Lateralization and Behavioral Correlation of Changes in Regional Cerebral Blood Flow With Classical Conditioning of the Human Eyeblink Response

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Schreurs, Bernard G., Anthony R. McIntosh, Marcel Bahro, Peter Herscovitch, Trey Sunderland, and Susan E. Molchan. Lateralization and behavioral correlation of changes in regional cerebral blood flow with classical conditioning of the human eyeblink response. J. Neurophysiol. 77: 2153–2163, 1997. Latency as a possible site underlying learning (see Thompson and Krupa 1994). In addition, evidence from clinical studies suggests that the human cerebellum may be crucial to eyeblink conditioning (Daum et al. 1993; Topka et al. 1993). There is also considerable evidence from both human and animal learning literature that primary sensory cortices are important in learning and memory beyond simple stimulus processing. Functional plasticity correlated with learning and memory has been demonstrated at the cellular level in single sensory cortical cells (Recanzone et al. 1992; Weinberger et al. 1990) and in cellular ensembles in subcortical and cortical maps (Gonzalez-Lima 1992; Harvey et al. 1986; Scheich et al. 1992). This plasticity has recently been shown to occur in the human auditory cortex with the use of positron emission tomography (PET) regional cerebral blood flow (rCBF) (Molchan et al. 1994). These data suggest that sensory systems can code, in parallel, the physical and behavioral properties of stimuli. Such findings were anticipated in classical conditioning studies of the marine mollusk, Hermissenda, where a pair of type B photoreceptors has been identified as the site of learning-specific change (e.g., Alkon 1983).

Recently, Molchan et al. (1994) showed that during classical conditioning of the human eyelid response there was a significant increase in rCBF in the primary auditory cortex and a significant decrease in rCBF in the cerebellum. A striking aspect of those data was the considerable laterality in the changes in blood flow. Specifically, changes in the auditory cortex were strongest on the side opposite to the air puff delivery, whereas cerebellar changes were strongest on the same side as the air puff. Interestingly, the tone was presented binaurally and observations of human eyelid conditioning of the right eye show that conditioned responses (CRs) are markedly bilateral (e.g., Hilgard and Campbell 1936). In addition to lateralized changes in auditory cortex and cerebellum, Molchan et al. (1994) identified a number of other areas including cingulate cortex, parietal cortex, and prefrontal lobes that are involved in eyelid conditioning.

INTRODUCTION

The search for neural substrates of learning and memory in model systems has identified a number of sites of plasticity. The use of well-controlled behavioral paradigms, such as classical conditioning, has permitted this search to be focused on a relatively small number of areas (e.g., Thompson 1986; for review see Schreurs 1989). For example, studies of classical conditioning of the rabbit nictitating membrane/eyelid response have focused attention on the cerebellum as a possible site underlying learning (see Thompson and Krupa 1994). In addition, evidence from clinical studies suggests that the human cerebellum may be crucial to eyelid conditioning (Daum et al. 1993; Topka et al. 1993). There is also considerable evidence from both human and animal learning literature that primary sensory cortices are important in learning and memory beyond simple stimulus processing. Functional plasticity correlated with learning and memory has been demonstrated at the cellular level in single sensory cortical cells (Recanzone et al. 1992; Weinberger et al. 1990) and in cellular ensembles in subcortical and cortical maps (Gonzalez-Lima 1992; Harvey et al. 1986; Scheich et al. 1992). This plasticity has recently been shown to occur in the human auditory cortex with the use of positron emission tomography (PET) regional cerebral blood flow (rCBF) (Molchan et al. 1994). These data suggest that sensory systems can code, in parallel, the physical and behavioral properties of stimuli. Such findings were anticipated in classical conditioning studies of the marine mollusk, Hermissenda, where a pair of type B photoreceptors has been identified as the site of learning-specific change (e.g., Alkon 1983).

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These data suggest that a form of learning as apparently simple as eyelblink conditioning appears to engage an extensive network of neural systems. Because the involvement of these areas was specific to the acquisition of the CR, the data of Molchan et al. (1994) lend support to the notion that learning and memory may involve the interactions among a number of neural systems and that any region has the potential to play a role depending on the requirements of the task (McIntosh and Gonzalez-Lima 1994b).

In an effort to examine further the marked differences between bilateral sensory input and motor output on the one hand, and the considerable laterality of rCBF changes on the other, we conducted an experiment in which subjects received binaural auditory stimulation and air puff delivery to the left eye, the eye opposite that used by Molchan et al. (1994) task?''). Although there will be some overlap in the regions identified in this approach, the present experiment sought to correlate changes in rCBF with behavior (e.g., CRs and latency of CRs) and elaborate on the complexity of changes in rCBF that occur with learning. Areas that showed strong relationships to the acquisition of the CR may suggest a special role for those areas in learning the behavior.

