

fMRI was used to study human brain activity during Pavlovian fear conditioning. Subjects were exposed to lights that either signaled painful electrical stimulation (CS+), or that did not serve as a warning signal (CS-). Unique patterns of activation developed within anterior cingulate and visual cortices as learning progressed. Training with the CS+ increased active tissue volume and shifted the timing of peak fMRI signal toward CS onset within the anterior cingulate. Within the visual cortex, active tissue volume increased with repeated CS+ presentations, while cross-correlation between the functional time course and CS- presentations decreased. This study demonstrates plasticity of anterior cingulate and visual cortices as a function of learning, and implicates these regions as components of a functional circuit activated in human fear conditioning. *NeuroReport* 10:3665–3670 © 1999 Lippincott Williams & Wilkins.

**Key words:** Anterior Cingulate; Echo-planar; Functional magnetic resonance imaging; Human; Learning; Visual cortex

## Functional MRI of human Pavlovian fear conditioning: patterns of activation as a function of learning

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### Introduction

Much of what is known about the neurobiological substrates of learning and memory comes from experimental investigations in laboratory animals. These studies frequently use Pavlovian fear conditioning in which a neutral stimulus (CS), usually a light or tone, is paired with a noxious stimulus (UCS) such as electric shock. Animals learn that the CS predicts the UCS as the stimuli are repeatedly paired, and this learning is manifested as changes in the timing, magnitude, or frequency of behavioral and autonomic responses [1]. Presentation of the CS alone will provoke the expression of a conditional fear response once learning has occurred.

Results from fear conditioning studies in laboratory animals suggest that the thalamus, amygdala, hippocampus and sensory cortex are involved in the acquisition and expression of the fear response, and implicate the amygdala as a principal site of CS–UCS convergence [2,3]. A number of fMRI studies have demonstrated human amygdala activation during differential fear conditioning and presentations of fearful faces and negatively valenced pictures [4–6]. Functional brain imaging using PET in humans suggests that there is an increase in frontal and temporal cortex activity following Pavlovian fear conditioning, and that these regions, as well as the visual and cingulate cortices, are activated by fearful stimuli [7–12]. Nevertheless, questions regarding the nature of

these learning-related changes in human brain activation remain. For example, learning may be demonstrated through changes in the timing of functional activation, the similarity between the pattern of neuronal firing and resulting metabolism and important environmental stimuli, or the amount of tissue engaged in the task. These questions have been difficult to address with PET because of limited temporal and spatial resolution and difficulties associated with extended repeated testing of individual subjects.

The current study used the excellent temporal and spatial resolution of fMRI to examine human brain activity during Pavlovian fear conditioning in healthy subjects. This neuroimaging technique is sensitive to variations in blood oxygenation and flow, and therefore can be used to localize regional brain neuronal activity during sensory, cognitive or motor tasks that produce local changes in cerebral blood oxygen levels [13,14]. The present study was designed to identify a subset of the neural structures involved in Pavlovian fear conditioning, characterize the pattern of functional activation within each region, and describe changes in the functional pattern as learning occurs.

### Materials and Methods

**Subjects:** Ten healthy, right handed subjects (4 male), ranging from 20 to 45 years of age (mean,

27.4 ± 2.9 years), were recruited for this study. Handedness was defined by the Edinburgh Inventory. Subjects were offered extra credit in psychology courses and \$6/h for their participation. All participants signed a consent form that was approved by the Institutional Review Boards of the University of Wisconsin-Milwaukee and Medical College of Wisconsin.

**fMRI:** Imaging was performed on a Bruker Biospec 3T/60 scanner equipped with three-axis local gradient coil and an endcapped quadrature birdcage RF coil using a gradient-echo echo-planar pulse sequence. Eight contiguous 8 mm axial slices were collected (TR = 2000 ms, TE = 27.2 ms, FOV = 24 cm, flip angle = 90°) in a series of 115 sequential images during each 230 second block of stimulus presentations. A standard, T1-weighted neuroanatomical MRI (TR = 400 ms, TE = 20 ms, FOV = 24 cm, flip angle = 22°) was obtained for each subject without the use of echo-planar (functional) imaging to serve as an anatomical map over which functional images were superimposed. Slice thickness and offset were equivalent for echo-planar and T1 scans. Functional images were manually overlaid upon anatomical images and offset parameters adjusted, if necessary, for best registration. Initial functional images from each successive block of conditioning trials were overlaid on T1 scans for each subject and any change in position due to subject movement from block to block was manually corrected.

