

Fear Conditioning in Humans: The Influence of Awareness and Autonomic Arousal on Functional Neuroanatomy

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Summary

The degree to which perceptual awareness of threat stimuli and bodily states of arousal modulates neural activity associated with fear conditioning is unknown. We used functional magnetic neuroimaging (fMRI) to study healthy subjects and patients with peripheral autonomic denervation to examine how the expression of conditioning-related activity is modulated by stimulus awareness and autonomic arousal. In controls, enhanced amygdala activity was evident during conditioning to both “seen” (unmasked) and “unseen” (backward masked) stimuli, whereas insula activity was modulated by perceptual awareness of a threat stimulus. Absent peripheral autonomic arousal, in patients with autonomic denervation, was associated with decreased conditioning-related activity in insula and amygdala. The findings indicate that the expression of conditioning-related neural activity is modulated by both awareness and representations of bodily states of autonomic arousal.

Introduction

Classical fear conditioning embodies rapid associative learning mediated through pairing of a previously innocuous stimulus (conditioned stimulus, CS) with an aversive or threatening “unconditioned” stimulus (US), such as a loud noise or mild electric shock (Davis, 1992; LeDoux, 1992, 1996). Following such pairing, the CS becomes predictive of US and elicits arousal responses previously associated with the US. Such learning can be indexed in autonomic or motor responses to the CS (now CS+), in the absence of CS-US pairing. These

behavioral markers represent simple response repertoires fundamental to adaptive survival, including threat signaling and escape behavior (LeDoux, 1996).

Evidence from experiments in animals, patient studies, and functional neuroimaging investigations indicate that acquisition of conditioned fear is dependent on the integrity of the amygdala, a medial temporal lobe structure involved in emotional memory processes (Davis, 1992; LeDoux, 1996; Bechara et al., 1995; Maren and Fanselow, 1996; Buchel et al., 1998; LaBar et al., 1998; Cahill and McGaugh, 1998; Cahill, 2000; Dolan et al., 2000). The basolateral amygdala nucleus is particularly implicated in supporting conditional learning of CS-US pairings (LeDoux, 1996; Maren and Fanselow, 1996; Fanselow and LeDoux, 1999). However, in some circumstances it may be difficult to dissociate the role of amygdala in fear conditioning from its role in mediating behavioral responses that index such learning (Cahill et al., 1999). Animal experiments suggest involvement of central amygdala nucleus in generation of autonomic arousal in response to conditioned stimuli (Kapp et al., 1981, 1992; LeDoux, 1992). Furthermore, patients with amygdala lesions do not develop autonomic responses (skin conductance changes) with fear conditioning, but nevertheless manifest autonomic arousal to *intrinsically* aversive stimuli (US) (Bechara et al., 1995; Tranel and Damasio, 1989).

In humans, fear conditioning can occur independently of conscious awareness. For example, if subjects are conditioned to an “unseen” visual stimulus, where rapid presentation and backward masking eliminate the conscious percept of stimulus identity, autonomic and behavioral measures show implicit learning of CS-US pairings (Ohman and Soares, 1993; Morris et al., 1998; Bunce et al., 1999). Cortical pathways are implicated in processing detailed information about stimulus identity and conscious awareness of the conditioned stimulus, whereas subcortical pathways to amygdala via structures such as thalamic pulvinar nucleus may support the expression of unconscious conditioning (LeDoux, 1996; Morris et al., 1997, 1998, 1999).

States of peripheral autonomic arousal are represented within brain regions that overlap those implicated in emotion (Critchley et al., 2000a,b; 2001a,b,c). Moreover, activity within areas involved in central autonomic control is modulated by feedback of peripheral autonomic responses (Critchley et al., 2001a). These autonomic arousal states bias emotional behavior and influence processes such as memory (e.g., Damasio et al., 1990; Cahill et al., 1994; Cahill and McGaugh, 1998). For example, amygdala is associated with memory enhancement for emotional material, a process thought to be mediated through the influence of states of arousal on amygdalo-hippocampal functional connectivity (Cahill et al., 1994; Cahill and McGaugh, 1998; Cahill et al., 1999). Peripheral autonomic arousal may also influence conditioning, as suggested by pharmacological and motivational manipulations in animals (Young et al., 1995; Maren et al., 1994).

The contribution of autonomic arousal to the central

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neural expression of fear conditioning in humans is unknown. One means of testing its influence would be to examine fear conditioning in the absence of autonomic responses. However, interventions such as pharmacological blockade of peripheral norepinephrine receptors have only a partial effect on autonomic arousal, as other axes of autonomic arousal remain functional, including sympathetic electrodermal activity (mediated cholinergically), and the withdrawal of parasympathetic tone.

We have studied a rare group of patients with pure autonomic failure (PAF), which represents a unique human lesion-deficit model of autonomic function (Critchley et al., 2001a). Patients with PAF cannot modulate their bodily state via the autonomic nervous system as a result of peripheral autonomic denervation, but have no other neurological deficit (sensory or motor). Peripheral autonomic denervation results in an inability to vasoconstrict during gravitational challenges (Mathias and Bannister, 1999; Mathias, 2000), a failure to generate increases in heart rate and blood pressure during physical or cognitive effort (Mathias and Bannister, 1999), a lack of sympathetic skin conductance responses to emotive, aversive, and orienting stimuli (Magnifico et al., 1998), and diminished pupil responses (Clark and Ewing, 1988). PAF is distinguished from central neurodegenerative causes of autonomic failure (e.g., multiple system atrophy, or Parkinson's disease with autonomic failure) by absent clinical and hormonal indicators of central neurological degeneration, and normal life expectancy (Mathias and Bannister, 1999). Similarly, gross and pervasive disturbances in attention or emotional functioning have not been suspected from clinical observations, although subtle deficits in subjective emotional experience are reported (Critchley et al., 2001a). PAF, where there is an absence of peripheral autonomic responses, provides a powerful model to test how autonomic responses influence brain activity associated with fear conditioning. Thus, in the present study, we address two fundamental questions regarding human fear conditioning. First, we directly examine the influence of level of perceptual awareness of a CS+ on regional brain activity during conditioning (Figure 1). Second, we examine how conditioning-related brain activity is modulated by peripheral autonomic arousal, i.e., the presence and absence of autonomic bodily states that are a *sine qua non* expression of classical conditioning.

Results

Behavioral Measures of Awareness and Conditioning

Subjects were required to judge whether or not they liked each face stimulus they viewed, by making a two-choice button-press response to each stimulus viewed during the scanned task. Subjects consistently ($86.8\% \pm 11\%$) judged the unmasked presentation of the two angry faces (unpaired CS+ and CS- stimuli) as negative in subjective affective valence (dislike), whereas when these angry faces were masked by neutral faces, they made "dislike" responses on $25\% \pm 22\%$ of such trials ($t(32) = 9.94, p < 0.001$). There were no significant differences in the number of "dislike" responses to the unmasked CS+ or CS- stimuli ($t(32) = 1.32, n.s.$) nor in

affective judgements of the neutral faces masking CS+ stimuli or CS- stimuli ($t(32) = 0.61, n.s.$). These findings support previous evidence (Esteves et al., 1994, 1994; Morris et al., 1998) indicating that the parameters we used for visual backward masking were associated with a lack of conscious awareness for the masked "unseen" face stimuli.