METHODS

Subjects

The subjects were 10 normal right-handed female volunteers (age 24.5 ± 0.85 yr, mean ± SE, range 21–30 yr) who participated after giving informed consent. Female subjects were used because the experimental equipment allowed little clearance from the scanner: the larger skulls of many males would not allow them and the equipment to pass into the scanner (Molchan et al. 1994). Subjects were given instructions that would not reveal the purpose of the experiment (neutral instruction set). The study was approved by appropriate institutional review boards at the National Institutes of Health.

Except where noted, the scanning methods, eyelid conditioning procedure, and regional analysis were identical to those employed by Molchan et al. (1994).

PET scans

PET scans were carried out with a Scanditronix PC2048-15B scanner (Scanditronix) which provides 15 transverse slices through the brain spaced 6.5 mm apart (center to center), with transverse resolution of 6.9 mm (full width at half maximum) and axial resolution of 5–6 mm. Emission scans were obtained after a bolus intravenous injection of 40 mCi (1 Ci = 37 GBq) of H$_2^{15}$O (half-life 123 s). Images were acquired over 60 s, starting when the bolus of radio tracer arrived in the head. Because of the near-linear relationship between rCBF and tissue counts accumulated over a brief scan period, the acquired images reflected relative changes in rCBF during different scan states. Six scans were conducted, separated by 12 min to allow for radioactive decay of the H$_2^{15}$O to <2% peak levels, except for the second and third scans, which were 19 min apart to allow sufficient time for conditioning to occur.

Eyeblink conditioning

Eyeblinks were detected with the use of a low-torque, rotary potentiometer (Litton) coupled to the upper eyelid. The potentiometer, together with a flexible tube that was positioned ~10 mm from the left cornea (i.e., eye opposite that used by Molchan et al. 1994) to deliver a 100-ms, 2-psi (1 psi = 6.89 kPa) air puff, was attached to the left side of a plastic mask, which served as the head-holding device for the PET scans. The 2-psi air puff served as the unconditioned stimulus (UCS) and was strong enough to elicit an eyelid blink reliably without being noxious. Earphones delivered a bilateral 80-dB, 1,000-Hz, 500-ms tone, which served as the conditioned stimulus (CS). The output of the potentiometer was amplified and recorded on a chart recorder (Gould model 2200), operated at a chart speed of 125 mm/s.

Two PET scans were performed during each of three different phases of stimulus presentation. The phases of stimulus presentation occurred in the following order: 1) explicitly unpaired presentations of the tone and air puff (unpaired control); 2) paired presentations of the tone preceding and coterminating with the air puff (classical conditioning); and 3) explicitly unpaired presentations of the tone and air puff (unpaired extinction). This third phase represents a change from the tone-alone extinction phase used by Molchan et al. (1994). The change was made to provide the same number of tone and air puff presentations in each phase and ensure greater comparability of the extinction phase with the unpaired control and paired phases. Consequently, in the present experiment, the number of tone and air puff presentations was the same for each of the three phases. During scans the intertrial intervals for paired trials averaged 15 s and the intertrial interval for unpaired trials averaged 8 s. Paired presentations of tone and air puff began after the second unpaired control scan and continued through the first and second paired scans and between the first and second unpaired extinction scans. The paired presentations that occurred between scans were separated by an average intertrial interval of 30 s.

A CR was defined as any eyelid closure >0.5 mm that occurred ≥150 ms after tone onset but before air puff onset. On explicitly unpaired extinction trials, a CR was defined as any eyelid closure >0.5 mm that occurred 150 ms after tone onset but before the end of the 1,200-ms observation interval. Responses that occurred <150 ms after tone onset were scored as alpha responses (unconditioned responses to the tone) and not counted as CRs (see for example, Gormezano 1966).

Image analysis

POSTPROCESSING. Images from each subject were linearly interpolated from the original 15 slices to 43, and then images from the second through sixth scan were registered to the first scan with the use of algorithms developed by Woods et al. (1992) to correct for head movement across the experiment. After registration, subject images were spatially transformed to an averaged PET image that conformed to the standard space (Friston et al. 1989, 1991a). Transformed images were then smoothed with a $20 \times 20 \times 12$ mm Gaussian filter to reduce individual differences in functional anatomy.

