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# Ongoing cumulative effects of single TMS pulses on corticospinal excitability: An intra- and inter-block investigation

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HIGHLIGHTS

- MEPs amplitude evoked by TMS is an indirect measure of corticospinal excitability.
- Single TMS pulses, delivered at random or fixed inter-trial interval (ITI), induce cumulative changes in neural activity.
- Temporal summation of neuronal depolarisation induced by several single TMS pulses increases MEPs amplitude.

ABSTRACT

*Objective:* To evaluate the effects of several single TMS pulses, delivered at two different inter-trial intervals (ITIs), on corticospinal excitability.
*Methods:* Twelve healthy volunteers participated in two experimental sessions, during which TMS pulses were delivered at random or at fixed ITIs. The TMS single pulse-induced modulation of corticospinal output (motor evoked potential amplitude – MEP) was evaluated on-line. Each session began with a baseline block, followed by 10 blocks, with 20 TMS pulses each. Intra- and inter-block effects were valuated using an ANOVA model, through nested random effect on subjects considering the subject-specific variability.
*Results:* The delivery of successive TMS pulses significantly changed both intra-block and inter-block cortical excitability, as demonstrated by an increase in the amplitude of MEPs ($p < 0.001$) and supported through trend analyses, showing a perfect linear trend for inter-block levels ($R^2 = 1$) and nearly linear trend for intra-block levels ($R^2 = 0.97$). The MEPs significantly increased when the TMS pulses were delivered at both random and fixed ITIs.
*Conclusions:* Single TMS pulses induce cumulative changes in neural activity during the same stimulation, resulting in a motor cortical excitability increase.
*Significance:* Particular attention should be taken when several single TMS pulses are delivered in research and clinical settings for diagnostic and therapeutic purposes.


## 1. Introduction

Transcranial magnetic stimulation (TMS) is both a tool to measure the state of the human motor cortex, using a single TMS pulse approach, and a tool to induce cortical excitability changes, using a repetitive TMS (rTMS) approach (Wagner et al., 2009). However, it has recently been suggested that single TMS pulses also induce significant changes in cortical excitability. Specifically, studies have shown that a single TMS pulse modulates brain activity, inducing cortical oscillations (Paus et al., 2001; Van Der Werf and Paus, 2006; Rosanova et al., 2009; Kawasaki et al., 2014) and neuronal activity changes (Moliadze et al., 2003; Funke and Benali, 2010; Stamoulis et al., 2011).

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In this context, we generally consider that "single TMS pulses", delivered at random or in fixed inter-trial intervals (ITIs), are commonly used to evaluate the corticospinal state and do not produce any changes in cortical excitability per se (Kiers et al., 1993; Pell et al., 2011; Julkunen et al., 2012). Nevertheless, the ITIs used in several experimental designs (generally between 0.15 and 0.3 Hz) do not guarantee the independence of a subsequent neurophysiological response from the previous response (Nielsen 1996; Schmidt et al., 2009). Therefore, the effects of several single TMS pulses, delivered in sequence, on corticospinal excitability are currently undervalued. Specifically, we might underestimate the dependence of a second single TMS pulse on the previous pulse. Support for this hypothesis comes indirectly from neuroimaging studies, reporting not only specific neuronal activation induced through a TMS pulse (Denslow et al., 2004; Hanakawa et al., 2009; Chen et al., 2013) but also a specific haemodynamic time course of the response (Bohning et al., 1999; 2000). However, direct evidence for changes in corticospinal excitability is lacking.

Although the after effects (off-line) induced through a short train of single pulse TMS have been well established, in terms of both haemodynamic (Allen et al., 2007) and blood oxygenation changes (Thomson et al., 2012), the effects of several single TMS pulses and more specifically the role of the ITIs on the ongoing enrollment of corticospinal excitability is still under-investigated. Only a recent study, investigating whether the single-trial MEP amplitude distribution was time invariant, has highlighted that the individual MEP amplitudes are strongly dependent on ITI (Julkunen et al., 2012). In this scenario, understanding the effect of the ITI on the functional state of cortical neurons during stimulation is key, considering that the single-pulse TMS approach is typically used as a tool to measure the corticospinal excitability state. The repeated application of TMS pulses over many trials at random or fixed intervals is commonly used to measure the corticospinal excitability state, although the effects of non-specific factors, such as habituation or anticipation to TMS pulses, on motor cortical excitability and on the subsequent corticospinal responses are yet under-investigated. In this study, we examined whether repeated single TMS pulses, delivered over the primary motor cortex at random and fixed ITIs, could induce ongoing changes of corticospinal excitability, measured as MEPs amplitude. Corticospinal excitability modulation was also evaluated within and between blocks. We hypothesised that TMS pulses, singularly delivered, but spaced by specific ITIs, could modulate the excitability of the resting human motor cortex, inducing cumulative changes in neural activity.

