Cerebellar theta burst stimulation dissociates memory components in eyeblink classical conditioning

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Abstract

The cerebellum plays a critical role in forming precisely timed sensory-motor associations. This process is thought to proceed through two learning phases: one leading to memory acquisition; and the other leading more slowly to memory consolidation and saving. It has been proposed that fast acquisition occurs in the cerebellar cortex, while consolidation is dislocated into the deep cerebellar nuclei. However, it was not clear how these two components could be identified in eyeblink classical conditioning (EBCC) in humans, a paradigm commonly used to investigate associative learning. In 22 subjects, we show that EBCC proceeded through a fast acquisition phase, returned toward basal levels during extinction and then was consolidated, as it became evident from the saving effect observed when re-testing the subjects after 1 week of initial training. The results were fitted using a two-state multi-rate learning model extended to account for memory consolidation. Transcranial magnetic stimulation was used to apply continuous theta-burst stimulation (cTBS) to the lateral cerebellum just after the first training session. Half of the subjects received real cTBS and half sham cTBS. After cTBS, but not sham cTBS, consolidation was unaltered but the extinction process was significantly impaired. These data suggest that cTBS can dissociate EBCC extinction (related to the fast learning process) from consolidation (related to the slow learning process), probably by acting through a selective alteration of cerebellar plasticity.

Introduction

The cerebellum is thought to play a critical role in motor learning and control. These two aspects have been integrated into the Motor Learning Theory (Marr, 1969; Albus, 1971), and the cerebellum has been proposed to act as a ‘forward controller’ predicting the consequences of learned motor acts and correcting intervening errors (Raymond et al., 1996; Ito, 2008). Multiple processes may contribute to motor skill acquisition, which usually proceeds through a rapid convergence toward a stable state before being consolidated into persistent memory (Lee & Schweighofer, 2009; Shadmehr et al., 2010). Recently, a model with different learning rates has been proposed to explain the cerebellar learning process (Smith et al., 2006).

The pavlovian eyeblink classical conditioning (EBCC) is an experimental paradigm widely used to investigate cerebellar functioning (Welsh & Harvey, 1991; Llinas et al., 1997). In EBCC, the three main operations attributed to the cerebellum are recognized: prediction; learning; and timing. The cerebellum has been shown to learn the temporal relationship between two stimuli and then to facilitate the generation of well-timed predictive responses (Perrett et al., 1993; Garcia & Mauk, 1998; Medina et al., 2002). In practice, a conditioned stimulus (CS; e.g. a sound) is repeatedly paired with an unconditioned stimulus (US; e.g. an electrical stimulus on the supraorbital nerve) eliciting the unconditioned response (UR; the eyeblink). After a certain number of pairings, the eyeblink is triggered by the CS alone just before the expected US, revealing that the association of CS and US has been learned and used to predict the occurrence of US at the appropriate time (CR; conditioned response). It should be noted that the cerebellum is necessary and sufficient when the CS precedes US and the two stimuli co-terminate (‘delay-EBCC’), while the hippocampus is also required when the CS precedes US and the two stimuli are separated by a free pause (‘trace-EBCC’). Finally, the EBCC is made of multiple components, namely acquisition, extinction and consolidation, which develop with multiple temporal dynamics and probably involve (at least in part) different cerebellar sub-circuits.

Most available information on EBCC has been obtained in experimental animals, in which cerebellar lesions prevented both delay-EBCC (Clark et al., 1984) and trace-EBCC (Woodruff-Pak et al., 1985; Kim, 1997) affecting all EBCC components (Hikosaka et al., 1995). This was clearly demonstrated in rabbits, in which the EBCC was prevented either by inactivation of the cerebellar cortex (Attwell et al., 2001, 2002), cerebellar nuclei (Knupa et al., 1993) or inferior olive (Welsh & Harvey, 1998). Recently, cerebellar cortical and
nuclear functions in EBCC have been temporally and spatially dissociated: the γ-aminobutyric acid (GABA)\(\alpha\) receptor agonist muscimol prevented the development of conditioned responses (CRs) when micro-injected immediately after training in the cerebellar cortex but not in the anterior interpositus nucleus (Attwell et al., 2002; Cooke et al., 2004). In addition, computational modeling has shown that the cerebellar cortex can account for the faster component and the deep cerebellar nuclei for slower components of EBCC learning (Medina & Mausk, 2000). Multiple plasticity sites have been suggested to balance synaptic weights in the circuit transferring initial associative learning from cortical to nuclear sites (Medina et al., 2001; Garrido et al., 2013).

