Modulation of 7 T fMRI Signal in the Cerebellar Cortex and Nuclei During Acquisition, Extinction, and Reacquisition of Conditioned Eyeblink Responses

Thomas M. Ernst ^{1,2,*} Markus Thürling,^{1,2} Sarah Müller,¹ Fabian Kahl,¹ Stefan Maderwald,² Marc Schlamann,^{3,4} Henk-Jan Boele,⁵ Sebastiaan K. E. Koekkoek,⁵ Jörn Diedrichsen,⁶ Chris I. De Zeeuw,^{5,7} Mark E. Ladd,^{2,8} and Dagmar Timmann¹

¹Department of Neurology, Essen University Hospital, Essen, Germany

²Erwin L. Hahn Institute for MRI, University of Duisburg-Essen, Essen, Germany

³Department for Diagnostic and Interventional Radiology and Neuroradiology, Essen University Hospital, University of Duisburg-Essen, Essen, Germany

⁴Department of Neuroradiology, University Hospital of Giessen, Justus-Liebig-University of Giessen, Giessen, Germany

⁵Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands

⁶Department for Computer Science, University of Western Ontario, London, Ontario, Canada

⁷The Netherlands Institute for Neuroscience, Royal Academy of Arts & Sciences, Amsterdam, The Netherlands

⁸Division of Medical Physics in Radiology, German Cancer Research Center, Heidelberg, Germany

Abstract: Classical delay eyeblink conditioning is likely the most commonly used paradigm to study cerebellar learning. As yet, few studies have focused on extinction and savings of conditioned eyeblink responses (CRs). Saving effects, which are reflected in a reacquisition after extinction that is faster than the initial acquisition, suggest that learned associations are at least partly preserved during extinction. In this study, we tested the hypothesis that acquisition-related plasticity is nihilated during extinction in the cerebellar cortex, but retained in the cerebellar nuclei, allowing for faster reacquisition. Changes of 7 T functional magnetic resonance imaging (fMRI) signals were investigated in the cerebellar cortex and nuclei of young and healthy human subjects. Main effects of acquisition, extinction, and reacquisition against rest were calculated in conditioned stimulus-only trials. First-level β values were determined for a spherical region of interest (ROI) around the acquisition peak voxel in lobule VI, and dentate and interposed nuclei ipsilateral to the unconditioned stimulus. In the cerebellar cortex and nuclei, fMRI signals were significantly lower in extinction compared to acquisition and reacquisition, but not significantly different between acquisition and reacquisition. These findings are consistent with

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: German Research Foundation; Contract grant number: DFG TI 239/10-1, 10-2; Contract grant sponsor: Dutch Organization for Medical Sciences, Life Sciences, and Social and Behavioral Sciences; Contract grant sponsor: ERC-adv and ERC Poc

***Correspondence to:** Thomas M. Ernst; Essen University Hospital, Department of Neurology, Hufelandstr. 55, 45147 Essen, Germany. E-mail: thomas.m.ernst@uni-duisburg-essen.de

Received for publication 17 June 2016; Revised 25 April 2017; Accepted 26 April 2017.

DOI: 10.1002/hbm.23641

Published online 5 May 2017 in Wiley Online Library (wileyonlinelibrary.com).

the theory of bidirectional learning in both the cerebellar cortex and nuclei. It cannot explain, however, why conditioned responses reappear almost immediately in reacquisition following extinction. Although the present data do not exclude that part of the initial memory remains in the cerebellum in extinction, future studies should also explore changes in extracerebellar regions as a potential substrate of saving effects. *Hum Brain Mapp* 38:3957–3974, 2017. © 2017 Wiley Periodicals, Inc.

Key words: associative learning; delay conditioning; cerebellar imaging; human; deep cerebellar nuclei

INTRODUCTION

A fundamental ability of the brain is to learn new stimulus–response associations, including motor, cognitive, emotional, and autonomic responses. Equally important, the brain is also able to extinguish previously learned associations if no longer needed. There is good evidence that the cerebellum contributes to the acquisition and retention of new associations [Christian and Thompson, 2003; Longley and Yeo, 2014; Thieme et al., 2013]. Classical eyeblink conditioning has been studied in greatest detail. The cerebellum is also known to be involved in the extinction of conditioned eyeblink responses [Bengtsson et al., 2007; Jirnhed et al., 2007; Ramnani and Yeo, 1996]. Comparatively few studies, however, addressed the underlying cerebellar mechanisms (De Zeeuw and Ten Brinke [2015], Hu et al. [2015] and Robleto et al. [2004] for reviews). Based on his early observations of spontaneous recovery in appetitive conditioning, Pavlov [1927] suggested that extinction involves a newly learned inhibition of the original association. Other learning theorists have proposed that extinction leads to the erasure of the original association [Rescorla and Wagner, 1972]. These two hypotheses are not mutually exclusive. Part of the initial memory may be erased within the cerebellum, whereas other parts are retained and are under newly learned inhibitory control of extracerebellar areas. The same extinction neural network may be at work, which is known to suppress the expression of conditioned fear responses [Milad and Quirk, 2012].

In addition to spontaneous recovery, phenomena such as reinstatement, renewal, and savings support the idea that the initial memory is at least partly preserved during extinction [Bouton, 2014; Todd et al., 2014]. After extinction, conditioned responses (CRs) return following the presentation of a single unconditioned stimulus (US; termed as reinstatement) or in a context that is different from the extinction context (termed as renewal) [Bouton and King, 1983]. Saving effects refer to the observation that reacquisition is faster after extinction than new learning [Napier et al., 1992]. After extinction, part of the initial memory may be retained in the cerebellar cortex, the cerebellar nuclei, or both (Robleto et al. [2004] for review). There is also experimental evidence that the initial memory in the cerebellar cortex is at least partly erased during extinction of conditioned eyeblink responses [Bengtsson et al., 2007; Jirnhed et al., 2007; Medina et al., 2002a].

Cerebellar hemisphere lobule VI and interposed nuclei have long been known to be critically involved in eyeblink conditioning [Attwell et al., 2001; Gerwig et al., 2005; McCormick and Thompson, 1984a]. It is generally agreed that conditioned stimulus (CS) information, for example, a tone, is transmitted to the cerebellum via the mossy and parallel fiber system, and that unconditioned stimulus (US) information, for example, an air puff, is transmitted via the climbing fibers from the inferior olive. Most scientists in the field would also agree that the Purkinje cells learn to pause in response to the CS, which is followed by less inhibition of the cerebellar nuclei and elicitation of the conditioned response (CR) in response to the CS [De Zeeuw and Ten Brinke, 2015; Ten Brinke et al., 2015]. The pausing of the Purkinje cell has been shown to be reversed during extinction and to reoccur during reacquisition [Jirnhed et al., 2007]. The inhibitory pathway from the cerebellar nuclei to the inferior olive is assumed to play an important modulatory role in not only the acquisition [Hesslow and Ivarsson, 1996] but also the extinction of conditioned responses. Pharmacological blocking of GABA receptors in the inferior olive prevented extinction in rabbits [Medina et al., 2002a] and direct stimulation of this pathway led to extinction of CRs in decerebrate ferrets [Bengtsson et al., 2007].

