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RESEARCH ARTICLE

Enhancement of single motor unit inhibitory responses to transcranial magnetic stimulation in amyotrophic lateral sclerosis

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Abstract In healthy human subjects, transcranial magnetic stimulation (TMS) applied to the motor cortex induces concurrent inhibitory and excitatory effects on motoneurone activity. In amyotrophic lateral sclerosis (ALS), a neurodegenerative disease affecting both cortical and spinal motor neurons, paired-pulse studies based on electromyographic (EMG) recording have revealed a decrease in TMS-induced inhibition. This suggested that inhibition loss may promote excito-toxicity in this disease. Against this hypothesis, an abnormally high incidence of inhibitory responses to TMS has been observed in the peristimulus time histograms (PSTHs) in ALS single motor unit studies. The disappearance of cortico-motoneuronal excitatory inputs might, however, have facilitated the detection of single motor unit inhibitory responses in the PSTHs. This question was addressed here using a new approach, where the strength of the excitatory and inhibitory effects of TMS on motoneurone activity was assessed from the duration of inter-spike intervals (ISIs). This analysis was conducted on single motor unit (MU), tested on healthy subjects and patients with ALS or Kennedy's disease (KD), a motor neuron disease which unlike ALS, spares the cortico-spinal pathway. MUs tested on KD patients behaved like those of healthy subjects unlike those tested on ALS patients. The present data reveal that in ALS, the TMS-induced inhibitory effects are truly enhanced during voluntary contractions and not reduced, as observed in paired-pulse TMS studies under resting conditions. The possible contribution of inhibitory loss to the physiopathology of ALS therefore needs to be reconsidered. The present data do not support the idea that inhibition loss may underlie excito-toxicity in ALS.

Keywords Motor unit · Transcranial magnetic stimulation · Inhibition · Motor neuron diseases · Inter-spike interval

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Introduction

Applying TMS to the motor cortex at sub-threshold intensity levels transiently depresses electromyographic (EMG) activity in healthy human subjects (Davey et al. 1994). At supra-threshold intensities, TMS induces a combination of excitatory and inhibitory responses, in the form of a motor evoked potential (MEP) followed by a silent period partly of cortical origin (Chen et al. 1999). EMG-based paired-pulse methods have been developed for investigating the inhibitory effects of TMS (Chen 2004). TMS conditioning pulses delivered at sub-threshold or supra-threshold intensity of either 1–5 or 50–100 ms before supra-threshold test pulses induce either early short-lasting or late long-lasting inhibitory effects, related to different sets of GABAergic cortical interneurons (Nakamura et al. 1997; Di Lazzaro et al. 1998).

In single motor unit studies on healthy subjects, purely inhibitory responses to TMS (i.e., not preceded by any excitatory responses) have been observed at sub- or near-threshold intensities, although much more rarely than excitatory responses (Palmer and Ashby 1992; Boniface et al.

1994; Brouwer and Qiao 1995; Maertens de Noordhout et al. 1999). The likelihood of purely inhibitory responses occurring at TMS intensities below the excitatory response threshold depends on the muscle tested (Palmer and Ashby 1992). In forearm and finger muscles for instance, purely inhibitory single MU responses to TMS have been rarely, if ever, observed in healthy subjects (Palmer and Ashby 1992; Classen and Benecke 1995; Garland and Miles 1997). In the corresponding motoneurones, TMS-induced inhibitory effects might be masked by particularly powerful cortico-motoneuronal EPSPs.

Beside PSTH analysis, changes in the duration of the inter-spike intervals (ISIs) have been used to investigate the inhibitory and excitatory effects of cortical stimulation on neuronal activity. The earliest evidence that cortical stimulation has inhibitory effects on human motor unit activity was actually provided by the finding that supra-threshold transcranial electrical stimulation induced ISI lengthening intermingled with ISI shortening, depending on the timing of the stimulation after a spike (Calancie et al. 1987). Later on, Garland and Miles (1997) confirmed that supra-threshold TMS applied to the motor cortex, too early after a motoneurone spike, to produce an excitatory response delayed the subsequent firing. Examining the ISI changes separately in TMS trials with and without excitatory responses should therefore provide an effective means of concurrently assessing the cortically-induced excitatory and inhibitory deficits in motor neuron diseases.

The pyramidal neurones from which cortico-motoneuronal inputs originate (upper motor neurons, UMNs) and the spinal and bulbar motoneurones (lower motor neurons, LMNs) are the primary targets of ALS, a fatal neuro-degenerative disease with a still unknown etiology (Bruijn et al. 2004; Boillee et al. 2006). In this disease, signs of hyper-excitability of the cortico-motoneuronal pathway have been reported to occur, in the form of abnormally low TMS intensity thresholds and/or abnormally large MEPs (Eisen and Swash 2001). These findings suggested that excito-toxic mechanisms may be involved in neuronal death in ALS (Heath and Shaw 2002). There is some evidence that cortical GABAergic processes might be impaired in ALS (Nihei et al. 1993; Lloyd et al. 2000; Petri et al. 2003; Maekawa et al. 2004). On this basis, it was proposed that loss of inhibition might contribute to the development of cortico-spinal hyper-excitability in ALS. Comforting this idea, most of the TMS paired-pulse studies performed on ALS patients have reported a decrease in effectiveness of both the early and late intra-cortical inhibitory processes (Yokota et al. 1996; Ziemann et al. 1997; Salerno and Georgesco 1998; Zanette et al. 2002; Vucic and Kiernan 2006). This contrasts markedly with the abnormally high occurrence of TMS-induced inhibitory responses observed in ALS patients' hand and forearm muscles in PSTH studies (Awiszus and Feistner 1993; Mills 1995; Attarian et al. 2006). The loss of cortico-motoneuronal excitatory inputs in these patients might, however, have facilitated the detection of single motor unit inhibitory responses in the PSTHs, limiting the possibility of detecting genuine changes in the effectiveness of the inhibitory processes.

To clarify this question, an ISI-based approach was implemented to analyze the effects of TMS on the firing time of single motor units tested on healthy subjects and patients with ALS. To evaluate the specificity of the alterations detected in ALS, this procedure was also applied to patients with KD, an inherited motor neurone disease affecting the bulbar and spinal motoneurones but sparing the cortico-motoneuronal pathway. With a view of detecting any pathological changes in the TMS-induced inhibitory effects apart from those affecting the excitatory cortico-motoneuronal pathway, the study was centered on MUs producing monosynaptic-like excitatory responses to TMS in healthy subjects and both groups of patients.

Population and methods

Ethical approval

The data used in the present study were a part of the dataset recorded in two previous studies (Attarian et al. 2006, 2008). All experiments were performed in keeping with the Helsinki Declaration. All the subjects gave their informed written consent to the experimental procedures, which were approved by the local Ethics Committee CCPPRB-Marseille I.

Clinical assessment and findings

The age of the patients with sporadic ALS (4 men and 1 woman), patients with KD (three men) and healthy subjects (five men and four women) ranged from 56 to 68, 44 to 60 and 54 to 63 years, respectively, with no significant differences between the groups (Kruskal Wallis test $P = 0.2$).

Clinical findings are summarized in Table 1. Beside neurological needle examination, manual muscle wrist extension tests (MMT) was performed to assess muscle strength, using the Medical Research Council score. The presence of a brisk tendon reflex or a Hoffmann sign on the right upper limb was taken to reflect UMN dysfunction, whereas fasciculation, areflexia or muscle atrophy were taken to reflect LMN deficits (Table 1).

