

Functional MRI of Cerebellar Activity During Eyeblink Classical Conditioning in Children and Adults

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Abstract: This study characterized human cerebellar activity during eyeblink classical conditioning (EBC) in children and adults using functional magnetic resonance imaging (fMRI). During fMRI, participants were administered delay conditioning trials, in which the conditioned stimulus (a tone) precedes, overlaps, and coterminates with the unconditioned stimulus (a corneal airpuff). Behavioral eyeblink responses and brain activation were measured concurrently during two phases: pseudoconditioning, involving presentations of tone alone and airpuff alone, and conditioning, during which the tone and airpuff were paired. Although all participants demonstrated significant conditioning, the adults produced more conditioned responses (CRs) than the children. When brain activations during pseudoconditioning were subtracted from those elicited during conditioning, significant activity was distributed throughout the cerebellar cortex (Crus I–II, lateral lobules IV–IX, and vermis IV–VI) in all participants, suggesting multiple sites of associative learning-related plasticity. Despite their less optimal behavioral performance, the children showed greater responding in the pons, lateral lobules VIII, IX, and Crus I, and vermis VI, suggesting that they may require greater activation and/or the recruitment of supplementary structures to achieve successful conditioning. Correlation analyses relating brain activations to behavioral CRs showed a positive association of activity in cerebellar deep nuclei (including dentate, fastigial, and interposed nuclei) and vermis VI with

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CRs in the children. This is the first study to compare cerebellar cortical and deep nuclei activations in children versus adults during EBC. *Hum Brain Mapp* 35:1390–1403, 2014. © 2013 Wiley Periodicals, Inc.

Key words: cerebellum; development; learning; memory; neuroimaging

INTRODUCTION

Eyeblink classical conditioning (EBC) is a widely used model of learning, in which a neutral conditioned stimulus (CS; e.g., a tone) and an unconditioned stimulus (US; e.g., a corneal airpuff) are temporally paired. One version of this procedure is delay conditioning, in which the CS precedes, overlaps, and coterminates with the US. Following repeated CS–US presentations, the CS alone reliably elicits a conditioned response (CR; e.g., an eyeblink) in anticipation of the US presentation, indicating that an association between the CS and US has been learned.

This well-characterized model system provides a powerful tool for studying the neural correlates of learning and memory. Through extensive laboratory animal investigations, the neural circuitry supporting EBC has been mapped in great detail [for review, see Christian and Thompson, 2003]. This line of research has produced overwhelming evidence that the cerebellum and associated structures are critically important for eyeblink conditioning. Specifically, contributions from the cerebellar cortex [Yeo, et al., 1984, 1985; Yeo and Hardiman, 1992], particularly in lateral lobule VI, and cerebellar deep nuclei [Lavond et al., 1984; McCormick and Thompson, 1984a,b; Lavond et al., 1985] have been well-documented.

EBC has also been used to investigate cerebellar development and the ontogeny of learning. Developmental studies of EBC have shown that older (24 days) rat pups conditioned at a faster rate compared to younger (17 days) rat pups [Stanton et al., 1992; Stanton, et al., 1998], reflecting specific ontogenetic changes in cerebellar circuitry [Freeman et al., 1995a; Freeman, 2010].

Developmental EBC studies in humans ranging from infants to older adults have focused mainly on behavioral measures of conditioning [Woodruff-Pak and Thompson, 1988; Knuttinen et al., 2001; Claflin et al., 2002; Herbert et al., 2003; Jacobson et al., 2008; Cheng et al., 2010; Stanton et al., 2010; Jacobson et al., 2011]. Although these studies offer significant insight on how development affects behavioral performance during eyeblink conditioning, little is known about how development affects cerebellar activity mediating human EBC. To date, investigations of cerebellar function in humans during EBC have been limited to studies of adult patients with cerebellar lesions [Gerwig et al., 2003, 2010] and adult neuroimaging studies [Molchan et al., 1994; Logan and Grafton, 1995; Blaxton et al., 1996; Schreurs et al., 1997; Ramnani, et al., 2000; Schreurs et al., 2001; Knuttinen et al., 2002; Cheng et al., 2008; Parker et al., 2012]. Findings from these studies support the premise that the

adult cerebellum is critically important for EBC. Functional magnetic resonance imaging (fMRI) studies in adults report activations in cerebellar lobule VI during EBC [Ramnani et al., 2000; Cheng et al., 2008]. In a PET study of older and younger adults [Schreurs et al., 2001], older adults showed decreased regional cerebral blood flow, but both populations demonstrated learning-related changes in the cerebellum. However, to date, no study has directly examined cerebellar activation patterns in children during EBC.

The lack of information relating to cerebellar function in children during EBC represents a significant gap in knowledge. By comparing activity patterns between the developing and adult brain, we can begin to address important questions concerning how and when this fundamental form of learning occurs. This study investigated how development affects cerebellar cortical and deep nuclear activity during delay eyeblink conditioning in children and adults using fMRI.

MATERIALS AND METHODS

Participants

Fourteen children and 16 adults participated in this study, which was conducted at the Cape Universities Brain Imaging Centre (CUBIC) in Cape Town, South Africa. Two children were excluded for failure to respond to the US, and 11 of the remaining 12 (91.7%) met criterion for conditioning. Three adults were excluded for failure to respond to the US and one for excessive spontaneous blinks during baseline. Of the remaining 12, nine (75.0%) met criterion for conditioning. The criteria for conditioning were one or more conditioning sessions with at least 25% CRs and 15% CRs above the level of blinking during pseudoconditioning. These exclusion criteria were modified from those we have used previously [Jacobson et al., 2008] to reflect the general reduction in % CRs observed in the scanner environment and were defined based on visual inspection of the behavioral data before any imaging analyses were performed. Exclusion rates did not differ for the children versus the adults ($\chi^2 = 1.20, P > 0.20$).

Data from the 11 children (seven male, four female; mean age = 11.5 years; range = 9.3–13.8) and nine adults (five male, four female; mean age = 24.9 years; range = 19.0–29.7) who met criteria for conditioning were included in the analyses. Neuroimaging data from one child's last two conditioning sessions were lost due to technical difficulties. None of the children or adults had previously participated in any eyeblink conditioning studies. All procedures were approved by the Human Investigation Committee at Wayne State University

School of Medicine and Faculty of Health Sciences Human Research Ethics Committee at the University of Cape Town.

Procedure

Behavioral apparatus

Stimulus presentation was controlled and behavioral data were recorded using a laptop computer interfaced to an NI USB-6218 data acquisition module running custom software developed under LabView version 7.1 (National Instruments, Austin, TX). Auditory stimuli were presented through the standard Siemens MR scanner headphones. A video (*The Adventures of Milo and Otis*) was projected without its soundtrack through a waveguide in-line with the bore of the magnet onto a rear projection screen positioned behind the bore of the magnet and viewed using an adjustable mirror attached to the single channel head coil. Standard laboratory safety goggles were modified by attaching the end of a polyethylene tube (Nalgene, Rochester, NY), which delivered an airpuff, and an MRI-compatible infrared sensor, which recorded eyeblinks. Airpuff delivery was controlled by a solenoid valve (Asco, Florham Park, NJ), and a fiber-optic probe (RoMack, Williamsburg, VA) measured the reflectance of infrared light from the left eye [Miller et al., 2005; Cheng et al., 2008].