## 2. Materials and methods

### 2.1. Subjects

Twelve healthy volunteers (6 females; ages 23.5 ± 4.3 years) participated in this study. None of the participants had a history of neurological, psychological or other relevant medical diseases, and these individuals were not taking CNS-active medication at the time of the experiments. None of the participants had any contraindication for TMS (Rossi et al., 2009), and all participants were right-handed according to the Edinburgh Handedness Inventory test (Oldfield, 1971). The study was approved through the Ethics Committee of IRCCS Centro San Giovanni di Dio, Fatebenefratelli, Brescia, Italy, and written informed consent was obtained from all participants before the experiment.

### 2.2. Experimental design

Each participant took part in two experimental sessions, during which the subjects received either single TMS pulses at a random ITI of 0.18–0.4 Hz, with one pulse between 5.5 and 2.5 s (one pulse every 4 s on average – Experiment 1); or single TMS pulses at a fixed ITI of 0.25 Hz (one pulse every 4 s – Experiment 2). The two experimental sessions were conducted on different days. The schedule was maintained across participants to control for potential circadian effects (Sale et al., 2007). Corticospinal excitability was investigated by recording MEPs from the abductor pollicis brevis (APB) of the left hand. The choice to evaluate the motor cortex of the non-dominant hemisphere was determined using the initial experimental protocol, and these data represented the control condition. Fig. 1 shows the experimental protocol.

To obtain baseline measurements, each experimental session was initiated with a baseline MEP block, followed by 10 experimental blocks. In each block, 20 single TMS pulses were applied with an intensity of 120% of the resting motor threshold (RMT). A 100-s break separated each TMS block.

During the experiment, the participants were seated on a comfortable armchair in a shielded sound-proofed room. During MEP recordings, the participants were instructed to keep their hands completely relaxed, while passively sitting and fixing their eyes on the visual target located directly in front of them. Each experimental session lasted approximately 60 min.

### 2.3. Motor cortical excitability

TMS-elicited MEPs were recorded to measure the motor cortical excitability of the left APB representation area. Single-pulse TMS was performed using a Magstim Super Rapid magnetic stimulator (Magstim Company, Whitland, UK) and a standard figure-of-eight shaped coil with an outer winding diameter of 70 mm that generates 2.2 T as a maximum output. The current waveform was biphasic and posterior-anterior directed. The coil was placed tangentially on the scalp with the handle pointing backwards and laterally, approximately 45° from the midline. The stimulation started at supra-threshold intensity. The optimal stimulus site to elicit MEPs in the left APB was selected after positioning the coil approximately over the central sulcus and moving it along the scalp in 0.5 cm steps in the right primary motor area. On the motor hot spot, RMT was assessed as the lowest stimulus intensity required to produce a response of at least 50 μV in amplitude in the relaxed muscle for at least five out of ten consecutive stimulations, at a resolution of 1% of the maximal stimulator output (Rossini et al., 1994, 2015). A TMS neuronavigation system (SofTaxic, EMS, Bologna, Italy) was used to ensure a high degree of reproducibility across separate experimental sessions (Cincotta et al., 2010; Carducci and Brusco, 2012).

Surface electromyographic (EMG) activity was recorded from the left APB muscle (Brain Products GmbH, Munich, Germany), with the active electrode mounted on the belly of the muscle and the reference electrode placed over the base of the metacarpal-phalangeal joint. EMG activity was monitored throughout the experiment to ensure complete muscle relaxation. The EMG signal was acquired using a band-pass filter at 0.1–1000 Hz and digitised at a sampling rate of 1 kHz using a 16 bit A/D-converter. The skin/electrode impedance was maintained below 10 kΩ. The data were analysed off-line using BrainVision Analyzer software (Brain Products GmbH, Munich, Germany).