In order to investigate the mechanisms and phases of EBCC in humans, cerebellar circuit function and plasticity can be modified by transcranial magnetic stimulation (TMS; Pascual-Leone et al., 1999; Walsh & Cowey, 2000; Grimaldi et al., 2013). In a recent work, cerebellar continuous theta burst stimulation (cTBS; a form of repetitive TMS (rTMS)) was shown to impair the acquisition of CRs in EBCC protocols (Hoffland et al., 2012). In the present work, we have delivered cTBS after associative learning had already occurred, in order to test whether the acquisition and consolidation processes could be dissociated (Attwell et al., 2002; Cooke et al., 2004). We found that cTBS delivered after a first learning session impaired extinction, which primarily engaged a fast adaptation process.

These observations were interpreted using a two-state learning model (Smith et al., 2006) suggesting that EBCC learning phases can be dissociated by cTBS in humans.

Materials and methods

Subjects

Twenty-two healthy volunteers participated in this study (mean age: 25.7 ± 2.49 years). All participants had no history of neurological, psychiatric or hearing disorders. All participants were naive to EBCC at the start of the study. The majority of volunteers were women; however, no gender differences have previously been reported in the literature in terms of effect of cTBS. Informed consent was obtained from all participants, and the study was approved by the local Ethics Committee and conducted in accordance with regulations laid down in the Declaration of Helsinki.

Set-up and experimental design

The experiment consisted of two EBCC sessions, separated by 1 week. Just after the end of the first EBCC session (5–10 min), a stimulation protocol was applied over the right cerebellum. A first group received a sham stimulation (SHAM, control group; 11 participants), while a second group received effective cTBS stimulation (cTBS group; 11 participants). Therefore, the first session will be called EBCC\textsubscript{pre} and the second session EBCC\textsubscript{post} with reference to the occurrence of stimulation.

The experimental design is summarized in Fig. 1. The subjects were comfortably seated on a chair in a quiet room with normal indoor lighting. Each EBCC session consisted of seven blocks: six acquisition blocks followed by one extinction block. The first nine trials of each EBCC acquisition block consisted of nine CS–US pairs, the 10th trial was US only and the 11th trial was CS only. The inter-trial interval was randomized between 10 and 30 s. The extinction block was composed of 11 trials where only CS was delivered. For all the blocks, the 12th one was a rest trial with no stimulus presentation.

The US was produced by stimulating the right supraorbital nerve percutaneously through a pair of Ag–AgCl cup electrodes with the cathode over the supraorbital foramen and the anode 2 cm above. Single, constant-current, square-wave electrical stimuli with a pulse width of 200 μs was delivered through an electrical stimulator Digitimer DS7 (Digitimer Ltd). The electrical stimulation intensity was subject-specifically adjusted in order to obtain a stable R2 response (i.e. it is the bilaterai component of UR), which occurs in humans between 20 and 50 ms after the US onset (Kimura et al., 1985). This US was preceded (inter-stimulus interval = 600 ms) by a tone (the CS) of 2 kHz and 400 ms duration presented bilaterally to the subject via binaural headphones at an intensity 50–70 dB above the individual hearing threshold (minimal sound pressure level of 80 dB; Hoffland et al., 2012). Short-latency ‘alpha’ eyelink responses (onset latency < 250 ms) sometimes occurred unconditionally in response to auditory stimuli and were not associative. Moreover, responses occasionally occurring within 250 ms from CS onset could be related to ’voluntary responses’ distinct from true CRs (Coleman & Webster, 1988), and were not scored as CRs.