Imaging apparatus

The MR scans were acquired on a 3T Allegra (Siemens, Erlangen, Germany) MRI scanner at the CUBIC. All children were prepared for scanning in a mock scanner where they listened to a recording of the scanner noises.

High-resolution T1-weighted structural MR images were acquired using a three-dimensional (3D) echo planar imaging (EPI)-navigated [Tisdall et al., 2012] multiecho MPRAGE [van der Kouwe et al., 2008] sequence that had been optimized for morphometric analyses using FreeSurfer software. Imaging parameters were: field of view (FOV): $256 \times 256 \text{ mm}^2$; 128 sagittal slices, repetition time (TR): 2,530 ms; echo time (TE): 1.53/3.21/4.89/6.57 ms; inversion time: 1,100 ms; Flip angle: 7° ; voxel size: $1.3 \times 1.0 \times 1.3 \text{ mm}^3$. The 3D EPI navigator provided real-time motion tracking and correction [Tisdall et al., 2012], which served to substantially reduce the presence of any motion artifacts in the structural imaging data.

A T_2^* -weighted gradient echo, EPI pulse sequence was used to collect 205 whole brain functional volumes (6:36 min) sensitive to blood oxygen level dependent contrast (TR 2,000 ms, TE 30 ms, 34 interleaved slices, slice thickness 3 mm, gap 1.5 mm, FOV $200 \times 200 \text{ mm}^2$, in-plane resolution $3.125 \times 3.125 \text{ mm}^2$) during the EBC acquisitions.

Presentation of stimuli

We used a conditioning procedure that produced significant learning in young adults [Cheng et al., 2008, 2010].

The CS tone was a binaural 1,000 Hz tone (95 dB), lasting 750 ms, that co-terminated with a 100 ms corneal airpuff to the left eye (10 psi measured at the delivery site). Trials were grouped into blocks: nine trials/block, 2 s/trial, 4 s inter-trial interval (ITI), such that each block lasted 34 s. Each session consisted of eight 9-trial blocks. Pseudoconditioning consisted of four sets of alternating tone alone and airpuff alone blocks. Conditioning blocks consisted of eight paired CS-US trials, plus a ninth CS-alone test trial. Blocks were separated by 16 s rest periods (Fig. 1A). The ITI in the current paradigm is similar to the ITI we used previously [Cheng et al., 2008] and was selected to ensure a sufficient number of trials to permit analysis of the fMRI data. These temporal parameters were also selected to provide enough trials to permit conditioning within a limited time period and to ensure participant comfort. For children, we performed five scans, one pseudoconditioning and four conditioning sessions. For adults, we performed three scans, one pseudoconditioning and two conditioning sessions. Children were administered two additional conditioning sessions to determine whether, with additional training, their conditioning performance would reach the level of the adults.

Once participants were fitted with the conditioning goggles and positioned in the magnet, they were asked to lie still and watch the video. They were instructed to pay attention to the video as best they could while distracting tones and airpuffs were presented. The children were given a 2-h lunch break between the second and third conditioning sessions.

Data Analysis

The topography of typical behavioral eyeblink responses collected inside the magnet is shown in Figure 1B. The 300-ms time period prior to presentation of the tone was used as a baseline. To determine trials during which CRs occurred, blink amplitudes during this baseline period were compared with the maximum blink amplitude during the 350 ms preceding the airpuff (Fig. 1C). The time window of 350-ms pre-US presentation was selected, in accordance with common conventions in the literature [Finkbiner and Woodruff-Pak, 1991; Jacobson et al., 2008, 2011], to exclude voluntary and alpha blink responses as CRs [Spence and Ross, 1959; Gormezano, 1966]. To qualify as a CR, the difference between the maximum blink amplitude during this 350-ms time window and the mean response amplitude during the baseline had to exceed three times the standard deviation of the mean during the 300-ms baseline period. Performance was expressed as the percentage of trials with valid CRs (% CR). This measure of learning was examined in a session (pseudoconditioning/session 1/session 2) by age (child/adult) repeated measures analysis of variance (ANOVA). Latency of the peak amplitude of the blink response (relative to onset of the 350 ms CR time window) was averaged across the CS

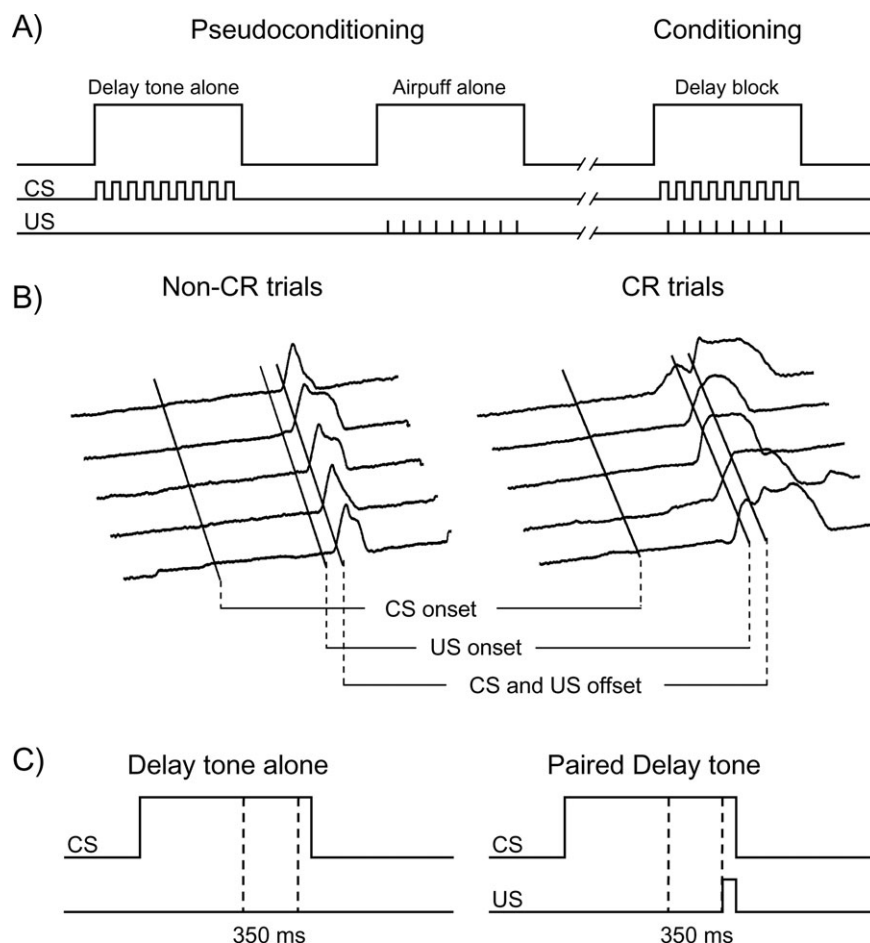


Figure 1.

