Published in final edited form as:

Cerebellum. 2012 December ; 11(4): . doi:10.1007/s12311-012-0377-3.

Eyeblink Conditioning in Healthy Adults: A Positron Emission Tomography Study

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Abstract

Eyeblink conditioning is a paradigm commonly used to investigate the neural mechanisms underlying motor learning. It involves the paired presentation of a toneconditioning stimulus which precedes and co-terminates with an airpuff unconditioned stimulus. Following repeated paired presentations a conditioned eyeblink develops which precedes the airpuff. This type of learning has been intensively studied and the cerebellum is known to be essential in both humans and animals. The study presented here was designed to investigate the role of the cerebellum during eyeblink conditioning in humans using positron emission tomography (PET). The sample includes 20 subjects (10 male and 10 female) with an average age of 29.2 years. PET imaging was used to measure regional cerebral blood flow (rCBF) changes occurring during the first, second, and third blocks of conditioning. In addition, stimuli-specific rCBF to unpaired tones and airpuffs ("pseudoconditioning") was used as a baseline level that was subtracted from each block. Conditioning was performed using three, 15-trial blocks of classical eyeblink conditioning with the last five trials in each block imaged. As expected, subjects quickly acquired conditioned responses. A comparison between the conditioning tasks and the baseline task revealed that during learning there was activation of the cerebellum and recruitment of several higher cortical regions. Specifically, large peaks were noted in cerebellar lobules IV/V, the frontal lobes, and cingulate gyri.

Keywords

Eyeblink conditioning; Motor learning; PET imaging; Frontal lobe; Extinction

Introduction

One of the best-understood models of motor learning is classical eyeblink conditioning, which involves the repeated presentation of a neutral conditioned stimulus (CS, tone) paired and co-terminating with an unconditioned stimulus (US, airpuff to the subject's eye). Initial exposure to paired tone and airpuff trials induces an airpuff-evoked reflexive eyeblink. Following repeated paired presentations, an eyeblink develops which precedes the airpuff. This eyeblink is produced from a learned association between the tone and the upcoming airpuff and is therefore considered a conditioned response (CR). It is known that the cerebellum plays a pivotal role in eyeblink conditioning.

Neuroimaging has become a popular approach to investigate brain regions essential for eyeblink conditioning in normal populations. Specifically, positron emission tomography (PET) and functional magnetic resonance (fMR) imaging have been used to explore motor learning-related changes in regional cerebral blood flow (rCBF) in both animals [1] and humans [2–5]. The eyeblink conditioning literature is complex and plagued by inconsistencies. However, there is a consensus from the majority of these fMR and PET imaging studies during eyeblink conditioning (with pseudoconditioning-specific activity subtracted) that the bilateral cerebellum is essential. Specifically, Blaxton et al. reported positive peaks in bilateral cerebellar lobules IV/V and VI with a negative peak in ipsilateral lobule VII using PET imaging [4]. Schreurs et al. also used PET imaging and revealed no positive cerebellar peaks but instead found negative peaks in ipsilateral cerebellar lobule VII and contralateral lobule VIII [3]. Molchan et al. is the only related study that did not find bilateral cerebellar peaks. Using PET, they only report a negative peak in ipsilateral cerebellar lobule IX [2]. In addition, bilateral cerebellar activity is supported by increases in human cerebellar glucose metabolism in bilateral lobules IX, vermal IV/V, and contralateral IV/V [6] and in animals [7] during eyeblink conditioning (nomenclature according to Schmahmann et al. [8]). When examining animal eyeblink conditioning, Miller et al. found negative peaks bilaterally in cerebellar lobules VI [1]. Neuroimaging studies are consistent with reports from several cerebellar lesion studies involving impaired eyeblink conditioning [9–12]. The most recent review of human lesion data reports deficits in eyeblink conditioning following cerebellar lesions in areas supplied by the superior cerebellar artery: lobules IV, V, and VI [11]. Interestingly, eyeblink conditioning is significantly more impaired on the lesion side than the nonlesion side.

There is great promise in studies using neuroimaging methods to study the progression of learning and we hypothesize that rCBF activations may differ depending on the stage of learning. There is no consistency in previous reports regarding the number of trials presented and this may account for the previous lobular variations in cerebellar peaks. All prior studies complete the eyeblink conditioning phase of the experiment before beginning neuroimaging. The current study is the first to analyze rCBF during different blocks of learning. PET imaging was the chosen methodology for this study because it provides quantitative data for analyzing significant changes in rCBF. In addition, eyeblink conditioning methodology involves a puff of air to the subject's eye which can produce large movement artifacts during MR imaging. PET scans are less susceptible to these artifacts. PET imaging also provides a less confining environment in comparison to MR scans which can produce a hindrance in learning. The eyeblink parameters used in this study are similar to previous reports with the exception of PET scans following each 15-trial block

of conditioning. This allows us for the first time to identify brain areas activated during three blocks of learning.