Based upon the El Escorial World Federation of Neurology criteria (Brooks 1994), three patients with suspected ALS (Table 1, nos. 4, 5, 6) and one patient with a bulbar onset (no. 7) showed clinical signs of LMN but no signs of UMN dysfunction in the right upper limb. One patient with

Table 1 Patients' characteristics and ALS diagnosis according to the El Escorial criteria (Brooks 1994)

Patient	Disease duration (months)	Diagnostic category (ALS)	Wrist extension MMT	Atrophy	Fasciculation	LMN	Stretch reflex	Hoffman sign	UMN
KD									
1	31	NA	5	+	+	+	±	–	–
2	60	NA	5	+	+	+	–	–	–
3	130	NA	5	–	+	+	–	–	–
ALS									
4	9	Suspected	5	–	+	+	+	–	–
5	12	Suspected	3.5	+	+	+	+	–	–
6	13	Suspected	5	+	+	+	+	–	–
7	16	Bulbar	4	–	+	+	+	–	–
8	30	Probable	5	–	–	–	+	–	–

NA non applicable, *UMN* upper motor neuron clinical signs in the right upper limb, *LMN* lower motor neuron signs in the right upper limb, *MMT* manual muscle rating using the MRC score

probable ALS (no. 8) showed no clinical signs of UMN or LMN in the right upper limb. All patients subsequently developed definite ALS and all but one (no. 8) died meanwhile. All these patients were taking riluzole.

The three patients with KD (mean disease duration: 73.7 ± 50.9 months) had an abnormally large number of tri-nucleoside CAG repeats in the exon-1 coding regions of the androgen receptor gene. They all had characteristic clinical LMN signs, including bulbar and limb weakness associated with muscle atrophy, fasciculation, depressed or absent tendon reflexes, and gynecomastia.

Instructions to subjects

The instructions to subjects, data recording procedures and basic methods of analysis used here have been described previously (Attarian et al. 2006). In short, the subjects were seated with their wrist immobilized in a U-shaped device maintaining the hand in a semi-prone position. They were instructed to produce an isometric wrist extension against a force transducer. The MUs tested on the wrist extensor muscles were kept firing continuously, with the help of visual and auditory feedback. MU responsiveness to TMS was tested on sequences lasting 2–3 min including 40–60 stimuli.

Data recording

MU activity and intramuscular EMG activity were recorded from the right extensor carpi radialis muscles (ECR) using a macro-single-fiber intramuscular electrode (Stålberg 1982) and a grounded reference electrode placed on the upper arm. MU spike trains were amplified and filtered (300–3,000 Hz). The surface EMG activity of ECR muscles was recorded using paired non-polarizable surface electrodes. The direct (DC) and filtered (AC, band-pass 0.1–1,000 Hz) force signal measured by the force transducer (15 mV/mN, linear range 0–20 N) was calibrated in Newtons (N). Single MU activity, EMG activity and wrist extension forces were sampled (30 , 5 , and 1 kHz, respectively) using the CED 1401-plus device and the Spike 2–5 software (CED, Cambridge, UK). The electrophysiological and biomechanical signals were digitized and stored on a computer for further analysis.

Stimulation procedure

As all the patients and healthy subjects were right-handed, TMS was delivered (1 stimulus every 3 s) to the left motor cortex via a Magstim 200 stimulator and a figure-of-8 coil (Magstim; Whitland, Wales, UK). The site at which a MEP was induced in the ECR muscles at the lowest stimulation intensity was marked on the skull so that the coil location could be constantly monitored. The resting motor threshold was taken to be the minimum stimulus intensity, eliciting a MEP in response to four out of ten stimulations (display gain, 50 μV/division). This intensity was used thereafter in the single motor unit tests. The median numbers of stimuli were 55, 50 and 58 in the case of healthy subjects and patients with ALS and KD, respectively.

Data analysis

MU spikes were discriminated off-line and converted into events using the Spikes 2–5. The few milliseconds after each stimulus during which the waveform discrimination process might have failed because of the stimulus artifact were closely examined. Any spikes obviously missed were included in the spike-event train a posteriori.

Motor unit functional characteristics

Macro-MUPs were extracted by applying the spike-triggered averaging procedure to the intramuscular EMG activity. The macro-MUP area (mV ms) was taken as an index to MU size. An index to the contractile force of each MU

was provided by the peak amplitude of the twitch (TA, mN), extracted by applying the spike-triggered averaging procedure to the net AC-filtered extension force, in the case of inter-spike intervals (ISIs) longer than 90 ms only (Schmied et al. 1999). MU contractile effectiveness was expressed as the twitch amplitude over the macro-MUP area (mN/mV ms). The MU discharge patterns were assessed in terms of the mean ISI and its coefficient of variation (ISI-CV% = standard deviation/mean ISI × 100) during the whole recording period, excluding any ISIs lasting more than 300 ms, which presumably included a pause in the MU tonic activity.

Motor unit responses to cortical stimulation

MU responsiveness to TMS was assessed by computing PSTHs using a Windows-based software program (AVE, E. Fetz and L. Shupe, Primate Center, University of Washington, Seattle, WA). PSTHs (1 ms-bins) were computed by collecting spike events, 100 ms before and 200 ms after the stimulus. Post-stimulus changes in MU activity were detected on the basis of positive inflexions in the cumulative sum (CUSUM, Ellaway 1978) relative to 80 ms pre-stimulus baseline. The limits of early peaks, attributable to excitatory cortico-motoneuronal inputs were marked by positioning two cursors on the edges of the CUSUM inflexions above the pre-stimulus baseline. The magnitude of the excitatory response (imp.stim^{-1}) was given by the sum of the bin counts above the baseline over the number of stimuli. The *Z* score test (Garnett and Stephens 1980) was used to characterize the significance of the MU excitatory responses to TMS. Only MUs showing significant peaks with *Z* scores greater than >2.58 ($P < 0.01$) were selected for the ISI analysis.

ISI analysis

As illustrated in Fig. 1, motoneurone spike trains and associated events were processed in three steps, in order (1) to take into account the non-physiological ISI lengthening which occurs whenever a spike has been masked by the stimulus artifact, (2) to take into account the conduction time from motor cortex to muscle, and (3) to analyze TMS trials with and without excitatory responses separately.

First a virtual artifact associated with a virtual stimulation marker was added off-line to the data file, 1.5 s after each real stimulus (Fig. 1a top). The virtual stimulation was associated with a time-window made to last as long as the PSTH trough caused by the artifact in the PSTH (5–7 ms). Any spikes occurring within the virtual artifact window were deleted (Fig. 1a bottom). This event was infrequent, and only a few spikes were removed (0–9 depending on the duration of the recording).

Depending on the timing of the stimuli after spikes and on the conduction time from cortex to muscle, the TMS-induced monsynaptic EPSP could actually affect the motoneurone ISI which followed that during which the stimulation was delivered (Fig. 1a). To properly analyze the effects of the EPSP on the timing of the next firing, the spike train had to be shifted by an interval ($\tau 1 + \tau 2$) corresponding to the conduction time from the cortex to the muscle. This was achieved by shifting the spike train backward by a period equal to the latency of the monosynaptic-like response in the PSTH (Fig. 1b). The peak appeared in the first 1 ms-bin after the stimulus in the PSTH (Fig. 1c), as presumably occurred in the spinal cord whenever the cortico-motoneuronal EPSPs triggered a spike.

The ISI analysis included an assessment of the duration of the five ISIs preceding the stimulus, that of the ISI during which the actual or virtual stimulus was liable to act (ISI_{stim}), and that of the following three ISIs ($ISI1_{post}$, $ISI2_{post}$, $ISI3_{post}$). The five pre-stimulus ISIs were averaged to obtain the control value ($ISI_{control}$). In each trial, the effects of TMS were revealed by the change in absolute duration of the corresponding ISIs (ISI_{stim}, $ISI1_{post}$, $ISI2_{post}$, $ISI3_{post}$) as compared to the $ISI_{control}$ and in percentage of $ISI_{control}$ (ISI_{stim}%, $ISI1_{post}$%, $ISI2_{post}$%, $ISI3_{post}$%).

In the third step (Fig. 1d–g), TMS trials were subdivided into two groups, in line with previous studies on healthy subjects (Mills 1988; Classen and Benecke 1995). The "peak" trials included all trials in which a spike was triggered within the PSTH peak delimited by the dotted area (Fig. 1d, e). The "no peak" trials included all trials where no spikes occurred during the PSTH peak (Fig. 1d, f). It was thus possible to assess the ISI changes involving excitation ("peak" trials) separately from those not involving excitation ("no peak" trials).