Study design/analysis and sample topography of eyeblink responses measured in the MRI scanner. **(A)** Pseudoconditioning consisted of alternating four delay tone alone and four airpuff alone blocks. Conditioning sessions consisted of eight blocks of paired CS-US trials. Adults received three separate sessions (1 pseudoconditioning + 2 conditioning) and children received five separate sessions (1 pseudoconditioning + 4 conditioning). **(B)** Typical response profiles are shown for non-CR and CR trials. Peak amplitude responses indicate maximal eye closure. **(C)** Dotted lines indicate the time window from which eyeblink responses were sampled.

tone alone trials (ninth trial in each conditioning block; to avoid UR contamination) to examine age-related differences in the timing of eyeblink responses. For pseudoconditioning, all nine tone alone trials from the four tone alone blocks were used in the peak latency analysis.

Structural and functional imaging preprocessing and statistical analyses were performed with Statistical Parametric Mapping (SPM2 and SPM8) software (Wellcome Department of Cognitive Neurology, London, UK). Preprocessing included motion correction, structural data coregistration, normalization, and smoothing. EPI functional images were realigned and resliced correcting for minor motion artifacts, and structural images were co-registered to the mean motion-corrected functional image for each participant. Cerebellar structural and functional data were isolated and normalized into standard stereotaxic space using the spatially unbiased atlas template (SUIT) of the human cerebellum

and brainstem [Diedrichsen, 2006], and the functional images were smoothed with a Gaussian filter (full-width half-maximum 5 mm). Given the children's age range, all structural and functional data were carefully inspected to ensure proper normalization and every child was found to be acceptable. Following SUIT transformation, voxel dimension was 2 mm³. The general linear model was used to estimate individual subject activations and a random-effects analysis was conducted on all subjects.

Contrasts were designed to investigate brain activity changes as a function of associative learning (conditioning versus pseudoconditioning) as well as age-related differences (children versus adults). To control for Type I error, Monte Carlo simulations [Forman et al., 1995] were performed, which indicated that activation clusters of at least 10 voxels were significant at a $P < 0.01$ (corrected) level. Activation clusters within the cerebellum surviving this

and brainstem [Diedrichsen, 2006], and the functional images were smoothed with a Gaussian filter (full-width half-maximum 5 mm). Given the children's age range, all structural and functional data were carefully inspected to ensure proper normalization and every child was found to be acceptable. Following SUIT transformation, voxel dimension was 2 mm³. The general linear model was used to estimate individual subject activations and a random-effects analysis was conducted on all subjects.

threshold and also previously reported to be involved during EBC were used in region-of-interest (ROI) analyses. These included a region in lateral lobule VI [Yeo et al., 1984; Ramnani et al., 2000; Cheng et al., 2008] and vermis VI. The latter was selected due to its involvement during event timing [Spencer et al., 2007] and the role of timing during CR expression [Gerwig et al., 2005]. Exploratory analyses were performed to examine the relations of additional large clusters of activity in left Crus II and right lobule VIII to learning [Gerwig et al., 2003; Plakke et al., 2007]. Coordinates for the peak response within each ROI were identified at the group level and served as the center of a 3D sphere (4 mm radius) that was created to sample individual subject mean brain activity in that ROI. This radius was chosen to restrict functional activity to within the anatomical boundaries of these regions. In addition to this functional ROI approach, anatomically derived ROIs were generated for the deep nuclei (which were subsequently isolated into the dentate, fastigial, and interposed nuclei) and the hippocampus using probabilistic maps [Amunts et al., 2005; Dimitrova et al., 2006; Diedrichsen et al., 2011], in light of the extensive evidence of the involvement of these regions in eyeblink conditioning [Berger et al., 1976; McCormick and Thompson, 1984a; Christian and Thompson, 2003]. Estimated mean and peak beta weights generated from ROI analyses were used as indices of brain activation. Mean and peak responses within the ROIs were examined in relation to behavioral performance to assess the relation between neural activation and behavioral CRs.

RESULTS

Behavioral Findings

Significant across session differences in eyeblink CRs (% CRs) were observed in both children and adults (Fig. 2). A repeated-measures ANOVA showed main effects of session, $F(1,18) = 42.63$, $P < 4.0 \times 10^{-6}$ and age, $F(1,18) = 7.94$, $P < 0.02$. The session \times age interaction fell short of statistical significance $F(1,18) = 3.52$, $P < 0.08$. Post hoc comparisons showed that child ($M \pm SD = 12.88 \pm 12.00\%$ CR) and adult (18.16 ± 6.31) performance did not differ during pseudoconditioning, $t(18) = 1.19$, $P > 0.20$, but the adults produced a significantly greater percentage of CRs than the children during both conditioning session 1 (49.77 ± 19.37 and 27.78 ± 13.00 , respectively), $t(18) = 3.03$, $P < 0.008$, and session 2 (55.81 ± 24.52 and 33.71 ± 17.15 , respectively), $t(18) = 2.37$, $P < 0.03$.

Within-group ANOVAs showed main effects of session for both the children, $F(1,10) = 6.19$, $P < 0.04$, and the adults, $F(1,8) = 17.97$, $P < 0.003$. Post hoc comparisons showed that both the children (P 's < 0.05) and the adults (P 's < 0.004) produced significantly more CRs during conditioning relative to pseudoconditioning. Importantly, there were no significant differences in percent unconditioned responses across sessions (pseudoconditioning: $M \pm SD = 87.44 \pm 4.75$, conditioning 1: 87.74 ± 2.01 , conditioning 2:

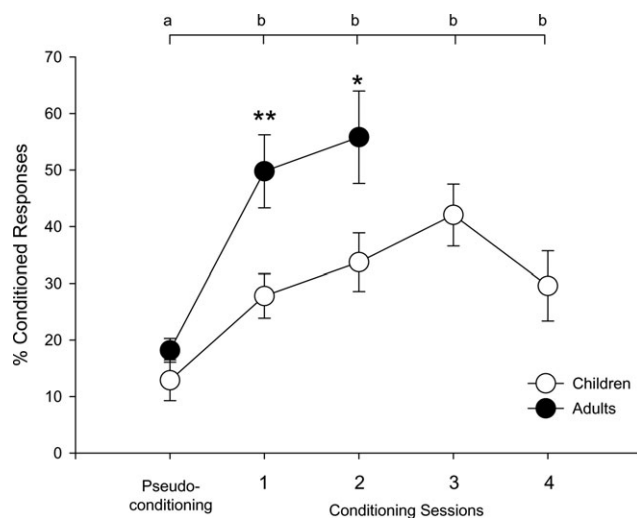


Figure 2.

