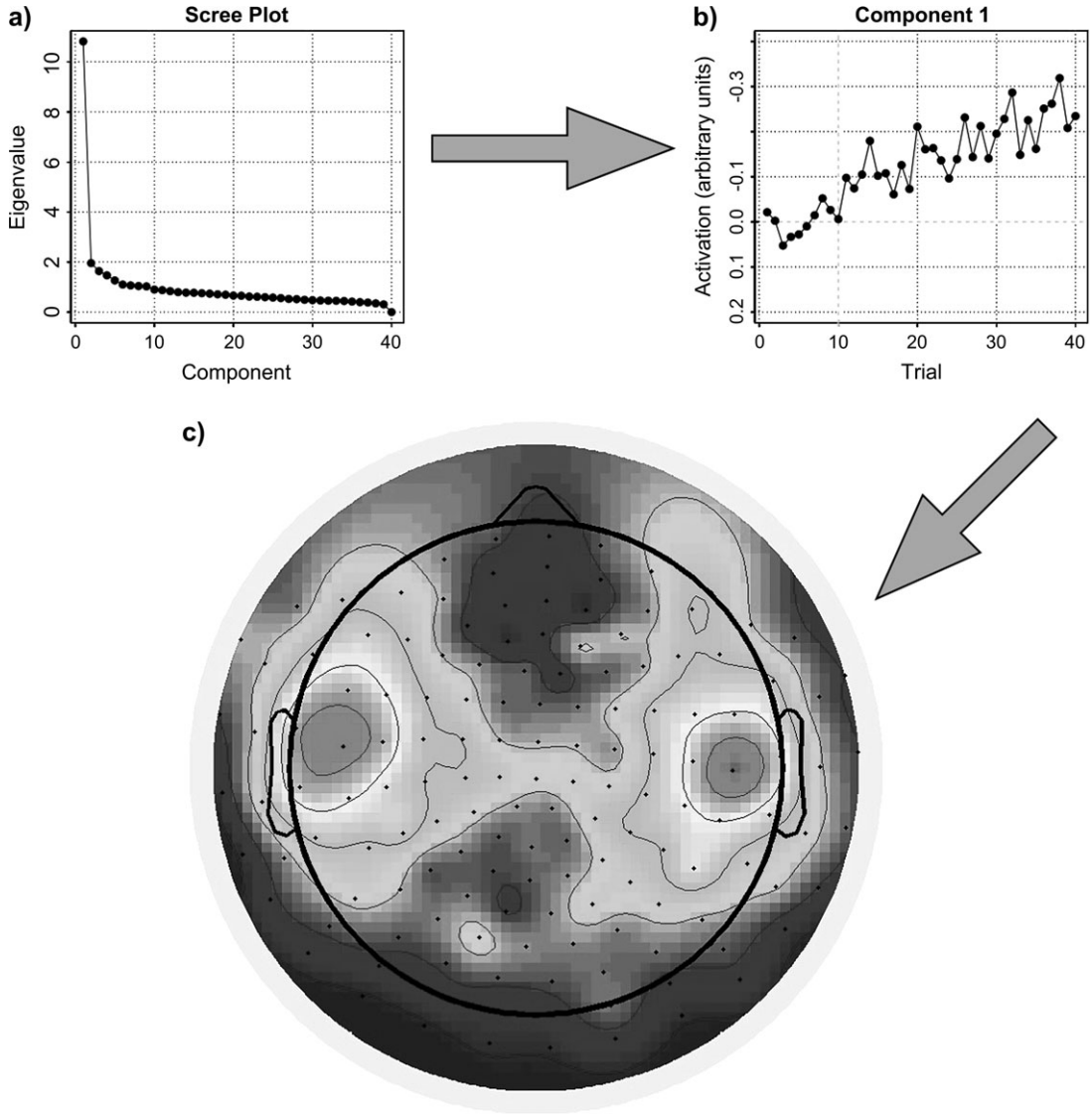

Figure 2. (*a*) Principal component analysis of a contrast of the crucial SSF response to the 2850-Hz tone with the other frequencies yields a single dominant component. (*b*) The time course is characterized by an increasing change starting during the acquisition phase (>trial 10). (*c*) Backprojection of the component activation to the sensor space indicates that this component is particularly marked by an amplitude increase over temporal sensors (blue–red coding values between 0–0.3). This result was utilized to form 2 sensor groups of interest (RTS and LTS).

