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RESEARCH ARTICLE

Superconditioning TMS for examining upper motor neuron function in MND

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Abstract

We used transcranial magnetic stimulation (TMS) of motor cortex, including a novel four-pulse *superconditioning* (TMSsc) paradigm, in repeated examinations of motor-evoked potentials (MEPs) in eight subjects with motor neuron disease (MND), including seven with amyotrophic lateral sclerosis (ALS). The goals were: (1) to look for evidence of cortical hyperexcitability, including a reduction in short-interval intracortical inhibition (SICI); and (2) to examine the utility of using TMSsc for quantifying upper motor neuron function during MND progression. Testing of abductor pollicis brevis (APB) and tibialis anterior (TA) muscles bilaterally was carried out every 3 months in MND subjects for up to 2 years; results were compared to those from a cohort of 15 control subjects. Measures of SICI were not significantly different between control and MND subjects for either APB or TA muscles. Other measures of cortical excitability, including TMS threshold and MEP amplitude, were consistent with *lowered* cortical excitability in MND subjects. Certain combinations of superconditioning TMS were capable of causing stronger inhibition or facilitation of MEPs compared to dual-pulse TMS, for both APB and TA target muscles. Moreover, there were multiple cases in which target muscles unresponsive to strong single-pulse TMS, whether at rest or when tested with an active contraction, showed an MEP in response to TMSsc optimized for facilitation. Our findings suggest that a multi-faceted neurophysiologic protocol for examining upper motor neuron function in MND subjects might benefit from inclusion of TMSsc testing.

Keywords Transcranial magnetic stimulation · TMS · Motor-evoked potential (MEP) · Motor neuron disease (MND) · Amyotrophic lateral sclerosis (ALS) · Motor cortex · Excitability · Upper motor neuron · Short-interval intracortical inhibition (SICI)

Introduction

Motor neuron disease (MND), and its more specific phenotype amyotrophic lateral sclerosis (ALS) is characterized by pathology within both upper and lower motor neurons of central motor pathways (Brooks et al. 2000). Traditional EMG/nerve conduction testing provides objective and reliable evidence for lower motor neuron involvement, once denervation/reinnervation of limb or bulbar muscles has ensued. However, there is no equivalent neurophysiology measure of upper motor neuron function that is widely considered to be both objective and reliable for diagnosing and/or following the progression of ALS (Turner et al. 2009; Mitsumoto et al. 2014; Bakkar et al. 2015).

Attempts to define abnormalities in central motor conduction in persons with ALS using non-invasive single-pulse transcranial magnetic stimulation (TMS) were largely unsuccessful (Schriefer et al. 1989; Enterzari-Taher et al. 1997; Mills 2003), in part because of the high variability in the amplitude of the resultant motor-evoked potential (MEP) (Bickford et al. 1987; Berardelli et al. 1991; Schady et al. 1991; Brasil-Neto et al. 1992; Kiers et al. 1993). Dual-pulse TMS using a subthreshold (i.e., conditioning) and suprathreshold (i.e., test) paradigm revealed both inhibitory and

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excitatory effects of the conditioning pulse on motor cortex excitability, depending on the interval between the two pulses. Very short intervals (1–5 ms) caused attenuation of the MEP, termed *short-interval intracortical inhibition* (SICI) (Kujirai et al. 1993; Di Lazzaro et al. 1998). Longer intervals (10–25 ms) resulted in a *larger* MEP, an effect termed *intracortical facilitation* (ICF) (Ziemann et al. 1996).

Reports of reduced SICI in persons with ALS are plentiful (Yokota et al. 1996; Ziemann et al. 1997; Caramia et al. 2000), attributed to loss of inhibitory cortical interneurons due to excitotoxicity (Enterzari-Taher et al. 1997; Ziemann et al. 1997; Stefan et al. 2001). Extending this argument, some groups have asserted that disruptions in SICI reflect a *hyperexcitable* motor cortex (Eisen et al. 1993; Ziemann et al. 1997). However, alterations in SICI have been reported in a host of disorders affecting central motor systems beyond ALS (Ziemann et al. 1997; Attarian et al. 2009), suggesting this is a non-specific effect. Finally, evidence of an *increase* in central inhibition in ALS was derived from TMS studies of motor unit recruitment (Schmied and Attarian 2008).

