Classical fear conditioning in functional neuroimaging

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Classical conditioning, the simplest form of associative learning, is one of the most studied paradigms in behavioural psychology. Since the formal description of classical conditioning by Pavlov, lesion studies in animals have identified a number of anatomical structures involved in, and necessary for, classical conditioning. In the 1980s, with the advent of functional brain imaging techniques, particularly positron emission tomography (PET), it has been possible to study the functional anatomy of classical conditioning in humans. The development of functional magnetic resonance imaging (fMRI) – in particular single-trial or event-related fMRI – has now considerably advanced the potential of neuroimaging for the study of this form of learning. Recent event-related fMRI and PET studies are adding crucial data to the current discussion about the putative role of the amygdala in classical fear conditioning in humans.

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Abbreviations

CR	conditioned response
CS	conditioned stimulus
efMRI	event-related fMRI
fMRI	functional magnetic resonance imaging
PET	positron emission tomography
SCR	skin conductance responses
TR	repetition time
US	unconditioned stimulus

Introduction

In classical conditioning, a previously neutral stimulus (the conditioned stimulus or CS), through temporal pairing with an unconditioned stimulus (US), comes to elicit a behavioural response previously associated with the US alone [1]. In fear conditioning, the US is aversive and the behavioural response is measured in terms of a dependent variable, such as autonomic responses (e.g. skin conductance responses [SCR] or heart rate). Hence, classical conditioning is a form of associative learning involving the formation of linkages between a neutral stimulus and a stimulus with innate behavioural significance.

In general, two broad categories of experimental approach can be distinguished in the literature: classical fear conditioning, using visual or auditory CS and aversive US

(e.g. foot-shock or loud noise); and classical conditioning of the nictitating membrane response (e.g. classical eye-blink conditioning), with a tone as the CS and an air-puff to the cornea as the US ([2–5]; see [6] for a related example of classical conditioning of the human flexion reflex).

We focus in our review on classical fear conditioning and highlight new data from event-related functional magnetic resonance imagery (fMRI) studies. To put these findings into context, we will discuss earlier important positron emission tomography (PET) work and then describe recent work using event-related fMRI and its application to studies of fear conditioning. We will discuss key differences between these new studies and older studies, especially those concerning the role of the amygdala. The special case of trace conditioning (Figure 1), in which the CS and US are separated in time — the subject of a recent fMRI study — will be discussed in some detail. We will close with a reconsideration of the ongoing controversy regarding the role of the amygdala in fear conditioning.

Early studies using PET

In one of the first $H_2^{15}O$ PET activation studies of aversive conditioning, subjects were scanned during an initial habituation phase during which the visual stimulus (a video-tape showing various snakes) was presented alone [7]. During the following acquisition phase, the subjects were ‘conditioned’ to the snake video by pairing it to electric shocks. Acquisition phases were not scanned. During a final extinction phase, snake videos were presented again but unpaired (i.e. identical to the habituation phase). The comparison of interest was between the habituation and the extinction phase, in which learning had taken place. The analysis of autonomic responses (i.e. SCR) confirmed that conditioning had occurred: subcortical activations were seen in the thalamus, hypothalamus and the mid-brain, and cortical activations were observed in the cingulate gyrus, the premotor cortex and bilateral parietal cortices. Cortical activations were mainly attributed to attentional mechanisms related to the salience of visual stimuli after conditioning.

Within the same year, a second PET study of classical fear conditioning was reported [8] using a similar paradigm — here, the CS were tones and US were electric shocks. Subjects were scanned during a habituation phase (CS alone) and during an extinction phase (CS alone) which immediately followed the acquisition phase (CS paired with US). Activations were seen in frontal and temporal cortices and were mainly right-lateralised. The most striking aspect of these studies was the absence of the expected amygdala activation.

Figure 1

Differences between delay and trace conditioning. (a) A schematic representation of CS-US timing during classical delay conditioning and during trace conditioning. (b) Activation of anterior cingulate and bilateral insulae for CS⁺ relative to CS⁻ (i,ii) and amygdala activation that decreased over time (iii,iv) were similar in both studies. Trace conditioning also activated the hippocampus (v), with a time-course similar to the amygdala. (i,iii) from [21^{••}]; (ii,iv,v) from [31^{••}].

