

Electromyography as a Recording System for Eyeblink Conditioning with Functional Magnetic Resonance Imaging

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Received March 29, 2001

This study was designed to develop a suitable method of recording eyeblink responses while conducting functional magnetic resonance imaging (fMRI). Given the complexity of this behavioral setup outside of the magnet, this study sought to adapt and further optimize an approach to eyeblink conditioning that would be suitable for conducting event-related fMRI experiments. This method involved the acquisition of electromyographic (EMG) signals from the orbicularis oculi of the right eye, which were subsequently amplified and converted into an optical signal outside of the head coil. This optical signal was converted back into an electrical signal once outside the magnet room. Electromyography (EMG)-detected eyeblinks were used to measure responses in a delay eyeblink conditioning paradigm. Our results indicate that: (1) electromyography is a sensitive method for the detection of eyeblinks during fMRI; (2) minimal interactions or artifacts of the EMG signal were created from the magnetic resonance pulse sequence; and (3) no electromyography-related artifacts were detected in the magnetic resonance images. Furthermore, an analysis of the functional data showed areas of activation that have previously been shown in positron emission tomography studies of human eyeblink conditioning. Our results support the strength of this behavioral setup as a suitable method to be used in association with fMRI. © 2002 Elsevier Science (USA)

INTRODUCTION

The eyeblink conditioning paradigm is a particularly well-understood model system to study the mechanisms of associative learning and memory in animals (Disterhoft *et al.*, 1977; Thompson *et al.*, 1976) and in

humans (Carrillo *et al.*, 1997; Daum *et al.*, 1993; Daum and Schugens, 1996; Gabrieli *et al.*, 1995; McGlinchey-Berroth *et al.*, 1997; Woodruff-Pak, 1988, 1993). Eyeblink conditioning requires that the subject associate a neutral stimulus, e.g., auditory conditioned stimulus (CS), with a behaviorally significant stimulus, e.g., a corneal airpuff unconditioned stimulus (US). The best understood eyeblink conditioning paradigm is the delay paradigm, which involves associating a tone CS that precedes, overlaps, and coterminates with a corneal airpuff US. Eyeblink conditioning in humans requires that eyeblink responses be monitored as the subject responds to the stimuli. The majority of the studies involving human eyeblink conditioning have used reflectance of infrared light as the method of choice to measure eyelid responses (Carrillo *et al.*, 1997; Clark and Squire, 1998). However, since the electronic components involved in the infrared measurement interfere with imaging in the MRI environment (Thompson *et al.*, 1994), we have developed a method to detect the electromyographic (EMG) activity of the eyelids (orbicularis oculi). In a study reported elsewhere, it has been shown outside of the magnet that the two techniques are equivalent in their ability to detect eyeblinks (Knuttinen *et al.*, 2001). The purpose of this study was to optimize the EMG approach as a suitable method for detecting eyeblink responses during fMRI, and to further develop a behavioral setup that can be used to study eyeblink conditioning in the magnet.

Functional MRI provides detailed images that reflect changes in cerebral blood flow, cerebral blood volume, and oxygenation induced by sensory, motor, or cognitive tasks (Cohen and Bookheimer, 1994; Kwong, 1995; LeBihan and Karni, 1995), including eyeblink conditioning (Knuttinen *et al.*, 2000; Preston *et al.*, 2000; Ramnani *et al.*, 2000). This imaging approach has served as a valuable tool in detecting patterns of hemodynamic changes in frontal cortex, cerebellum, and hippocampus and associated temporal lobe structures while subjects are performing a variety of learning

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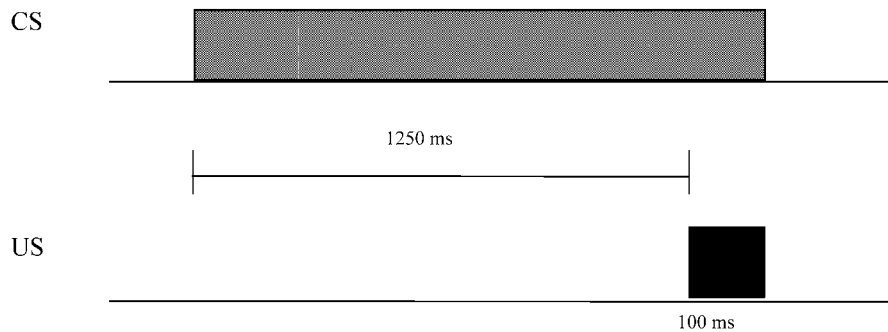


FIG. 1. The electromyogram for eyeblink detection methodology was tested in the delay 1250-ms conditioning paradigm. This paradigm consisted of a tone (CS), indicated by the hatched gray region, that overlapped and coterminated with the corneal airpuff unconditioned stimulus (US), indicated in black.

tasks (Demb *et al.*, 1995; Desmond *et al.*, 1997; Gabrieli *et al.*, 1996, 1997; Poldrack *et al.*, 1998; Wagner *et al.*, 1997). Eyeblink conditioning is well-suited for neurobiological analyses with fMRI due to the use of appropriate unpaired control (pseudoconditioning) procedures that can distinguish between areas of the brain that are responding to sensory stimuli (e.g., auditory and somatosensory cortices) and those that are selectively activated under paired learning conditions by comparing pseudoconditioning and conditioning trials in the same subject. Subjects can also return for retesting over a period to examine the processes underlying memory consolidation. The dynamic characterizations of fMRI allow for visualization of the hemodynamic activity (during and after associative learning) in the circuitry mediating learning with a good degree of spatial and temporal localization.

