Brain Stimulation xxx (2014) 1–7

Contents lists available at ScienceDirect

Brain Stimulation

journal homepage: www.brainstimjrnl.com

Original Research

Jitter of Corticospinal Neurons During Repetitive Transcranial Magnetic Stimulation. Method and Possible Clinical Implications

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ARTICLE INFO

Article history:
Received 7 November 2013
Received in revised form
8 April 2014
Accepted 1 May 2014
Available online xxx

Keywords:
Transcranial magnetic stimulation
Jitter
Single fiber
Motor tract
Evoked potential

ABSTRACT

Background: Repetitive transcranial magnetic stimulation (rTMS) of the motor cortex activates corticospinal neurons mainly through the depolarization of cortico-cortical axons belonging to interneurons of superficial layers.
Objective: We used single-fiber electromyography (SFEMG) to estimate the "central jitter" of activation latency of interneural pools from one pulse of TMS to another.
Methods: We evaluated 10 healthy subjects and one patient with multiple sclerosis. By recording SFEMG evoked activity from the left first dorsal interosseous (FDI), we first used a standard repetitive electrical 3 Hz stimulation of the ulnar nerve at the wrist to calculate the mean consecutive difference from at least 10 different potentials. The same procedure was applied during 3 Hz repetitive TMS of the contralateral motor cortex. The corticospinal monosynaptic connection of the FDI and the selectivity of SFEMG recording physiologically justified the subtraction of the "peripheral jitter" from the whole cortico-muscular jitter, obtaining an estimation of the actual "central jitter."
Results: All subjects completed the study. The peripheral jitter was 28 μs ± 6 and the cortico-muscular jitter was 344 μs ± 97. The estimated central jitter was 343 ± 97 μs. In the patient the central jitter was 846 μs, a value more than twice the central jitter in healthy subjects.
Conclusion: Current results demonstrate that the evaluation of the central component of the cumulative cortico-muscular latency variability in healthy subjects is feasible with a minimally invasive approach. We present and discuss this methodology and provide a "proof of concept" of its potential clinical applicability in a patient with multiple sclerosis.

Introduction

Transcranial magnetic stimulation (TMS) [1] is a tool of choice to study noninvasively the functionality of the corticospinal pathway in the intact human [2–5]. Using a near-threshold intensity of stimulation, each pulse of TMS activates corticospinal neurons trans-synaptically, through the firing of cortico-cortical axons belonging to interneurons of superficial cortical layers [6–8]. A spatio-temporal summation of their excitatory post-synaptic potentials is necessary to permit corticospinal motoneurones (MNs) to discharge. The evoked descending volleys are recordable by epidurally implanted electrodes at spinal level [repeated indirect (I)-waves at near-threshold stimulation and an early direct (D)-wave following high-intensity TMS] [9]. The temporal summation of these waves along the various corticospinal fibers impinging upon each individual spinal MN generates the related motor evoked potential (MEP), which is recordable from contralateral target muscles.

Pietro Caliandro and Simone Rossi contributed equally to this work.

Disclosure of financial interests and potential conflicts of interest: This work has been partially supported by the European Commission with the Collaborative Project no. 248587, "THE Hand Embodied," within the FP7-ICT-2009-4-2-1 program "Cognitive Systems and Robotics."

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1935-861X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.brs.2014.05.001

Epidural recordings provided important advances in understanding the physiology of brain activation following TMS, although they have been carried out only in patients requiring invasive therapeutic implants (i.e., mostly for chronic pain relief) or monitoring recordings during spinal neurosurgery, rather than in healthy subjects or other kind of patients. Therefore, several physiological questions still remain open: these are linked with the many possible interactions between the currents induced in the brain by TMS pulses and the complexity of cortical and/or spinal neural circuits. Indeed, these are composed, besides corticospinal output neurons, of both excitatory and inhibitory networks [10–12] including cell bodies and axons of different size, location, orientation and function [13]. Finally, differences in nervous impulse propagation along corticospinal tracts of different diameter and conduction properties should also be considered.

We aimed to investigate TMS physiology in healthy subjects with a method, applicable also in patients to get insights into corticospinal pathophysiological function. We reasoned that recording the TMS-evoked electromyographic activity by single muscle fibers, thanks to the exclusive relationship that each single muscle fiber has with a given motoneuron, might offer a better physiological window of cortical physiology than a surface recorded MEP, which includes a submaximal compound potential activity [14,15], unless complex and time-consuming collision techniques are used, as the triple stimulation technique [15].