Behavioral evidence for conditioning was observed in six control subjects and four patients with PAF. These subjects made significantly faster response times to CS+ compared to CS- stimuli (Figure 2). Note that because of autonomic dysfunction, standard measures such as acquisition of differential electrodermal responses were inappropriate. Three control subjects and four PAF patients did not show significant response time differences between CS+ and CS- stimuli, either as a main effect or in an interaction with time, and were therefore classed as not having conditioned during the task. There was no significant difference in the rate at which conditioning successfully occurred in the four PAF subjects and six controls, whose discriminatory response times to CS+ and CS- stimuli followed the same pattern of early establishment and maintenance across the experiment (Figure 2B).

Fixed Effects Analysis of Conditioning in Control Subjects

A key question relating to conditioning is whether neural mechanisms supporting CS-US associative learning differ depending on whether the stimuli are consciously or implicitly perceived. A fixed effects factorial analysis was conducted on data from the six control subjects who showed behavioral conditioning, to examine main effects of conditioning, awareness, and the interaction between conditioning and awareness. First, we tested for differential regional activity between CS+ and CS- stimuli as a main effect, i.e., including both masked and unmasked stimuli. Significant activity in this analysis was observed in right amygdala (Talairach coordinates of peak; 16, 2, -24, $p < 0.05$, corrected) (Figure 3A, Table 1). No brain regions showed significantly more activity to CS- compared to CS+ stimuli.

We next tested for a main effect of conditioning as a function of time, as previous neuroimaging studies of conditioning (employing different paradigms) suggest a time-dependent decrease in magnitude of differential BOLD responses to CS+ versus CS- stimuli in amygdala. No brain regions reached corrected (voxel level) significance in the time-dependent contrast of CS+_(masked and unmasked) versus CS-_(masked and unmasked) events. However, activity in anterior cingulate/medial prefrontal cortex and superior cerebellar vermis survived cluster level significance, indicating that responses in these regions reflect a time-dependent differentiation between CS+ and CS- events (Table 1). No brain regions showed significantly more activity to CS- compared to CS+ stimuli in this time-dependent manner.

In the main effect of masking, i.e., the comparison between unmasked and masked CS+ and CS- stimuli, we found no differences in regional activity that survived corrected significance ($p < 0.05$) for whole brain or predicted regions of interest. However, time-dependent effects were observed in right middle temporal gyrus ex-

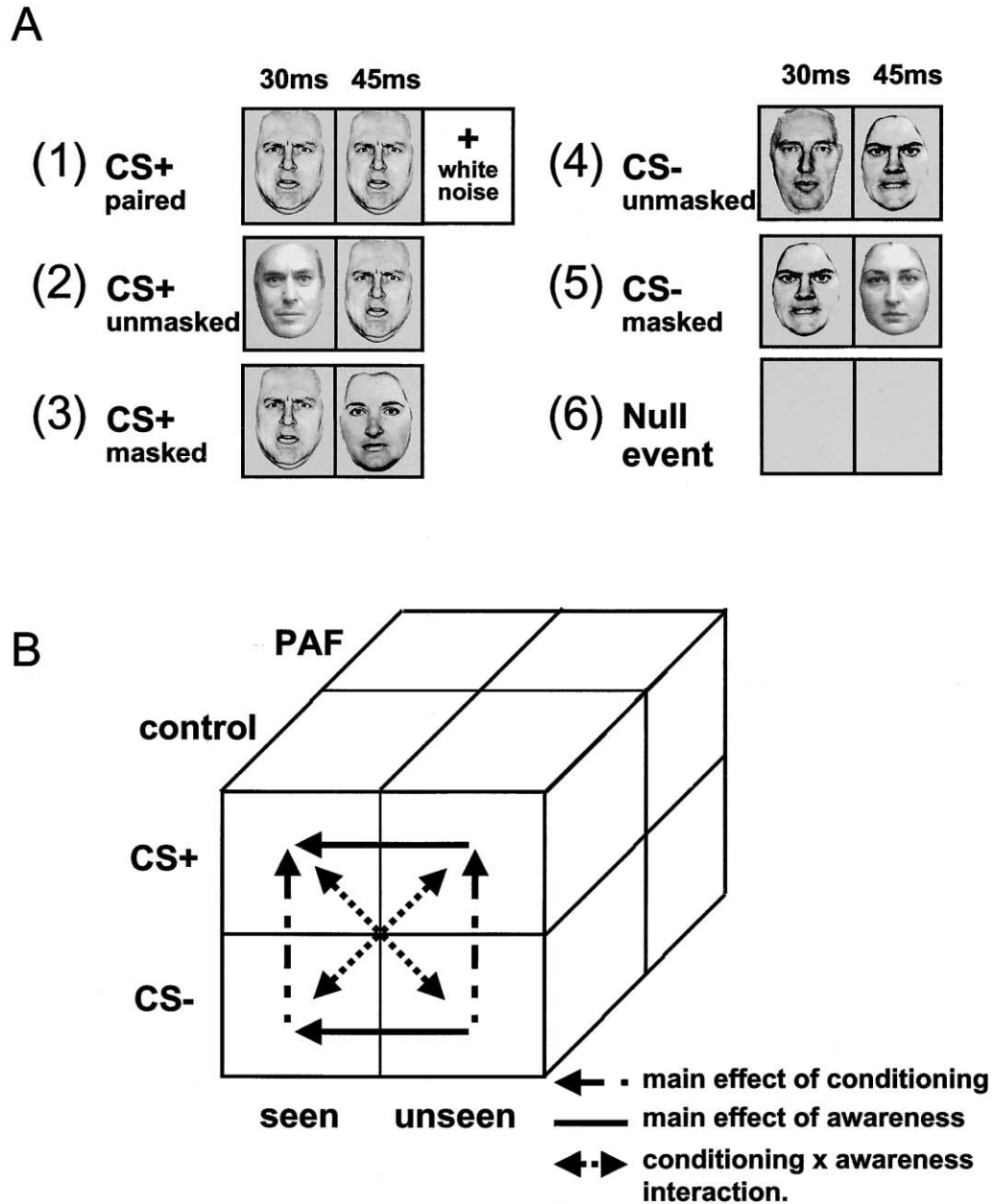


Figure 1. Stimuli and Experimental Design

(A) Stimuli. Six event types were visually presented pseudorandomly in a balanced design across subjects. These consisted of (1) CS+ stimulus, an angry face, paired with an aversive white noise (CS+_{paired}). The same CS+ stimulus was also presented without an associated noise (CS+_{unpaired}) in (2) unmasked (CS+_{unmasked}) and (3) masked (CS+_{masked}) trials. Visual backward masking was used to control the subjects' awareness of the stimuli. Subjects were consciously aware of the unmasked stimuli, but not of the masked stimuli. Thus, to mask a CS+ stimulus, the CS+ face was presented briefly (30 ms) then immediately replaced by a stimulus from a set of neutral faces, presented for 45 ms. For the unmasked conditions, the order of CS+ and neutral face stimuli was reversed. Similar methods were used to present (4) unmasked and (5) masked trials of a CS- stimulus (a different angry face, that was never paired with the aversive noise). Lastly, (6) null events were included in the task design.