METHODS

Subjects

Four healthy young volunteers (two males, two females, average age = 27) recruited from the Northwestern University community served as subjects. Informed consent was obtained for participation in the study in keeping with guidelines approved by the institutional review board. Subjects received neutral instructions that would not reveal the nature of the association between the tone and airpuff during the experiment. In addition, a movie, *Milo and Otis*, was played to minimize boredom and prevent the dissipation of arousal during the experimental session. All subjects received payment for their participation.

Behavioral Design and Delivery

The apparatus used for delivery of the stimulus was a modified version of that used for eyeblink conditioning in the rabbit (Akase *et al.*, 1994; Thompson *et al.*, 1994). The conditioning paradigm used to test the methodology of EMG detection was the delay 1250-ms

eyeblink conditioning paradigm outlined in Fig. 1. This paradigm has been shown to elicit robust learning in both young and aging subjects (Knuttninen *et al.*, 2001). The CS was a 1350-ms, 85-dB, 1000-Hz, 5-ms rise/fall tone delivered binaurally over nonmagnetic acoustically shielded earphones (Avotec, Jensen Beach, FL). Auditory stimulus calibration occurred prior to testing each subject using a constant tone and a Radio Shack Realistic sound level meter (No. 33-2050) with "C" weighting and slow response settings. The US was a 4-psi 100-ms corneal puff of nitrogen gas delivered to the right eye. A nitrogen tank was placed within the control room and connected to a three-way solenoid valve (Fluid Process Controls, Burr Ridge, IL, 24 V dc, No. 3823A2TVTF6). The airpuff stimulus was calibrated with a digital pressure gauge (R&D Separations, Rancho Cordova, CA). The CS and US coterminated. The trigger signal was received from the computer running Lab View software. Stiff plastic tubing attached to the tank measuring 25 ft in length was passed through a waveguide into the magnet room. The delay between the command pulse for the solenoid to open and the occurrence of the puff at the eyelid was measured using reflected light from a piece of tissue paper at the end of the delivery system. This delay was then entered into the computer and the US onset time was adjusted to maintain the proper timing of the tone and airpuff. The end of the plastic tubing was fitted with a corrugated drinking straw and pipet tip that was attached to the head coil for precise alignment and delivery of the airpuff to the center of the right eye. The air pressure at the pipet tip was calibrated before each subject was trained to ensure a 4-psi airpuff was being delivered.

Subjects received an initial block of 30 explicitly unpaired random presentations of tone-alone and airpuff-alone trials to test for pseudoconditioning. These were presented prior to 60 paired presentations of the tone and airpuff. The intertrial interval for all trials ranged from 16 to 20 s. A long intertrial interval was used to allow the hemodynamic response to return to baseline before the next trial. The initial pseudocondi-