In addition, another novel aspect of this study is that similar to assessing blocks of learning, rCBF associated with three blocks of extinction is investigated. Two studies investigated extinction and while one found no significant cerebellar peaks [2], the second study (from the same group) reported a significant peak in rCBF in the contralateral cerebellar lobule VIII [3]. The experimental design used in these studies consisted of tone-alone extinction where rCBF produced during pseudoconditioning was subtracted. This design fails to control for the US/UR-specific activity present during pseudoconditioning. We improved upon this design by following each block of extinction with imaged pseudoconditioning trials. This allows subtraction of the original pseudoconditioning phase (before any learning/extinction) from the pseudoconditioning phase immediately following extinction. Results of this subtraction indicate extinction-dependent peaks in rCBF.

Methods and Materials

Subjects

A total of 20 healthy subjects were recruited through the University of Iowa Mental Health Clinical Research Center. There were 10 males and 10 females with a group age mean of 29.2 years (range, 20–48 years), and a standard deviation of 9.22 years. They were screened using an abbreviated version of the Comprehensive Assessment of Symptoms and History to rule out a history of psychiatric disorder. All subjects had normal, self-reported hearing, no identified abnormalities on neurological exam, and 19 of the 20 subjects were right-handed.

Eyeblink Conditioning

For eyeblink conditioning, subjects wore headphones for delivery of the tone CS (75 dB, 1 kHz) and eyeglasses equipped to deliver the airpuff-unconditioned stimulus (US: 5 psi) to the left eye. The eyeglasses also contained an infrared photo beam system for recording eyeblinks. Stimuli presentation was computer controlled and the eyeblink response, recorded as eyeblink amplitude, latency, and peak, was digitized and stored for subsequent analysis (San-Diego Instruments Eyeblink Conditioning System™). Subjects were given a session of pseudoconditioning to gather baseline information which involved unpaired tones and airpuffs for a total of 10 trials with five CS- and five US-alone. A total of 45 paired CS/US trials were presented to the subjects during the acquisition phase of the experiment. These trials were divided into three 15-trial blocks of conditioning. PET imaging was performed for the last five trials in each block (see “PET imaging” section below). The tone CS was presented for 500 ms and coterminated with the 100 ms US producing a 400 ms interstimulus interval for eyeblink conditioning. The interval between the trials ranged from 8 to 16 s ($M=12$ s). Following the 45 acquisition trials, subjects received 90 extinction trials consisting of three 30-trial blocks of extinction. In each 30-trial block of extinction, 20 CS-alone trials were given and then PET imaging was performed for the last 10 pseudorandom, five CS and five US alone trials.

Behavioral Analyses

A CR was counted if the eyeblink amplitude exceeded 10 % of the subject's baseline UR amplitude (based on the mean UR amplitude for 10 US-alone trials presented prior to acquisition trials) and if the eyeblink response occurred between 200 and 400 ms after the CS onset. Eyeblinks fitting this criterion were encoded as a CR and those occurring after 400 ms were encoded as a UR. CR incidence (%) and CR onset latency (ms) were then calculated for the responses. In addition, overall eyeblink onset time was calculated for all trials included in the experiment. Eye-blinks occurring prior to 200 ms after CS onset were

counted as “alpha” responses (i.e., short-latency, tone-evoked nonassociative responses). Trials where an alpha response was present were excluded from data analysis.

CR incidence was modeled as a binary outcome (yes/no) and onset time was modeled as a continuous outcome. Since each subject was tested multiple times under varying experimental conditions, repeated measurements of CR characteristics obtained from the same subject were correlated. Linear regression or ANOVA models are not well suited for analysis of correlated data. Therefore, generalized estimating equation modeling was applied in the analysis of CR incidence, onset time, peak, and amplitude [13, 14]. For these analyses, experiment phase, age, and sex were included as covariates and the first-order autoregressive variance–covariance structure (AR (1)) was used, based on model selection criteria, to model the correlation between repeated measures from the same subject. Linear contrasts of interest on the variable experiment phase were constructed to test for relevant hypotheses. A significance level of 0.05 was used for any hypothesis testing in the paper. All statistical analyses were carried out with SAS v9.2 (Cary, NC, USA). Age over 40 is known to significantly impair eyeblink conditioning; however, age as a covariate revealed no significant effect in this sample.

PET Imaging

Methods for acquisition and processing of the [^{15}O]water PET imaging data have been previously described [15]. MR scans were obtained for each subject with a standard T1-weighted three-dimensional SPGR sequence on a 1.5 T GE Signa (General Electric, Milwaukee, WI, USA) scanner (TE=5; TR=24; flip angle=40; NEX=2; FOV=26; matrix=256×192; slice thickness, 1.5 mm). PET data were acquired after the bolus intravenous administration of [^{15}O] water (50 mCi) using a GE 4096-plus whole-body scanner. A preliminary scout injection provided an initial estimate of the bolus arrival time which was then utilized to optimize the timing of imaging [15]. Imaging of the last five trials of each block of acquisition and extinction was timed to start 10 s before the arrival of the [^{15}O]water bolus in the brain. Images were acquired in 20–5 s frames. Based on time–activity curves over major cerebral arteries, the eight frames reflecting the 40 s after bolus transit were selected and summed [15, 16]. The summed image was reconstructed in 2-mm voxels in a 128×128 matrix using a Butterworth filter (expressed in PET counts). Injections were repeated at approximately 15-min intervals [17, 18].