Behavioral findings in children and adults. A significantly greater percentage of CRs was exhibited during conditioning relative to pseudoconditioning, indicating that both groups learned the CS-US association. Between session contrasts: % CRs in each session labeled “b” was significantly greater (at $P < 0.05$) than during pseudoconditioning, which is labeled “a.” Between group contrasts: adults produced significantly more CRs than children during sessions 1 and 2 (** $P < 0.01$; * $P < 0.05$).

84.71 ± 2.42), $F(1,18) = 0.24$, $P > 0.20$, or between children (87.55 ± 2.16), and adults (85.52 ± 4.70), $F(1,18) = 0.18$, $P > 0.20$. Thus, these behavioral findings indicate that, although all participants met criteria for conditioning and produced similar rates of unconditioned responses, the adults produced more CRs than the children.

Repeated-measures ANOVA on latency of peak responses on CS alone trials showed a main effect of session, $F(1,17) = 54.83$, $P < .001$ but not age, $F(1,17) = 1.31$, $P > 0.20$. The session \times age interaction fell short of statistical significance, $F(1,17) = 3.87$, $P < 0.07$. Post hoc comparisons showed that child and adult performance did not differ during pseudoconditioning, $t(17) = 1.36$, $P > 0.19$, but the children’s peak latency (715.91 ± 81.63 ms) was significantly longer than the adults (458.56 ± 69.27 ms) during conditioning session 1, $t(18) = 2.34$, $P < 0.04$, but not during session 2, $t(18) = 0.86$, $P > 0.20$.

Neuroimaging Findings

Initial imaging analyses focused primarily on differences in brain activity between pseudoconditioning and conditioning session 1, because that was when initial learning occurred (Fig. 2). Whole cerebellar analyses of children and adults revealed structures that demonstrated significantly ($P < 0.01$, corrected) greater responses during conditioning session 1 relative to pseudoconditioning (Table I). Significant cerebellar areas of activation in children (Fig. 3)

TABLE I. Significant activations during Session 1 relative to Pseudoconditioning

a) Children							
Hemisphere	X	Y	Z	SPM {Z}	N Vox	Brain region	
Right	26	-48	-48	4.8	572	Lobule VIII	
	26	-52	-58	3.7		Lobule VIII	
	18	-42	-54	3.5		Lobule IX	
	6	-64	-30	3.3		Lobule VIII	
	20	-86	-24	3.4		Crus I	
	18	-68	-42	3.2		Lobule VIII	
	44	-58	-30	2.9		Crus I	
	26	-56	-30	3.3		Lobule VI	
	18	-58	-48	2.9		Lobule VIII	
	Left	-42	-58	-46		3.7	65
-30		-38	-34	4.1	50	Lobule VI	
-24		-68	-28	3.9	42	Lobule VI	
-2		-48	-16	3.1	28	Vermis IV,V	
-18		-42	-50	3.2	24	Lobule IX	
-22		-30	-28	3	21	Lobule IV, V	
-44		-60	-24	3.3	20	Crus I	
-16		-44	-46	2.8	13	Lobule IX	
2		-66	-18	3	12	Vermis VI	
-12		-50	-38	3	10	Lobule IX	
-6		-44	-54	2.9	10	Lobule IX	
b) Adults							
Right		44	-44	-54	3.1	45	Lobule VIII
	30	-40	-54	2.9	Lobule VIII		
	52	-56	-28	2.7	Crus I		
	16	-76	-38	3.3	Crus II		
Left	46	-58	-42	3.1	12	Crus II	
	-16	-34	-18	2.9		21	Lobule IV, V

MNI coordinates of activation maxima in the cerebellum (Schmahmann et al., 2000) in children (a) and adults (b). Regions listed were thresholded at a minimum cluster size of 10 voxels and z-scores of $P < 0.01$ (corrected). Indented entries represent local maxima within the main cluster.

included lateral lobules IV, V, VI, VIII, IX, Crus I and II, and vermis IV, V, and VI. Although the adults activated fewer cerebellar areas, most of those overlapped with areas activated in the children (e.g., lobule VIII, Crus I).

Between-group comparisons between children and adults also showed significant age-related differences in cerebellar activations (Fig. 4). Although the adults produced more CRs, the children showed significantly ($P < 0.01$, corrected) greater activation in the pons, cerebellar lateral lobule VIII, lateral lobule IX, Crus I, and vermis VI compared to adults (Table II). By contrast, the adults did not show any areas of activation that were significantly greater than the children's during conditioning session 1.

Relation of Neuroimaging Data to Behavior

Correlation analyses relating brain activity to behavior were performed to identify regions that may contribute to the expression of CRs. Mean activation within two functionally (lateral lobule VI and vermis VI) and five anatomically

(cerebellar deep nuclei, dentate nucleus, fastigial nucleus, interposed nuclei, and hippocampus) defined ROIs was examined in relation to % CRs within each session (Table III), and mean activity within these ROIs during session 1 was also examined in relation to % CRs during each of the subsequent conditioning sessions (Table IV).

Mean activity in left and right deep nuclei and vermis VI in session 1 was significantly correlated with CRs during conditioning session 1 in the children (Table III, Figs. 5 and 6). Mean activity within each of the cerebellar subnuclei (dentate, fastigial, and interposed) was also significantly correlated with CRs during session 1 in children. Scatter plots with regression lines show the strong, positive relation of mean activity in left cerebellar deep nuclei and each of the subnuclei to % CRs during session 1 (Fig. 5). When session 1 brain activity was examined in relation to subsequent behavioral performance (sessions 2–4), activity in the left deep nuclei and vermis VI was also significantly correlated with CRs during session 2 in the children (Table IV). Significant correlations between CRs during session 2 and cerebellar subnuclei activity in session 1 were only detected in the left dentate and right interposed nuclei while correlations with the left interposed and right dentate nuclei approached significance.

In adults, mean activity in the left dentate nucleus was significantly correlated with CRs during session 1 but the correlation for all other regions fell short of statistical significance (Table III). Finally, age of children and adults was not significantly correlated with either behavioral CRs or brain beta weights (r 's ranged from -0.06 to $+0.25$ all P 's > 0.20).

DISCUSSION

This is the first fMRI study to examine the neural substrates underlying EBC in children, compared with adults. Both children and adults demonstrated successful conditioning in the MRI environment. Although both groups produced similar rates of unconditioned responses, the adults produced more CRs than the children. Significant learning-related activity was distributed throughout the cerebellar cortex in all participants, suggesting multiple sites of plasticity. Despite higher levels of behavioral conditioning in the adults, the children showed greater activity in multiple areas within the cerebellar cortex, suggesting that they may require greater activation and/or the recruitment of supplementary structures to achieve successful conditioning. The strong correlations between the imaging data and the learned behavioral responses further emphasized the importance of the cerebellar deep nuclei and vermis VI in the expression of conditioned eyeblink responses.