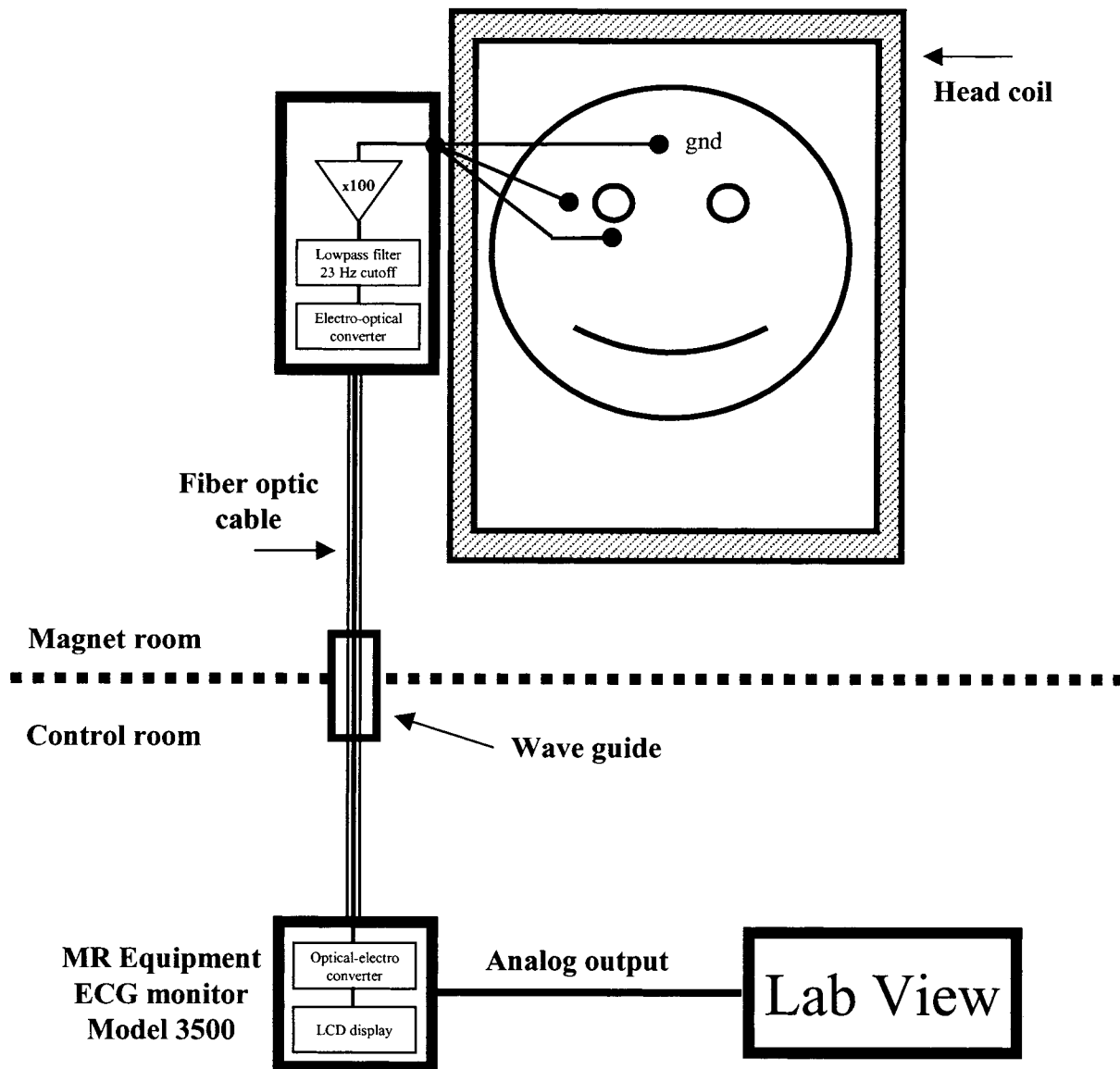


FIG. 2. Block diagram showing the location of the electrodes and the electric/optical converters. The specifics of the signal conditioning are given. Note that the biopotential signal is primarily an optical signal while inside the magnet room. This reduces the potential for distortion of the measured electromyographic signal.

tioning trials served as a control baseline condition to determine unconditioned response (UR) amplitudes, basal rates for eyeblinks to CS-alone presentations, and the hemodynamic response to sensory stimuli (tone and airpuff) alone. A smaller number of unpaired trials were used due to the robust sensory response evoked by the stimuli and to avoid latent inhibition effects, which can delay acquisition of learning (Siddle *et al.*, 1987). A parametric behavioral study has demonstrated that relatively fast and robust learning curves occur after the conditioning trials begin using this procedure (Carrillo *et al.*, 1997). It was necessary for the pseudoconditioning trials to precede conditioning since baseline measurements taken after the con-

ditioning would not have been at baseline level. They would have been affected by conditioning and would have measured extinction processes.

Eyeblink Detection

The detection of the eyeblinks was accomplished by using a device designed to monitor the electrocardiogram (ECG) signal in the MRI environment (Physiologic Monitor No. 3500, MR Equipment, Bayshore, NY). This device uses fiberoptic technology to reduce the noise induced during the scanning procedure. A block diagram of the setup is shown in Fig. 2.

Eyeblinks were recorded via radiolucent ECG electrodes (Red Dot No. 2570, 3M, St. Paul, MN) placed around the orbicularis oculi muscle of the right eye. Prior to electrode placement, the area was cleaned by the experimenter with NuPrep gel (Grass Instrument Division of Astro-Med Inc., West Warwick, RI) to decrease skin resistance. One electrode was placed 1 cm lateral to the outer canthus and a second was placed 1 cm below the right eye. The ground electrode was aligned at the center of the subject's forehead. There was no overt motion of the electrodes during the eyeblink. The ECG monitoring device used a method similar to that described by Felbinger *et al.* (1996) to detect and conduct the signal out of the magnet room. In short, the electrical potentials were detected via the electrodes and fed to the electro-optical converter, which was located just outside of the head coil. The converter was located as close as possible to the subject's head to minimize the length of the carbon-fiber wires (2 at 12.5 in. and one at 9.25 in.) leading to the electrodes to reduce the amount of interference induced by the MRI scanner and far enough away to eliminate any susceptibility artifacts. The converter used a low-pass filter (23-Hz cutoff) and an amplifier with a gain of 100 to improve the signal strength before conversion to an optical signal (see Fig. 2). The two biopotentials, referenced to the ground electrode, were multiplexed onto the same fiberoptic cable. The fiberoptic cable was passed through a waveguide in the RF shielding of the magnet room. Once outside of the magnet room, the optical signal was converted back to an electrical signal without any conditioning. The analog electrical signal (pin 11 referenced to pin 1 on the physiologic monitor) was converted to a digital signal using custom software written in Lab View (National Instruments, Austin, TX).

Lab View was used to coordinate the stimulus presentation and processing of the behavioral response, which was sampled at 5 kHz. The postprocessing in Lab View consisted of full wave rectification and integration with a 10-ms time constant to generate a pseudoanalog signal (see Fig. 3). The mean and standard deviation (SD) of the 500-ms baseline waveform were calculated. A conditioned response was one that exceeded the mean baseline value by 4 SD for a minimum of 10 ms prior to US onset.

Noise Assessment of the ECG Device

It is imperative to minimize the amount of noise induced in the fMRI signal time series data. Therefore, before implementing the study described, a noise assessment of the ECG device was completed. This consisted of a functional run with and without the device present. The experimental runs used the same imaging parameters as the fMRI acquisition except that the number of volumes was limited to 128 after the signal

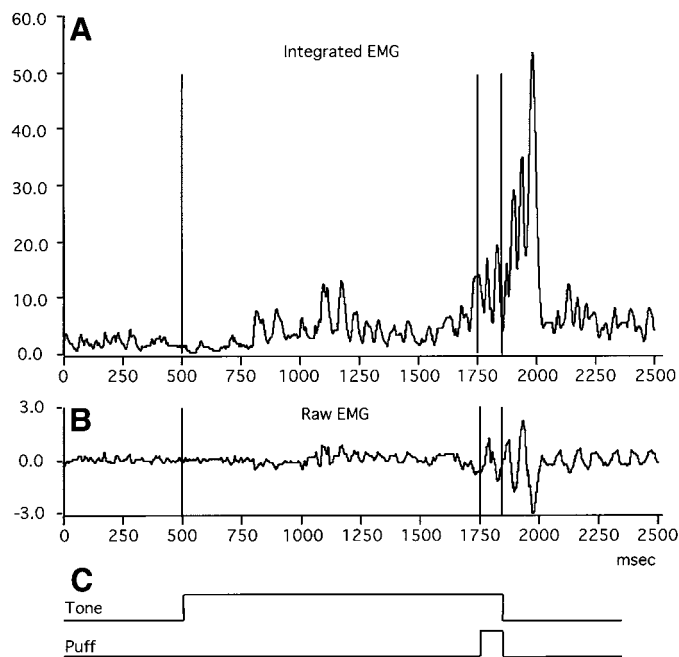


FIG. 3. Example of a conditioned response recorded inside the magnet. (A) Rectified and integrated ($\tau = 10$ ms) electromyographic activity for a single trial. The amplitude is shown in relative arbitrary integrated units. (B) The "raw" electromyographic signal used to generate the signal in (A). The amplitude is shown as increases and decreases in relative voltage. (C) Relative timing of the tone conditioning stimulus and airpuff unconditioned stimulus.

