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Potentiation of Short-Latency Cortical Responses by High-Frequency Repetitive Transcranial Magnetic Stimulation

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Veniero D, Maioli C, Miniussi C. Potentiation of short-latency cortical responses by high-frequency repetitive transcranial magnetic stimulation. *J Neurophysiol* 104: 1578–1588, 2010. First published July 14, 2010; doi:10.1152/jn.00172.2010. It is generally accepted that low- and high-frequency repetitive transcranial magnetic stimulation (rTMS) induces changes in cortical excitability, but there is only indirect evidence of its effects despite a large number of studies employing different stimulation parameters. Typically the cortical modulations are inferred through indirect measurements, such as recording the change in electromyographic responses. Recently it has become possible to directly evaluate rTMS-induced changes at the cortical level using electroencephalography (EEG). The present study investigates the modulation induced by high-frequency rTMS via EEG by evaluating changes in the latency and amplitude of TMS-evoked responses. In this study, rTMS was applied to the left primary motor cortex (MI) in 16 participants while an EEG was simultaneously acquired from 29 scalp electrodes. The rTMS consisted of 40 trains at 20 Hz with 10 stimuli each (a total of 400 stimuli) that were delivered at the individual resting motor threshold. The on-line modulation induced by the high-frequency TMS was characterized by a sequence of EEG responses. Two of the rTMS-induced responses, P5 and N8, were specifically modulated according to the protocol. Their latency decreased from the first to the last TMS stimuli, while the amplitude values increased. These results provide the first direct, on-line evaluation of the effects of high-frequency TMS on EEG activity. In addition, the results provide a direct demonstration of cortical potentiation induced by rTMS in humans.

INTRODUCTION

Repetitive transcranial magnetic stimulation (rTMS) offers the unique advantage of manipulating brain activity for a period that may outlast the stimulation itself. Most studies investigating the impact of rTMS on cortical activity have been performed on the primary motor cortex (MI)—primarily because an MI stimulation of adequate intensity evokes activation of the corticospinal tract and results in a muscle twitch called a motor evoked potential (MEP). In this case, the effects of rTMS are easily quantifiable, and cortical changes can be inferred from the modulation of MEP amplitude. Nevertheless, MEPs are the result of complex events and necessarily depend on the state of different elements along the corticospinal pathway. Indeed the stimulation site is at least two synapses away from the muscle (Siebner and Rothwell 2003) and direct recordings of descending volleys from the corticospinal system in humans indicate that TMS predominantly activates the intracortical circuits in MI (Lemon 2002). Moreover cortical

stimulation induces periodic activity (I-waves) that has a complex relationship with MEP amplitude (Di Lazzaro et al. 2007). The resulting outcome at the muscle level thus depends on both the excitability of different synapses and the repetitive, complex activation.

Based on the results of these motor studies, there is general agreement that the effects of rTMS are frequency-dependent. High-frequency rTMS (≥ 5 Hz) produces a local increase in cortical excitability as revealed by a concurrent increase in MEP amplitude (Maeda et al. 2000; Pascual-Leone et al. 1994). Low-frequency rTMS (≤ 1 Hz) has the opposite influence on brain excitability and is associated with a decrease in MEP amplitude (Chen et al. 1997; Maeda et al. 2000). More recent studies indicate that the modulatory influence of rTMS may be more complex than previously reported and point to an interaction among different stimulation parameters, such as frequency, intensity, number of stimuli and the interval between the trains delivered during the rTMS session (Fitzgerald et al. 2006; Houdayer et al. 2008; Siebner and Rothwell 2003).

One of the primary limitations of motor studies is the need to determine whether changes in MEPs amplitude are due to a modulation of cortical or peripheral excitability—or both. There has been some evidence of lasting effects in central circuits from motor studies employing paired-pulse paradigms, which are designed to evaluate the excitability of distinct inhibitory and facilitatory cortical circuits. These studies indicate a frequency-dependent effect on the two phenomena (for a review, see Fitzgerald et al. 2006). Nevertheless, some of these studies also found a concurrent, overall modulation in spinal excitability when prolonged periods of stimulation were applied (Quartarone et al. 2005).

In recent years, there has been growing interest in a new approach that combines TMS with other imaging techniques, such as positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and electroencephalography (EEG) (Bestmann et al. 2008; Ilmoniemi and Kicic 2010; Miniussi and Thut 2010; Siebner et al. 2009; Thut and Pascual-Leone 2010). The major advantage of this approach is that physiological signals generated at the cortical level are recorded, and thus it does not rely on a mixed central-peripheral signal. This new approach also opens the possibility of assessing whether rTMS induces changes in brain regions that are functionally connected to the targeted area (Bestmann et al. 2008; Ilmoniemi et al. 1997; Massimini et al. 2005).

Although the combination of TMS with PET or fMRI may be preferable because of the high spatial resolution, EEG has been shown to be a particularly sensitive tool for detecting TMS-induced activation. Indeed it has been reported that

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predominant EEG activity can be reliably determined when the stimulation intensity is set at 40% of the motor threshold (Komssi et al. 2007), whereas fMRI fails to detect any change at 80% of the motor threshold (Bohning et al. 1999). Moreover, imaging techniques relying on metabolic changes have poor temporal resolution, but EEG can reveal electrophysiological changes that occur at the millisecond time scale and can shed light on the temporal window during which profound, function-related and TMS-induced neural events are thought to occur (Bonato et al. 2006; Komssi and Kahkonen 2006).

When TMS-EEG coregistration experiments are designed according to the “inductive” approach (Miniussi and Thut 2010), TMS-evoked potentials (TEPs) are measured from the scalp while the subject is in a resting state. These experiments aim to study the reactivity of a target area (usually tested with single-pulse TMS) using the amplitude of the TEPs to test the overall state of the stimulated cortex (Komssi et al. 2004, 2007). Analogously to MEPs, TEPs are quantifiable markers of the state of the brain that are directly generated and recorded from the cortex (Miniussi and Thut 2010).

Using various recording systems, several TMS-induced responses have been described thus far, some beginning 5–10 ms after the TMS pulse (Bonato et al. 2006; Ilmoniemi et al. 1997; Kahkonen et al. 2004; Komssi et al. 2002, 2004; Lioumis et al. 2009). Despite the reproducibility of most of these TMS-induced responses, the factors that modulate these components remain unclear and therefore the functional meaning and origin of each deflection are not known. To fully exploit the potential of TEPs application, it is necessary to understand their functional meaning and characterize their behavior using different TMS protocols and patho-physiological conditions.

To our knowledge, only two studies investigated the effects of high-frequency rTMS on TEPs features. Esser and colleagues (2006) delivered single-pulse TMS after conditioning MI with rTMS, thus using an off-line approach. They found that high-frequency rTMS induced an increase in the amplitude of several TEPs and that this modulation was primarily due to a change in premotor area activity. A second study by Hamindi et al. (2010), applying trains of rTMS at 10 Hz over postcentral gyrus and superior parietal lobule, demonstrated a quadratic relation between most of evoked response amplitude and number of delivered stimuli.