Behavior

Behavioral EBC studies have demonstrated that humans ranging from infants as young as 5 months to older adults show successful conditioning [Woodruff-Pak and

Conditioning Session 1 > Pseudoconditioning in Children

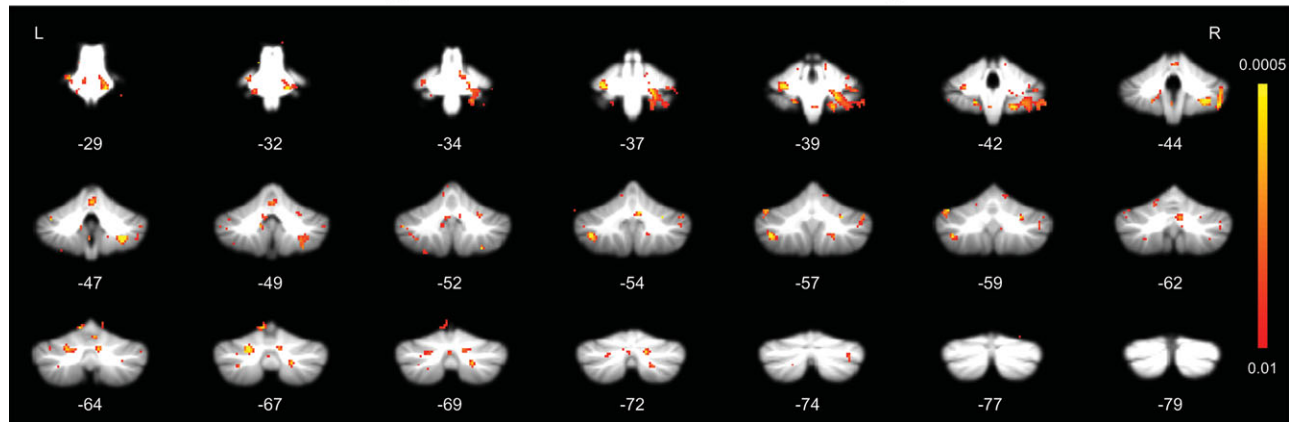


Figure 3.

Whole cerebellum analyses showing regions with significantly greater responses during session I of conditioning relative to pseudoconditioning in children.

Thompson, 1988; Knutten et al., 2001; Clafin et al., 2002; Herbert et al., 2003; Jacobson et al., 2008; Cheng et al., 2010; Stanton et al., 2010; Jacobson et al., 2011]. In the current study, children and adults demonstrated significantly greater % CRs during conditioning compared to pseudoconditioning, suggesting both groups learned the CS-US association. Adults showed higher levels of conditioning than children, even though the children received twice as many trials, providing evidence of continued developmental change from child to adulthood in this domain. Importantly, although their learning was not as robust as adults, children showed significant conditioning inside the MRI environment.

The current behavioral findings, coupled with results from other studies on how aging affects conditioning

[Woodruff-Pak and Thompson, 1988; Knutten et al., 2001; Clafin et al., 2002; Herbert et al., 2003; Jacobson et al., 2008; Cheng et al., 2010; Stanton et al., 2010; Jacobson et al., 2011], suggest that EBC performance is optimal during young adulthood and less robust during childhood and in older populations. The present findings in children extend our knowledge on EBC performance over the human lifespan.

Learning-Related Changes in Brain Activity

Laboratory animal work has identified the cerebellar cortex as one important site of plasticity for EBC, particularly lateral lobule VI [Yeo et al., 1984, 1985; Lavond and

Children > Adults in Conditioning Session 1

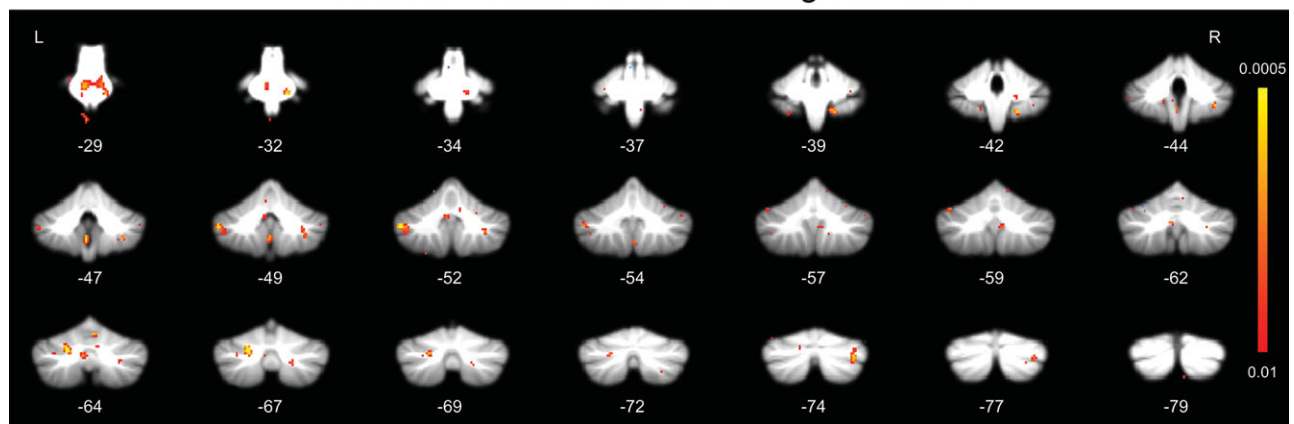


Figure 4.

Whole cerebellum analyses showing regions with significantly greater response in children relative to adults during session I of conditioning (minus pseudoconditioning).

TABLE II. Greater activations in children relative to adults during Session 1

Hemisphere	X	Y	Z	SPM [Z]	N Vox	Brain region
Right	8	-26	-30	3.9	166	Pons
	32	-46	-48	3.2	27	Lobule VIII
	34	-76	-36	3.3	25	Crus I
	16	-42	-54	3.4	19	Lobule IX
Left	2	-66	-18	3.1	13	Vermis VI
	-50	-52	-40	3.8	62	Crus I
	-22	-68	-32	3.7	41	Crus I
	-4	-48	-50	3.3	25	Lobule IX
	-8	-64	-36	3.2	23	Lobule VIII
	-6	-52	-32	2.7	12	Lobule IX

MNI coordinates of activation maxima in the cerebellum (Schmahmann et al., 2000) in children relative to adults. Regions listed were thresholded at a minimum cluster size of 10 voxels and z-scores of $P < 0.01$ (corrected).

Steinmetz, 1989; Yeo and Hardiman, 1992; Harvey et al., 1993; Nolan and Freeman, 2006]. Rats with damage to Purkinje cells in the cerebellar cortex were impaired in the acquisition and extinction of eyeblink conditioning [Nolan and Freeman, 2006], and aspirations of lateral lobule VI were found to impair normal acquisition of eyeblink conditioning in rabbits [Yeo et al., 1984, 1985]. In humans, conditioning levels in patients with damage to lateral lobule VI were reduced compared to controls [Gerwig et al., 2003], and fMRI findings showed that this area of

cortex responds during delay EBC [Ramnani et al., 2000; Cheng et al., 2008]. The present data revealed two distinct foci of activation within left lateral lobule VI for the children (Table I), suggesting multiple regions of plasticity within this lobule. This finding is consistent with evidence that partial lesions to this structure may not be sufficient to eliminate conditioning completely [Yeo and Hardiman, 1992; Harvey et al., 1993].