Trials with virtual stimuli were subdivided in the same way. As virtual stimuli were used to evaluate the non-physiological ISI lengthening which may occur whenever a spike has been missed due to the stimulation artifact, only the "no peak" virtual trials were included in the following calculations, and referred to as "virtual" trials. Indeed, the presence of spikes within the PSTH peak following the actual as well as the virtual stimulation provided evidence that the stimulus artifact had not affected the spike discrimination accuracy.

The effects of TMS on the next firing were next investigated in the trials defined as "peak", "no peak" and "virtual" trials. In each of the "peak" and "no peak" trials, the strength of the TMS effects was assessed on the basis of the absolute duration of ISI_{peak} and ISI_{nopeak} (Fig. 1d) and their relative change, expressed as $ISI_{peak}\% = (ISI_{peak} – ISI_{control})/ISI_{control} * 100$ and as $ISI_{nopeak}\% = (ISI_{nopeak} - ISI_{control})/ISI_{control} \times 100$. In each "virtual" trial, the non-physiological effects of the artifact were assessed in the same way on the basis of the duration of $ISI_{virtual}$ (Fig. 1a) and its

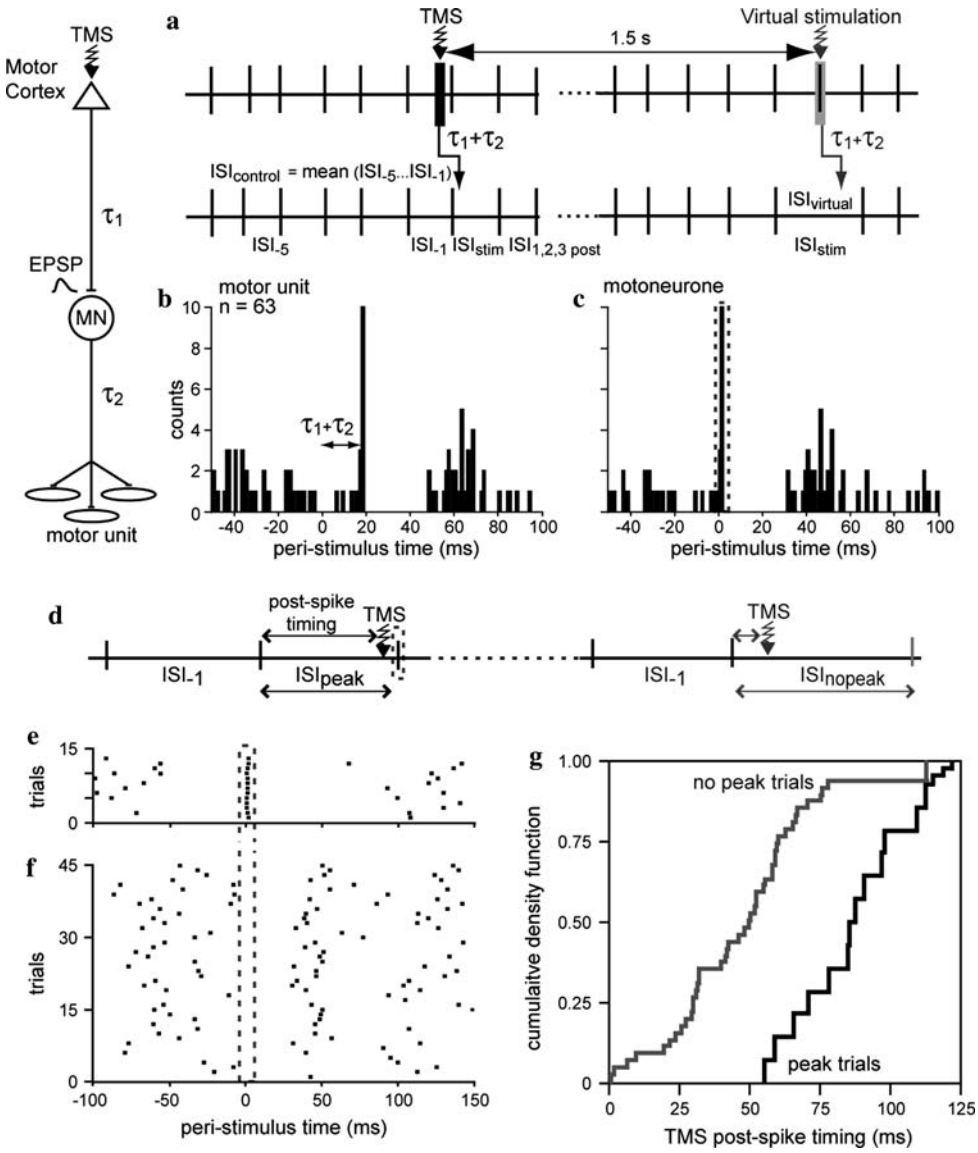

Fig. 1 In the spike train (**a** *top*), the *black area* shows the limits of the TMS artifact detected in the original MU recording. Any spikes occurring within these limits will be missed, leading to non-physiological ISI lengthening. This was mimicked by introducing a virtual artifact (*gray area*) off-line after each stimulation with a delay of 1.5 s. In order to analyze the actual impact of the TMS-induced monosynaptic EPSP on the motoneurone activity, the spike train was shifted backward (**a** *bottom*) by the interval $\tau 1 + \tau 2$, given by the latency of the peak in the PSTH computed with the original spike train (**b**). The PSTH computed with the shifted spike train is shown in **c**. The intervals between the stimulation and the previous spike (post-spike interval) and the duration of the corresponding ISI were measured in each trial (**d**) and are denoted by ISI_{peak} or ISI_{nopeak} depending on whether TMS was followed or not by a spike within the limits of the PSTH peak (*dotted area* **c**). The corresponding trials are shown in the raster-displays **e** and **f**, respectively. *Graph* **g** shows the cumulative density function of the TMS post-spike intervals in the "peak" and "no peak" trials (*black* and *gray curves*, respectively)

change, expressed as $ISI_{virtual}\% = (ISI_{virtual} - ISI_{control})/ISI_{control} \times 100$.

In order to determine the influence of the previous firing time on the ability of TMS to affect the next firing time, the interval between the TMS and the previous spike (post-spike timing) was assessed in each of the TMS "peak" and "no peak" trials (Fig. 1d, g). The distribution of the post-spike intervals associated with "peak" and "no peak" trials (black and gray curves, respectively in Fig. 1g) could be thus compared between healthy subjects and patients. To take any differences between the pre-stimulus firing rates into account, each post-spike timing was also expressed as a percentage of the $ISI_{control}$ (post-spike timing (%) = post-spike timing/$ISI_{control} \times 100$).

Median values [quartile deviation = (upper-lower quartiles)/2] and the corresponding box plot were used to describe the ISI variables pooled across trials per MU in each group, and across MUs per group because many of these variables were not normally distributed. The median values of $ISI_{control}$, that of ISI_{stim} including all ISI_{peak} and ISI_{nopeak} trials and that of $ISI1_{post}$, $ISI2_{post}$, $ISI3_{post}$ were assessed after pooling all the data obtained on each MU. Likewise, under the three ("peak", "no peak" "and virtual") conditions, median $ISI_{control}$, ISI_{peak}, ISI_{nopeak}, $ISI_{peak}\%$, $ISI_{nopeak}\%$, post-spike timing, post-spike timing (%), $ISI_{virtual}$ and $ISI_{virtual}\%$ values were obtained on each MU. Lastly, under "no peak" conditions, the net effect of TMS on the next firing time was assessed by subtracting the median $ISI_{virtual}\%$ from the median $ISI_{nopeak}\%$ values.

Statistical analysis

Statistical analyses were performed by conducting nonparametric tests (JMP software, SAS Institute Inc, Cary).