had reached steady state. A phantom was used to assess the overall noise and a human volunteer was used to determine if the noise was detectable above that induced by physiological processes in the brain. The electrodes were placed on the phantom in a fashion that simulated the method described above. In the volunteer study, the electrodes were applied as described. During the experiment no behavioral stimuli were given to detect the noise associated with the device. The subject was asked to lie quietly during the procedure.

The time course data were analyzed to determine if the variance changed with the presence of the ECG device. It is possible to use the χ^2 statistic to test the hypotheses about the variance. However, if one assumes that the data are normal and a sufficiently large sample is used ($n > 40$), it is possible to use a Z statistic to test the differences in variance (Devore, 1982). This statistic can be written as

$$Z = \frac{S - \sigma_0}{\sigma_0 / \sqrt{2n}},$$

where S and σ_0 are the standard deviations of the two different measures, and n is the number of samples. To control for Type I errors, an $\alpha = 0.01$ was used that

corresponds to a Z value of 2.33. The number of samples in the time course (n) was 128 and the variance was derived from the region of interest (ROI) in each data set. The variance for the data set without the device present was the reference value, σ_0 . A $|Z| > 2.33$ would indicate that the device had a significant effect on the time course data. The ROI was located at the level and in the same hemisphere (right) as the electro-optical converter, since the maximum noise is assumed to occur nearest to the converter. In the volunteer, the ROI included both gray and white matter, while excluding the ventricles. Similar analysis was completed on the phantom data. A power spectral analysis was conducted on the same data similar to that described by Zarahn *et al.* (1997) to observe changes in the noise spectrum due to the presence of the ECG device. The results of the power spectral analysis were reviewed qualitatively.

Scanning Procedures

Imaging was conducted at Northwestern University Medical School on a Siemens Vision 1.5-T scanner equipped with whole-body EPI gradients and a transmit/receive quadrature head coil. Head movement was minimized with the use of a vacuum pillow (Vac-Fix, Toledo, OH) and restraint calipers built into the head coil. These devices help to keep movement minimized to < 1 mm total excursion during the functional run (20 min) and the intervolume deviation to submillimeter deviations (Parrish *et al.*, 1998). fMRI data were collected using an EPI sequence with parameters of TR = 2000 ms, TE = 40 ms, FOV = 240 mm, matrix of 64×64 , and 24 axial slices of 6-mm thickness. Images of whole brain were acquired with slices oriented parallel to the anterior commissure–posterior commissure (AC/PC) plane. Imaging consisted of two functional runs: a pseudoconditioning run, followed by a conditioning run. The pseudoconditioning run included a total of 60 unpaired trials. The subsequent conditioning run included a total of 60 paired trials, which lasted approximately 20 min. High-resolution 3D anatomical MRI data were collected at the end of the functional series.

Behavioral Data Analysis

The criteria for an eyeblink response to be classified as a conditioned response (CR) required the integrated signal to: (1) be greater than or equal to 4 SD above the baseline for a minimum duration of 10 ms; (2) occur more than 100 ms after tone onset (to correct for voluntary responses); and (3) remain above baseline throughout the CS–US interval before blending into the unconditioned response (UR). Eyeblinks that returned to baseline before US onset were defined as alpha responses. In general, these alpha responses tended to be of short latency and duration. The same

response criteria were applied to the pseudoconditioning tone-alone trials to that portion of the trial prior to when the US would have occurred. Following each training session, the computer generated a graphical depiction and a tabulated summary of the eyeblink responses for each trial. The overall percentage of trials with CRs was determined for each subject. The data were also used to determine differences in the amount of conditioning (percentage CRs) between the pseudoconditioning and conditioning trials.

Functional Data Analysis

Image data were transferred to an HP workstation for analysis using SPM97 software (Wellcome Department of Cognitive Neurology, London, UK). Time series data were acquisition timing corrected and motion corrected. For the standard analysis, a time series of each voxel was correlated with a reference waveform and transformed into a Z score map (Friston *et al.*, 1994, 1995). A model of the expected magnetic resonance signal response was created by convolving the stimulus with a canonical hemodynamic response function (Friston *et al.*, 1995). In this experiment, the modeled stimulus period included both the tone and airpuff, which lasted less than 2 s. The general linear model was used to determine the statistical significance on a voxel-by-voxel basis (Friston *et al.*, 1994). In addition, the response from the pseudoconditioning phase was subtracted from that of the conditioning phase to show only differences in areas underlying associative learning. Activation maps of the difference data were made using an uncorrected threshold of $P < 0.001$ and an extent threshold of 3 voxels.