### 2.4. Corticospinal excitability analysis

Changes in corticospinal excitability induced through TMS were evaluated using MEP amplitudes as the dependent variable. Firstly, the continuous EMG signal was divided into epochs (from 400 ms before, to 400 ms after) for each TMS pulse and subsequently baseline corrected (50 ms before the TMS pulse). Epochs containing muscle artefacts were rejected (overall 5.5% of epochs). The

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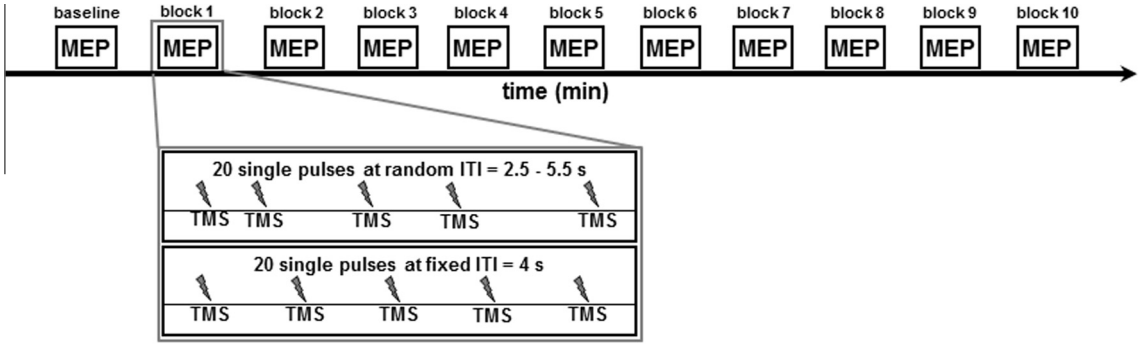


**Fig. 1.** Schematic representation of the experimental procedure. Two experimental sessions were performed: *Experiment 1* – single transcranial magnetic stimulation (TMS) pulses delivered at random inter trial intervals (ITIs) of 0.18–0.4 Hz (time range: 5.5–2.5 s); *Experiment 2* – single TMS pulses delivered at fixed ITIs of 0.25 Hz (every 4 s). Each experimental session was initiated with a baseline motor evoked potential (MEP) block, followed by 10 experimental blocks. In each block, 20 single TMS pulses were applied with an intensity of 120% of the resting motor threshold. A break of 100 s separated each TMS block.

amplitude of each MEP was measured peak-to-peak, and the mean value was calculated for the baseline and each experimental block. The RMT for the APB muscle was 60.3 ± 6.8 (mean ± standard deviation) of the maximum stimulator output. Within and between blocks analyses were performed on MEP amplitudes. Because the MEP amplitude is a reliable and physiologically relevant indicator of the effectiveness of a single TMS pulse and the primary outcome measure of TMS protocols (for a review see Ziemann et al., 2008), we focused on this measurement in the present study.

### 2.5. Statistical analysis

Before comparing the MEPs amplitude across conditions, we examined the Gaussian distribution of the MEPs. The mean, standard deviation and median of the distribution of all MEPs were 1413.4, 1076.5 and 1121.1 μV, respectively, showing an evident positive skewness. Considering the non-Gaussian distribution of the MEPs, confirmed using the Shapiro–Wilk test ($W = 0.89$, $p < 0.001$), the amplitude values were transformed using the Box-Cox transformation (Fox, 1997).

Subsequently, a three-way repeated measures ANOVA was conducted on the MEPs amplitude to examine the effect of the following factors: *ITI effect* (random and fixed), *inter-block effect* (11 levels) and *intra-block effect* (20 levels). Specifically, with regard to the large variability of MEP within each subject, we considered this aspect in the ANOVA model thus, the random effect was specified on individual subjects to control for subject-specific heterogeneity. Furthermore, considering the structure of the experimental design (10 experimental blocks with 20 single TMS pulses within each block), the random effects on the subjects were specified as nested random effects, i.e., the subject variability was modelled within the intra-block factor levels that, in turn, were nested within the inter-block levels. This design, in which each level of the inter-block factor is present in one level of the intra-block factor, facilitates the proper evaluation of the intra-block effect at different levels of inter-block factors, by splitting the variance error into different components: the inter-block variance was computed in relation to the general MEP average value, and the intra-block variance was computed in relation to the MEP average value within each inter-block (McDonald, 2009).

