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Changes in intracortical circuits of the human motor cortex following theta burst stimulation of the lateral cerebellum

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ABSTRACT

Objective: The cerebellum takes part in several motor functions through its influence on the motor cortex (M1). Here we applied the theta burst stimulation (TBS) protocol, a novel form of repetitive Transcranial Magnetic Stimulation (rTMS) over the lateral cerebellum. The aim of the present study was to test whether TBS of the lateral cerebellum could be able to modulate the excitability of the contralateral M1 in healthy subjects.
Methods: Motor evoked potentials (MEPs) amplitude, short intracortical inhibition (SICI), long intracortical inhibition (LICI) and short intracortical facilitation (SICF) were tested in the M1 before and after cerebellar continuous TBS (cTBS) or intermittent TBS (iTBS).
Results: We found that cTBS induced a reduction of SICI and an increase of LICI. On the other hand, cerebellar iTBS reduced LICI. MEPs amplitude also differently vary following cerebellar stimulation with cTBS or iTBS, resulting decreased by the former and increased by the latter.
Conclusions: Although the interpretation of these data remains highly speculative, these findings reveal that the cerebellar cortex undergoes to bidirectional plastic changes that modulate different intracortical circuits within the contralateral primary motor cortex.
Significance: Long lasting modifications of these pathways could be useful to treat various pathological conditions characterized by altered cortical excitability.

1. Introduction

The cerebellum takes part in several motor functions through its influence on the motor cortex and corticospinal outputs (Eccles et al., 1967; Ito, 2001 for a review). Purkinje cells (PC), the output neurones of the cerebellar cortex, have inhibitory connections with the deep cerebellar nuclei (DCN), which have a disynaptic excitatory pathway through the ventral thalamus to the motor cortex (Allen and Tsukahara, 1974; Kelly and Strick, 2003 among others). Inhibitory PC output results in a reduction of excitatory output from DCN to the motor cortex that leads to modification of motor control. Furthermore, cerebellar PC exhibit unique features of synaptic plasticity. In animal models, when two inputs, one from a climbing fiber and the other from a set of granule cell axons, are repeatedly associated in PC, the input efficacy of the granule cell axons in exciting the PC is persistently depressed (LTD see Ito, 2001, 2002). On the other hand, granule cells excitation may be persistently enhanced following theta burst or prolonged high frequency (100 Hz) electrical stimulation of the mossy fibers, indicating the occurrence of glutamatergic long term potentiation (LTP) (Kase et al., 1980; Maffei et al., 2002, 2003; D'Angelo et al., 1999, 2001; Lev-Ram et al., 2003; Coesmans et al., 2004; Jörntell and Hansel, 2006). These mechanisms are crucial for spatial distribution of plasticity, local network activity and long-range modulation of different neural sites (D'Angelo et al., 2005).

In humans, activity in the cerebello-thalamo-cortical pathway has been demonstrated non-invasively trough electrical (Ugawa et al., 1991;Ugawa et al., 1994) or transcranial magnetic stimulation of the cerebellum (Ugawa et al., 1995; Pinto and Chen, 2001). A single TMS pulse applied over the lateral cerebellum 5–7 ms before magnetic stimulation of the primary motor cortex (M1) causes inhibition of the motor-evoked potential (MEP) produced by motor cortical stimulation (cerebellar inhibition-CBI). A recent study used

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magnetic cerebellar stimulation to investigate connections between the cerebellum and intracortical circuits within the contralateral M1 tested with paired pulse TMS (ppTMS) protocols. Daskalakis and coworkers (2004) reported that cerebellar stimulation is able to modulate both inhibitory and excitatory neurones in the human motor cortex, since magnetic stimulation of the cerebellum at different intensities changed the activity of short intracortical inhibition (SICI), intracortical facilitation (ICF) (Kujirai et al., 1993; Ridding et al., 1995; Ziemann et al., 1996; Rothwell, 1997; Chen et al., 1998; Roshan et al., 2003; Chen, 2004), and long intracortical inhibition (LICI) (Valls-Sole et al., 1992; Wassermann et al., 1996; Ziemann et al., 1998; Hanajima et al., 2002) in the contralateral M1. Such intracortical circuits are taught to reflect the activity of distinct GABAergic and Glutamatergic interneurones (Chen, 2004).

Furthermore, recent investigations showed that when repetitive TMS (rTMS) is applied over the cerebellum at low frequency (1 Hz), long lasting changes occur in the excitability of the contralateral M1. Following cerebellar rTMS, MEPs were suppressed up to 30 min and ICF was concurrently modified (Oliveri et al., 2005; Fierro et al., 2007). Indeed, the same procedure interfered with the execution of cognitive tasks, presumably modulating cerebello-thalamo-cortical circuits targeting different cortical areas such as contralateral prefrontal and parietal cortices (Torriero et al., 2004, 2007; Koch et al., 2007; Oliveri et al., 2007).

Moreover, the potential of rTMS as a tool to induce plastic changes in humans has been recently demonstrated trough the development of the new theta burst stimulation (TBS) protocol (Huang et al., 2005), a novel form of rTMS that employs very low intensity to increase or decrease motor cortical excitability in healthy subjects for up to 20 min after the end of stimulation (Huang et al., 2005). In analogy with the well known protocols able to induce LTP or LTD in animal brain slices (Hess and Donoghue, 1996), TBS makes use of brief trains of high frequencies of stimulation (up to 50 Hz) to induce focal long-lasting changes in cortical excitability. Continuous TBS (cTBS) was able to decrease the excitability of the primary motor cortex, activating LTD-like mechanisms, while the opposite effect was induced when the brief trains were intermittent (iTBS).

On the basis of the previous works in humans, showing that cerebellar rTMS is able to induce persistent changes in the excitability of contralateral motor cortex (Oliveri et al., 2005; Fierro et al., 2007) and taking account of the previous investigations in animals showing the existence of both LTP- and LTD-like mechanisms in the cerebellum (Ito, 2001; Ito, 2002; Kase et al., 1980; Maffei et al., 2002, 2003; D'Angelo et al., 1999, 2001), in this study we hypothesized that if the novel TBS protocols were applied over the cerebellum, they could be able to activate different plastic mechanisms and therefore induce opposite changes of specific intracortical circuits in the interconnected contralateral motor cortex. In analogy with the results obtained with cerebellar low frequency 1 Hz rTMS (Oliveri et al., 2005; Fierro et al., 2007), cTBS, a procedure known to have similar inhibitory effects when applied over the primary motor cortex as 1 Hz rTMS, could increase the excitability of the contralateral motor cortex. On the other hand, iTBS should induce opposite effects. Therefore, we applied TBS on the lateral cerebellum and we tested possible changes in the excitability of the contralateral M1. MEPs amplitude and different inhibitory and facilitatory intracortical circuits (SICI, LICI, SICF) were measured before and after cerebellar continuous TBS (cTBS) or intermittent TBS (iTBS).

2. Methods

2.1. Subjects

Twenty healthy volunteers (ten men and ten women, range 20–37 years old) participated in this study. All subjects were right handed based on the Edinburgh Handedness Inventory. Written informed consent was obtained from all subjects. The experimental procedures used here were approved by the local Ethics Committee and were carried out in accordance with the Declaration of Helsinki.