Based on computer simulation and reversible inactivation of the cerebellar nuclei (that is, interposed nuclei) and inferior olive in rabbits, Medina et al. [2001, 2002a] proposed the following mechanism underlying extinction and savings: one long-standing common belief is that long-term depression (LTD) at the parallel fiber–Purkinje cell synapse underlies acquisition, as a result of co-activation of climbing and parallel fiber inputs (e.g., Christian and Thompson [2003] and Linden and Connor [1993] for reviews). By the way of the inhibitory nucleo-olivary pathway described above, at the same time, excitation of the cerebellar nuclei inhibits climbing fiber output of the inferior olive. During presentation of the CS-only extinction trials, there will be lack of excitation of the inferior olive because of the missing US, and also inhibition of the inferior olive via the cerebellar nuclei. As a consequence, climbing fiber output may fall below spontaneous activity of the inferior olive which triggers extinction [Medina et al., 2002a; cf. Ten Brinke et al., 2015]. In that case Purkinje cells are activated by the parallel fibers alone which is thought to be followed by long-term potentiation (LTP)

at the parallel fiber-Purkinje cell synapse, which in turn may increase inhibition of the nuclei and reduce the size and occurrence of CRs. In an accompanying study, Medina et al. [2001] included the mossy fiber–nuclear cell synapse in their model of extinction. LTP at the mossy fiber–nuclear cell synapse is another locus of plasticity which has been related to acquisition of conditioned eyeblinks (Freeman and Steinmetz [2011]; Pugh and Raman [2008]; Zhang and Linden [2006] for review). A central assumption of the learning model used by Medina et al. is that plasticity in the cerebellar cortex has to occur first and is a prerequisite for subsequent plasticity at the mossy fiber–nuclear cell synapse (Mauk [2000] and Mauk et al. [2014] for reviews). This rule applies to acquisition and extinction. The authors proposed that extinction-related LTP in the cerebellar cortex will promote LTD and reverse acquisition-related LTP at the mossy fiber to nuclear cell synapse, but with a temporal delay. Preserved memory in the cerebellar nuclei is assumed to explain savings. Recently many different forms of synaptic and intrinsic plasticity have been found in the cerebellar cortex underlying motor learning including eyeblink conditioning (Gao et al. [2012] for review; D'Angelo et al. [2015, 2016] and De Zeeuw and Ten Brinke [2015] for recent review). It has even been questioned whether LTD at the parallel-fiber Purkinje cell synapse contributes to eyeblink conditioning at all [Johansson et al., 2015; Schoneville et al., 2011]. The general idea that the initial plasticity is reversed in the cerebellar cortex during extinction, but remains in the nuclei, may still be valid.

Simultaneous recordings of activity within the cortex and nuclei are one possible way to further test the hypothesis of reversed bidirectional learning within the cerebellar cortex and retained memory in the cerebellar nuclei during extinction. Medina's model predicts increased activation of the nuclei and decreased activation of the Purkinje cells in the cerebellar cortex during acquisition [Medina et al., 2001]. During extinction, increased activity of the Purkinje cells and therewith inhibition of nuclear activity is presumed. During reacquisition, Medina's model predicts decreased cortical activity and increased activity of the cerebellar nuclei. Nuclear activity develops on a faster time scale compared to the initial acquisition, because memory has been retained.

Few studies attempted to simultaneously record neuronal activity in the cerebellar cortex and nuclei. Gould and Steinmetz [1996] performed multiple-unit neural simultaneous recordings in the cerebellar cortex and nuclei (i.e., lobule VI and adjacent ansiform lobule, i.e., Crus I according to Schmahmann et al.'s [1999] nomenclature, and interposed nuclei) during acquisition and extinction of conditioned eyeblink responses. During acquisition, there was a learning-related increase of neuronal activity in both the cerebellar cortex and nuclei. This activity declined during extinction in the cerebellar nuclei, but remained largely the same in the cerebellar cortex. Although this has been interpreted as evidence of savings within the cerebellar

cortex but not the cerebellar nuclei [Robleto et al., 2004], a decrease of neuronal activity in the cerebellar nuclei during extinction is also predicted by the Medina model. In the multiunit recordings, however, it cannot be excluded that cells other than Purkinje cells contributed to neuronal activity in the cerebellar cortex. Gould and Steinmetz [1996] performed additional single-unit recordings from Purkinje cells in lobule VI and adjacent ansiform lobule. They found that 2/3 of the Purkinje cells, which showed changes of firing rates related to acquisition, increased their firing rates, whereas 1/3 decreased their firing rates during acquisition. During extinction, the authors found that a large part of the Purkinje cells kept increased firing rates (41%), others returned to baseline (28%). In addition, there were Purkinje cells, which changed from decreased to increased firing rates (19%) and some which changed from baseline to decreased firing rates (12%). The Purkinje cells that changed from decreased firing rates in acquisition to increased firing rates in extinction agree with Medina's model. Ten Brinke and colleagues recently applied stringent criteria to identify eyeblink cells in the cerebellar cortex and verified their contribution to the conditioning and extinction process with trial-by-trial analysis [De Zeeuw and Ten Brinke, 2015; Ten Brinke et al., 2015], solving most of the ambiguities described above. In short, they demonstrate that it is the suppression of the simple spike activity of the eyeblink Purkinje cells and the enhanced firing of molecular layer interneurons and olivary neurons that can explain the conditioning behavior.

To further investigate whether learning related plastic changes are retained in the cerebellar cortex, in the cerebellar nuclei or both cerebellar activation was assessed during acquisition, extinction and reacquisition of conditioned eyeblinks in young and healthy human subjects using ultra-high-field functional magnetic resonance imaging (7 T fMRI). Our group used ultra-high-field MRI in the past to image activation of the deep cerebellar nuclei, including the interposed nuclei, in humans [Thürling et al., 2015]. In the case that learning-related plastic changes are reversed during extinction, fMRI activity in the cerebellar cortex and/or nuclei was expected to be different in extinction compared to late acquisition and reacquisition. In the case that learning-related plastic changes are maintained in the cerebellar cortex and/or nuclei during extinction, fMRI activity was expected to be different between initial acquisition and reacquisition phases.

METHODS

Subjects

A total of 43 young and healthy subjects performed the experiment. The experiment was prematurely terminated in 10 subjects because of extensive movements ($n = 5$, e.g., sneezing, hiccups), sleepiness ($n = 1$), obvious lack of conditioned responses ($n = 1$), or technical errors ($n = 3$). From

Figure 1.

Conditioning protocol. A total of 240 trials were applied in 6 episodes of 40 trials each [duration ~13 min per episode, pauses between episodes not displayed, randomized intertrial intervals (ITI) within episode: 16–20 s]. In acquisition and reacquisition phase, 30% CS-only trials were interspersed between the paired CS–US trials in a randomized pattern.

the remaining 33 subjects, 4 subjects were excluded from final statistical analysis because conditioned response rate was below spontaneous blink rate during acquisition (see subsequent section *Eyeblink Conditioning* for details). Correspondingly, a total of 29 subjects (16 males, 13 females, mean age 24.0, SD 4.0 years, range: 18–34 years) were included in data analysis. All subjects were right-handed based on the Edinburgh handedness inventory [Oldfield, 1971]. Informed consent was obtained from all participants. This study was approved by the local ethics committee.

Eyeblink Conditioning

Eyeblink conditioning was performed after anatomical MR image acquisition with the subject lying supine inside the magnet bore. The custom-build 7 T-MR-compatible conditioning setup used was described previously [Thürling et al., 2015]. In brief, infrared reflecting markers were attached to the subject's upper eyelids (one on the right and one on the left). The movement of one of the two markers was tracked online using infrared lighting, a high-speed infrared sensitive camera (frame rate individually adapted to optimize marker detection, mean frame rate 31.1, SD 4.9 Hz) and a control computer. The US, an air puff administered to the outer corner of the right eye, was delivered through plastic tubing attached to the head coil. Air puff maximum peak pressure at the nozzle was 19 kPa (2.7 psi, measured with a sealed nozzle). The CS, a tone at 1.5 kHz, was delivered using an MR-compatible loudspeaker system placed at the magnet bore entrance and could be clearly differentiated from the scanner background noise throughout the whole experiment. Additionally, a visual CS consisting of a green LED light being switched off while the tone was playing was applied.

Light sources, stimulus LED, and video camera were positioned in the room above the scanner. Light reached the markers and video recordings were made via a two-mirror system. The dim, point-like stimulus LED was visible near the center of the test subject's field of view, and differed in color, position, size, and visible light intensity from the infrared lights. The scanner room was darkened except for the infrared lights and the green stimulus LED.

