

Conditions for enhancing the encoding of an elementary motor memory by rTMS



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HIGHLIGHTS

- Co-administration of low frequency primary motor cortex stimulation with rTMS enhances formation of motor memories.
- The enhancing effect of rTMS on the formation of motor memories depends on the frequency and time of the stimulus in relationship to the onset of the training movements.
- The training related improvement in kinematics above naïve levels supports the notion of M1 engagement in events leading to improved motor skills.

ABSTRACT

Objective: Motor learning results in changes of movement representation in primary motor cortex (M1) a process involving long-term potentiation (LTP). Pairing motor training with repetitive transcranial magnetic stimulation (rTMS) of M1 enhances the formation of a motor memory. Here we determined the effect of pairing M1 stimulation and the execution of training movements at different times and frequencies on the formation of a motor memory.

Methods: Formation of a motor memory was defined as increases in motor evoked potentials (MEP) of the training agonist (extensor carpi ulnaris muscle, ECU) and increases in peak acceleration of the trained movements that last more than 60 min. Training consisted of auditory-paced ballistic wrist extension movements (30 min, 0.5 Hz) paired with 0.1, 0.25 or 0.5 Hz subthreshold rTMS. The rTMS pulse was applied at either the onset, 100 ms prior to or 300 ms after the onset of training movement related increases in electromyographic (EMG) activity of ECU. This was compared to a Sham condition.

Results: Only 0.1 Hz rTMS applied at the onset of the training related increase in ECU-EMG activity resulted in increases in MEP amplitudes and peak acceleration when compared to the Sham.

Conclusions: The formation of motor memory is enhanced above the naïve level by co-administration of low frequency rTMS at the time of execution of training movements.

Significance: These results indicate the importance of time and frequency of rTMS in these settings and should be considered in the design of rehabilitation treatment strategies using rTMS.

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1. Introduction

Practice induces primary motor cortex (M1) plasticity, a process involved in motor learning in humans (Pascual-Leone et al., 1994;

Karni et al., 1995; Classen et al., 1998; Bütefisch et al., 2000; Muellbacher et al., 2002b), non-human primates (Nudo et al., 1996b) and rodents (Riout-Pedotti et al., 1998). One proposed mechanism of learning-induced cortical plasticity is that synaptic strength of cortical horizontal connections is modified through long-term potentiation (LTP) and long-term depression (LTD) (Donoghue et al., 1996; Riout-Pedotti et al., 2000; Sanes and Donoghue, 2000). One technique that has been used to induce LTP in M1 is Hebbian type stimulation. According to Hebb, "any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other" (Hebb, 1949). Hebbian or associative LTP has been generated by pairing stimulation of cortical afferents with depolarization or stimulation-induced firing of the targeted postsynaptic pyramidal tract neuron (PTN) (Baranyi and Feher, 1981; Baranyi and Szente, 1987; Iriki et al., 1991) and by pairing stimulation of thalamo-cortical as well as cortico-cortical fibers with stimulation of horizontal intracortical fibers in cortical layers II/III (Iriki et al., 1991; Hess and Donoghue, 1994, 1996; Sanes and Donoghue, 2000). In humans, the importance of the temporal relationship between afferent stimulation and discharging of pyramidal tract neurons was demonstrated in different experimental settings using peripheral nerve stimulation and/ or low frequency repetitive transcranial magnetic stimulation (rTMS) of M1 in healthy adults (Stefan et al., 2000; Wolters et al., 2003; Bütefisch et al., 2004; Kujirai et al., 2006; Thabit et al., 2010; Mrachacz-Kersting et al., 2012) and patients after stroke (Bütefisch et al., 2011). When TMS is applied to M1 at intensities below the motor threshold (termed *subthreshold* stimulation), it stimulates the intracortical connections targeting pyramidal tract neurons (PTNs) but does not result in their discharge (Day et al., 1987; Di Lazzaro et al., 2004). It can, therefore, be used to evoke inputs to targeted PTNs. We have previously demonstrated in a NMDA receptor activation dependent motor learning paradigm (Bütefisch et al., 2000) that *subthreshold* rTMS of M1 during the execution of training movements (voluntary cortical drive eliciting discharges of PTNs) is more effective in enhancing motor memory formation when compared to random *subthreshold* rTMS stimulation (Bütefisch et al., 2004). The importance of time was confirmed in other studies using different experimental paradigms (Stefan et al., 2000, 2002; Kujirai et al., 2006; Mrachacz-Kersting et al., 2012). Mrachacz-Kersting et al. demonstrated in subjects imagining movements that M1 plasticity was induced when peripheral nerve afferent stimulation arrived at M1 during the imagined execution but not during the imagined preparation or the imagined completion of the movement (Mrachacz-Kersting et al., 2012). Similarly, in the paradigm used by Stefan et al., referred to as paired associative stimulation (PAS), pairing of *suprathreshold* TMS of M1 and electrical peripheral stimulation of somatosensory afferents resulted in long lasting, input specific increases in M1 excitability when given in a way that the inputs to M1 arrive approximately at the same time (Stefan et al., 2000). These increases in M1 excitability depend on NMDA receptor activation suggesting that LTP-like mechanisms may underlie the cortical plasticity induced by PAS (Stefan et al., 2002). Taken together, there is some evidence in human M1 that Hebb's rules (the related characteristics of co-operatively and associativity) apply to human motor cortex (on a system level) (Stefan et al., 2000, 2002; Wolters et al., 2003; Kujirai et al., 2006; Mrachacz-Kersting et al., 2012) and that an LTP-like process is operating in this type of plasticity.

In contrast to pairing paradigms (PAS) where peripheral nerve afferent stimulations are applied at discrete time points (Stefan et al., 2000, 2002; Wolters et al., 2003; Kujirai et al., 2006), pairing rTMS with movement related M1 activity (Bütefisch et al., 2004; Thabit et al., 2010) differs in several aspects. First, in movement related paradigms, activation of PTNs is achieved by voluntary

muscle activity while in the PAS paradigms suprathreshold rTMS is necessary (Kujirai et al., 2006). Second, executing movements results in sensory feedback to M1, thereby providing additional afferent input to the targeted PTN neurons. Third, the frequencies of movement execution are higher in motor learning paradigms (1–0.5 Hz) than the frequency used for peripheral nerve afferent stimulation (0.05–0.2 Hz) resulting in rTMS paired and unpaired movements (Bütefisch et al., 2004). In the present study we tested the effect of different frequencies and times of pairing training movements and *subthreshold* rTMS of the corresponding M1 on the encoding of a motor memory. Training movements were executed at a fixed rate of 0.5 Hz frequency, a rate effective in inducing M1 plasticity (Muellbacher et al., 2002a,b; Ziemann et al., 2004) and paired with the rTMS pulse at a ratio of 1:1, 1:2 and 1:5 by applying rTMS at 0.5, 0.25 or 0.1 Hz frequency. The rTMS pulse was applied at the onset of movement execution (0 ms in reference to the movement onset), during the preparation of the movement (–100 ms in reference to the movement onset) and after completion of the movement (+300 ms in reference to the movement onset) (Stefan et al., 2000; Wolters et al., 2003; Bütefisch et al., 2004; Mrachacz-Kersting et al., 2012). We tested the hypothesis that rTMS applied to M1 is most effective in enhancing the formation of a motor memory when rTMS occurs at the onset of the movement execution in a 1:1 ratio (rTMS at 0.5 Hz frequency) as this is reminiscent of the settings used for Hebbian-type stimulation in cellular in vitro and in vivo experiments (Baranyi and Feher, 1981; Baranyi and Szente, 1987; Iriki et al., 1991; Hess and Donoghue, 1994, 1996; Sanes and Donoghue, 2000) and human studies using PAS protocols or the pairing of imagined movements and peripheral nerve stimulation (Stefan et al., 2000; Kujirai et al., 2006; Mrachacz-Kersting et al., 2012).

2. Methods

The experiments were approved by the Institutional Review Board at West Virginia University, and conducted according to the Declaration of Helsinki. All subjects gave their written informed consent.

2.1. Subjects

Nine subjects (6 female, age 65.22 ± 8.33 years (55–79 years) participated in this single blinded, randomized, placebo controlled study. These subjects fulfilled the following inclusion criteria: ages 55–80 years with no previous history of neurological or psychiatric diseases and a normal neurological examination, normal MRI of the brain (see below for details) and normal cognitive function (Table 1, see below for details), no intake of CNS active drugs, no contraindication to TMS or MRI, ability to meet the criteria of the inclusion experiment (see below), and ability to give informed consent. All subjects were right handed according to the Edinburgh inventory for handedness (Oldfield, 1971). Because the information expected to be gained from these data are also important for the understanding of motor control in the injured brain, such as after stroke,

Table 1

Repeatable Battery for the Assessment of Neuropsychological Status (Randolph et al., 1998): the mean, SD and range of scores for each subtest and total score are given.