2.2. EMG recording

Motor-evoked potentials were recorded bilaterally from the first dorsal interosseous (FDI) muscles using 9 mm diameter, Ag–AgCl surface cup electrodes. The active electrode was placed over the muscle belly and the reference electrode over the metacarpophalangeal joint of the index finger. Responses were amplified with a Digitimer D360 amplifier (Digitimer Ltd, Welwyn Garden City, Herts, UK) through filters set at 20 Hz and 2 kHz, then recorded by a computer using SIGNAL software using a sampling rate of 5 kHz per channel (Cambridge Electronic Devices, Cambridge, UK).

2.3. Paired pulse transcranial magnetic stimulation protocols

Single and paired TMS of the motor cortex of both hemispheres were performed with a 9 cm figure-of-eight coil and two Magstim 200 stimulators (The Magstim Company, Whitland, UK) connected via two Bistim modules. The magnetic stimuli had a nearly monophasic pulse configuration, with a rise time of ~100 μs, decaying back to zero over ~0.8 ms. For paired pulse protocols the output of each of the two pairs of Magstim 200 stimulators was connected to the TMS coil using a y cable. The coil was placed at the optimal position for eliciting MEPs from the contralateral FDI muscle. The optimal position was marked on the scalp with a felt pen to ensure identical placement of the coil throughout the experiment. The handle of the coil pointed backward and was perpendicular to the presumed direction of the central sulcus, about 45 deg to the midsagittal line. The direction of the induced current was from posterior to anterior and was optimal to activate the motor cortex trans-synaptically (Werhahn et al., 1994).

The resting motor threshold (RMT) was defined as the lowest intensity that produced MEPs of >50 μV in at least five out of 10 trials with the muscles relaxed (Rossini et al., 1994). The active motor threshold (AMT) was defined as the lowest intensity that produced MEPs of >200 μV in at least five out of 10 trials when the subject made a 10% of maximum contraction using visual feedback (Rothwell, 1997). Determination of RMT and AMT were done in step width of 1% of MSO. SICI and ICF were tested using paired TMS with a subthreshold conditioning stimulus (CS) preceding a suprathreshold TS (Kujirai et al., 1993; Rothwell, 1997). Subthreshold CS stimulus was set at 80% AMT while the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. ISIs of 1, 2, 3, 5, 7, 10 and 15 ms were utilized to test SICI and ICF. LICI was tested following the protocol adopted by Valls-Sole et al. (1992). The intensity of TS were adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. The intensity of CS was set at 120% RMT. The CS preceded the TS by 100 and 150 ms. SICF was tested with TS set at 130% RMT followed by a subthreshold CS adjusted at an intensity of 90% RMT. ISIs of 1.0, 1.3, 2.1, 2.5, 3.3 and 4.1 ms were used (Ziemann et al., 1998; Cattaneo et al., 2005).

2.4. Repetitive transcranial magnetic stimulation

A MagStim Super Rapid magnetic stimulator (Magstim Company, Whitland, Wales, UK), connected with a figure-of-eight coil with a diameter of 90 mm was used to deliver rTMS over the scalp site corresponding to the lateral cerebellum. The magnetic stimulus had a biphasic waveform with a pulse width of about 300 μs. During the first phase of the stimulus, the current in the centre

of the coil flowed toward the handle. Three-pulse bursts at 50 Hz repeated every 200 ms for 40 s (equivalent to "continuous theta burst stimulation, cTBS" in Huang et al. (2005) were delivered at 80% AMT over left lateral cerebellum (600 pulses). In the intermittent theta burst stimulation pattern (iTBS), a 2 s train of TBS is repeated 20 times, every 10 s for a total of 190 s (600 pulses). AMT was tested over the motor cortex of the left hemisphere. TMS was applied over the lateral left cerebellum using the same scalp co-ordinates (1 cm inferior and 3 cm left to the inion) adopted in previous studies, in which MRI reconstruction and neuronavigation systems showed that cerebellar TMS in this site predominantly target the posterior and superior lobules of the lateral cerebellum (Koch et al., 2007; Fernandez Del Olmo et al., 2007). Although cerebellar stimulation has been originally performed with a double cone coil (Ugawa et al., 1995) we used the figure-of-eight coil, since this approach has been adopted in previous investigations in which cerebellar rTMS was shown to be effective in modulating the excitability of the contralateral motor cortex (Oliveri et al., 2005; Fierro et al., 2007). The coil was positioned tangentially to the scalp, with the handle pointing superiorly. This orientation is able to modulate contralateral M1 excitability (Oliveri et al., 2005) and to interfere with cognitive functions such as procedural learning and sub-second time perception when a 1 Hz rTMS paradigm is adopted (Torriero et al., 2004, 2007; Koch et al., 2007). The exact coil position was marked by an inking pen to ensure an accurate positioning of the coil throughout the experiment. The stimulating coil was held by hand and coil position was continuously monitored throughout the experiment.

2.5. Experimental design (Fig. 1)

This study involved seven experiments, that were carried out in different days, at least one week apart. Subjects were randomly allocated to the different experiments.

2.6. Experiment 1: effects of cTBS of the lateral cerebellum on MEPs, SICI and ICF circuits

In ten subjects MEPs, SICI and ICF of the right M1 were tested in different blocks before and after cTBS protocol applied over the left lateral cerebellum. The order of presentation of the blocks was pseudorandomized across subjects. Twenty MEPs were recorded before, 1, 15, 30 and 60 min after TBS. Before cTBS, the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. Measurements were made on each individual trial. The mean peak-to peak amplitude of the MEP was calculated off-line for each block. For SICI and ICF the TS given alone, or the TS preceded by the CS at various interstimulus intervals (ISIs) were intermixed randomly in one block. In each block seven conditions were randomly intermingled: TS alone (MEP) and CS + TS (conditioned MEP for each six different ISIs: 1, 3, 5, 7, 10 and 15 ms). Two blocks were recorded before and following TBS, (after that MEPs alone were recorded, 3 min after TBS). Before and after TBS the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. CS intensity was set at 80% AMT. Ten responses were collected for paired conditioned MEP for each ISI and 20 for test stimulus alone with a total number of 80 trials in each block. The inter-trial interval was set at 5 s (±10%), for a total duration of approximately 7 min. Measurements were made on each individual trial. The mean peak-to peak amplitude of the conditioned MEP at each ISI was expressed as a percentage of the mean peak-to-peak amplitude size of the unconditioned test pulse in that block. The order of blocks was pseudorandomized across subjects.

In five subjects TMS was applied over the left M1 ipsilateral to cerebellar stimulation. MEPs and SICI curve were recorded in a separate session before and after cTBS.

2.7. Experiment 2: effects of cTBS applied on the lateral cerebellum on LICI and SICF circuits (Fig. 2)

In twelve subjects (eight of whom took part in Exp. 1) LICI and SICF circuits of the right M1 were tested in different blocks before and after cTBS applied over the left lateral cerebellum. The order of presentation of the blocks was pseudorandomized across subjects. Recordings started 1 min after TBS for the first block and 8 min after TBS for the second block. For LICI circuit the TS given alone, or the TS preceded by the CS at various interstimulus intervals (ISIs) were intermixed randomly in one block. In each block four conditions were randomly intermingled: TS alone (MEP) and CS + TS (conditioned MEP for each different ISIs: 100 and 150 ms). Two blocks were recorded before and after TBS. Before each block, the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. CS intensity was set at 120% RMT. Ten responses were collected for paired conditioned MEP for each ISI and 20 for test stimulus alone with a total number of 50 trials in each block. The inter-trial interval was set at 5 s (±10%), for a total duration of approximately 5 min. Measurements were made on each individual trial. The mean peak-to peak amplitude of the conditioned MEP at each ISI was expressed as a percentage of the mean peak-to-peak amplitude size of the unconditioned test pulse in that block.