(B) Design. In control subjects, analysis took the form of a 2 × 2 factorial design, testing for the main effects of conditioning and masking (awareness of CS+ stimuli), and also conditioning × masking interaction. This was performed using a fixed effects model. A second level random effects approach was used to examine differences between controls and PAF patients (i.e., presence and absence of autonomic responses) in separate analyses for seen (unmasked) and unseen (backward masked) CS stimuli. We also tested for an interaction between presence and absence of autonomic responses and masking for conditioning-related activity (CS+ versus CS-).

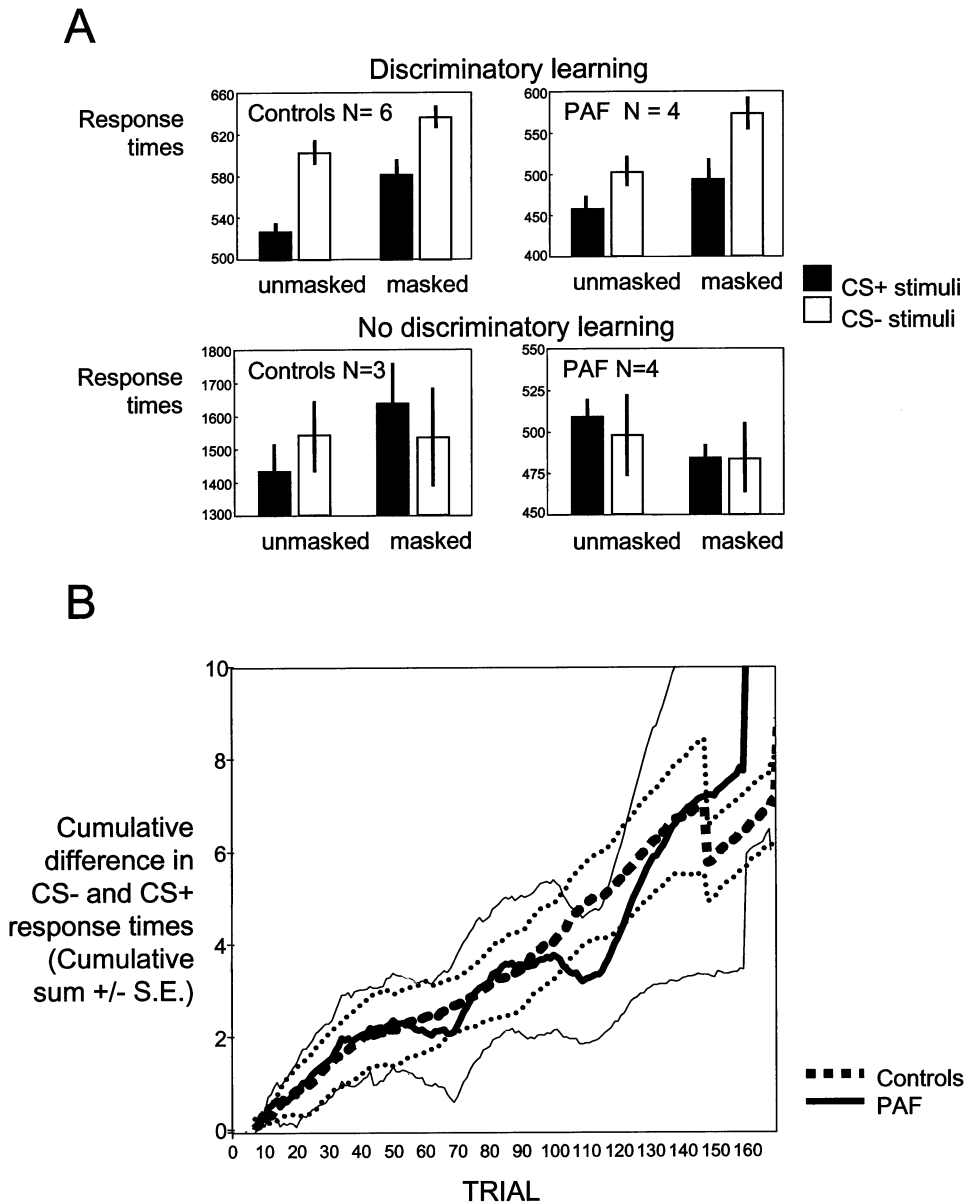


Figure 2. Behavioral Indexing of Conditioning

(A) Response times to stimuli. Subjects were divided into four groups according to diagnosis (healthy control or PAF) and whether or not they showed facilitation of response times to CS+ versus CS- stimuli (our behavioral index of conditioning). Bar charts show the different group mean (and standard error of) responses times to masked and unmasked CS+ stimuli (unpaired with US) and masked and unmasked CS- stimuli. Six controls and four PAF subjects showed significant differential response times to CS+ and CS-. The remaining three controls and four PAF subjects did not differentiate significantly between CS+ and CS- stimuli in their response times.

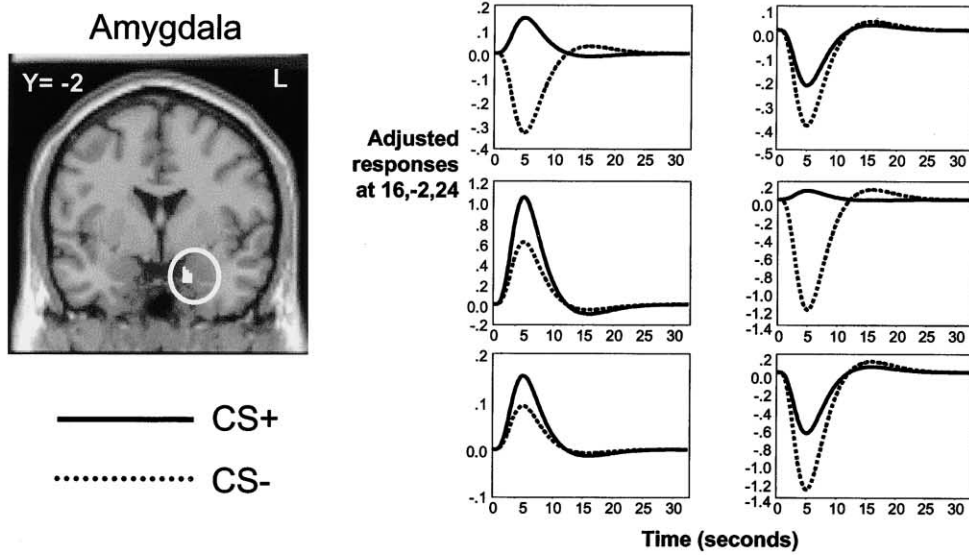
(B) Learning rates in control and PAF subjects who conditioned. To illustrate that controls and PAF subjects acquired the conditional discrimination at similar rates, the cumulative difference in response times (response to CS- minus response time to CS+ / sum of response times to CS+ and CS-) \pm standard errors of the two groups are plotted against the trial number for controls (N = 6, dotted lines) and for PAF patients (N = 4, solid lines). Both groups show similar rates of acquisition and maintenance of the differential contingent responses, consistent with intact central mechanisms for conditioning in PAF subjects.