PET Image Analyses

The anterior commissure–posterior commissure (AC–PC) plane was identified and used to realign MR images of all subjects to a standard position. The PET image for each subject was fit to their MR scan with AIR [19]. Images from each injection were checked for head movement and individually re-fit as needed [20, 21]. To reduce anatomic variability, the PET images were normalized, then smoothed with an 18-mm Hanning filter [22]. An average MR was generated by transforming subjects' MRI into a Talairach bounding box. The PET images were brought to standardized space using the transform from the MR. Images were then analyzed using the locally developed software package BRAINS (Brain Research: Analysis of Images, Networks, and Systems) [23, 24] and statistical analysis was performed using an adaptation of the method of Worsley [16, 18, 22]. Within-subject subtractions of relevant injections were used in the computation of voxel-by-voxel *t* tests of the changes in count images. The *t* threshold was adjusted to correct for the large number of voxel-by-voxel *t* tests performed, the lack of independence between voxels, and the resolution of the processed PET images [16, 18, 25]. Results were visualized overlying the group's averaged MR scans [18].

This study analyzed rCBF during six basic subtractions: the three blocks of acquisition and extinction with unpaired tone and airpuff-specific activity (pseudoconditioning control task) subtracted. In these analyses, significant changes in rCBF are labeled as positive peaks (greater activity in the experimental task) or negative peaks (less activity in the experimental task). Tabled data report the brain regions with significant changes in rCBF. Individual significant peaks are reported with their corresponding three-dimensional [20] coordinates (x, y, z), corresponding Brodmann's area (BA) when appropriate, size (cc), and the highest t value ($t_{\max}$, representing the "peak"). Following methods traditionally used in our laboratory, included peaks contain at least 50 continuous voxels that have a t value threshold of >3.0 . Talairach coordinates are used to indicate the side of the brain in standard radiological convention as if you are looking at the patient so that the left side of the image is the right side of the brain and the right side of image is the left side of the brain.

Results

Eyeblink Conditioning Behavioral Data

Following repeated paired presentations of the tone and airpuff, 19 subjects (one subject was unable to complete the study, so those data were not included) showed an increase in CR incidence (Fig. 1a, CR incidence; conditioning). Specifically, CRs significantly increased from 40.90 ± 15.10 % during the first acquisition block to 64.40 ± 16.38 % in the second (Fig. 1a, Acq; $\chi^2=5.79, p=0.0161$). When comparing the first and third blocks, the CR% significantly increased even more to 66.74 ± 15.24 % ($\chi^2=6.33, p=0.0119$). There was no significant difference between the second and third blocks of acquisition. As a result of learning, eyeblink onset timing decreased representatively (Fig. 1b, eyeblink onset; conditioning). There was a statistically significant decrease in the onset latency between the first and second blocks of conditioning of 387.29 ± 19.78 – 349.20 ± 18.55 ms (Fig. 1b, Acq; $\chi^2=6.17, p=0.0130$) as well as the first and third blocks of 334.79 ± 20.00 ms ($\chi^2=8.76, p=0.0031$). Similar to CR incidence, eyeblink latency did not significantly differ between the second and third blocks of conditioning. Analyses of CR onset time (as opposed to overall eyeblink onset), CR peak, and CR amplitude revealed no significant differences between each block of conditioning (Fig. 2a and b).

Eyeblink Extinction Behavioral Results

Repeated presentation of the CS-alone produced rapid extinction of CRs (Fig. 1a, CR incidence; extinction). CR incidence during the third block of acquisition (66.74 ± 15.24 %) dramatically declined to 25.67 ± 12.29 % during the first extinction block (Fig. 1a, $\chi^2=9.46, p=0.0021$), and although extinction blocks do not differ significantly from each other, CRs continued to decrease into the second and third blocks (21.22 ± 10.60 and 19.33 ± 12.03 %). As CR expression decreased due to extinction, latency increased in the CRs that were still expressed (Fig. 1b, eyeblink onset; extinction). During the last block of conditioning, subject's eyeblinks occurred with a latency of 334.78 ± 20.00 ms which significantly increased to an average of 401.24 ± 14.49 ms following extinction (Fig. 1b, $\chi^2=6.27, p=0.0123$). Similar to CR incidence, eyeblink latency did not significantly differ between the three blocks of extinction (Fig. 1b).

Eyeblink Conditioning Imaging Results

PET imaging revealed significant changes in rCBF during eyeblink classical conditioning that differed significantly from those observed during the unpaired tone and airpuff phase (pseudoconditioning). Changes in rCBF that exceeded our significance level of 0.005 are reported as either positive ($t_{\max}$ greater than 3.0) or negative ($t_{\max}$ less than -3.0) peaks in rCBF that vary in location as acquisition and extinction occur. Table 1 reports rCBF peaks by the three acquisition blocks and Table 2 reports peaks during extinction.