STATISTICAL ANALYSIS. The characterization of learning-related changes in rCBF was performed with the use of an analytic approach involving a univariate test (Molchan et al. 1994) followed by a new, more sensitive multivariate image analysis. Specifically, Statistical Parametric Mapping software (SPM, version 4) was used to assess task-related activity changes pixel by pixel (Molchan et al. 1994). After this a multivariate analysis, known as Partial Least Squares, identified distributed patterns of regional activity that most related the experimental design (PLS) (Bookstein et al. 1996; Streissguth et al. 1993; see McIntosh et al. 1996 for the extension to image analysis). This approach can be seen as using the SPM analysis for regional tests ("is this region significantly activated across tasks?") and the PLS analysis to test distributed systems ("are there systems that as a whole are related to the task?"). Although there will be some overlap in the regions identi-
fied by each analysis, the main distinction is that the PLS analysis works at the system level, whereas the SPM analysis works at the regional level. Each of these analyses is explained more fully below.

SPM. A repeated-measures analysis of variance procedure was performed on a voxel-by-voxel basis with an analysis of covariance adjustment for variations in global blood flow (Friston et al. 1991a,b). There were no systematic differences in global cerebral blood flow between tasks [F(5,54) = 2.10, P > 0.05], so the validity of the analysis of covariance adjustment was confirmed. Pairwise comparisons and linear and quadratic trend analyses were conducted across the three different phases of the experiment (significance level of P < 0.001). Linear increases or decreases in rCBF across the phases of the experiment might indicate sensitizing or habituating effects that resulted from stimulus presentation and/or duration in the scanner. In contrast, quadratic trends in rCBF would follow the associative relationship between tone and air puff across the three phases of the experiment (i.e., unrelated, related, and then unrelated again, see Molchan et al. 1994).

PLS ANALYSIS. PLS analysis operates on the interrelation among two or more “blocks” of matrices and seeks to obtain a new set of variables that optimally relates the blocks with the use of the fewest dimensions. In the present application, one block contains matrices of the functional PET images for all subjects in all experimental conditions, a second block consists of contrasts coding the experimental design, and a third block contains the percent CRs or CR latencies. Analyzing the second and third blocks against the first, PLS can carry out image-wide PET activation analysis and extract brain-behavior covariances and correlations. Within the space of image descriptors, the findings of PLS are carried by singular images, computed multivariate optima somewhat related to the previously introduced eigenimages of Friston et al. (1993). The singular images are spatial patterns of pixel contents representing pixel-by-pixel covariances with task or behavior. The numerical values within the singular images are called saliences.

A second set of descriptors derived from PLS are the scores. For the images, the scores are obtained by the summed cross-product of the singular image with each subject’s image. For a particular singular image, each subject will have a single score in each scan condition. These scores represent the relation of that subject to the singular image, and, when examined by task, can be used to assess the importance of the singular image.

STATISTICAL SIGNIFICANCE. Averaging the scores within a task and comparing these averages between tasks provides an indication of whether a particular spatial pattern significantly relates to the contrasts. A descriptive index of this discrimination can be obtained by the use of something like a one-way analysis of variance; however, the scores may be biased and therefore conventional parametric statistics may not be appropriate. The inferential interpretation of the task discrimination by the scores obtained by PLS was assessed with the use of a permutation test (Edgington 1980; McIntosh et al. 1996). For the present application, the test involves randomly reordering the rows of the design matrix and redoing PLS and analysis of variance with the new ordering. At each permutation, the statistic is compared with the obtained statistic for the original data. The obtained statistic is assigned a probability value based on the number of times a statistic from the permuted data exceeds the obtained value. With the use of this method, the significance of the PLS scores can be assessed without relying on the distributional assumptions underlying most conventional parametric statistical methods. The permutation test used in this application was a multiple regression approach to analysis of variance (Pedhazur 1982). The collection of condition contrast vectors (or behavioral measures) was used as independent variables and the scores as dependent variables.

BRAIN-BEHAVIOR RELATIONS. In many neuroimaging studies, behavioral measures are obtained when subjects are engaged in the experiment. Aside from ensuring that subject performance is within expectation, behavioral measures can also provide important information relating brain activity with observed behavior. A cross-correlation of subject behavioral measures and brain activity within task is straightforward. However, with the use of PLS, not only can this correlation be examined, but changes in brain-behavior correlations across tasks can be assessed also. Of particular interest in this analysis was whether similar brain regions were identified as showing a relation to behavior as those regions identified in the analysis of changes in rCBF. The analysis differs from that concerned with experimental design. Rather than the covariance of brain and design contrasts, the cross-correlation matrix contains the relation between the behavioral measure and brain within each condition of interest (e.g., in the case of 2 scans, the matrix would have 2 cross-correlation vectors). The resulting singular images thus indicate whether there are common patterns of brain-behavior relations between the conditions by extracting singular images that can potentially represent commonalities or differences where they exist. This form of PLS analysis is more similar to applications in teratology (Streissguth et al. 1993).