**Electrical stimulus:** A customized AC (60 Hz) source was used to provide electrical stimulation (UCS) through two aluminum surface electrodes (2 cm diameter) over the right tibial nerve above the medial malleolus. Participants individually determined the intensity of the electrical stimulation by rating practice trials from 0 to 5 (0 = no sensation, 5 = painful but tolerable). Stimulus intensity was determined with the subject recumbent within the scanner and remained constant throughout the experiment.

**Visual stimulus:** A red 25 W light bulb was flashed (0.5 Hz) for 10 s as the conditional stimulus (CS). The light bulb was positioned 10° to the right side of the subject's focal point, housed in a black plastic light fixture and mounted on a wood frame 5.5 m from the scanner. The CS was viewed with a pair of prism glasses that were attached to the RF coil directly above the subjects' eyes to direct their field of vision toward a fixation point.

**Scanning protocol:** Participants were assessed in a single 2 h session. After completing health and

handedness questionnaires, participants were randomly assigned to one of two groups (paired or control). Paired group subjects received co-terminating CS (10 s) and UCS (0.5 s; ITI = 30 s, ISI = 0 s) presentations during all training blocks. Control subjects received explicitly unpaired CS and UCS (ITI = 30 s, ISI = 15 s) presentations on all blocks. Following standard T1 weighted anatomical scans, all subjects were presented five blocks (five trials per block) of the CS and UCS while functional images were obtained. Subjective pain ratings were obtained after each stimulus block.

**fMRI analysis:** All functional image analyses were performed using AFNI v2.2. [15]. fMRI data were movement corrected and registered onto T1 anatomical images. Activated regions were identified through cross-correlation analysis with a phase-shifted reference function (square waveform canonical) representing the temporal pattern of visual stimulation (see Fig. 1) [16]. A threshold correlation coefficient of 0.40 ( $p < 0.001$ ) was used to eliminate signal changes that did not have a strong relationship to the reference function. Voxels with signal changes passing the 0.40 correlation threshold were then directly registered upon T1 anatomical scans obtained during the same imaging session.

Three regions of interest (anterior cingulate, retrosplenial and visual cortices) were selected for further analysis based on initial visual inspection of group differences in activation, and were anatomically defined in three dimensions using a human brain atlas [17] in conjunction with the T1-weighted images for each individual subject. The anterior cingulate was defined as the cortical area surrounding the cingulate sulcus, anterior to the precentral sulcus and immediately ventral, rostral and dorsal to the genu of the corpus callosum. The retrosplenium was defined as cortex posterior to the splenium of the corpus callosum, anterior to the parieto-occipital fissure, superior to the calcarine sulcus and inferior to the subparietal sulcus. The primary visual cortex was defined as the tissue surrounding the calcarine fissure, inferior to the parieto-occipital fissure, medial to the intra-occipital gyrus and superior to the lingual gyrus. The amount of tissue engaged by the task was measured by tallying the total number of voxels passing the 0.40 correlation threshold within each region of interest (ROI). In addition, the functional data from all voxels within a ROI were averaged to obtain a single time course that was representative of the temporal pattern of activation within that ROI. The maximum correlation coefficient between the phase-shifted canonical and the averaged time course from all voxels within a ROI was utilized as an index of the similarity between