Recent studies using PET

A more recent PET study employed a differential classical conditioning design [9]. Emotionally expressive faces (CS) were conditioned by pairing with an aversive burst of white-noise (US). Instead of comparing pre-acquisition (habituation) and post-acquisition (extinction) scans, this study employed a differential aversive classical conditioning paradigm. The comparison of interest was between scan data acquired in response to the CS⁺ (i.e. the CS paired with the aversive US) and CS⁻ (the CS never followed by the US) in the scan period following the acquisition (i.e. during extinction). Out of four different faces, two were designated CS⁻ and the others two CS⁺. Each face was presented in three emotional expressions (happy, fearful and neutral). Autonomic responses were indexed by SCR. Comparison of CS⁺ versus CS⁻ showed activation in the pulvinar and the antero-lateral thalamus. In a post-hoc analysis, regions in the brain that correlated significantly with the response in the pulvinar, as highlighted in the main analysis, were identified. Regions showing a significant temporal correlation with the right pulvinar included the right amygdala, the basal forebrain and bilateral fusiform gyri. Although identified through a post-hoc analysis, this was the first functional imaging study to demonstrate the involvement of the amygdala in human classical fear conditioning—this involvement would be predicted on the basis of numerous animal and human lesion studies [10–13].

In a follow-up study by the same group, using differential conditioning with auditory CS (200 Hz and 8000 Hz), the primary aim was to identify plastic changes in the tonotopic auditory cortex, occurring as a consequence of learning [14[•]]. Indeed, the authors were able to demonstrate learning-related modulation of auditory cortex responses to identical tones solely as a function of conditioning. A post-hoc analysis, similar to the one performed in the previous study, showed correlations between modulated areas of primary auditory cortex and bilateral amygdalae. Other regions showing learning-related activity that correlated with auditory cortex function included the anterior cingulate gyrus and bilateral insulae.

All classical conditioning paradigms described so far presented a CS⁺ overtly. However, this overt presentation is not a necessity for learning, as conditioned responses can also be achieved if a CS is presented outside of awareness [15]. The simplest paradigm for presenting a visual stimulus without awareness is to use a technique called 'backward masking'. If a face stimulus (CS) is presented for a very short time (30 ms), and then immediately followed by presentation of a different face stimulus (mask), subjects will report only seeing the mask while remaining unaware of the target face (CS). This masked emotional learning paradigm was employed in a H₂¹⁵O PET study [16^{••}] which showed that the amygdala was only activated during the CS⁺ scans but not during the CS⁻ scans. The authors further observed an interesting hemispheric difference: the

right amygdala was activated to a greater extent than the left amygdala if the CS⁺ was presented out of awareness, whereas this was reversed (i.e. greater activation in left than in right amygdala) when the CS⁺ was presented consciously. This involvement of the amygdala in the expression of classically conditioned responses to stimuli presented outside of awareness is consistent with a previous study showing amygdala activation by masked presentation of emotionally expressive faces [17]. This most recent study has important implications for the role of the amygdala in classical conditioning. Not only does it replicate earlier behavioural reports showing that classical conditioning does not require awareness of the relationship between CS and US, but more importantly it shows that processing of this relationship, even in the case in which subjects are not aware of it, involves the amygdala.

fMRI studies

Event-related fMRI

fMRI offers improved spatial and temporal resolution in comparison with PET. The temporal resolution of fMRI was further improved by the introduction of single-trial or event-related fMRI (efMRI) [18,19]. In simple terms, this technique is analogous to the recording and analysis of event-related potentials in electrophysiology, where different stimuli are presented and the responses sampled repeatedly over time. Event-related fMRI provides an ideal context for studying the neurobiology of classical conditioning. For the first time, scientists have at their disposal a means of measuring the responses evoked by single stimuli or single stimulus categories (e.g. CS⁺ or CS⁻).