The conditioning protocol used is illustrated in Figure 1. A standard delay protocol was used established by Gormezano and Kehoe [1975]. The CS duration was set to 550 ms and the US (duration 100 ms) was applied 450 ms after CS onset. Owing to temporal constraints in MRI acquisition the experimental protocol was divided into 6 consecutive episodes of 40 trials each (duration: ~13 min). Episodes were distributed between these 3 phases: acquisition phase (3 episodes), extinction phase (1 episode), and reacquisition phase (2 episodes). The intertrial interval (ITI) within each episode was randomized between 16, 18, and 20 s (mean set to 18.0 s). In acquisition phase (120 trials) and reacquisition phase (80 trials), paired CS–US events were interspersed randomly with CS-only events at a ratio of 7:3 (70% reinforcement). CS-only events were distributed in a randomized fashion, with 1–3 paired CS–US events (mean 2.2) between each 2 consecutive CS-only events. During extinction phase (40 trials), a CS-only event was presented in each trial. All ITIs and order of events were the same for all subjects. The time between episodes was 0.1 and 3.4 min (mean 1.4, SD 0.6 min) for most of the episodes (96%). In 4% of the cases, the time between episodes was longer due to technical difficulties (5.3–20.6 min, mean 12.9, SD 6.1 min). To avoid subjects dozing off, the intercom was used to address subjects between episodes and they were asked to stay alert.

Marker movements were monitored online and analyzed offline semiautomatically with a custom-made software based on LabVIEW (Release 2012, National Instruments, Austin, TX) and MATLAB (Release 2014a, The MathWorks, Inc., Natick, MA). For each trial, a marker recording with a duration of 2 s was saved, with all CS applied at 500 ms and all US applied at 950 ms after the start of recording. Timing was scaled to US onset at $t = 0$ ms. A 500 ms baseline was evaluated at the start of each trial. Blink onsets were defined as the moment the blink amplitude changed 3 standard deviations from the mean baseline value. The spontaneous blink rate was calculated from the number of blinks in all baseline intervals. Mean spontaneous blink rate during acquisition phase was 35.1 SD, 18.6 blinks per minute (bpm) for all 33 subjects tested and 36.7 SD, 18.6 bpm for the 29 included in data analysis (see below).

Data were manually inspected for occasional loss or misdetections of the marker position (e.g., due to low light intensity or rapid movement) in single frames, seldom in two or more adjacent frames, on a trial-by-trial basis. Falsely detected marker positions were manually corrected. For each episode, relative eyelid closure was scaled based on the distance between the mean marker position with eyes open (0% eyelid closure) and the mean marker position with eyes closed (100% eyelid closure) in each acquisition and reacquisition episode. Trials that did not show a clear eyes-open or eyes-closed position were excluded from the respective mean value calculation. For the extinction phase, the mean distance of the last acquisition and the first reacquisition episode was used. Trials with spontaneous eyeblinks in the baseline, that is, immediately before CS presentation were considered as not-conditioned. Eyeblinks starting earlier than 150 ms after the CS-onset were considered reflexive to the CS and not counted as conditioned responses (α -response) [Woodruff-Pak, 1997]. These trials with either spontaneous blinks or α -blinks were kept in the analysis, but were treated as trials without a CR (regardless of the presence or absence of a CR). Four subjects who displayed a CR incidence rate (expressed as CRs per minute) in paired acquisition trials below the mean -1 SD group spontaneous blink rate (4.3, 8.6, 14.3, and 15.7 bpm) were excluded from further analysis.

As outlined below, fMRI analysis was based on CS-only trials in the acquisition and reacquisition phases to avoid interference with MRI signal related to the US and UR in paired trials. Behavioral and MR data analyses were based on the identical set of trials. Data analyses included all CS-only trials. Six CS-only trials per block were chosen to have large-enough fMRI signal per block. Therefore, focus of behavioral analysis was on CS-only trials using the same block sizes. For further illustration, behavioral data are also presented and analyzed in paired trials. We decided to use 7 CS-US paired trials per block (which covers half of the time scale compared to blocks of 6 CS-only

trials), because the learning curves are shown in more detail. This resulted in 4 paired CS-US blocks and 2 CS-only blocks for each of the 3 acquisition and 2 reacquisition episodes and 7 blocks for the single extinction episode, with the last block covering only 4 CS-only events (Fig. 1). Relative CR incidence was calculated as the percentage of trials displaying a CR in each block.

As a measure of response size and timing precision, the eyelid movement onset and the maximum slope of the respective eyelid marker curves (i.e., the maximum eyelid closing speed prior US onset, given in percentage eyelid closure per unit time) were determined for each trial with a CR. Mean values were calculated for each of the blocks described above.

For statistical analysis, CR parameters were compared using analysis of variance (ANOVA) with repeated measures. Acquisition effects were determined using acquisition blocks as within subjects' factor ($n = 6$ in CS-only trials and $n = 12$ in paired CS-US trials). To assess extinction effects, the last 4 acquisition blocks and the last 4 extinction blocks were compared using phase (acquisition vs extinction) and block ($n = 4$) as within subjects' factors. Saving effects were determined comparing the first four acquisition blocks with the (first) 4 saving blocks using phase (acquisition vs savings) and block ($n = 4$) as within-subjects' factor. $P < 0.05$ was considered significant. Greenhouse-Geisser correction was applied when appropriate. The same statistical analyses were performed using spontaneous blink rate assessed during the baseline period as dependent variable. All statistical tests were performed using SPSS (Release 22.0, IBM, Armonk, NY).

MRI Acquisition

All MR images were acquired with the subjects lying supine inside a whole-body MRI scanner operating at 7 T (Magnetom, Siemens Healthcare, Erlangen, Germany) equipped with a 32-channel transmit-receive head coil (Nova Medical, Wilmington, MA). To homogenize the radio frequency excitation field (B1), two pads filled with high-permittivity fluid were placed on either side of the each subjects' upper neck [Teeuwisse et al., 2012]. Subject head movement was reduced using wedge-shaped cushions tightly applied to the subjects' neck. For anatomic reference, structural T1-weighted (T1w) MR images of the entire head were acquired using a sagittal oriented magnetization prepared rapid gradient echo (MPRAGE) sequence with an adiabatic wideband uniform rate smooth truncation (WURST) preparation pulse [Wrede et al., 2012]. Imaging parameters were set as follows: TR, 3500 ms; TE, 2.13 ms; TI, 1100 ms; flip angle, 7°; bandwidth, 210 Hz/pixel; 256 slices; acquisition matrix, 256×224 ; parallel acceleration factor, 2; phase and slice partial Fourier factors, 6/8; TA, 6:15 min; and isotropic voxel size, 1.0 mm.

Functional MRI acquisition was performed in 6 consecutive episodes of 380 volumes each using a two-

dimensional echo planar imaging (EPI) sequence with sinc-shaped excitation pulses and sinusoidal readout gradients (provided by Siemens Healthcare, Erlangen, Germany). Covering the whole cerebellum, the field of view (FOV) was a coronal, slightly angulated slab with the dimensions $220 \times 220 \times 72 \text{ mm}^3$. Further imaging parameters were selected as follows: TR, 2000 ms; TE, 27 ms; flip angle, 76° ; bandwidth, 1008 Hz/pixel; 33 slices; acquisition matrix, 110×110 ; parallel acceleration factor, 2; phase partial Fourier factor, 7/8; TA, 13 min; and isotropic voxel size, 2.0 mm with 10% distance factor between slices. Ghost artifacts were reduced by the application of local (instead of global) phase correction and the selection of “feet to head” ($F \gg H$) for the phase encoding direction.