RESULTS

For all subjects, the EMG signal was successfully recorded during fMRI scanning using the 3M electrodes, NuPrep gel, and physiologic monitor (see Fig. 3). The EPI sequence added minimal artifacts to the EMG signal and the filtering reduced them even further as demonstrated in Fig. 3. Figure 4 shows the artifact-free signals obtained from a learner and a non-learner, while their eyeblink responses were being monitored in the magnet. Furthermore, the EMG signal clearly detected differences between trials in which the subject showed a conditioned response (eyeblink) to the tone and those trials in which the subject did not show such responses. In all four of our subjects, the EMG signals were suitable for detecting eyeblink measurements, which correlated with the behavioral paradigm being used, i.e., EMG detection of eyeblinks (URs) was present on all trials and in all subjects when an airpuff was given. The EMG electrodes, carbon fiber leads, and electro-optical converter located outside of the head coil (see Fig. 2) did not produce any visually

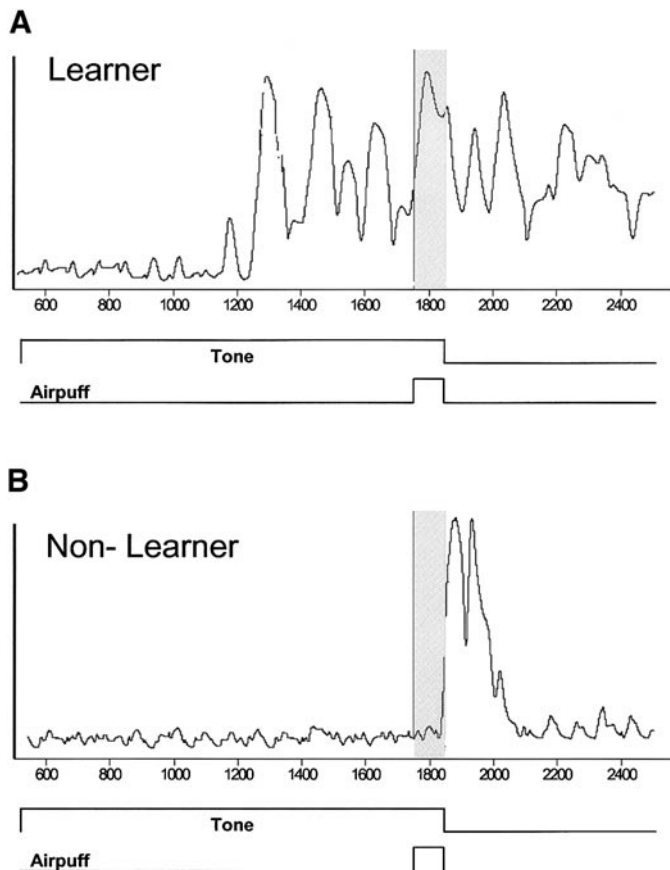


FIG. 4. Example of filtered, rectified, and integrated electromyographic tracings in the delay 1250-ms task. The electromyographic response was taken from a single trial during the conditioning run, while magnetic resonance images were being acquired. Tone onset occurred at 500 ms, and subsequently overlapped and coterminated with the US. The gray-shaded area represents the area of the US stimulus presentation (1750–1850 ms). Each trial was monitored for a duration of 2500 ms. Note the relatively low level of baseline activity in both the top and bottom panels, indicating minimal disturbance of the electromyographic signal by the magnetic resonance pulse sequences. (A) Typical conditioned response observed in two subjects who were able to acquire the delay 1250-ms task. (B) Typical response elicited by a subject who was unable to acquire the delay 1250-ms task even after receiving 60 conditioning trials. Note the lack of response to the tone CS. The subject did, however, show a robust UR.

detectable susceptibility artifacts in the images acquired. However, it was our experience that there was noticeable signal loss in the brain areas adjacent to the converter, if the converter was placed within the head coil.

The time series of signal intensity from the ROI with the mean removed and the power spectrum generated from the phantom data are shown in Fig. 5. Using the statistical analysis described in the Methods section, a Z score was calculated to determine if the variance of the signal intensity with and without the ECG device present was the same. The time course of the phantom data with the mean removed is shown in Fig. 5A. The

Z scores were 1.2 and 0.24 for the phantom data and the human data, respectively, indicating there was no statistical difference. Therefore, the ECG device did not add any detectable levels of noise to the images. A lower Z score indicated that the time series were from the same population. The Z score for the human data was much lower than that for the phantom data, which indicated that the time series with and without the ECG device were nearly identical. It is hypothesized that this is due to the presence of physiologic noise that tends to dominate the fMRI time course. Furthermore, the power spectrum from the phantom data demonstrates the relative power of the noise at the frequencies properly sampled by the TR of 2 s (Fig. 5B). These results indicate that the presence of the ECG device does not alter the noise spectrum. The same was also true for the human data.

All subjects reported that they were able to hear the tone through the nonmagnetic headphones and were able to feel the airpuff, which remained aimed at the right eye when observed at the end of the imaging session. The motion correction results demonstrated that subjects moved less than 1 mm during an entire imaging run (20 min). In addition, it did not appear that there was any stimulus-correlated head motion.

Two subjects were able to condition, i.e., learn to associate the CS with the subsequent US, at levels of 60 and 80% CRs by the end of the conditioning run. The conditioning run was later divided into three blocks of 20 trials to look at acquisition of learning, i.e., an increase in CRs across a given session. The percentage of CRs was greater during the second and third blocks of conditioning trials (50 and 70%) in these learners than after the first block of conditioning (20%), indicating that substantial associative learning did occur as trials progressed (Fig. 6). Two subjects were not able to condition: one had progressively smaller URs and reported having fallen asleep during the experimental session (<10% CRs), and the other was unable to reach a level beyond 20% CRs (either inside or outside of the magnet). Therefore, the lower conditioning level cannot be attributable to factors associated with the scanning environment. All subjects reported being comfortable throughout the training session.