RESULTS

Behavior

Figure 1 shows sample responses to the tone and air puff during the course of the experiment. From top to bottom, the figure shows an unconditioned response to the tone (alpha response); an unconditioned response to the air puff; a CR during pairing of the tone and air puff; and a combination of alpha response and CR during pairing.

Figure 2 shows the percentage of CRs and alpha responses during each scan and for 6-min blocks of tone–air puff pairings between scans. Overall, Fig. 2 shows a marked increase in CRs during the course of the experiment and a decrease in alpha responses to a relatively low, stable level. In particular, the figure shows that during the unpaired control scans (UP1 and UP2) the tone elicited alpha responses of 43.5 and 22.7%, respectively, and that responses that met the latency and amplitude criteria for CRs occurred at a frequency of 11 and 6.7%, respectively. During the first three blocks of pairings, alpha responses decreased from 17.4 to 10.9%, whereas CRs showed significant acquisition, increasing from 35.7% during the first block (B1) to 59.8% by the third block B3, F(2,19) = 6.81, P < 0.01. During the 1-min period of the first paired scan (P1), CRs were at 28.5%. Acquisition of CRs continued during the next two training blocks (B4, B5), increasing from 49.5 to 62.5%. During the 1-min period of the second paired scan (P2), CRs were at 30.5%. CRs asymptoted at ~61% during the next two blocks of paired trials (B6, B7). The first unpaired extinction scan (EX1) yielded responding at a level of 39.5%. Responding during the final two blocks of paired training trials (B8, B9) rose from 47 to 52.3%. The second unpaired extinction scan (EX2) yielded 58% CRs.

Overall statistical analysis of CR latency was limited by the relatively low frequency of responses during the unpaired scans (UP1 and UP2). However, at a descriptive level, responses did decrease in latency from a mean of 370 ms during the first and second unpaired scans to a mean latency of 312 ms during the first and second paired scans. With the
increase in CR frequency during training, response latencies could be assessed statistically and were found to decrease slightly from 330 to 290 ms during the first three blocks of pairings (linear trend), $F(1, 9) = 3.13, P > 0.05$. During the scans, CR latencies increased significantly from a mean of 312 ms for the paired scans (P1 and P2) to a mean value of 372 ms for the unpaired extinction scans EX1 and EX2, $F(1, 9) = 14.80, P < 0.005$.

The CR frequency data suggest that acquisition of CRs occurred during the training trials but that performance of CRs was at a somewhat lower level during the scans. One possible reason for the apparent difference was a change
in some of the background stimulus conditions (context) between training trials and scans. For example, the stimuli associated with injection of the isotope such as movement and noise involved in preparing the dose and the physical sensation of the injection, although constant across scans, were not present during the blocks of training trials. Such changes in context have been proposed to reduce performance of CRs without necessarily affecting learning (e.g., Bouton 1993; Myers and Gluck 1994). This interpretation is supported by an examination of CR latencies that did not change between pairings and scans, averaging 313 ms during paired training and 312 ms during the paired scans.

**SPM image analysis of classical conditioning**

Results from the comparison of averaged rCBF during the paired and unpaired control scans are summarized in Table 1 and the corresponding difference images are depicted in Fig. 3, top. Each of these areas showed not only a significant difference in rCBF between the paired and unpaired control phase but also a significant quadratic trend across the three phases of the experiment. Consequently, the changes in rCBF in these areas followed the associative relationship between tone and air puff. Inspection of the figure and table shows both increases and decreases in rCBF as a function of conditioning. There were significant increases in the primary auditory [Brodmann area (BA) 42; 56, −8, 8], auditory association (BA 22; 30, −44, 8), and temporoparietal cortices (BA 18; −32, −86, 8). All of these increases, with the exception of the increase in temporoparietal cortex, occurred in the right hemisphere (i.e., contralateral to the side of UCS presentation). In contrast, there were a number of decreases that occurred in the left hemisphere and others that occurred bilaterally. Specifically, there were decreases in the left prefrontal cortex (BA 47; −42, 32, −12) and bilateral decreases in the cerebellar cortex (42, −72, −28 and −16, −72, −28) and the temporal poles (e.g., BA 38; 24, 14, −28).

A number of other areas showed significant changes in rCBF between the paired and unpaired control phases and a significant linear trend across the three phases of the experiment. Areas with increases in rCBF included the brain stem (−20, −36, −28), left anterior cingulate (−20, −2, 32), and right posterior cingulate (12, −30, 28). Decreases in rCBF were noted in the left hippocampus (−28, −4, −16), medial cerebellum (−30, −68, −20), left medial temporoparietal lobe (−40, −38, −12), and superior temporal lobe (−58, −50, 20).