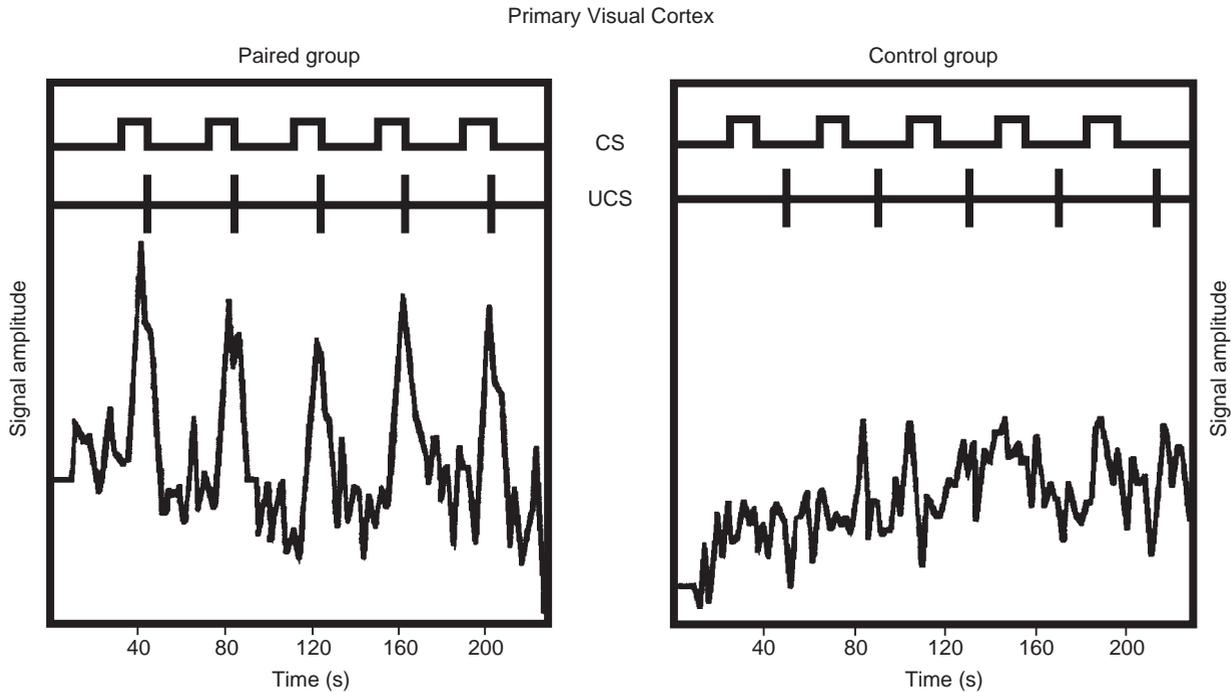


FIG. 1. CS and UCS waveforms and MR signal from a single voxel within the primary visual cortex of individual subjects, representative of the paired and control groups on block 5. Cross-correlation of the phase-shifted reference function (CS waveform) and MR signal yields correlation coefficients that are used to threshold data.

the temporal pattern of CS presentations and the ROI time course (cross-correlation). The timing of CS driven activation was measured by shifting the reference canonical to obtain the maximum correlation with the ROI time course, then inferring the number of seconds activation was delayed from CS onset (phase-delay). Repeated measures analysis of variance (ANOVA), with *a priori* significance threshold representing a 0.05 probability level, was completed on voxel count, cross-correlation, and phase-delay measures to compare group differences and changes in activation across training blocks.

## Results

Mean UCS intensity delivered to the paired ( $3.06 \pm 0.52$  mA) and control ( $3.59 \pm 1.46$  mA) groups did not significantly differ ( $t(9) = 0.31$ ,  $p > 0.05$ ). Repeated measures ANOVA indicated no significant differences in subjective pain ratings of the UCS between groups (paired =  $3.92 \pm 0.18$ , control =  $3.58 \pm 0.26$ ;  $F(1,8) < 1$ ) or across training blocks ( $F(4,32) = 1.64$ ).

Presentation of the visual and electrical stimulation produced large stimulus-related changes in functional activation that differed between groups and across training blocks within the anterior cingulate and visual cortices. Figure 2 shows representative activation from one paired group subject during training. Notice that the number of active voxels

within the anterior cingulate and visual cortices increased across the five stimulus blocks. The pattern of activation varied for each ROI across training (see Fig. 3). Repeated measures ANOVA showed that voxel count within the anterior cingulate was greater for paired than control subjects as demonstrated by a significant group effect ( $F(1,8) = 9.49$ ,  $p \leq 0.05$ ). The group by block interaction was not significant ( $F(4,28) = 1.11$ ). However, while group differences were not significant on Blocks 1 and 2 ( $t(18) = 1.79$ ,  $p > 0.05$ ), voxel count was greater for paired than control subjects on blocks 4 and 5 ( $t(18) = 2.34$ ,  $p < 0.05$ ). In addition, significant group differences in phase-delay were demonstrated within this region, such that paired group activity occurred earlier relative to CS onset than that of the control group ( $F(1,8) = 36.94$ ,  $p \leq 0.05$ ). No cross-correlation differences were demonstrated within the anterior cingulate ( $F(1,8) = 2.13$ ).