To this aim, we developed a method combining single-fiber electromyography (SFEMG) to evaluate the neuro-muscular jitter occurring after stimulation of the peripheral nerve at 3 Hz (s-SFEMG) and the repetitive TMS (rTMS), also at 3 Hz, of the contralateral motor cortex (cortico-muscular jitter). We defined "cortico-muscular jitter" the jitter occurring after rTMS and peripheral jitter the jitter generated at the end-plate after peripheral nerve stimulation. Through the subtraction of the peripheral jitter from the whole cortico-muscular jitter, we estimated the component of the cortico-muscular jitter due to central mechanisms rather than to end-plate transmission. We used the expression "central jitter" to refer to the central component of the cumulative cortico-muscular jitter.

Previous studies have already investigated the jitter of corticospinal neurons following transcranial magnetic [16–19] and electric single-pulse stimulation [16,20,21] in healthy humans and in some patients with neurological disorders [17,18], although most of these studies used single motor unit estimation rather than SFEMG recordings [17–19,21]. They provided evidence of predominantly monosynaptic transmission of the descending volley at the spinal level, and of occurrence of jitter mainly in spinal neuron when using electric transcranial stimulation [16,20]. Moreover, Zarola and colleagues provided an elegant experimental evidence for the transsynaptic activation of corticospinal neurons following single-pulse TMS using a circular coil [16].

We originally hypothesized that jitter is taking place also following rTMS, both in healthy subjects and neurological patients. Therefore, we verified the feasibility of a new method to calculate exclusively the central component of the cortico-muscular jitter. This last issue is not negligible if we consider that end-plate transmission may account for a great variability of the cortico-muscular jitter mainly in patients with peripheral nerve damage. Here we introduce this new methodology and provide an applicative example in a patient with multiple sclerosis (MS).

Methods

Ten healthy fully right-handed subjects (5 females, 5 males; mean age 28.5, range 23–34 years), all volunteers, naïve to the purpose of the experiment, were included after the approval of the procedure by the Ethical Committee of the participating Institutes. All were neurologically normal and denied the use of drugs or alcohol in the days preceding the experiment.

The protocol was also carried out on a patient (male, 24 years old) suffering for four years from a relapsing-remitting multiple sclerosis (MS). He was currently treated with Natalizumab at standard dose and timing (300 mg administered monthly) for two years, without side effects. His neurological examination at the time of the neurophysiological evaluation showed: nystagmus in all gaze directions and bilateral slight dysmetria; paraparetic gait (but he was able to walk without help for about 500 m) with bilateral Babinski sign; weakness in his right upper arm. Tetrahyperreflexia, prevailing in the right side, with clonus in his right lower foot. Expanded Disability Status Scale (EDSS) [22] score was 4. He also complained of severe fatigue, indexed by a score of 5 at the Fatigue Severity Scale (FSS) [23]. Upper motor function as assessed with NineHole Peg Test [24], were symmetrical (left hand: 28.5 s; right hand 26 s). At neurophysiological examinations, he had a normal central motor conduction time (measured with the standard "F-wave" method) for the left hand (6.3 ms) and a slightly increased central motor conduction time for the right hand (7.2 ms) and bilaterally for the lower limbs (19.8 ms and 20.5 ms). The magnetic resonance, which excluded gadolinium-enhanced acute brain and spinal lesions at the time of neurophysiological testing, showed multiple bilateral lesions in the subcortical white matter, in the pons in the posterior third of the corpus callosum and in the left cerebellar hemisphere.

Healthy subjects and the patient gave a written informed consent to the study, after being instructed that they could interrupt the recording session whenever they wanted. Subjects set comfortably in a reclining chair, keeping their arm fully relaxed and their hands pronated on a support providing a fully natural position.