tending into insula (58, -8, 10; 62, -8, -12; $p < 0.05$, corrected), representing greater responses to unmasked than masked stimuli at early time periods compared to later in the experiment (Table 1). No regions responded preferentially to the “unseen” masked CS stimuli compared to the unmasked stimuli in the interaction with time.

To test whether or not the same neural architecture

supports conditioning to “seen” and “unseen” stimuli, we next examined for an interaction between conditioning (CS+ versus CS-) and awareness (masked versus unmasked stimuli). Greater conditioning-related activity when stimuli are “seen,” compared to when stimuli were “unseen,” was observed in right middle temporal gyrus, extending into anterior superior temporal gyrus (62, -2, -8; voxel level, $p = 0.08$; cluster level, $p <$

A



B

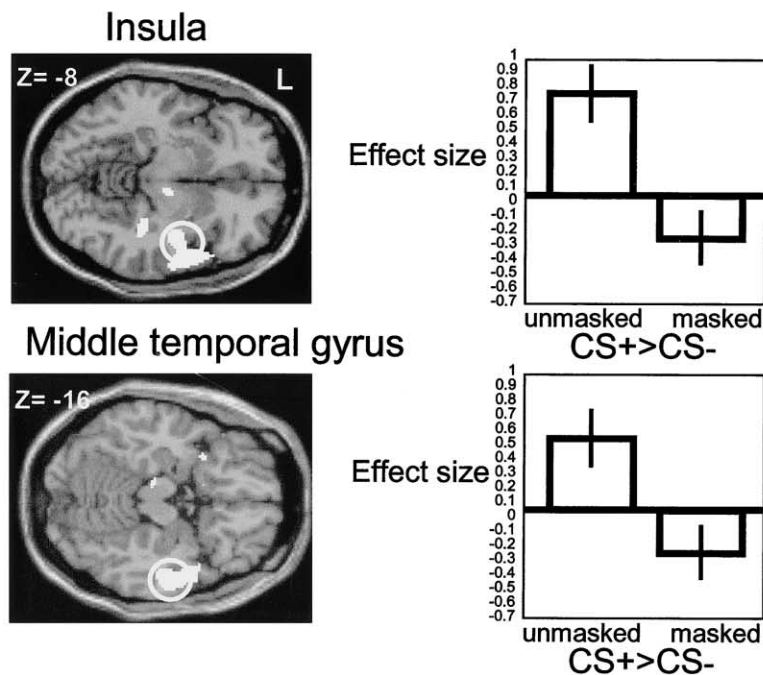


Figure 3. Conditioning in Healthy Subjects

(A) Main effect of conditioning. In a fixed effects analysis of control subjects who demonstrated behavioral conditioning ($N = 6$), a significant main effect of CS+ versus CS- stimuli (unmasked and masked) was observed in the right amygdala (coordinates of peak differential response, 16, -2, 24). The figure shows the location of this activation, with group data plotted on a normalized template image, and the individual differential fitted hemodynamic responses to CS+_(masked and unmasked) (full lines) and CS-_(masked and unmasked) (dotted lines) stimuli, for each of the six subjects.

(B) Interaction between conditioning and masking in healthy subjects. Activity in insula and right lateral temporal cortices reflected the interaction between awareness (i.e., unmasked versus masked stimuli) and conditioning (CS+ versus CS- stimuli), i.e., (CS+ versus CS-)_{unmasked} - (CS+ versus CS-)_{masked}. The figure maps this activity, thresholded at $p < 0.001$ (uncorrected) for presentation purposes, on horizontal sections of a normalized template brain image with the height in millimeters above the anterior commissure (z-coordinate) in the top left corner. Adjacent to the brain map are corresponding bar graphs of the contrast of parameter estimates for peak interaction-related activity for both regions. The effect size reflects the percentage (\pm standard error) difference in amplitudes of hemodynamic responses corresponding to the designated contrast (adjusted for confounds) within insula cortex and lateral temporal lobe cortex for the contrasts of CS+ versus CS- for masked and unmasked stimuli that make up the interaction.

Table 1. Activity Relating to Conditioning and Masking in Control Subjects in whom There Was Behavioral Conditioning

Site	Side	Coordinates	T Value
Main Effect of Conditioning CS+ versus CS-			
Amygdala	R	16, 2, -24	3.65 ^a
Conjunction of Unmasked and Masked Conditioning			
Parahippocampal gyrus/inferior amygdala	R	20, -8, -30	Minimum T = 2.07 ^a
Main Effect of Conditioning, CS+ versus CS- Interaction with Time			
Paracingulate/medial prefrontal cortex	Bilateral	10, 52, 18	4.01 ^b
Cerebellar vermis	Midline	-6, -46, -8	3.85 ^b
Main Effect of Awareness, Unmasked > Masked Stimuli, Interaction with Time			
Insula	R	58, 8, 10	3.94 ^a
Middle temporal gyrus	R	62, -8, -12	3.84 ^b
Interaction of Awareness and Conditioning (CS+ > CS-) _{unmasked} > (CS+ > CS-) _{masked}			
Middle temporal gyrus/superior temporal gyrus	R	62, -2, -8	4.19 ^b
Insula	R	42, -8, -8	3.61 ^a
Insula	L	36, -12, 4	3.45 ^a

p < 0.05, corrected. ^ap < 0.05, corrected for region of interest. ^bp < 0.05, corrected for whole brain at cluster level.

0.001, corrected) (Figure 3B). Differential activity (p < 0.05, corrected for small volume) was also observed bilaterally in insula cortex (42, -8, -8; -36, -12, 4,) for the same contrast, indicating greater insula activity during “seen” CS+ presentations. No areas showed time-dependent modulation of conditioning-by-awareness related activity.

Differences between Controls and PAF Patients in Activity Related to Conditioning

A random effects analysis of all 17 subjects (PAF and controls, irrespective of whether they behaviorally conditioned) was conducted to determine if the presentation of face stimuli elicited predictable activity in temporal lobe face regions. CS- face stimuli were compared to null events. Increases in activity related to the processing of faces was observed in bilateral parietal lobe (p < 0.05 cluster level) and in regions of fusiform and inferior temporal gyral cortex (x, y, z coordinates; 42, -52, -22, T = 3.95 and 64, -46, -4, T = 4.50, respectively, p < 0.05, corrected for small volume of regions of interest). No significant difference in activity within these or other areas was observed in a comparison of PAF patients and controls even when we restricted the analysis to subjects showing behavioral conditioning. These findings indicate that PAF patients and controls had equivalent BOLD responses to face stimuli.