	Mean	SD	Range
Total scale	113.00	6.35	105–121
Immediate memory	109.75	9.85	90–126
Visual spatial	112.37	7.99	105–126
Language	104.37	10.25	92–127
Attention	110.75	12.02	100–135
Delayed memory	109.75	8.38	99–122

and age impacts processes involved in motor control (Mattay et al., 2002; Ward and Frackowiak, 2003; Talelli et al., 2008), a middle aged population was selected for the present study.

2.2. Overview of the experiments

According to our hypothesis we aimed to apply low frequency rTMS to M1 in such a way that rTMS pulses arrive while M1 is active in generating the training movements. Specifically, presence of M1 activity was estimated by movement-related increases in electromyographic (EMG) activity in muscles supporting the movement (Figs. 1 and 2). The temporal relationship between M1 PTN discharges and onset of the execution of the movement were previously established in non-human primates (Kalaska, 2009). Subjects were asked to execute wrist extension movements in response to an auditory stimulus at a rate of 0.5 Hz frequency, a rate that effectively induces M1 plasticity (Muellbacher et al., 2002a,b; Ziemann et al., 2004). The effect of pairing these training movements with 6 different rTMS protocols on formation of motor memory was tested in a single exposure cross-over experimental design. In all conditions, rTMS was applied to M1 contralateral to the training hand. To test the effect of time of rTMS pulse in relation to the training movement, 0.1 Hz rTMS was applied to target the extensor carpi ulnaris (ECU) muscle, a muscle supporting the training movement at the beginning of movement execution. For rTMS at movement onset the pulses were applied when EMG-ECU activity exceeded baseline (0.1 Hz rTMS_0 ms, Fig. 2), for rTMS during movement preparation the pulses were applied 100 ms prior to the increase in ECU EMG activity over baseline (0.1 Hz rTMS_−100 ms) and for rTMS after completion of the movement pulses were applied after cessation of movement related increases in ECU EMG activity (0.1 Hz rTMS_300 ms). While this experimental set up does not allow the precise timing at the cellular level and M1 activity in the present experimental setting

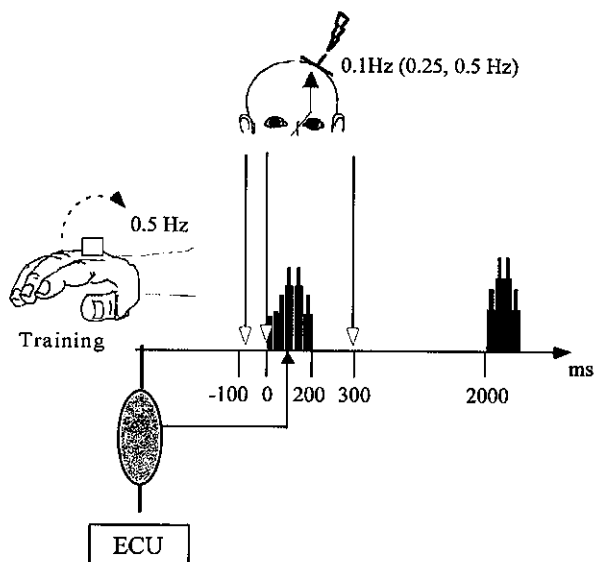


Fig. 1. Experimental stimulation conditions: in 6 experiments, rTMS or Sham stimulations were applied at different times and frequencies during the execution of ballistic wrist extension movements. The movements were auditory paced at 0.5 Hz frequency. RTMS and Sham stimulations were triggered by movement related increases in electromyographic (EMG) activity of the muscle supporting the training movement (training agonist, extensor carpi ulnaris, ECU). RTMS was delivered at either −100 ms, 0 ms, or +300 ms in reference to the onset of the EMG burst of the ECU muscle (time points are indicated by the open arrows). RTMS applied at 0 ms was also delivered at higher frequencies of 0.25 and 0.5 Hz (frequencies are indicated next to the symbol for the TMS coil). Sham stimulation was applied at 0.1 Hz frequency at 0 ms.

likely consists of both, discharging and refractory PTN, it is suited to test the effect of time across major domains.

In experimental paradigms for Hebbian type stimulation (Stefan et al., 2000, 2002; Wolters et al., 2003; Kujirai et al., 2006; Mrachacz-Kersting et al., 2012) afferent stimulation was applied at 0.1 Hz. This is consistent with the finding from *in vitro* and *in vivo* data of Baranyi et al. where the pairing of afferent input stimulation and discharging of the M1 pyramidal tract neurons is greatest when the afferent input is paired with the PTN discharge at frequencies 0.1–0.3 Hz (Baranyi and Feher, 1981; Baranyi and Szente, 1987; Baranyi et al., 1991). In the present study, we tested the rTMS_0 ms condition at three frequencies: 0.1 Hz (0.1 Hz rTMS_0 ms), 0.25 Hz (0.25 Hz rTMS_0 ms), and 0.5 Hz (0.5 Hz rTMS_0 ms). As training movements were executed at a fixed rate of 0.5 Hz, a rate effective in inducing M1 plasticity (Muellbacher et al., 2002a,b; Ziemann et al., 2004) (see below for details), rTMS at these frequencies resulted in a pairing ratio of 1:5 (0.1 Hz rTMS_0 ms), 1:2 (0.25 Hz rTMS_0 ms) or 1:1 (0.5 Hz rTMS_0 ms). These results were compared to Sham stimulation (see below for a detailed description, Fig. 1).

For the purpose of determining the effects of these six different stimulation conditions (0.1 Hz rTMS_0 ms, 0.1 Hz rTMS_−100 ms, 0.1 Hz rTMS_300 ms, 0.25 Hz rTMS_0 ms, 0.5 Hz rTMS_0 ms, Sham) on the formation and retention of an elementary motor memory, training related effects on MEP amplitudes and peak acceleration of the trained movement were measured prior to the training (baseline), immediately after training (post1) and again 30 (post2) and 60 min (post3) after completion of training (see “TMS and kinematic measures” for details). These measures were chosen because of the evidence that training induced increases in the MEP amplitude associated with improved performance or changes in the kinematics of movements reflect motor learning (Bütefisch et al., 2000; Muellbacher et al., 2001; Ziemann et al., 2004). All subjects participated in all six different stimulation conditions with an intersession interval of at least 48 h. The order of stimulation conditions was randomized and the subjects were unaware of the experimental purpose of the study.

2.3. MRI of the brain

High-resolution T1 weighted anatomical images were collected on a 3T GE scanner with the following parameters: Spoiled gradient recalled (SPGR) acquisition; FOV = 240; matrix 256 × 256; slice = 1.5 mm; 124 slices. The MRI of the brain was reviewed by an experienced board certified neurologist for possible structural abnormalities. Once anatomic normality of the brain was established, the MRI of the brain was reconstructed in Brainsight (Brainsight, Rogue Research, Montreal, Canada) and served as each subjects' reference for the coil position across experiments and within each experiment.

2.4. Neuropsychological testing

The Repeatable Battery for the Assessment of Neuropsychological Status (Randolph et al., 1998) was administered to all subjects to establish normality of cognitive function (see inclusion criteria). The test is composed of 5 subtests and a total score, each with a mean of 100 and a standard deviation of 15. We determined that any score of 85 or lower on any of the subtests or the total score would suggest abnormal cognitive function and would exclude a subject. All subjects included in this study scored above 85 on all the subtests and the total score. In fact the lowest score on any subtest was 90, which is above the 25th percentile. The mean, standard deviation and range of scores for each subtest and total score are summarized in Table 1.

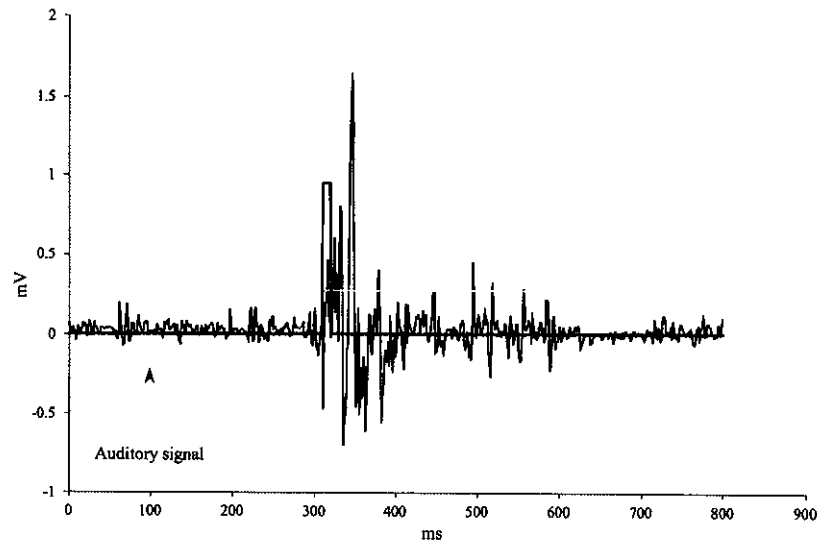


Fig. 2. Determining the onset of the movement related EMG activity in ECU muscle using a pre-set threshold. In this single subject EMG recording of the muscle supporting the training movement (extensor carpi ulnaris muscle, ECU), the onset of movement related EMG activity is clearly identified by the software program using a pre-set threshold of about 10–20% maximum muscle contraction EMG amplitude (horizontal bar in grey). RTMS application was triggered (TTL signal is indicated) when the amplitude of the EMG activity of the ECU muscle exceeded the pre-set threshold. The recording started 100 ms prior to the auditory go signal (arrow).