Procedures of recording and peripheral stimulation

A four-channel Synergy, Medelec electromyography version 11.1 was used for all recordings. The software for stimulated SFEMG provided by the manufacturer was used to analyze single-fiber muscle responses. A bipolar surface electrical stimulator (cathode in distal position and anode proximal, inter-electrode distance 2.2 cm) was used to stimulate the left ulnar nerve at the wrist. The stimulation producing the greatest amplitude of the conventional Compound Motor Action Potential (CMAP) recorded from the left first dorsal interosseous (FDI) muscle was first determined for each subject (silver disc electrodes of 0.99 cm in diameter were used). Filter settings were 3 Hz–10 kHz. We then used a 3 Hz repetitive nerve stimulation (RNS) with a supramaximal stimulus, 15% greater than the stimulation intensity producing the maximal CMAP amplitude and recorded from FDI by an SFEMG needle electrode. Each train of RNS was composed by 100 pulses (pulse duration was 0.1 ms).

The SFEMG needle is a specially constructed concentric needle electrode used to record action potentials in individual muscle fibers. The features of the SFEMG technique result from the small recording surface of the needle (25 microns in diameter) [25]. During SFEMG recordings, filters were set at 2 kHz (high-pass) and 10 kHz (low-pass) [26] both during electrical stimulation and rTMS. In each single subject, both during peripheral and cortical stimulation, we recorded 10 single-fiber potentials each from a different site of registration in the FDI muscle, and we analyzed at least 50 stimuli for each single-fiber. The recording sites were changed by slight movements of the needle without necessity of multiple insertions in the muscle. The criteria used for an acceptable recording were: sharp, spiky, and fast rise time; only potentials with a rise time of <0.3 ms and an amplitude of >200 μV were accepted for analysis. The jitter was measured at the rise phase of the potentials.

For each site of recording, we analyzed only potentials with the constant shape. The jitter was calculated as the mean consecutive difference (MCD) for each single-fiber potential using the standard software provided by the manufacturer. Moreover, we calculated the mean MCD (mMCD) for the overall 10 single-fiber potentials for both types of stimulus (i.e., peripheral and cortical). We first recorded 10 end-plate potentials during electrical RNS and then we collected 10 single-fiber potential after rTMS. In order to match the relative non-selectivity of stimulation of rTMS with the peripheral activation of axons, we decided to stimulate the ulnar nerve by surface stimulator, rather than with a near-nerve technique.

Procedures of brain stimulation and neuronavigation

A standard eight-shaped focal coil connected with a biphasic magnetic stimulator (SuperRapid, Magstim, Whitland, UK), with 2.0 T as maximal output, was used for rTMS and for searching the individual threshold of stimulation, defined according to International standards [27] on the "hot spot" for the left FDI muscle. The choice of the FDI muscle was motivated by the fact that the corticospinal pyramidal neurons to hand intrinsic muscles establish monosynaptic connections with the spinal motoneuron [28].

The hot spot was marked on the scalp to allow the same coil positioning during the experiments. Throughout the experiment, the coil was positioned on the right hemiscalp hot spot, with the handle pointing backwards and at about 45° from the midline. It was fixed in that position with a mechanical arm, and an experimenter checked continuously its stability. A navigated stimulation system [SofTaxic optically-tracked (EMS, Italy)] was also used in three of the subjects. This system allowed the exact repositioning of the TMS coil within and across experimental sessions, thus minimizing the variability of corticospinal output induced by each TMS pulse. The software uses passive spherical markers applied both on the coil and on the subjects' head. Marker positions were recorded by an optical digitizer (Polaris Vicra, NDI, Canada) and reproduced on the computer screen which provided three dimensional online information on the initial and actual coil placements, by displaying any difference in spatial coil location and orientation (three rotation angles) respect to the initial pulse, with a tolerance of less than 2 mm for each dimension [29]. Such a procedure minimizes the variability of TMS-induced electric fields directly measured within a scalp model [30].

Once the individual resting excitability threshold was defined, the intensity of stimulation was increased by about 10%–20%, and rTMS at 3 Hz was used to evaluate the cortico-muscular jitter. The intensity of stimulation was different among subjects in order to achieve the highest possible stability (different from one subject to another) for the SFEMG potentials, while the not standard rTMS stimulation frequency was used to match the timing of central stimulation with the well-established frequency for the repetitive electrical stimulation of the nerve. The magnetic stimulator triggered simultaneously both the electromyograph used for SFEMG recordings and the one used for safety reasons (see later). Each train of rTMS lasted no more than 60 s (180 pulses). Each subject underwent a maximum of ten 60-s trains (1800 pulses). The inter-train interval was at least 3 min. Such a relatively long trains of rTMS were necessary to collect a sufficient number of single-fiber motor evoked potentials (at least 500 valid pulses, corresponding to 50 SFEMG MEPs for each muscular fiber) to compute a statistically reliable jitter. Then, we calculated the MCD of the latencies of each SFEMG MEP.