Moreover, we examined whether the differences in the ANOVA reflected a specific tendency of modulation using a trend analysis at both the inter-block and intra-block levels, which evaluated whether any trend could be detected. For this purpose, we performed a non-parametric smoothing spline analysis (Ramsay and Silverman, 2005) using the Akaike Information Criterion method for the selection of the smoothing parameters. The smoothing spline method estimates the trend curves based on a flexible data-driven procedure that, free of any a priori specification of the type of relationship (e.g., linear, quadratic, cubic) facilitated the detection of the true type of relationship between variables.

Subsequently, based on spline results, we applied the best fitting regression model to the data to confirm the type of trend observed from spline curves and evaluated the slope of the detected trend. To determine the trend type, linear models were applied to the estimated spline function values as dependent variables and time points (from 1 to 11 for inter-block or from 1 to 20 for intra-block) as independent variables. To evaluate the slope of the detected trend, linear models, inter and intra-block MEP values (as the dependent variable) and time points (as the independent variable) were used. The goodness of fit of the regression models was evaluated using the coefficient of determination, $R^2$.

All statistical analyses were conducted using *R*: A language and environment for statistical computing, version 3.03 (R Core Team, 2013; R foundation for Statistical Computing, Wien; www.r-project.org). The level of statistical significance was set at $p < 0.05$.

## 3. Results

The three-way repeated measures ANOVA showed significant effects of the *intra-block effect* ($F_{19,4307} = 3.59$, $p < 0.001$) and of the *inter-block effect* ($F_{1090} = 4.83$, $p < 0.001$) on MEPs amplitude. No significant effect was observed for the ITI effect ($F_{111} = 0.26$, $p = 0.619$) or for the interactions (all $p > 0.05$).

Post-hoc analyses (Fisher's LSD correction) on the inter-block effects showed that the MEPs in the blocks from 2 to 11 were larger than the MEPs in the baseline block, and five of these differences were significant (block 2, block 3, block 6, block 8, $p < 0.050$, and block 11 $p < 0.010$). Similarly, the MEP amplitude increased from the first to the last stimulation within blocks, i.e., intra-block effect (Fig. 2). Therefore, the repeated delivery of single TMS pulses changed the cortical excitability both within the same block of stimulation and between consecutive blocks.

The results of the trend analyses further supported the effect of a single TMS pulse. The estimated smoothing spline curves revealed substantial linear trends for both inter-block and intra-block levels (Fig. 3). Specifically, the application of linear regression models generated perfectly linear trends ($R^2 = 1$) for inter-block levels at both ITI sessions, and nearly linear trends were observed for intra-block levels at random ($R^2 = 0.97$) and fixed ($R^2 = 0.99$) ITI sessions.

The evaluation of the strength of these tendencies was estimated as the slope coefficients of the linear regression models, showing results of 0.03 ($p = 0.008$) and 0.05 ($p < 0.001$) for intra-block levels at random and fixed ITI sessions, respectively, and

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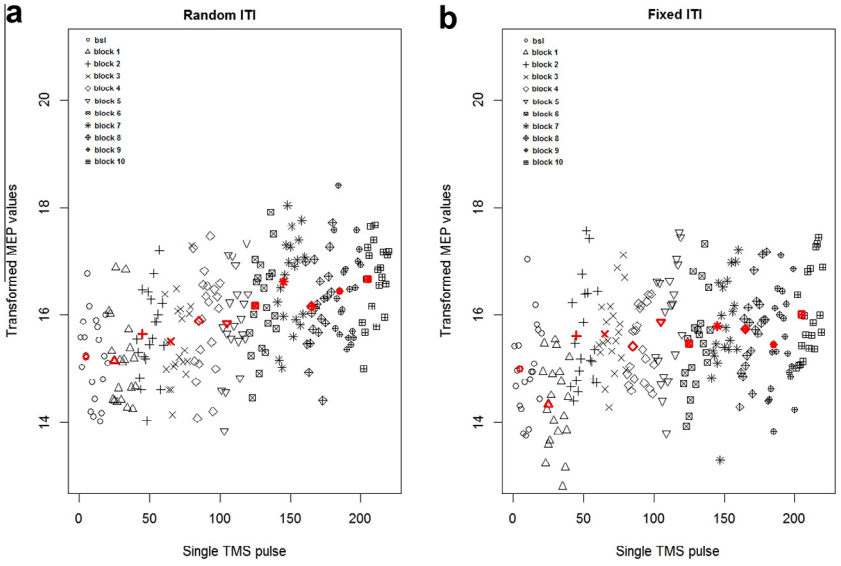