2.5. TMS and kinematic measures

Subjects were comfortably seated in a dental chair surrounded by a frame that carried a coil holder to assist with the application of TMS to the brain (Brainsight, Rogue Research, Montreal, Canada). The subject's right arm was positioned in a molded armrest with the subject's forearm immobilized and the wrist freely movable. Movements of the hand were recorded with a 2D accelerometer mounted on the dorsum of the hand (Bütefisch et al., 2000), 3 cm distal to the styloid process of the ulna. Acceleration of wrist extension movements in the two main movement planes (extension/flexion; abduction/adduction) was recorded.

EMG activity (bandpass 1 Hz–1 kHz) was recorded from the ECU, a forearm muscle supporting the training movement and the flexor carpi ulnaris (FCU), a forearm muscle antagonistic to the training movement using surface electrodes (11 mm diameter) in a belly-tendon montage and a data acquisition system (LabVIEW, National Instruments, CA, USA). The raw EMG was sampled and digitized at a rate of 5 kHz and stored on a PC for off-line analysis. TMS was delivered through a figure of eight-shaped coil (7 cm wing diameter) using two Magstim 200 stimulators connected via a Bistim module (Magstim Company, UK). The coil was placed tangentially to the scalp and rotated 45° away from the midline. The current induced in the brain was therefore directed posterior-anteriorly, approximately perpendicular to the central sulcus, which is the optimal condition for activating the corticospinal tract transynaptically (Werhahn et al., 1994; Kaneko et al., 1996). Stimuli were delivered to the optimal site for stimulating the contralateral ECU muscle, and the site was marked on the subject's reconstructed MRI of the brain. At this hot spot, the resting motor threshold (MT), defined as the minimum stimulus intensity to evoke an MEP of >50 μ V in at least five of ten trials (Rossini et al., 1994), was determined to the nearest 1% of the maximum stimulator output (MSO) for ECU and FCU muscles. Obtaining measures for both muscles in the hot spot of the ECU muscle was done because of the impact of time after completion of the training on the outcome measures (Liepert et al., 1998; Bütefisch et al., 2000). After identifying the resting MT for both muscles, MEPs were elicited by single TMS

pulses at increasing stimulus intensities (Devanne et al., 1997; Ridding and Rothwell, 1997). Stimulation started at the next lower 5% level of subjects MT and the absence of a measurable MEP was confirmed. If any measurable MEPs were seen at that level, intensity was decreased by 5% MSO to confirm complete absence of MEP responses. Intensities were then increased in increments of 5% of MSO up to 80% of MSO. At each intensity, 10 stimuli were given at an ISI of 5 s. Responses were set to zero for intensities below the intensity that demonstrated absence of measurable MEP to a level of 35% MSO (see data analysis, Fig. 3).

Throughout the experiment, the stimulating coil was kept in a constant position with respect to the subject's head using the reconstructed image of the subjects' brains (Brainsight, Rogue Research, Montreal, Canada). Timing and intensity of stimuli were controlled by PC-based software (STIMM, James Long Company). As the time since completing the training has an impact on our outcome measures and applying intensities at random order would have increased the time required for data collection, stimuli were applied at increasing intensities. In a recent study by Pearce et al. comparing randomized and ordered application of different intensities, revealed similar results for both approaches (Pearce et al., 2013).

For kinematic measures of ballistic wrist extension movements subjects were asked to extend the wrist as quickly as possible in response to an auditory signal. At each time point (pre, post1, post2 and post3) 5 measurements were taken at an inter-trial interval of 5 s.

The endpoint measures of this study were the motor training related increases in the mean MEP amplitudes of the stimulus response curve for the ECU muscle and the peak acceleration of wrist extension movements. Measures obtained immediately after completion of the training were used as an estimate of the training effect on memory formation, while measures obtained at 60 min after completion of the training were used to estimate the longevity of the training effect. According to our stated hypothesis, we expected a long lasting increases in MEP amplitude of the ECU muscle and peak acceleration of wrist extension movements when rTMS was applied at the onset of movement execution.

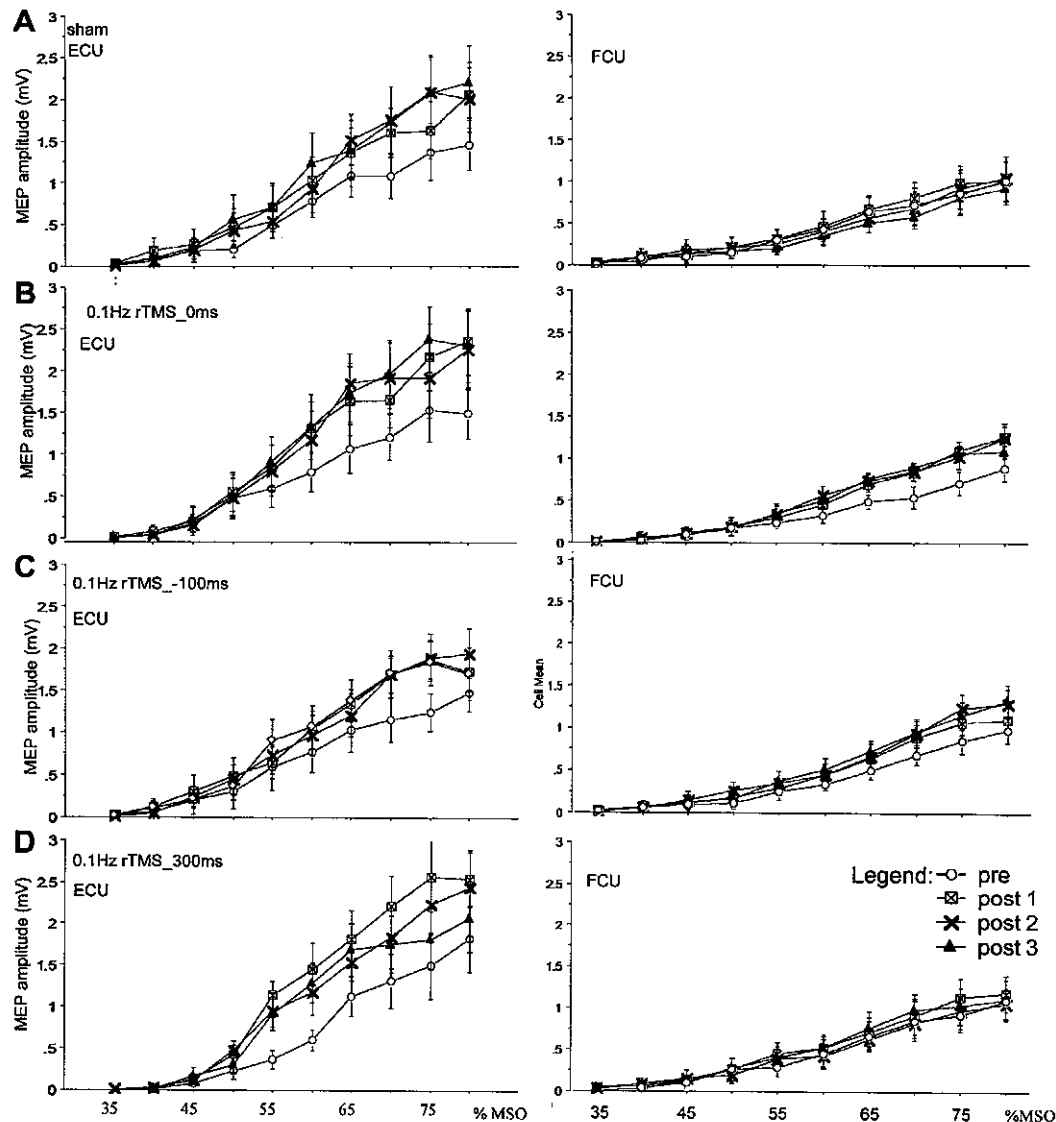


Fig. 3. Effects of rTMS applied at different times during training on stimulus response curves of ECU and FCU muscles: MEPs were recorded prior to training (pre, open circle), immediately after training (post1, cross in square), 30 min (post2, cross) and 60 min (post3, triangle) after completion of the training. RTMS intensities are expressed as percentage of maximum stimulator output (%MSO). Sham stimulation was applied to primary motor cortex at 0.1 Hz frequency at the onset to the EMG (Sham, A), rTMS was applied to primary motor cortex at 0.1 Hz frequency at the onset to the EMG (0.1 Hz rTMS_0 ms, B), 100 ms prior to the onset of EMG (0.1 Hz rTMS_-100 ms, C), and 300 ms after the onset of EMG (0.1 Hz rTMS_300 ms D). Training related increases in MEP amplitudes of ECU muscle were seen for all conditions (A–D). This was not seen in the FCU muscle, a muscle antagonistic to the training movement. Repeated measures ANOVA revealed statistically significant four way interaction of condition * intensity * type of muscle * time ($F = 1.34$, $p = .032$) supporting a differential effect of the interventions that is different for each muscle (see Tables 4 and 5 for detailed statistics). Means $\pm$ SE are given.