Positive and negative peaks in rCBF during conditioning and extinction were consistently found in the frontal lobes, cingulate gyri, and the cerebellum (Table 1, acquisition peaks). The frontal lobe consistently showed bilateral positive peaks in the superior and medial gyri with BA 8 activated during all blocks of conditioning. The frontal lobes also showed activity in the medial frontal gyrus in contralateral BA 10 (second acquisition block) and ipsilateral BA 11 (third acquisition block). The contralateral inferior frontal lobe (BA 47) was the only area with a negative peak during the third block of conditioning. The ipsilateral posterior cingulate (BA 30) increased rCBF during the first block of conditioning, the bilateral anterior (BA 32) and posterior cingulate (BA 29) had increased rCBF during second block of conditioning and the contralateral posterior (BA 31) and ipsilateral cingulate (BA 29, 32) had increased rCBF during the third block. The contralateral cingulate (BA 33, 21, 24) also showed negative peaks during all blocks of conditioning. During all three blocks of acquisition, positive peaks were found in the contralateral cerebellar lobules IV and V (Fig. 3, positive) along with a negative peak during the first block of conditioning in vermal lobules IV and V (Fig. 3, negative). During the second block, another negative peak was found vermally in lobules I and II (not illustrated).

Extinction Imaging Results

Extinction also revealed dynamic changes in rCBF in areas similar to those reported during learning (Table 2, extinction peaks). The frontal lobe had positive rCBF peaks in BA 8 during all blocks of extinction (contralateral, first and third; ipsilateral, second block). These peaks spread throughout several gyri: the superior, medial, and middle frontal gyri. In addition, contralateral BA 10 revealed positive peaks during the first and third blocks of extinction in the superior, medial, and middle frontal gyri. In addition, contralateral BA 6 showed positive peaks during the second and third block of extinction as well as contralateral BA 9 and ipsilateral BA 45 during the third block of extinction. Negative peaks were also found during the second extinction block in contralateral BA 25 in the medial frontal gyrus. Similar to the third block of conditioning, the inferior frontal lobe (BA 47) showed a negative peak during the first (ipsilateral) and third (contralateral) blocks of extinction. There was also significant activity in the cingulate gyrus with positive peaks during the first block of extinction in the anterior midline (BA 32) and right posterior (BA 31), but this activity disappeared during the second block, and during the third block there were negative peaks in the left cingulate gyrus (BA 33 and 24). During the first block of extinction, right cerebellar lobules IV/V and left cerebellar lobule VIII showed positive peaks (Fig. 4). There were no significant cerebellar peaks during the second extinction block but the third block recruited an increase in rCBF in vermal IV/V as well as the right lobule VIII (Fig. 5). In addition, there was a negative peak in right cerebellar lobule VI during the third block of extinction (Fig. 5).

Discussion

The present study reports human eyeblink conditioning-dependent changes in cerebellar rCBF which both supports and extends previous reports. It novelly analyzes level of learning-related rCBF by imaging eyeblink conditioning following three blocks of learning. The cerebellar PET imaging results presented here reveal positive rCBF peaks in the contralateral cerebellar lobules IV/V throughout all blocks of conditioning compared to during unpaired pseudoconditioning. This indicates that the vermal and contralateral cerebellum play a role in eyeblink conditioning from the point of naïveté to that of being well-learned. Negative peaks were also found in the bilateral cerebellum in the during early (vermal lobules IV/V) and middle conditioning (lobules I/II).

Previous studies have identified areas of the brain essential to eyeblink conditioning by imaging rCBF following learning. However, humans acquire conditioned responses within

the first few paired presentations with an average of 43 % during the first conditioning session (41 % CRs reported here) [2–4, 6]. As a result, human learning-dependent rCBF is obscured by methods involving one imaging session following learning. The methodology used in this study uniquely captures the rCBF recruited during the first block of learning and allows comparison with the later two blocks.

The activations in contralateral cerebellar lobules IV/V reported here are supported by Blaxton et al., who also found significant peaks in lobule VI [4]. However, they found major ipsilateral contributions of these lobules which are absent in our study. Several additional neuroimaging studies have shown bilateral cerebellar involvement in eyeblink conditioning in both animals [1] and humans [1–3]. Human cerebellar lesions to areas supplied by the superior cerebellar artery: lobules IV, V, and VI, reveal deficits in eyeblink conditioning bilaterally (although subjects were significantly more impaired on the lesion side) [11]. Bilaterality in lobules IV/V is also confirmed by increases in cerebellar glucose metabolism during learning in humans [6] and rats [7]. There are no studies supporting the negative peaks in lobules I/II reported here but Blaxton et al. report a negative peak in ipsilateral lobule VII [4]. Schreurs et al. also reports negative peaks in ipsilateral cerebellar lobule VII and contralateral lobule VIII [3] while Molchan et al. report a negative peak in ipsilateral cerebellar lobule IX [2]. Finally, MR in animals reveals negative peaks bilaterally in cerebellar lobules VI [1]. Considering the evidence for bilateral cerebellar involvement in eyeblink conditioning, the minimal ipsilateral cerebellar activation in our study is perplexing. However, combining the results presented here and those from previous studies, a general conclusion is that rather than having one static location responsible for learning and memory, a collaboration between cerebellar structures is likely. Although there is overwhelming evidence that the cerebellum plays an essential role in associative learning during eyeblink conditioning [26], it may also contribute to performance of the conditioned response [27, 28]. Thus, some of the changes in cerebellar rCBF may reflect changes in CR performance as well as associative learning.