Table 2 In the three groups, MU functional characteristics are given as means (±SDs)

	Control $N = 60$	ALS $N = 49$	KD $N = 33$	Control versus ALS	Control versus KD	ALS versus KD
TMS excitatory threshold (%)	46.5 (2.2)	41.1 (3.0)	42.4 (3.9)	NS	NS	NS
Peak latency (ms)	22.3 (4.7)	23.8 (5.7)	16.3 (1.7)	NS	$P < 0.01$	$P < 0.0001$
Peak duration (ms)	3.1 (1.3)	4.2 (1.8)	4.4 (2.1)	$P < 0.0001$	$P < 0.0001$	NS
Peak area (imp per stimulus)	0.26 (0.10)	0.29 (0.10)	0.31 (0.15)	NS	NS	NS
Macro-MUP area (mV.ms)	1.2 (0.6)	2.9 (2.3)	8.6 (3.6)	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Twitch (mN)	11.2 (10.4)	15.6 (11.6)	46.8 (39.5)	NS	$P < 0.0001$	$P < 0.001$
Twitch/macro-MUP (mN/ms.mv)	8.8 (6.4)	7.7 (7.0)	7.5 (7.3)	NS	NS	NS
Background ISI (ms)	84.3 (18.8)	77.6 (24.1)	91.3 (19.2)	NS	NS	NS
Coefficient of variability (%)	21.3 (5.6)	38.3 (14.8)	39.2 (7.5)	$P < 0.0001$	$P < 0.0001$	NS

The TMS parameters (resting motor threshold, PSTH peak latency, duration and area), the electrical (macro-MUP), mechanical (twitch) and firing parameters (background ISI, ISI variability) of the MUs are compared across groups (Kruskal–Wallis test, *P* significance levels, *NS* nonsignificant difference)

Within each group of subjects, the impact of TMS on the successive firing times was assessed using the Wilcoxon matched-pairs test to make paired comparisons between the median $ISI_{control}$, ISI_{stim}, $ISI1_{post}$, $ISI2_{post}$ and $ISI3_{post}$ values obtained with TMS and virtual stimulation. The Kruskal–Wallis test was used to compare the relative ISI changes (ISI_{stim}%, $ISI1_{post}$%, $ISI2_{post}$%, $ISI3_{post}$%, ISI_{peak}%, ISI_{nopeak}%), and the TMS post-spike timings (%) corresponding to "peak" and "no peak" trials across the three groups and to make post hoc comparisons between two groups. Non-parametric regression analyses were performed to assess the co-variation between the various parameters using the Spearman coefficient of correlation (ρ), and linear regression lines are given as an illustration only. In all these tests, significance level was taken to be $P < 0.05$. The motor units' electromechanical properties (macro-MUP area, twitch amplitude), the characteristics of the PSTH peaks (latency, duration, amplitude), and the firing properties (background ISI and coefficient of variation) are given in the text and in Table 2 in terms of mean values and standard deviations (±SD).

Results

The ISI-based assessment of TMS effects was performed on a total number of 49, 33 and 60 MUs tested on ALS patients, KD patients and healthy subjects, respectively. The functional characteristics of these three groups of MUs are given in Table 2. As previously noted in the larger populations of MUs originally tested (Attarian et al. 2006), there were no consistent differences in the TMS resting motor thresholds between patients with ALS or KD and healthy subjects (30–50, 30–55 and 35–60% of the maximum stimulator output, respectively). According to the selection criteria, the size of the PSTH peaks did not differ significantly between the three groups.

Global impact of TMS on successive MU firing times

With each MU, the global impact of TMS on four consecutive motoneurone firing events was assessed upon pooling the results of all TMS trials. In Fig. 2, the median $ISI_{control}$, ISI_{stim}, $ISI1_{post}$, $ISI2_{post}$, and $ISI3_{post}$ values obtained with each MU are connected by gray lines, while the median values obtained upon pooling all the MUs in the healthy group, KD group, and ALS group are connected by black lines (Fig. 2a–c, respectively).

The median $ISI_{control}$, ISI_{stim}, $ISI1_{post}$, $ISI2_{post}$, and $ISI3_{post}$ values obtained with each MU in the TMS trials were compared with the corresponding values obtained in the virtual stimulation trials, as shown in Table 3.

In both the healthy and KD groups, TMS did not induced any consistent change in ISI_{stim}. Therefore, in these two groups, the only means of detecting the effect (excitatory or inhibitory) of TMS on the subsequent motoneurone firing time consisted in analyzing the "peak" and "no peak" trials separately. In ALS, however, the net lengthening effect of TMS on ISI_{stim} apparent in Fig. 2c, was confirmed in comparison with the virtual stimulation (Table 3). Accordingly, the relative change in ISI_{stim} was found to be abnormally large in the ALS group (Kruskal–Wallis test, $P = 0.004$; Fig. 2d) in comparison with both the healthy and KD groups, in whom no consistent change was detected (Table 3).

The only significant effect of TMS on the subsequent ISIs ($ISI1_{post}$, $ISI2_{post}$, and $ISI3_{post}$) was a slight lengthening of $ISI1_{post}$ (Table 3), which occurred with a similar strength in healthy subjects and in patients with KD or ALS (Fig. 2e).

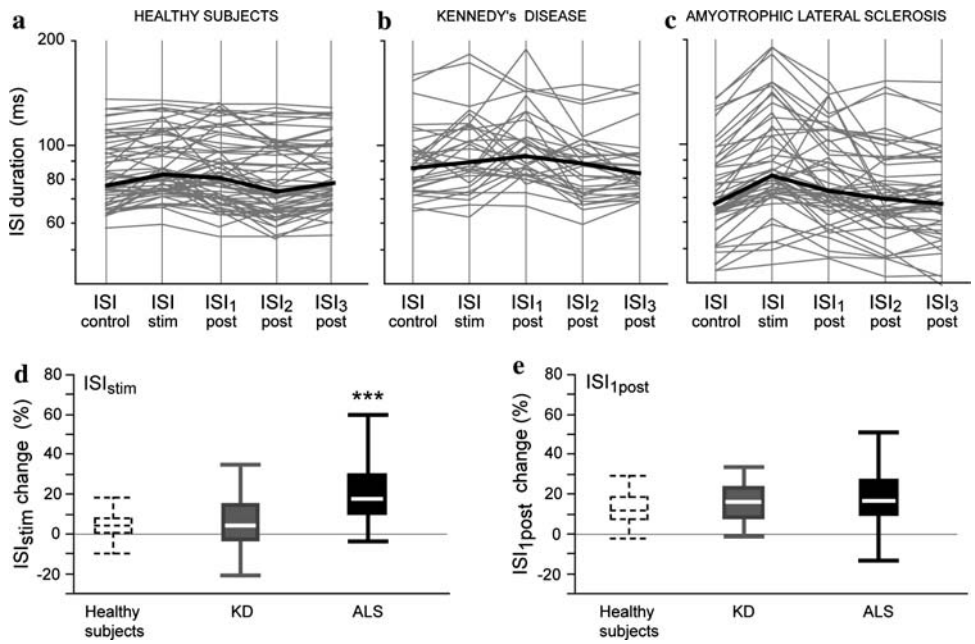

Fig. 2 The global impact of TMS on ISI duration is shown for each MUs in healthy subjects, KD patients and ALS patients (**a**, **b**, **c**, respectively). The *gray lines* connect the median values of the ISIs preceding the stimulation ($ISI_{control}$), the ISI during which the stimulation was delivered (ISI_{stim}) and the three subsequent ISIs ($ISI1_{post}$, $ISI2_{post}$, $ISI3_{post}$) obtained upon pooling all the TMS trials. The *black lines* connect the median ISI values obtained to the whole population of MUs tested in each group. The relative changes in ISI_{stim} ($ISI_{stim}\% = (ISI_{stim} - ISI_{control})/ISI_{control} \times 100$) and $ISI1_{post}$ ($ISI1_{post}\% = (ISI1_{post} - ISI_{control})/ISI_{control}*100$) are compared across groups (**d** and **e**, respectively, Kruskal–Wallis test, *** $P = 0.004$)