2.5. Training and rTMS protocols

Because the objective of the present study was to enhance motor learning, we chose a training paradigm that has been demonstrated to be effective in inducing motor learning related M1 plasticity (Bütefisch et al., 2000; Muellbacher et al., 2002b; Ziemann et al., 2004). Accordingly, subjects performed auditory paced ballistic wrist extension movements (no specific pre-set training angle was given) at 0.5 Hz frequency for three blocks of 10 min duration with 2–3 min of rest in between training blocks (30 min, 900 movements total). Subjects were instructed to return to the baseline position by relaxation as confirmed by EMG (see Figs. 1 and 2). This training was chosen based on results from studies of similar ballistic movements that demonstrated training related improvement of kinematics (Bütefisch et al., 1995;

Muellbacher et al., 2002a) and an increase of MEP amplitude (Muellbacher et al., 2002a). Mechanisms underlying these changes include an LTP-like mechanism as seen in three different experiments, using either drugs that interfere with NMDA receptor activation or increase GABAergic inhibition (Bütefisch et al., 2000) or a stimulation technique that produces LTP-like phenomenon but fails to induce LTP-like phenomenon after training (Muellbacher et al., 2002b; Ziemann et al., 2004).

The number of movement executions was selected based on results of both primate and non-primate studies demonstrating formation of a motor memory after similar (Muellbacher et al., 2002a) or fewer repetitions in a training task (Nudo et al., 1996a). Acceleration and EMG signals of training movements were recorded at 1 kHz. Quality of the motor training, as defined by the accuracy and consistency of training movements, was monitored

on-line by one investigator. Great care was taken that subjects moved in response to the auditory stimulus. Occasionally the onset of the auditory stimulus was anticipated and subjects started to move early or movement kinematics indicated changes in movement angle. In these situations the subject was encouraged to perform better. Additionally, quality and consistency of a random sample of 120 training movements (40 movements per 10 min training block) was measured off-line by calculating the direction, the dispersion of directions and the magnitude of the first peak acceleration of these training movements. The measures for direction and dispersion were derived from the length of the mean of the individual vectors in the unit circle. In a unit circle, a mean vector of 1 means that the direction of the individual vectors were identical, while a mean vector close to 0 means maximal dispersion (Batschelet, 1981).

For all stimulation conditions with the exception of the Sham condition, rTMS was administered through a 70 mm figure-of-eight, air-cooled coil connected to the Magstim Super Rapid (Magstim Company, UK). While the subject was engaged in the execution of training movements, rTMS was applied to M1 contralateral to the training hand. Sham stimulation was applied through an air-cooled Sham coil, a coil with the same physical appearance as the real rTMS air-cooled coil. Stimulation through the Sham coil does result in a very small magnetic field at all intensities up to 100% MSO. Depending on the set intensity of the stimulator, the Sham coil produces a click of different intensity that mimics the auditory perception of being stimulated with the real air-cooled coil. The following rTMS protocols were tested (see Fig. 1 and below for details): (1) rTMS applied at 0.1 Hz frequency, 100 ms prior to the onset of EMG activity in a muscle supporting the training movements (ECU) (0.1 Hz rTMS_{-100 ms}); (2) rTMS applied at 0.1 Hz frequency at the onset (0 ms) of EMG activity of ECU muscle (0.1 Hz rTMS_{0 ms}); (3) rTMS applied at 0.1 Hz frequency at 300 ms after the onset of EMG activity of ECU muscle (0.1 Hz rTMS_{300 ms}); (4) Sham stimulation applied at 0.1 Hz frequency at the onset of EMG activity in ECU muscle (Sham); (5) rTMS applied at 0.25 Hz frequency at the onset (0 ms) of EMG activity of ECU muscle (0.25 Hz rTMS_{0 ms}) and (6) rTMS applied at 0.5 Hz frequency at the onset (0 ms) of EMG activity of ECU muscle (0.5 Hz rTMS_{0 ms}).

The onset of the movement-related EMG activity in ECU was defined as an increase of EMG amplitude above a preset threshold of about 10–20% of the maximal amplitude of the movement-related EMG burst in the ECU muscle and detected online by a custom made LabView program (Figs. 1 and 2). For the 0.1 Hz rTMS_{-100 ms} condition, the reaction time for each training trial was estimated based on the time between the auditory “go” signal and the onset of movement related EMG activity. The mean reaction time of the 4 training trials preceding the training trial that was targeted for rTMS was calculated online (rTMS was applied in one of five movements). The rTMS pulse was applied 100 ms prior to the estimated EMG onset of the fifth trial. This was procedure was repeated in subsequent trials throughout the training.

Effects of rTMS were compared to Sham stimulation applied at 0.1 Hz where subjects performed a motor training without any cortical stimulation (Sham). In this condition, a Sham stimulus was applied at the onset of the training movement (0 ms) using a figure of eight, air cooled Sham coil.

In all stimulation conditions, rTMS was applied at 80% resting motor threshold (MT, determined for the Magstim Super Rapid (Magstim Company, UK) an intensity that activates cortico-cortical connections targeting pyramidal tract neurons (Day et al. 1987), but does not discharge pyramidal tract neurons as indicated by the absence of a recordable MEP (Di Lazzaro et al., 1998c). To verify subthreshold intensity and accurate timing of the rTMS pulses, EMG recordings were analyzed off-line. EMG was inspected for the presence of MEP amplitudes and the actual timing of the rTMS

pulse in reference to the movement-related EMG burst was measured (Fig. 2).

2.5.1. Inclusion experiment

Prior to entering the main experiment, all subjects underwent an inclusion experiment to determine the ability of TMS to elicit a measurable MEP of >100 μ V and an increase in MEP amplitude with increasing stimulus intensity (up to 100% of MSO) of at least 20% over MEP amplitude at MT (Wittenberg et al., 2003) as well as the ability to perform the training movements. The experimental set-up was similar to the main experiment (see above for details).

2.6. Data analysis and statistical methods

Data analysis of MEP amplitude and kinematic measures was performed by personnel blind to the stimulation condition. Because of the rTMS stimulus artifact, analysis of the training data was not blinded. MEP amplitudes were measured off-line. Recordings with increased EMG background activity were excluded from further analysis. Peak-to-peak MEP amplitudes were determined and their mean calculated for each stimulus intensity.

2.6.1. Stimulus response curve (SRC)

Comparability of corticospinal excitability prior to training (pre) in the different conditions was tested by comparing the SRCs in separate one-way ANOVAS. The effect of the different times of rTMS pulse applied during the training on SRCs was evaluated with repeated measures ANOVA. MEP amplitudes of ECU and FCU muscles were the dependent variables, stimulation condition (0.1 Hz rTMS_{0 ms}, 0.1 Hz rTMS_{-100 ms}, and 0.1 Hz rTMS_{300 ms}, Sham), time of measurement (pre, post1–post3), type of muscle (ECU and FCU), and intensity of the stimulator output (intensity 35–80% of MSO) were the independent variables. Subject was the random factor. The effect of frequency of rTMS on the SRCs of the ECU muscle was tested using repeated measures ANOVA. MEP amplitudes of ECU muscle were the dependent variables, stimulation condition (0.1 Hz rTMS_{0 ms}, 0.25 Hz rTMS_{0 ms}, 0.5 Hz rTMS_{0 ms}, Sham), time of measurement (pre, post1–post3), and intensity of the stimulator output (intensity 35–80% of MSO) were the independent variables. Subject was the random factor.

For post-hoc testing, for each stimulation condition, the subject's mean MEP amplitudes at each intensity (35–80% MSO) were normalized to the subject's maximum mean MEP amplitude obtained prior to training (pre) (Muellbacher et al., 2002b). To test for the effect of training in each condition, the normalized MEP amplitudes of pre and post1 testing were compared using a paired *t*-test. Training related changes in MEP amplitude were expressed as differences between the pre- and post-measurements in each condition and at each intensity (normalized MEP_{post1} – normalized MEP_{pre} (Δ MEP_{post1}) and normalized MEP_{post3} – normalized MEP_{pre} (Δ MEP_{post3})). For each stimulation condition, Δ MEPs at intensities of 35–80% MSO were then averaged (mean Δ MEP). According to our hypothesis, we compared the mean Δ MEPs of the different stimulation condition with Sham with a *t*-test associated with the method of orthogonal contrast. The alpha level was decreased to 0.01 to correct for multiple comparisons. We expected Δ MEP amplitudes to be greater in 0.1 Hz rTMS_{0 ms} when compared to Sham. Further, we expected the Δ MEP_{post3} to be smaller in 0.1 Hz rTMS_{300 ms} when compared to Sham.