In this vein, the present work aims to investigate the on-line effect of high-frequency rTMS (20 Hz) on the motor area. Using TMS-compatible EEG equipment (Veniero et al. 2009), it was possible to focus on the modulation induced by each stimulus in the train, recording from 4 to 50 ms poststimulus. We hypothesized that high-frequency rTMS would enhance the cortical response of early components through potentiation induced by the rapid sequence of stimuli.

METHODS

Subjects

Sixteen healthy, right-handed subjects [9 female, mean age: 23.4 ± 4.5 yr] participated in this study after giving their written informed consent. None of the participants had any contraindication to rTMS or any neurological, psychiatric, or other relevant medical problems (Rossi et al. 2009). The protocol was performed in accordance with ethical standards and was approved by the Ethical Committee at the IRCCS San Giovanni di Dio Fatebenefratelli.

Procedure

Subjects were seated in a comfortable armchair in a dimly illuminated, electrically shielded, and sound-proof room with their hands pronated in a relaxed position. During the experiments, subjects were required to focus on a central fixation point. Two blocks of sham magnetic stimuli (sham1 and sham2) interleaved with one block of magnetic stimulation (real) were delivered. The entire experimental session lasted ~80 min; the experimental EEG recording spanned 34 min.

TMS

TMS pulses were delivered using a SuperRapid transcranial magnetic stimulator connected to four booster modules and a double 70 mm standard figure-eight coil (Magstim, Whitland, UK) that generates 2.2 T as a maximum stimulator output (MSO). The coil was placed tangentially to the scalp with the handle pointing backwards and laterally at about a 45° angle away from the midline, approximately perpendicular to central sulcus. Each experimental session started with the coil positioning. To find the motor hot spot, the coil was moved in steps of ~0.5 cm in the fronto-central region of the scalp. The hot spot was defined as the point where TMS induced the maximum MEP from the relaxed abductor digiti minimi (ADM) muscle of right hand. After the target area was found, the coil was stabilized in the same position by means of a mechanical support that consisted of a holding arm (Magic arm Manfrotto, with 2 large clamps) and a heavy duty tripod. Once the coil was immobilized, the resting motor threshold (RTM) was determined, defined as the lowest stimulus intensity, which produced in the ADM muscle at least five MEPs of 50 μ V of 10 consecutive stimuli (Rossini et al. 1994).

Each subject underwent an experimental session consisting of three 10 min blocks of rTMS. That is a block of sham-TMS (sham1) was followed by a block of real-TMS (real) and a second sham-TMS (sham2), separated by a rTMS-free interval of 2 min to allow coil replacement. Sham stimulations were performed with a real coil that was turned over, and a 30-mm-thick plywood shield, of the same shape and size as the coil, was fastened to the coil and placed against the electrodes (Harris and Miniussi 2003; Rossi et al. 2007). Magnetic stimuli were delivered at 100% of RMT over left MI at 20 Hz repetition rate so that a total of 400 stimuli were divided in 40 trains of 10 stimuli (0.45 s train duration), separated by an inter-train interval of 14.55 s duration as can be seen in Fig. 1.

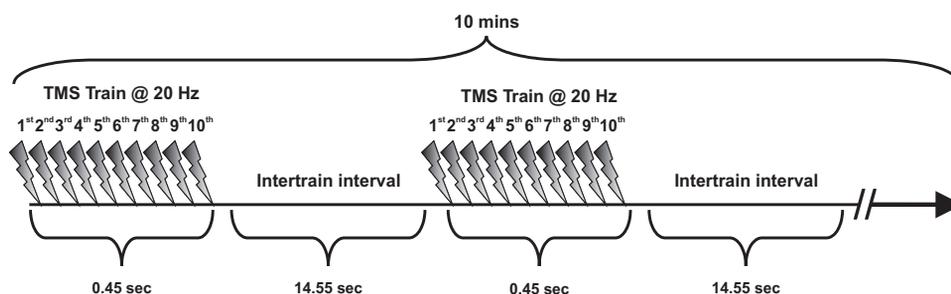


FIG. 1. Transcranial magnetic stimulation (TMS) protocol. Trains of 10 stimuli were delivered over the left primary motor cortex (MI) with an inter-stimulus interval of 50 ms, thus repetitive TMS (rTMS) frequency was 20 Hz. A total of 40 trains was applied with an inter-train interval of 14.55 s. Each block lasted 10 min.

EEG recordings

TMS-compatible EEG equipment (BrainAmp 32MRplus, Brain-Products GmbH, Munich, Germany) was used for recording TEPs from the scalp. The EEG was continuously acquired from 29 recording sites (Fp1, Fp2, F7, F3, Fz, F4, F8, FC5, FC1, FC2, FC6, T7, C3, Cz, C4, T8, CP5, CP1, CP2, CP6, P7, P3, Pz, P4, P8, O1, Oz, O2, Iz) using electrodes mounted on an elastic cap. Additional electrodes were used as ground and reference. The ground electrode was positioned in Fpz, while linked mastoid served as the active reference for all electrodes. MEPs were collected from right and left ADM via surface electrodes in belly tendon montage. The signal was band-pass filtered at 0.1–1,000 Hz and digitized at a sampling rate of 5 kHz. To minimize overheating of the electrodes proximal to the stimulating coil, TMS-compatible Ag/AgCl sintered ring electrodes were used, consisting of rings of 2 mm thickness, with inner and outer diameters of 6 and 12 mm, respectively. Skin/electrode impedance was maintained <5 k Ω for cortical and electromyographic (EMG) recordings. Horizontal and vertical eye movements were detected by recording the electrooculogram (EOG). The voltage between two electrodes located to the left and right of the external canthi recorded horizontal eye movements. The voltage difference between reference electrodes, and electrodes located beneath the right eye recorded vertical eye movements and blinks. To reduce auditory contamination of EEG induced by coil clicks, white noise (~90 dB) was played through insert earphones during the entire experiment.

MEP recordings

MEPs were collected from right and left ADM via Ag/AgCl surface electrodes in belly tendon montage. The signal was band-pass filtered at 0.1–1,000 Hz and digitized at a sampling rate of 5 kHz. Skin/electrode impedance was maintained <5 k Ω .

Control experiments

To ensure that the TEPs recorded in the first milliseconds after the magnetic pulse were cortical in origin and not caused by or contaminated by other sources (i.e., facial muscle activity), a separate series of control experiments was performed. Each experimental session was separated from the main experiment by ≥ 4 wk. Two subjects underwent a second session that followed the same recording procedures as the main experiment. In addition to the previous EEG-TMS setup, two bipolar needle electrodes were inserted into the main muscle mass of the left temporal muscle, ipsilateral to the hot spot. Additionally, seven single pulses were delivered to one of the subjects with the coil close to the temporal muscle, roughly positioned over T7. Finally, at the end of the main experimental session, an additional single-pulse session was performed with a double, 50 mm, figure-eight custom coil; this coil was positioned over the ADM cortical hot spot.