resulting in a significant interaction ($F_{1,346}$ = 19.93, P < 0.001). Neither main nor interaction effects involving hemisphere were significant (all F values < 1.8, P > 0.17), confirming the impression that the amplitude changes for the high and low tones are similar across hemispheres. Thus, the main feature distinguishing between CS+ and CS– related activity is the differentiation between the high tones. To scrutinize this in more detail, activity related to the 500-Hz tone of the CS+ and CS– was subtracted from the activity related to its respective high tone (Fig. 3*b*), to reflect activity solely related to the processing of the high tone. For the ipsilateral (left) hemisphere, amplitudes for both tones exhibited a similar increase during acquisition and extinction. In the contralateral (right) hemisphere, activity related to the tones develops similarly over the first 15 trials of the acquisition phase (A1–A3) but then starts to diverge toward the end of the acquisition (A4). The mixed-effect model revealed an overall effect for condition ($F_{1,346}$ = 8.14, P < 0.006) and time ($F_{1,346}$ = 21.59, P < 0.001). The first effect reflects an approximately 22% stronger enhancement for the 2850-Hz as compared with the 1200-Hz tone (means ± SE: 0.38 ± 0.02 vs. 0.31 ± 0.02). However, not all interactions reached a level of statistical significance (all F values < 1.9, P > 0.16). In order to increase the power of the analysis, we calculated planned contrasts according to our hypothesis (i.e., amplitudes for 2850 > 1200 Hz). For the left hemisphere, no time interval showed significantly greater activation for the relevant CS+ cue (2850 Hz; P values > 0.09). Contrasts for the right hemisphere indicated a significantly enhanced amplitude for the 2850-Hz tone at the end of the acquisition (A4; P < 0.01) and the beginning of the extinction (Ext1; P < 0.03). This is marked in a box in the upper panel of Figure 3*b*. Within this time window, relative enhancement for the CS+ was about 62% stronger than for the CS– in the right hemisphere as compared with only 18% in the left hemisphere.