2.6.2. Peak acceleration of wrist extension movements

To evaluate the comparability of peak acceleration prior to the training (pre) across conditions, peak acceleration prior to the training (pre) was tested using separate one-way ANOVAS. The effect of rTMS applied at different times (0.1 Hz rTMS_{0 ms}, 0.1 Hz rTMS_{-100 ms}, and 0.1 Hz rTMS_{300 ms}) and frequencies (0.1 Hz

rTMS_0 ms, *0.25 Hz rTMS_0 ms*, *0.5 Hz rTMS_0 ms*) on the peak acceleration of the wrist extension movements were tested in two separate mixed model ANOVAs.

For post-hoc testing, training related changes in peak acceleration were expressed as the differences between post1–pre measurements (peak acceleration_{post1} – peak acceleration_{pre}, Δ peak acceleration_{post1}) and post3–pre measurements (peak acceleration_{post3} – peak acceleration_{pre}, Δ peak acceleration_{post3}). According to the stated hypothesis, the following post-hoc contrasts were calculated using a *t*-test. For each stimulation condition (*0.1 Hz rTMS_0 ms*, *0.1 Hz rTMS_–100 ms*, *0.1 Hz rTMS_300 ms*, *0.25 Hz rTMS_0 ms*, *0.5 Hz rTMS_0 ms*), Δ peak accelerations were compared to the corresponding Δ peak accelerations in the Sham condition. The alpha level was decreased to 0.01 to correct for multiple comparisons. According to our hypothesis, we expected Δ peak accelerations to be greater in *0.1 Hz rTMS_0 ms* when compared to Sham. Further, we expected the Δ peak accelerations_{post3} to be smaller in *0.1 Hz rTMS_300 ms* when compared to Sham.

2.6.3. Training characteristics

Training characteristics were evaluated for each 10 min block of training (train 10, train 20 and train 30). EMG recordings were inspected for presence of MEP amplitudes to ensure subthreshold stimulation. Separate repeated measures ANOVA were used to test the effect of time (train 10–30) and type of training (*0.1 Hz rTMS_0 ms*, *0.1 Hz rTMS_–100 ms*, *0.1 Hz rTMS_300 ms*, *0.25 Hz rTMS_0 ms*, *0.5 Hz rTMS_0 ms*, Sham), on peak acceleration, dispersion of training angle and angle.

3. Results

3.1. Effect of time of rTMS pulse in reference to the onset of EMG on SRC and peak acceleration

3.1.1. Baseline measures of corticospinal excitability

Prior to training (baseline), measures of corticospinal excitability (MT, SRC) and kinematics (peak accelerations) were similar across the four different experimental conditions probing the effect of time of rTMS on plasticity (*0.1 Hz rTMS_0 ms*, *0.1 Hz rTMS_–100 ms*, *0.1 Hz rTMS_300 ms*, Sham; Tables 2 and 3). Further, stimulus intensities for rTMS during the training were similar across conditions (Tables 2 and 3).

3.1.2. Training characteristics

3.1.2.1. Training kinematics. While the angles and dispersions of training angles did not differ significantly across conditions (Tables 3 and 4), the magnitude of the first peak acceleration of the training movements was different across conditions ($F = 3.28$, $p = .038$, Table 3 for detailed statistics). However, post-hoc comparisons did not reveal any statistically significant differences in peak acceleration (Table 3). There was no change in the movement kinematics during the training when tested as a function of training time (lack of significant effects for training time (10, 20, 30 min of train-

ing) and interaction between training time and condition, (Table 3)). To test for a cumulative effect of training on the plasticity measure MEP amplitude, the amplitudes at baseline and post1 were compared. In all conditions, increases in MEP amplitude at post1 over baseline were significant at the 0.05 level confirming the presence of a cumulative training effect on this plasticity measure (paired *t*-test: *0.1 Hz rTMS_–100 ms*: $p = 0.04$; *0.1 Hz rTMS_300*: $p = 0.0001$; *0.1 Hz rTMS_0 ms*: $p = 0.03$; Sham: $p = 0.003$).

3.1.2.2. Time of the TMS pulse in reference to the EMG. Off-line inspection of the ECU EMG data during the training revealed absence of discernible MEPs and the occurrence of the actual rTMS pulse at 10.79 ± 1.90 in the *0.1 Hz rTMS_0 ms* condition, at -71.20 ± 6.44 in the *0.1 Hz rTMS_–100 ms* condition, at 313.05 ± 1.52 in the *0.1 Hz rTMS_300 ms* condition, and at 16.61 ± 8.99 ms in Sham (Table 4). As expected, the greatest difference between the targeted time and the actual time was seen in the *–100 ms* condition as the rTMS pulse was estimated based on the average of the preceding four movement onset times (time interval between the auditory stimulus and the onset of the movement which includes the reaction time). All other conditions were independent of the reaction time. There was a significant effect for of EMG-TMS time ($F = 905.02$, $p < .0001$, Table 3 for detailed statistics) indicating that this variable is indeed different across stimulation conditions (Table 3).

3.1.3. Formation of a motor memory

Training alone (Sham) or when combined with rTMS applied at either *–100*, *0*, *+300 ms* in reference to the onset of the EMG of the ECU muscle resulted in an increase in the MEP amplitudes of the ECU muscle when measured immediately post-training (post1, Figs. 3A–D and 4A) and 60 min after completion of the training (post3, Figs. 3A–D and 4A). This was not seen in the muscle antagonistic to the training movements (FCU, Fig. 3A–D). This differential effect of training on MEP amplitude as a function of time is statistically significant as indicated by the statistically significant 4 way interactions between condition * intensity * type of muscle * time ($F = 1.34$, $p = .032$, Table 3). The main effects for type of muscle ((ECU and FCU muscles); $F = 31.817$, $p = .0005$), intensity ((MSO 35–80% MSO); $F = 37.64$, $p < .0001$) and time (pre, post1, post3); $F = 11.16$, $p \leq .0001$) and interactions between type of muscle * intensity ($F = 19.28$, $p < .0001$), type of muscle * time ($F = 6.90$, $p = .0015$), time * intensity ($F = 4.62$, $p < .0001$), type of muscle * time * intensity ($F = 3.53$, $p < .0001$) were also statistically significant (see Table 3 for details). For post-hoc comparisons, training related changes in MEP amplitudes were expressed as differences between the pre- and post-training (post1) normalized MEP amplitudes evoked at intensities of 35–80% of MSO in each condition (Δ MEP). Next, Δ MEP of *0.1 Hz rTMS_0 ms*, *0.1 Hz rTMS_–100 ms*, and *0.1 Hz rTMS_300 ms* were compared to Δ MEP in the Sham condition. *0.1 Hz rTMS_0 ms* resulted in an increase in Δ MEP when compared to Sham immediately after the training (post1, $p = .046$, Table 3) which was no longer significant after correction for multiple comparisons. This effect

Table 2

Measurements of corticospinal excitability at baseline: motor threshold and intensity of rTMS is given as % of maximum stimulator output (MSO). Mean $\pm$ SE. ECU: extensor carpi ulnaris muscle, FCU: flexor carpi ulnaris muscle. There were no significant differences at baseline.

Training conditions	MT _(Bistim setting, 2 Magstim)		MT _(rapid Magstim)	Intensity of rTMS
	ECU	FCU	ECU	
Sham	49.00 $\pm$ 8.65	50.89 $\pm$ 9.27	60.00 $\pm$ 2.71	48.25 $\pm$ 2.24
0.1 Hz rTMS_0 ms	50.33 $\pm$ 8.14	52.22 $\pm$ 8.57	62.63 $\pm$ 3.31	50.12 $\pm$ 2.65
0.1 Hz rTMS_–100 ms	51.88 $\pm$ 7.30	54.00 $\pm$ 6.95	66.00 $\pm$ 3.04	52.71 $\pm$ 2.41
0.1 Hz rTMS_300 ms	50.44 $\pm$ 7.28	52.67 $\pm$ 9.17	60.63 $\pm$ 3.25	48.00 $\pm$ 2.60
0.25 Hz rTMS_0 ms	50.78 $\pm$ 2.39	51.78 $\pm$ 2.41	61.63 $\pm$ 2.73	49.75 $\pm$ 2.60
0.5 Hz rTMS_0 ms	49.56 $\pm$ 2.62	51.11 $\pm$ 2.61	60.13 $\pm$ 3.41	48.00 $\pm$ 2.76

Table 3

Results of pre-planned comparisons for effect of rTMS timing.