Random effects analyses, comparing PAF patients with controls, were used to determine how an absence of peripheral autonomic responses influences the expression of conditioning-related regional brain activity. Age and sex were included as covariates of no interest in these comparisons. Simple effects of conditioning to “seen” and “unseen” CS stimuli, and the interaction between conditioning and awareness, were examined in separate analyses. In the “seen” condition, PAF subjects (in whom there was behavioral evidence of conditioning) showed significantly less activity in the contrast of CS+ versus CS- stimuli in bilateral amygdalae, right insula, and right anterior hippocampus when compared to control subjects who showed conditioning (p < 0.05, corrected, Figure 4, Table 2). Notably, reduced right insula activity in PAF patients during unmasked conditioning seemed to overlap anatomical regions associ-

ated with the interaction between awareness and conditioning in controls. Consequently, the degree of overlap was tested by creating a mask image from a factorial analysis of conditioning in control subjects (thresholded at T > 3.1). This showed a peak of insula activity common to both analyses (x, y, z coordinates, 44, -4, 0, p < 0.05, corrected for mask image volume). In a comparison of controls versus PAF patients for “unseen” masked faces, no differences in activity survived threshold significance (Table 2). There were no group differences in time-dependent analyses for any of the above contrasts.

Finally, to determine how awareness and autonomic arousal interact during conditioning, we tested for significant (p < 0.05, corrected) between group differences in the interaction between conditioning and awareness (i.e., the three-way interaction between arousal, awareness, and conditioning). A region of right posterior insula showed significantly reduced activity in PAF patients, compared to controls, during unmasked conditioning [(CS+ > CS-) _{unmasked}] compared to masked conditioning [(CS+ > CS-) _{masked}]. Thus, in contrast to increased insula activity to unmasked CS+ stimuli in control subjects, the absence of autonomic arousal in PAF subjects was associated with attenuation of awareness-related differential responses to CS+ compared to CS- stimuli in right posterior insula (Table 2, Figure 5).

Discussion

Fear conditioning is a simple form of associative learning, manifest across species, that provides a powerful model for understanding emotional processing (e.g., LeDoux, 1996). The present study addressed two questions relating to neural activity underlying conditioned fear in humans; namely, how subjective awareness and states of bodily arousal influence regional brain activity during fear conditioning. Cortical regions including superior temporal, anterior insula, and adjacent orbitofrontal cortices are modulated by level of awareness of a conditioned stimulus. Absent autonomic responsivity, and therefore blunting of arousal-related somesthetic representations, is associated with attenuated conditioning-related activity in insula, amygdalae, and right anterior hippocampus, an effect most pronounced for unmasked stimulus presentation.

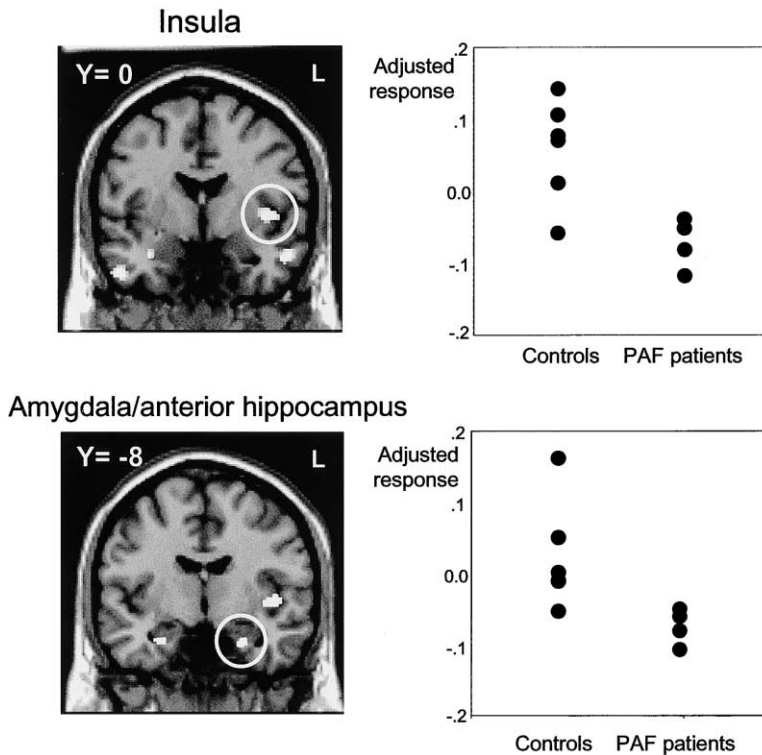


Figure 4. Reduced Activity in PAF Patients, Relative to Controls, during Conditioning to Unmasked Stimuli

The influence of autonomic arousal on conditioning was determined by random effects comparison of regional brain activity associated with CS+ versus CS- in controls and PAF subjects who demonstrated behavioral conditioning. Significant differential activity between the groups is shown superimposed upon a normalized structural template image for the contrast CS+_{unmasked} versus CS-_{unmasked} in right insula and bilateral amygdalae ($p < 0.001$, uncorrected for presentation purposes) with plots of adjusted responses in insula and amygdala for controls and PAF patients.

Amygdala activity during conditioning was modulated by arousal, but not by conscious awareness of CS+ stimuli. In contrast, conditioning-related insula activity was modulated by both peripheral arousal and awareness. These effects are consistent with a proposal that the insula is involved in representing states of awareness related to external threat as well as in representing internal states of arousal (Critchley et al., 2001c). The methodology used to demonstrate implicit learning and subliminal conditioning in many human studies has been strongly criticized (e.g., Shanks and St. John, 1994; Lovibond and Shanks, 2002). Nonetheless, there is increasing evidence from a variety of experimental situations, for example behavioral (Ohman and Soares, 1993) and

functional imaging studies in both healthy subjects (Morris et al., 1999; Vuilleumier et al., 2001; Whalen, et al., 1998) and patients with hemianopia (Morris et al., 2001), that conditioning and amygdala responses to threat stimuli are expressed in the absence of conscious awareness of stimulus occurrence. Our observations in control subjects provide further evidence for attention-independent amygdala activity during conditioning. In the absence of awareness of target stimulus occurrence, it has been proposed that subcortical structures such as the pulvinar nucleus may provide the anatomical connectivity necessary for subliminal processing of such stimuli (Morris et al., 1999), a proposal consistent with animal evidence for both cortical and subcortical pathways underlying fear conditioning (LeDoux, 1992, 1996). In our study, attention-related differences during conditioning were observed in insula and adjacent lateral temporal cortices, and together these findings suggest a hierarchy in the functional organization of structures that support evolutionarily adaptive learning. In this scheme, the amygdala acts as an involuntary interface between threat and body response with the insula supporting representations of the response to external threat and body states. The latter proposal includes the idea that the insula provides an important component of the matrix through which the subjective representation of emotion, so-called feeling states, is expressed.