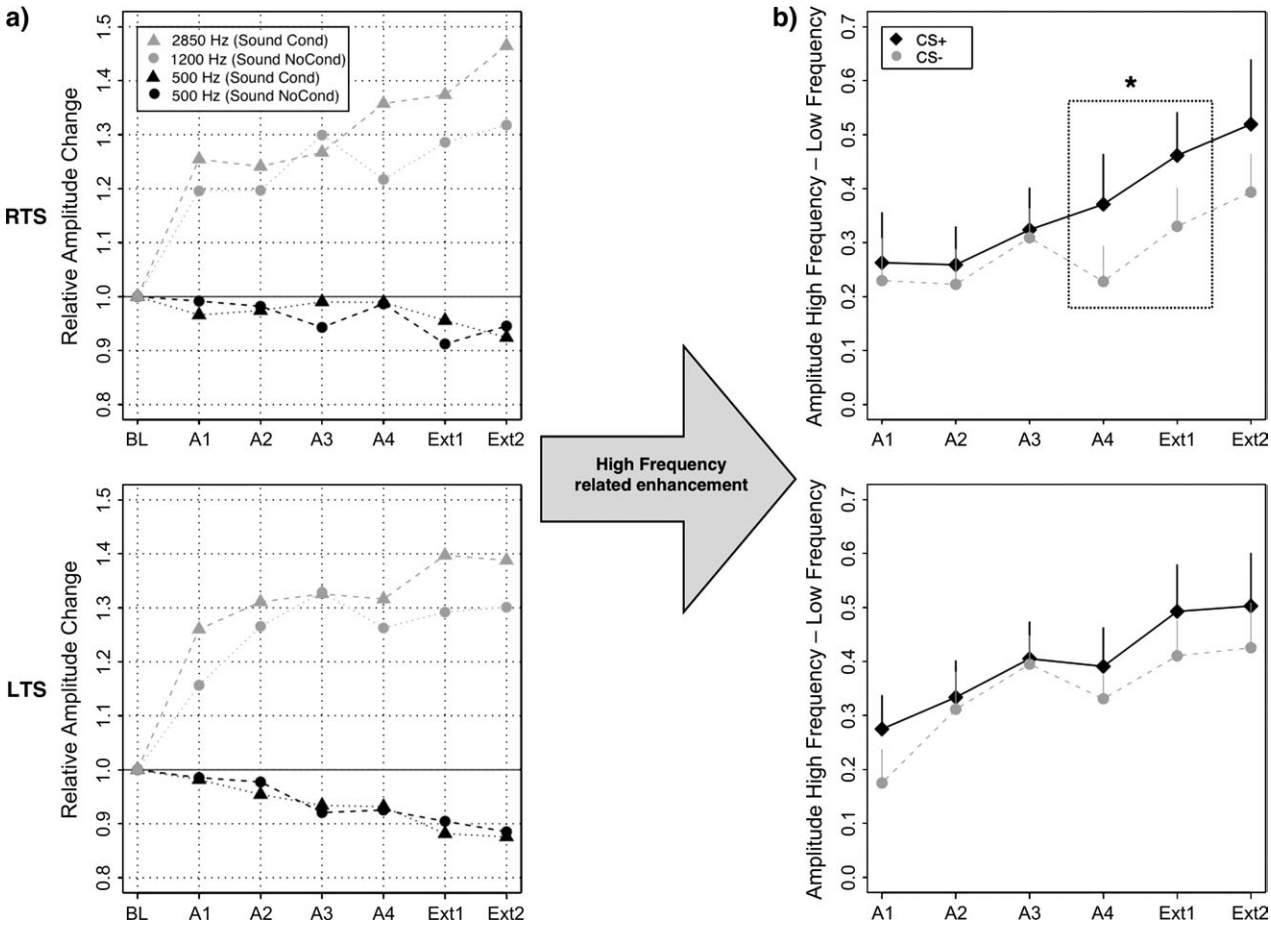

Figure 3. (*a*) The left panel shows separately for RTS and LTS the evolvement of the amplitudes for the 4 sounds relative to the 10 baseline trials. Amplitudes for the high frequencies increase rapidly within 5 trials (A1), whereas amplitudes for the 500-Hz tones remain unchanged or show a slight decrease. (*b*) Contrasting the SSF amplitudes of the high tones against amplitude evoked by their respective 500-Hz tone as seen in the right panel, a differentiation between the 2 information-carrying high tones can be seen toward the end of the acquisition and beginning of extinction at the contralateral RTS.

Heart Rate

Changes in heart rate relative to baseline (preacquisition; trials 1–10; filled circles in Fig. 4*a*,*b*) were collapsed from 0 to 7.5 s post stimulus onset. The results are depicted in Figure 4*a*,*b* for the CS+ and the CS–, respectively. For both types of stimuli, it is evident that heart rate decelerates during the first half of the acquisition phase (trials 11–20; filled squares), with this pattern appearing a little more pronounced for the CS+. During the second half of the acquisition (trials 21–30; filled diamonds) and extinction (trials 31–40; filled triangles), heart rate changes return toward baseline levels. Furthermore, the heart rate response is in general characterized by an early decelerative component (until ~4 s) and a later less-pronounced slowing of heart rate (from ~4 s onward). Thus, planned comparisons using *t*-test were carried out between the baseline condition and the other experimental phases for 2 time windows (0.5–4 and 4.5–7.5 s) separately (Fig. 4*c*,*d*). This analysis revealed that for the early time window (0.5–4 s), a significant deceleration was only present for the early acquisition trials (trials 11–20) of the CS+ (t_{15} = 2.30, P = 0.036). The corresponding response for the CS– failed to reach statistical significance (t_{15} = 1.66, P = 0.12). During the late time window (4.5–7.5 s), a significant deceleration relative to the baseline was observable for the CS– (t_{15} = 2.23, P = 0.04) as well as a trend for the CS+ (t_{15} = 1.77, P = 0.096) for the early acquisition trials. All other contrasts were not significant (t < 1.32, P > 0.42).

Furthermore, high frequency–related SSF enhancements (see section above) for 2 time windows (one early during acquisition, the other spanning the end of acquisition and beginning of extinction; i.e., where the SSF effects were found to differentiate between CS+ and CS–) were also correlated with heart rate changes (10 baseline trials subtracted). For the heart rate, we also chose 2 time windows, one for the first and second half of the acquisition each. The results are summarized in Table 1. The most striking finding is a strong association (r = 0.62 and r = 0.67) between heart rate during the first half of the acquisition (T11–20 in Table 1) and the late SSF effects (T26–35). This means that the greater the acceleration (or the less deceleration was present) in heart rate for the CS+, the higher was the CS+ related SSF enhancement (see Fig. 5*a*). Note that the heart rate effect appeared earlier than the neurophysiological effect, implying that early peripheral conditioning to some extent predicts subsequent cortical conditioning.