In addition to showing that the functional images acquired were not contaminated by artifact from the EMG methodology, an analysis was done in all subjects to investigate the brain regions that became activated during conditioning. Activation maps for conditioning were generated by using cognitive subtraction of the pseudoconditioning (both CS and US) baseline from the conditioning trials to remove baseline and nonassociative sensory activation.

Figure 7 depicts the brain areas in the learners that were significantly more active during the paired tone–airpuff trials than during the pseudoconditioning unpaired trials. As shown, areas of significant activation

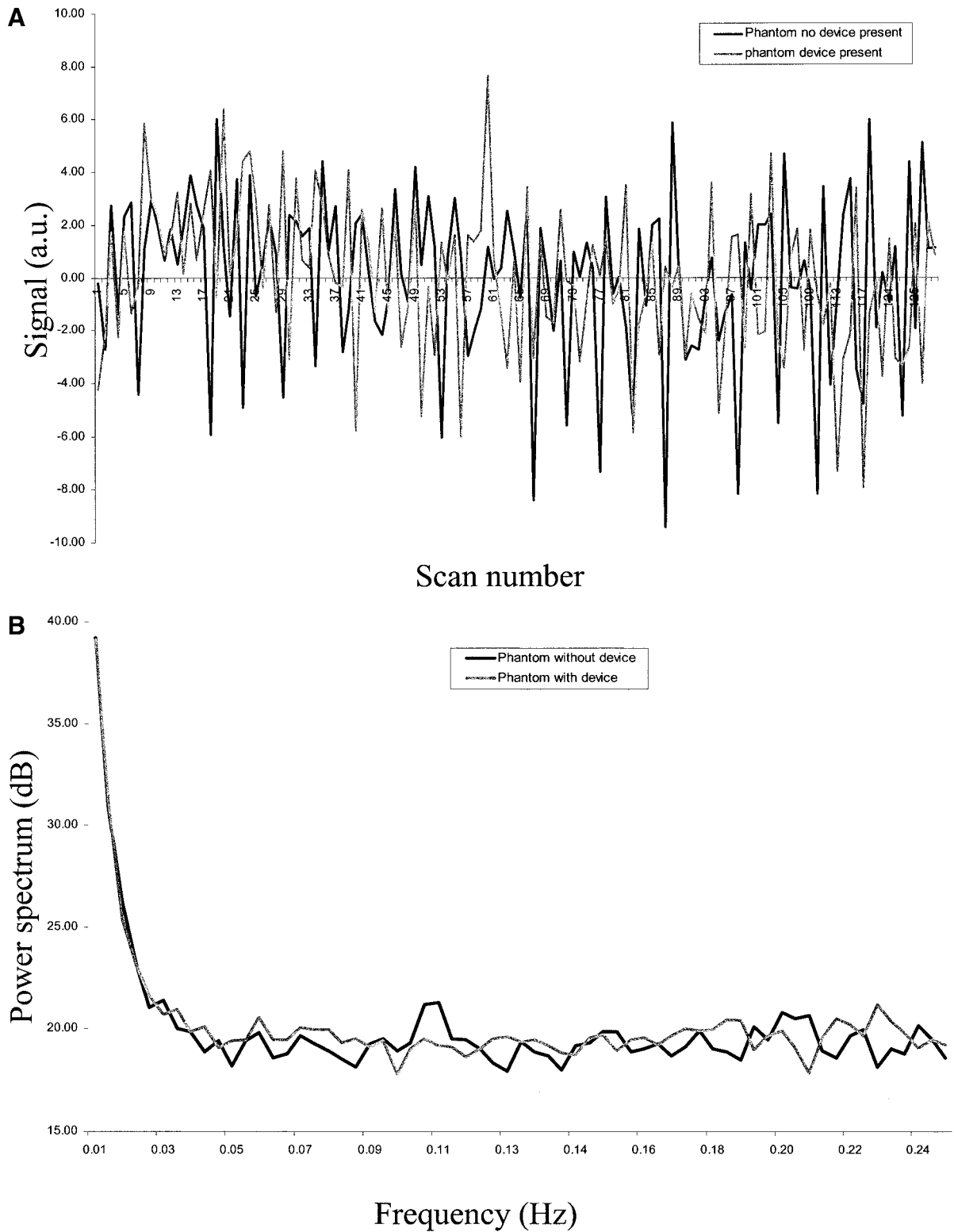


FIG. 5. Noise analysis of the time series data from a phantom with and without the ECG device. (A) Time course of the magnetic resonance signal intensity with and without the device present is shown. Using the analysis described in the text, the Z score was 1.2, which is less than the critical Z of 2.33 needed to determine if the device caused a significant amount of noise. (B) Power spectrum of the noise. Only frequencies (0–0.25 Hz) that are properly sampled by the $TR = 2$ s are shown. Note that the two curves are nearly identical, indicating that there is no change in the noise spectrum or any increase in the noise due to the presence of the ECG device.

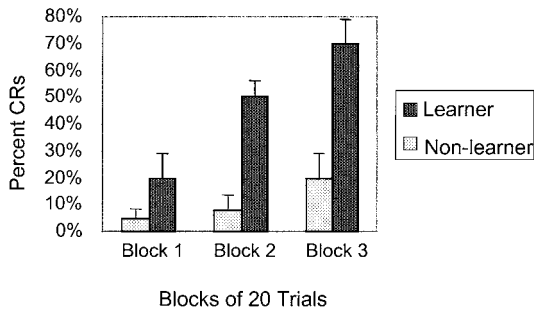


FIG. 6. Performance of learners versus nonlearners in the delay 1250-ms eyeblink paradigm. The conditioning run was subdivided into three blocks of 20 trials (for a total of 60 conditioning trials) to observe the rates of learning in the single run. Learners were able to reach a level of 70% CRs by the end of conditioning. The nonlearners, however, were unable to reach a level past 20% CRs.

included bilateral cerebellum, auditory cortex, basal ganglia, posterior cingulate, and bilateral frontal cortex. The two nonlearners showed activations in auditory and visual cortex during the pseudoconditioning control condition, but neither of these two subjects showed any areas of activation in the conditioning minus pseudoconditioning.