To further verify if a decrease in the short latency TEPs was present after a low-frequency stimulation protocol, the data already published by Bonato et al. (2006) were analyzed, dividing the 600 stimuli delivered at 1 Hz frequency rate, in three blocks of 200 stimuli each (following the logic of Brignani et al. 2008). Information about experimental setting has been published elsewhere (Bonato et al. 2006).

Analysis

This study aimed to investigate the modulation induced by the “summation effect” of each stimulus within the train; thus TEPs and MEPs were averaged based on the occurrence of stimulus in the train. All 40 responses to each of the 10 stimuli were averaged with respect to the position of the stimulus in the train. In other words, the first stimulus in the train was averaged with all other stimuli occupying the same position in the following trains. The same procedure was

repeated for remaining stimuli (from the 2nd to the 10th). For TEPs, analyses were performed on the amplitude and latency values from 14 scalp sites near the stimulation site (F3, F4, FC5, FC6, FC1, FC2, C3, C4, CP5, CP6, P3, P4, CP1, CP2).

A separate three-way ANOVA with repeated measures was performed to evaluate the amplitude and latency of each component with the following factors: *stimulus* (from 1st to 10th), *side* (ipsilateral vs. contralateral to magnetic stimulation) and *electrode position* (F3/4, FC5/6, FC1/2, C3/4, CP5/6, P3/4, CP1/2). As with the TEPs, MEP amplitude was calculated in relation to the occurrence of the stimulus in the train. Based on recordings from the right ADM, the size of the MEPs evoked by each of 10 stimuli of rTMS was measured peak to peak (μ V), averaged, and expressed as ratio of the MEP amplitude obtained after the first stimulus in the train (Lorenzano et al. 2002). One-sample *t*-test were used to reveal if any of the changes found in MEPs amplitude recorded after each stimulus (from 2nd to 10th) differed significantly from the baseline (i.e., MEPs amplitude elicited by the 1st stimulus of each train). For TEPs analysis, the normal distribution was tested using the Kolmogorov–Smirnov test (for all $P > 0.2$). The Huynh–Feldt ϵ correction factor was applied to compensate for the possible effects of nonsphericity in the compared measurements. The correction factor reduces the degree of freedom of the usual *F*-test; only the corrected probability values are reported. Post hoc tests were performed to investigate significant effects using the Bonferroni correction when appropriate in the case of multiple comparisons. All of the statistical analyses were performed with statistical data analysis software (Statsoft).

RESULTS

Subjects did not report any adverse effects of the stimulation. The mean stimulation intensity was 62% of the MSO and ranged from 44% to 75% MSO.

The real TMS over MI evoked a sequence of EEG responses consisting of deflections with alternating positive and negative polarity; this response began in the first few milliseconds after the magnetic pulse, as can be seen in Fig. 2. After the TMS-induced artifact (Veniero et al. 2009), four distinct TEPs were recorded, as previously reported by other authors (Bonato et al. 2006; Esser et al. 2006; Komssi et al. 2004; Paus et al. 2001). All electrodes recorded two components, a positive deflection peaking at 5 ± 0.7 ms (P5) and a negative deflection peaking at 8 ± 1 ms (N8). These peaks were characterized by a large amplitude that was maximal at the scalp position corresponding to the hot spot, and they were particularly apparent over FC5 and C3, which were the electrodes nearest to the point stimulated in most of the subjects.

The positive TEP, P30 (30 ± 4 ms), and the following negative deflection, N45 (40 ± 3.2), were clearly detectable at scalp sites situated over the midline, particularly over the vertex (Cz). Because N8 was characterized by a huge negative deflection that was especially prevalent near the stimulated site, it must be noted that the EEG signals took several milliseconds to return to the baseline level, suggesting that N8 may have partially hid subsequent responses. This effect was particularly evident in the recording sites near the hot-spot, FC5, C3, and CP5. Moreover, P30 and N45 were not clearly identifiable in most subjects after the first stimulus of the train—possibly because P5–N8 amplitude increased with the number of delivered stimuli (see following sections). Thus we analyzed P30 and N45 modulation only at the vertex (Cz). In this case, a one-way ANOVA with factor stimulus (from 1st to 10th) was performed.

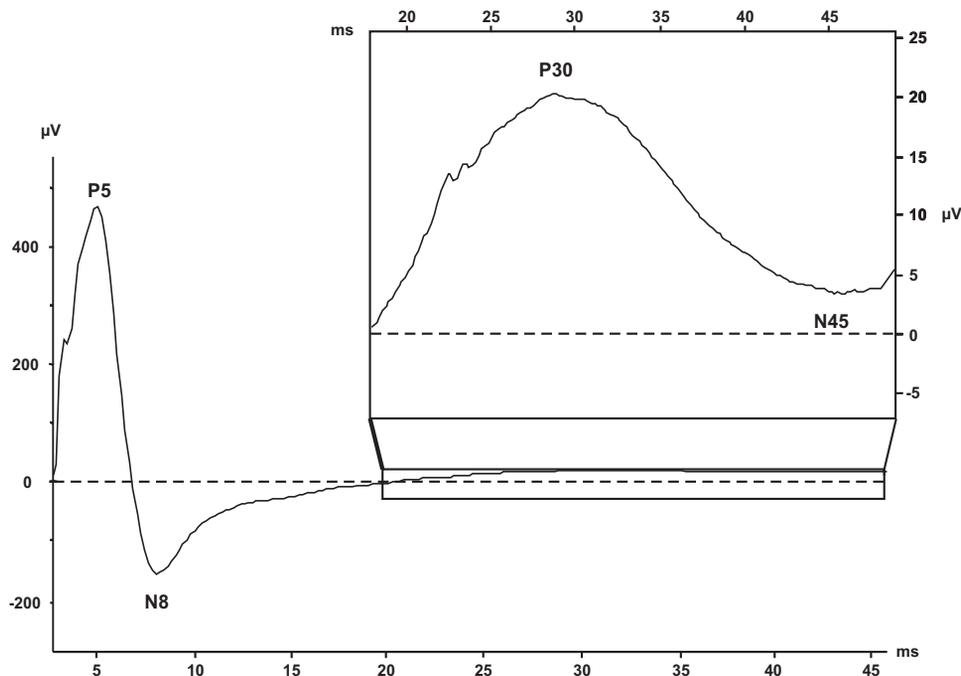


FIG. 2. TMS-evoked potentials (TEPs) recorded from the vertex (Cz) in 1 representative subject during the real TMS condition. The waveform represents the average of the responses evoked by the 1st stimulus of 40 trains. The TEPs recorded from 20 to 45 ms are magnified for clear visualization.