The frontal lobes and cingulate gyri also show dynamic fluctuations in rCBF throughout learning. In this study, the frontal lobes and cingulate gyri showed massive activations during conditioning and extinction, implicating a reciprocally connected cerebellar–forebrain circuit in tasks traditionally thought to rely solely on one of these areas. BA 8 in the superior and medial frontal gyri showed positive rCBF peaks which seemed to alternate between left and right hemispheres during conditioning. This area is located in the dorsolateral frontal cortex and is made up of the presupplementary motor cortex and the premotor cortex which function in motor learning and motor action planning. During the first block of conditioning, the contralateral prefrontal cortex (BA 10) also showed a positive peak. This finding is similar to that of Blaxton et al. who also showed bilateral BA 10 and 11 peaks during conditioning [4]. This study also reports a negative peak in the prefrontal cortex in BA 47 during the third block of conditioning which replicates the finding by Schreurs et al. [3] and was reported by Blaxton et al., but they found a positive peak during learning [4]. Two studies also report negative prefrontal peaks in BA 44/45 [4]. There was dramatic activity (both positive and negative peaks) noted in the bilateral cingulate gyri during all blocks of learning and memory. This finding is similar to that of Blaxton et al. who reported a positive peak in the anterior cingulate during conditioning [4].

A straightforward interpretation of these results is that human eyeblink circuitry includes not only the cerebellum but also the frontal lobes and cingulate gyri. An example of this hypothesis is illustrated in Fig. 2, middle, sagittal image. In this one image, you can see simultaneous activation of the cerebellum, anterior cingulate, and frontal lobe. However, this may be an erroneous conclusion as neuronal modulation has been reported during tasks for which the recorded neurons are not necessary. The hippocampus in animals is an example of

a forebrain area that is known to have perfect neuronal correlates of learning during eyeblink conditioning [29–33] and yet not be required for learning to occur [34]. Although the hippocampus undergoes modulatory changes during conditioning, lesions do not impair eyeblink conditioning indicating proper hippocampal function is not necessary for learning to occur. Therefore, areas with increased rCBF during eyeblink conditioning as presented here and by Blaxton et al. may show differences between correlates of learning and not inconsistencies eyeblink neural circuitry/substrates [4].

Finally, rCBF associated with three blocks of tone-alone extinction was investigated. Following acquisition, initial presentation of tone-alone extinction trials continue to elicit CRs but following repeated presentation CRs decrease and the subjects completely extinguish. Extinction involves active loss of CR expression due to context-dependent (presence of the CS and US) inhibitory learning. The learned association between the tone and airpuff remains which is supported by reports of faster reacquisition and immediate restoration of CRs following subsequent CS and US paired presentation [35, 36]. The experimental design used by our study involved each block of extinction followed by imaged unpaired tone and airpuff pseudoconditioning trials. One downside to this design is incomplete extinction due to the attempt to re-associate once the airpuff is reinstated. As a consequence the 30 extinction trials (double that presented during conditioning) were presented per block to allow for complete extinction before each imaging session. This design allowed subtraction of the original pseudoconditioning phase (before any learning/extinction) from the pseudoconditioning phase immediately following each block of extinction. Two studies examined eyeblink extinction using PET imaging but in both of these experiments tone-alone extinction peaks were examined versus paired activations [2, 3]. This design fails to control for the US/UR specific activity present during pseudoconditioning. Results from one study found no significant cerebellar peaks [2], while the second study (from the same group) reported a significant peak in rCBF in the contralateral cerebellar lobule VIII [3].

During the first block of extinction, the present study found contralateral cerebellar lobules IV and V positive peaks but there was also an ipsilateral positive peak in lobule VIII. There were no significant peaks during the second block but lobules IV and V reappeared during the last extinction block as well as a negative peak in lobule VI. The positive peak in lobule VIII disappeared during the second extinction block and was replaced by a contralateral negative peak in a similar location of lobule VIII during the last extinction block. This peak is similar to that reported by Schreurs et al. [3]. Peaks were also found in superior, medial, middle, and inferior frontal lobes during extinction indicating the forebrain systems may modulate the cerebellum during extinction to produce extinction-dependent behavioral inhibition. Specifically, the frontal lobes recruited were similar to those recruited during learning BA 8, 10, 47 but extinction also involved areas 6, 9, 25, 45. As noted above, area 8 functions in motor learning and motor action planning and areas 9, 10, and 11 are part of the prefrontal cortex which is associated with memory. There was also significant activity in the cingulate gyrus but significantly less than during learning. The majority of the activity was located in the anterior cingulate.

Summary

The current study investigated the role of the cerebellum in level of learning-specific eyeblink conditioning in humans using positron emission tomography. Our findings suggest that learning-specific neuronal activation occurs in several structures distributed throughout the normal brain. In particular, there are large contributions of the cerebellum, frontal lobes, and cingulate gyri.