Safety aspects

It is worth noting that the combination of intensity, frequency, and number of pulses used here is not included in the last available version of the safety guidelines for TMS use in clinical practice and research, which lacks information in the range of stimulation between >1 Hz and <5 Hz [31]. Therefore, a strict monitoring of subjects was necessary: rTMS was stopped whenever required by the subject or in case of spread of excitation at cortical level, as revealed by a couple of surface electrodes placed on the left biceps and deltoid muscles. To this purpose, an additional 4-channel electromyograph (Phasis, Esa-Ote Biomedica, Florence, Italy) was used. Monitoring the appearance of MEPs in a proximal muscle when the coil is placed on the optimal position ("hot spot") to elicit hand muscle twitches, is considered the best warning toward the occurrence of an epileptic seizure [31].

In addition, at experimental debriefing subjects were required to list eventual side effects and to rate the discomfort of the whole procedure.

Estimation of the "central jitter" and data analysis

The described procedure including the peripheral study, neuronavigation and rTMS sessions lasted about 1 h. Single-fiber muscle responses, obtained either by peripheral or cortical stimulation, were stored on the hard disk of the electromyograph (a four-channel Synergy, Medelec) and analyzed off-line. The criteria used for an acceptable recording were: sharp, spiky, and fast rise time. We analyzed only potentials with constant shape. After calculation of the mMCD for both peripheral and cortical stimuli in each subject, the cortico-muscular mMCD and the peripheral mMCD were compared by Mann-Whitney U-test. The level of significance was set at $P < 0.05$. Since the jitter is mathematically a standard deviation value, it represents the square root of the mean of the squared deviations of the observed values from their mean, so the central jitter (expressed in μs) must be estimated by the following formula: $\sqrt{(\text{cortico-muscular jitter}^2 - \text{peripheral jitter}^2)}$ and not by a simple difference between cortico-muscular and peripheral jitter.

Results

At experimental debriefing, half of the subjects experienced minor side effects, mainly concerning discomfort due to local pain at the point of insertion of the needle in the muscle. Despite the relatively high intensity of stimulation and the length of the rTMS trains (see methods) all subjects completed the study; four of them complained of slight, transient aching at the point of scalp stimulation.

In one of the subjects, a spread of excitation at cortical level was detected by the appearance of stable MEPs from the biceps and deltoid muscles, despite the targeting of the hot spot for the FDI muscle. This occurred toward the end of the session, when a sufficient number of SFEMG MEPs (about 450) had been already collected. Therefore, data from this subject (subject C of Table 1, a 30 year old female) have been included in the analysis. However, rTMS was immediately stopped in order to prevent the eventual occurrence of a seizure. The subject did not report any complication thereafter.

Table 1 shows the mMCD values after peripheral and cortical repetitive stimulation, and the difference between the two values for each subject (i.e., the estimated central jitter). The cortico-muscular jitter was significantly higher than the peripheral jitter ($P < 0.001$, Mann–Whitney U-test). In the overall sample, the mean peripheral jitter was 28 μs ± 6 and the mean cortico-muscular jitter was 344 μs ± 97. The mean estimated central jitter was 343 ± 97 μs.

Figure 1 shows SFEMG potentials recorded after electrical stimulation of the nerve and Fig. 2 shows SFEMG potentials after rTMS of the brain in a healthy subject. Moreover, the figures show the histograms of the discharge latencies. As demonstrated in the

Table 1
Peripheral, cortico-muscular and estimated central jitter in the ten normal subjects.

Subjects	Peripheral jitter (mMCD in μs)	Cortico-muscular jitter (mMCD in μs)	Central jitter (mMCD in μs)
A	29	218	216
B	30	312	310
C	39	553	552
D	20	309	308
E	33	368	366
F	32	257	255
G	28	356	355
H	24	381	381
I	23	423	423
L	20	266	265

Fig. 2, the distribution of latencies is generally bi-modal after rTMS; we rarely observed one cluster of latencies. The repetitive nerve stimulations generate one cluster of latencies (Fig. 1).