Recently, two studies using efMRI to investigate classical conditioning appeared simultaneously in the literature [20•,21•]. Both experiments investigated aversive classical delay conditioning. The study by LaBar and colleagues [20•] used different geometric shapes as CS and an electric shock as US. Single-trial fMRI was used to study acquisition and extinction separately. To increase the sampling rate (i.e. decrease the repetition time [TR]), these authors acquired only a limited set of coronal slice images, centred on the amygdala. Although technical difficulties prevented the recording of online autonomic data, the paradigm provided reliable conditioning in subjects studied in a separate experiment outside the scanner. The authors found increased signal in the amygdala evoked by the CS⁺. Amygdala activation was enhanced during initial acquisition of conditioned responses. During extinction, a similar picture emerged, with amygdala activations being greater during the early phase of extinction. Additional cortical activations were found in the anterior cingulate. The observation of decreases in amygdala activation over time was in accordance with electrophysiological findings in rats showing a similar temporal pattern during acquisition [22].

In the second study using efMRI, the authors introduced a random phase-shift between stimulus presentation and image acquisition, making it possible to obtain whole-brain

coverage with 48 slices [21•]. Each CS was presented for 3s before the US followed. The possible confound of US-induced blood oxygen-level dependent changes was circumvented by introducing a 50% partial reinforcement strategy. The face-designated CS⁺ was not always (i.e. 100% reinforcement) followed by the US, but only in half of the CS⁺ trials (i.e. 50% partial reinforcement). The experiment therefore included CS⁺ trials that were not followed by a US, which allowed the investigators to measure CS⁺-related evoked hemodynamic responses without the possibly confounding effect of the US (CS⁺ unpaired). CS were neutral faces and the US was an aversively loud 1 kHz tone. Again, differentially evoked signals (i.e. a greater signal in response to the CS⁺ compared to that evoked by the CS⁻) were found in the anterior cingulate cortex and the amygdala. Additional activations were seen in motor-related brain regions (e.g. the premotor cortex), and these were interpreted as an expression for the 'readiness' to escape an aversive situation. The most striking similarity between this study and the study by LaBar *et al.* [20•] was the observation that differential CS⁺-related responses decrease over time.

These two independent studies underline a common network for aversive conditioning in humans that includes the amygdala and the anterior cingulate cortex. Although the stimuli, the scanning details and the study parameters (TR, slice orientation, modality of the US [footshock [20•] versus loud tone [21•]]) differed between the two studies, both highlight the amygdala as a central unit in the processing of the CS-US contingency. Most importantly, both studies reveal that activation of the amygdala decreases over time.

Blocked fMRI

A 'blocked' fMRI paradigm, whereby different conditions are presented within a block, has also been used to study differential classical conditioning in humans [23•]. The authors examined differences in classical conditioning between social phobics and a control group. Using fMRI, the authors were able to analyse signal changes related to the CS before the US was administered. Imaging of CS-related responses was started after 60 learning trials and represented the retention of the association rather than its acquisition. A region-of-interest analysis (i.e. focussing on areas expected to be activated) revealed significantly increased activity in the amygdala for social phobics compared to control subjects. More precisely, control subjects showed decreased amygdala responses, whereas social phobia subjects showed a signal increase for CS⁺ compared to CS⁻ stimuli. Amygdala deactivations in control subjects seem to be contradictory to the previously discussed studies [20•,21•], in which amygdala activation rather than deactivation was observed. This contradiction can be resolved by the fact that the present study [23•] investigated CS⁺ trials during late conditioning (i.e. retention). The earlier studies investigated subjects during acquisition and retention, and they showed learning-related activation only during early acquisition and a similar CS⁺-related deactivation

tion during later stages [21••]. It would now be of considerable interest to study social phobias during acquisition. Combining the findings of all these studies, one would predict that social phobias do not show the ‘physiological’ decrease of amygdala activation over time. This might be related to amygdala pathology *per se* or to the failure of a control-mechanism exerted over the amygdala.