Electromyographic (EMG) recordings

EMG activity was recorded, using Ag–AgCl cup electrodes, from both the orbicularis oculi muscles (OO), for eyelink detection, and from the first right dorsal interosseous muscles (FDI), for motor-evoked potentials (MEPs) detection used to set the TMS threshold (Rothwell, 1997). OO EMG activity was recorded with the active electrode on the lower eyelid and the reference electrode approximately 3 cm distant on the lateral canthus (Kimura et al., 1985). FDI EMG activity was recorded with the active and the reference electrodes arranged in a classical belly-tendon montage. EMG raw signals were amplified and band-pass filtered (20 Hz to 3 kHz) using a Digitimer D360 amplifier, digitized at a sampling rate of 5 kHz (CED 1401 laboratory interface) for on-line visual display.

TMS

A monophasic Magstim stimulator connected to a figure-of-eight coil placed over the left primary motor cortex that delivers a single-pulse TMS was used to elicit a MEP of ~1 mV peak-to-peak amplitude from the right FDI muscle.

A MagStim Super Rapid magnetic stimulator (Magstim, UK\textsuperscript{TM}), connected with a figure-of-eight coil, was used to deliver rTMS according to the cTBS protocol. cTBS consisting of three-pulse bursts at 50 Hz repeated every 200 ms given in a continuous train lasting 40 s (600 pulses) was delivered at 80% of the active motor threshold (AMT), found on the FDI (Huang et al., 2005). The AMT was defined as the lowest intensity evoking five MEPs of at least 200 μV in 10 consecutive trials while subjects maintained a low-level tonic contraction (20% of maximal voluntary contraction) in FDI muscle. cTBS was applied over the right lateral cerebellum using the same scalp co-ordinates (1 cm inferior and 3 cm right to the inion) adopted in previous magnetic resonance imaging studies showing that this site targets the posterior lobules of the lateral cerebellum (Del Olmo et al., 2007). The coil was positioned tangentially to the scalp (Koch et al., 2008).

Sham stimulation was delivered through the figure-of-eight coil angled at 90° with only the edge of the coil resting on the scalp. Stimulus intensity was set only at 40% AMT. This stimulation intensity along with the tilted arrangement of the coil, while ineffective in inducing any cortical activation or unpleasant sensations,
ensures an adequate noise and scalp sensation (Koch & Rothwell, 2009; Brusa et al., 2012).

Data analysis

The CRs were detected from the EMG recordings in acquisition and extinction blocks analysed on a trial-by-trial basis quantifying the number of CRs (#CRs) occurring within each block. CR onset was marked at the earliest point at which EMG activity began to rise from EMG baseline level, in a time window from 300 ms before US onset to the US onset itself. CRs were defined as EMG activity lasting at least 50 ms (Gerwig et al., 2005; Fig. 2).

Statistical analysis was performed using factorial ANOVA test (STATISTICA®) in each session, with BLOCK and GROUP as independent categorical factors. Then, in order to determine the statistical significance of differences in pairwise comparisons, the multiple comparison Bonferroni test (least significant difference) was performed.

In all tests, the level of statistical significance was preset to $P < 0.01$. Unless otherwise stated, all results are indicated as mean ± standard error of the mean (SEM).