The technique was also tested in a patient suffering from a relapsing-remitting form of multiple sclerosis (MS). Figure 3 shows that the cortico-muscular jitter in this patient (mMCD: 874.32 μs) was definitely higher than that corresponding to the upper limit observed in healthy subjects (mMCD: 553 μs) (Table 1). The same applies to the estimation of the "central jitter": in the patient it was 846 μs ($\sqrt{([\text{cortico-muscular } 875\ \mu s]^2 - [\text{peripheral } 28\ \mu s]^2)}$), a value more than twice the mean central jitter in healthy subjects (about 340 μs), in which the highest value was 552 μs (Table 1). The mMCD during peripheral nerve stimulation in the patient (22 μs) was comparable to that found in healthy subjects (28 ± 6 μs). It is worth noting that in the patient up to 4 clusters of latencies appeared. Most salient clinical features (see Methods section for additional clinical details) of the patient were the presence of a slight right hemiparesis, nystagmus in all gaze directions, marked fatigue (Fatigue Severity Scale: 5) and current therapy with Natalizumab at standard dosage. However, neurophysiological recordings were carried out from the left FDI muscle, which had a normal value of the central motor conduction from the cortex to the spinal cord (i.e., central conduction time 6.3 ms).

Discussion

Current results demonstrate that the evaluation of the central component of the cumulative cortico-muscular latency variability in healthy subjects is feasible with a minimally invasive approach, which is limited to the insertion of an SFEMG needle in an intrinsic hand muscle. To achieve this goal, we took advantage of an extremely selective recording (i.e., from single muscles fibers) associated to a relatively non-selective peripheral and cortical stimulation. The selectivity of recording of the SFEMG needle and the monosynaptic cortico-motoneuronal connection at spinal level for the target FDI muscle (Ghez [28]) represent the physiological background allowing this procedure.

The obtained central jitter could theoretically be generated in the cortex, along the corticospinal fibers and/or in the spinal neuron. Taking into account that near-threshold TMS excites axons lying in superficial layers of the cortex, mainly belonging to excitatory and inhibitory interneurons [6,10–13,32], a first likely explanation accounting for the central jitter is a different timing of recruitment of disparate interneural pools fired from one TMS stimulus to another, conveying their not completely synchronous inputs on the corticospinal neurons as a final common pathway. We have also to consider the possibility that multiple I-waves descending in the corticospinal tract evoke separate, but summating excitatory post-synaptic potentials in the spinal MN and that the latency of initiation of a discharge at the spinal MN, and therefore the latency of the SF discharges, will vary depending on the excitability of the MN itself. This hypothesis is supported by the bi-modal distribution of latencies of the SFEMG potentials recorded after magnetic brain stimulations [10,19].

Additional mechanisms possibly contributing to central jitter should be considered. First, because of the convergence of many corticospinal axons on a single spinal motoneuron [33], asynchronous