For SICF circuit the TS given alone, or the TS followed by the CS at various interstimulus intervals (ISIs) were intermixed randomly in one block. In each block seven conditions were randomly intermingled: TS alone (MEP) and CS + TS (conditioned MEP for each six different ISIs: 1, 1.3, 2.1, 2.5, 3.3 and 4.1 ms). Two blocks were recorded before and after TBS. Before each block, the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. CS intensity was set at 90% RMT. Ten responses were collected for paired conditioned MEP for each ISI and 20 for test stimulus alone with a total number of 70 trials in each block. The inter-trial interval was set at 5 s (±10%), for a total duration of approximately 6 min. Measurements were made on each individual trial. The mean peak-to peak amplitude of the conditioned MEP at each ISI was expressed as a percentage of the mean peak-to-peak amplitude size of the unconditioned test pulse in that block.

2.8. Experiment 3: effects of cTBS applied on the neck muscles on MEPs, SICI and LICI circuits

Since previous studies demonstrated that rTMS may partially modulate the corticospinal output through a spinal mechanism involving activation of peripheral nerve fibers (Gerschlager et al., 2002), we performed a control experiment in six subjects in which the coil was placed over the left neck area 5 cm below the area where cerebellar stimulation had been performed ('posterior neck stimulation'). The handle of the coil pointed upwards, which induced an upward current in the brain during the reversal phase of the biphasic stimulus.

In six subjects (four of whom participated in Exp. 1 and 2) MEPs, SICI, ICF and LICI of the right M1 were tested in different blocks before and after cTBS protocol applied over the left neck muscles. The order of presentation of the blocks was pseudorandomized across subjects.

2.9. Experiment 4: effects of high intensity cTBS applied on the lateral cerebellum on MEPs, SICI and LICI circuits

To verify whether the effects induced by cTBS at 80% AMT were intensity dependent we performed a further control experiment in

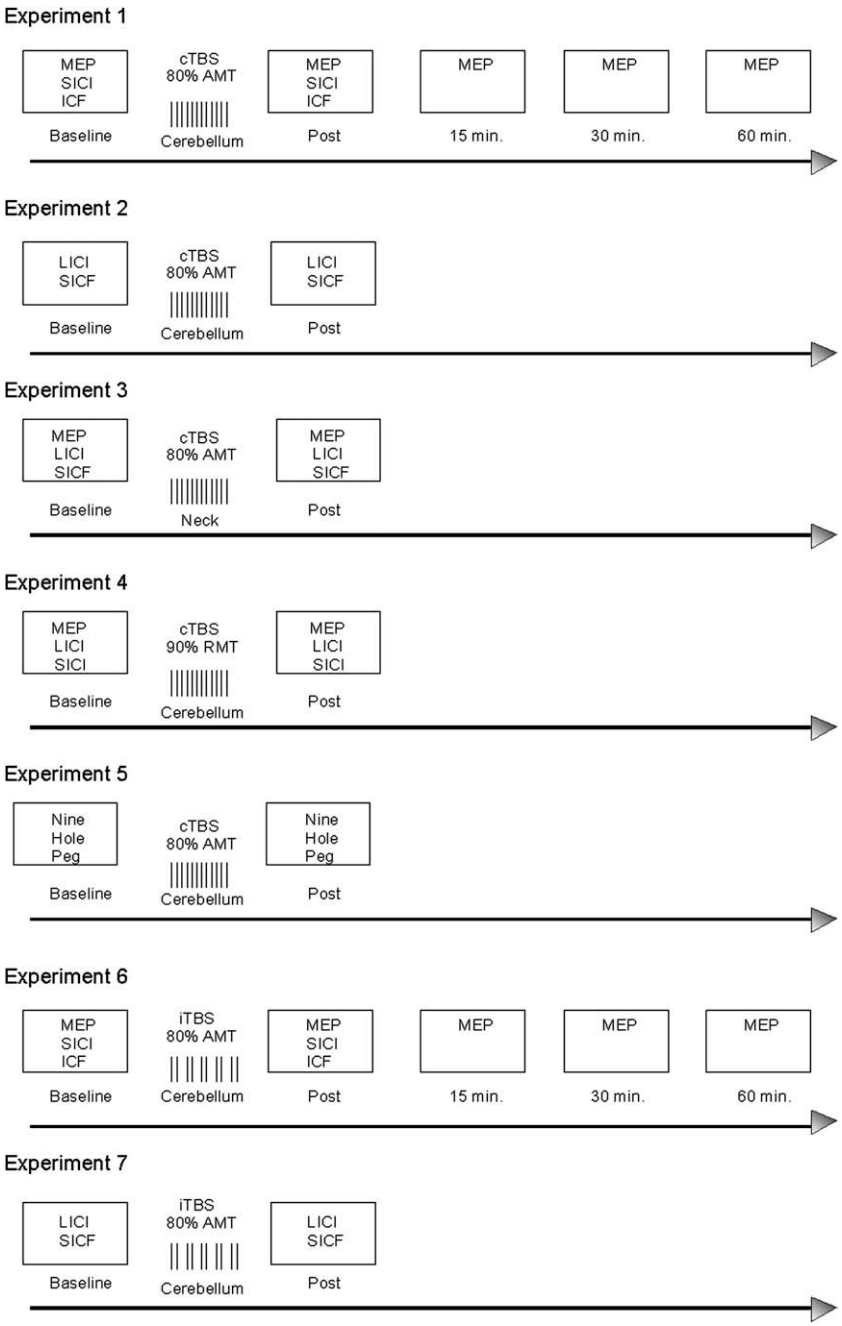

Fig. 1. Schematic representation of the different experiments performed in this study.

which the intensity of cTBS was set at 90% RMT on the basis of previous studies in which it was effective in modulating the excitability of the contralateral motor cortex (Oliveri et al., 2005; Fierro et al., 2007). In six subjects (four of whom participated in Exp. 1 and 2 MEPs, SICI, ICF and LICI of the right M1 were tested in different blocks before and after cTBS protocol applied over the left lateral cerebellum. The order of presentation of the blocks was pseudorandomized across subjects.