Image Analysis

The functional imaging data were analyzed using SPM 8 (Wellcome Department of Cognitive Neurology, London, UK) running on MATLAB (Release 2014a, The MathWorks, Inc., Natick, MA). As described previously [Thürling et al., 2015], SPM default brightness threshold was set from 0.8 to 0.1 to avoid signal dropouts within the hypointense cerebellar nuclei. Functional volumes were corrected for slice timing and realigned to the first image to correct for head motion. Owing to the restricted FOV in functional volumes, computational coregistration of T1w and functional volumes yielded inappropriate results; hence, registration of functional volumes to the structural T1w volume was manually inspected and corrected. Using a general linear model, event-related first-level analysis was performed with the unsmoothed functional volumes. Events were blocked in sets of either 7 paired CS-US events or 6 CS-only events, resulting in 4 paired CS-US blocks and 2 CS-only blocks for each of the 3 acquisition and 2 reacquisition episodes and 7 blocks for the single extinction episode, with the last block covering only 4 CS-only events (cf. Figure 1). Head motion data from functional volume realignment was used as regressors to compensate for effects of motion (6 regressors, 3 translational, and 3 rotational corrections).

Structural T1w volumes were segmented and individual cerebellar masks were generated using the spatially unbiased atlas template of the human cerebellum (SUIT) and the functions supplied by the SUIT toolbox for SPM [Diedrichsen, 2006] (<http://www.icn.ucl.ac.uk/motorcontrol/imaging/suit.htm>). To optimize cerebellar coverage, masks were manually corrected using MRICron software (release June 2013, <http://www.sph.sc.edu/comd/rorden/mricron>). Using the SUIT toolbox and employing the DARTEL algorithm individual normalization of the segmented volumes to the SUIT, cerebellar probability maps were calculated. Normalization was then applied to the first-level contrasts masked explicitly by the cerebellar masks generated earlier.

Similarly, another ROI-based normalization was calculated for the cerebellar nuclei. As the nuclei offer low T1, but strong T2* contrast, nuclear bean-shaped masks were

manually drawn not on the anatomical T1 images, but on the mean functional volumes generated during realignment using MRICron software. Using these ROI drawn on the mean functional volume together with the T1w anatomical volume, ROI-based (multimodal) DARTEL normalization to a 7 T dentate-interposed nuclei template [Thürling et al., 2015] was performed using the function “Normalization with dentate ROI” supplied by the SUIT toolbox [Diedrichsen et al., 2011]. Again, normalization was applied to the first-level contrasts masked explicitly by the individual nuclear masks.

For group analysis, normalized first-level contrasts were smoothed using an isotropic Gaussian kernel of 4 mm FWHM for the cortical contrasts and 2 mm FWHM for the nuclear contrasts. As stated above, group analysis was performed for CS-only trials only to exclude fMRI signal changes related to the US and UR. First, one-sample *t* tests were calculated to provide general information of activation patterns within the whole cerebellum in each of the three phases.

For each phase, a first-level contrast was generated over all CS-only blocks within the phase and divided by the number of blocks. Main effects of acquisition, extinction, and reacquisition against rest in the CS-only trials were calculated based on these mean first-level contrasts over the corresponding blocks. Small volume correction was applied using the SUIT cerebellar template and the nuclear template, respectively. To correct the significance level for multiple comparisons, permutation tests (with 1000 permutations) were used [Winkler et al., 2014]. Spontaneous blink rate was included as a covariate. Second, to depict the pattern of signal change across acquisition, extinction, and reacquisition, mean β values of the normalized and smoothed first-level contrasts were determined for two regions of interest (ROI): (1) a spherical ROI (radius 6 mm) around the acquisition peak voxel in the right lobule VI and (2) the right dentate and interposed nuclei. ANOVA with repeated measures was used to perform the same statistical comparisons as for behavioral CR parameters. A mixed model was used which allowed to incorporate spontaneous blink rate as a time-varying covariate (http://www.ats.ucla.edu/stat/spss/faq/repeat_covar.htm).

RESULTS

Behavioral Eyeblink Conditioning Data

Mean group CR incidences across blocks in the three learning phases are displayed in Figure 2a. Mean CR incidences in paired CS-US trials and CS-only trials showed a similar time course. CR incidences increased during the first half of the acquisition phase and plateaued at a level of approximately 50% during the second half of this phase. In the extinction phase, CR incidences declined and reached a level below 10% after ~20 trials. CR reacquisition occurred very fast on the scale of the block size. CR incidences were similar to the late acquisition phase

Figure 2.

Results of behavioral data analysis. (a) Mean group percentage CR incidences and (b) mean group maximum slope and standard errors versus the mean trial index of all trials grouped within each block. Mean trial index is used to display complete block data in the correct temporal order of events. For example, a CS-only block of trials with the trial indices 3, 7, 9, 13, 16, and 18 would be displayed at mean trial index 11. Behavioral data are grouped in blocks of 6 trials for the CS-only trials and in

blocks of 7 trials for the paired CS–US trials, corresponding to the blocks chosen in fMRI evaluation. Error bars indicate standard errors. Note that maximum slope in the last extinction blocks is based on few remaining CRs. (c) Averaged eyelid marker traces in CS-only trials in a single subject. Mean traces correspond to the blocks described above. [Color figure can be viewed at wileyonlinelibrary.com]

already within the first block (saving effects). The time course of CR acquisition, extinction, and reacquisition is further illustrated in Figure 3 showing the mean group number of conditioned responses on a single-trial basis. Both paired CS–US and CS-only trials are shown, the latter indicated in red. The individual mean CR incidence calculated over both paired and CS-only trials together averaged to 45.5 SD, 23.4% in the third acquisition episode ($CR_{plateau}$). $CR_{plateau}$ limits of 63% and 37% were calculated to quantitatively describe saturation (in acquisition and reacquisition) and decay (in extinction) curves. The number of trials needed to reach the limits $1 - 1/e$ ($=63\%$) $CR_{plateau}$ and $1/e$ ($=37\%$) $CR_{plateau}$ correspond to the “lifetimes” in a simple exponential model for saturation or decay. Acquisition needed an average of 15.6 SD 22.9 trials to reach a level of 63% $CR_{plateau}$, and extinction needed an

average of 6.3 SD 6.8 trials to fall below 37% $CR_{plateau}$. The reacquisition of CR needed only 3.4 SD 2.2 trials to reach 63% $CR_{plateau}$ (individual CR values calculated using a gliding average over 4 trials).

Subjects significantly increased the number of conditioned responses across blocks in the acquisition phase. ANOVA with repeated measures revealed a significant block effect [CS-only trials: $F_{(5,140)} = 6.63$, $P < 0.001$; paired CS–US trials: $F_{(6,82,191,09)} = 6.25$, $P < 0.001$]. CR incidences were significantly less in the last 4 blocks of extinction both compared to the last 4 blocks of acquisition and to the (first) 4 blocks of reacquisition in CS-only and paired trials (phase effect: all P values < 0.001). Comparing CS-only trials in the last 4 acquisition blocks with the last 4 extinction blocks showed a significant block effect [$F_{(3,84)} = 4.981$, $P = 0.003$] and a significant block by phase

Figure 3.

Mean group number of conditioned responses (a,b) and spontaneous blinks in the baseline period (c) on a single-trial basis in the acquisition, extinction, and reacquisition phases. (a) Responses counted as conditioned responses in the behavioral analysis (i.e., trials without spontaneous blinks in baseline or

preceding α -blink, a very conservative approach). (b) All responses in the critical CS–US time window (150 ms after CS onset until US onset). CS-only trials are indicated as red dots. [Color figure can be viewed at wileyonlinelibrary.com]

interaction [$F_{(3,84)} = 8.638$, $P < 0.001$], related to the learning related increase in acquisition (Fig. 2a). All other block effects and block by phase interactions in CS-only and paired trials were not significant. Considering the 7 extinction blocks alone, CR incidence showed a significant decline [$F_{(2.73,76.47)} = 17.467$, $P < 0.001$; ANOVA with repeated measures, Greenhouse Geisser correction].

Finally, ANOVA revealed significant saving effects. In CS-only trials, CR incidences in the 4 saving blocks were significantly increased compared to the first 4 acquisition blocks [phase effect: $F_{(1,28)} = 22.099$, $P < 0.001$], and the

block by phase interaction was close to significance [$F_{(3,84)} = 2.328$, $P = 0.08$]. The block effects were not significant. Similarly, in paired CS–US trials, CR incidences in the first 4 saving blocks were significantly higher compared to the first 4 acquisition blocks [phase effect: $F_{(1,28)} = 23.635$, $P < 0.001$]. The phase by block interaction was close to significance [$F_{(3,84)} = 2.422$, $P = 0.072$]. The block effect was not significant.