Pre-planned comparisons	DF	F	p
<i>Corticospinal excitability and kinematics at baseline</i>			
Separate one way ANOVAs			
DV: MEP amplitudes (SRC), IV: training condition	3, 24	Ns	
DV: MEP amplitudes (SRC), IV: intensity of TMS	9, 72	35.01	<.0001
DV: MEP amplitudes (SRC), IV: type of muscle	1, 8	51.12	<.0001
DV: peak acceleration, IV: training condition		Ns	
<i>Training characteristics</i>			
4 separate repeated measures ANOVAs: DV: peak acceleration, angular dispersion, angle, EMG-TMS time; rm IV: training time, condition.			
Significant results: DV: peak acceleration, rm IV: condition DV: EMG-TMS time, rm IV: condition. Post hoc comparisons: t-test; all comparisons	3, 72	3.28	.03
	3, 72	905.02	<.0001
		Ns	
2 Separate repeated measures ANOVAs: DV: MT of ECU, intensity of rTMS; rm IV: condition			
		Ns	
<i>Post training measurements</i>			
Mixed model ANOVA: DV: peak acceleration,			
IV: time (pre, post1–3), conditions (0.1 Hz			
rTMS_0 ms, 0.1 Hz rTMS_–100 ms, 0.1 Hz			
rTMS_+300 ms, Sham)			
Condition * time	9, 72	6.91	<.001
<i>Post hoc comparisons: t-test</i>			
Peak acceleration Sham vs 0.1 Hz rTMS_0 ms at post1	16	2.837	<.01
Peak acceleration Sham vs 0.1 Hz rTMS_0 ms at post3	16	3.312	<.01
Peak acceleration Sham vs 0.1 Hz rTMS_–100 ms post1 & 3 and 0.1 Hz rTMS_+300 ms post1 & post3		Ns	
<i>Repeated measures ANOVA: DV: MEP amplitude rm IV</i>			
Muscle	1, 8	31.17	.0005
Time (pre, post1–3)	3, 24	11.16	<.0001
Intensity (35–80%)	9, 72	37.64	<.0001
conditions		Ns	
<i>Significant interactions</i>			
Muscle * time	3, 24	6.90	.0016
Muscle * intensity	9, 72	19.28	<.0001
Time * intensity	27,	4.62	<.0001
	216		
Muscle * time * intensity	27,	3.53	<.0001
	216		
Muscle * time * intensity * conditions	81,	1.34	.032
	648		
<i>Post hoc comparisons for ECU: t-test</i>			
Δ MEP 0.1 Hz rTMS_0 ms vs Δ MEP Sham, post1	72	4.11	.046
Δ MEP 0.1 Hz rTMS_0 ms vs Δ MEP Sham, post3	72	6.92	.01
Δ MEP 0.1 Hz rTMS_–100 ms and Δ MEP 0.1 Hz			
rTMS_300 ms vs Δ MEP Sham, post1 and 3		Ns	
Post hoc comparisons for FCU: t-test: all comparisons		Ns	

DV: dependent variables, IV: independent variables, rm: repeated measures.

Table 4

Training kinematics and timing of the TMS pulse during the training. Peak acceleration (accel.) is expressed in g. Training angle (angle) is given in degrees of a 360 degree circle. Angular dispersion (dispersion) is expressed as length of unit vector. TMS-EMG time interval (time of TMS) is the time of TMS pulse in reference to the onset of EMG burst of ECU muscle. The means ± SE are given for all data. There was no statistically significant difference of training parameters between conditions.

Training conditions	Accel.	Angle	Dispersion	Time of rTMS
Sham	1.13 ± .07	323.33 ± 2.51	.994 ± .002	16.61 ± 8.99
0.1 Hz rTMS_0 ms	1.25 ± .05	319.97 ± 2.13	.995 ± .001	10.79 ± 1.90
0.1 Hz rTMS_–100 ms	1.31 ± .06	322.92 ± 2.41	.993 ± .010	–71.20 ± 6.44
0.1 Hz rTMS_300 ms	1.15 ± .05	322.74 ± 2.77	.996 ± .002	313.05 ± 1.52
0.25 Hz rTMS_0 ms	1.15 ± .13	322.49 ± 3.93	.995 ± .001	13.92 ± 1.54
0.5 Hz rTMS_0 ms	1.09 ± .05	315.81 ± 2.98	.993 ± .003	14.80 ± 1.82

was long-lasting as indicated by the statistically significant difference of these measures 60 min after completion of the training (post3: $p = .01$, Table 3). In contrast, increased Δ MEP amplitude of the ECU muscle in the other conditions did not reach statistical significance when compared to Sham (Table 3). Inspection of Fig. 4 shows that in the 0.1 Hz rTMS_300 ms condition the MEP amplitude differs from all other conditions because after an initial increase of the MEP amplitude, there is a decrease of the MEP amplitude over time (Figs. 3D and 4A). Although the effect did

not reach statistical significance, this indicates that the mechanism of the initial increase in MEP amplitude is different compared to the 0.1 Hz rTMS_0 ms condition. Similar comparisons for FCU muscle indicated no significant effects of the training on the MEP amplitude (Table 3).

The different stimulation conditions had a different effect on the peak acceleration as a function of time as indicated by the significant interaction between condition and time ($p < .001$, Table 3). For post-hoc comparisons, increases in peak acceleration were expressed as

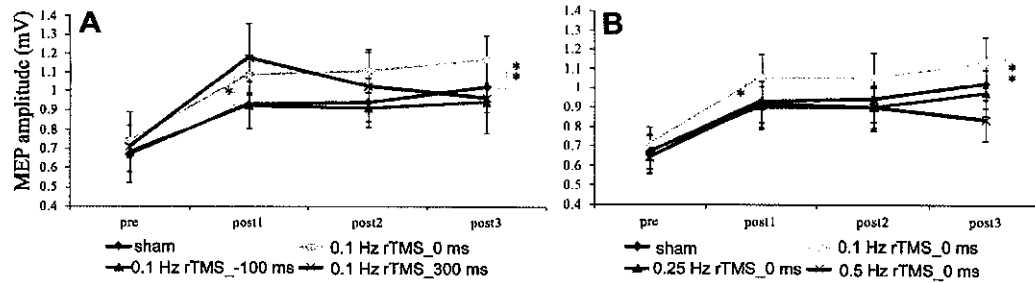


Fig. 4. Effects of different times (A) and frequency (B) of rTMS on MEP amplitude of ECU muscle. (A) Training combined with rTMS at 0.1 Hz frequency at the onset and after completion of the training movement (0.1 Hz rTMS_0 ms and 0.1 Hz rTMS_300 ms) were most effective in increasing MEP amplitude (post1). This effect was long lasting for 0.1 Hz rTMS_0 ms while wearing off in the 0.1 Hz rTMS_300 ms condition. (B) Increasing the frequency of rTMS applied at the onset of the movement did not produce any further increase over baseline. * $p < 0.05$. Mean $\pm$ SE.

differences between pre- and post1 and post3 (post1–pre, post3–pre, Fig. 5). Post-hoc comparisons revealed that 0.1 Hz rTMS_0 ms was superior to Sham in increasing peak acceleration when measured immediately after completion of the training (post1, Fig. 5A, $p < .01$) and also 60 min. after completion of the training (Fig. 5B, $p < .01$). The other conditions did not produce increases in peak acceleration post-training (see Fig. 5A and B, post1 and post3) that were statistically significant different from Sham, Figs. 3–5.

3.2. Effect of different frequencies of rTMS (0.1, 0.25 and 0.5 Hz) on SRC and peak acceleration:

3.2.1. Baseline measures of corticospinal excitability

At baseline, measures of corticospinal excitability (MT and SRC) and kinematics (peak accelerations) were similar across the four different experimental conditions probing the effect of rTMS fre-

quency applied at the onset of movement related EMG activity (0.1 Hz rTMS_0 ms, 0.25 Hz rTMS_0 ms, 0.5 Hz rTMS_0 ms, Sham; Tables 2 and 5).