The two-state consolidated model of EBCC learning

The EBCC kinetics were modeled by extending the two-state multirate model (Smith et al., 2006; Shadmehr et al., 2010) to include the consolidation process occurring between EBCCpre and EBCCpost (‘two-state consolidated model’). The net adaptation is made up of the sum of two processes, fast and slow. The fast process $(x_f)$ responds strongly to large errors but has poor retention, while the slow process $(x_s)$ responds weakly to small errors but retains information well.

$$x(n + \Delta n) = A \cdot x(n) + B \cdot e(n)$$

$$y(n) = C \cdot x(n) + D$$

where the $x$ vector is composed by the two state vectors $(x_s$ and $x_f)$:

$$x(n) = \begin{bmatrix} x_s(n) \\ x_f(n) \end{bmatrix}$$

$B_f$ and $B_s$ are the learning rates ($B_f > B_s$), $A_f$ and $A_s$ are the retention factors ($A_f < A_s$), $n$ is the block and $\Delta n$ is the discrete block increment. The error $e(n)$ is the difference between the motor output $y(n)$ and the ‘optimal’ state dependent from the imposed task $f(n)$, so that $e(n) = y(n) - f(n)$. In the ideal case, $f(n) = 100\%$ (10 trials) of CRs in the acquisition blocks (CS + US, blocks 1–6), while $f(n) = 0\%$ of CRs in the extinction block (CS only, seventh block) for both EBCCpre and EBCCpost sessions. The free parameters $A_s$, $A_f$, $B_s$ and $B_f$ were initialized to the values reported for force-field experiments (Smith et al., 2006):

$$A = \begin{bmatrix} 0.99 & 0 \\ 0 & 0.75 \end{bmatrix}; B = \begin{bmatrix} 0.04 \\ 0.4 \end{bmatrix}$$

The model input was the error $[e(n)]$ and the model output $[y(n)]$ was the predicted #CRs for each block. The model was first applied

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to all data obtained in the EBCC-pre blocks irrespective of the subject group in order to obtain optimal parameter estimates. Data fittings were elaborated using Matlab functions implementing an iterative prediction-error minimization method in order to adjust the free parameters of the model to their optimal values. The two processes were initialized to zero level (‘block 0’), corresponding to a naive behavior, in the EBCC-pre session. By having to account for two sequential learning sessions separated by a week, during which a remarkable maintenance of the learning level attained during the first session was observed, the model was extended to include a consolidation mechanism, which is a memory transition from a temporary fast state to a stable slow state during the inactive period, i.e. outside the context of the task. Given that a fast extinction driven by the fast process does not cancel the learnt associative task, we have considered the late stable acquisition performance as the motor memory installed and consolidated in-between the two sessions. Thus, the initial condition of the slow learning process in EBCC-post was set at the net learning level attained at the last block of EBCC-pre acquisition (block 6). Then, while maintaining model parameters fixed, the model was extrapolated in order to predict the EBCC-post session. The goodness-of-fit was evaluated through a regression test ($R^2$ and RMSE) between the experimental data (block values) and the model points.

Results

In these experiments we have investigated the role of the cerebellum in associative learning during a two-session EBCC protocol. This protocol was designed in order to allow the subjects to learn the association of a tone with an electrical stimulus of the right supraorbital nerve. Learning occurred in a first training session (EBCC-pre) and the subjects were then re-tested using the same protocol 1 week later (EBCC-post). The underlying hypothesis is that the cerebellum learns on two different time-scales, so that the cerebellar cortex operates as a fast learning module, while deeper structures, like the cerebellar nuclei, operate as a slow learning module (Medina et al., 2001). We tested this hypothesis by interfering with the cerebellar adaptation processes using cerebellar cTBS between the two EBCC sessions. The results were interpreted using a two-state multi-rate model integrating two learning processes with different sensitivities to error and different retention strengths (Smith et al., 2006).

Acquisition, extinction and consolidation of the CR in EBCC

The EBCC experiments were well tolerated by all subjects. The threshold current that elicited an eyeblink was 1.9 ± 0.3 mA. A stimulus intensity of 12.2 ± 8.1 mA was used and maintained throughout the experimental sessions. The cTBS protocol was well tolerated by all subjects and no adverse effects were reported. The mean AMT was 41.0 ± 2.8% of maximum stimulator output. The subjects were randomly divided into two groups, one undergoing sham stimulation and the other undergoing cerebellar cTBS stimulation.