Similar changes across the three learning phases were present considering the maximum slope of the conditioned responses in CS-only and paired trials (Fig. 2b,c). During

acquisition, the maximum slope became significantly steeper across blocks [block effect; CS-only trials: $F_{(2,391,59,768)} = 4.865$, $P = 0.008$; paired CS-US trials: $F_{(4,168,100,042)} = 4.431$, $P < 0.001$]. The slope steepness declined during extinction, and was significantly less in the last 4 extinction blocks compared both to the last 4 acquisition blocks and to the (first) four savings blocks in CS-only and paired trials (phase effect: all P values < 0.05). Block effects and block by phase interactions were not significant. Considering the 7 extinction blocks alone, slope steepness also declined across blocks (Fig. 2b), but this decline was not significant [$F_{(2,02,14,20)} = 1.494$, $P = 0.204$]. Finally, maximum slope was steeper in the (first) 4 saving blocks compared to the first 4 acquisition blocks [phase effect, CS-only trials: $F_{(1,25)} = 12.332$, $P = 0.002$; paired CS-US trials: $F_{(1,25)} = 7.520$, $P = 0.011$]. Block effects and phase-by-block interactions were not significant. Considering CR onset, there was no significant change across blocks and between phases (all P values > 0.094).

Spontaneous Blink Rate

Spontaneous blink rate was assessed during the baseline period and compared between the three learning phases (Fig. 3c). Mean spontaneous blink rate (expressed as mean number in the baseline period of 500 ms prior CS onset) was less in extinction and reacquisition phases compared to acquisition phase (mean spontaneous blink count in acquisition: 0.32, SD 0.17; extinction: 0.24, SD 0.17; reacquisition: 0.26, SD 0.15). There was no significant decline across the 6 acquisition blocks [block effect: $F_{(5,140)} = 1.288$, $P = 0.272$; ANOVA with repeated measures]. Comparison between acquisition and extinction phase and between acquisition and reacquisition phase showed significant phase effects [$F_{(1,28)} = 5.542$, $P = 0.026$ and $F_{(1,28)} = 6.611$, $P = 0.016$, respectively], but no block or phase-by-block interaction effects (all P values > 0.136). Mean spontaneous blink rate was not significantly different between extinction and reacquisition phases (all P values > 0.252). Note that because it could not be excluded that higher spontaneous blink rate in baseline had an impact on fMRI signal in acquisition, fMRI analysis was performed using spontaneous blink rate as covariate.

fMRI Data: Cerebellar Cortex

Cerebellar cortical activation during CS-only trials is displayed in Figure 4 as group main effect of the whole acquisition (a), extinction (c), and reacquisition (d) phases against rest (one sample t test, $P < 0.05$, permutation corrected). To allow for more direct comparison between initial acquisition and reacquisition phases, acquisition is also shown considering the first 4 blocks only (b). Throughout the three phases, the general pattern of activation was the same. The most pronounced activation was found in the more lateral parts of right lobule VI (ipsilateral to the presentation of the US) with some extension into Crus I and

additional activation of left lobule VI. Significant activation was also present in more intermediate parts of lobule VI, again predominantly on the right, and in more midline structures of the anterior lobe. Finally, significant activation was observed in the more intermediate parts of Crus I extending into Crus II, lobule VIIb and lobule VIIa bilaterally. A summary of activations of the cerebellar cortex in the three learning phases is given in Table I.

Mean β values were compared between blocks and learning phases in a spherical ROI centered at the activation peak in the lateral right lobule VI ($x = 38$ mm, $y = -51$ mm, $z = -28$ mm). Figure 5a shows that mean β values were comparable in the acquisition and reacquisition phases, but showed a decline in the extinction phase. Mean β values were already high in the first acquisition block and showed no significant change across the 6 acquisition blocks [$F_{(5,139,6)} = 0.185$, $P = 0.968$]. Mean β values were significantly lower in the last 4 extinction blocks both compared to the last 4 acquisition blocks [phase effect; $F_{(1,196,5)} = 19.216$, $P < 0.001$] and compared to the 4 reacquisition blocks [$F_{(1,194,8)} = 14.043$, $P < 0.001$]. Block and phase-by-block effects were not significant (P values > 0.107). Mean β values were not significantly different comparing the first 4 acquisition blocks and the 4 saving blocks [phase effect; $F_{(1,196,6)} = 1.238$, $P = 0.267$]. Block and phase-by-block effects were not significant (P values > 0.364).

fMRI Data: Cerebellar Nuclei

Figure 6 displays the results of one-sample t tests of the main effect of the whole acquisition (a), extinction (c), and reacquisition (d) phases against rest on the level of the cerebellar nuclei (one-sample t test). Acquisition is also shown considering the first 4 blocks only (b). During acquisition and reacquisition phases, significant activation was observed within the right interposed nuclei extending in the dorsal dentate nuclei ($P < 0.05$, permutation corrected). Additional activations were found in the left dorsal dentate nuclei and in the more ventral parts of the dentate nuclei bilaterally. During the extinction phase, activation was present at a trend level ($P < 0.01$, uncorrected). A distinct focus of activation was observed in the right interposed nucleus. A summary of activations of the cerebellar nuclei in the three learning phases is given in Table II.

Similar to mean β values in lobule VI, mean β values in the right dentate and interposed nuclei were comparable in the acquisition and reacquisition phases, but showed a decline in the extinction phase (Fig. 5b). Similarly, mean β values were already high in the first acquisition block and showed no significant change across the 6 acquisition blocks [$F_{(5,139,7)} = 0.172$, $P = 0.972$]. Mean β values were significantly lower in the last 4 extinction blocks both compared to the last 4 acquisition blocks [$F_{(1,196,2)} = 6.272$, $P = 0.013$] and compared to the 4 reacquisition blocks [$F_{(1,192,9)} = 5.39$, $P = 0.021$]. Finally, mean β values were not significantly different comparing the first 4 acquisition

Figure 4.

Cerebellar cortical activations in CS-only trials in (a) the whole acquisition phase (6 blocks), (b) the acquisition phase considering the first 4 blocks only, (c) the whole extinction phase (7 blocks), and (d) the whole reacquisition phase (4 blocks) mapped on coronal slices of the SUIT maximum probability template [Diedrichsen et al., 2009]. All images are thresholded at $P < 0.05$ (permutation corrected; one sample t test). Spontaneous blink rate was used as

covariate. Slice positions are indicated in the sagittal view on the right hand side. Positions are presented in SUIT coordinates; cerebellar lobules labeled in Roman numerals according to Schmahmann et al. [1999]; left and right cerebellar hemisphere are marked with L and R, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

blocks and the 4 saving blocks [$F_{(1,196,6)} = 0.078$, $P = 0.780$]. None of the block and phase-by-block effects were significant (P values > 0.831).

fMRI Signals Comparing Trials With and Without CRs

To show that fMRI signals in CS-only trials in which a CR is performed reflect more than performance of the CR (i.e., learning), cerebellar activation in trials with and without a CR were compared in the acquisition phase. Trials

with spontaneous blinks prior the CS and α -blinks were separately modeled in first-level evaluation, but excluded from further analysis. Based on one-sample t tests, activation appeared to be more pronounced in the cerebellar nuclei, particularly the right interposed nuclei, and lobule VI in trials with CRs compared to trials without CRs (Supporting Information, Fig. 1 and Tables 1 and 2). These differences, however, were not significant neither based on two-sample t tests ($P < 0.05$, permutation corrected), nor comparing mean β values in the 2 ROIs (P values > 0.05 ; two-sided paired t test).