The scalp distribution maps obtained from the averaged activity of the first stimulus (Fig. 3) show that P5 was associated with a strong, localized positivity that corresponded to activity in the stimulated cortex and surrounding sites. This positivity evolves into an inverse pattern, displaying an equally strong negativity that peaks at ~ 10 ms (N8) after the TMS pulse. The third component (P30) had a clear dipolar distribution on the scalp and was still characterized by a sustained negative activation of the left MI and a positive component over the central and right electrodes, representing a nearly symmetrical activation of the contralateral MI (Cz-C4). Thereafter the negative component moved more anterior and reached its maximum activation when it was approximately over FC1; this activation also extended toward the contralateral frontal region. At the same time, this negative potential expanded to parietal and occipital sites in both the ipsi- and contralateral hemispheres. The positive component of the dipole, found ~ 30 ms after TMS, extended to temporal and parietal sites contralateral to the hot spot (Fig. 3). This dipole pattern also characterized N45.

TEPs modulation induced by rTMS

Figure 4A shows the effect of rTMS on P5 and N8 amplitude and latency over the C3 electrode. On both components, the train of stimuli caused a gradual increase in the amplitude, which reached its lowest and highest values at the first and the last stimulus of the train, respectively. The inverse pattern occurred with latency values, which reached their minimum at the 10th stimulus. P30 and N45 were not modulated by the stimulation, showing no change in latency or amplitude. Statistical analyses, which are detailed in the following sections, substantiated these observations.

Latency

The ANOVA performed on P5 latency revealed a significant main effect of stimulus [$F(9,90) = 41.5, P = 0.00$], indicating a reduction in latency over the course of the train. Post hoc tests indicated that rTMS caused a gradual and significant shortening of P5 latency from the first to the third stimulus (1st = 5.55 ms, 2nd = 5.41 ms, 3rd = 5.26 ms vs. 4th–10th ranging from 5.20 to 5.09 ms, all $P < 0.05$). After the third stimulus,

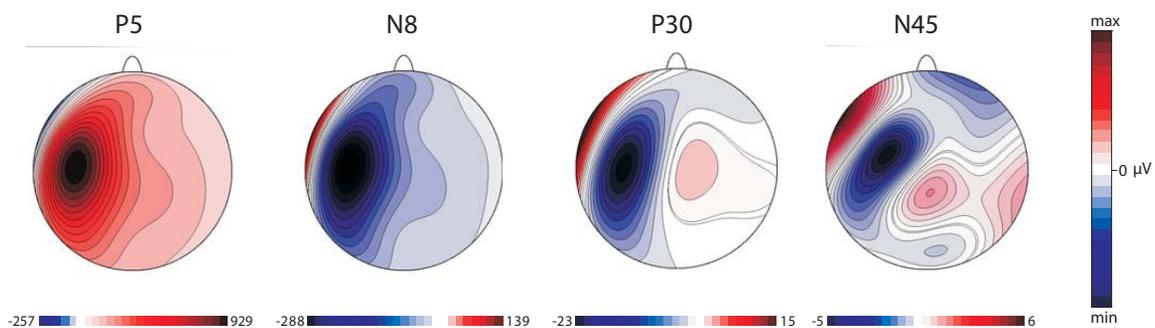


FIG. 3. Topography maps displaying the voltage distribution at specific time points corresponding to the TEPs latencies, P5, N8, P30, and N45, as reported in the text. Maps were computed from a grand average of TEPs elicited from the first stimulus of 40 trains during the real condition. Color scaling is indicated on the right. Numbers at the bottom of the maps indicate the minimum and maximum voltage (μV) of each response.

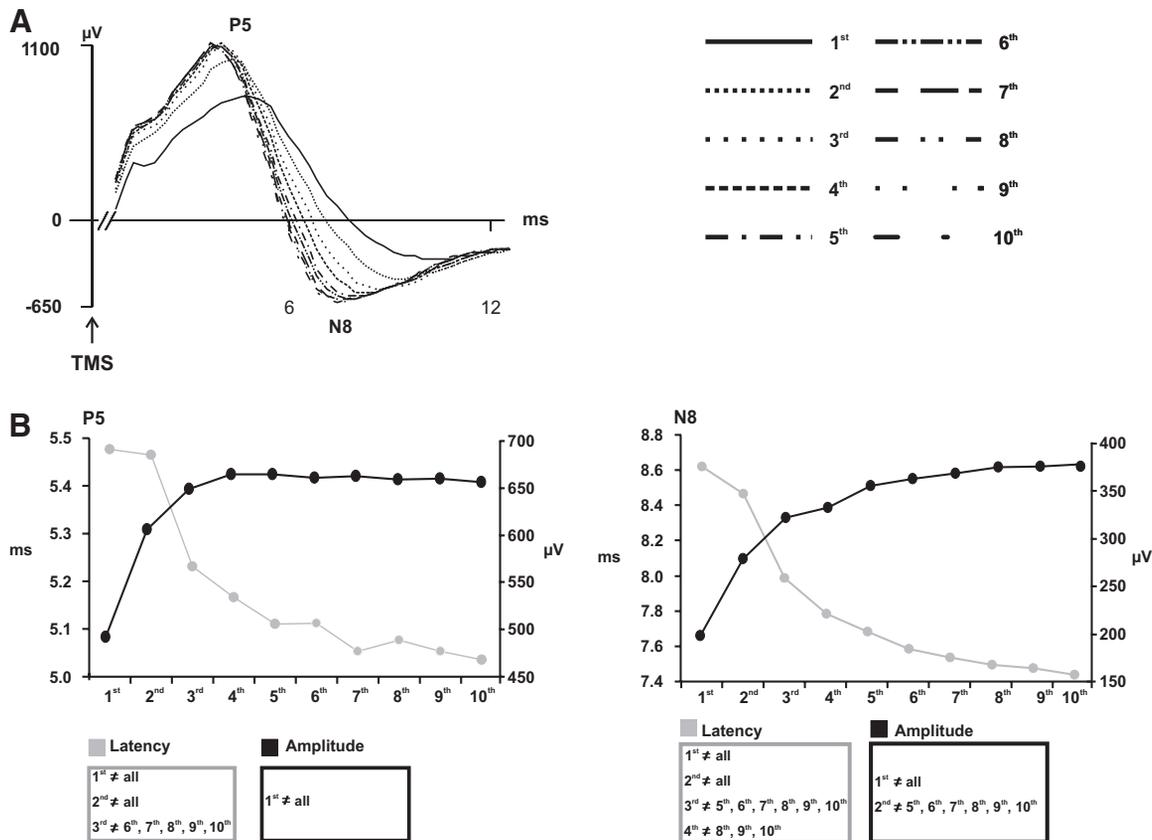


FIG. 4. Effects of high-frequency stimulation on P5 and N8 amplitude and latency. *A*: grand average obtained from all subjects for each stimulus for C_3 electrode. Both components decreased in latency and increased in amplitude in relation to the occurrence of the stimulus in the train. Each line (waveform) represents TEPs induced by each stimulus of the 20 Hz train (1st to 10th). *B*: results of ANOVA performed on latency and amplitude values for P5 and N8. In the boxes significant effects ($P < 0.05$) for latency (gray line) and amplitude (black line) are reported. The x axis in both panels specifies the stimulus position in the train. On the left side the latency values (ms) and on the right side the amplitude value (μV) are shown.

the latency values were no longer modified by successive stimuli, indicating that the latency values reached a plateau ($P > 0.05$); see Fig. 4*B*.