An example of the EBCC learning process in a subject tested with sham stimulation is shown in Fig. 3A, along with the CRs pattern during the 12 trials of the first, sixth and seventh blocks. In EBCC-pre, the learning process started from a naive state (no CR) and passed through an acquisition phase, during which the proportion of CRs gradually increased and eventually stabilized at a value of about 80%. Then the CRs disappeared during extinction. In EBCC-post, the CRs appeared immediately with high probability and the proportion of CRs remained high (about 80%) thereafter. Finally, the CRs disappeared again during extinction. This behavior indicated that a consolidation process progressed during the week of washout between the EBCC-pre and EBCC-post, so that the association of the US and CS was saved.

An example of the EBCC learning process in a subject tested with cerebellar cTBS is shown in Fig. 3B. The EBCC-pre session looked very similar to that of subjects undergoing sham stimulation. In the EBCC-post session, the first block showed CRs reversal, but in the seventh block CRs extinction was reduced. Therefore, cTBS was more likely to interfere with the fast rather than the slow learning process.

These results were in line with the hypothesis that different mechanisms took part in the EBCC learning process. The existence of a fast rapidly reversible learning process emerged during the acquisition and extinction phases. The existence of a slower process emerged as consolidation. The coexistence of two processes proceeding at different rates resembled EBCC learning in rabbits (Medina et al., 2001) and force-field learning in humans (Smith et al., 2006).

In order to evaluate the consistency of observations reported in Fig. 3, the analysis was extended over the entire data set, and was analysed using multiple ANOVA statistics.

During EBCC-pre, both groups showed a similar increase in the number of CRs from the first to sixth block, followed by a decrease of CRs in the seventh block reflecting the extinction phase. ANOVA analysis yielded a significant effect of the BLOCK factor (BLOCK effect: $N = 7; df = 6; F = 5.6; P < 0.001; \eta^2$ partial = 0.51), but not of the GROUP factor (GROUP effect: $N = 2; df = 1; F = 6.3; P = 0.015$), indicating that the two groups could be considered as taken from the same population.

During EBCC-post, ANOVA analysis yielded a significant effect of the BLOCK factor (BLOCK effect: $N = 7; df = 6; F = 5.6; P < 0.001; \eta^2$ partial = 0.18) as well as of the GROUP factor (GROUP effect: $N = 2; df = 1; F = 8.9; P = 0.003; \eta^2$ partial = 0.055), Fig. 4. Out of the 91 pairwise comparisons performed by the Bonferroni test, only 12 comparisons resulted in significant differences: these comparisons were those between the seventh block of the SHAM group with blocks 1–6 of the SHAM group, and the comparisons between the seventh block of the SHAM group with blocks 1–6 of the cTBS group. The only distinguishable block was the extinction block in the SHAM group, which then determined the difference observed between groups. These post hoc findings mean that the extinction block of the cTBS group was not significantly different from the preceding six acquisition blocks, indicating that extinction was impaired by cTBS.

It should be noted that CRs always occurred before the US and CS was saved. The anticipation time with respect to US onset was: cTBS in EBCC-pre, 45.9 ms; cTBS in EBCC-post, 45.9 ms; SHAM in EBCC-pre, 9.2 ms; SHAM in EBCC-post, 9.2 ms; cTBS in EBCC-pre, −188.15 ± 8.47 ms; cTBS in EBCC-post, −190.96 ± 19.07 ms. The CR latencies were consistent with previous EBCC studies in humans (Hoffland et al., 2012). The factorial ANOVA on the anticipation times for EBCC-pre revealed no significant effects associated either to BLOCK or to GROUP factors (BLOCK effect: $N = 7; df = 6; F = 1.19; P = 0.31$; GROUP effect: $N = 2; df = 1; F = 0.74; P = 0.39$). The factorial ANOVA on anticipation times for EBCC-post revealed no significant effects associated to BLOCK factor (BLOCK effect: $N = 7; df = 6; F = 0.25; P = 0.96$), while the GROUP factor was significant (GROUP effect: $N = 2; df = 1; F = 11.66; P < 0.001; \eta^2$ partial = 0.08), revealing that CRs were even more anticipated in the SHAM group. However, the Bonferroni post hoc analysis did not reveal any significant difference associated with specific group/block combinations.
A two-state consolidated model of learning predicts EBCC data