The observation that feedback of peripheral states of autonomic arousal enhances activity within the amygdala during fear conditioning provides empirical evidence for models of long-term memory enhancement by emotional material (Cahill et al., 1994; Cahill and McGaugh, 1998; Cahill, 2000). These models emphasize the role of amygdala activity, in the context of arousal states, in facilitating mnemonic consolidation. It should be noted that the absence of arousal was also associ-

Table 2. Effect of Autonomic Arousal on Conditioning to Unmasked and Masked Stimuli

Site	Side	Coordinates	T Value
Controls > PAF Subject for (CS+ > CS-) _{unmasked}			
Orbitofrontal cortex	L	-24, 40, -6	9.75
Insula	R	44, -2, 2	6.00 ^a
	R	38, -14, 2	4.72 ^a
Amygdala	R	28, 2, -14	5.26 ^a
	L	-34, 4, 29	5.08 ^a
		-30, -4, -24	4.78 ^a
Anterior hippocampus		26, -10, -26	5.03 ^a
Controls > PAF Subjects for (CS+ > CS-) _{masked}			
No significant differences			
Interaction: Controls > PAF for Aware versus Unaware Conditioning			
Controls[(CS+ > CS-) _{unmasked}] > (CS+ > CS-) _{masked}] > PAF[(CS+ > CS-) _{unmasked}] > (CS+ > CS-) _{masked}]			
Posterior insula	R	42, -4, 14	4.56 ^a

$p < 0.05$, corrected. ^a $p < 0.05$, corrected for small volume of region of interest.

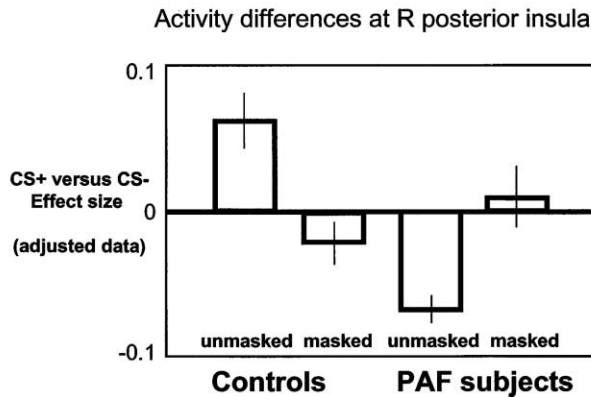
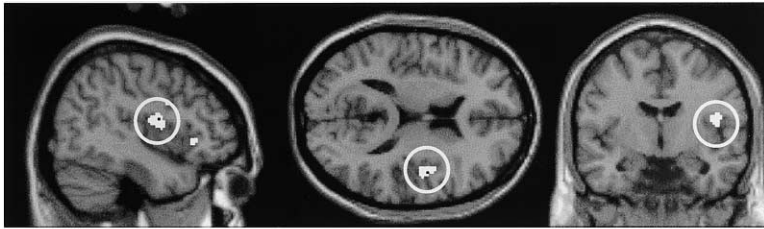


Figure 5. Regional Activity Differences Relating to the Three-Way Interaction of Arousal \times Awareness \times Conditioning

The three-way interaction between arousal, awareness, and conditioning (i.e., Controls $((CS^+_{unmasked} \text{ versus } CS^-_{unmasked}) - (CS^+_{masked} \text{ versus } CS^-_{masked})) \text{ versus PAF}((CS^+_{unmasked} \text{ versus } CS^-_{unmasked}) - (CS^+_{masked} \text{ versus } CS^-_{masked}))$) was associated with regional activity differences characterized by an attenuation of activity in the absence of autonomic arousal (PAF) in right insula during conditioning to unmasked stimuli compared to masked stimuli, where conditioning is indexed by $CS^+ > CS^-$. Thus, autonomic arousal enhances awareness-related activity during conditioning. Interaction-related activity is plotted on sagittal, axial, and coronal sections of a normalized template brain, highlighting insular activity differences. The bar chart shows effect sizes for components contributing to the interaction. The magnitude of effects is shown for comparisons between CS^+ versus CS^- , with and without awareness, in PAF patients and controls. The later were derived from individual subject analyses of regional activity insula sites reflecting the three-way interaction. It can be seen that the three-way interaction is driven by enhanced insula responses to the consciously perceived threat (unmasked CS^+), relative to the CS^- stimuli in controls and a reduction in insular activity to the consciously perceived threat in the absence of autonomic responses in PAF patients.

ated with attenuation of activity in anterior hippocampus, an observation consistent with the idea that emotional states, mediated via arousal, also modulate mnemonic processing within the hippocampus (Cahill and McGaugh, 1998). In the present study, both PAF patients and controls who conditioned exhibited similar learning curves during conditioning, indicating that central mechanisms mediating conditioning were intact and exist even in the absence of associated autonomic activity. However, further larger studies of PAF patients are required to determine if absent autonomic arousal may have more subtle behavioral effects on interactions between autonomic responses and memory, including and long-term correlates of fear conditioning.

One explanation for the observed difference between PAF patients and controls may have a basis in the different neural populations within the amygdala region. An absence of peripheral autonomic arousal may lead to a shift toward representation of “safe” CS^- stimuli rather than threat CS^+ stimuli within amygdala subregions. Conditioned stimuli, predicting aversive outcomes, typically engender enhanced amygdala responses associated with somatic, autonomic, and endocrine signs of fear. However, the amygdala may also be involved in learning about positively valenced stimuli (Davis and Whalen, 2001). Thus, in PAF, where there is reduced contextual representation of aversiveness consequent upon absent peripheral autonomic responses, the signaling of positively valenced stimuli may assume greater precedence, resulting in the observed decrease in differential amygdala responses to CS^+ and CS^- stimuli, most evident during conscious appraisal of the stimuli.