TABLE I. Summary of cortical activations

Index	Location	Side	SUIT coordinates/mm			Cluster size/mm ³	Peak <i>t</i> value
Acquisition phase							
1	Extended cluster	Bilateral				49161	
	Lobule VI	Right	38	−51	−28		11.39
	Lobule VI	Left	−37	−62	−25		9.60
	Lobule VIIb	Right	11	−70	−37		9.07
	Lobule VI	Right	10	−66	−12		8.84
	Lobule VI	Left	−6	−76	−23		8.72
2	Lobule Crus I	Left	−10	−40	−28	31	5.11
3	Lobule I-IV	Right	11	−40	−28	27	4.98
Acquisition phase: first four blocks							
1	Extended cluster					40261	
	Lobule VI	Right	34	−52	−30		9.45
	Lobule Crus I	Left	−37	−62	−26		8.93
	Lobule Crus I	Left	−14	−72	−31		8.67
	Lobule VIIb	Right	11	−71	−38		8.15
	Lobule VI	Right	12	−68	−13		7.77
2	Lobules I-IV	Left	−10	−40	−28	47	5.57
3	Lobules I-IV	Right	11	−40	−28	99	5.41
Extinction phase							
1	Extended cluster	Bilateral				16646	
	Lobule VI	Right	10	−70	−15		8.37
	Lobule Crus II	Left	−10	−74	−40		8.15
	Lobule VI	Right	35	−51	−29		7.40
	Lobule VIIb	Right	10	−69	−37		6.38
	Lobule VI	Left	−33	−55	−32		6.15
2	Lobule I-IV	Left	−10	−41	−29	36	5.90
3	Lobule VIIa	Right	33	−49	−45	182	5.09
4	Lobule Crus II	Right	25	−68	−40	24	4.73
5	Lobule V	Left	−6	−59	−7	29	4.51
Reacquisition phase							
1	Extended cluster	Bilateral				24839	
	Lobule VI	Left	−6	−73	−14		9.62
	Lobule VI	Right	39	−50	−27		8.83
	Lobule Crus II	Left	−23	−71	−42		8.25
	Lobule VI	Left	−36	−53	−30		7.79
	Lobule Crus I	Right	10	−71	−31		7.13
2	Lobule Crus II	Right	41	−47	−45	387	5.67
3	Lobule Crus I	Left	−30	−80	−37	61	5.37
4	Lobule VI	Left	−31	−38	−25	25	4.81
5	Lobules I-IV	Left	−3	−52	−17	105	4.76
6	Lobules I-IV	Right	7	−54	−2	25	4.48

Table displays cortical activation clusters larger than 20 mm³ thresholded at $P < 0.05$ permutation corrected ($t > 4.2$). Spontaneous blink rate was used as covariate. Within larger clusters with multiple local maxima of activation, only maxima with a minimal distance of 20 mm to a larger activation maximum are listed. Activations are shown based on all blocks per phase (i.e., 6 blocks of six CS-only trials in acquisition, 7 blocks in extinction, and 4 blocks in reacquisition) and the acquisition phase considering the first 4 blocks only.

DISCUSSION

The young and healthy subjects included in this study were able to acquire conditioned eyeblink responses in the 7 T MRI scanner. They showed significant extinction and fast recovery of conditioned responses in a subsequent reacquisition phase. During acquisition, a significant increase of fMRI signal was observed in the 2 ROIs in the cerebellar cortex and nuclei which decreased during extinction and reappeared during reacquisition. Data are

in accordance with the hypothesis that at least part of the initial cerebellar memory is reversed during extinction of conditioned eyeblink responses. However, no significant difference in fMRI signal was found between initial acquisition and reacquisition phase.

The reversal of activation in the cerebellar cortex and nuclei during extinction and rereversal during reacquisition is consistent with bidirectional learning. It cannot explain, however, why conditioned responses reappear

Figure 5.

Mean β values of (a) a spherical ROI centered at the cortical activation peak at ($x = 38$ mm, $y = -51$ mm, $z = -28$ mm) and (b) the whole right nuclear template. β values calculated for blocks of 6 (last extinction block: 4) consecutive CS-only events as described above. Error bars indicate standard errors. [Color figure can be viewed at wileyonlinelibrary.com]

almost immediately in reacquisition trials following extinction. Part of the initial memory may be retained in the cerebellum which is inhibited during extinction. Inhibition may be mediated via extracerebellar neural circuits. Memory may also be stored in extracerebellar areas during extinction and contribute to saving effects. These different possibilities are discussed in more detail below.

Performing the experiment in the environment of the MRI scanner had little impact on the learning rates. Subjects did not perform worse compared to subjects with a similar age distribution previously tested outside the MRI scanner using the same paradigm [Ernst et al., 2016]. CR incidences during acquisition, extinction, and reacquisition were very similar in both studies (see Fig. 2 in Ernst et al.

[2016]). The finding of very fast recovery of conditioned responses is well known in the literature, not only in experiments performed on the same day, but also following more extended periods of time [Gerwig et al., 2010; Smit et al., 2008; Timmann et al., 2005]. Relearning has also been found to occur within a limited number of trials in animal data [De Zeeuw and Ten Brinke, 2015; Jirenhed et al., 2007]. For example, Jirenhed et al. [2007] reported that Purkinje cell CRs (i.e., pauses) were back within 5 paired CS-US reacquisition trials following extinction in the decerebrate ferret.

During acquisition, the fMRI signal increased in both the cerebellar cortex and the cerebellar nuclei. No significant decreases were observed. Findings are in good accordance with a previous 7 T fMRI study of our group which also found parallel increases of the fMRI signal within the cerebellar cortex and nuclei during CR acquisition [Thürling et al., 2015]. An increase of activity of the cerebellar cortex and nuclei has also been reported from the beginning of acquisition in earlier recording studies in rabbits [Berthier and Moore, 1990; Gould and Steinmetz, 1996; McCormick and Thompson, 1984b]. The fMRI signal in the cerebellar cortex has been shown to reflect activity related to synaptic input and not activity of the principal neurons per se [Lauritzen et al. [2012] for review]. As already discussed in more detail in Thürling et al. [2015], LTD at the parallel fiber–Purkinje cell synapse would be expected to cause decreased fMRI signal, which was not observed. In recent years, many different forms of synaptic and intrinsic learning-related plasticity have been reported in the cerebellar cortex [Gao et al. [2012] for review; D'Angelo et al. [2015, 2016] for recent review]. It has even been questioned whether LTD is a necessary requirement for acquisition of conditioned eyeblinks [Johansson et al., 2015; Schonewille et al., 2011]. Increased fMRI signal in the cerebellar cortex may, for example, reflect LTP at the mossy fiber to granule cell synapse. As genetic blockage of molecular layer interneurons affects eyeblink conditioning [Ten Brinke et al., 2015], synaptic input of interneurons is another possibility. Furthermore, a recent study showed a plastic and excitatory feedback loop from the cerebellar nuclei to the granule cells and Golgi cells during eyeblink conditioning in mice [Gao et al., 2016]. The underlying physiology of the fMRI signal in the cerebellar nuclei is little understood. LTP at the mossy fiber to nuclear cell synapse is one possible explanation of increased fMRI signal in the nuclei [Freeman and Steinmetz [2011], Pugh and Raman [2008], and Zhang and Linden [2006] for review].

During extinction, fMRI signal was present in the same areas as during acquisition. Whereas fMRI signal in the two regions of interest (lobule VI and interposed/dentate nuclei) were similar at the end of acquisition and the beginning of extinction, the signal was significantly less at the end of extinction. At the end of the extinction phase, fMRI dropped below the fMRI signal during acquisition both within the cerebellar cortex and the nuclei. As yet, our previous 7 T study is the only other human fMRI

Figure 6.