3.2.2. Training characteristics

RTMS was set to be applied at the onset (0 ms) of EMG activity in the ECU muscle. Off-line analysis of training data (EMG and TMS stimulation artifact) revealed absence of MEPs and the occurrence of the actual rTMS pulse at 10.79 ± 1.90 in the 0.1 Hz rTMS_0 ms condition, at 13.92 ± 1.54 ms in the 0.25 Hz rTMS_0 ms condition, at 14.80 ± 1.82 ms in the 0.5 Hz rTMS_0 ms condition, and at 16.61 ± 8.99 ms in Sham (Table 4). The training angles, dispersion of angles and first peak acceleration and intensity and time of rTMS pulses did not differ significantly across conditions (Tables 2, 4 and 5). As indicated by the lack of significant effects for training time (10, 20, 30 min of training) and interaction between training time

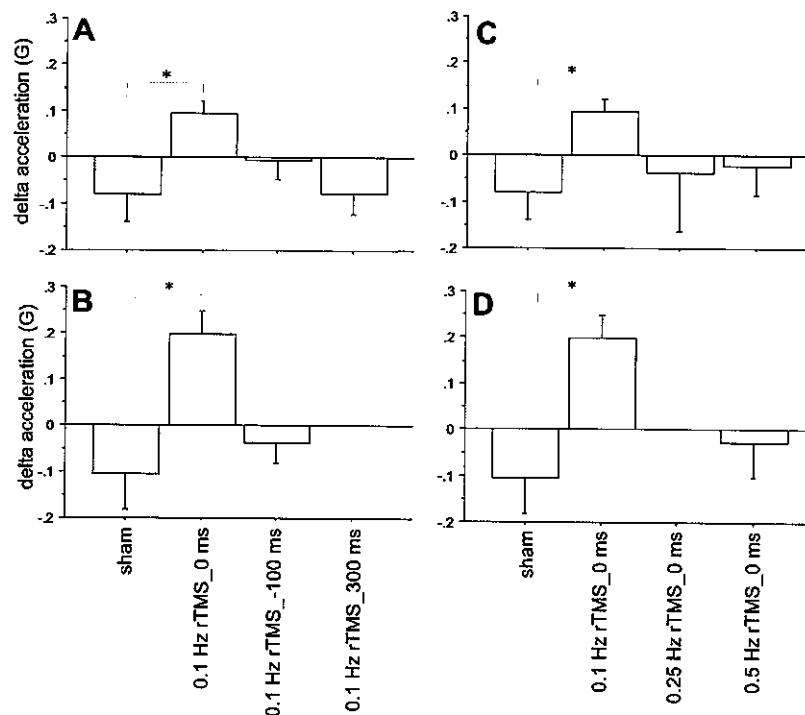


Fig. 5. Effects of different times and frequencies of rTMS pulse during the training on peak acceleration of wrist extension movements. Acceleration of 5 ballistic wrist extensions was recorded prior to training (pre), immediately after training (post1), and again 30 min (post2) and 60 min (post3) after completion of the training. Training related changes in peak acceleration are expressed as the differences between post1–pre (Δ peak acceleration, (A) and (C)) and post3–pre (Δ peak acceleration, (B) and (D)). Training combined with rTMS at 0.1 Hz frequency at the onset (0.1 Hz rTMS_0 ms) resulted in increases in peak acceleration after the training (A) and (C) that were long lasting (B) and (D) and superior to Sham. These increases were not seen in the other conditions. * $p < .01$. Mean $\pm$ SE.

Table 5
Results of pre-planned comparisons for effect of rTMS frequency.

Pre-planned comparisons	DF	F	p
<i>Corticospinal excitability and kinematics at baseline</i>			
4 separate one way ANOVAs			
DV: MEP amplitudes (SRC), IV: training condition		Ns	
DV: MEP amplitudes (SRC), IV: intensity of TMS	9, 72	27.02	<.0001
DV: MEP amplitudes (SRC), IV: type of muscle	1, 8	18.62	<.0001
DV: peak acceleration, IV: training condition		Ns	
<i>Training characteristics</i>			
4 separate repeated measures ANOVAs: DV: peak acceleration, angular dispersion, angle, EMG-TMS time, rm IV: training time, condition. No significant results		Ns	
2 Separate repeated measures ANOVAs: DV: MT of ECU, intensity of rTMS; rm IV: condition.		Ns	
<i>Post training measurements</i>			
Mixed model ANOVA			
DV: peak acceleration,			
IV: time (pre, post1–3), conditions (0.1 Hz			
rTMS_0 ms, 0.1 Hz rTMS_–100 ms, 0.1 Hz			
rTMS_+300 ms, Sham)			
Condition * time	9, 72	2.05	.046
Post hoc comparisons: t-test: all comparisons were ns		Ns	
<i>Repeated measures ANOVA: DV: MEP amplitude rm IV</i>			
Muscle	1, 8	22.97	.001
Time (pre, post1–3)	3, 24	10.34	.0001
Intensity (35–80%)	9	22.07	<.0001
Conditions		Ns	
Significant interactions: Time * intensity	27, 216	6.75	<.001
Post hoc comparisons for ECU and FCU muscle: t-test: all comparisons were ns		Ns	

DV: dependent variables, IV: independent variables, rm: repeated measures.

and condition training did not result in statistically significant changes when tested for each 10 min increment of training time (Table 5). To test for a cumulative effect of training on plasticity measure MEP amplitude, a paired *t*-test was performed. In all conditions, increases of MEP at post1 over baseline were significant at the 0.05 level confirming a training effect on this plasticity measure (paired *t*-test: 0.25 Hz_rTMS_0: $p = .03$; 0.5 Hz_rTMS_0: $p = .01$; 0.1 Hz_rTMS_0: $p = 0.02$; Sham: $p = 0.003$).

3.2.3. Formation of a motor memory

TMS applied at 0 ms in reference to the onset of the EMG of ECU muscle at the higher frequencies of 0.25 and 0.5 Hz frequency (0.25 Hz_rTMS_0 ms, 0.5 Hz_rTMS_0 ms) resulted in increases in the MEP amplitudes of ECU muscle when measured immediately post-training (post1, Fig. 4B). This was not seen in FCU muscle (data not shown). This differential effect of training on MEP amplitude as a function of time is statistically significant as indicated by the significant main effects for the variables type of muscle ($F = 22.97$, $p = .001$), intensity ($F = 22.07$, $p < .0001$) and time ($F = 10.34$, $p = .0001$). There were significant interactions between time * intensity ($F = 6.75$, $p < .001$). See Table 5 for details.

For post-hoc testing, Δ MEP was calculated in each condition for each subject (see above for details). There were no significant differences between Δ MEP of 0.25 Hz_rTMS_0 ms, and 0.5 Hz_rTMS_0 ms when compared to Δ MEP in the Sham condition (Sham) and 0.1 Hz_rTMS_0 ms. TMS applied at higher frequencies had no significant effect on peak acceleration when compared to Sham and 0.1 Hz_rTMS_0 ms (Fig. 5C and D, Table 5).

4. Discussion

The main two new findings of this study were first, that the timing and frequency of rTMS applied to M1 while subjects were engaged in performing a training movement and had different effects on the formation and retention of an elementary motor

memory (MEP amplitude and peak acceleration) and second, that this type of training improved the kinematics of the trained movement above naïve levels, a finding that would support the notion of the formation of motor memories within M1 as an initial first step in events leading to improved motor skills (Muellbacher et al., 2002b) (see below). Although we do not provide direct evidence for the discharge of the targeted PTNs at the time of the TMS pulse, the temporal relationship between M1 PTN discharges, EMG activity and movement execution was previously established in non-human primates (Kalaska, 2009). In these simultaneous recordings of pyramidal tract neuron discharges, EMG activity and movement kinematics, neuronal discharges preceded the onset of movement-related EMG activity, and movement by 100–200 ms prior to EMG onset (Crammond and Kalaska, 2000). In the present study, the increases in EMG activity of the agonist prior to the onset of the ballistic movement provide therefore indirect evidence of PTN activity. Further, subthreshold TMS applied to M1 stimulates intracortical connections targeting pyramidal tract neurons (Day et al., 1987; Rothwell, 1997; Di Lazzaro et al., 1998a, 2002a) and can therefore be used as means to stimulate M1 afferents targeting pyramidal tract neurons. Evidence from direct epidural recordings of the spinal cord by Di Lazzaro et al. suggests that indeed subthreshold rTMS activates primarily a low threshold subpopulation of cortical neurons in proximity to the targeted hot spot (Di Lazzaro et al., 2002a).

The lack of any significant rTMS related effects on the MEP of the FCU muscle, a muscle antagonistic to the training movement would support the notion of a specific effect for ECU muscle and would argue against an overall increase in M1 excitability. Further, because there is evidence that motor learning occurs in the motor cortex (Bütefisch et al., 2000; Muellbacher et al., 2002b; Kalaska, 2009) the reported differences in rTMS-training intervention related MEP amplitudes of ECU and FCU muscles likely reflect differential excitability changes within the movement representation of the motor cortex. The net effect of such changes in excitability could be training-induced strengthening of the intracortical

neuronal ensembles generating outputs supporting the training movements.