**SPM image analysis of extinction**

Results from the comparison of averaged rCBF during the paired and unpaired extinction scans are summarized in Table 2 and the corresponding difference images are depicted in Fig. 3, bottom. Inspection of the figure shows both increases and decreases in rCBF as a function of unpaired extinction. There was an increase in rCBF that also contained a quadratic trend in the right cerebellar cortex (16, −68, −28). Decreases in rCBF during unpaired extinction that were also featured in a quadratic trend analysis occurred in the left temporoparietal lobe (BA 18; −34, −84, −4) and the right superior and transverse temporal cortices (BA 22; 48, −12, 0, and BA 42; 50, −6, 4, respectively). Finally, areas that showed both a decreased rCBF during unpaired extinction and a linear trend included the right inferior temporal cortex (42, −66, 0), the right anterior cerebellar cortex (46, −32, −24), and the right inferior parietal lobe (40, −30, 32).

**PLS analysis**

Helmert orthogonal contrasts were used for the design matrix in the PLS analysis. This simply codes for the total degrees of freedom for the experimental effects (total number of scans minus 1), rather than performing pairwise contrasts as used in SPM. It is important to note that the outcome of the PLS analysis would be identical regardless of the exact contrasts used so long as all the degrees of freedom were represented (McIntosh et al. 1996).

The PLS results are presented in Fig. 4, A and B. The first two singular images (spatial patterns) were retained from the analysis. As in the SPM analysis, these images reflected a linear and quadratic trend across the experiment and are best illustrated in the graphs of scores at the bottom of Fig. 4, A and B. The graph in Fig. 4A shows that the first pattern represented a linear decrease across the experiment, whereas

**Table 1. Comparison of averaged rCBF during paired scans vs. unpaired control scans**

<table>
<thead>
<tr>
<th>Gyral Location or Area</th>
<th>BA</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>Z Value</th>
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<tbody>
<tr>
<td><strong>A. Regions showing significant increases in rCBF during classical conditioning</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Superior temporal (right)</td>
<td>22</td>
<td>30</td>
<td>−44</td>
<td>8</td>
<td>4.49</td>
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<tr>
<td>Lateral temporoparietal (left)</td>
<td>18</td>
<td>−32</td>
<td>−36</td>
<td>8</td>
<td>3.34</td>
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<tr>
<td>Transverse temporal (right)</td>
<td>42</td>
<td>56</td>
<td>−8</td>
<td>8</td>
<td>3.27</td>
</tr>
<tr>
<td><strong>B. Regions showing significant relative decreases in rCBF during classical conditioning</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superior temporal pole (right)</td>
<td>38</td>
<td>24</td>
<td>14</td>
<td>−28</td>
<td>4.80</td>
</tr>
<tr>
<td>Cerebellar cortex (right)</td>
<td>42</td>
<td>−72</td>
<td>−38</td>
<td>−12</td>
<td>3.96</td>
</tr>
<tr>
<td>Cerebellar cortex (left)</td>
<td>−16</td>
<td>−72</td>
<td>−28</td>
<td>−12</td>
<td>3.90</td>
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<td>Inferior temporal pole (left)</td>
<td>20</td>
<td>−42</td>
<td>−14</td>
<td>−28</td>
<td>3.46</td>
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<tr>
<td>Inferior prefrontal lobe (left)</td>
<td>47</td>
<td>−42</td>
<td>32</td>
<td>−12</td>
<td>3.36</td>
</tr>
</tbody>
</table>

*Gyral location or area and Brodmann area designation (BA) are listed. Stereotaxic coordinates (mm) (Talairach and Tournoux 1988) of foci showing both maximal between-condition differences and significant quadratic trend are shown. Z values were obtained by transformation of t-statistics (Friston et al. 1991b). rCBF, regional cerebral blood flow.*
FIG. 3. Z-transformed difference images for the comparison of the averaged paired minus unpaired control scans (top) and averaged unpaired extinction minus paired scans (bottom). The atlas plates (Talairach and Tournoux 1988) show the location (in mm) of the brain section relative to a line connecting the anterior and posterior commissures. Images are in the transverse plane: anterior is at top and left is at left. Only slices in which areas showed both statistically significant differences and quadratic trend are shown. Statistically significant increases in regional cerebral blood flow (rCBF) are graded from red to yellow with red at the threshold of $P \leq 0.001$. Statistically significant decreases in rCBF are depicted in blue, which corresponds to a threshold of $P < 0.001$. The stereotaxic coordinates for the maximum differences are listed in Tables 1 and 2.
The second pattern (Fig. 4B) follows a quadratic trend. It is important to note that the quadratic trend was not explicitly coded in the design matrix; rather, it was extracted by the analysis. The PLS suggests that the strongest experimental trend was linear across scans, but that there was also a nonlinear function (i.e., the quadratic trend) that was statistically independent of the linear change.