Table 3 In the three groups of subjects, the median (quartile deviation) values of the ISI preceding ($ISI_{control}$), that during which the stimulation was delivered (ISI_{stim}) and that of the three-three subsequent ISIs ($ISI1_{post}$, $ISI2_{post}$, $ISI3_{post}$) obtained with TMS are compared with the corresponding ISIs obtained with virtual stimulation (Wilcoxon matched-pairs test)

		TMS (ms)	Virtual (ms)	Wilcoxon test
Healthy subjects $n = 61$	$ISI_{control}$	77.0 (14.1)	76.3 (12.7)	NS
	ISI_{stim}	82.7 (13.3)	79.8 (13.1)	NS
	$ISI1_{post}$	80.7 (14.9)	75.1 (14.9)	$P = 0.001$
	$ISI2_{post}$	73.4 (17.2)	75.4 (13.6)	NS
	$ISI3_{post}$	78.3 (15.2)	76.8 (13.3)	NS
Kennedy's disease $n = 33$	$ISI_{control}$	88.1 (11.6)	88.4 (12.8)	NS
	ISI_{stim}	89.6 (17.8)	93.6 (12.1)	NS
	$ISI1_{post}$	92.3 (14.9)	82.2 (14.9)	$P < 0.0001$
	$ISI2_{post}$	88.5 (13.3)	84.3 (11.0)	NS
	$ISI3_{post}$	83.0 (9.1)	82.9 (10.5)	NS
Amyotrophic lateral sclerosis $n = 49$	$ISI_{control}$	68.1 (12.3)	67.5 (13.0)	NS
	ISI_{stim}	82.5 (19.7)	73.2 (13.8)	$P = 0.02$
	$ISI1_{post}$	72.5 (18.2)	66.7 (18.2)	$P < 0.0001$
	$ISI2_{post}$	70.4 (13.7)	67.2 (10.9)	NS
	$ISI3_{post}$	67.9 (14.8)	67.3 (11.2)	NS

P significance levels

NS non significant difference

TMS post-spike timings associated with "peak" versus "no peak" trials

As illustrated in Fig. 1g in a healthy subject, the timing of the stimulation after the previous motoneurone spike (TMS post-spike timing) differed greatly between "peak" and "no peak" trials. As shown in Fig. 3, the post-spike timings corresponding to "peak" trials were consistently shorter than those corresponding to "no peak" trials in the three groups of subjects.

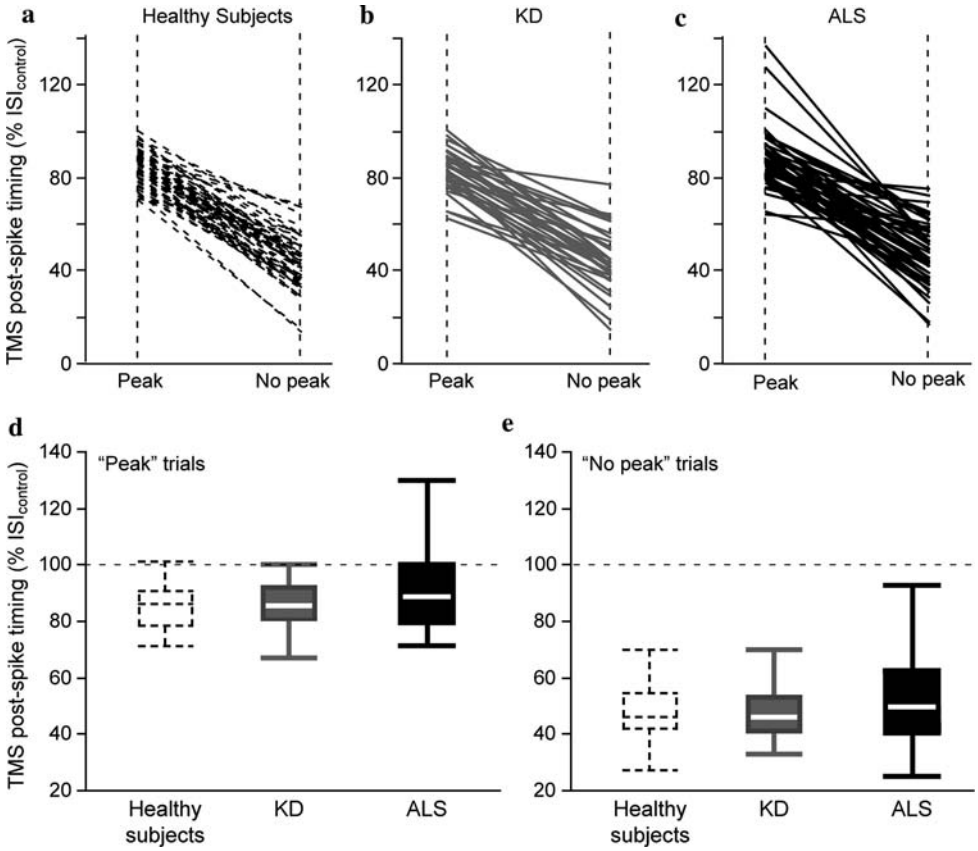

Fig. 3 The median values of the post-spike timings obtained in each of the MUs tested in healthy subjects and patients with KD or ALS (**a**, **b** and **c**, respectively) upon pooling the "peak" trials are connected with those obtained in the corresponding "no peak" trials. No consistent differences were observed upon performing the Kruskal–Wallis test across groups to compare the TMS "peak" and "no peak" post-spike timings (**d** and **e**, respectively) obtained in healthy subjects, and patients with KD or ALS (*white*, *gray* and *black box plots*, respectively)

In most of the MUs tested on healthy subjects and KD or ALS patients (Fig. 3a, b, c, respectively), the "peak" trials occurred when TMS was delivered near the time at which the next spike was expected to occur after an interval greater than 60% of the $ISI_{control}$ value, whereas the "no peak" trials occurred when TMS was delivered much earlier after a spike. No consistent differences were observed in the across-group comparisons of the TMS post-spike intervals observed in either "peak" or "no peak" trials (Fig. 3d, e, respectively).

Excitatory versus inhibitory effects of TMS on next MU firing time

As shown in Fig. 4, TMS had opposite effects on the next firing time in "peak" and "no peak" trials versus trials with virtual stimulation in the case of three MUs tested on a healthy subject (Fig. 4a–c), a KD patient (Fig. 4d–f), and an ALS patient (Fig. 4g–i). In each plot, the gray lines connect the $ISI_{control}$ value to that of the ISI during which the stimulation was applied to each of the "peak" (Fig. 4a, d, g), and "no peak" trials (Fig. 4b, e, h) and to each of the virtual stimulation trials (Fig. 4c, f, i).

With these three MUs, most of the single trial ISI_{peak} values were shorter than the corresponding $ISI_{control}$ indicating that TMS had advanced the next spike (i.e., excitatory effects). This was confirmed by the shortening of the median ISI_{peak} value (Fig. 4a, d, g). By contrasts, many of the single trial ISI_{nopeak} values were longer than the corresponding $ISI_{control}$, indicating that TMS had delayed the next spike (i.e., inhibitory effects). This was confirmed by the lengthening of the median ISI_{nopeak} (Fig. 4b, e, h). In the "virtual" trials, the weak impact of the stimulation artifact (reflecting the few spikes which had been missed) was revealed by the slight lengthening of median $ISI_{virtual}$ values as compared to $ISI_{control}$ (Fig. 4c, f, i).

The median of the net ISI changes induced by TMS with respect to the $ISI_{control}$ obtained with each MU after pooling the "peak" trials (ISI_{peak}) are plotted in Fig. 5a–c with respect to the net changes obtained in the "no peak" trials (ISI_{nopeak}) in the case of healthy subjects and KD and ALS patients, respectively.