Contingency Awareness

On average, the entire group scored 11.06 (SE = 0.62) on our questionnaire (range 8–16). All participants assured us that they heard 2 different tones. In order to determine the extent to

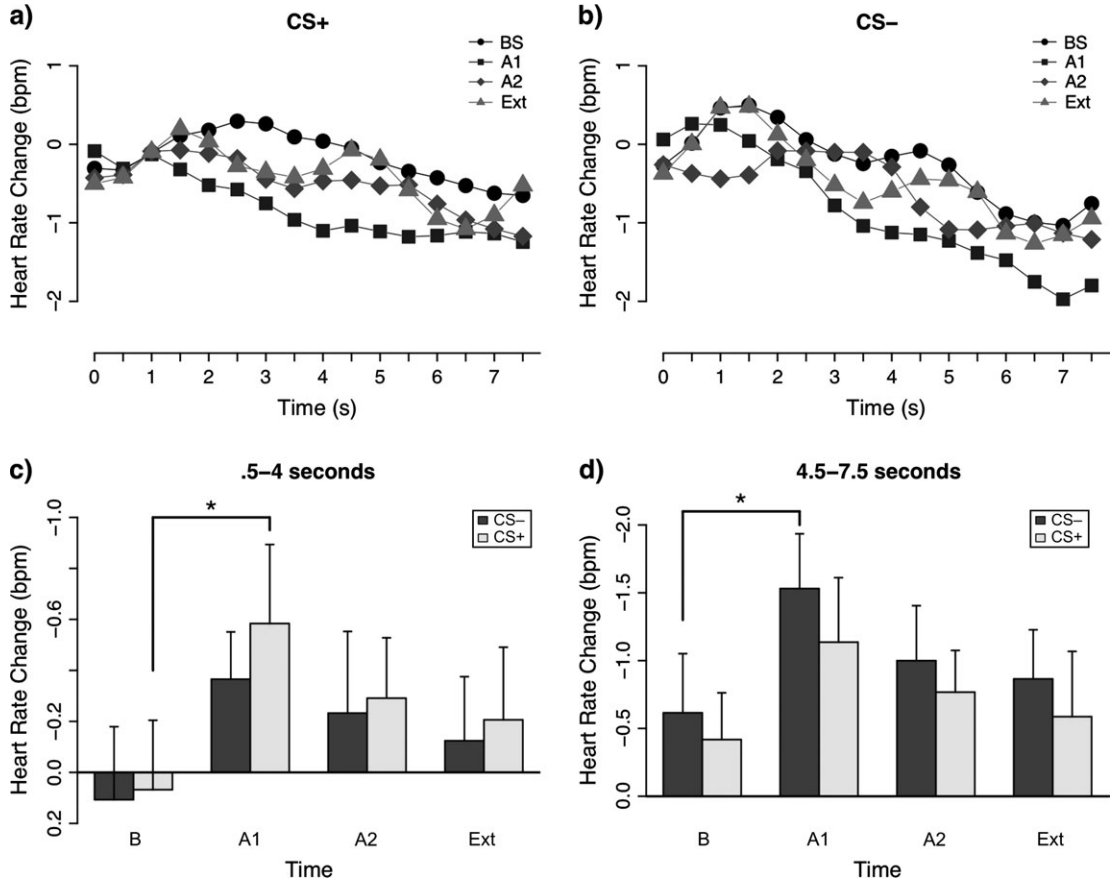

Figure 4. Heart rate responses for different experimental phases following either CS+ (*a*) or CS− presentation (*b*). Most responses can be characterized by a deceleration compared with the 10 baseline trials (filled circles). This is particularly pronounced for the heart rate responses during the first 10 acquisition trials (filled squares). (*c*) Whereas for the CS+ the deceleration can be found more for the early part of the heart rate response (0.5–4 s), it is (*d*) more evident for the late part (4.5–7.5) during CS− presentation.