The ANOVA performed on N8 latency indicates a similar effect. The analysis showed a significant main effect of stimulus [$F(9,90) = 50.1$, $P < 0.001$], suggesting that the responses were faster when evoked by the stimuli delivered during the second half of the train. In this case, there was a more gradual modulation because, as indicated by post hoc comparisons, the N8 latencies evoked by the first through third stimuli were 8.72, 8.38, and 8.05 ms, respectively (latency for the 4th–10th stimuli ranged from 7.85 to 7.5 ms, all $P < 0.05$). Finally, there was a significant reduction when comparing the 4th stimulus to the 8th–10th stimuli (8th and 9th = 7.56, 10th = 7.5 ms, all $P < 0.05$). Post hoc analyses showed no difference among all of the other pulses (all $P > 0.2$). The statistical results are summarized in Fig. 4*B*. The results of the ANOVA performed on P30 and N45 latency showed no effect of stimulus [P30: $F(9,90) = 0.59$, $P = 0.58$; N45: $F(9,90) = 0.06$, $P = 0.08$].

Amplitude

The statistical analysis performed on P5 amplitude revealed a significant main effect of stimulus [$F(9,90) = 13.4$, $P < 0.001$] and side [$F(1,10) = 20$, $P = 0.001$], as well as a significant stimulus \times side interaction [$F(9,90) = 12.9$, $P <$

0.001]. Post hoc comparisons revealed that 20 Hz TMS induced an increase in P5 amplitude during the train delivery—particularly over the left stimulated hemisphere. Indeed, TMS induced a fast and sustained increase in P5 amplitude that began at the second stimulus of the train, which induced a larger cortical response relative to the first stimulus (1st = 491 μV vs. 2nd–10th stimuli = 652 μV , $P < 0.001$). No difference was found in the other comparisons ($P > 0.5$). The significant effect of side revealed that the left hemisphere (ipsilateral to the TMS stimulation) showed larger responses relative to the right hemisphere (mean = 962.9 vs. mean = 308, $P = 0.001$). Finally, the two-way interaction stimulus \times side indicated that the P5 recorded in the left hemisphere differed from successive evoked responses only after the second stimulus (1st = 748 μV , 2nd = 912 μV , 3rd–10th stimuli ranging from 978 to 994 μV , $P < 0.001$).

Like the P5 component, the N8 amplitude clearly increased over the course of the train. Statistical analysis showed a main effect of stimulus [$F(9,90) = 13.6$, $P = 0.002$] and side [$F(1,10) = 15.5$, $P = 0.002$]. The effect of stimulus was primarily caused by an increase in the amplitude starting at the third stimulus. As shown in Fig. 4*B*, the responses evoked by the first and second stimuli were significantly smaller relative to the amplitude values recorded after the remaining stimuli (1st = 195 μV vs. 2nd = 268 μV , $P = 0.000$; vs. 3rd–10th stimuli ranging from 311 to 373 μV , $P < 0.01$). A difference

between the hemispheres (side) also emerged, indicating stronger responses over the left sites, ipsilateral to the stimulation, relative to the nonstimulated side (left side = 531 μV vs. right side = 134 μV , $P = 0.004$). Moreover, significant stimulus \times side [$F(9,90) = 12.6$, $P = 0.003$] and stimulus \times electrode interactions [$F(9,90) = 12.6$, $P = 0.041$] as well as a significant three-way interaction, stimulus \times side \times electrode [$F(54,540) = 3.47$, $P = 0.025$], emerged. Taken together, these results reveal the topographic specificity of the rTMS effects. N8 amplitude increased consistently over the electrodes surrounding the left MI (FC5, C3, CP5), whereas no significant modulation was found over the contralateral side ($P > 0.5$). In the left hemisphere, the N8 recorded from FC5 displayed the strongest effect during the first half of the train ($P < 0.001$) until C3 reached the same amplitude values as the fifth stimulus (amplitude over FC5 vs. amplitude over C3, $P = 1.00$). Moreover, C3 exhibited the most complex modulation of the cortical sites. In other words, the N8 amplitude increase was slower and reached its maximum at the fourth stimulus. Finally, P30 showed no change in amplitude values [$F(9,90) = 0.03$, $P = 0.06$]. The same was true for N45 [$F(9,90) = 0.06$, $P = 0.07$].

Sham stimulations

In contrast to real TMS, sham1 and sham2 stimulations of the left MI failed to elicit a recordable MEP or relevant EEG responses.

MEPs

Clear modulation of MEPs amplitude was present as can be seen in Fig. 5. The rTMS trains delivered to the ADM motor area elicited MEPs in the contralateral ADM target muscle; these MEPs gradually increased in amplitude over the course of the train. Nevertheless, after the effect induced by the initial pulses, a "fatigue" effect was evident between the fourth and the eighth pulses of the train. This effect was highly variable between subjects; some subjects reached the maximum response at the third pulse, while, in others, the maximum response was present at the sixth pulse. The results indicated a significant rTMS-induced increase in MEP amplitude in the second ($t = 2.29$, $P = 0.04$), third ($t = 2.59$, $P = 0.03$), fourth ($t = 2.81$, $P = 0.02$), seventh ($t = 5.33$, $P < 0.001$), and eight

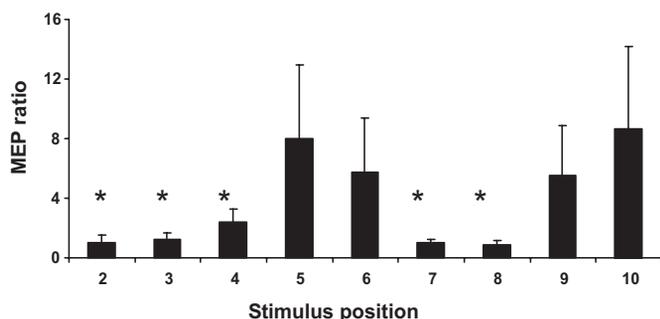


FIG. 5. MEP modulation during the train delivery. MEPs amplitude elicited by the stimuli in the train (from 2nd to 10th) are expressed as a ratio of the MEP amplitudes evoked by the 1st stimulus of the train, represented by 0. Bars marked with an asterisk are significantly different from values elicited by the 1st stimulus, as emerged from t -test analysis (all $P < 0.05$). Vertical lines indicate SEs.