References

1. Miller MJ, Chen NK, Li L, Tom B, Weiss C, Disterhoft JF, et al. fMRI of the conscious rabbit during unilateral classical eyeblink conditioning reveals bilateral cerebellar activation. *J Neurosci*. 2003; 23:11753–11758. [PubMed: 14684877]
2. Molchan SE, Sunderland T, McIntosh AR, Herscovitch P, Schreurs BG. A functional anatomical study of associative learning in humans. *Proc Natl Acad Sci*. 1994; 91:8122–8126. [PubMed: 8058767]
3. Schreurs BG, McIntosh AR, Bahro M, Herscovitch P, Sunderland T, Molchan SE. Lateralization and behavioral correlation of changes in regional cerebral blood flow with classical conditioning of the human eyeblink response. *J Neurophysiol*. 1997; 77:2153–2163. [PubMed: 9114262]
4. Blaxton TA, Zeffiro TA, Gabrieli JDE, Bookheimer SY, Carrillo MC, Theodore WH, et al. Functional mapping of human learning: a positron emission tomography activation study of eyeblink conditioning. *J Neurosci*. 1996; 16:4032–4040. [PubMed: 8656296]
5. Ramnani N, Toni I, Josephs O, Ashburner J, Passingham RE. Learning- and expectation-related changes in the human brain during motor learning. *J Neurophysiol*. 2000; 84:3026–3035. [PubMed: 11110829]
6. Logan CG, Grafton ST. Functional anatomy of human eyeblink conditioning determined with regional glucose metabolism and positron emission tomography. *Proc Natl Acad Sci*. 1995; 92:7500–7504. [PubMed: 7638220]
7. Plakke B, Freeman JH, Poremba A. Metabolic mapping of the rat cerebellum during delay and trace eyeblink conditioning. *Neurobiol Learn Mem*. 2007; 88:11–18. [PubMed: 17468019]
8. Schmahmann JD, Doyon J, McDonald D, Holmes C, Lavoie K, Hurwitz AS, et al. Three-dimensional MRI atlas of the human cerebellum in proportional stereotaxic space. *NeuroImage*. 1999; 10:233–260. [PubMed: 10458940]
9. Gerwig M, Kolb FP, Timmann D. The involvement of the human cerebellum in eyeblink conditioning. *Cerebellum*. 2007; 6:38–57. [PubMed: 17366265]
10. Gerwig M, Hajjar K, Dimitrova A, Maschke M, Kolb FP, Frings M, et al. Timing of conditioned eyeblink responses is impaired in cerebellar patients. *J Neurosci*. 2005; 25:3919–3931. [PubMed: 15829644]
11. Timmann D, Drepper J, Frings M, Maschke M, Richter S, Gerwig M, et al. The human cerebellum contributes to motor, emotional and cognitive associative learning. A review. *Cortex*. 2010; 46:845–857. [PubMed: 19665115]
12. Timmann D, Brandauer B, Hermsdorfer J, Ilg W, Konczak J, Gerwig M, et al. Lesion-symptom mapping of the human cerebellum. *Cerebellum*. 2008; 7:602–606. [PubMed: 18949530]
13. Burton P, Gurrin L, Sly P. Extending the simple linear regression model to account for correlated responses: an introduction to generalized estimating equations and multi-level mixed modelling. *Stat Med*. 1998; 17:1261–1291. [PubMed: 9670414]
14. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: a generalized estimating equation approach. *Biometrics*. 1988; 44:1049–1060. [PubMed: 3233245]
15. Hurtig RR, Hichwa RD, O'Leary DS, Ponto LLB, Narayana S, Watkins GL, et al. Effects of timing and duration of cognitive activation in [O-15]water pet studies. *J Cereb Blood Flow Metab*. 1994; 14:423–430. [PubMed: 8163584]
16. O'Leary DS, Andreasen NC, Hurtig RR, Hichwa RD, Watkins GL, Ponto LLB, et al. A positron emission tomography study of binaurally and dichotically presented stimuli: effects of level of language and directed attention. *Brain Lang*. 1996; 53:20–39. [PubMed: 8722897]
17. Andreasen NC, O'Leary DS, Cizadlo T, Arndt S, Rezai K, Ponto LLB, et al. Schizophrenia and cognitive dysmetria: a positron-emission tomography study of dysfunctional prefrontal–thalamic–cerebellar circuitry. *Proc Natl Acad Sci U S A*. 1996; 93:9985–9990. [PubMed: 8790444]
18. Andreasen NC, O'Leary DS, Arndt S, Cizadlo T, Hurtig R, Rezai K, et al. Short-term and long-term verbal memory—a positron emission tomography study. *Proc Natl Acad Sci U S A*. 1995; 92:5111–5115. [PubMed: 7761457]