Areas identified in the linear pattern were dorsal premotor cortex (BA 6; −12, 22, 40) and orbitofrontal cortices (BA 11; 20, 36, −12, and −16, 40, −12), which followed a linear decrease, and the lateral cerebellum (−26, −68, −20) and anterior temporal cortices (BA 20; 40, −18, −24, and −34, 0, −24), which showed linear increases. For the second pattern, the left inferior (BA 47; −48, 36, −4) and middle prefrontal cortices (BA 9; −42, 18, 24) and middle cerebellum (2, −72, −28) all followed a U-shaped function, whereas the left parahippocampal gyrus near the uncus (BA 35; −22, −22, −20), bilateral temporoccipital cortices (BA 22/21; 38, −56, 8 and −42, −60, 8), and right auditory cortex (BA 41/42; 42, −12, 4) was negatively related to this U-shaped pattern. The second pattern therefore identified areas that were specifically related to the change in the associative relationship between the CS and UCS, independent of effects accounted for by the first pattern. Moreover, the pattern corresponds very closely to the quadratic trend observed in the SPM analysis as well as identifying the middle prefrontal cortices and the hippocampus. Variation in the location of the maximal regions of change is related to the different sources of error variance in the univariate SPM versus multivariate PLS (see Arndt et al. 1995 for further discussion of this issue).

Permutation tests were performed to determine whether the trends observed in the graphs in Fig. 4 were of substantive importance. Tests were performed on the regression of linear and quadratic trends on the PLS scores. Not unexpectedly, the $R^2$ value for the regression of the linear trend on the first PLS was highly significant ($R^2 = 0.65, P < 0.001$), but this was not the case for the quadratic trend ($R^2 = 0.05, P > 0.05$). The opposite was the case for the second pattern (linear $R^2 = 0.02, P > 0.10$; quadratic $R^2 = 0.39, P < 0.001$).

**Behavioral correlations**

The PLS analysis of correlations of the brain-behavior relations was performed for the two paired scans and the two unpaired extinction scans. Analyses of percent CRs and CR latency were each performed separately. In both cases, there were strong similarities in the singular images identified by looking at either percent CRs or latency measures, with some notable exceptions.

For percent CRs, the first singular image identified a pattern of brain regions commonly correlated to percent CRs across both paired scans and the first unpaired extinction scan (correlation of scores and percent CRs = 0.7 and 0.56 for paired scans, and 0.86 for the 1st extinction scan). The percent CRs measured in the second extinction scan showed no relation to these areas (correlation = 0.04). Permutation tests on the subject scores for this pattern and percent CRs were significant ($R^2 = 0.537, P < 0.001$). Permutation tests indicated that the subsequent singular images did not appear to be of substantive importance and were not considered.

The dominant areas on the singular image that showed positive saliences (increased rCBF with increased CRs) included middle cerebellum (6, −50, −28), left dorsal premotor cortex (BA 6/9; −10, 30, 24), right middle cingulate (BA 24; 12, −20, 36), and right superior temporal cortex (BA 22; 40, −46, 8). Areas showing the opposite relation to CRs (negative saliences or decreased rCBF with increased CRs) were left inferior prefrontal cortex (BA 45/47; −38, 24, 0), left middle prefrontal cortex (BA 9; −30, 14, 32), and right inferior parietal cortex (BA 40; 30, −26, 24).

Analysis of latency measures also resulted in only one significant singular image ($R^2 = 0.6550, P < 0.001$), with all paired and unpaired extinction scans showing equal loadings on the singular image (correlations 0.684 and 0.78 for paired scans, and 0.729 and 0.722 for extinction scans). Interestingly, the areas defined in the singular image were the same as those identified in the CR analysis, with the exception of right superior temporal and left inferior and middle prefrontal cortices.

In summary, analyses of both behavioral measures identified regions that were also present in the analysis of design effects. The middle cerebellum was identified across all analyses, whereas the left inferior (BA 45/47) and middle (BA 9) prefrontal and right superior temporal (BA 22) cortices were identified by the design and percent CR analysis only. The possible significance of these differences will be discussed below.