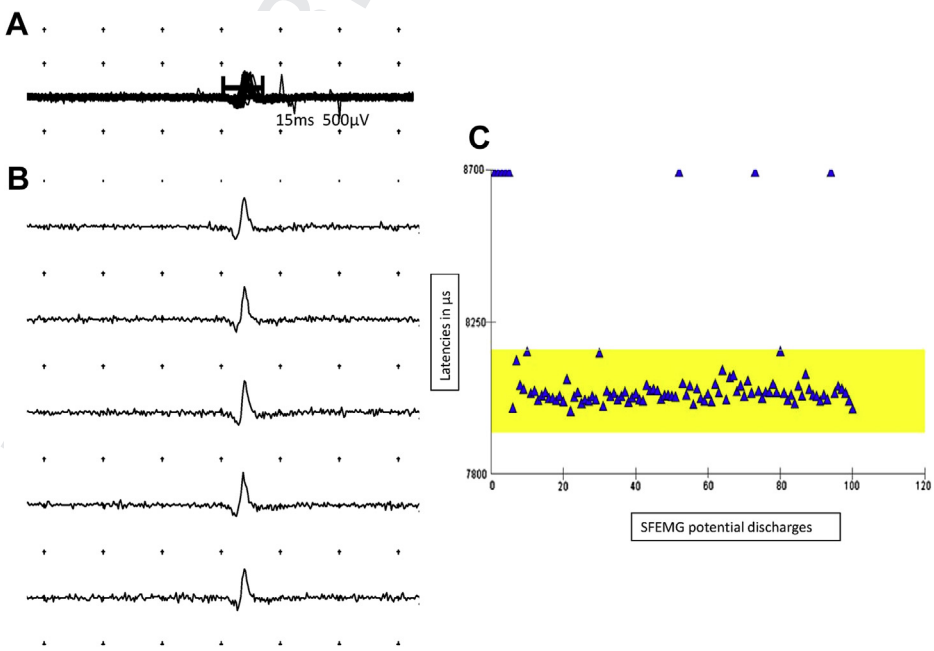

Figure 1. An SFEMG potential after electrical nerve repetitive stimulations and the consecutive discharge latencies in a healthy subject. Panel A: superimposed discharges of an SFEMG potential. Panel B: a magnification of individual discharges. Panel C: discharges used to calculate the MCD of the SFEMG potential (triangles in the yellow area) and discharges excluded from the analysis (triangles outside the yellow area). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

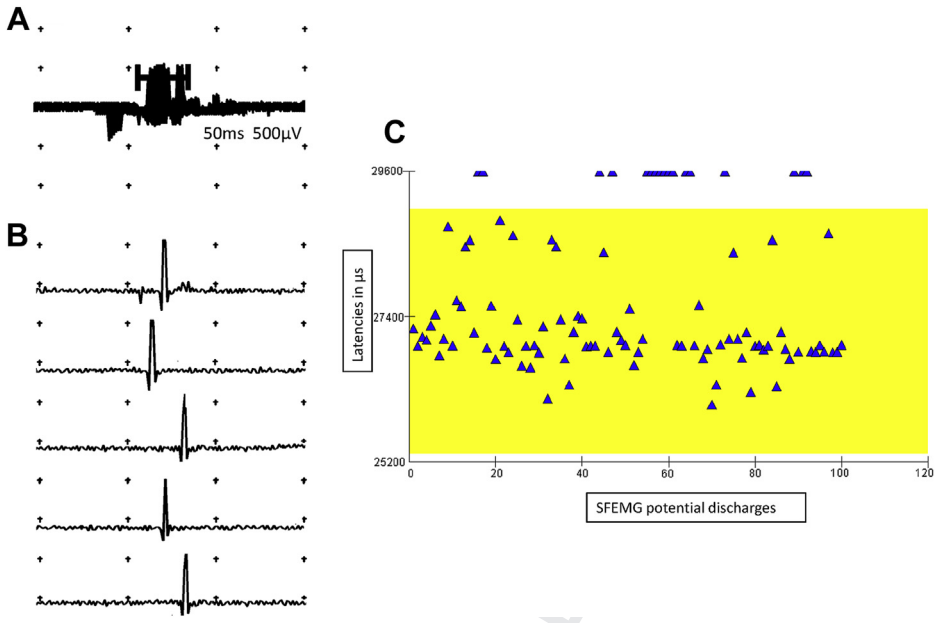

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Figure 2. An SFEMG potential after magnetic brain repetitive stimulations and the consecutive discharge latencies in a healthy subject. Panel A: superimposed discharges of an SFEMG potential. Panel B: a magnification of individual discharges. Panel C: discharges used to calculate the MCD of the SFEMG potential (triangles in the yellow area) and discharges excluded from the analysis (triangles outside the yellow area). Note the dual latency distribution shown in upper left in superimposed mode. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activation of corticospinal neurons of different size and conduction velocity from one TMS pulse to another might cause a time shift in the activation of the spinal MN. This possibility is very unlikely in the adopted experimental setting, since there is evidence of a monosynaptic connection between the cortical and the spinal MN for the FDI muscle [28]. Second, it has also been shown in cat and monkey that a substantial portion of corticospinal excitation on forelimb MNs is mediated by interneurones located in the C_3–C_4 segments, which are denoted as "C_3–C_4 propriospinal neurons." Propriospinal neurons project monosynaptically to MNs and contribute to cortico-motoneuronal disynaptic excitation [34], a mechanism which has been described also for human upper limb MNs [35]. Therefore, the possibility of non-monosynaptic, propriospinal component in the SFEMG MEP, needs to be considered, although two main arguments make this possibility unlikely: 1) there is no evidence in monkey and man that individual propriospinal neurons