Figure 3 demonstrates that while tissue volume within the primary visual cortex did not change in paired subjects, it decreased in controls with repeated stimulus presentation as demonstrated by a significant group  $\times$  block interaction ( $F(4,28) = 8.58$ ,  $p \leq 0.05$ ). Voxel count within the visual cortex was similar ( $t(18) = 0.39$ ,  $p > 0.05$ ) for both groups as training began (Blocks 1 and 2), and remained constant across training blocks in paired subjects ( $t(18) = 1.06$ ,  $p > 0.05$ ) while decreasing in controls ( $t(18) = 3.14$ ,  $p < 0.05$ ), such that a significantly

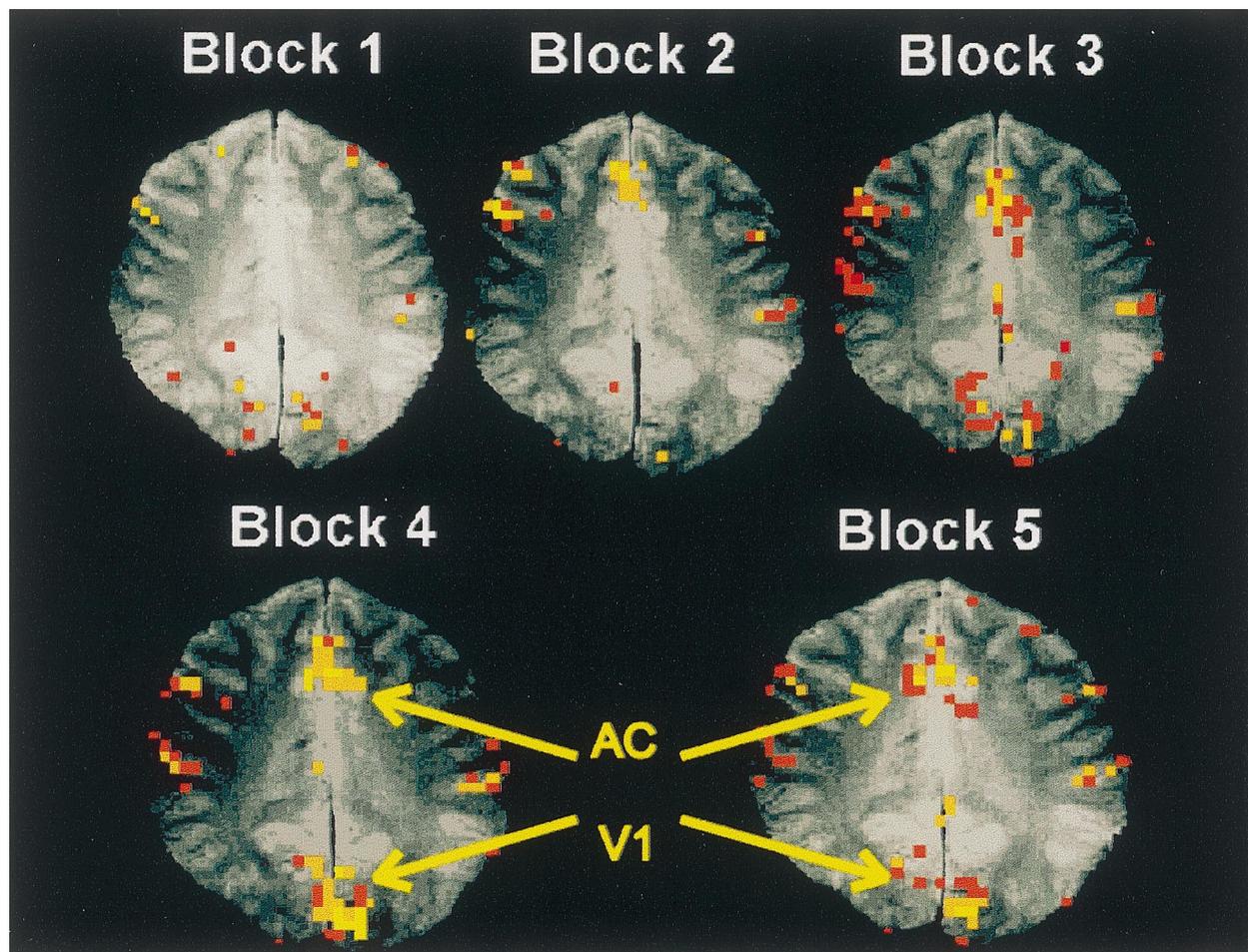


FIG. 2. Functional images from one representative paired group subject, registered onto axial T1 anatomical scans. The images demonstrate increases in the amount of active tissue across training blocks within the anterior cingulate (AC) and primary visual (V1) cortices.

( $t(18) = 3.61$ ,  $p < 0.05$ ) larger amount of active tissue was demonstrated in paired than in control subjects by the end of training. No differences were observed in the phase-delay of visual cortex activity ( $F(1,8) < 1$ ). However, significant group differences in cross-correlation were demonstrated within this region ( $F(1,8) = 7.92$ ,  $p < 0.05$ ). As shown in Fig. 3, the cross-correlation values remained constant in the paired group, while decreasing in control subjects. Finally, no differences were observed within the retrosplenial cortex on any measure ( $F(1,8) < 1$ ). The absence of group differences within the retrosplenium suggests that these measures of functional activity are not contaminated by shock-related artifact.

## Discussion

Results from this experiment demonstrate learning-related changes in activation within the anterior cingulate and primary visual cortices. Although both regions showed learning-related changes in activa-

tion, the specific pattern of those changes differed. Within the anterior cingulate, the amount of active tissue increased as a function of repeatedly pairing CS–UCS trials, but did not change with unpaired light and shock presentations. Further, the timing of paired group activation moved toward CS onset relative to control subjects as learning progressed. Lesion and physiological assessment of anterior cingulate function suggests that this region plays a modulatory role in autonomic activity and internal emotional states [18], and may facilitate but not be necessary for learning affective behavior [19]. Learning-related changes in anterior cingulate activation are consistent with theories of Pavlovian conditioning that suggest that this type of learning may be related to changes in attention [20] as well as with functional imaging studies that have demonstrated increased cerebral blood flow within the anterior cingulate cortex related to attentional processes [21,22].

Within the primary visual cortex, learning was reflected through changes in the volume of active