**Fig. 2.** Intra-block mean MEP values of all subjects in the baseline block and for each experimental block. (a) Random and (b) fixed ITIs. The mean MEP value for each block is indicated in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

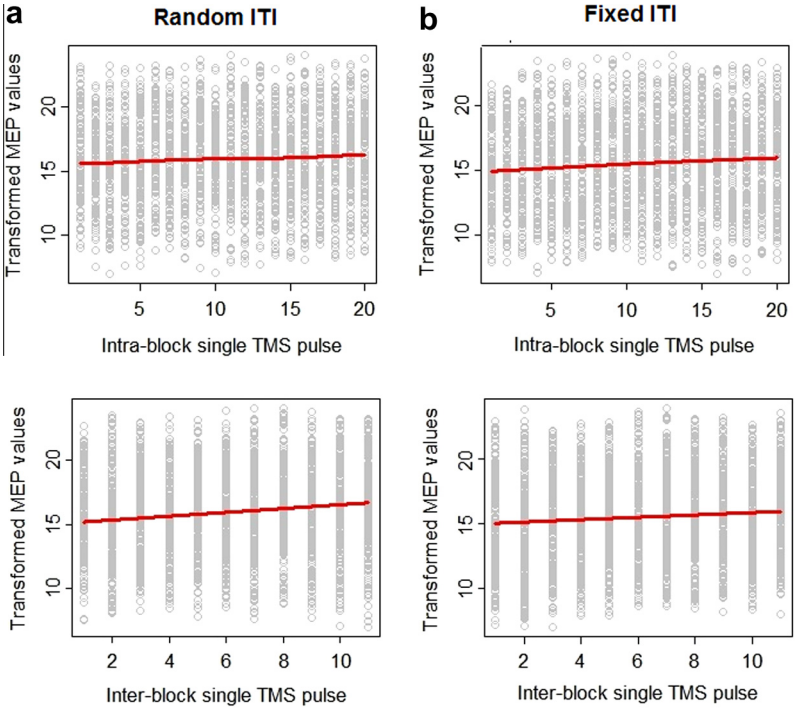


**Fig. 3.** Scatter plot and estimated smoothing-spline curves of single MEP values for intra- and inter-block effects. (a) Random and (b) fixed ITIs. In both cases, the curves (solid red lines) show positive linear trends across single TMS pulses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.15 ($p < 0.001$) and 0.09 ($p < 0.001$) for inter-block levels at random and fixed ITI sessions (Fig. 4). Furthermore, the slope coefficients of random and fixed ITI sessions of intra-block levels were significantly different ($p = 0.051$), whereas no significant difference in the slope coefficient between ITI conditions in the inter-block ($p = 0.058$) were observed.

## 4. Discussion

In the present study, we reported that the application of single TMS pulses at low frequency of stimulation, a protocol that is commonly used to evaluate motor cortical excitability, induced changes in corticospinal excitability independently of the temporal pattern of the TMS pulses (random vs. fixed ITI). Specifically, the proper evaluation of the changes in MEPs, evaluated through repeated measure ANOVA with nested random effects, showed an amplitude increase in both inter and intra-stimulation blocks. These results were strongly supported by the linear trend analysis showing that the delivery of successive TMS pulses significantly changed both intra-block and inter-block cortical excitability.