We did not observe significant modulation of amygdala responses with time, a feature of previous fMRI conditioning experiments (Buchel et al., 1998; Labar et al., 1998). However, the study differs from these previous reports in a number of critical aspects. Firstly, we reinforced only 30% of CS^+ trials, compared to the study of Buchel et al. (1998) where 50% of CS^+ trials were reinforced or the study of La Bar et al. (1998) where 100% of CS^+ trials were reinforced during the acquisition phase of conditioning. Secondly, the intertrial interval in the present study was much shorter at 5 s compared to earlier event-related studies (30 s in the study of LaBar et al. [1998], mean stimulus presentation time 10 s; 10 s in the study of Buchel et al. [1998], with a 3 s stimulus presentation time). Thus, the sparse reinforcement schedule, short stimulus duration, and increased rate of stimulus presentation may have minimized adaptation of amygdala responses during our experiment. A third methodological distinction is the use of an operant task, a valence judgment of the faces, absent from both these earlier event-related studies of conditioning. Differences are also apparent in comparison to studies using positron emission tomography (PET), such as those of Morris et al. (1998, 1999), where right-sided amygdala activity was more strongly associated with unaware processing of conditioned stimuli, compared to left-sided activity for “seen” unmasked CS^+ stimuli. The lack of laterality, compared to PET findings, may reflect methodological differences between blocked PET experiments (measuring regional cerebral blood flow changes) and event-related fMRI designs. However this remains speculative, and mechanisms underlying

such between study differences remain elusive. Other issues relating to amygdala activity during fear conditioning, for example age and gender differences, were not the focus of the present study although possible influences of gender have previously been raised in relation to amygdala activity in memory tasks (Canli et al., 2000). We observed no direct effect of age or gender on our results. We also directly controlled for these possible influences on group differences between PAF patients and controls by inclusion of age and gender as covariants of no interest in group analyses.

Modulation of activity within insula was observed in the interaction between awareness (masking) and autonomic arousal. Activation of the insula has been previously reported as a time-independent association of conditioning to unmasked faces (Buchel et al., 1998) and is implicated in more general representations of aversive stimuli (Phillips et al., 1997; Casey et al., 2001). Insular cortex is also implicated in autonomic control and representation (Cechetto and Saper, 1990; Oppenheimer et al., 1992; Critchley et al., 2000a, 2000b, 2001a, 2001b). The present study extends these observations by showing a commonality in modulation of a region of insular cortex by awareness and autonomic arousal. Further, activity in an adjacent insular region was also highlighted in the interaction between awareness and arousal. Thus, awareness of threat stimuli, representation of internal bodily states of arousal, and their interactions are associated with modulation of insula activity. These findings suggest that the insula may support phenomena related to awareness of both external threat and of internal bodily states which index the occurrence of an aversive stimulus. In this framework, insular cortex may play a crucial role in supporting feedback representation of peripheral autonomic arousal that provides input to conscious awareness of emotional states. We suggest that such awareness of autonomic change may provide a core component of what is termed feeling states.

In conclusion, this study examined the influence of stimulus awareness and peripheral autonomic states of arousal on regional brain activity during fear conditioning. Conditioning-related neural activity in insula and lateral temporal cortices, but not amygdala, was modulated by awareness. There was attenuation of conditioning-related activity in amygdala and insula in the absence of feedback of peripheral states of autonomic arousal. These findings suggest partial segregation within the neural system mediating fear conditioning, into representations of eliciting threat stimuli and of associated bodily states of arousal.

Experimental Procedures

Subjects and Paradigm

We studied 17 adult subjects, who included 9 healthy volunteers and 8 patients with the clinical diagnosis of pure autonomic failure (PAF). PAF patients were diagnosed after detailed investigation at the Autonomic Unit, National Hospital for Neurology and Neurosurgery. Each patient had a greater than 5 year history of symptoms of acquired peripheral autonomic failure, including postural (orthostatic) hypotension, with no other sensory or motor symptoms. Clinical and pathological features of PAF summarized above have been described in detail elsewhere (Mathias and Bannister, 1999; Mathias, 2000). The age range of the healthy controls (range, 25–67

years; mean \pm SD, 39 years \pm 14; 3 women, 6 men) spanned those of the patients (range, 55–66 years; mean \pm SD, 62 years \pm 4; 5 women, 3 men). Age was treated as a nuisance covariate in analyses of between group effects. Written informed consent was obtained from all subjects for the study, which was approved by the Joint Ethics Committee of the Institute of Neurology and National Hospital for Neurology and Neurosurgery.

Subjects were scanned when performing a conditioning task in which they viewed repeated presentations of face stimuli (neutral or angry faces derived from a standard emotional test battery [Ekman and Friesen, 1976]). A total of 180 events of 6 event types were shown in a randomized order. There were 30 presentations of an angry face, presented for 75 ms and followed immediately after by auditory delivery (100 db, 500 ms) of a loud unpleasant white noise burst (unconditioned stimulus, US). These trials thus represented a CS/US pairing. Consequently, associative conditional learning during the task resulted in this CS becoming the CS⁺. In addition to these 30 “CS⁺_{paired}” presentations, the same CS⁺ was presented in unpaired trials. In 30 trials, the face was presented for 30 ms then followed by a neutral face, presented immediately, for 45 ms. In effect, the neutral face backward masked the angry face and subjects were unaware of seeing the angry face, reporting the neutral face alone. The parameters for masked presentation of these facial stimuli have been established in earlier studies (Morris et al., 1998, 1999). We refer to these unpaired trials as CS⁺_{masked}. In a further 30 trials, the unpaired CS⁺ was presented for 45 ms (as the mask), following a 30 ms presentation of a neutral face. Here subjects reported seeing the CS⁺ but were unaware that they had seen the masked neutral face. We therefore refer to these trials as CS⁺_{unmasked}. A second angry face (different identity to the CS⁺) was never paired with an aversive tone and therefore became the CS⁻. This face was presented either unseen, masked by a neutral face in CS⁻_{masked} trials, or following the 30 ms presentation of a neutral face in CS⁻_{unmasked} trials.

Neutral faces for masked and unmasked trials were taken from a set of 30 neutral faces, half of which were derived from the Ekman series (Ekman and Friesen, 1976). Face stimuli were presented in grayscale and were cropped to remove nonfacial details such as hair and clothing (Figure 1). The identities of the CS⁺ and CS⁻ angry faces were switched pseudorandomly between subjects. In addition to these five event types, there were 30 occurrences of null events, where no face stimulus was presented. An interstimulus interval of 5000 ms was used throughout the task. Subjects were instructed to attend to an occurrence of a face and asked to make a 2 choice button-press response, based on whether they liked or disliked the face stimulus. Subjects were not guided further about how to make this judgment.

Functional and Structural Imaging

A 2 Tesla Magnetom VISION MRI system (Siemens, Erlangen, Germany) equipped with a head volume coil was used to acquire contiguous axial multislice T2*-weighted echoplanar images (echo time, 40 ms) that covered almost the whole brain (32 slices; slice thickness, 3 mm; giving 14.4 cm vertical field of view). The effective repetition time (TR) was 3.2 s per volume. To minimize head movement during scanning, subjects were restrained with bi-temporal foam pressure pads. Three hundred image volumes were obtained in each subject over 16 min, covering the period of task performance. In each subject, a 3D structural MRI was also acquired using an enhanced T1-weighted MPRAGE sequence (TR/TE/TI/NEX 9.7/4/600/1, flip angle 12°, matrix size 256 \times 192, FOV 256 \times 192, yielding 120 sagittal slices and a slice thickness of 1.5 mm with in plane resolution of 1 mm \times 1 mm).