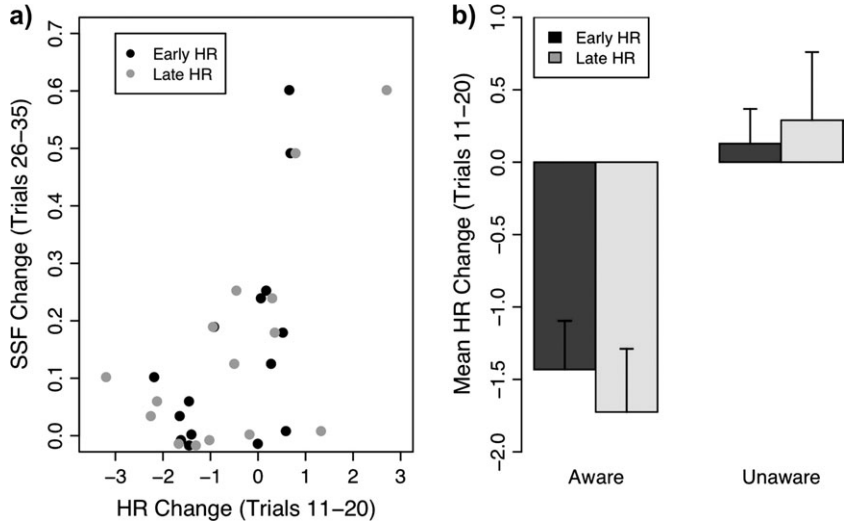

Figure 5. (*a*) Less heart rate deceleration (i.e., relative acceleration) in response to the CS+ tone is positively correlated with enhanced SSF response to the high-frequency tone of the CS+ as compared with the CS−. Note that the heart rate changes precede the SSF changes. (*b*) Splitting the sample into 2 groups via median split of the contingency awareness scores shows that this variable is reflected in the heart rate response to the CS+ tone. Relatively unaware subjects do not exhibit decelerative response that more aware subjects do.

which contingency awareness is reflected by the heart rate, we created 2 groups via a median split (cut-off 10.5). The average awareness score was 13.13 for the aware and 9 for the unaware group (t_{14} = 6.19, P < 0.001). Groups did not differ in terms of valence and arousal ratings of the stimulus categories. The results for the comparisons regarding heart rate are summarized in Table 2. The first thing to note is that contingency awareness is more strongly related to heart rate responses following CS+

presentation (P values ranging from 0.002 to 0.1) as compared with presentation of CS– (P values ranging from 0.36 to 0.43). Furthermore, this differentiation was more pronounced for the first half of the acquisition. As depicted in Figure 5*b*, this effect is due to a pronounced heart rate deceleration of the contingency aware group, whereas heart rate of the contingency unaware subjects remains relatively unchanged. No significant correlations between SSF and contingency awareness could be detected. When, however, the SSF data described in the section above (referring to the high frequency–related enhancements of the CS+ and CS– sounds) are analyzed again including the factor awareness, then additionally condition × awareness ($F_{1,340}$ = 6.35, P < 0.012) and time × awareness × hemisphere interactions ($F_{1,340}$ = 6.50, P < 0.012) are revealed. The first effect (see Fig. 6*a*) arises from the fact that only contingency unaware subjects show a clear cortical distinction between the high-frequency components of the CS+ and CS– sounds. For this group, a greater cortical response is seen following CS+ presentation as compared with CS–. Concerning the time × awareness × hemisphere interaction, separate mixed-effects models for each hemisphere reveal that a significant time × awareness interaction is present only for the left hemisphere ($F_{1,170}$ = 4.45, P < 0.04). As can be taken from Figure 6*b*, the increase over the acquisition and extinction blocks is steeper for the unaware group, whereas the increase of the cortical response in the aware group is flatter.

Table 1
Correlation of HR changes with high-frequency related SSF effects

Heart rate	SSF difference			
	T11–20		T26–35	
	r	*P*	*r*	*P*
T11–20				
Early				
CS+	0.37	0.16	0.62	0.009
CS−	0.21	0.43	0.02	0.94
Late				
CS+	0.49	0.05	0.67	0.004
CS−	0.15	0.58	0.01	0.96
T21–30				
Early				
CS+	0.36	0.16	0.53	0.03
CS−	−0.17	0.51	−0.22	0.40
Late				
CS+	0.66[a]	0.004	−0.56[b]	0.02
CS−	−0.29	0.26	−0.23	0.38

[a]Without outlier: r = 0.06, P = 0.83.
[b]Without outlier: r = 0.11, P = 0.68.