Assuming that the magnitude of the corticospinal response, evaluated as the MEP amplitude, depends on the intrinsic

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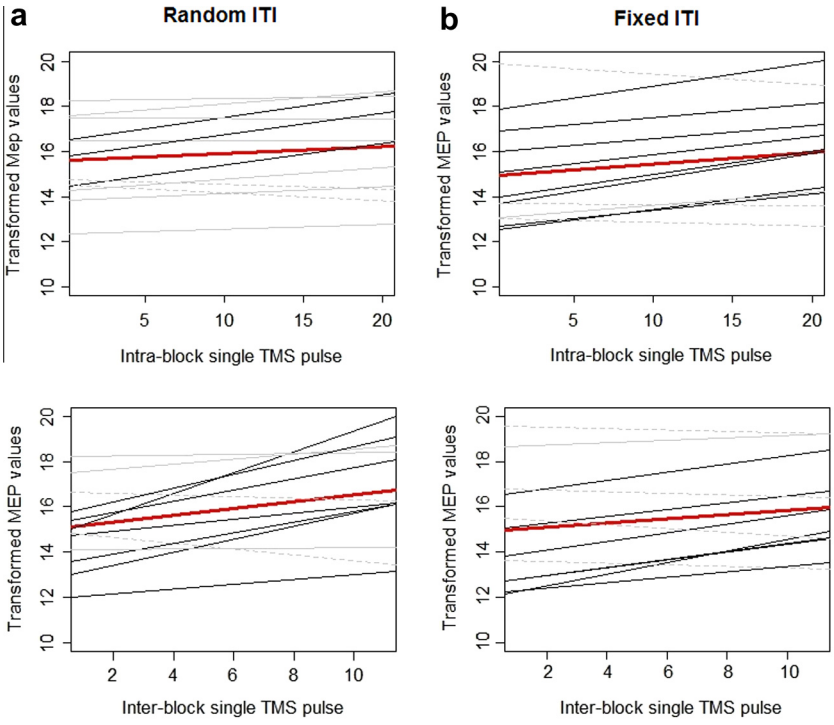


**Fig. 4.** Linear trends analysis of the MEP values for the intra- and inter-block effects observed from each subject. (a) Random and (b) fixed ITIs. The red lines represent the trends for overall subjects, computed as the means of the 12 regression lines. The black and grey lines indicate single subject trends: the black lines refer to subjects with significantly positive trends; the grey lines refer to subjects with positive trends that were not significant; and the dashed grey lines refer to subjects with negative trends that were not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

excitability of the neurons in the motor pathway, we hypothesised that the sequential delivery of single TMS pulses modulated corticospinal excitability, depolarising the potential of the membrane, resulting in an increase in the firing rate with a consequent increase in the neuronal activation (Julkunen et al., 2012). The slope increase observed in the present study could reflect the summation of the neuronal depolarisation induced over time through several single TMS pulses. A potential explanation could be that the cumulative effect of several single TMS pulses induces a charge accumulation in the stimulated neuronal tissue. A single TMS pulse could prime subsequent pulses by priming background excitability toward an increase in excitability. Thus, the effects induced through several TMS pulses were additive, resulting in a greater change in neural activity than obtained with a single pulse. These results are consistent with the cumulative modulation induced through the prolonged application of single-pulse TMS on neurodynamic parameters, encoded in terms of cortical EEG (Stamoulis et al., 2011).

Neurophysiological evidence (Ilmoniemi et al., 1997; Noguchi et al., 2003; Allen et al., 2007; Bestmann, 2008), and a recent near-infrared spectroscopy study, highlighting a rapid haemoglobin concentration increase up to 3–6 s after single-pulse TMS (Furubayashi et al., 2013), supports our finding that a single TMS pulse modulated neuronal activation. Specifically, the application of a single TMS pulse with an inter-stimulus interval of approximately 4 s, as in our fixed and random protocols, repetitively depolarising the cortico-spinal tract neurons and modulating the cortical activation pattern, could have determined the final increase of corticospinal excitability. Evidence that a single TMS pulse induces an activation peak in a specific time window after single-pulse TMS has also been reported through the time-course analysis of functional magnetic resonance imaging signals (Bohning et al., 1999; Shitara et al., 2011). These data suggest that the TMS, eliciting neural and haemodynamic changes, although for short-term after the pulse, could itself produce phenomena associated with the induction of spike timing-dependent modulation (for a review see Feldman, 2012). Therefore, a potential mechanism underlying the observed MEP changes could be the continuous elevation of neural activity induced through sequential TMS pulses (Allen et al., 2007) that promote a corticospinal excitability modulation by reducing the synaptic firing threshold. Thus, repeated single TMS pulses might depolarise the neuronal membrane and induce the pre-activation of motor cortical synapses, determining an increase in the peripheral outcome. A potential mechanism underlying this corticospinal increase was identified as a short-term synaptic enhancement. However, considering that the MEP amplitude reflects the status of excitatory horizontal axons, corticospinal neurons, spinal motoneurons, and muscle fibres and the efficiency of the excitatory synapses connecting these structures, the increase of this motor outcome could highlight a general change in the motor pathway. Therefore, these findings cannot discriminate whether TMS-induced modulation is determined through spinal circuitry changes controlled via cortico-spinal tract axons or exclusively through cortical interneurons networks, representing a limitation of the present study.