($t = 3.13$, $P = 0.01$) responses relative to the baseline (i.e., the first stimulus in the train). To verify whether the amplitude of P5 or N8 corresponded to the amplitude of the MEPs, the Pearson's correlation coefficient ($P < 0.05$) was calculated for the amplitude of the MEPs evoked by each stimulus and the amplitude of P5 and N8 evoked by the corresponding stimulus over FC5, C3 and CP5 (i.e., the electrodes positioned over MI, where the peak TEPs amplitude was recorded). Importantly, no significant correlation was found.

Control experiments

To test whether P5 and N8 were caused by an activation of the ipsilateral trigeminal nerve root or the temporal muscle, a second recording session was performed with two subjects. This recording session directly recorded the EMG activity via needle electrodes inserted into the left temporal muscle. The results indicated that over cortical sites, the primary results of the main experiment were replicated, showing a similar modulation of P5 and N8. Nevertheless, an activation was recorded from the temporal muscle that appeared at the same latency (5 and 8 ms) as the cortical responses. In contrast to the similarity in latency, the magnitude of the recorded signal was not consistent; the cortical response reached a maximal amplitude of ~ 400 μV , while the EMG-evoked activity was < 20 μV (i.e., 20 times smaller than the cortical response). It was unclear whether the signal recorded from the needle electrodes was a simple effect of volume conduction. While this possibility seems reasonable, the opposite effect is also possible: the signal recorded from the cortical electrodes may simply record the muscle twitch. Nevertheless when stimulation was performed over the temporal muscle (in the T7 area), a clear, EMG response was recorded with a stable latency, that is 20 Hz TMS was not able to modulate the amplitude or latency of the responses within the train. Interesting results also come from comparison between the responses recorded from C3 and from the needle electrodes after a cortical or a direct muscle stimulation (Fig. 6A). It can be seen that moving the stimulating coil toward the temporal muscle caused a decrease in the amplitude of signal recorded from C3 and a concurrent increase in amplitude of signal recorded from the muscle. In the session performed with the smaller (50 mm) coil, the cortical components were still present and had a maximum amplitude equal to the one found in the main experiment (408 μV).

Low-frequency stimulation

To further characterize P5 and N8, data from a previous study applying 1 Hz rTMS have been analyzed. However, in the Bonato et al. (2006) different acquisition parameters were implied (i.e., sampling rate 2.5 kHz and band-pass filter at 0.1–500 Hz) that produce a modification of TMS artifact in the first milliseconds after the magnetic pulse and a consequent spread in length over the signal (Veniero et al. 2009). We found that the signal recorded from four subjects was characterized by a slow EEG recovering, thus the first components were not detectable. Nevertheless in two subjects, we were able to identify P5 and N8. When comparing the first block of stimulation (from stimuli 1 to 200) to the following ones (201–400 and from 401 to 600), a decrease in both component was evident despite no statistical analysis was performed since of the small sample size (Fig. 6B).

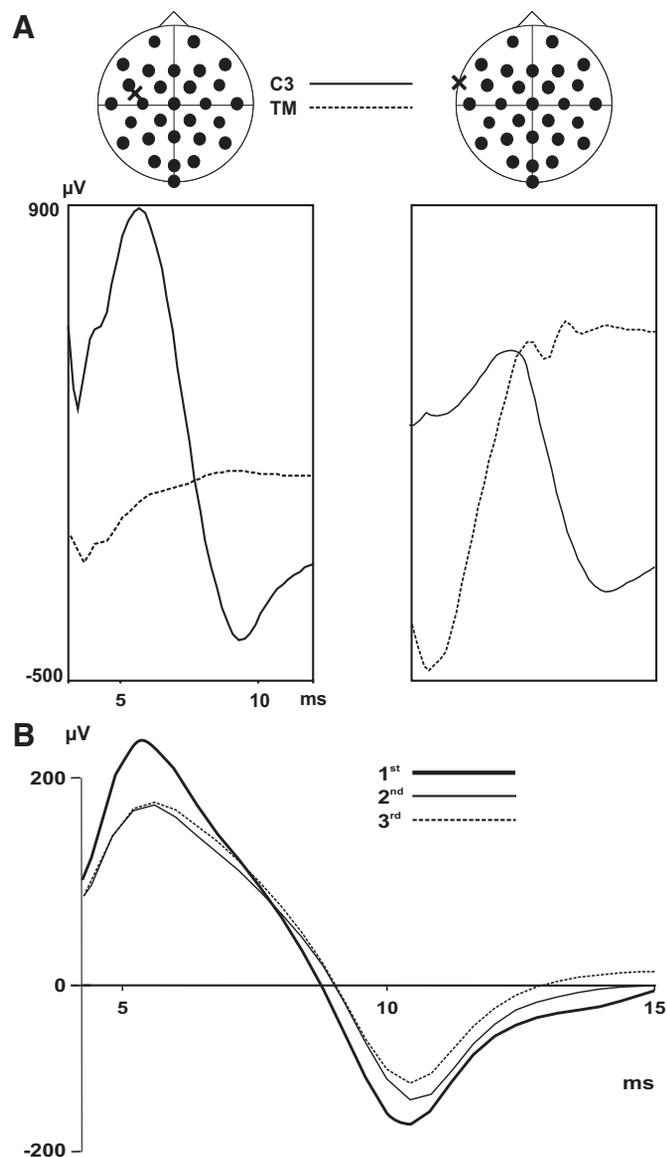


FIG. 6. Control experiments. *A*: comparison between hot spot (C3) and temporal muscle (TM) stimulation. Each line represents the average of several pulses delivered to 1 subject. *Left*: the responses over C3 and TM as a consequence of the hot-spot stimulation are shown. *Right*: the results of direct TM stimulation is depicted. *B*: effects of low-frequency stimulation on P5 and N8. Each line (waveform) represents the grand average obtained from 2 subjects, dividing 600 stimuli in three blocks of 200 stimuli each (1st to 3rd): 1–200, 201–400, 401–600. Both components decreased in amplitude as a function of number of delivered stimuli.