2.10. Experiment 5: effects of cTBS applied on the lateral cerebellum on hand dexterity

The nine-hole pegboard task was used to measure possible effects of cerebellar cTBS on hand dexterity. cTBS was applied over the left lateral cerebellum with the same protocol as in experiment 1. The nine-hole pegboard task is typically altered in patients with cerebellar lesions (Haggard et al., 1995; Johnson-Greene et al., 1997; Miall and Silburn, 1997), as well as in normal subjects following transient inhibition of the cerebellum (Miall and Christensen, 2004). Six subjects (that took part in Exp. 1 and 2) were first trained in performing the nine-hole pegboard task for 5 consecutive trials using left and right hand in alternate trials. Inter-trial interval was 60 s. For each trial, the subject began with the hand resting beside the peg-board, which was held steady on the table with the other hand. The experimenter started the trial with a verbal ready-steady-go command, and timed the trial with a digital stopwatch. After 5 training trials, we recorded the baseline performance for each subject in another 5 trials. The evaluation was repeated immediately after cTBS (real or sham), and 15 min later. For sham cTBS the coil was positioned over the same scalp site, but

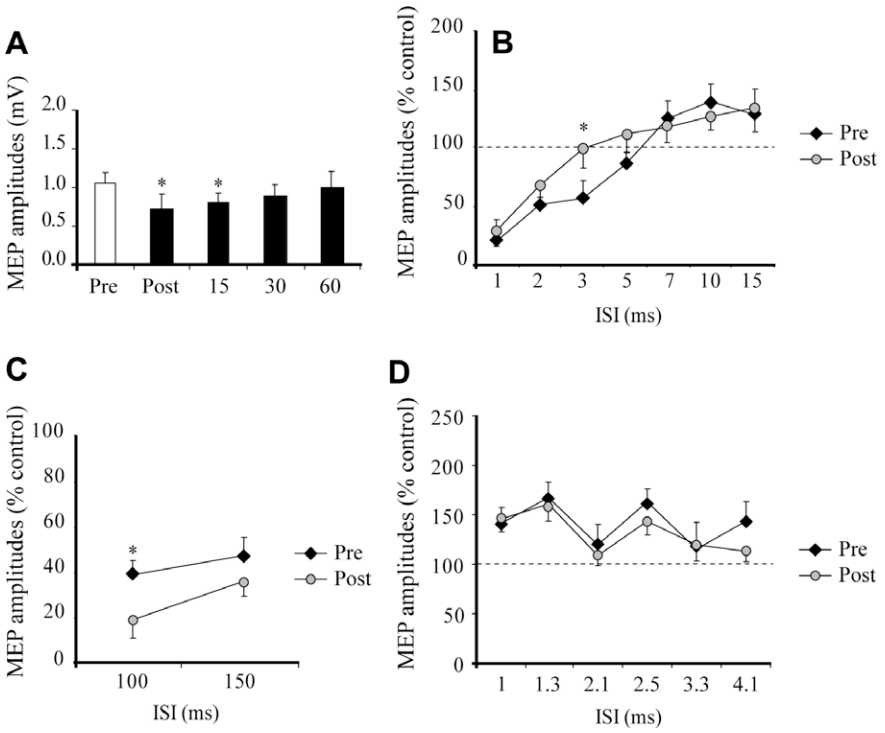

Fig. 2. (A) Effects of cerebellar cTBS on MEPs amplitude obtained from contralateral M1. Following cTBS there was a reduction of MEPs amplitude that lasted up to 15 min. (B) cerebellar cTBS modulated SICI circuits in contralateral M1, attenuating intracortical inhibition at ISI = 3 ms. (C) Effects of cerebellar cTBS on LICI circuits. Following cTBS there was an increase of LICI at ISI = 100 ms. (D) cerebellar cTBS did not modulate SICF circuits in contralateral M1. Errors bars indicate 1 SEM. Asterisks indicate $p < 0.05$.

angled away so that no current was induced in the brain. The order of presentation of the conditions (sham or real TBS) was pseudorandomized across subjects.

2.11. Experiment 6: effects of iTBS applied on the lateral cerebellum on MEPs, SICI and ICF circuits

In ten subjects (six of whom took part in Exp. 1 and 2) MEPs, SICI and ICF of the right M1 were tested in different blocks before and after iTBS protocol applied over the left lateral cerebellum. The same procedure as in experiment 1 was adopted.

2.12. Experiment 7: effects of iTBS applied on the lateral cerebellum on LICI and SICF circuits

In ten subjects (six of whom took part in Exp. 1 and 2) LICI and SICF circuits of the right M1 were tested in different blocks before and after iTBS applied over the left lateral cerebellum. The same procedure as in experiment 2 was adopted.

2.13. Experiment 8: effects of a single conditioning magnetic pulse applied over the lateral cerebellum on MEPs amplitude

Since cerebellar stimulation has been originally performed with a double cone coil with single conditioning magnetic pulse that induced an inhibition of the MEPs evoked from the contralateral motor cortex (cerebellar inhibition-CBI; Ugawa et al., Ann Neurol 1995), we performed this control experiment ($n = 8$) to confirm that the figure-of-eight coil may consistently activate the lateral cerebellum. The CS (intensity = 90% RMT) preceded the TS by 3, 5, 7, 10, 15 and 20 ms. The intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI. For CS, TMS over the lateral left cerebellum was applied using the same scalp co-ordinates (1 cm under and 3 cm left to the inion) adopted in previous experiments. The coil was positioned tangentially to the scalp, with the handle pointing superiorly. The current in the coil was directed upward, which induced downward current in the cerebellar cortex. Ten responses were collected for paired conditioned MEP for each ISI and 20 for test stimulus alone with a total number of 60 trials in each block. The inter-trial interval was set at 5 s (±10%). Measurements were made on each individual trial. The mean peak-to peak amplitude was analyzed for the TS and the conditioned MEP at each ISI. Moreover in the same subjects we also tried to verify whether magnetic stimulation using a figure-of-eight coil at foramen magnum level stimulates the pyramidal tract at brain stem and elicits MEPs from hand muscles as reported previously with double cone coil (Ugawa et al., 1995).

2.14. Statistical analysis

The effects of cTBS or iTBS of the left cerebellum on the size of MEPs evoked from right M1 were measured on the mean peak-to-peak amplitude of MEPs. The mean amplitude values were analyzed with a repeated measures analyses of variance (ANOVA) with time as within-subjects main factor. In experiment 1, 3, 4 and 6 the TBS effects on SICI were analyzed through different ANOVAs for each protocol (iTBS or cTBS) with TIME (pre vs. post TBS) and ISI (1 ms vs. 2 ms vs. 3 ms vs. 5, vs. 7, vs. 10 vs.15 ms) as main factors were performed on the mean peak-to-peak amplitude of the unconditioned TS. In experiment 2, 3, 4 and 7 the TBS effects on LICI were analyzed with separate ANOVAs for each protocol (iTBS or cTBS) with TIME (pre vs. post TBS) and ISI (50 ms vs. 100 ms vs. 150 ms) as main factors were performed on the mean peak-to-peak amplitude of the unconditioned TS. In experiment 2 and 7 the TBS effects on SICF were analyzed with separate ANOVAs for each protocol (iTBS or cTBS) with TIME (pre vs. post TBS) and ISI (1.0 ms vs. 1.3 ms vs. 2.1 ms vs. 2.5 ms. vs. 3.3 ms vs. 4.1 ms) as main factors were performed on the mean peak-to-peak amplitude of the unconditioned TS. In experiment 5, the effects of cerebellar cTBS on hand dexterity was tested with an ANOVA

performed on subjects' mean times with rTMS (cTBS vs. sham), TIME (basal vs. post TBS vs. 15 min. post TBS), HAND (left vs. right) and BLOCK as main factors.

When a significant main effect was reached, Duncan's post hoc test were employed to characterize the different effects of the specific ISIs. For all statistical analyses, a p value of <0.05 was considered to be significant. Mauchley's test examined for sphericity. The Greenhouse–Geisser correction was used for non-spherical data.