19. Woods RP, Grafton ST, Watson JD, Sicotte NL, Mazziotta JC. Automated image registration: II. Intersubject validation of linear and nonlinear models. *J Comput Assist Tomogr.* 1998; 22:153–165. [PubMed: 9448780]
20. Talairach, JTP. Co-planar stereotaxic atlas of the human brain. New York: Theme Medical Publishers; 1998.
21. Ted, Cizadlo. Image registration issues in the analysis of multiple injection 15O H₂O PET studies: BRAINFIT. *Proc SPIE.* 1994; 2186:423–430.
22. Arndt S, Cizadlo T, Andreasen NC, Zeien G, Harris G, Oleary DS, et al. A comparison of approaches to the statistical analysis of [O-15]H₂O pet cognitive activation studies. *J Neuropsych Clin Neurosci.* 1995; 7:155–168.
23. Andreasen NC, Cohen G, Harris G, Cizadlo T, Parkkinen J, Rezai K, et al. Image-processing for the study of brain structure and function—problems and programs. *J Neuropsych Clin Neurosci.* 1992; 4:125–133.
24. Andreasen NC, Cizadlo T, Harris G, Swayze V, Oleary DS, Cohen G, et al. Voxel processing techniques for the antemortem study of neuroanatomy and neuropathology using magnetic resonance imaging. *J Neuropsych Clin Neurosci.* 1993; 5:121–130.
25. Andreasen NC, Oleary DS, Cizadlo T, Arndt S, Rezai K, Watkins L, et al. Remembering the past —2 facets of episodic memory explored with positron emission tomography. *Am J Psychiatry.* 1995; 152:1576–1585. [PubMed: 7485619]
26. Freeman JH, Steinmetz AB. Neural circuitry and plasticity mechanisms underlying delay eyeblink conditioning. *Learn Mem.* 2011; 18:666–677. [PubMed: 21969489]
27. Welsh JP, Harvey JA. Cerebellar lesions and the nictitating membrane reflex: performance deficits of the conditioned and unconditioned response. *J Neurosci.* 1989; 9:299–311. [PubMed: 2913208]
28. Jimenez-Diaz L, Navarro-Lopez JD, Gruart A, Delgado-Garcia JM. Role of cerebellar interpositus nucleus in the genesis and control of reflex and conditioned eyelid responses. *J Neurosci.* 2004; 24:9138–9145. [PubMed: 15483132]
29. Berger TW, Rinaldi PC, Weisz DJ, Thompson RF. Single-unit analysis of different hippocampal cell types during classical conditioning of rabbit nictitating membrane response. *J Neurophysiol.* 1983; 50:1197–1219. [PubMed: 6644367]
30. Disterhoft JF, Coulter DA, Alkon DL. Conditioning-specific membrane changes of rabbit hippocampal neurons measured in vitro. *Proc Natl Acad Sci U S A.* 1986; 83:2733–2737. [PubMed: 3458232]
31. Daum I, Channon S, Canavan AGM. Classical conditioning in patients with severe memory problems. *J Neurol Neurosurg Psychiatry.* 1989; 52:47–51. [PubMed: 2496204]
32. Woodruff-Pak DS. Eyeblink classical conditioning in H.M.: delay and trace paradigms. *Behav Neurosci.* 1993; 107:911–925. [PubMed: 8136067]
33. Gabrieli JDE, McGlinchey-Berroth R, Carrillo MC, Gluck M, Cermak LS, Disterhoft JF. Intact delay-eyeblink classical conditioning in amnesia. *Behav Neurosci.* 1995; 109:819–827. [PubMed: 8554707]
34. Schmaltz LW, Theios J. Acquisition and extinction of a classically conditioned response in hippocampectomized rabbits (*Oryctolagus cuniculus*). *J Comp Physiol Psychol.* 1972; 79:328–333. [PubMed: 5026000]
35. Falls, WA. Extinction: a review of theory and the evidence suggesting that memories are not erased with nonreinforcement. In: O'Donohue, W., editor. *Learning and behavior therapy*. Boston: Allyn and Bacon; 1998. p. 205-229.
36. Robleto K, Poulos AM, Thompson RF. Brain mechanisms of extinction of the classically conditioned response. *Learn Mem.* 2004; 11:517–524. [PubMed: 15466302]

Fig. 1.

Behavioral group data for CR incidence and eyeblink onset for acquisition and extinction during each experimental block. All data points represent group means $\pm$ SEM ($n=20$). **a** CR incidence (%). Presentation of paired tone and airpuff trials results in classical eyeblink conditioning which increases with each subsequent block. During tone-alone extinction trials the subject's CR incidence gradually declines. **b** Eyeblink onset (ms). As a consequence of the increase in CR incidence, eyeblink onset timing decreases. These data include the onset time of both CRs and missed (UR only) trials. During the loss of CRs in the extinction blocks, eyeblink onset times increase. The responses observed during the

control blocks of fixation and baseline are normal spontaneous eyeblinks with varying onset times averaging around 360 ms. *Fix*: fixation (no stimuli), *baseline*: baseline pseudoconditioning period of unpaired (*UP*) tones and airpuffs, *conditioning*: (*CC*) paired tones and airpuffs, *extinction*: (*EX*) tone-alone trials. Blocks of conditioning and extinction: first (CC1/EX1), second (CC2/EX2), third (CC3/EX3)

Fig. 2.

Behavioral group data for the properties of the CRs including onset time, peak, and amplitude during each conditioning block. All data points represent group means $\pm$ SEM ($n=20$). a CR onset and peak time (ms). CR onset and the peak of these responses do not change significantly following paired tone and airpuff trials. b CR amplitude (mm). CR amplitude also does not change significantly as a result of learning

Cerebellar lobule IV/V peaks during the first block of acquisition

Fig. 3. Positron emission tomography (PET) peaks during the first block of eyeblink conditioning. The images chosen show the first block of conditioning with the rCBF during the baseline, pseudoconditioning phase (unpaired tones and airpuffs) subtracted. The *top row of images* is an example of the positive cerebellar peaks while the bottom row represents the negative peaks. The positive peak shown is from the first conditioning block and is representative of those in both the second and third blocks so those peaks are not shown. The middle positive image in Fig. 3 also illustrates the location of significant frontal and cingulate peaks in rCBF. For each illustration (Figs. 3–5) there are three orthogonal views per row with transaxial on the left, sagittal in the middle, and coronal on the right. *Green crosshairs* are used to show the location of the slice. Images follow radiological convention and show location as if facing the patient where the left side of the image represents the patient's right side. The statistical maps of the PET, showing the regions where the two groups differed significantly at the 0.005 level, are superimposed on a composite MR image derived by averaging the MR scans from the subjects. Regions in *red/yellow tones* indicate greater activation during the experimental block and regions with *blue* indicate deactivations or more activation during the control phase. The statistical results are portrayed using the value of the associated t statistic, which is shown on the color bar on the right. The images are referred to as “t maps” showing all voxels in the image which exceed a display threshold