DISCUSSION

To address the question of how a 20 Hz rTMS modulates EEG activity, the responses to each stimulus of the high-frequency train were compared. We used an EEG recording apparatus that allowed us to evaluate the cortical signal starting at 5 ms after the TMS pulse without any prolonged saturation of the signal—despite the short inter-stimulus interval (50 ms). The real TMS delivered over MI elicited (in a time window of 50 ms poststimulus) four distinct cortical responses with latency and scalp distributions that matched those of TEPs described in previous studies (Bonato et al. 2006; Esser et al. 2006; Komssi et al. 2002; Litvak et al. 2007). Our protocol induced changes in the amplitude and latency of P5 and N8,

but no significant effects occurred for P30 and N45. The 20 Hz rTMS induced a rapid increase in the amplitude of the short-latency responses; only a few stimuli were sufficient to increase the amplitude of these TEPs to a level that was no longer statistically modifiable. The same was true for latency values, which decreased to a minimum after three or four pulses as shown in Fig. 4*B*. The induced effects showed topographic specificity, being maximal at the scalp position that corresponded to the hot-spot and areas immediately around it. Nevertheless these components appeared for all electrodes with an amplitude that decreased as the distance from the targeted region increased. These responses displayed a stable latency, indicating an effect of volume conduction that has reported in other EEG-TMS studies (Van Der Werf and Paus 2006).

P5 and N8

The nature of the P5 and N8 responses requires further considerations. These components are recorded as large signal deflections, and, although they have been described in previous papers, the present study is the first to report modulation of these components. Although it is usually assumed that TMS simultaneously activates a large assembly of neurons, one could argue that because of their amplitude, P5 and N8 may represent spurious muscle activation and that the short-latency TEPs are thus not cortical in origin. The TMS may activate facial muscles, particularly the temporal one, which is the broader facial muscle close to MI, via cortical stimulation. Although humans exert cortical control of the trigeminal innervated muscles through bilateral corticobulbar projections to motoneuron pools, it has been repeatedly shown that TMS focused over the face area evokes bilateral excitatory and inhibitory phenomena. However, these responses are predominantly contralateral, being larger than those evoked in the ipsilateral muscle, suggesting a stronger contralateral projection (for a review, see Nordstrom 2007). Moreover with the exception of the digastric muscle, preactivation of the target muscle is essential when TMS is used to elicit MEPs in the lower facial muscles (Cruccu et al. 1989; Macaluso et al. 1990), suggesting that a remarkable high intensity of stimulation is needed to induce a response. The hot spot generated by a focal coil is ~9 cm lateral to the vertex and 4 cm anterior to the interaural line (Jaberzadeh et al. 2008; Macaluso et al. 1990). It is usually distinct from the hot spot that evokes finger movements of the contralateral hand (Cruccu et al. 1997). However, it must be noted that this position is not far from FC5. In summary, although P5 and N8 latency fit the latency reported for cortical activation of temporal and masseter muscles (from 7 to 10 ms) (Cruccu et al. 1997; Dubach et al. 2004; Gooden et al. 1999; Jaberzadeh et al. 2008), their maximal amplitude was not recorded over contralateral sites, but ipsilaterally to TMS. Moreover, the lack of contraction that could have been monitored with EEG recordings and the coil positioning, closest to C3 or CP5 and still able to evoke EMG responses in the ADM, leads us to conclude that P5 and N8 were not elicited by cortical activation of efferent corticobulbar pathways.

Another possibility is that TMS directly activates the ipsilateral trigeminal nerve root. In this case, an ipsilateral MEP (i.e., M response by direct stimulation of the alpha motoneurons) may be generated in the facial muscles in most of the

subjects without preactivation but at a very high intensity if stimulation (Cruccu et al. 1997). However, we were remarkably far from the position that would elicit such an activation, and the TMS field strength decreases quickly with distance from the coil. Generally, the position that elicits ipsilateral trigeminal nerve root activation is similar to that used for cortical stimulation of facial muscles, and the mean latency of this activation is 3–3.5 ms after the TMS pulse (Cruccu et al. 1997; McMillan et al. 2001). If TMS directly activates the ipsilateral trigeminal nerve, one would conclude that the TMS artifact hid the first part of the ipsilateral MEP and that P5 may represent the second peak of a MEP. However, this conclusion is not well supported. In addition, the maximum response should have been recorded during the control experiment from the needle electrodes directly inserted into the temporal muscle—this was not the case. Nevertheless, the second problem raises a major question: why did repetitive activation of the trigeminal root induce significant modulation of temporal MEP amplitude and latency as found in the main experiment? This finding seems to indicate a cortical origin for these components.

Finally, one could argue that TMS directly activates the temporal muscle, but it seems unlikely that application of TMS to the motor area with a smaller, 50 mm coil would induce a direct muscle contraction. Moreover, direct stimulation of the temporal muscle during the control experiment elicited a very stable latency, which contrasts with the latency modulation found in the main experiment. The last consideration emerged from the results of an experiment (Miniussi et al. 2010) that was designed to explore the effect of premotor 1 Hz rTMS sessions on MI excitability. In this experiment, we found a reduction in both components after the conditioning session. Such an effect has been ascribed to an exclusively cortical phenomena (Munchau et al. 2002). We therefore consider P5 and N8 to be cortical potentials even if we cannot totally exclude other sources.

In a previous work by Hamidi et al. (2010), rTMS was delivered at 10 Hz over the parietal cortex, and the modulation induced within the train was evaluated. The authors reported a quadratic relationship between peaks amplitude and pulse number, with the exception of the first TEP, peaking at 4 ms after TMS pulse. Despite that the latency of the first short-latency component was very similar to P5, its amplitude was several times lower. This observation is, however, not sufficient to conclude for an artifactual response. Indeed it is well known that different cerebral areas show a different sensitivity to TMS, the primary motor cortex showing larger amplitude responses when compared with other areas (Kahkonen et al. 2004, 2005). In the same vein, as recently discussed by Veniero et al. (2009), dissimilarities between present results and those by others could be due to different acquisition parameters. The decrease in the amplitude of the signal recorded in the first milliseconds after the pulse by means of filters induces slowing down recovery by some milliseconds congruent with the used filter. Due to these reasons in the reanalyzed data of Bonato et al. (2006), the P5 and N8 components were recognized only in two subjects. Nevertheless in these data a decrease in amplitude as function of number of stimuli was present. These results are important because the opposite outcome of low- versus high-frequency stimulation suggests that P5 and N8 does not simply reflect a muscle activation.

Finally, beside the filtering, other technical issues could be the reason of differences among the present study and the previous ones. Indeed, in most of EEG-TMS coregistration studies, a sample and hold circuit is used, blocking the amplifiers output for some milliseconds. Despite this circuit, EEG analysis has been often limited, starting from 7/8 ms (Komssi et al. 2002; Litvak et al. 2007) or to even longer intervals after the pulse.

Cortical modulation

The earliest recorded cortical pattern showed a strong positivity centered over the motor area ipsilateral to the stimulation; this pattern was followed by a similar component with reversed polarity. In a paper by Esser et al. (2006), source localization of the activity occurring at 5 ms revealed a source located in MI. The following peak, N8, may correspond to the strong, negative peak at 10 ms described in previous studies, a response with a scalp distribution that indicates activation of motor areas (Bonato et al. 2006; Kahkonen et al. 2004; Litvak et al. 2007). In particular, Litvak et al. (2007) proposed the ipsilateral premotor cortex as the source of this second component. The topographic distribution and opposite modulation as a consequence of low- and high-frequency rTMS of the P5 and N8 components supports the idea that these components represent a direct response of the brain area stimulated by TMS, a response that, especially in the case of N8, may involve connected motor areas anterior to MI. This hypothesis is supported by studies that found a maximum interaction between the premotor cortex and MI activity at 8–10 ms (Mochizuki et al. 2004).