DISCUSSION

Electromyography as a recording system allows for excellent detection of eyeblink responses during echo planar functional imaging that is equivalent to eyeblink detection in conditioning experiments outside of the magnet. This experimental setup confirms the findings of Felbinger *et al.* (1996) that showed that there are no additional artifacts on the EMG signal imposed by echo planar sequences. These artifacts are minimized by the short carbon-fiber wires, eliminating any loops, and the placement of the electro-optical converter just outside the head coil. Figure 5 demonstrates that there is no added noise generated by the physiologic monitor. Further analysis of the noise data demonstrated that physiologic noise from the subject dominated the noise present in the data. The behavioral setup described in this article, therefore, allows for adequate and safe monitoring of behavioral eyeblink responses that occur during classical eyeblink conditioning and serves as a helpful tool that may be used in other behavioral experiments that require the monitoring of eyeblinks.

Unilateral measurements of eyeblink responses is adequate in most eyeblink conditioning experiments; however, two physiologic monitors could be used to monitor bilateral eyeblinks, if desired. The use of fiberoptic for the transmission of signal outside the magnet bore also decreases the potential of electromagnetic artifacts. The electrodes were placed at least 1 cm away from the outer canthus of the eye, to avoid the potential risk of heating hazards (which did not occur

in our experiments). This placement is adjacent to the orbicularis oculi muscle and has been successfully used in behavioral experiments conducted outside of the magnetic resonance environment (Knuttinén *et al.*, 2001). Given the magnitude of the EMG responses obtained in the subjects in this experiment, it is clear that this electrode placement was sufficient for the robust detection of eyeblink responses.

Our results from this methodological study also showed that classical eyeblink conditioning serves as a useful behavioral paradigm to be used in conjunction with event-related fMRI, which allows for the targeting of specific memory processes. Our findings of the learning-specific activations are generally consistent with results reported previously in positron emission tomography (PET) studies (Blaxton *et al.*, 1996; Logan and Grafton, 1995; Molchan *et al.*, 1994; Schreurs *et al.*, 1997). For example, there is substantial precedent for the observation of changes observed in cerebellum. There is a significant amount of animal literature that documents the importance of the role of the cerebellum as a convergence point for the CS and the US in eyeblink conditioning (Lavond *et al.*, 1993; Schreurs *et al.*, 1995) and in the processing of the temporal relationship between stimuli critical for associative learning (Topka *et al.*, 1993; Perret *et al.*, 1993). Both subjects who demonstrated an increase in conditioned responses during the training session showed increased activation in the ipsilateral cerebellum in the paired state compared with the unpaired control condition. This indicates that the changes were specifically related to learning, rather than to the nonspecific aspects of the experimental protocol. These data are consistent with the previous literature of human lesion studies that have demonstrated impaired conditioning in patients with cerebellar damage (Solomon *et al.*, 1989) and with rabbit studies that show an essential role for the ipsilateral cerebellum in eyeblink conditioning (McCormick and Thompson, 1984; Krupa *et al.*, 1993). One of our subjects showed substantial blood flow increases in bilateral cerebellum that are also consistent with studies in rabbits that have shown that bilateral behavioral responses occur when the airpuff is delivered to one eye (Disterhoft *et al.*, 1977). Furthermore, although unilateral lesions of cerebellar cortex impair conditioning, considerable transfer of training does occur when rabbits are subsequently trained on the non-lesioned and previously naïve side (McCormick *et al.*, 1982; Yeo *et al.*, 1985; Lavond *et al.*, 1994).

Patterns of learning-specific regional activation in the two subjects who learned were also observed in CS sensory auditory cortex, basal ganglia, and posterior cingulate. Conditioning-specific activation of the auditory cortex contralateral to the side of the airpuff has previously been documented in other functional anatomical studies of associative learning in humans (Bahro *et al.*, 1999; Molchan *et al.*, 1994; Schreurs *et al.*