Discussion

The present study attempted to elucidate the temporal dynamics of neuromagnetic auditory SSR during aversive (differential delay) conditioning using 40-Hz amplitude-modulated tones. In the context of the conditioning literature, a novel approach was taken by simultaneously presenting 2 tones modulated at different rates (frequency tagging). The CS+ and the CS– shared one common carrier frequency (500 Hz), being differentiated by their high-frequency component. This approach allows conclusions about the specificity of responses within trials by including an irrelevant (unpredictive) stimulus, an important marker of the specificity of learning (Weinberger 2004). Whereas the steady-state activity for the high (informative) frequencies increased rapidly within the first 5 trials over LTS and RTS, the brain response for the low (uninformative) frequency gradually decreased over the course of the experiment. A slower separation between the 2 higher frequencies differentiating the CS+ and the CS– was seen toward the end of the acquisition and at the beginning of the extinction phase (trials 26–35) over RTSs contralateral to the stimulated ear. This conforms to the majority of previous studies in humans and animals, which show that stimuli of greater motivational salience elicit greater neuronal responses. The rapid differentiation between the irrelevant low tone and the high tones is in accordance with animal studies cited in the Introduction (Edeline et al. 1993; Fritz et al. 2003; Weinberger 2004). It is worth to underline that the LTS also showed some discriminative effects (high vs. low), even though stimuli were presented to the left ear. This means that certain conditioning-relevant

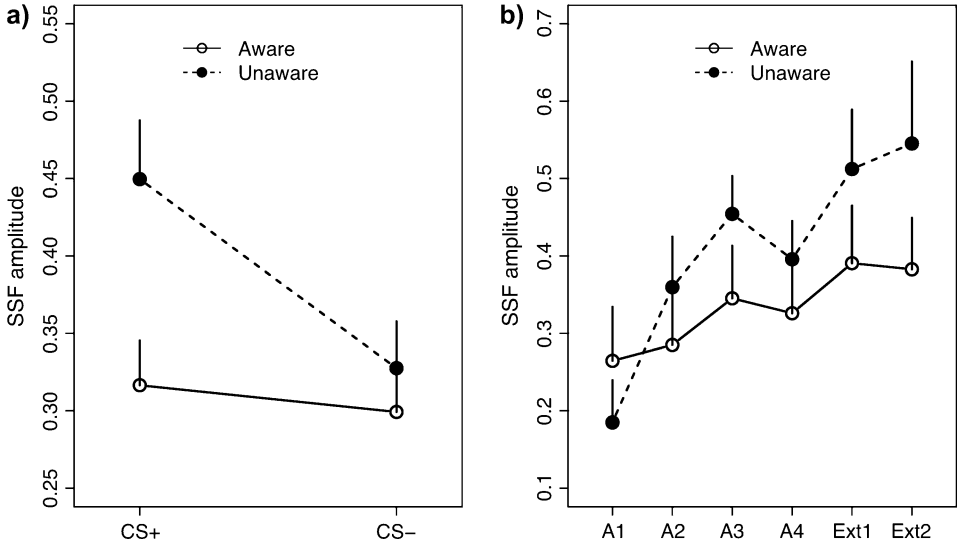

Figure 6. (*a*) Although aware and unaware subjects show similar strength of brain responses to presentation of CS−, they strongly differ for the CS+. Unaware subjects exhibit a marked differentiation of auditory cortical responses between the sound conditions, whereas aware subjects do not. (*b*) The unaware group also have a steeper increase of the SSF amplitude in general over LTS (ipsilateral to sound presentation) than aware subjects.

Table 2
Differences in HR depending on contingency awareness

	Heart rate							
	T11–20				T21–30			
	Early		Late		Early		Late	
	CS+	CS−	CS+	CS−	CS+	CS−	CS+	CS−
Aware	−1.43 (0.33)	−0.74 (0.41)	−1.73 (0.44)	−1.25 (0.57)	−1.05 (0.27)	−0.65 (0.44)	−1.05 (0.27)	−0.71 (0.40)
Unaware	0.13 (0.24)	−0.20 (0.39)	0.29 (0.47)	−0.58 (0.59)	0.36 (0.61)	−0.02 (0.57)	0.35 (0.61)	−0.06 (0.61)
t-test (*P* value)	0.002	0.36	0.007	0.43	0.10	0.39	0.05	0.39