The EBCC data were interpreted using a two-state multi-rate model (Smith et al., 2006; Shadmehr et al., 2010) characterized by the combination of a fast and a slow learning process and extended to include a consolidation process (‘two-state consolidated model’; Fig. 5). Both fast and slow learning processes in the EBCCpre model started from zero, assuming that learning was elicited from a naïve level (see Fig. 3). Consolidation in the model occurred through an update of the slow state to the level reached at the end of acquisition in EBCCpre. Parameter optimization was based on the ensemble of EBCCpre data, as no GROUP effect was observed, yielding:

$$A = \begin{bmatrix} 0.88 & 0 \\ 0 & 0.68 \end{bmatrix}; B = \begin{bmatrix} 0.088 \\ 0.36 \end{bmatrix}$$

The model showed that, when the CS–US pairs were presented, CR generation in the first blocks was driven mainly by the fast process, then, as acquisition progressed, learning was taken over by the slow process. Finally, extinction was largely driven by the fast process. Correlation analysis confirmed the goodness of the model in fitting the data ($R^2 = 0.97$; RMSE = 11%). Each model point lied within the standard deviation interval of each corresponding data point. It should be noted that the $A$ and $B$ parameters obtained by fitting were similar to those reported for force-field experiments (Smith et al., 2006), supporting that the two-state multi-rate model could be generalized to EBCC.

In order to verify whether the model could also account for EBCCpost after consolidation (i.e. after setting the initial CR to the level attained during the sixth block of EBCCpre), the same parameters optimized during EBCCpre were used to extrapolate the model to EBCCpost.

The results reported above showed the significant GROUP effect (SHAM and cTBS) for the EBCCpost. Thus, data of the two groups were compared separately to the model. For the SHAM group, the model fitting was robust and highly significant ($R^2 = 0.99$; RMSE = 5.1%). For the cTBS group, the model fitting was slightly less robust and with a higher averaged distance between model and data points ($R^2 = 0.96$; RMSE = 14%). Apparently, the only model point outside the experimental data variability range was the seventh block of the cTBS group, with a 32% distance from the mean data value.

Therefore, a two-state consolidated model effectively accounted for responses in a two-session EBCC paradigm and identified the deviation in EBCC extinction caused by cerebellar cTBS.

Discussion

This work, by combining results obtained by applying cTBS over the cerebellum with a two-state learning model, shows that EBCC
A two-state consolidated model explains EBCC

The EBCC learning kinetics were explained adopting a two-state multi-rate model modified to save memory between the EBCCpre and EBCCpost sessions. Interestingly, the rate constants and the relative proportions of the two learning components in EBCC experiments were similar to those obtained in force-field experiments (Smith et al., 2006), suggesting a general applicability of this cerebellar learning scheme to motor adaptation in different tasks.

The multi-rate model correctly predicted learning dynamics occurring before and after the consolidation process (Kojima et al., 2004). The model implied that consolidation occurred through a growth of the slow process, which collected also the motor memory component ascribed to the fast process. Hence, in parallel, during this motor memory transfer, the fast process decreased going back to zero level. This complex restructuring of memory suggested that the CS-US association was actively ‘re-called’, in line with observations indicating that extinction primarily involves only a temporary inhibition of the original memory (Rescorla, 1996). It should also be noted that, in the model, the fast process was driven by large errors, while the slow process was driven by small errors. The specific effect of cTBS on the fast processes is consistent with the observation that, in the vestibular-ocular reflex, inhibition of Purkinje cell activity affected only the adaptation mechanisms engaged by large errors (Boyd et al., 2006).