3. Results

3.1. Experiment 1

The procedure was well tolerated by all subjects. Before cTBS the mean RMT of the right M1 was 38 ± 3.5% (mean ± SD) MSO. After cTBS it was 39 ± 4.2% MSO. No significant change was found at paired t-test analysis. We found that after cTBS MEP amplitude was significantly reduced (ANOVA with TIME as main factor: $F(1,9) = 7.22$; $p < 0.05$). (Fig. 2A). In comparison with baseline, post hoc analysis showed that this effect was evident 1 min ($p < 0.05$) and 15 min after cTBS ($p < 0.05$), while it vanished at 30 min. Cerebellar stimulation with cTBS modified the SICI circuits over contralateral M1 as demonstrated by a two way ANOVA performed on mean percentage of change in respect to TS. It is important to notice that for SICI measures (and for all the paired pulse experiments of this study) the intensity of TS was adjusted to evoke a MEP of approximately 1 mV peak to peak in the relaxed left FDI before and after TBS. For unconditioned TS MEPs amplitude pre and post TBS were respectively 1.12 ± 0.31 mV and 1.18 ± 0.23 mV. There was a significant ISI main factor ($F(1,6) = 35.70$; $p < p < 0.05$) and a significant TIME - ISI interaction ($F(6,54) = 3.55$; $p < 0.05$). Post hoc analysis showed that changes occurred at ISI = 3 ms ($p < 0.05$) in which SICI was reduced (Fig. 2B). The amount of change in percentage of unconditioned MEP amplitude induced by cTBS did not correlated with percentage of change of SICI at ISI = 3 ms ($R = 0.61$; $p = 0.07$).

No changes were observed over the ipsilateral M1 for both MEPs amplitude and SICI circuits.

3.2. Experiment 2

We found in this experiment that cerebellar cTBS was also effective in modulating LICI circuits in contralateral M1. For unconditioned TS MEPs amplitude pre and post TBS were respectively 0.96 ± 0.33 mV and 0.91 ± 0.22 mV. ANOVA analysis performed on mean percentage of change in respect to TS showed that there was a significant ISI main factor ($F(2,22) = 7.42$; $p < 0.05$) and a significant TIME × ISI interaction ($F(2,22) = 3.55$; $p < 0.05$). Post hoc analysis showed that LICI was increased after cerebellar cTBS at ISI = 100 ms ($p < 0.05$) (Fig. 2C). The amount of change in percentage of MEP amplitude induced by cTBS did not significantly correlated with percentage of change of LICI at 100 ms ($R = 0.26$; $p = 0.41$).

Analysis of SICF circuits showed that there was a significant effect of ISI ($F(5,55) = 6.15$; $p < 0.05$), but no significant interaction TIME × ISI. (Fig. 2D).

3.3. Experiment 3

The procedure was reported to induce some discomfort due to contraction of the neck muscles. When applied over the lateral neck muscles, cTBS did not change the excitability of contralateral M1 as shown by ANOVA analyses performed on MEPs, SICI and LICI curves. There was a significant ISI main effect for SICI ($F(2,10) = 5.23$; $p < 0.05$) and LICI ($F(2,10) = 7.42$; $p < 0.05$), but not TIME × ISI interaction (Fig. 3A–C).

3.4. Experiment 4

When cTBS was applied over the lateral cerebellum at 90% RMT the procedure was reported to induce some pain and discomfort due to contraction of the neck muscles. High intensity cTBS (90% RMT) induced similar effects as low intensity cTBS (80%AMT). We found that after cTBS MEP amplitude was significantly reduced ($t = 3,4$; $p = 0.02$) (Fig. 4A). Cerebellar stimulation with cTBS modified the SICI circuits over contralateral M1 as demonstrated by a two way ANOVA performed on mean percentage of change in respect to TS. For unconditioned TS, MEPs amplitude pre and post TBS were respectively 1.22 ± 0.32 mV and 1.17 ± 0.27 mV. There was a significant ISI main factor ($F(1,5) = 13,01$; $p < 0.001$) and a significant TIME × ISI interaction ($F(6,30) = 3,21$; $p < 0.05$). Post hoc analysis showed that changes occurred at the 3 ms ($p < 0.05$) in which SICI was reduced. (Fig. 4B).

We found in this experiment that high intensity cerebellar cTBS significantly modulated LICI circuits in contralateral M1. For unconditioned TS, MEPs amplitude pre and post TBS were respectively 1.07 ± 0.26 mV and 1.19 ± 0.31 mV. ANOVA analysis revealed a significant TIME main factor ($F(1,5) = 6,87$; $p < 0.05$) but failed to show any significant TIME × ISI interaction (Fig. 4C).

Subsequent analyses were performed to compare possible different effects induced by cTBS at lower (80% AMT) or higher intensity (90% RMT). First we determined that for both SICI and LICI measures the two baseline evaluations did not differ using t-test analysis in the same subjects that took part in the different experiments (for SICI at 3 ms: $p = 0.42$; for LICI at 100 ms: $p = 0.65$).

Furthermore the mixed ANOVA failed to reveal any significant INTENSITY × TIME × ISI interaction either for SICI ($F(6,t-t30) = 0,97$; p = n.s) and for LICI circuits ($F(1,5) = 0.33$ p = n.s.),

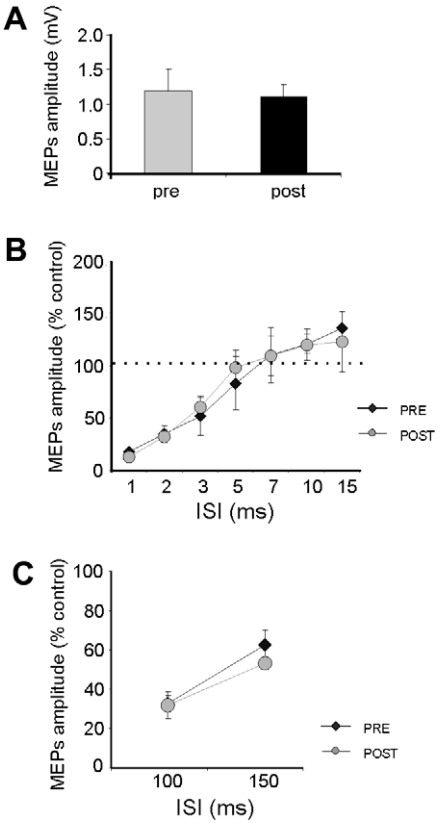

Fig. 3. No effects were found after neck cTBS on MEPs amplitude (A), SICI (B) LICI circuits (C) recorded from contralateral M1.