mechanisms appear to act bilaterally even in cases of monaural stimulation. Conditioning studies comparing groups with exclusive left versus right stimulus presentation and measuring electrophysiological responses of both hemispheres simultaneously seem promising in answering the question of what is laterality dependent (i.e., based on the ear chosen for presentation) and independent (i.e., mechanisms that act independently of the ear chosen for presentation). To our knowledge, this is the first study in humans to show the dynamics of auditory cortical Pavlovian conditioning at such a temporal resolution. Yet in contrast to the invasive animal studies, MEG does not allow us to differentiate whether a best frequency shift (i.e., more neurons active), an increased coherent activation, or a response amplification of neurons previously coding the high frequencies took place. Nevertheless, the rapidity of the changes makes it likely that—disregarding the detailed plastic process—a modulation of the balance of preexisting excitatory and/or inhibitory connections took place. One potential mechanism that could be active is that normally subthreshold (silent) horizontal inputs become suprathreshold by means of selective attention. Several electroencephalographic/MEG studies from different modalities show that selective attention to certain stimuli increases brain responses in primary sensory areas (Herrmann and Knight 2001; Keil et al. 2005; Stolarova et al. 2005). Due to the fact that the CS+/CS– common low frequency did not show the same increments as for the high frequencies, we conclude that the effects reported here are not due to general sensitization (e.g., enhanced arousal) but represent associative learning.

Yet, some comments need to be made regarding the slower discrimination between the high frequencies as compared with animal data: 1) In contrast to animal studies, it is clear to the subject that no real threat is posed by the stimuli and the participant can discontinue the experiment at any time. 2) A limitation of our study is that the temporal parameters chosen were not optimal. It is well established that associations between 2 events become more likely when they are contiguous in time, with the CS preceding the US by a small amount of time. Apart from the CS–US interval contiguity between 2 events is also established via the interval between trials. For associative learning, the interval between CS and US should ideally be markedly shorter than the interval between the trials (Rescorla 1988). This was not the case for our study (the end of the US being closer to the onset of the CS of the next trial than to the one of the actual trial) and may have interfered to some extent with learning. Even though CS and US partly overlapped (which should promote association within one trial) and learning could be demonstrated in this experiment, future studies following similar design principles as outlined here should extend the intertrial interval. 3) Generally it is not clear what effects compound stimuli as used in this study would have on conditioning-related effects in animals, making a point-to-point comparison difficult at the moment.

An interesting aspect of our results is that there is some evidence from the heart rate data that differentiation between the 2 informative high tones must have occurred earlier than is reflected in the SSF data. Generally an increased brain response for the CS+ tone relative to the CS– tone was marked by a relative heart rate acceleration toward the CS+. This is in line with previous reports by Moratti and Keil (2005) who showed that subjects with accelerated heart rate responses to the CS+ during the acquisition phase showed increased visual steady state–evoked fields during the extinction phase. Similarly, our results show that heart rate effects preceded effects seen in the neuromagnetic data, that is, early changes in heart rate during acquisition were not reflected in the concomitant steady-state data but rather in SSF changes later during acquisition. This result indicates that refined processing of auditory cortical areas may not be required for an auditory stimulus to gain behavioral relevance. Indeed, a very recent study on the visual P300 (Klostermann et al. 2006) found that target detection on the thalamic level preceded analogous cortical responses by ~150 ms and motor reaction by ~70 ms. But also a conditioning study by Edeline et al. (1990) showed conditioning-related increases in neural activity are seen first in the magnocellular medial geniculate prior to changes in the auditory cortex. Microstimulation of this area following an auditory CS has also proven to be an effective US (Cruikshank et al. 1992). In the present work, projections from the nonlemniscal part of the medial geniculate body to the amygdala could have initialized autonomic (fear) responses and by the cholinergic path of the nucleus basalis influence processing of CS+ within primary auditory cortex (Weinberger 2004). Of course this remains speculative at this stage and cannot be resolved with our data at hand.