Differential responding in the adult cerebellar cortex has been reported in several neuroimaging studies on human EBC [Molchan et al., 1994; Logan and Grafton, 1995; Blaxton et al., 1996; Schreurs et al., 1997; Ramnani et al., 2000; Knuttinen et al., 2002; Cheng et al., 2008; Parker et al., 2012], although PET studies have produced varying results—decreases in one study [Molchan et al., 1994] and increases in another [Logan and Grafton, 1995]. Using fMRI and the cerebellar SUIT template [Diedrichsen, 2006], the present study was able to improve localization and report the spatial extent of activation within the cerebellar cortex (Tables I and II). Although Ramnani et al. [2000] showed increased fMRI activation in lateral lobule VI in adults, the adults in the current study did not show learning-related activity in this area. This lack of differential activity may reflect a more rapid acquisition due to the relatively simple conditioning protocol. The present study used a single-cue conditioning protocol and reinforced 8/9 trials within each block, whereas Ramnani et al. [2000] used a differential conditioning protocol and a 50% reinforcement rate. The greater activation levels in children,

TABLE III. Relation of mean brain activity to percent conditioned responses within each session

		Structure					
<i>a) Children</i>							
Session	Left Deep Nuclei	Right Deep Nuclei	Left Lobule VI	Right Lobule VI	Vermis VI	Left Hippocampus	Right Hippocampus
1	0.78**	0.65*	0.38	0.08	0.88***	0.37	0.20
2	0.48	0.47	-0.04	0.11	0.27	0.30	0.34
3	0.37	0.36	0.41	0.49	0.40	0.57†	0.35
4	-0.17	0.08	-0.02	0.10	0.03	-0.15	-0.07
Session	Left Dentate	Left Fastigial	Left Interposed	Right Dentate	Right Fastigial	Right Interposed	
1	0.73*	0.77**	0.67*	0.65*	0.67*	0.79**	
<i>b) Adults</i>							
Session	Left Deep Nuclei	Right Deep Nuclei	Left Lobule VI	Right Lobule VI	Vermis VI	Left Hippocampus	Right Hippocampus
1	0.53	0.02	0.08	0.53	0.31	0.51	-0.20
2	-0.03	0.05	0.07	0.36	0.32	0.40	0.18
Session	Left Dentate	Left Fastigial	Left Interposed	Right Dentate	Right Fastigial	Right Interposed	
1	0.73*	-0.13	0.13	0.09	0.06	0.18	

Pearson correlation values between mean brain activity and behavioral performance within each session. Mean activity in the deep nuclei and vermis VI significantly correlated with conditioned responses during session 1 in children. Activity in the sub-nuclei (dentate, fastigial, and interposed) were also significantly correlated during session 1 in children.

† $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Bold values indicate correlation coefficients that were significant or approached significance.

TABLE IV. Relation of mean brain activity in session 1 to percent conditioned responses in each of the sessions

		Structure						
<i>a) Children</i>		Left Deep Nuclei	Right Deep Nuclei	Left Lobule VI	Right Lobule VI	Vermis VI	Left Hippocampus	Right Hippocampus
Session								
1		0.78**	0.65*	0.38	0.08	0.88***	0.37	0.20
2		0.67*	0.57[†]	0.48	0.37	0.66*	0.45	0.32
3		0.40	0.42	0.70*	0.13	0.29	0.42	0.22
4		0.05	0.09	0.21	-0.11	0.03	-0.01	-0.07
Session		Left Dentate	Left Fastigial	Left Interposed	Right Dentate	Right Fastigial	Right Interposed	
1		0.73*	0.77**	0.67*	0.65*	0.67*	0.79**	
2		0.65*	0.26	0.54[†]	0.58[†]	0.36	0.65*	
<i>b) Adults</i>		Structure						
Session		Left Deep Nuclei	Right Deep Nuclei	Left Lobule VI	Right Lobule VI	Vermis VI	Left Hippocampus	Right Hippocampus
1		0.53	0.02	0.08	0.53	0.31	0.51	-0.20
2		0.31	-0.11	0.51	0.50	0.38	0.53	-0.03
Session		Left Dentate	Left Fastigial	Left Interposed	Right Dentate	Right Fastigial	Right Interposed	
1		0.73*	-0.13	0.13	0.09	0.06	0.18	
2		0.53	-0.37	-0.22	-0.03	-0.13	-0.11	

Pearson correlation values between mean brain activity during session 1 and behavioral performance in each of the sessions. Session 1 activity in the left deep nuclei and vermis VI significantly correlated with conditioned responses during sessions 1 and 2 in children.

[†] $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Bold values indicate correlation coefficients that were significant or approached significance.

who do not learn as well as adults, may reflect an active learning process and are consistent with previous fMRI studies using more complex designs [Ramnani et al., 2000; Cheng et al., 2008].

Gerwig et al. [2010] reported that patients with focal and degenerative lesions to lateral lobule VI showed deficits in CR acquisition even after multiple training sessions, indicating that this region is involved in the learning of the CS-US association. The rapid acquisition by the adults in the current study makes it difficult to detect changes in the fMRI signal related to an active learning process. The lack of lateral lobule VI activation in the adults, therefore, does not rule out an important role for this area during acquisition but rather suggests that CR expression, in well-trained participants, does not critically rely on this area. It is possible that if the adult's acquisition rate were reduced (similar to that of the children), acquisition-related activity would have been detected in this area.

Large, significant learning-related activations in right lobule VIII were found in all participants, and age-related comparisons showed that children activated this region bilaterally more than adults. Gerwig et al. [2003] found that patients with lesions in the inferior cerebellar cortex (e.g., lobule VIII) were not as impaired during EBC as patients with lesions to the superior cerebellar cortex (e.g., lobule VI). Thus, lobule VIII may play a supportive, but not critical role during EBC. These authors also found that

relative to unilateral cerebellar cortical lesions, bilateral lesions produced modest (but not statistically significant) impairments during EBC, and animal studies suggest that contralateral cerebellar cortical lesions do not affect eyeblink conditioning [Freeman, et al., 1995b]. In this study, bilateral cerebellar cortical activations in children were detected in lobules VI, IX, and Crus I (Table I). In conjunction with lesion data [Freeman et al., 1995b; Gerwig et al., 2003], bilateral cerebellar activation in PET work [Logan and Grafton, 1995; Blaxton et al., 1996], and the presence of bilateral eyeblink CRs [Disterhoft et al., 1977; Campolattaro and Freeman, 2009], the present neuroimaging findings support the idea that contralateral regions of the cerebellar cortex may play a modulatory but not necessary role during EBC. The widespread fMRI activations in the current study suggest that delay EBC engaged multiple structures throughout the cerebellar cortex [Plakke et al., 2007; Gerwig et al., 2010].