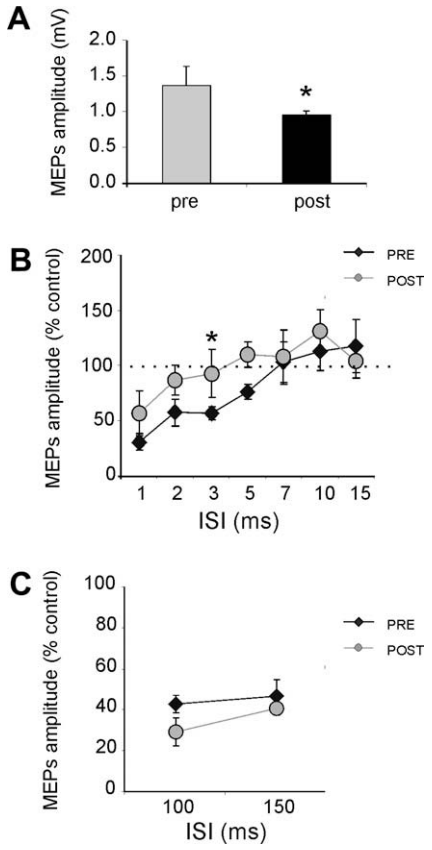

Fig. 4. (A) Effects of cerebellar cTBS at 90% RMT on MEPs amplitude obtained from contralateral M1. Following cTBS at 90% RMT there was a reduction of MEPs amplitude. (B) cTBS modulated SICI circuits in contralateral M1, inducing a significant decrease at ISI = 3 ms. (C) Effects of cerebellar cTBS on LICI circuits recorded from contralateral M1. After cTBS there was an increase of LICI at ISI = 100 ms. Errors bars indicate 1 SEM. Asterisks indicate $p < 0.05$.

showing that the two different experimental sessions induced the same effects.

3.5. Experiment 5

An ANOVA performed on mean times recorded from each subjects performance in the nine-hole pegboard task did not show any significant effects for the main factors of rTMS (cTBS vs. sham), TIME (basal vs. post TBS vs. 15 min. post TBS), HAND (left vs. right) and BLOCK as main factors (Fig. 5).

3.6. Experiment 6

When iTBS was applied over the lateral cerebellum we observed that the mean RMT of the right M1 did not change (37 ± 4.4% MSO. vs. 38 ± 5.6% MSO). We found that following cerebellar iTBS MEP amplitude was significantly increased (ANOVA with TIME as main factor: $F(1,9) = 4.22$; $p < 0.05$) (Fig. 6A). In comparison with baseline, post hoc analysis showed that this effect was evident immediately after ($p < 0.05$), and 15 min after iTBS ($p < 0.05$) while it vanished after 30 min. The bidirectional changes induced by different protocols of TBS were confirmed by a subsequent mixed ANOVA comparing the data obtained in Exp. 1 and 6 with TBS protocol (cTBS vs. iTBS) and TIME as main factor, showing a significant TBS protocol × TIME interaction ($F(2,9) = 8.02$; $p < 0.05$).

Cerebellar stimulation with iTBS modified the SICI circuits over contralateral M1 as demonstrated by a two way ANOVA performed on mean percentage of change in respect to TS. For unconditioned TS, MEPs amplitude pre and post TBS were respectively 1.18 ± 0.29 mV and 1.12 ± 0.37 mV. There was a significant ISI main factor ($F(1,6) = 19.65$; $p < 0.05$) and a significant TIME × ISI interaction ($F(6,54) = 2.96$; $p < 0.05$). Post hoc analysis showed that changes occurred at the 15 ms ISI ($p < 0.05$) in which ICF was reduced (Fig. 6B). The amount of change in percentage of unconditioned MEP amplitude induced by cTBS did not correlated with percentage of change of SICI at ISI = 15 ms ($R = 0.41$; $p = 0.26$). No changes were observed over the ipsilateral M1 for both MEPs amplitude and SICI circuits.

3.7. Experiment 7

In this experiment, we observed that cerebellar iTBS was effective in modulating LICI circuits in contralateral M1. For unconditioned TS, MEPs amplitude pre and post TBS were, respectively, 1.03 ± 0.21 mV and 1.08 ± 0.16 mV. A two way ANOVA performed on the mean percentage of change in respect to TS showed that there was a significant ISI main factor ($F(2,18) = 6.38$; $p < 0.05$) and a significant TIME × ISI interaction ($F(2,18) = 3.21$; $p < 0.05$). Post Hoc analysis showed that LICI was reduced after cerebellar iTBS at ISI = 100 ms ($p < 0.05$) (Fig. 6C). The amount of change in the percentage of MEP amplitude induced by iTBS did not significantly correlate with the percentage of change of LICI at 100 ms ($R = 0.18$; $p = 0.65$). No change was observed in SICF circuits following cerebellar iTBS. ANOVA showed that there was a significant effect of ISI ($F(5,45) = 11,74$; $p < 0.05$), but no significant interaction TIME × ISI (Fig. 6D).

3.8. Experiment 8

In this control experiment we found that a single magnetic CS applied over the cerebellum at 90% RMT with the figure-of-eight coil was able to induce an inhibitory effect on the contralateral motor cortex. The ANOVA performed on mean MEPs amplitude values showed a main effect of ISI ($F(1,7) = 3.14$; $p < 0.05$). Post hoc analysis showed that MEP amplitude was reduced in comparison with TS at both ISIs of 5 ms (TS = 1.16 ± 0.11 mV; CS = 0.84 ± 0.12 mV; $p < 0.05$) and 7 ms (TS = 1.06 ± 0.11 mV; CS = 0.88 ± 0.14 mV; $p < 0.05$), but not at later ISIs of 10, 15 and 20 ms (Fig. 7). When we tried to stimulate the pyramidal tract at brain stem using the figure of eight coil with the same parameters, we failed to observe any significant activation of such deeper structure.

4. Discussion

Our data show that when different TBS protocols are applied over the lateral cerebellum in healthy subjects, they exerts profound changes within the intracortical circuits in the contralateral motor cortex. We found that cerebellar cTBS induced a reduction of MEPs amplitude. Moreover it decreased SICI (at ISI = 3 ms) and increased LICI (at ISI = 100 ms) circuits. On the other hand, cerebellar iTBS provoked an increase of MEPs amplitude and reduced LICI circuits (at ISI = 100 ms).

We speculate below that these changes may reflect the modulation of different intracortical circuits within the motor cortex driven by activation of cerebello-thalamo-cortical pathways.

5. Mechanisms for cerebellar stimulations

The physiology of cerebellar-thalamo-cortical pathway activated by a single magnetic stimulus has been recently clarified. It has been proposed that a single CS activates the Purkinje cells of the superior cerebellum; this results in an inhibition of the dentate nucleus, which is known to exert a background tonic facilitatory

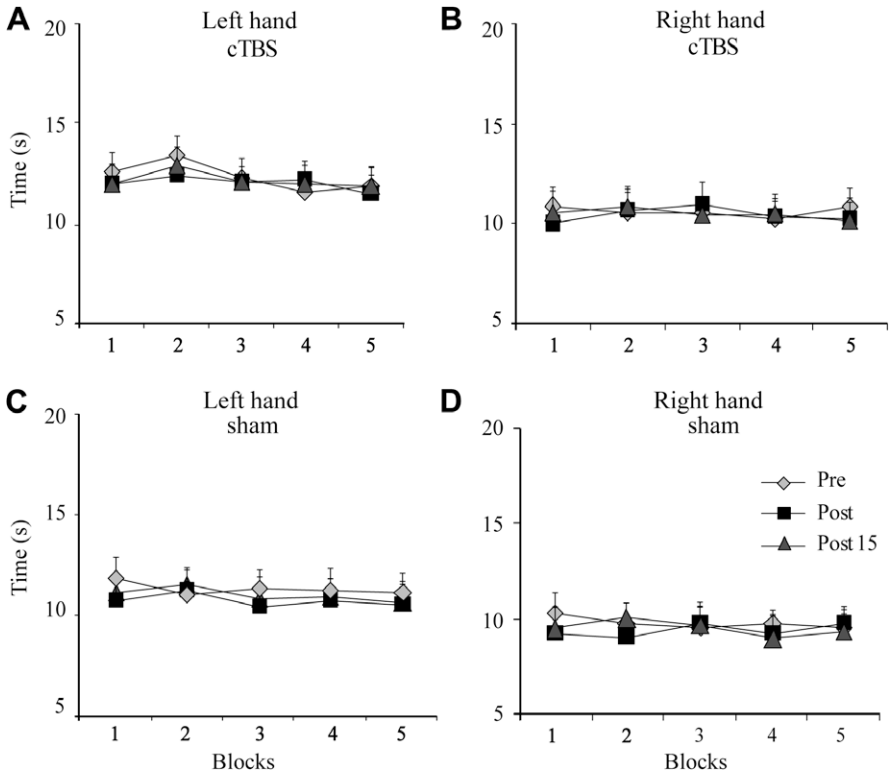

Fig. 5. *c*TBS (real or sham) applied over the left lateral cerebellum did not change subjects' performance in the nine-hole pegboard task for both left (A, C) or right hand (B, D).