**Discussion**

The present experiment replicates and extends our previous findings in which classical conditioning of the eyelink

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**Table 2. Comparison of averaged rCBF during unpaired extinction scans vs. paired scans**

<table>
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<tr>
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<td>16</td>
<td>−68</td>
<td>−28</td>
<td></td>
<td>3.44</td>
</tr>
<tr>
<td>Lateral temporoccipital (left)</td>
<td>18</td>
<td>−34</td>
<td>−84</td>
<td>−4</td>
<td>4.01</td>
</tr>
<tr>
<td>Superior temporal (right)</td>
<td>22</td>
<td>48</td>
<td>−12</td>
<td>0</td>
<td>3.81</td>
</tr>
<tr>
<td>Transverse temporal (right)</td>
<td>42</td>
<td>50</td>
<td>−6</td>
<td>4</td>
<td>3.72</td>
</tr>
</tbody>
</table>

Details and abbreviations as in Table 1.
response produced increases in rCBF in the primary auditory cortex opposite the side of air puff delivery and decreases in rCBF in the cerebellar cortex on the same side as the air puff (Molchan et al. 1994). A number of others areas associated with aspects of learning and memory were also found to change as a function of classical conditioning of the eyelink response. In particular, there were increases in rCBF in auditory association cortex and temporoccipidal cortex, and decreases in the temporal poles and inferior prefrontal lobe.

The more sensitive multivariate PLS analysis complemented the SPM analysis and expanded it by identifying the middle prefrontal cortices and the hippocampus as areas involved in the associative process. Moreover, the analysis of behavioral correlations with the use of the PLS analysis provided information about how rCBF in identified areas was related to the acquisition of CRs. By focusing on two behavioral parameters, percent CRs and CR latency, we were able to identify key regions that were common to the design and behavior analyses, namely the middle cerebellum. A more detailed examination of the brain-behavior relations also demonstrated that the left inferior and middle prefrontal and superior temporal cortices were specifically related to percent CRs, but not CR latency. These latter areas were also identified in the task PLS. The convergence of area identification across the analyses suggests that these regions were central to the learning and maintenance of the associative relation. Regions identified only in the behavior PLS could be argued to represent only performance, but the overlap with areas identified in the design analysis suggests that the cerebellum, left prefrontal, and superior temporal cortices played special roles in identifying the temporal relation between the CS and US (revealed in the task analysis) and then organizing the motor system(s) for the appropriate learned response (revealed in the behavior analysis). Consequently, this analysis elaborates on the quadratic trend in rCBF across tasks noted in the cerebellum and left prefrontal and superior temporal cortices that reflected the changes in those structures as the relationship between tone and air puff changed. In other words, there is a consensus across analyses that the cerebellum and left prefrontal and superior temporal cortices appear to be involved in learning the CR and not just performing it.

Different parts of the prefrontal lobe were identified by PLS as showing either changes in activation or correlations with behavior—dorsal prefrontal area 45/47. These areas are anatomically distinct and do have different connection patterns (Pandya and Yeterain 1990). These areas may be involved in different parts of the associative process (e.g., execution of CR, identification of CS-UCS relationship). Among many other things, the dorsal prefrontal cortex has been identified as playing some role in associative behaviors. Indeed, it has been demonstrated that patients with frontal lobe damage have difficulty in associative tasks (conditional associative learning, CAL task, Petreides 1985). Although eyelink conditioning may be somewhat simpler than the CAL task, our data do support an important role for dorsal prefrontal cortex that spans all stages of associative learning. The inferior prefrontal cortex, on the other hand, was identified in our previous experiment (Molchan et al. 1994) and has been anatomically linked to the temporal lobes (Pandya and Yeterain 1990), leading to the supposition that this region may play a key role in the sensory association between CS and UCS. The dorsal prefrontal cortex, by virtue of its strong ties to premotor and motor cortices, may act to coordinate the sensory information with the motor response. In our previous study, the prefrontal activity was right lateralized, ipsilateral to the side of the UCS. Here too the prefrontal involvement was lateralized to the side of UCS presentation.

An important feature to note in the PLS analysis was that the linear “time” effects were depicted on a singular image that was orthogonal to the pattern depicting the change in the associative relation of the tone and air puff. Therefore areas in this singular image are those that most closely followed the change in the associative relation independently of any linear temporal effects. In other words, the analysis was able to separate the associative changes (learning) from nonassociative changes (habitation, sensitization) that took place over the course of the experiment. Left prefrontal cortex, middle cerebellum, left parahippocampal gyrus, and bilateral temporopolar and right auditory cortices were areas that seemed specifically related to the associative changes across the experiment.