Further support for the obtained results was provided in a study on cortical cellular actions induced through a single TMS pulse (Moliadze et al., 2003). During spontaneous activity, the TMS induces excitatory activity during the first 500 m after the pulse, followed by the inhibition and subsequent enhanced activity associated with spontaneous fluctuations in activity via burst-like discharges (Funke and Benali, 2010). A similar mechanism might explain the present data, during both fixed and random ITIs of TMS pulses, in which pulses delivered during rebound excitation might increase the corticospinal outcome. Once the depolarising threshold was exceeded, the excitatory processes would become predominant and subsequently depolarise a larger number of long projecting neurons (Hanakawa et al., 2009), determining an

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increase in the corticospinal outcome and vice versa. Thus, depending on the time between two TMS pulses, we might induce excitatory, or in some cases inhibitory, effects on cortical excitability. Moreover, regarding the effects observed between blocks, it is possible that the neuronal recruitment in the first block could affect recruitment in the subsequent block, determining the carry-over effects on cortical excitability, evaluated in terms of short-term after effects.

These results provide further evidence that a single TMS pulse affects neural activity, influencing the background activity in the targeted region, compatible with an activity-dependent model of plasticity. Albeit, these data do not support the involvement of timing-dependent plasticity, as the excitability changes were not temporal resolution-specific, with modulation effects observable in both, random and fixed ITI conditions.

A limitation of the study is that two very short ITIs were examined. A possible extension to this study that would be useful to pursue is the examination of the effects induced by TMS pulses delivered at longer ITIs, though a recent study has demonstrated that longer ITI (10 s) does not affect cortical excitability in terms of MEPs amplitude changes (Julkunen et al., 2012). However, the primary goal of our study was to investigate the difference between random and fixed ITIs on the ongoing effects of single TMS pulses on corticospinal excitability. In this regard, the independence of our results by ITI conditions, and more specifically the corticospinal modulations observable during the single TMS pulses delivered both at random and at fixed intervals, allow us to exclude the involvement of additional and non-specific factors such as habituation or anticipation to TMS pulses.

Finally, the current study might have been improved by the inclusion of a sham condition, to exclude non-specific effects, e.g., the sound of TMS pulse that could affect the arousal level of the subjects. Nevertheless, if arousal was an issue such effect should have been present mainly in the first part of the experiment and not towards the end, with an corticospinal excitability increase observed only during the first blocks followed by a decrease of MEPs amplitude, instead of the linear corticospinal excitability increase highlighted in our study.

Moreover, we investigated the expression of a transient alteration in the efficacy of existing synapses in terms of on-line changes, and only further studies with longer lasting evaluation of corticospinal excitability could evaluate the potential involvement of plasticity mechanisms.

Although indirectly, an increase in MEP amplitude during the delivery of several single TMS pulses is supported through the results observed during a control/sham stimulation condition (Chaieb et al., 2011). However, particular attention is required when TMS is applied as a tool for corticospinal excitability evaluation (Bailey et al., 2014). The findings obtained in the present study are partially consistent with the findings of Julkunen et al. (2012), showing that individual single-trial MEP amplitudes were strongly affected through both ITIs and the response block number during the stimulation train. Another recent study, in which a random interval between TMS stimuli was used, reported a significant change in the slope over time in some participants (using a simple linear regression analysis) (Cuypers et al., 2014). In the present study, we focused on this result, highlighting a linear increase in the cortical excitability in the majority of subjects.

The increase in the corticospinal excitability could represent a further variable that should be considered in the assessment of corticospinal activity at rest. Therefore, particular attention should be taken when several MEPs are recorded in research and clinical settings for diagnostic and therapeutic purposes.