Cerebellar nuclear activations in CS-only trials in (a) the whole acquisition phase (6 blocks), (b) the acquisition phase considering the first 4 blocks only, (c) the whole extinction phase (7 blocks), and (d) the whole reacquisition phase (4 blocks) mapped on coronal slices of a previously published dentate (d, light gray) and interposed (i, dark gray) nuclei template [Thürling et al., 2015]. Images for acquisition (a, b) and reacquisition (d)

phase are thresholded at $P < 0.05$ (permutation corrected; one sample t test), whereas extinction phase images (c) are thresholded at a trend level of $P < 0.01$ (uncorrected, one sample t test). Spontaneous blink rate was used as covariate. All positions are presented in SUIT coordinates; left and right cerebellar hemispheres are marked with L and R, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

study which included extinction trials. The number of CS-only trials interspersed in acquisition and the number of extinction trials, however, was less than in this study, and no direct comparison between acquisition and extinction phases had been made. Similar to this study, areas involved in acquisition and extinction largely overlapped. Miller et al. [2003] used fMRI to examine eyeblink conditioning in rabbits. They included 15 CS-only trials after a total of 300 paired acquisition trials. Extinction phase was short, and the aim was to study retention rather than extinction. Differences between the end of the acquisition phase and the beginning of the CS-only phase were small. Differences were observed only at the level of the contralateral interposed nuclei which lacked activation during the CS-only phase, in line with the present findings. There are three human studies using positron emission tomography (PET) which included an extinction phase. PET has a lower temporal and spatial resolution than fMRI, and does not allow for an event-related design. Subtraction analysis between blocks of two different conditions has to be

performed. In the study by Molchan et al. [1994], regional cerebral blood flow (rCBF) was compared in CS-only extinction trials with rCBF in CS-US paired acquisition trials. No significant increases or decreases were observed within the cerebellum related to extinction. At first sight, data seem to contradict the present findings and one could argue that activity in the cerebellar cortex did not change in extinction compared to acquisition. During extinction, however, an average of 40% CR incidence is reported. That is extinction was incomplete. In the two other PET-studies, to allow for direct comparison between an extinction phase and an initial phase with explicitly unpaired CS and US trials, US-only trials were included in the extinction scans [Parker et al., 2012; Schreurs et al., 1997]. This, however, leads to reinstatement and no clear conclusions on extinction can be drawn. In fact, in Schreurs et al. [1997], CR rate was higher during extinction than acquisition scans.

The present data partially support the hypothesis put forward by Medina et al. that acquisition-related plasticity

TABLE II. Summary of nuclear activation

Index	Location	Side	SUIT coordinates/mm			Cluster size/mm ³	Peak <i>t</i> value
Acquisition phase							
1	Extended cluster					605	
	Dentate nucleus	Left, dorsocaudal	−14	−67	−34		6.30
	Dentate nucleus	Left, dorsocaudal	−12	−64	−31		5.61
	Dentate nucleus	Left, ventrocaudal	−22	−59	−38		5.45
	Dentate nucleus	Left, dorsocaudal	−12	−59	−34		5.11
	Dentate nucleus	Left, ventrorostral	−20	−54	−39		4.54
	Dentate nucleus	Left, ventrorostral	−15	−50	−39		4.18
2	Extended cluster					489	
	Dentate nucleus	Right, ventrocaudal	20	−65	−38		4.90
	Interposed nucleus	Right	11	−56	−29		4.81
	Dentate nucleus	Right, dorsocaudal	13	−66	−34		4.63
	Dentate nucleus	Right, dorsocaudal	13	−59	−33		4.62
	Dentate nucleus	Right, dorsocaudal	11	−63	−31		4.48
	Dentate nucleus	Right, ventrocaudal	13	−61	−37		4.30
3	Dentate nucleus	Right, ventrorostral	17	−50	−40	8	3.98
Acquisition phase: first four blocks							
1	Extended cluster					461	
	Dentate nucleus	Left, dorsocaudal	−14	−67	−34		5.49
	Dentate nucleus	Left, ventrorostral	−21	−53	−39		4.82
	Dentate nucleus	Left, ventrorostral	−22	−57	−39		4.78
	Dentate nucleus	Left, dorsocaudal	−13	−63	−31		4.70
	Dentate nucleus	Left, ventrorostral	−15	−50	−39		4.52
	Dentate nucleus	Left, dorsocaudal	−12	−59	−32		4.36
	Dentate nucleus	Left, ventrocaudal	−21	−61	−36		4.21
2	Extended cluster					248	
	Dentate nucleus	Right, ventrocaudal	20	−65	−38		4.57
	Dentate nucleus	Right, dorsocaudal	11	−64	−32		4.14
	Interposed nucleus	Right	10	−56	−29		4.00
	Dentate nucleus	Right, dorsocaudal	13	−59	−33		3.92
	Dentate nucleus	Right, ventrocaudal	13	−61	−37		3.77
	Dentate nucleus	Right, ventrocaudal	24	−60	−37		3.49
3	Dentate nucleus	Right, ventrorostral	17	−50	−40	16	4.01
Extinction phase							
1	Dentate nucleus	Left, dorsocaudal	−14	−67	−34	10	4.27
2	Dentate nucleus	Right, ventrocaudal	15	−67	−36	19	3.97
3	Dentate nucleus	Right, ventrocaudal	21	−64	−38	7	3.93
4	Dentate nucleus	Left, ventrorostral	−12	−48	−37	12	3.92
5	Dentate nucleus	Left, ventrocaudal	−17	−65	−38	12	3.66
6	Interposed nucleus	Right	12	−52	−32	3	3.54
7	Dentate nucleus	Right, dorsocaudal	12	−65	−31	19	3.50
8	Dentate nucleus	Right, ventrorostral	13	−49	−37	10	3.48
9	Dentate nucleus	Left, dorsocaudal	−9	−67	−33	1	3.27
Reacquisition phase							
1	Extended cluster					78	
	Interposed nucleus	Right	11	−55	−30		4.63
	Dentate nucleus	Right, dorsocaudal	14	−59	−35		4.03
2	Dentate nucleus	Left, dorsorostral	−10	−58	−33	86	4.42
3	Dentate nucleus	Right, dorsocaudal	20	−62	−35	5	3.45
4	Interposed nucleus	Left	−9	−53	−27	1	3.21

Table displays nuclear activation clusters thresholded at $P < 0.05$ permutation corrected ($t > 3.2$). Spontaneous blink rate was used as covariate. Within larger clusters with multiple local maxima of activation, only maxima with a minimal distance of 4 mm to a larger activation maximum are listed. Dentate nucleus quadrants chosen as previously described by Küper et al. [2011]. Activations are shown based on all blocks per phase (i.e., 6 blocks of six CS-only trials in acquisition, 7 blocks in extinction, and 4 blocks in reacquisition) and the acquisition phase considering the first 4 blocks only.

is nihilated during extinction in the cerebellar cortex, but retained in the cerebellar nuclei, allowing for faster reacquisition. Although the model by Medina et al. [2001, 2002a] worked with the assumption of LTD during acquisition which is reversed to LTP during extinction, which is not supported by the present data, the present findings are consistent with the notion that at least part of the initial memory is reversed during extinction which is rereversed during reacquisition. Similarly, the model predicts that activity of the cerebellar nuclei is inhibited during extinction, which is also consistent with decreased fMRI signal in the nuclei during extinction. We were unable, however, to find an equivalent of retained memory in the cerebellum; that is, we did not observe any difference in fMRI signal in the nuclei or cerebellar cortex comparing early acquisition and reacquisition. One possible explanation for this negative finding is that extracerebellar areas make a significant contribution to savings. Memory of the learned association may be stored outside the cerebellum, and part of this memory may be preserved during extinction. Animal data show that the hippocampus, amygdala, and prefrontal cortex are involved in acquisition of delay and trace conditioned eyeblinks to various extents [Boele et al., 2010; Mintz and Wang-Ninio, 2001; Weible et al., 2000]. Similarly, neuroimaging studies show acquisition-related changes of activity in cortical and subcortical areas, including the hippocampus, prefrontal areas, striatum, and auditory cortex [Blaxton et al., 1996; Knutten et al., 2002; Logan and Grafton, 1995; Molchan et al., 1994; Parker et al., 2012; Schreurs et al., 1997]. Recent findings [De Zeeuw and Ten Brinke, 2015; Jirenhed et al., 2007], on the other hand, provide evidence for cerebellar savings. They found that the acquisition-related pausing of the Purkinje cell rapidly reoccurred during reacquisition. Given that plastic changes during acquisition appear to be distributed and involve more areas than the cerebellum, it would be surprising if the cerebellar cortex would be the only place of savings. As yet there are no animal or neuroimaging studies that directly assessed the contribution of extracerebellar areas to saving effects. Field of view was restricted to the cerebellum in this study. It would be of interest to include the whole brain in future high-resolution fMRI studies.