Nearly all the MUs tested on healthy subjects (Fig. 5a), KD patients (Fig. 5b) and ALS patients (Fig. 5c) showed a shortening of ISI_{stim} in comparison with $ISI_{control}$ (i.e., negative changes) in the "peak" trials, which revealed the excitatory effects of TMS. Although there was some variability among patients, no significant differences were found in the TMS ISI shortening effects (Fig. 5d) in the across-group comparison.

In the "no peak" trials, most of the MUs tested on healthy subjects (Fig. 5a), KD patients (Fig. 5b), and or on ALS patients (Fig. 5c) showed an ISI lengthening in comparison with the $ISI_{control}$ values, which revealed the inhibitory

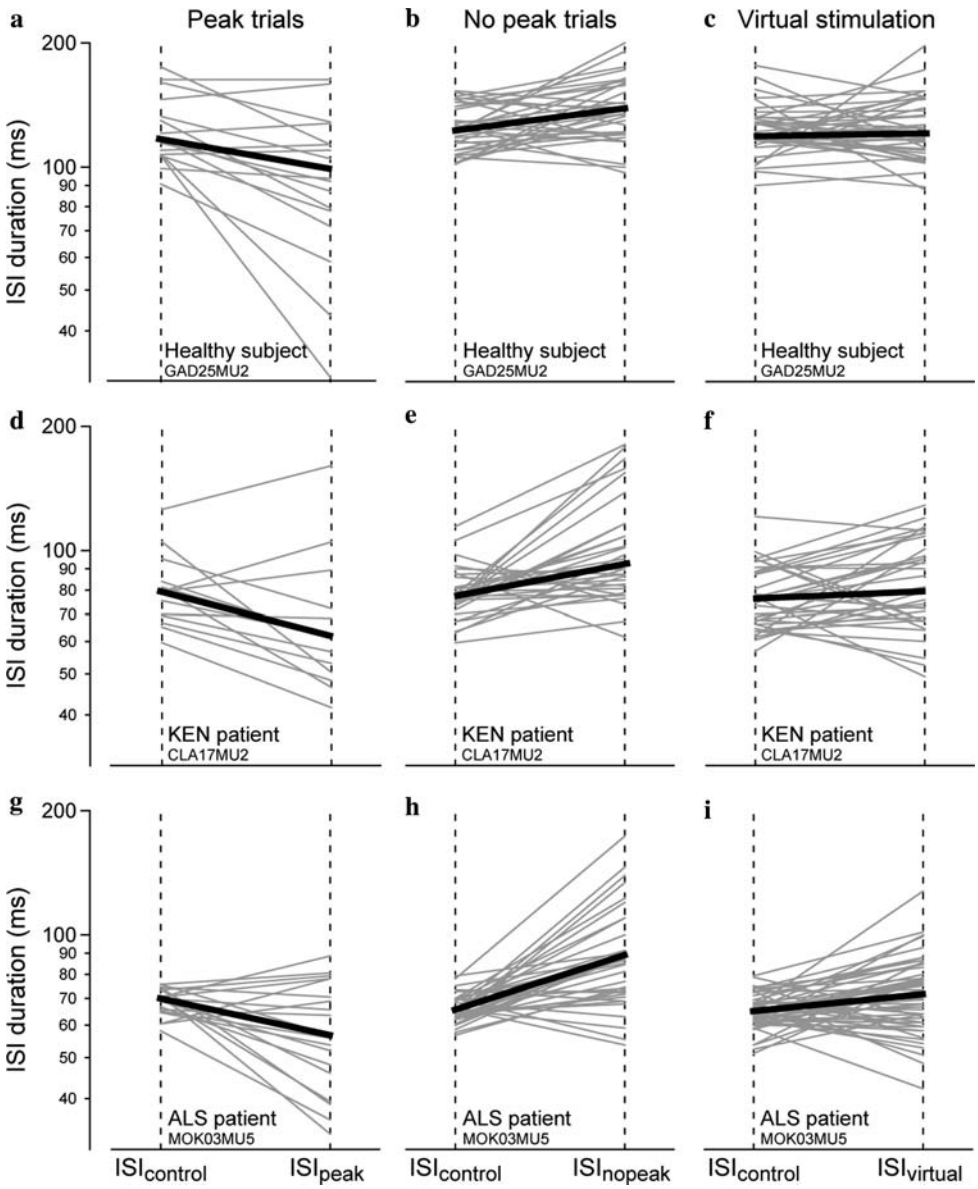

Fig. 4 The impact of TMS and virtual stimulation on ISI duration in the case of three MUs tested in a healthy subject (**a**, **b**, **c**), a patient with KD (**d**, **e**, **f**), and a patient with ALS (**g**, **h**, **i**). In each case, ISI duration is plotted at each of the trials involving excitatory responses (**a**, **d**, **g**), no excitatory responses (**b**, **e**, **h**) and virtual stimulation (**c**, **f**, **i**). In each plot, *gray lines* connect the duration of the $ISI_{control}$ to that of the ISI_{peak}, ISI_{nopeak}, and $ISI_{virtual}$ recorded in a single trial and the *black lines* connect the median durations obtained upon pooling all the trials

effects of TMS. Much greater dispersion was observed, however, in the ALS group, where many MUs showed an abnormally marked ISI lengthening (Fig. 5c). Accordingly, the ISI lengthening in the "no peak" trials (Fig. 5d) was significantly greater in ALS patients than in healthy subjects and KD patients (Kruskal–Wallis test, $P = 0.004$).

These data confirmed that the delay induced by TMS on the next firing time observed on ISI_{stim} in ALS patients but not in the other two groups (Fig. 2d) was a genuine inhibitory effect occurring independently of any changes in the excitatory effects of TMS.

Correlations between the ISI shortening induced by TMS and the PSTH peak area

In order to relate the ISI-based and PSTH-based assessments of TMS excitatory effects, the median value of the changes in ISI_{peak} obtained with each MU after pooling the "peak" trials was plotted versus the corresponding PSTH peak area (Fig. 6).

With both healthy subjects and KD patients (Fig. 6a, b), the close correlation observed between the two indices indicates that the most marked ISI shortening effects were associated with the largest PSTH peaks ($\rho = -0.45$, $P = 0.0003$ and $\rho = -0.58$, $P = 0.0004$, respectively). This correlation was completely absent in ALS patients (Fig. 6c, $\rho = -0.07$, $P = 0.6$).

Discussion

The present results provide a quantitative picture of the inhibitory and excitatory effects on motor unit firing behavior induced by applying juxta-threshold TMS on the