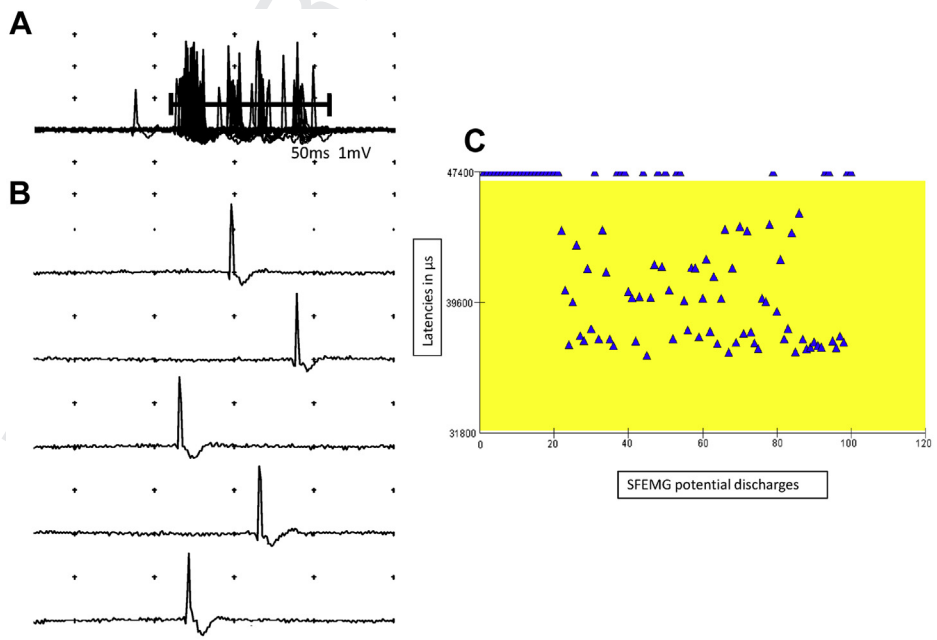

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Figure 3. An SFEMG potential after magnetic brain repetitive stimulations and the consecutive discharge latencies in the patient with MS. Panel A: superimposed discharges of an SFEMG potential. Panel B: a magnification of individual discharges. Panel C: discharges used to calculate the MCD of the SFEMG potential (triangles in the yellow area) and discharges excluded from the analysis (triangles outside the yellow area). Note the multimodal latency distribution shown in upper left in superimposed mode. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

project to intrinsic hand muscles (see Ref. [35]); 2) even assuming that these projections exist, they hardly may transmit corticospinal volleys in a resting condition, since in this condition the C_3–C_4 propriospinal system is under strong feed-forward inhibition [36].

An even less likely possibility is that the jitter is entirely generated in the spinal MNs. The central jitter is about twice the mean jitter of H-reflex in healthy subjects, which ranges between 138 μs and 186 μs in the different studies carried out on limb muscles [37–39]; moreover, the H-reflex jitter is considered to be influenced by a disynaptic pathway (inhibitory interneuron-α MN) when a maximum stimulus is used to elicit the reflex response [37]. So, one could argue that the central jitter we measured is due to a pathway more complex than a disynaptic connection. Obviously, it has to be considered that we applied magnetic stimulation delivered at scalp level, and this definitely excludes an exclusive origin of the central jitter in the spinal MNs.

Summarizing, the central jitter is probably due to cortical and spinal mechanisms; the former mainly involving the activation of cortical interneurons, the later mainly involving the summation of different I-waves generating excitatory post-synaptic potentials in the spinal MN.

Previous studies combining SFEMG and TMS looked at the cortico-muscular jitter after single-pulse stimulations of the motor cortex, both in healthy subjects (1016, [18–20]) and in patients affected by central [17,18] or peripheral demyelination (Magistris et al. [15]). In these studies, which however used single motor unit recordings rather than SFEMG, the cortico-muscular jitter was taken as a whole, without disentangling the contribution of the actual central component from the peripheral one. The latter is not negligible, accounting for about 10–15% of the variability in the subjects of the present study (see Table 1). Such a component of the variability of the cortico-muscular jitter may be even larger in patients with peripheral neuropathies due to the demyelination [15,17].