Related studies

A recent report of an fMRI study also dealt with classical conditioning, although this was an implicit and not a prime focus of the report [24•]. The authors’ intention was to study pain and the anticipation of pain. The painful stimulus (thermal pain) was termed the US and the signal predicting pain — the colour — the CS+. The main finding was in accordance with other studies of classical conditioning, showing activation of anterior cingulate cortices and the insulae, although the field of view did not include the medial temporal lobe. A comparison between activations in response to real pain (US), in comparison to anticipated pain (CS), revealed an anterior posterior dissociation, with activation to real pain lying anterior to activations evoked by anticipated pain.

Trace conditioning studies

All of the experiments described so far used ‘delay’ conditioning in which there is no interval between the CS and the US. The other common variant within classical conditioning paradigms is ‘trace’ conditioning. Trace conditioning differs from delay conditioning in the temporal relationship between the CS and the US. In delay conditioning, the US is presented at the end of the CS so that they overlap temporally. In trace conditioning, there is a gap between the offset of a CS and onset of a US (Figure 1). The term trace conditioning stems from the idea that a ‘memory trace’ is necessary to ‘bridge the gap’ between the CS and the US so that associative learning can take place [1]. Lesion studies [25–28] and studies of amnesic patients [29,30] suggest a critical role for the hippocampus in classical conditioning if the CS is not immediately followed by the US.

A recent study investigated the neuronal basis of trace fear conditioning in humans using efMRI [31••]. The CS was a non-aversive tone and the US was an aversively loud 1 kHz tone. The onset of the US followed the CS after a trace period of 1 s. Although the modality of the CS changed from visual to auditory, the evoked hemodynamic responses in anterior cingulate cortex, insula and amygdala were consistent with a previous study [21••]. More importantly, in this new study, evoked hemodynamic responses in the anterior hippocampus were observed, in accordance with the predictions from lesion studies. Interestingly, activation of the anterior hippocampus showed a similar decrease over time as the amygdala in this and a previous study [21••].

Conclusions

Functional neuroimaging studies have confirmed and extended findings from lesion studies, identifying the sub-

cortical and cortical structures that are involved in human classical conditioning. In particular, new imaging techniques (e.g. efMRI) reveal striking temporal patterns within medial temporal lobe activations. In all fear conditioning paradigms, the amygdala shows a decrease of CS+-evoked hemodynamic responses over time — in trace conditioning, such decreases are seen in both the hippocampus and the amygdala. These observations have a direct impact on the present controversy about the role of the amygdala in classical fear conditioning. One view suggests a role for medial temporal lobe structures in modulating or enabling associative changes in cortex. The hypothesis is that brain systems that mediate learning, in which the amygdala plays a central role, do so by enabling or permitting associative plasticity that encodes sensory contingencies that are being acquired. Following acquisition, the learned association will be expressed at a cortical level reflecting changes in synaptic connection strengths [32]. However, once the association has been learned, there is no need for further permissive modulation of plasticity, and systems mediating it disengage (e.g. a decline in amygdala activation).

In the alternative view, the amygdala is regarded as a rapid subcortical information processing unit that is continuously involved in the processing of the CS (and US) in aversive classical conditioning [13]. To assess behaviourally relevant (i.e. dangerous) stimuli, processing speed can be vital for the organism. A subcortical system including the amygdala would be considerably faster compared to a more complex cortical network. However, it should be emphasised that in this view, the fast unspecific subcortical system with a deliberately high false alarm rate is constantly ‘under supervision’ of the more specific cortical system.

Although more recent event-related fMRI studies seem to favour the former view, the findings are not conclusive and the controversy persists. Responses evoked by a paired CS+ (i.e. the CS+ is paired with the US) show activation in the amygdala with no decreases over time [31••]. It is conceivable that different nuclei of the amygdala show a different temporal involvement during conditioning. This observation would unify both seemingly unreconcilable theories, by highlighting different properties of different sub-regions (e.g. nuclei) of the amygdala. Such an ongoing activation in response to the US is in accordance with the latter view, hypothesising a continuous involvement of the amygdala in aversive classical conditioning. Interestingly, the location of this continuous activation within the amygdala was different from that of the activation that decreased over time.

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