A methodological issue should be considered as a possible limitation of the present study. We used a biphasic TMS pulse delivered with a posterior-anterior directed current in the brain, which induces at least two currents of inverse direction, leading to the transient depolarisation of corticospinal cells sufficient for triggering neuronal spikes (Arai et al., 2005). The application of biphasic TMS pulse, inducing a more complex cortical pattern and activating several types of motor cortical outputs, reflecting a longer waveform duration and higher stimulus pulse amplitude (Di Lazzaro et al., 2001; Sommer et al., 2006), could result in a more powerful stimulation, thereby eliciting neuronal activation (Pell et al., 2011). Thus, the repetitive activation of cortical neurons induced through a single biphasic TMS pulse could result in a major cumulative activation with consequent corticospinal modulation. Further studies evaluating the cumulative effects of monophasic single pulses on cortical excitability are needed to understand the effect of TMS pulse morphology on motor response (Delvendahl et al., 2014).

To explain our results a relevant point to consider is the role of the TMS frequency. In our study, a very low frequency of stimulation (0.25 Hz) has been applied, compared to the 1 Hz frequency often employed in rTMS protocols. By applying this protocol we found an effect that it is not in line with the motor cortex excitability inhibition, usually observed after low frequency rTMS (Chen et al., 1997). We hypothesise that this discrepancy could be determined by a complex interaction between multiple factors. First, the effects of rTMS on motor cortex excitability can vary depending on the stimulation frequency, ranging from stable MEP amplitude with a stimulation frequency of 0.1 Hz to a decrease at 0.9 Hz stimulation (Chen et al., 1997). The second factor is the intensity of stimulation. Inhibitory effects can be observed after low frequency repetitive stimulation at a relatively low intensity (90% RMT), whereas contradictory effects have been found with suprathreshold intensities (Pal et al., 2005; Heide et al., 2006; Daskalakis et al., 2006), as used in our study. Finally, the number of the applied pulses should be considered, with long term depression phenomena observable only after long periods of stimulation (600–1500 pulses) (for review see Hoogendam et al., 2010; Pell et al., 2011). Notwithstanding, our results are partially in agreement with previous studies that reported cortical excitability changes within stimulation train at low frequency (for a review, see Fitzgerald et al. (2006)).

In the present study, the TMS was delivered not in a standard continuous protocol, but in a block design, suggesting that breaks during the stimulation could have determined the observed effect (Rothkegel et al., 2010). Further studies comparing the ongoing cortical excitability during a continuous or block protocol could determine the functional relevance of the break.

The results could also be partially explained through an increase in the MEP size following several minutes of hand inactivity (Todd et al., 2006). This aspect might have partially contributed to the observed changes, although we hypothesised that the final outcome observed in terms of cortical excitability change substantially resulted from the experimental intervention itself, depending not only on the number of TMS stimuli applied but also by the continuous delivery of these events.

Reliable MEP amplitudes have principally been reported with not more than an average of ten MEP responses per blocks, although moderate reliability was also obtained with five MEPs per block (Kamen, 2004; Christie et al., 2007; Doeltgen et al., 2009; Bastani and Jaberzadeh, 2012). However, most of these studies used twenty trials to evaluate the mean MEP amplitude in multiple independent sessions. Based on these results, studies that include the recording of more MEPs per block and a large number of blocks for data collection should consider the cumulative effects of single TMS pulses and the ITIs used. The common use of the average responses for a block could conceal the observed trend in individual trials, both within and between blocks.

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## 5. Conclusions

Resting MEP amplitude has been used to provide an index of baseline corticospinal responsiveness, representing the default characteristic of the pathway from motor cortex to muscle (Carroll et al., 2011). Although the Guidelines of the International Federation of Clinical Neurophysiology (Rothwell et al., 1999) suggested that the optimal stimulation rate should be more than 3 s between consecutive stimuli, the corticospinal excitability increase observed in these experiments indicates that the use of this protocol could represent an important experimental variable that should be considered in the assessment of corticospinal activity at rest. High or low frequency TMS paradigms are typically used to induce specific synaptic modulations between cortical neurons. However, herein, we report the first use of a single TMS pulse protocol to induce cortical excitability modulation during the same stimulation.

## Conflict of interest

Nothing to declare.

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