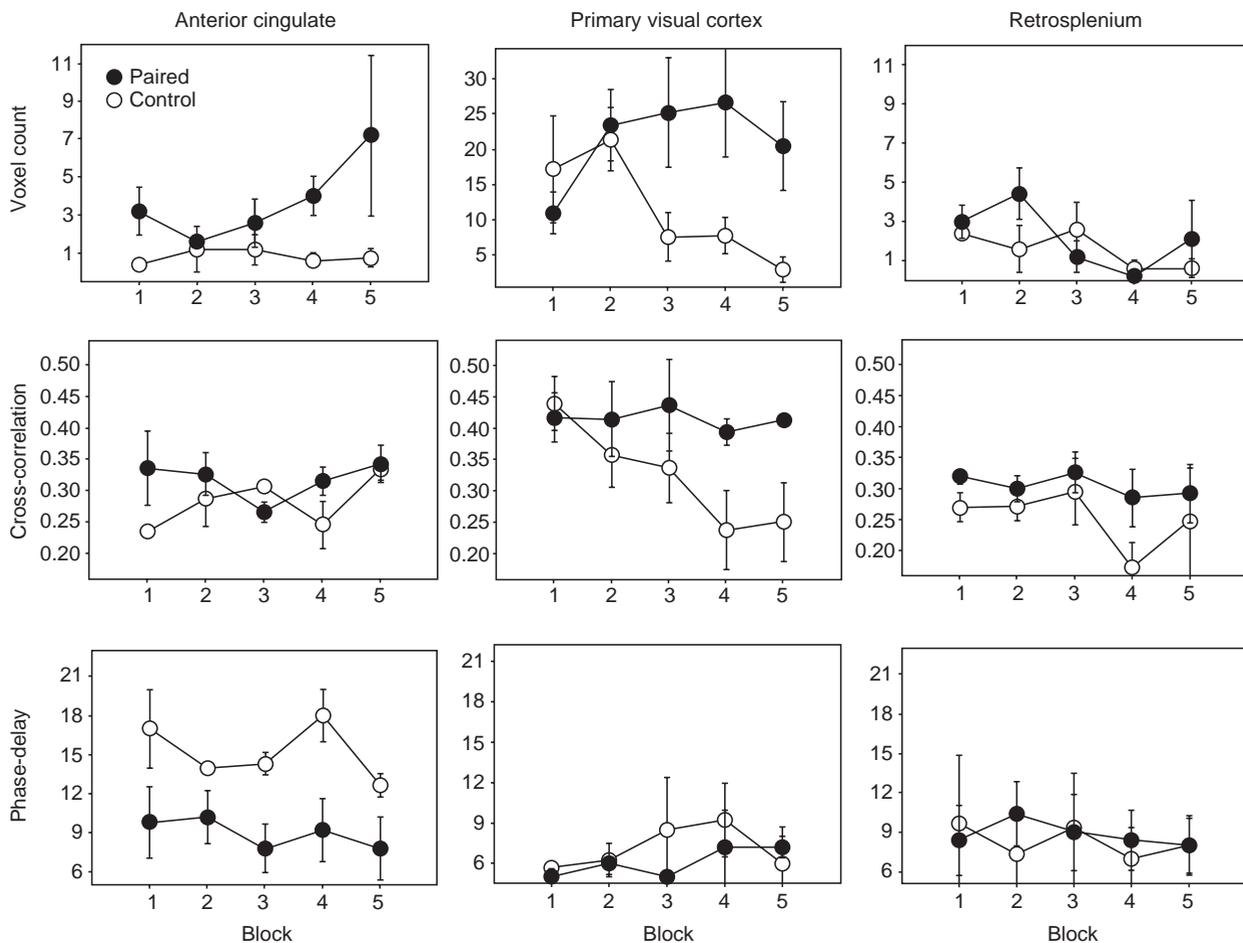


FIG. 3. Voxel count, cross-correlation and phase-delay measures of functional activity within the anterior cingulate, primary visual and retrosplenial cortices. The pattern of learning-related change varied between anterior cingulate (voxel count and phase-delay), primary visual (voxel count and cross-correlation) and retrosplenial (no learning-related changes) cortices.

tissue and the similarity of the functional time-course to the pattern of CS presentations, but not through changes in the timing of activation. The volume of active tissue was maintained in paired subjects over the course of training, while it decreased in controls. The similarity of the functional time-course to the pattern of CS presentations remained constant over training in paired subjects, while it decreased in control subjects. This result is consistent with the suggestion that an orienting response to the CS should habituate without reinforcement [23]. The (presumed) orienting response within the visual cortex of paired subjects may have been preserved by pairing CS and UCS, while this response habituated in controls.

Because no behavioral CR data were monitored during this study, we cannot address the relationship between functional brain activation and behavioral expression of learning. However, research in progress from our laboratory, in which galvanic skin response was monitored during fMRI, suggests that

the CS+, but not CS- provokes a fear response [24,25]. Although, no activation was found in the present study within regions previously implicated in fear conditioning studies with laboratory animals (amygdala, hippocampus and thalamus), this certainly does not preclude a role of these and other brain regions in human conditional fear. Our parameters may not have produced adequate images of the medial temporal lobe to detect amygdala activation, and other related conditioning paradigms may better demonstrate learning-related changes in brain activity within these regions [24,25]. In addition, the method used to define ROIs in this study can be improved upon with the use of higher resolution anatomy and three dimensional data reconstruction, which were not available at the time these data were collected. The potential for some limited variation in ROI measurement due to movement and other factors increases the chances of not detecting structures with more subtle patterns of CS-related activity (Type II error). These improvements will allow

ROIs to be defined using the Talairach coordinate system and will offer better control over variance due to brain size and head orientation.