Fig. 4.
Positive PET peaks in lobules IV/V and VIII during the first block of eyeblink extinction

Cerebellar positive and negative peaks during the third block of extinction

Fig. 5. Positive PET peaks in lobules IV/V, VI, and negative peak in lobule VIII during the third block of eyeblink extinction

Table 1
Areas of significant rCBF changes during the three extinction blocks with unpaired activity subtracted

Acquisition peaks																			
		First Block vs. UP						Second Block vs. UP						Third Block vs. UP					
		x	y	z	BA	cc	t max	x	y	z	BA	cc	t max	X	y	z	BA	cc	t max
Frontal lobe	Superior frontal gyrus	-20	26	48	8	0.1	3.498	13	38	44	8	1	4.626	-26	27	48	8	1.5	4.388
	Medial frontal gyrus	17	34	-3	10	1.8	4.355	-8	25	-15	11	0.2	3.609						
	Inferior frontal gyrus	6	33	42	8	0.1	3.31												
Cingulate gyrus	Anterior cingulate gyrus	-4	9	22	33	0.6	-4.671	-1	36	25	32	0.9	4.101	25	27	-12	47	0.5	-3.741
								14	-12	34	24	0.4	-3.562						
	Cingulate gyrus	5	-16	39	21	0.4	-4.078	7	-12	34	24	0.4	-3.562	6	-44	29	31	0.9	4.081
Cerebellum		-14	-63	13	30	0.1	3.28							6	-12	34	24	0.3	-3.663
	Posterior cingulate gyrus							13	-42	11	29	0.2	3.456	-17	11	36	32	0.1	3.291
	Lobules I/II							0	-39	-14	-	0.2	3.467	-10	-50	6	29	0.2	3.472
	Lobules IV/V	18	-46	-14	-	0.6	3.873	18	-47	-14	-	0.2	3.572	19	-47	-14	-	0.6	4.066
		1	-47	-20	-	0.3	-3.993												

Areas with significant positive and negative peaks are organized by their general lobular location, then by a more specific location within the lobule, and finally in descending order of size. In addition, rCBF changes are reported in terms of their location in Talairach coordinates (x, y, z), corresponding Brodmann's area when appropriate, blob size (cc), and Tmax. Ipsilateral localizations are identified by peaks with a negative x-Talairach coordinate which indicates the left side of the brain and contralateral localizations are identified by a positive x-coordinate indicating the right side

Table 2

Areas of significant rCBF changes during the three extinction blocks with unpaired activity subtracted

Extinction peaks																			
		First block vs. UP						Second block vs. UP						Third block vs. UP					
		x	y	z	BA	cc	t max	x	y	z	BA	cc	t max	x	y	z	BA	cc	t max
Frontal lobe	Superior frontal gyrus	42	22	48	8	1.1	4.089												
		21	54	19	10	0.1	3.263												
	Medial frontal gyrus	5	31	41	8	1.9	4.39	16	13	-15	25	0.2	-3.516	1	30	39	8	0.2	3.451
		Middle frontal gyrus	43	48	-3	10	4.7	4.565	-32	25	44	8	0.3	3.53	28	2	48	6	1.7
								33	-1	42	6	0.2	3.348	33	46	-5	10	0.2	3.477
	Precentral frontal gyrus							46	-5	25	6	0.2	3.348	38	6	39	9	0.3	3.446
	Inferior frontal gyrus	-20	31	-9	47	0.7	-3.686							20	13	-15	47	1.3	-4.214
Cingulate gyrus	Anterior cingulate gyrus	0	37	25	32	0.5	3.9							-54	22	8	45	0.4	3.828
		-1	29	-3	24	0.2	-3.448							-2	9	22	33	0.4	-3.675
	Cingulate gyrus	4	-50	29	31	0.2	3.542							-2	26	22	24	0.3	-3.571
Cerebellum	Lobules IV/V	39	-52	-23	-	0.3	3.624												
	Lobule VI													0	-62	0	-	0.5	3.704
	Lobule VIII	-26	-75	-31	-	1.1	3.957							18	-58	-17	-	1.1	4.581
													11	-74	-27	-	0.2	-4.683	

Areas with significant positive and negative peaks are organized by their general lobular location, then by a more specific location within the lobule, and finally in descending order of size. In addition, rCBF changes are reported in terms of their location in Talairach coordinates (x, y, z), corresponding Brodmann's area when appropriate, blob size (cc), and Tmax. Ipsilateral localizations are identified by peaks with a negative x-Talairach coordinate which indicates the left side of the brain and contralateral localizations are identified by a positive x-coordinate indicating the right side