Another possible explanation for the present findings is that learned associations are suppressed during extinction via a newly learned inhibition in extracerebellar areas. Kalmbach and Mauk [2012] observed that both the cerebellum and forebrain areas contribute to extinction of trace conditioned eyeblinks in rabbits. Likewise, PET studies report extinction-related changes of regional cerebral blood flow (rCBF) in cerebral areas including the prefrontal cortex [Molchan et al., 1994; Parker et al., 2012]. In extinction of conditioned fear, there is good evidence that prefrontal areas and the hippocampus are involved in learned inhibition of the amygdala during extinction in a context-dependent manner [Milad and Quirk [2012] and Orsini

and Maren [2012] for reviews). New inhibitory learning has also been suggested to contribute to extinction of conditioned eyeblinks (Hu et al. [2015] and Robleto et al. [2004] for reviews), but this has never been studied in detail. The same extinction neural network may be at work, which is known to suppress the expression of conditioned fear responses [Milad and Quirk, 2012]. For example, Hu et al. [2015] have recently proposed that extinction of conditioned eyeblinks is mediated via the rmPFC (a possible homolog of vmPFC in humans) and the amygdala. Inhibition of the amygdala may decrease the reactivity of the pontine nuclei (and therewith the cerebellum) to the CS. Reduced salience of the CS may result in (or contribute to) extinction.

The present findings do not rule out that part of the initial memory is retained within the cerebellum. In extinction, the drop of CR incidence appeared to be faster and more pronounced than the drop of the fMRI signal (β values) particularly in the cerebellar cortex. The most parsimonious interpretation is that the remaining fMRI activity is related to residual CRs and/or the CS. Activity related to the residual CR may be related to motor performance and/or the extinction process. It cannot be excluded, however, that it reflects possible saving effects.

Although relearning was significantly faster than learning, both learning and relearning occurred within a limited number of trials in the tested human subjects. In the acquisition phase, the steepest learning curve is seen in the first 25 trials. In reacquisition, it took only a couple of trials and the number of conditioned responses was back to the level at the end of the acquisition phase (Fig. 3). Because the available signal to noise ratio did not allow analysis in single trials, our fMRI analysis was based on CS-only trials with 6 CS-only trials per block. These 6 CS-only trials were interspersed between 14 paired CS-US trials in acquisition and reacquisition phase. Thus, the steepest learning curve took place within the very first block of CS-only trials and may explain why we did not find a significant increase of fMRI signal across blocks. We cannot exclude that this explains the lack of different signal comparing acquisition and reacquisition. Finally, spontaneous blink rate was higher in acquisition compared to extinction and reacquisition. One may argue that higher spontaneous blink rate in baseline had an impact on fMRI signal in acquisition, and contributes to the decline of fMRI signal in extinction. However, it cannot explain the increase of fMRI signal in reacquisition compared to extinction phase. Furthermore, considering spontaneous blink rate as covariate did not change fMRI results.

Our findings are based on CS-only trials, thus activation related to the unconditioned response had no impact on the results. Differences between acquisition, extinction, and reacquisition phases cannot be attributed to the CS impact either, because this was the same in the 3 phases. The number of conditioned responses, however, differed between the conditions, and was less in the extinction

phase compared to the acquisition and reacquisition phases. Thus, similar to every eyeblink conditioning imaging study so far published, it cannot be decided to what extent motor performance explained changes in fMRI activity. Because CR responses were higher in reacquisition compared to initial acquisition, however, it appears unlikely that fMRI signal reflects motor performance only. Furthermore, comparing fMRI signal in CS-only trials with and without CRs showed no significant differences in the acquisition phase. Thus, at least part of the fMRI signal is likely related to plastic changes which develop during learning and are independent from CR performance. In CS-only trials, on the other hand, a US is expected but does not occur. A sensory prediction error signal may also contribute to the fMRI signal [Ramnani et al., 2000].

fMRI activation was seen in more areas in the cerebellar cortex and nuclei bilaterally than the 2 ROIs. Data are in good accordance with recent findings in mice showing acquisition related plastic changes in the anterior interposed bilaterally, but also in the posterior interposed and the lateral nucleus (i.e., dentate nucleus in humans) [Boele et al., 2013]. Similarly, Crus I and II have been shown to play an additional role in acquisition [Hardiman and Yeo, 1992]. The general pattern of activations did not differ between the three phases. This finding suggests that areas involved in acquisition are also involved in extinction and reacquisition of conditioned responses, and likely correspond to cerebellar areas related to the control of eyeblink (see Fig. 3 in Dimitrova et al. [2002]). Although animal recording studies cannot give a full map of cerebellar areas involved in the three learning phases, they provide good evidence of bidirectional learning at the level of individual Purkinje cells [Jirenhed et al. 2007]. Bidirectional learning implicates that the areas involved in acquisition are also involved in extinction (and reacquisition).

Although the general pattern of activations were the same, activated areas tended to be larger in acquisition compared to reacquisition (see voxel count in Tables I and II). One possible interpretation is that the reduction in cortical and nuclear areas corresponds to saving effects. In early eyelid conditioning, however, the response to the aversive stimulus is more diffuse and involves unspecific fear responses [Medina et al., 2002b]. Concomitant conditioned fear responses are suppressed in later acquisition, and may therefore lead to more extended activation of the medial and lateral cerebellum in early acquisition than in reacquisition [Lange et al., 2015]. Learned timing and shaping of the discrete skeletal motor response (i.e., the eyeblink) in early acquisition may also involve more extended areas. Finally, although awareness is not a prerequisite of short-delay eyeblink conditioning [Clark and Squire, 1998], there are likely concomitant cognitive processes activating the posterolateral cerebellar hemisphere and ventral dentate nucleus which may be more prominent in early acquisition compared to reacquisition.

On the other hand, based on the fear conditioning literature, one may expect that additional cerebellar areas contribute to extinction. As stated above, prefrontal areas and hippocampus are known to be of part of the neural network related to extinction of conditioned fear [Milad and Quirk, 2012]. Because there are known anatomical and functional connections between the neocerebellum and prefrontal cortex and hippocampus [Liu et al., 2012; Strick et al., 2009], one may expect activation of additional posterolateral hemisphere/dentate areas in the cerebellum during extinction. This, however, was not the case. Because context dependency is stronger during extinction than acquisition (at least in learned fear), additional activation of the posterolateral cerebellum may be more prominent in case extinction takes place in a context different from the acquisition context and/or during renewal (i.e., return of a previously extinguished learned response in a context different from the extinction context). This will be of interest to investigate in the future.

The authors like to emphasize that the ROI-based analysis of fMRI signals was exploratory and not inferential, and results need to be confirmed in future studies. 7T fMRI scanning methods need to be optimized to reduce the observed intra- and interindividual variability in fMRI signal.

In conclusion, the reversal of activation in the cerebellar cortex and nuclei during extinction compared to acquisition is consistent with bidirectional learning; that is, an erasure of the initial memory during extinction. The present findings, however, cannot explain the observed behavioral saving effects. Saving-related cerebellar activation in the very first reacquisition trials may have been missed, and results need to be confirmed in future studies. Furthermore, it will be of interest to study in more detail to which extent extracerebellar regions contribute to savings.

ACKNOWLEDGMENT

The authors like to thank Javier F. Medina (Baylor College of Medicine, Houston) for his valuable advice and fruitful discussions.

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