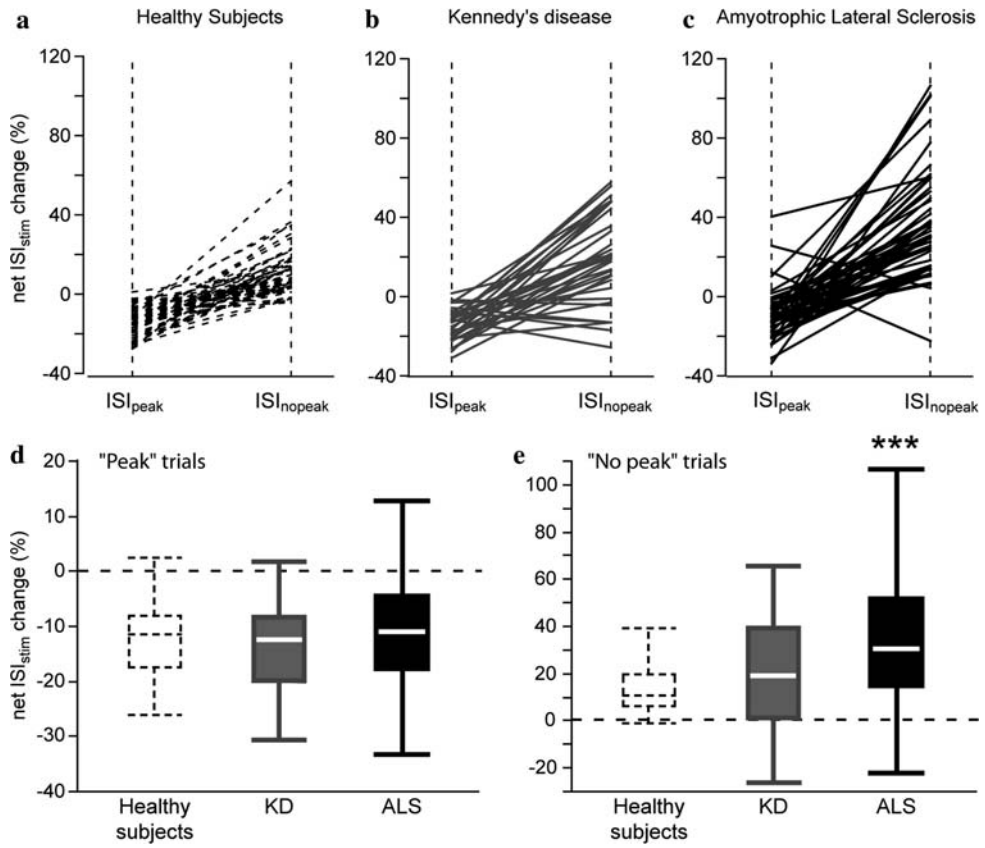

Fig. 5 Excitatory versus inhibitory effects of TMS were assessed by comparing the changes in the ISI during which TMS was delivered (ISI_{stim}), depending on the presence or absence of excitatory responses to TMS ("peak" trials vs. "no peak" trials). In each of the MUs tested in healthy subjects and patients with KD or ALS (**a**, **b** and **c**, respectively), the median values of the net changes in ISI_{stim} (referred to the corresponding $ISI_{control}$) obtained in the "peak" trials are connected to those obtained in the corresponding "no peak" trials. No consistent differences were observed upon performing the Kruskal–Wallis test to compare the net changes in ISI_{stim} obtained in the "peak" trials (**d**) across the populations of MUs tested in healthy subjects and patients with KD or ALS (*white*, *gray* and *black box plots*, respectively). By contrast, the net changes in ISI_{stim} associated with "no peak" trials (**e**) were abnormally marked in the ALS group (Kruskal–Wallis test, *** $P < 0.0001$)

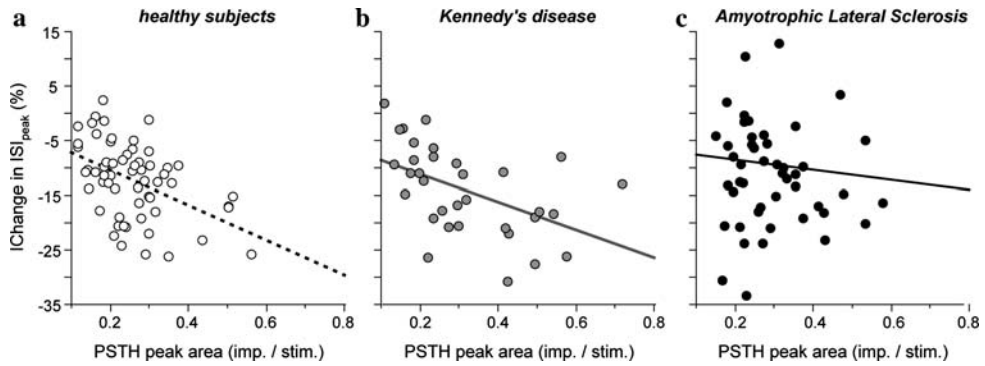

Fig. 6 Correlations between the indices reflecting the excitatory impact of TMS, based on PSTH and ISI analysis. The median value of the change in ISI_{peak} (ordinate) is plotted versus the corresponding PSTH peak area (abscissa) obtained with each MU tested in healthy subjects and patients with ALS or KD (**a**, **b** and **c**, respectively). In healthy subjects and patients with KD, very similar correlations were observed between the two indices. In ALS, this correlation was no longer present (**c**)

motor cortex of healthy subjects. Upon analyzing trials giving excitatory responses in the PSTHs separately, TMS was found to advance the first motor unit firing after the stimulation, and the resulting ISI shortening was found to covary with the amplitude of the PSTH peak. By contrast, in the trials with no excitatory responses, TMS was found

to delay the first firing time. The motor units tested on KD patients behaved very similarly, whereas those tested on ALS patients differed from those of healthy subjects in two ways: (1) the amounts of TMS-induced excitation, assessed in terms of ISI shortening and PSTH peak amplitude were no longer correlated, and (2) the TMS-induced inhibitory impact assessed in terms of ISI lengthening was significantly stronger. This effect was restricted to the first MU firing event occurring after the stimulation.

ISI-based versus PSTH-based analysis of TMS excitatory effects

In this study, MUs were selected from a previous database obtained from healthy subjects and patients with motor neuron diseases (Attarian et al. 2006, 2008), on the basis of the presence of an excitatory response to TMS in the PSTHs. In all three groups of subjects, the ISI-based analysis including all trials failed to show the presence of excitatory effects of TMS in the form of ISI shortening. The ISI shortening induced by TMS on the next MU firing event was clearly apparent, however, upon sorting out the "peak" trials contributing to the PSTH excitatory responses. Under these conditions, the amount of the ISI shortening induced by TMS was similar in all three groups of subjects. In both healthy subjects and KD patients (in whom the cortico-motoneuronal pathway is intact), a significant correlation was found to exist between the effectiveness of the TMS-induced monosynaptic EPSPs in terms of the PSTH peak amplitude and the resulting ISI shortening. This correlation was no longer present in ALS patients. This suggests that the integrative properties of the motoneurones tested on these patients may have been functionally impaired. Another possibility might be that the amplitude of the TMS-induced EPSPs may have varied across trials. Depending on the EPSP amplitude, TMS would in this case trigger spikes after a wide range of post-spike intervals, leading to various ISI shortening values. This would weaken the correlations between the changes in firing time (ISI-shortening) and the changes in firing probability (PSTH peak amplitudes). The fact that these correlations were lacking only in ALS patients indicates that the ISI-based analysis may uncover the occurrence of subtle abnormalities which are not detectable in the PSTHs.

TMS-induced inhibitory effects on single MU firing activity in healthy subjects

Upon pooling all the TMS trials, there was no evidence of any change in the timing of the first firing after the stimulation. This suggests that the excitatory and inhibitory effects of TMS expected to occur on the basis of previous studies (Calancie et al. 1987; Boniface et al. 1994; Garland and Miles 1997) were balanced out. Net inhibitory effects were observed, however, on the second firing event after the stimulation. This effect does not seem to be specific to cortical stimulation, however, as it has been reported to follow Ia EPSPs producing similar PSTH peaks (Mattei and Schmied 2002). In both cases, the lengthening of the second ISI after the cortical and Ia stimulation was particularly prominent in the "peak" trials. This nonspecific effect could therefore reflect at least partly the occurrence of AHP summation after ISI_{stim} shortening (cf. Mattei and Schmied 2002).

In the "no peak" trials, the inhibitory effects of TMS on the subsequent MU firing were clearly present in all three groups of subjects. This was observed, while consistent MEPs were detected by averaging the EMG activity of the muscle tested. The ISI lengthening observed in the "no peak" trials may therefore reflect the contribution of spinal inhibitory processes triggered by the excitatory responses of other MUs, such as recurrent inhibition and/or processes associated with muscle twitch, such as Ib autogenic inhibition and Ia excitation release processes. It might also reflect a shunting effect of the cortico-motoneuronal EPSP occurring too early in the AHP to advance the subsequent threshold crossing. If this were the case, a similar ISI lengthening should be expected to occur after any monosynaptic EPSPs, whatever their origin. This hypothesis can be ruled out, however, since no ISI lengthening has ever been observed in the previous study in which juxta-threshold monosynaptic EPSPs generated by tendon taps yielded PSTH peaks of a similar amplitude to those recorded here (Mattei and Schmied 2002). Consequently, the delayed motoneurone firing induced by TMS in the "no peak" trials must involve mechanisms resulting specifically from the motor cortex stimulation applied.