In the patient with MS, the central jitter was more than twice the mean central jitter value found in healthy subjects (about 800 μs), while the peripheral jitters were similar. Increased variability of single motor unit discharge in patients with MS has been already observed [15,17,18]. Many factors may theoretically account for the increase of the central jitter in the patient reported here: a corticospinal lesion can be excluded due to the normal central conduction time and absence of lesions on that pathway at MRI. However, subclinical central demyelination increasing phase cancellation of the descending volleys (Boniface et al. [17]; Magistris et al. [15]) cannot be excluded. The cerebellar dysfunction might have altered cortical excitability of the stimulated motor cortex though a dysfunction at some level of the cerebello-thalamo-cortical connecting fibers in the white matter [40,41]. Finally, it has been proposed that central fatigue, which is one of the most common and disabling symptoms in MS [42], might partly depend by a dysfunction of motor output [43]: increased central jitter (i.e., less synchronous corticospinal firing) might play a relevant role in this sense and could explain the increased variability of MEP latency reported even with normal CMCT in the target muscle in MS patients [44]. It is clear that these speculations should be verified in larger studies on patient populations, which also are necessary to clinically validate the proposed approach.

We adopted rTMS instead of single-pulse TMS. Such a strategy has both advantages and disadvantages. Certainly, the time required to collect a sufficient number of trials for a reliable statistical evaluation of the jitter is remarkably reduced, thereby making the discomfort induced by the needle inserted in the muscle more tolerable. However, the potential subjective discomfort due to the TMS-induced local pain and scalp sensation is greater during rTMS than during single-pulse stimulation. The possibility that small displacements of the coil throughout the session could account for the observed variability is unlikely: first, the intensity of TMS pulses was well above resting motor threshold, which makes the stimulation more efficient but less selective, thereby less sensitive to small coil displacements. Second, results are extremely consistent between subjects, including those in which neuronavigation was used. The displacements of the SF needle during rTMS are possible but they cannot influence jitter measurements because only potentials with the same shape and amplitude were computed. When the needle displacements occur, they cause a great variability in the shape and amplitude of the recorded potentials, which were excluded from the analysis.

A potential biasing factor of rTMS, which consists on the delivery of regularly spaced TMS pulses at different frequencies, should consider the increasing bulk of evidence indicating that after-effects on cortical excitability can take place: the continuous application of rTMS at <1 Hz decreases the excitability of the stimulated cortical networks, while rTMS at >5 Hz tends to increase it (see Ref. [31]). The use of higher frequencies of rTMS, which in principle might reduce even more the total experimental time, is precluded by safety recommendations [31]. The 3 Hz rTMS should in principle prevent the occurrence of inhibitory or facilitatory effects within the relatively long rTMS trains of the current study that might have *per se* biased the magnitude of the cortical jitter. Anyway, current results fill the gap in the range >1 Hz/<5 Hz rTMS of the last available safety tables [31], and suggest that this protocol should be carried out with caution, in presence of medically qualified personnel, and that examined subjects have to be strictly monitored as far as possible spread of excitation at cortical level is concerned.

Of course, also the intensity of stimulation has a role in determining the effect of rTMS on motor cortical excitability, for example 2 and 6 Hz rTMS delivered at an intensity of 80% of active motor threshold reduce cortical excitability, while stimulation at 70% and 90% of active motor threshold had no significant effect on MEP magnitude [45]. Meanwhile, since the suprathreshold intensity at 3 Hz rTMS on cortical excitability is still unknown, we cannot exclude some influence on jitter measurement. In any case, a suprathreshold intensity is mandatory to record stable SFEMG potentials.

Finally, the use of rTMS instead of single pulses makes the length of this method suitable for clinical applications aimed to investigate pathophysiological mechanisms of central fatigue, lesional or degenerative processes of the central nervous system. Future studies should consider the possibility to use pharmacological challenges, based on the administration of drugs with a well-defined mechanism of action, to determine which of the neurotransmitter or neuromodulator systems are implicated at interneuronal level [46] in the regulation of the cortical jitter.

Acknowledgments

Authors thank Drs Giovanni Bianco, Alberto De Capua, Nicola R. Polizzotto and Giuseppe Greco for their participation to experimental recordings and preliminary discussions in an early phase of the study.

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