Subject Monitoring and Debriefing

During scanning, the response times for subjects to make a preference (like/dislike) judgement to each stimulus were recorded to provide an operant measure of conditioning. After scanning, subjects were asked a series of questions to determine if they were aware of masked stimuli. Consistent with earlier studies (Morris et al., 1998), no subject reported consciously perceiving more than one face when masked stimuli were presented during scanning. Preference judgements made by the subjects were used as corroborative

rative evidence for the efficacy of masking. Response time data were tested at an individual level for a main effect of conditioning ($CS^{+}_{unpaired}$ versus $CS^{-}_{unpaired}$ stimuli across whole experiment). On the basis of these results, the subjects were divided into four groups: control subjects who showed behavioral evidence of conditioning (shorter response times to CS^{+} compared to CS^{-}), PAF subjects who conditioned, controls who did not condition, and PAF subjects who did not condition. For control and PAF subjects who showed this facilitation of responses to CS^{+} faces, a measure of the time course of learning was derived from response times across the experiment. Firstly, interpolated time courses for response times to unpaired CS^{+} and CS^{-} stimuli were calculated for each subject and the difference expressed as a proportion of the sum of the times to give a measure of facilitation of CS^{+} response on each "trial". Mean and standard error of the CS^{-} to CS^{+} difference were derived across control and PAF subjects and the cumulative means (\pm SEM) plotted to give comparative rates of acquisition and maintenance of discriminatory learning.

Imaging Data Analysis

Image processing and statistical analyses were performed using SPM99, developed and supported by the Wellcome Department of Cognitive Neurology, London (<http://www.fil.ion.ucl.ac.uk/spm/spm99.html>). For individual subjects, all volumes were realigned to the first volume. Residual motion artifacts were eliminated by regressing the time course of each voxel on a periodic function of the estimated movement parameters. To account for different sampling times of different slices, voxel time series were interpolated using Sinc interpolation and resampled using the slice at the anterior-posterior commissural line as the reference. The functional images were subsequently spatially normalized to a standard $T2^{*}$ template using nonlinear basis functions (Friston et al., 1995). The $T1$ -weighted structural image acquired in each subject was coregistered to a mean image created from the functional volumes, and then spatially normalized to standard coordinates using the same transformation as for the functional images. Functional data were smoothed using an 8 mm (full width at half maximum) isotropic Gaussian kernel to compensate for residual variability after spatial normalization and to permit application of Gaussian random field theory to provide for corrected statistical inference.

An event-related analysis was performed to identify differences in evoked hemodynamic response to each stimulus. Asynchrony between stimulus presentations and scan volume acquisitions enabled jittered sampling of time points over the course of hemodynamic responses to each stimulus type. Firstly, each subject's data were analyzed individually. Regressors for each stimulus type, including null events, were modeled as delta functions at the time points of each presentation convolved with a canonical hemodynamic response function. Also, because neurophysiological evidence (LeDoux, 1996) and evidence from event-related fMRI studies (Buchel et al., 1998; LaBar et al., 1998) indicate that conditioning is associated with rapid changes in differential amygdala responses, we tested for time \times event interactions using additional regressors created by multiplying initial regressor amplitudes by a zero mean exponential function with a time constant one quarter of the session length (220 s). Thus regressors for null, CS^{+}_{paired} , $CS^{+}_{unmasked}$, CS^{+}_{masked} , $CS^{-}_{unmasked}$, and CS^{-}_{masked} event types and their corresponding time \times event interactions were entered into multiple regression analyses.

In a fixed effects analysis, we examined data from those control subjects who showed clear behavioral effect of conditioning ($N = 6$). Using a factorial approach, we tested for activity corresponding to (1) main effect of conditioning (i.e., contrast for $CS^{+}_{unmasked}$ and CS^{+}_{masked} versus $CS^{-}_{unmasked}$ and CS^{-}_{masked}); we also undertook a conjunction analysis between the individual contrasts for unmasked and masked conditioning to test for significant commonalities ($CS^{+}_{unmasked} - CS^{-}_{unmasked}$ and $CS^{+}_{masked} - CS^{-}_{masked}$); (2) main effect of awareness (i.e., contrast for $[CS^{+}$ and $CS^{-}]_{unmasked}$ stimuli and $[CS^{+}$ and $CS^{-}]_{masked}$ stimuli); (3) the interaction between conditioning and awareness (i.e., $[CS^{+}_{unmasked} - CS^{-}_{unmasked}] - [CS^{+}_{masked} - CS^{-}_{masked}]$). Finally, we tested for time \times event interactions for (4) main effect of conditioning; (5) main effect of awareness; and (6) modulation of the interaction between conditioning and awareness.

In a second set of analyses, we determined individually for each of the 17 subjects (controls and PAF patients) activity relating to

(1) main effect of processing CS^{-} faces (CS^{-}_{masked} and $unmasked - null$ events), (2) conditioning to unmasked stimuli ($CS^{+}_{unmasked} - CS^{-}_{unmasked}$), (3) conditioning to masked stimuli ($CS^{+}_{masked} - CS^{-}_{masked}$), and their interaction with time, and (4) the interaction between conditioning and awareness ($(CS^{+}_{unmasked} - CS^{-}_{unmasked}) - (CS^{+}_{masked} - CS^{-}_{masked})$). Appropriate linear contrasts for these comparisons generated a t-statistic for each voxel, which (transformed into Z-statistics) constitute a statistical parametric map (SPM). These SPMs were then entered into random effects analyses at the second level (one SPM per subject) to determine generalizable effects across the subject group, and to explore the basis of variance across observed effects. To establish that predictable activity occurred in all subject during task performance, t test was performed across all 17 subjects to determine activity relating to the main effect of processing CS^{-} faces. Small volume corrections were applied to temporal lobe cortical regions corresponding to specific processing of faces. A group comparison of PAF patients and controls, across all subjects and then constrained to subjects exhibiting behavioral conditioning, was performed to test for group differences in this main effect of processing faces. In random effects comparisons of conditioning-related activity, individual data (from contrasts (2) to (4) above) were entered into four groups at the second level, corresponding to controls who showed conditioning, controls who did not condition, patients who conditioned, and patients who did not condition. The inclusion of subjects who did not condition in the analytic design enhanced estimates of error variance, though comparisons are reported only for PAF patients and controls who exhibited behavioral conditioning. In addition, putative effects of age and gender were controlled for by including these factors as covariates of no interest in group comparisons of conditioning-related responses. Significant activity corrected for multiple comparisons across the whole brain is reported at $p < 0.05$. Additionally, for medial temporal lobe regions, specifically amygdala (Morris et al., 1998; Buchel et al., 1998), and for insula (Buchel et al., 1998), where we had regionally specific hypotheses related to conditioning, correction for multiple comparisons was based on the volume of interest and the smoothness of the underlying SPM (Worsley et al., 1996). In all cases, the threshold was set at $p < 0.05$ (corrected). Data are reported using the x,y,z coordinate system of Talairach and Tournoux (1988), representing distance in mm lateral, anterior and vertical to the anterior commissure.

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