drive onto the contralateral M1 through synaptic relay in the ventral lateral thalamus (Middleton and Strick, 2000; Dum and Strick, 2003); this leads in turn to a disfacilitation of the contralateral M1, due to a reduction in dentato-thalamo-cortical facilitatory drive (Ugawa et al., 1994, 1997; Pinto and Chen, 2001; Daskalakis et al., 2004; Reis et al., 2008). Along this vein, we may speculate that, in the current study, low intensity cerebellar TBS induced different plastic changes in PC or in local interneurones, mainly

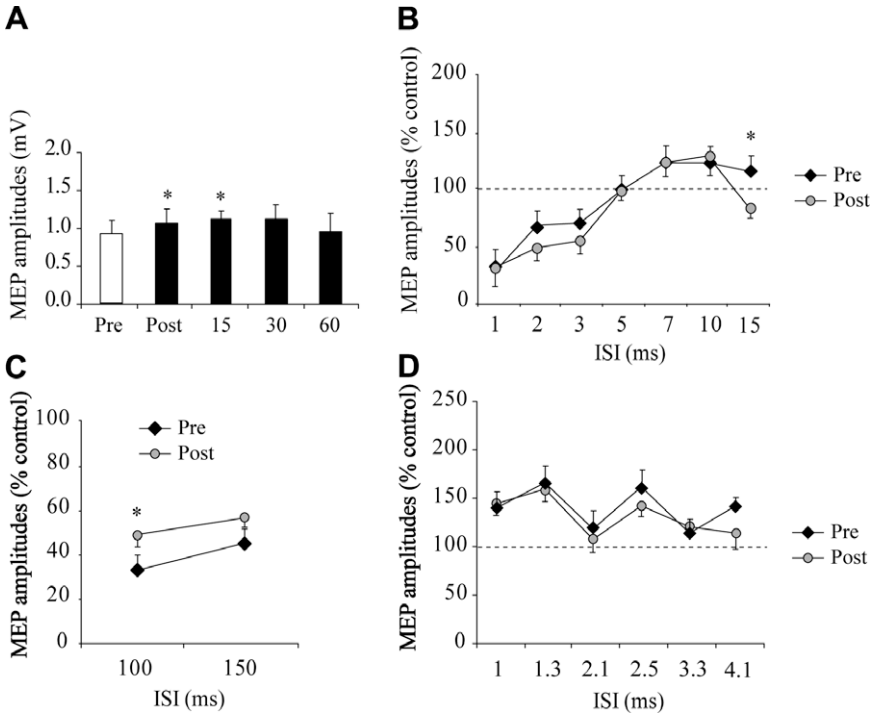

Fig. 6. (A) Effects of cerebellar iTBS on MEPs amplitude obtained from contralateral M1. Following iTBS there was an increase of MEPs amplitude that lasted up to 15 min. (B) cerebellar iTBS modulated ICF circuits in contralateral M1. There was a reduction of ICF at ISI = 15 ms. (C) Effects of cerebellar iTBS on LICI circuits recorded from contralateral M1. Following cTBS there was a reduction of LICI at ISI = 100 ms. (D) cerebellar iTBS did not modulate SICF circuits in contralateral M1. Errors bars indicate 1 SEM. Asterisks indicate $p < 0.05$.

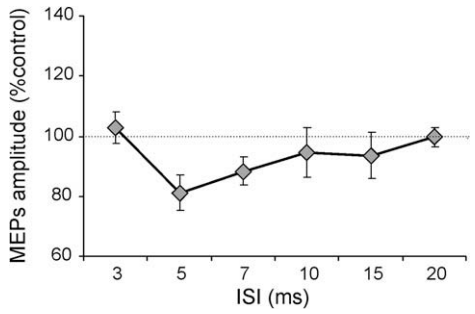

Fig. 7. Effects of a single magnetic pulse applied over the left lateral cerebellum with the figure-of-eight coil on the amplitude of MEPs from the contralateral M1. Inhibition was observed at ISIs of 5 and 7 ms.

affecting the ones with lower thresholds of excitability. Briefly, each PC receives excitatory input from a single climbing fibre. The synapses of mossy fibres, granule cells and PC are subject to modulation by GABAergic inhibitory interneurons, including Golgi cells, stellate and basket cells, Lugaro cells and unipolar brush cells. In particular the synapses of the Golgi cells in the granule layer contribute to the structure of the mossy fibre glomerulus and stellate/basket cells of the molecular layer that are stimulated by parallel fibres and inhibit PC excitability (Evans, 2007 for a review). The PC axons are the sole output of the cerebellar cortex and form inhibitory synapses with neurons in the deep cerebellar nuclei. Although we cannot exclude that cerebellar TBS could have provoked changes in the membrane excitability of the cerebellar cortex neurons, this protocol possibly induced pre and post synaptic interactions at the Purkinje cell synapse (Kase et al., 1980; Ito, 2001, 2002; Maffei et al., 2002, 2003; D'Angelo et al., 1999, 2001; Evans, 2007) and therefore decreased or increased the output of dentate nucleus, leading to subsequent changes in the excitability of the contralateral primary motor cortex. Given the very low intensity of stimulation adopted with TBS protocols (80% AMT), it is conceivable that stimulation was relatively focal and affected mainly the superficial layers of the cerebellar cortex. While it seems surprising that the lateral cerebellum could be activated by TBS at so low intensity, it has to be considered that the same procedure was able to modulate the excitability of the primary motor cortex without inducing any activation of the pyramidal output, thereby modulating transinaptically the excitability of the pyramidal neurons (Huang et al., 2005). Similarly in the current study it is possible that TBS activated sub-populations of interneurons with lower threshold of excitability without activating directly the cerebellar output toward the dentate nucleus. Additional potentially useful information would be obtained using same paradigm in patients with cerebellar dysfunctions (Ugawa et al., 1997; Liepert et al., 1998, 2004) in which we could expect different or absent changes induced by cerebellar TBS.