Differential activation within the cerebellar deep nuclei was not seen in any conditioning versus pseudoconditioning comparisons in the present study. This is consistent with the majority of neuroimaging investigations of human eyeblink conditioning [Molchan et al., 1994; Blaxton et al., 1996; Schreurs et al., 1997; Ramnani et al., 2000; Schreurs et al., 2001; Knuttinen et al., 2002; Cheng et al., 2008; Parker et al., 2012]. Given that the only study to report cerebellar deep nuclear activation during

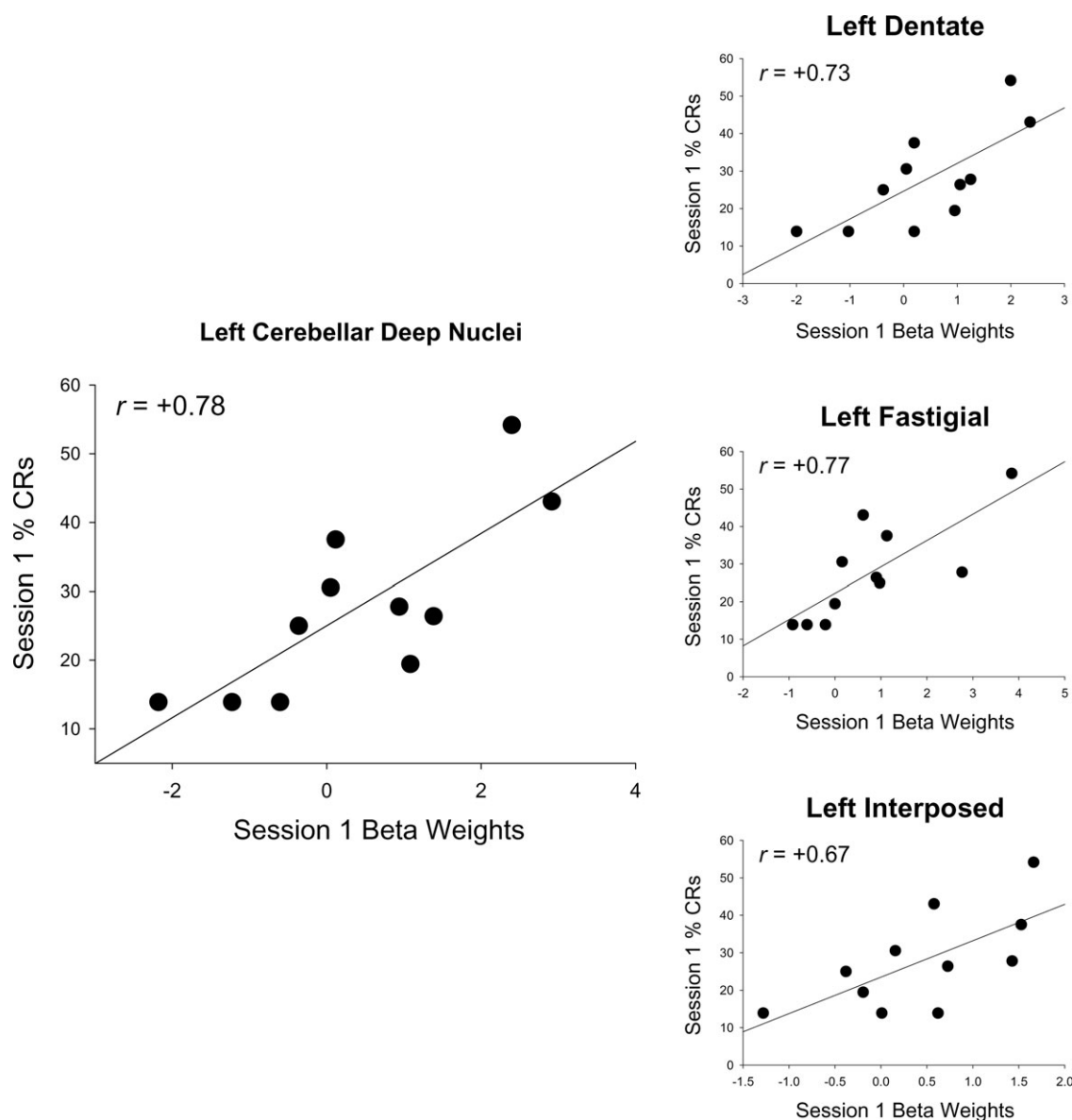
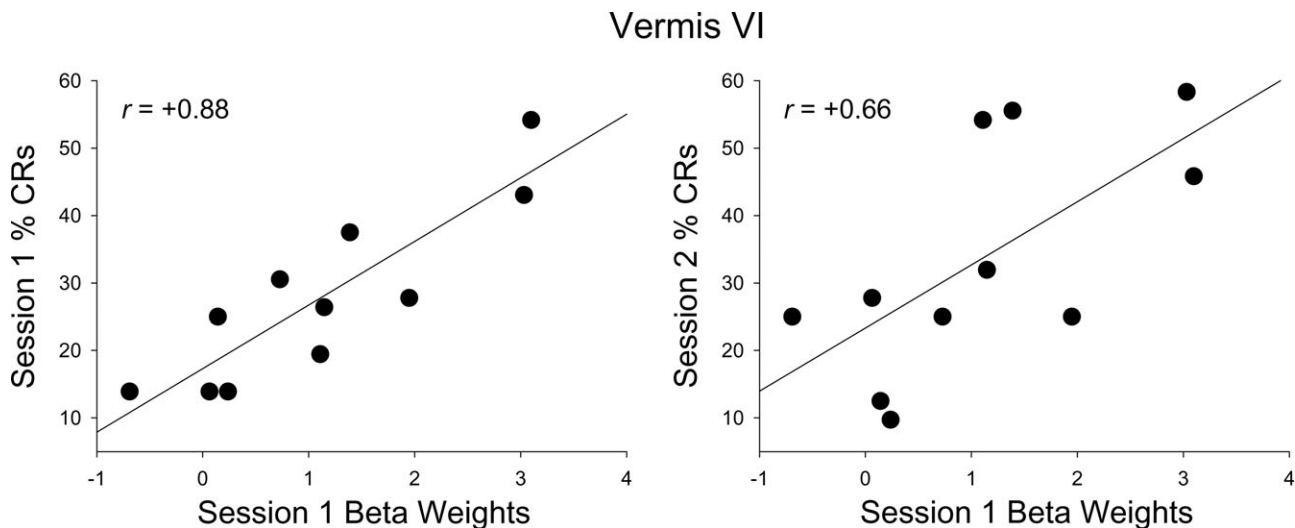


Figure 5.