The present study assessed human brain activity during acquisition of conditional fear, which includes both CS and UCS presentations. Thus subjects would be expected to express unconditional reactions to the electrical stimulation as well as gradually develop a CR over the course of training. The extent to which the differential patterns that we observed are specific to the presentation of both classes of stimuli within a block is not clear. Preliminary results indicate that patterns of activation seen when the CS is repeatedly presented alone in extinction are similar to those observed here [25].

## Conclusion

We demonstrated learning-related changes in human brain activation using a Pavlovian fear conditioning paradigm. Our findings suggest that learning is reflected through changes in the timing of activation, the amount of tissue activated, and the similarity of the functional time course to the temporal pattern of environmental stimuli. Comparison of the acquisition functions between ROIs and across measures of activation shows that the pattern of changes in activity varies between regions involved in the task such that a unique pattern of learning-related change was demonstrated for each region. Additionally, the nature of change varies between ROIs and across

functional measures such that learning was reflected through dynamic changes in the amount of active tissue within the anterior cingulate and primary visual cortices, while maintenance of the strength of cross-correlation between the reference waveform and functional time-course was reflective of learning in visual cortex.

## References

1. Rescorla R. *Annu Rev Neurosci* **11**, 329–352 (1988).
2. LeDoux J. *Annu Rev Psychol* **46**, 209–235 (1995).
3. Davis M. *J Neuropsychiatry Clin Neurosci* **9**, 382–402 (1997).
4. LaBar K, Gatenby J, Gore J et al. *Neuron* **20**, 937–945 (1998).
5. Whalen P, Rauch S, Etcoff N et al. *J Neurosci* **18**, 411–418 (1998).
6. Irwin I, Davidson R, Lowe M et al. *Neuroreport* **7**, 1765–1769 (1996).
7. Hugdahl K, Berardi A, Thompson W et al. *NeuroReport* **6**, 1723–1728 (1996).
8. Fredrikson M, Wik G, Annas P et al. *Psychophysiology* **32**, 43–48 (1995).
9. Fredrikson M, Wik G, Fischer H et al. *NeuroReport* **7**, 97–101 (1995).
10. Reiman E, Fusselman M, Fox P et al. *Science* **243**, 1071–1073 (1989).
11. Wik G, Fredrikson M, Ericson K et al. *Psychiatry Res* **50**, 15–24 (1993).
12. Zohar J, Insel T, Bernman K et al. *Arch Gen Psychiatry* **46**, 505–510 (1989).
13. DeYoe E, Bandettini P, Neitz J et al. *J Neurosci Methods* **54**, 171–187 (1994).
14. Cohen M and Bookheimer S. *Trends Neurosci* **17**, 268–277 (1994).
15. Cox R. *Comp Biomed Res* **29**, 162–173 (1996).
16. Bandettini P, Jesmanowicz A, Wong E et al. *Magn Reson Med* **30** (1993).
17. Duvernoy H. *The Human Brain: Surface, Three-dimensional Sectional Anatomy and MRI*. New York: Springer-Verlag, 1991.
18. Devinsky O, Morrell M and Vogt B. *Brain* **118**, 279–306 (1991).
19. Gabriele M, Kubota Y, Sparenborg S et al. *Exp Brain Res* **86**, 585–600 (1991).
20. Pearce J and Hall G. *Psychol Rev* **106**, 532–552 (1980).
21. Paus T, Petides M, Evans A et al. **70**, 453–469 (1993).
22. Posner M, Petersen S, Fox P et al. *Science* **240**, 1627–1631 (1988).
23. Stern J and Walrath L. *Psychophysiology* **14**, 334–342 (1977).
24. Knight D, Smith C, Stein E et al. *Soc Neurosci Abstr* **23**, 209 (1997).
25. Knight D, Smith C, Cheng D et al. *Soc Neurosci Abstr* **24**, 1523 (1998).

ACKNOWLEDGEMENTS: This work was supported by NIMH fellowship MH11722 (D.C.K.), NIDA grant DA09465 (E.A.S.), NIMH grant 50864 (F.J.H.) and a grant from the McDonnell Foundation (F.J.H.). Portions of this work were presented at the 1996 meeting of the Society for Neuroscience in Washington, DC.

**Received 7 July 1999;**  
**accepted 23 September 1999**