There are two recent imaging studies of human eyelid conditioning (Blaxton et al. 1996; Logan and Grafton 1995), which provide significant confirmation of some of our own data as well as identifying some differences. Logan and Grafton (1995) examined glucose metabolism and found increases in a number of regions including the cerebellum, hippocampus, striatum, temporal gyrus, and occipitotemporal fissure. Blaxton et al. (1996) examined rCBF and found increases in striatum and hippocampus, and decreases in cerebellum. Clearly, a number of areas, most notably the cerebellum and hippocampus, were identified to be specifically involved during eyelink conditioning in all of the imaging studies. Areas such as the striatum, temporal gyrus, and temporopolar cortex were found to be important in at least two of the studies. Differences in imaging methods (e.g., glucose metabolism vs. rCBF, different fields of view; the Logan and Grafton study positioned the PET camera more ventrally), behavioral paradigms (massed vs. continuous training), and conditioning levels may account for some of the between-study differences in brain areas and differences in the direction of changes observed in the imaging of human eyelink conditioning. These factors may also combine with sampling different time windows during the acquisition of the CR. It is possible that certain areas are recruited at different times during acquisition and may not be identified depending on what portion of the acquisition
curve is sampled. It may be possible to remedy these discrepancies either by obtaining more scans during different stages of acquisition, or to use a method that affords better temporal resolution to follow the acquisition curve continuously (e.g., evoked response potentials, functional magnetic resonance imaging).

It is interesting to note that there are some differences between the present study and our previous one. First, the extent of the activations and deactivations does not appear as large in the present study as those of Molchan et al. (1994). Second, there were several areas identified in the Molchan et al. (1994) study (e.g., striatum and parietal and insular cortices) that did not appear in the present experiment and several areas identified in the present experiment (e.g., hippocampus, temporopolar cortex, temporal poles) that were not found in the original study. Some of these differences may be explained by the fact that the overall levels of conditioning were somewhat lower in the present study (62.5% CRs) than in our earlier experiment (73.7% CRs). However, all of the areas identified across our two studies have been found to be involved in associative and/or attentional processes (e.g., inferior temporal cortex, Miyashita 1993; occipitotemporal cortex, Walsh and Perrett 1994), so the differences between studies may reflect the phasic involvement of different areas during different stages of response acquisition. For example, all of the areas identified in our present and previous study were also identified in recent imaging studies of human eyeblink conditioning (see above), which examined the brain with the use of a different radiotracer (Logan and Grafton 1995) and at different points during the acquisition of the conditioned eyeblink response (e.g., Blaxton et al. 1996; Logan and Grafton 1995).

Replication is a necessary part of scientific investigation and it has met with variable success in PET. Investigations of seemingly similar paradigms do not often yield the same results. For example, activation studies of the Stroop attention task have not consistently identified the same areas, and it appears to be a function of trial presentation rate (George et al. 1994; Pardo et al. 1990). Working memory tasks have been used extensively in PET with some discrepant results. However, a recent study by Haxby et al. (1995) found that different areas become active if working memory load is parametrically manipulated, suggesting that some of the specific regions engaged in working memory are not fixed. All of these studies suggest that regional participation in different functions may vary much more depend on the specific requirements of the task. The commonalities and discrepancies in regional involvement across different experiments concerning similar phenomena should be taken as an advantage of functional neuroimaging studies. The ability to explicitly quantify the different strategies used by the brain to perform seemingly similar functions is not attainable through classic approaches of neuroscientific investigation, such as ablation methods. These methods can characterize the dysfunctions arising from damage, but are not able to identify the distributed system(s) that underlie normal performance.

Thus the significance of the present findings turns, in part, on the replication of our previous finding of a conditioning-specific increase in rCBF in the auditory cortex (Molchan et al. 1994). This lends support to a growing body of literature suggesting that primary sensory cortices have an important role in associative learning beyond simple stimulus processing (Bakin and Weinberger 1990; Gonzalez-Lima and Scheich 1986; Harvey et al. 1986; Molchan et al. 1994). This point is reinforced by our observation that presentation of a binaural tone again resulted in predominantly unilateral change in auditory cortex. Moreover, the fact that changes in rCBF in the auditory cortex occurred on the side opposite the air puff rather than simply in the left auditory cortex (Molchan et al. 1994) strengthens the idea that sites of learning-specific modification are not fixed at a specific anatomic location but are functionally related to the nature of the stimuli involved and the task required. The decrease in rCBF bilaterally in the cerebellar cortex also replicates our earlier finding and adds further evidence to the involvement of the cerebellar cortex in eyelid conditioning. Indeed, the data lend support to a growing body of imaging literature suggesting that the cerebellum is involved in learning and memory (e.g., Andreasen et al. 1995; Blaxton et al. 1996; Logan and Grafton 1995). Finally, the present findings lend further credence to the notion that normal brain functioning during associative learning involves cooperative interactions among a number of brain areas (McIntosh and Gonzalez-Lima 1994a; Molchan et al. 1994).

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