4.1. Timing of the rTMS pulse with reference to the execution of the training movement

In the present study, M1 stimulation was most effective in increasing the formation and retention of a motor memory when the stimulus occurred at movement onset (0.1 Hz rTMS_0 ms). Stimulation during movement preparation (0.1 Hz rTMS_–100 ms) was not different from Sham. This would suggest that the enhancing effect on MEP amplitude and acceleration demonstrated for the 0.1 Hz rTMS_0 ms condition is likely more associated with M1 activity related to the execution of the motor command than to the preparation of the movement. Further stimulation after completion of the movement (0.1 Hz rTMS_300 ms) produced only a short lasting facilitatory effect on MEP amplitude and no improved kinematics. This indicates that there was no specific effect on formation of motor memory over the naïve level in these conditions. These results are consistent with the finding by Mrachacz-Kersting et al. (2012) demonstrating a facilitatory effect on M1 excitability when afferent M1 stimulation by means of afferent peripheral nerve stimulation arrives at M1 during the imagined execution of the movement but not during imagined preparation or after imagined completion of the movement (Mrachacz-Kersting et al., 2012). The results are also compatible with results by Stefan et al. (2000, 2002), where low frequency (0.2 Hz) pairing of *suprathreshold* rTMS of M1 and electrical peripheral stimulation of somatosensory afferents was used to stimulate two afferents converging on targeted PNTs. In their study plastic changes were observed when pairing of afferent stimulation was timed to occur synchronously at the targeted PTNs. Similar to our results, stimulation at an inter-stimulus time interval of >100 ms did not produce any enhancing effect. In this regard, the results share similarities with results of Hebbian type stimulation on a cellular level where pairing of thalamo-cortical afferent stimulation with stimulation-induced firing of the targeted postsynaptic PTN increased the probability of LTP induction when applied at an interstimulus interval of <50 ms (Baranyi and Szente, 1987).

The importance of timing was recently confirmed by another group using rTMS and movement related M1 activity (Thabit et al., 2010). In their study, *suprathreshold* rTMS was applied to M1 at –50, +50, +100 and +150 ms in reference to the onset of the practiced movement. A facilitatory effect of TMS on the MEP amplitudes was observed when the TMS pulse arrived 50 ms prior to the onset of the movement. The comparison of their results with the results of the present study is limited because of the different time intervals tested and the *suprathreshold* rTMS intensity which will likely result in stimulation of a different group of cortical neurons (Di Lazzaro et al., 2002a,b; Kujirai et al., 2006). Further, in the movement paradigm used by Thabit et al., the executed movements failed to produce motor learning as indicated by the lack of changes in the kinematics of the practiced movements and the short lasting increase in the MEP amplitudes (15 min).

While our results support the notion of the importance of the timing of events (stimulation of intracortical connections targeting PTN neurons and M1 activity), there are several limitations imposed by the experimental set up and the fact that measurements were taken at the system level as opposed to the cellular level in the work by Baranyi and Szente (1987). In contrast to the stimulation of thalamo-cortical afferents used by Baranyi et al., the cortico-cortical neurons targeted in the present study are one subset of neurons stimulated by TMS. TMS-elicited discharge might occur via various local networks. Further, corticospinal neuron discharge in M1 is associated with various aspects of the motor output as well as somesthetic input.

Although the intensity of rTMS was comparable across the different stimulation conditions (Table 2), the voluntary muscle contraction in conditions where rTMS was applied at the onset of ECU EMG activity (0.25 Hz rTMS_0 ms, 0.5 Hz rTMS_0 ms and 0.1 Hz rTMS_0 ms) likely affected the level of excitability of the stimulated neuronal population. Specifically, there is evidence from epidural recordings of descending volleys in conscious humans that voluntary contraction increases the size and number of descending volleys elicited by TMS (Di Lazzaro et al., 1998b). Further, rTMS during volitional activity activates a different subpopulation of intracortical neurons (Kujirai et al., 2006). However, the absence of MEP amplitudes in the conditions where rTMS was given at the onset of the movement (see above) and the lack of enhancing effect with rTMS at higher frequencies (0.25 Hz rTMS_0 ms and 0.5 Hz rTMS_0 ms) would argue against the possibility of voluntary activity related increases in neuronal excitability as the main underlying mechanism for the enhancing effect seen in the 0.1 Hz rTMS_0 ms.

4.2. Frequency of the rTMS pulse

Our experimental set-up differed from other pairing paradigms in intact human M1 (Stefan et al., 2000, 2002; Bütefisch et al., 2004; Ziemann et al., 2004; Kujirai et al., 2006) and *in vivo* experiments by Baranyi and Szente (1987), Baranyi et al. (1991) as pairing of rTMS and movement execution at 0.5 Hz was most effective when rTMS occurred at 0.1 Hz (0.1 Hz rTMS) which resulted in a 1:5 rTMS/movements ratio. The pairing at different ratios or higher frequencies has not been tested in human M1 before and therefore comparisons to results of other studies are limited. In the paradigms used by Stefan et al. and Thabit et al. pairing occurred at 0.2 Hz and was reported to be effective for the induction of paired associative plasticity (Stefan et al., 2000, 2002; Kujirai et al., 2006) or increases in M1 excitability (Thabit et al., 2010). The results of the present study seems to be in contrast to our hypothesis based on Hebb's rule where post-synaptic activity is preceded by pre-synaptic activity and movement related M1 activity without rTMS stimulation should have weakened the rTMS related effects. The present data does not provide any information that could explain the mechanisms underlying these findings. In future experiments we will determine M1 excitability between the occurrences of the rTMS stimuli to explore the possibility of relative refractoriness of the stimulated neuronal network, a phenomenon that could explain the superiority of low frequency rTMS over the higher frequencies in this paradigm. Another possible explanation for the present results are the effects of 0.1 Hz subthreshold rTMS on GABAergic and glutamatergic function (Ziemann et al., 1998). In their study subthreshold rTMS at 0.1 Hz frequency was effective in enhancing deafferentation related M1 plasticity, decreased short interval intracortical inhibition (SICI) and increased intracortical facilitatory activity (ICF) (Ziemann et al., 1998). Because SICI is controlled by GABA and glutamate, the authors proposed that 0.1 Hz subthreshold rTMS resulted in down-regulation of GABAergic function or up-regulates glutamatergic function or both (Ziemann et al., 1998). As we did not measure SICI and ICF we cannot provide evidence to substantiate this claim.

4.3. Limitations of the study

Due to the non-specific resolution of the method, it would be difficult to categorically demonstrate the characteristic of input specificity. In fact, in addition to the effects of direct stimulation, rTMS also influences remote but interconnected regions of the brain as a result of functional connectivity between cortical and sub-cortical regions (Bestmann et al., 2003). In the present study significant stimulation related increases in MEP amplitude were

only seen in ECU muscle, a muscle supporting the training and not seen in the FCU muscle, a muscle antagonistic to the training movement (Figs. 3 and 4). Because the time since the completion of the rTMS intervention will affect the obtained measures, the MEPs for both muscles ECU and FCU were elicited from the ECU hot spot. Although the finding of the FCU muscle are therefore limited a similar approach was used in previous experiments where TMS was applied to the location in M1 that elicits isolated thumb movements. Specifically, MEPs evoked by stimulating this location were recorded from the flexor pollicis brevis and extensor pollicis brevis, muscles that function as agonist and antagonist in a motor training task of the thumb. These recordings demonstrated very distinct changes depending on whether the muscle acted as agonist or antagonist despite the limitation of the site of stimulation (Bütefisch et al., 2000, 2002; Sawaki et al., 2002; Bütefisch et al., 2004). The lack of changes in FCU muscle would therefore support the notion that indeed, rTMS related increases in M1 plasticity were induced in M1 areas representing the trained movement. Although there is no direct evidence in the present study that plasticity was induced at the cortical level, rTMS related increases of this type of plasticity likely occur at the cortical level because sub-threshold TMS does not result in descending volley (Di Lazzaro et al., 1998a, 2002a, 2004). Further, previous studies using a similar training paradigm demonstrated that practice dependent plasticity (Classen et al., 1998; Muellbacher et al., 2002b) and the early phase consolidation of motor learning (Muellbacher et al., 2002b) occurs at the level of the cortex and is blocked by drugs that interfere with the activation of NMDA receptors or increase GABAergic transmission (Bütefisch et al., 2000).

In summary, we have demonstrated that the formation of motor memory is enhanced by co-administrating rTMS at the time of execution of training movements and that this type of training improved the kinematics of the trained movement above naïve levels, a finding that would support the notion of the formation of motor memories within M1 as an initial first step in events leading to improved motor skills. These results can be used to improve the designs of new rehabilitation treatment strategies using rTMS during motor training (Bütefisch et al., 2011). While the employed experimental set up has limitations pertaining to the lack of specificity of the stimulated neurons and their connections as well as of the origin of M1 discharges, the observed enhancing effect of rTMS applied at the onset of the training movement on the formation of a motor memory shares similarities with Hebbian or associative LTP as increases in MEP amplitude and peak acceleration evolved rapidly, were persistent, depended on the timing and frequency of the afferent input with reference to the discharge of the pyramidal tract neurons and were topographically specific. Further experiments are needed to examine the mechanisms in more detail and to completely rule out the possibility that non-Hebbian cellular mechanisms operates in this type of plasticity.

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