In aggregate, the model supports the hypothesis that cerebellum-driven learning arises from plastic mechanisms with at least two different response rates. Their superposition can explain acquisition, extinction and consolidation. Fast and slow processes would be updated simultaneously from motor learning errors, supporting a parallel architecture of motor memory (Lee & Schweighofer, 2009).

The effects of cTBS on EBCC

The ability of cerebellar cTBS to dissociate fast from slow EBCC components was likely to depend on the localization and properties of the TMS stimuli. Both the cerebellar cortical circuit and the deep cerebellar nuclei could play a crucial role in delay-EBCC, while the hippocampus is specifically involved in trace-EBCC (Kim, 1997; Mauk, 1997).

Cerebellar TMS is a reliable method to investigate cerebellar functions (Grimaldi et al., 2013), provided that the stimulation threshold is carefully defined using rectified EMG, and that current direction and stimulation site are accurately chosen (Ugawa et al., 1995; Shirota et al., 2011). Here, TMS pulses were applied at sub-threshold intensities in a lateral region previously demonstrated to affect EBCC (Del Olmo et al., 2007; Koch & Rothwell, 2009). Moreover, recent works have shown that TMS can generate distinct effects on visual motion detection when applied to the lateral cerebellum with respect to vermis, without causing significant activation of the nearby occipital visual cortex (Cattaneo et al., 2014). Therefore, we expect that the effects of cerebellar TMS on EBCC occurred through an interference with neuronal functions in the visuo-motor area of the lateral cerebellum. Several experimental works have shown that TMS can be used to investigate the functionality of the cerebello-thalamo-cortical pathway (Ugawa et al., 1995). rTMS delivered over the lateral cerebellum was shown to induce long-lasting changes in cortical excitability (Popa et al., 2010) and to determine relevant behavioral changes in healthy subjects, such as decreasing performance accuracy during paced-finger-tapping-tasks (Theoret et al., 2001), reducing time perception in cognitive tasks (Koch et al., 2007; Oliveri et al., 2007), and reducing the number of errors in a variety of cognitive tasks.
of category switches and number of words during phonemic fluency tasks (Arasanz et al., 2012).

As far as the stimulation pattern is concerned, cTBS has been reported to affect long-term synaptic plasticity in the cerebral cortex (Huang et al., 2005) and potentially also in the cerebellum (Colnaghi et al., 2011). Therefore, application of cTBS could have affected specific mechanisms of cerebellar plasticity. Experiments in rats in vitro and in vivo have shown that theta rhythms have remarkable impact on cerebellar cortex functioning. The cerebellar cortical circuit is resonant in the theta-band and selectively amplifies responses at 3–7 Hz (Gandolfi et al., 2013). Moreover, theta-stimulation patterns induce forms of long-term potentiation (LTP) and long-term depression (LTD) both at the mossy fiber-granule cells synapse (D’Angelo et al., 1999, 2005) and at the parallel fiber–Purkinje cell synapse (Koekkoek et al., 2003; De Zeeuw & Yeo, 2005). It is therefore expected that cTBS caused plastic changes in the cerebellar cortical circuit, as also suggested for saccadic eye movement adaptation (Colnaghi et al., 2011), eventually causing a long-lasting disorganization of EBCC mechanisms.

Thus, our results are consistent with the hypothesis that the fast reversible phase of EBCC learning occurs in the cerebellar cortex, while the persistent memory is stored into deeper structures, for example in the deep cerebellar nuclei. As a corollary, cTBS affected memories based on large magnitude errors, i.e. it altered the fast process operations by unbalancing its learning and retention. Alternatively, cTBS made the stored motor memory hard to reverse.