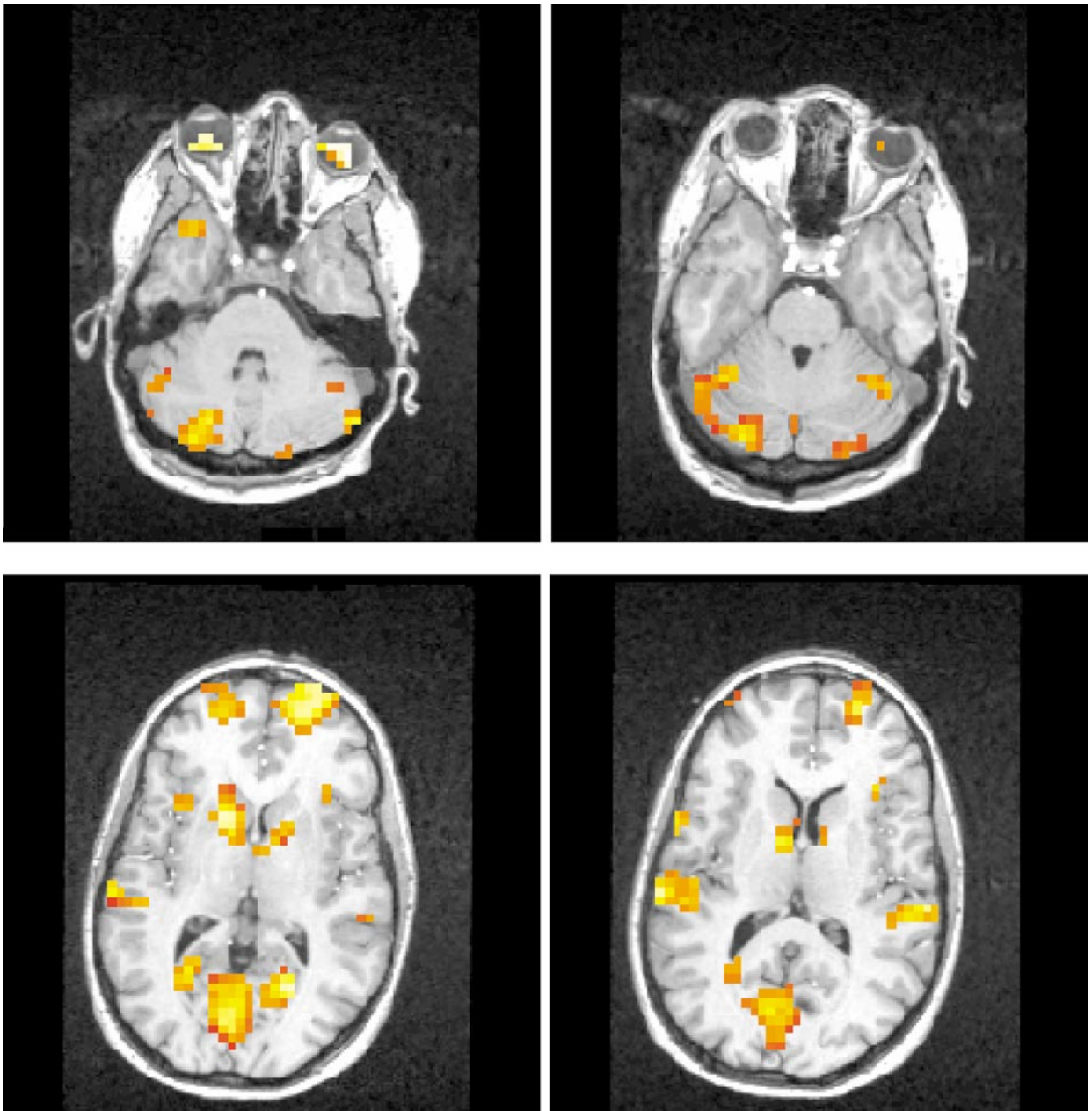


FIG. 7. The activation maps for conditioning were generated by subtracting the pseudoconditioning (CS and US alone) baseline from the conditioning trials (CS and US paired) to remove sensory-related activation. The pseudoconditioning maps were constructed from the 30 unpaired random presentations of tone alone and airpuff alone trials. Areas of increased activation relative to the pseudoconditioning baseline include: areas of cerebellum and caudate, cingulate, temporal, and frontal cortex. The nonlearners showed activity in the pseudoconditioning run, but both of these subjects showed no areas of activation in the subtraction maps.

al., 1997). Lesions of the basal ganglia have been reported to impair the eyeblink response in rabbits (Kao and Powell, 1988), and basal ganglia activation has been observed in PET studies of human eyeblink conditioning (Blaxton *et al.*, 1996). Cohen and Eichenbaum (1993) suggest the basal ganglia, in association with the cerebellum, is an important contributor to the formation of associations between related events, such as the CS and US. One important area we found to be

substantially activated was the posterior cingulate. The role of the cingulate region is heterogeneous and includes processes of attention, learning, and memory (Mesulam, 2000; Gabriel, 1990). Bahro *et al.* (1999) showed an increase in regional cerebral blood flow in the area of posterior cingulate with PET, which replicated their previous PET study findings (Molchan *et al.*, 1994). Our findings support their work and the hypothesis that the cingulate region is a component of

the paralimbic system. In particular, the posterior cingulate region receives the majority of hippocampal projections (Mesulam, 2000), thereby making it an important contributor in learning and memory tasks. In addition, animal studies have also documented the cingulate cortex to be reciprocally connected with all sensory cortices, making it an ideal area for the integration of stimulus associations (Gabriel, 1990; Paperna and Malach, 1991). The results we obtained in our conditioned subjects indicate that the methods outlined here offer a new and exciting approach with which to further examine the processes that may underlie associative learning in humans.

In summary, the goal of this study was to adapt and optimize a methodological approach for human classical eyeblink conditioning to be used in conjunction with event-related fMRI. This study indicates that eyeblink conditioning can be done using fMRI, despite the complex nature of the behavioral setup within the magnet. In addition, this methodology has recently been used to record behavioral responses without causing artifacts using a 3-T scanner (Preston *et al.*, 2000).

Although several PET studies have examined differences in blood flow in the human brain following conditioning, those studies are limited in their spatial resolution and repeatability (due to radioactivity) as compared with fMRI. The approach we describe here offers the opportunity to further extend the human eyeblink conditioning studies through the use of fMRI to better visualize those brain regions that show hemodynamic alteration during associative learning.

Finally, the analysis described in this study may be further modified to investigate other components of the learned response. One example would be to look at specific behavioral performance in individual learning trials, i.e., identify trials on which subjects produced a CR and contrast them with trials without CRs. In addition, this method is a valuable tool to look at memory consolidation since the same subjects can return for imaging after a period of time. Theoretically, brain regions activated during recall of the consolidated memories can be visualized.

ACKNOWLEDGMENTS

The authors thank S. J. Weiss and M. M. Oh for their assistance with this project.

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