The next component, P30, is often described as clear positivity over fronto-central electrodes (Bonato et al. 2006; Komssi et al. 2002), representing an interhemispheric spread of activation. Bonato et al. (2006) proposed that subcortical pathways are involved in this activity. This hypothesis is partially confirmed by modeling analysis that located the source in relatively deep structures (Litvak et al. 2007). Finally, as already described, N45 was a dipole centered over the stimulation site and has been traditionally located in the sulcal part of MI (Komssi et al. 2004; Paus et al. 2001). P30 and N45 were not significantly modulated by the high-frequency rTMS protocol. Interestingly, although their scalp distribution is characterized by engagement of the contralateral hemisphere, a sustained activation peaking over MI was always present. Some studies have excluded the possibility that P30 may be related to changes in the activation of MI because the P30 amplitude neither correlates with TMS intensity (Paus et al. 2001) or is modulated by low-frequency rTMS over MI (Van Der Werf and Paus 2006). The hypothesis that N45 originates in MI, which is supported by the findings of Paus et al. (Paus et al. 2001; Van Der Werf and Paus 2006), conflicts with the finding that suboptimal stimulation of MI does not induce any change in the features of N45 (Bonato et al. 2006). Moreover, if N45 was related to MI activation, one would expect to observe modulation during the 20 Hz TMS protocol, which was not the case. However, we should use caution when interpreting P30 and N45 because the high voltage corresponding to N8 may hide the following TEPs and thereby prevent clear evaluation of P30 and N45 modulations. In summary, these results suggest that high-frequency rTMS has a focal effect because it exclusively modulates the responses representing the activation of

ipsilateral motor areas. Additionally, because the scalp distribution maps suggest that this activation lasts for the entire time window (50 ms), our findings indicate a summation effect of cortical activity induced by each stimulus.

Although we can only speculate about its inhibitory or facilitatory nature, the enhanced amplitude and decreased latency suggest a strengthening of cortical activity. P5 and N8 may represent distinct phenomena given that the TMS has been shown to elicit distinct episodes of enhanced and suppressed activity at the cortical level (Allen et al. 2007; Moliadze et al. 2005). In particular, the N8 component may represent an inhibitory process initiated by the premotor area given that a magnetic pulse delivered over the premotor cortex at 8–10 ms prior to a second stimulus over MI can reduce the MEPs amplitude (Mochizuki et al. 2004). Furthermore, when MI is directly stimulated via an electrical pulse, inhibition can be detected within 10 ms of the stimulus (Krnjevic et al. 1966).

A previous EEG-TMS study has reported the existence of an evoked potential in children that is elicited by single-pulse TMS at 105% of motor threshold. This response had a mean amplitude of $136 \pm 73.8 \mu\text{V}$ (Bender et al. 2005), which is similar to the amplitude of the potential elicited by the first stimulus of the train in the N8 component ($185 \mu\text{V}$). In accordance with the explanation proposed by Bender (2005), and considering the findings from neuronal firing recording during single-pulse and repetitive TMS, which reported a cycling increase and decrease in the number of neuronal spikes (Allen et al. 2007; Moliadze et al. 2003), it is possible that rTMS produces a sudden and strong synchronized neuronal discharge represented by P5 and N8. Thereafter, TMS would generate a suppression of activation. Thus lower amplitude TEPs are recorded and a synchronization pattern is observed, as represented, for example, by N100 (not analyzed here because of our short time window).

We found that rTMS induces an increase in the MEPs amplitude, but this modulation was not significant for all of the responses to the train. There was a large difference between subjects in the timing of the maximum amplitude MEP in the contralateral ADM target muscle. Some subjects reached the maximum MEP response after the third pulse, whereas others reached the maximum after the fourth or fifth pulse. The lack of a clear correlation between MEPs and TEPs amplitude may be caused by several factors. First, the cortical components related to motor activation may be evoked in the first few milliseconds after the TMS pulse and would thus be hidden by a TMS-induced artifact. This hypothesis is in accordance with the results of recordings from epidural space that identified I-waves appearing 1–1.4 ms later than the volley, recruited by electrical anodal stimulation, with a latency of 2–2.6 ms (Di Lazzaro et al. 1999). The P5 component was still expected to correlate with MEP modulations because its latency resembled one of the later I-waves (Di Lazzaro et al. 2004). Alternately, the discrepancy could be explained by the contribution of neural structures located at noncortical levels—especially at the spinal level—such as circuits involved in the recurrent inhibition of spinal motoneurons. TMS-related changes in plasticity are usually derived from changes in MEP amplitude. However, as already noted, this method investigates an event that does not reflect the true nature of cortical activity, which involves at least two or three synapses. Whatever the reason for the discrepancy, this finding suggests that MEP modulation might not always be a reliable measurement of cortical excitability.

It has to be noted that a modulation of MEPs amplitude is usually tested at suprathreshold intensity and after the end of rTMS train. Few studies investigated how the final outcome—a decrease or increase—builds up within the train with opposite results (for a review, see Fitzgerald et al. 2006). As recently proposed by Maki and Ilmoniemi (2010), more complex indexes could be necessary to find the cortical correlate of cortico-spinal activation. Indeed they found that peak-to-peak amplitude of the components recorded between 15 and 30 ms after the pulse correlates with MEPs, but when the correlation is measured at single trial level.

Although we were not primarily interested in the duration of rTMS effects and did not attempt to measure them, the high rate of stimulation and the modulation of P5 and N8 are consistent with a timing-dependent form of plasticity (Allen et al. 2007). Esser et al. (2006) have previously described an induced increase in the amplitude of some TEPs that resulted from LTP induction after application of a single-pulse TMS and conditioning the MI with 5 Hz TMS. In the present study, rTMS modulations were investigated on-line and similar results were found—although our activations suggest strong involvement of the stimulated MI. The mechanisms underlying this change in the cortical response may rely on the modulation of synaptic transmission or on the long-term integrative properties of the motor cortex neurons (Miller et al. 2008). In the study by Esser et al. (2006), however, most of the LTP-related variations were ascribed to the ipsilateral premotor cortex. Investigation of the direct effects of TMS on brain responses would advance our understanding of how these effects may be related to modifications in functional and behavioral performance.

In conclusion, the present study is the first to investigate on-line modulation induced by high-frequency TMS with a focus on the contribution of each delivered stimulus. The results provide a direct demonstration of cortical potentiation induced by high-frequency rTMS in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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