### Insight on EBCC mechanisms

The involvement of the cerebellar cortex in EBCC was previously suggested by experiments in which the GABA\(_A\) receptor agonist muscimol was infused to transiently inactivate local circuit functions in rats. Infusion of muscimol in the posterior cerebellar cortex (lobule HVI) was effective after short (5–45 min; Attwell et al., 2002) but not after longer delays (90 min; Cooke et al., 2004). Conversely, muscimol infusion in the anterior interpositus nucleus just after training was poorly effective. These experiments suggested that learning was transferred quite early from a cortical into a nuclear neuronal site. Accordingly, the effect of cTBS was related to TMS applied just after training (5–10 min) and affected the transient phase of learning.

The cellular mechanisms of EBCC learning are thought to depend on long-term synaptic plasticity at cortical and deep cerebellar nuclei (DCN) synapses (D’Angelo, 2014). The parallel fiber–Purkinje cells synapse is strategically located at the convergence between the mossy fiber–parallel fiber pathway (carrying the CS) and the climbing fiber pathway (carrying the US). Another site of convergence is the DCN, which collects both mossy fiber and climbing fiber signals, in addition to being modulated by Purkinje cells. At both sites, long-term synaptic plasticity has been suggested to play important roles in EBCC (De Zeeuw & Yeo, 2005). In particular, cortical plasticity has been associated with the fast learning process, and DCN plasticity with the slow learning process ( Medina & Mauk, 2000; Garrido et al., 2013). Thus, the effect of cTBS is compatible with disruption of cortical rather than DCN plasticity.

Given the distributed nature of cerebellar cortical plasticity, a working hypothesis is that cTBS operated at multiple cortical sites (D’Angelo, 2014): (i) in the granular layer, on N-methyl-D-aspartate (NMDA) receptor-dependent LTP and LTD at the mossy fiber–granule cell synapses as well as on long-lasting changes in granule cell intrinsic excitability; (ii) in the molecular layer, on various forms of NMDA receptor-independent LTP and LTD at parallel fiber–Purkinje cell synapses, at climbing fiber–Purkinje cell synapses, at molecular interneuron synapses as well as on long-lasting changes in Purkinje cells intrinsic excitability. Some drugs may therefore be used to dissect the underlying mechanisms in humans in vivo: for example, nimodipine (a calcium antagonist) was shown to enhance EBCC acquisition and retention (Deyo et al., 1990; Straube et al., 1990; Solomon et al., 1995), memantine (an NMDA channel antagonist) is supposed to re-activate the fast EBCC learning process (Klyubin et al., 2011) and modafinil (an ampakine potentiating AMPA receptor functions) could enhance LTP and modify the TBS effects on cortical plasticity. Moreover, EBCC simulations using advanced mechanistic models of the cerebellar networks incorporating multiple plasticity mechanisms (Garrido et al., 2013) could help in defining the possible patterns of alterations leading to the specific EBCC impairment caused by cTBS.

### Conclusions and perspectives

This work suggests that a fast phase of EBCC affected by cTBS is controlled by the lateral posterior cerebellum through a mechanism related to long-term synaptic plasticity in the local microcircuit. The combination of cTBS experimental results with a multi-rate model suggests that EBCC conforms to a dynamic and distributed learning scheme, whose synaptic mechanisms could be dissected using pharmacological tests and mechanistic cerebellar network models (Garrido et al., 2013). EBCC analysis and modeling could eventually be used to investigate the integrity of cerebellar functions in subjects with cerebellar deficits (Christian & Thompson, 2003).

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### Abbreviations

AMT, active motor threshold; CR, conditioned response; CS, conditioned stimulus; cTBS, continuous theta burst stimulation; DCN, deep cerebellar nuclei; EBCC, eyeblink classical conditioning; EMG, electromyogram; FDI, first dorsal interosseous; GABA, \(\gamma\)-aminobutyric acid; LTD, long-term depression; LTP, long-term potentiation; MEP, motor-evoked potential; NMDA, N-methyl-D-aspartate; OO, orbicularis oculi; rTMS, repetitive transcranial magnetic stimulation; TMS, transcranial magnetic stimulation; UR, unconditioned response; US, unconditioned stimulus.

### References