Also, the amount of contingency awareness as assessed at the end of the experiment is reflected in the amount of heart rate "deceleration." It is interesting that the time window for this effect overlaps with the one described above and may seem contradictory at a first glance. But it does fit with previous reports showing enhanced trait anxiety and avoidance behavior in unaware subjects (Chan and Lovibond 1996; Grillon 2002). Contingency awareness may therefore be seen as a kind of moderating variable. For unaware subjects, the presentation of the CS+ still carries new and important information and they react with heart rate acceleration and stronger increase in SSF amplitude. In contrast, for aware subjects, the presentation of the CS+ bears less novel information and less resources are

required to process this sound. This interpretation resembles the notion proposed by the Rescorla-Wagner model (Rescorla and Wagner 1972) that the associative strength between CS+ and US depends on how surprising the association between these 2 is. Indeed, the SSF amplitude increases across trials (Fig. 6*b*) for the unaware group appears steeper, implying that learning in this group progresses throughout the experiment. Probably aware subjects are knowledgeable of the CS–US contingencies earlier, so no additional learning takes place. In this context, awareness can be seen as a variable that influences surprise. To clarify this question, however, we would have needed to assess contingency awareness on a trial-by-trial basis, a shortcoming of the present study. From a clinical viewpoint, it is interesting to emphasize that unaware subjects were those who produced a heart rate response, indicative of a stronger defensive (sympathetic) reaction, whereas subjects more aware of the contingencies exhibited a response typical for orienting reaction (heart rate deceleration). It would have been informative to have included scores of an anxiety questionnaire in this study in order to see whether the groups differ on this variable. Previous works support this notion (Chan and Lovibond 1996; Grillon 2002). Another finding in favor of this interpretation comes from Sanchez-Navarro et al. (2006), who showed that elicitation of a defensive heart rate acceleration is a stable response disposition across 2 conditions: loud noise and aversive pictures. Assuming that a difference could exist between high- and low-anxiety subjects concerning awareness, it will also be an interesting undertaking for future studies to investigate in more detail the degree to which different memory systems are involved in these 2 groups while processing CSs. It is well established that contingency awareness involves neural structures involved in declarative memory (i.e., hippocampus and neocortex; see Clark et al. 2002), in addition to implicit memory. From the present study, one could speculate that declarative knowledge of the relations between stimuli might be helpful for reducing fear responses. Another possible interpretation is that for subjects exhibiting a defensive heart rate response, the CS+ attains a greater degree of salience, boosting activity in auditory cortex, but distracting from fine details concerning the relationship between stimuli, leading to relatively higher contingency unawareness scores. This is in line with the notion that changes in neuronal response magnitude within the auditory cortex reflect formation of "physiological memory" for salient stimuli (Weinberger 2004). A logical argument against this interpretation is that no differences concerning ratings of the aversive pictures could be identified between the aware and unaware group, implicating that they had a similar salience. This could, however, also be due to a ceiling effect because the pictures chosen can be qualified as among the most unpleasant of the IAPS. At this point, it is also important to point out that the results concerning contingency awareness should be treated with caution and that our discussion of this matter is largely on a hypothetical level. The reason for this is that, even though an established approach similar to the one published by Clark and Squire (1998) has been taken, awareness was not manipulated directly but defined on a post hoc basis. Our findings will need to be complemented by studies with experimentally induced levels of awareness.

Overall, this study demonstrates the power of the high signal-to-noise ratio of SSRs, by which it is possible to dynamically monitor changes over the course of an experiment (the basic methodology and its comparison with more conventional auditory steady-state approaches will be described elsewhere). This study demonstrates rapid changes (within 5 trials) of auditory cortical responses in a conditioning paradigm, therefore partially confirming findings from animal studies in humans. However, differential responses for the CS+/CS– high frequencies developed more gradually than in these animal studies, possibly due to some limiting factors of the study (see above). Furthermore, our work replicates important effects reported previously in the conditioning literature that 1) similar to Moratti and Keil (2005), only subjects showing heart rate acceleration appeared to exhibit central differentiation between CS+ and CS–. This peripheral physiological measure could perhaps relate to a stronger amygdala activation in more unaware subjects, which subsequently drives cortical plasticity, for example, via the nucleus basalis. Even though a functional amygdala seems necessary for the central and behavioral changes induced by aversive conditioning (Bechara et al. 1995), this does not necessarily mean that this is expressed in peripheral physiological measures as recently shown by Critchley et al. (2002) in participants with peripheral autonomic denervation. 2) Behaviors indicative of anxiety are related to reduced knowledge of CS–US contingencies. These 2 facts shown by us and others implicate that enhanced responses in the auditory cortex for CS+ may not necessarily be reflected in enhanced contingency awareness, which was exactly what we found.

Notes

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