Furthermore previous investigations showed that even with higher intensities of stimulation (90% RMT) the effects of rTMS over the lateral cerebellum with the same coil shape and orientation may not be ascribed to muscle twitches and propioceptive activation. For instance, when a standard figure-8 coil was placed over the right neck area lateral to the C7 vertebra at an intensity sufficient to evoke a small twitch in neck and shoulder muscles equivalent to that seen when the stimulus was over the cerebellum, rTMS did not interfere with rhythmic finger movements, while cerebellar rTMS disrupted such motor task (Fernandez Del Olmo et al., 2007). Moreover in the current study we adopted a figure-of-eight coil to stimulate the cerebellum, while previous studies mainly used a double cone coil with a single conditioning pulse applied over the cerebellum being able to inhibit the contralateral M1 (Ugawa et al., 1995). To confirm that the cerebellum can be activated magnetically with the figure-of-eight coil we performed a control experiment testing the effects of a single magnetic pulse at 90% RMT, instead of TBS, over the excitability of the contralateral M1. We found that this procedure was able to induce a significant inhibition of the contralateral M1, although smaller than the previously reported CBI (Ugawa et al., 1995), suggesting that different coils may have different effects on the excitability of the cerebellum.

However the current study presents some limitations and therefore did not provide full convincing evidence to support the hypothesis that magnetic stimulation at very low intensity using a figure-of-eight coil activates superficial layers of cerebellar cortex. First we failed to obtain MEPs following stimulation of pyramidal tract at the brainstem. Moreover, we did not test neither the effects of single pulse conditioning over cerebellum at very low intensity using a figure-of-eight coil in patients with cerebellar dysfunction, nor the effects of coil position as described by Ugawa et al. (1995).

6. Cerebellar cTBS modulates GABAergic circuits

The changes in SICI and LICI circuits of the contralateral M1 following cTBS could reflect the modulation of gamma-aminobutyric acid (GABA) circuits. This hypothesis raises from recent investigations showing that a single dose of the specific GABA(B) receptor agonist baclofen increases LICI and decreases SICI (McDonnell et al., 2006). These authors proposed that the increase of LICI is most plausibly explained by facilitation of GABA(B) receptor mediated IPSP(B) in corticomotoneuronal neurons. In fact, previous works with intracellular recordings from cortical neurons showed that, in contrast to GABA(A) receptor mediated IPSPs (IPSP(A)), IPSP(B) typically last several hundreds of milliseconds (Connors et al., 1988; McCormick, 1989; Avoli et al., 1997) and can be mimicked by application of baclofen (McCormick, 1989). Therefore, McDonnell et al. (2006) suggested that in LICI protocol facilitation of IPSP(B) by baclofen leads to stronger hyperpolarization of the pyramidal cells 100 ms after the conditioning pulse, and that this was associated with stronger inhibition of the conditioned MEP.

The role of GABA(A) receptors in mediating SICI has been widely documented with paired-pulse TMS protocols (Di Lazzaro et al., 2000, 2005; Ilic et al., 2002).

SICI has a duration of few ms (Hanajima et al., 1998) similar to IPSP(A) (Connors et al., 1988; McCormick, 1989; Avoli et al., 1997), and is enhanced by benzodiazepines which are allosteric positive modulators of the GABA(A) receptor (Di Lazzaro et al., 2000, 2005; Ilic et al., 2002). Furthermore, SICI is reduced in the presence of LICI (Sanger et al., 2001), since pre-synaptic GABA(B) receptors could induce auto-inhibition on inhibitory interneurons. According to this, a decrease of SICI was observed after the ingestion of the GABA re-uptake inhibitor tiagabine (Werhahn et al., 1997) and baclofen (McDonnell et al., 2006), in which a specific role of GABA(B) receptors in controlling GABA release from inhibitory interneurons was documented.

Consistently with these premises, we could speculate that the SICI reduction at ISI = 3 ms following cerebellar cTBS in our study may be the consequence of activation of presynaptic GABA(B) receptors, while LICI changes may be due to increased GABA(B) receptor mediated inhibitory post-synaptic potentials. Noticeably, in our study the reduction of SICI was evident at ISI = 3 ms and LICI increase was found specifically at ISI = 100 ms. The same ISIs were tested in the study by McDonnell et al. (2006). In particular when SICI is recorded with an ISI = 3 ms, this is thought to produce clear inhibition of the test response (Kujirai et al., 1993; Ziemann et al.,

1996; Hanajima et al., 2003). SICI occurs in two phases at intervals around 1 and 2.5–4 ms but only the later phase is thought to measure true GABA(A) receptor mediated synaptic inhibition while refractoriness contributes to the early phase (Fisher et al., 2002; Hanajima et al., 2003). Moreover, SICF may contaminate SICI, but this facilitation occurs only at discrete intervals that typically spare the ISI of 3 ms, although ISI of 2 ms is less likely to be affected by SICF (Ziemann et al., 1998). Therefore, the finding that in our study clear modulation of SICI following cerebellar cTBS was observed at ISI = 3 ms reinforces the idea that changes were induced in GABA (B) presynaptic receptors.

The results of the cTBS protocol, revealing a decrease of MEPs amplitude obtained from the contralateral M1, were partially in contrast with the findings obtained in the previous studies, in which 1 Hz rTMS was applied over the cerebellum and an increase of MEPs amplitude was observed (Oliveri et al., 2005; Fierro et al., 2007). In this context, the direction of changes induced by cTBS is surprising and unexpected. On the basis of the original description of Huang et al. (2005) of the effects of TBS over the motor cortex, it would be intuitive to assume that cTBS of the lateral cerebellum might have comparable effects to 1 Hz rTMS. However, it has to be considered that the effects observed here depend on the modulation of polysynaptic pathways, involving both excitatory and inhibitory synapses. For instance, several lines of evidence showed that high frequency stimulation of the cerebellum leads to changes in thalamic synaptic plasticity, and these results have been interpreted as a neural substrate underlying movement adaptation in adult animals (for a review Aumann, 2002). Therefore, although this interpretation is highly speculative, it is possible that the high frequency stimulation protocol adopted in this study could have induced multiple plastic changes at different levels such as the cerebello-thalamic synapses (Aumann et al., 2000) and the thalamo-cortical synapses (Baranyi et al., 1991; Iriki et al., 1991), finally leading to different changes in the excitability of the primary motor cortex in comparison to the one induced by low frequency rTMS. In this regard, it has to be noticed that changes following cerebellar TBS in individual SICI and LICI measures did not correlate with MEPs amplitude. Therefore, it is likely that different pathways may be activated by TBS, leading to the described alterations.

Further investigations testing the effects of single bursts applied over the lateral cerebellum on the excitability of the contralateral M1 could be useful to clarify these complex interplays.

7. Cerebellar iTBS reduces LICI

Cerebellar iTBS did not affect SICI circuits but reduced LICI = 100 ms. Concerning LICI, iTBS had opposite effects in comparison with cTBS, inducing a reduction of GABA(B) intracortical circuits. This could partially explain the increase in MEPs amplitude observed after cerebellar iTBS. Taken together with the results obtained with cTBS (reduction of LICI), these findings suggest that the cerebellar projections to the contralateral M1 have strong interplay with GABA(B) intracortical circuits. We may speculate that the cerebellar-thalamo-cortical projections activated by cerebellar rTMS may directly contract synapses with the GABA(B) interneurons modulating the efficacy of these inhibitory circuits.

8. Conclusions

In conclusion we demonstrated that, although the interpretation of the data remains highly speculative, different protocols of cerebellar TBS may activate underlying cerebello-thalamo-cortical pathways that are linked with distinct intracortical M1 circuits.

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