Relation of mean activation in left cerebellar deep nuclei to CRs in children. Left panel shows that activity in the left cerebellar deep nuclei was positively correlated with CRs in session 1. Smaller graphs on the right show this region separated into dentate, fastigial, and interposed nuclei and show that session 1 activity within these subnuclei also correlated with CRs.

human EBC used PET [Logan and Grafton, 1995], it is possible that current fMRI techniques and analytic approaches may not be optimal for characterizing activity in these regions. The lack of differential activity in the present study may also be due to significant elevated responding in the deep nuclei elicited during pseudoconditioning. Unpaired CS-US presentations appear to be sufficient to activate the deep nuclei due to stimulation of the afferent mossy fiber (pons) and climbing fiber (in-

ferior olive) pathways that transmit the CS and US, respectively [Mauk et al., 1986; Steinmetz et al., 1986]. Alternatively, although the deep nuclei have been identified as a critical site of plasticity in EBC in laboratory animals [Lavond et al., 1984; McCormick and Thompson, 1984a,b; Lavond et al., 1985; Christian and Thompson, 2003], it is possible that the relative role of the deep nuclei in the circuitry subserving this form of learning differs between species.

**Figure 6.**

Relation of mean activation in vermis to CRs in children. Activity in the vermis was positively correlated with CRs in sessions 1 and 2.

Age-Related Changes in Brain Activity

As indicated earlier, despite adults' producing more CRs, children showed greater activation in several regions, including the pons and cerebellar cortical structures (Fig. 4 and Table II). Conversely, adults did not show greater activation in any cerebellar region. The pons are part of the essential neural circuitry underlying EBC and serve as a pathway for auditory CS afferents [Christian and Thompson, 2003]. The increased cerebellar activation exhibited by children may represent a more active learning process given their lower conditioning levels as compared to adults. The children's more distributed activation maps compared to adults (Tables I and II) support the idea that children may recruit supplementary structures to achieve successful conditioning, which is consistent with studies showing that children exhibit more extensive and diffuse activations to achieve the same level of performance as adults in other neuroimaging tasks as well [Rivera et al., 2005; Meintjes et al., 2010; De Guio et al., 2012].

Behavioral analyses of the latency of peak responses showed that relative to children, adults' peak responses occurred earlier; that is, closer to when the US would have been presented on the nonreinforced CS tone alone trials during conditioning session 1. Despite their poorer timing, children showed greater activation in vermis VI (Table II), which has been linked to cerebellar-mediated timing in finger tapping studies [Spencer et al., 2007; Stoodley et al., 2010]. Timing of the blink response is critically important in EBC, as poorly timed responses do not fully protect the eye from the airpuff. The significant correlations between activity in vermis VI and CRs during sessions 1 and 2 further confirm the importance of the cerebellar cortex in event timing [Perrett, et al., 1993; Ivry et al., 2002; Gerwig et al., 2005; Spencer et al., 2007] in EBC (Fig. 6). Left ante-

rior lobules IV and V were also more active in all participants. The anterior cerebellar cortex in adult rabbits has been implicated in CR timing [Perrett et al., 1993], which becomes more accurate between adolescence and adulthood in the rat [Brown et al., 2006].

Neurodevelopmental changes presumably mediate the age-related changes in brain activation during EBC seen in this study. Whole brain volume is not likely to account for these changes because children (7–11 years) have approximately 95% of the volume of the adult brain [Caviness et al., 1996]. However, nonlinear changes in cerebral gray and white matter volume continue throughout the lifespan [Sowell et al., 2003]. Although the greatest changes in myelination in the cerebellar peduncles occur during the first 36 months of human development, a gradual increase in myelination continues through age 11 years [Saksena et al., 2008]. The mechanisms mediating the developmental changes seen here require further investigation.

Behavior and Neuroimaging

The analysis examining the relation between neuroimaging data and behavioral CRs highlighted several regions. Activity in the lateral cerebellar cortex was related to learning rates in children and adults, but these correlations fell short of statistical significance in this small sample. For example, moderate correlations were seen between activation in left lobule VI and session 1 performance in children (Table III) and between right lobule VI activation and session 1 performance in adults (Table III). Other large clusters of activity that were examined in relation to children's CR performance but did not reach statistical significance in this small sample were left Crus II ($r = +0.24$, $P = 0.49$) and right lobule VIII ($r = +0.35$, $P = 0.29$).

Children's mean activity in the cerebellar deep nuclei in session 1 was significantly correlated with CRs in sessions 1 and 2 (Table IV). The relation between brain activity during session 1 and behavioral CRs during session 2 suggests that brain processes associated with initial CS-US learning may be predictive of future performance. Deep nuclear activity is critically involved in the acquisition and expression of the behavioral conditioned eyblink response in laboratory animals [McCormick and Thompson, 1984a, b]. Although the interposed nuclei have been identified as a critical site of plasticity in animal EBC studies [Lavond et al., 1985], activity within all three subnuclei (dentate, fastigial, and interposed) was significantly correlated with conditioned responding in children in this study (Fig. 5). Significant learning-related activation was not detected in the interposed nuclei in adults in this and previous fMRI eyblink conditioning studies [Ramnani et al., 2000; Knutinen et al., 2002; Cheng et al., 2008], possibly due to iron accumulation in this region associated with normal development. The MRI signal within the deep cerebellar nuclei is susceptible to iron deposits, and the variability of mean signal intensities in this region also significantly increases with age [Maschke et al., 2004]. An fMRI study in rabbits showed increased activation in the anterior interposed nuclei during eyblink conditioning [Miller et al., 2003], and human cerebellar deep nuclei activity, as assessed with PET, was correlated with conditioned eyblink responses [Logan and Grafton, 1995]. This is the first fMRI study to show that activity in the interposed nuclei was significantly correlated with human eyblink conditioned responding.

Activity in only the left dentate nucleus was significantly correlated with CRs in adults. A secondary analysis revealed that if peak responses within the ROI are used as the index of brain activity, strong positive correlations with behavior are found in the left ($r = +0.86$, $P < 0.003$), but not right ($r = +0.20$, $P > 0.20$), deep nuclei. These correlations seem to be largely driven by peak activity within the left dentate nucleus ($r = +0.73$, $P < 0.03$). It was somewhat unexpected that the dentate and not interposed nuclei showed activity correlated with CRs. Although this specific nucleus is not thought to be crucial for EBC, animal recordings from and lesions to the dentate-interposed nuclear complex [Bracha et al., 1994; McCormick and Thompson, 1984a] suggest that the dentate activity detected in the present study may contribute to, but is probably not necessary for, EBC.

Limitations

Limitations of the present study include the relatively small sample. Certain correlations reported in Tables III and IV might have reached statistical significance given more power. Another limitation is that our children's age range (9.3–13.8 years), although narrow, may have encompassed more than one stage of neurodevelopment. Finally,

the MRI environment, including loud noise and discomfort associated with lying in the bore of the scanner during learning, may have contributed to the relatively low levels of conditioning in this study.

Summary

This is the first fMRI investigation of the neural substrates underlying EBC to compare responses in children and adults. Thus, it is the first study to begin to address important questions regarding the ontogeny of eyblink conditioning, including how the developing brain processes this fundamental learning task. Additional studies are needed to determine when during development brain activity and behavioral responses begin to resemble more mature brain function. These questions may further aid in the diagnoses and treatment of neurodevelopmental disorders known to affect EBC, such as fetal alcohol syndrome [Jacobson et al., 2008, 2011].

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