TMS delayed the next MU spike when the stimulation was delivered as early as 20 ms after the previous spike, that is, during the first half of the motoneurone AHP, as previously observed (Calancie et al. 1987; Boniface et al. 1994; Garland and Miles 1997). This suggests the involvement of late and/or long lasting inhibitory events affecting the next crossing of the firing threshold, which can be expected to occur 40–110 ms later in the case of motor units discharging ISI values ranging from 60–130 ms, as in the present study.

The first possible explanation is the occurrence of motoneurone IPSPs induced by motor cortex stimulation. In primates, besides innervating motoneurones monosynaptically, collaterals originating from the same cortico-spinal axons have been found to innervate Ia interneurons (Jankowska et al. 1976). Intracellular recordings on monkey motoneurones have revealed that the monosynaptic EPSPs generated by applying electrical microstimulation to the motor cortex were followed by IPSPs after a disynaptic

delay in most of the motoneurones tested (Lemon et al. 1987). Assuming that TMS induces a similar combination of EPSPs followed by IPSPs in human motoneurones, the IPSPs might delay the next spike whenever the stimulation is delivered too early during the AHP for the EPSPs to advance the next crossing of the firing threshold, as observed in the present study. In these conditions, the IPSPs induced by a stimulus as early as 20 ms after a spike would have to last long enough to affect the subsequent firing event, which can occur anything up to 100 ms later. This idea does not fit the rather short duration of Ia IPSPs (Jankowska et al. 1976), but it does not rule out the contribution of other longer lasting IPSPs.

Another possible explanation for the prolonged inhibitory effects of TMS on motor unit firing activity is that of a transient withdrawal of the motoneurone's next excitatory drive (i.e., disfacilitation), as previously suggested (Boniface et al. 1994; Davey et al. 1994). This withdrawal might involve cortical inhibitory processes, recurrently activated by collaterals of the pyramidal tract capable of shutting down the cortical contribution to the motoneurone excitatory drive most effectively (Trevelyan and Watkinson 2005). This could also involve the activation of feed-forward inhibitory pathways controlling the transmission of cortico-spinal and proprioceptive excitatory inputs to motoneurones via propriospinal relays (Nicolas et al. 2001; Nakajima et al. 2000). The possibility that the spinal presynaptic inhibitory networks modulating the effectiveness of motoneurone excitatory proprioceptive assistance may be involved seems quite unlikely, as TMS has been found to reduce spinal presynaptic inhibition (Valls-Sole et al. 1994; Iles 1996).

TMS-induced inhibitory effects in patients with motoneurone diseases

The MUs tested in KD and ALS patients showed abnormally large macro-MUPs and produced abnormally large twitch forces indicating that in both groups re-innervation had occurred subsequent to motoneurone death. The effectiveness of the MU electromechanical coupling given by the ratio between the twitch amplitude and the macro-MUP area remained normal, indicating that functional compensation was still efficient as observed previously with MUs still responsive to TMS in ALS patients and with all MUs tested in KD patients (Attarian et al. 2006). Another particularity of the MUs tested in both groups of patients was their abnormally high discharge variability (cf. Attarian et al. 2006). Despite these common features, MUs tested in KD behaved similar to those tested in healthy subjects with regard to the inhibitory effects of TMS, unlike those tested in ALS. The fact that the inhibitory gain was not observed in KD patients indicates that the loss of spinal motoneurones common to both diseases was not a prerequisite for these abnormalities to occur.

In line with previous studies, showing that the inhibitory response rates increased in ALS patients' PSTHs, (Awiszus and Feistner 1993; Mills 1995; Attarian et al. 2006), the inhibitory effects of TMS on the next MU firing time were found to be enhanced in these patients. This contrasts strongly with the decrease in early and late intracortical inhibition, observed in ALS using TMS paired-pulse procedures (Yokota et al. 1996; Ziemann et al. 1997; Salerno and Georgesco 1998; Zanette et al. 2002; Vucic and Kiernan 2006). The methods differ greatly, however, the rather long duration of the inhibitory effects induced by TMS on motoneurone firing behavior is reminiscent of the time course of long-latency intra-cortical inhibition observed in paired pulse TMS studies on ALS patients. In these studies, however, the TMS intensities ranged from 120 to 130% of the resting threshold values (Salerno and Georgesco 1998; Zanette et al. 2002). These intensities are much higher than the juxta-threshold intensity used in the present single motor unit study. Moreover, the tonic activity of the cortical and/or spinal pre-motoneuronal afferent networks is expected to differ, depending on whether the subjects are at rest as in the paired-pulse studies or performing voluntary contractions, as in the present study. Another difference is that in TMS paired-pulse studies, the effects of the conditioning pulse are assessed from the size of the test MEP, taken to reflect the effectiveness of the fast cortico-motoneuronal pathway alone; whereas in the present single motor unit study, the single TMS pulses used were liable to affect various components of the motoneurone voluntary drive, including fast and slow cortico-motoneuronal monosynaptic pathways as well as non-monosynaptic pathways. Finally, one intriguing fact about the paired-pulse method is its ability to show up very similar alterations in a wide range of pathologies, which raises questions about its interpretation (Chan et al. 2002). By contrast, the inhibitory abnormalities observed in the present study were restricted to the ALS group.

The motor units tested in healthy subjects and patients with ALS or KD produced similar excitatory responses, and no consistent differences in PSTH peak amplitude or ISI shortening were observed between the three groups. Consequently, the stronger TMS-induced inhibitory effects detected in ALS patients cannot be ascribed to a loss of excitation. Moreover, the fact that this was observed in the presence of apparently normal excitatory responses suggests that the inhibitory gain might be an early event in ALS. Interestingly, in a longitudinal paired-pulse study, the decreases in TMS-induced short-latency and long-latency intra-cortical inhibition were reported to occur only in the later stages of ALS (Zanette et al. 2002). The loss of cortical inhibitory interneurons observed post-mortem in ALS

patients (Nihei et al. 1993; Maekawa et al. 2004), thought to possibly promote excito-toxic processes, might therefore be associated with the final stages of the disease.

As discussed above, the ISI lengthening induced by TMS may reflect the action of IPSPs reported to follow the cortico-motoneuronal EPSPs in response to motor cortex stimulation (Lemon et al. 1987). In this case, the greater ISI lengthening observed here in ALS patients may reflect an enhancement of spinal inhibitory transmission. The best known inhibitory interneurons in the spinal cord are the glycinergic and GABAergic Renshaw cells mediating the recurrent inhibition of the spinal motoneurones and the glycinergic Ia interneurons mediating reciprocal inhibition between pools of antagonist muscles (Rekling et al. 2000). EMG studies have shown that the efficacy of the recurrent inhibition as well as that of the presynaptic inhibition tested at rest, are reduced in ALS patients (Raynor and Shefner 1994; Drory et al. 2001). The effectiveness of Ia reciprocal inhibition in ALS has not yet been investigated to our knowledge. As far as the hyper-excitability hypothesis is concerned, it is worth noting that a gain in glycinergic transmission might promote hyper-excitability by enhancing glutamatergic transmission (Plaitakis 1990).

As suggested above, TMS might also act through cortical or sub-cortical inhibitory networks and transiently decrease the motoneurone excitatory drive. The enhanced inhibitory effects of TMS on the motor unit activity observed in ALS patients may therefore reflect either greater excitability or greater effectiveness of the cortical or sub-cortical inhibitory networks down-regulating the motoneurone excitatory drive during voluntary contractions and/or greater susceptibility of the motoneurones to a transient drop in the excitatory drive.

Whatever the mechanisms involved, the increased inhibition of single motor unit activity induced by TMS in ALS patients counter-balances the decrease reported to occur in paired pulse studies on resting patients. The possible contribution of inhibitory loss to the physiopathology of ALS therefore needs to be reconsidered. In light of the present data, it seems unlikely that inhibition loss may promote excito-toxicity in ALS.

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