On the basis of these uncertainties, there were two goals in this study. First, could we replicate the findings of *hyperexcitability* in the motor cortex of persons with MND/ALS, using traditional single- and dual-pulse TMS measures? Second, would our recently described advantages of a novel form of *four-pulse* TMS for examining inhibition and excitation in the motor cortex in control subjects (Calancie et al. 2018) carry over to a cohort of subjects with MND/ALS? This novel approach uses a three-pulse conditioning train and the resultant *temporal summation* of synaptic input to enhance the conditioning effect; we have termed this approach *superconditioning*, to differentiate it from the traditional single-conditioning pulse used in dual-pulse TMS.

Methods

We studied eight subjects with MND (60.0 ± 7.0 years; Table 1). Seven had a formal diagnosis of ALS, and one was diagnosed with Progressive Muscular Atrophy (PMA). For comparison, we studied 15 control subjects (55.2 ± 4.5 years), whose responses to superconditioning TMS (TMSsc) have been presented elsewhere (Calancie et al. 2018). All subjects provided their informed consent for this IRB-approved protocol, and also filled out a TMS-screening tool (Keel et al. 2001). Subjects with MND were recruited from a tertiary care facility. The Revised El Escorial Airlie House (Brooks et al. 2000) and Awaji (de Carvalho et al. 2008) criteria were used for diagnosis. All patients underwent EMG/nerve conduction study of

Table 1 Subject demographics

Subject#	Sex	Age (years)	Type	Disease dur (m)	ALSFRS-R		# of evals
					(Initial)	(Final)	
1	M	63	PLS	102	40	41	3[a]
2	M	51	Bulbar	20	37	31	4[b]
3	M	70	Limb—UMN	36	39	37	7[a]
4	M	54	Bulbar	29	35	27	8[a]
5	M	71	Limb—LMN	108	43	40	5[a]
6	M	59	PMA	156	40	41	5
7	F	60	Bulbar	13	42	35	3
8	F	58	Limb—LMN	22	40	27	6[a]

Age (years) at initial evaluation. Disease duration (months) from earliest symptom onset to first study evaluation. ALSFRS-R scores at initial and final study evaluations. Number of evaluations carried out for each subject. At their initial lab testing, all subjects had a formal diagnosis of ALS, with the exception of subject #6. In his case, he underwent clinical examination ~ 6 m before his final lab visit, and at that time still did not meet the Revised El Escorial diagnostic criteria for ALS (Brooks et al. 2000), and spinal muscular atrophy was ruled out (de Carvalho et al. 2007). At study enrollment, subject #1—who had been stable for many months—showed a decline with significant lower motor neuron involvement, and was, therefore, reclassified as PLS with progression to ALS. Three subjects ('Bulbar') were initially diagnosed with Progressive Bulbar Palsy presenting with dysarthria and dysphagia. Of these, subjects #2 and #4 were anarthric at screening, and subjects #4 and #7 developed severe dysphagia requiring feeding tube placement (between visit #2 and #3 for both). For subject #3, genetic testing for Hereditary Spastic Paraplegia revealed no evidence for genetic mutation. Four subjects (number 2, 4, 6, and 7) have died at this writing

Primary diagnosis ('type') was within the spectrum of *MND* motor neuron disease, *PLS* primary lateral sclerosis, *Bulbar* Progressive Bulbar Palsy, *PMA* Progressive Muscular Atrophy, *ALS* (limb onset, upper motor neuron [limb—UMN] or lower motor neuron [limb—LMN] predominant)

[a]One evaluation was missed, so that ~ 6 months elapsed from one evaluation to the next

[b]Three contiguous evaluations were missed, so that ~ 12 months elapsed from one evaluation to the next

bilateral upper and lower limbs. Upper motor neuron findings included Hoffman sign, crossed adductor response, increased tone, pseudobulbar affect, brisk jaw jerk, and palmomental reflexes. Lower motor neuron findings of severe weakness, muscle atrophy, and fasciculations also included EMG signs of active and chronic denervation/reinnervation in multiple upper and lower limb muscles, with particular attention paid to first dorsal interosseous and tibialis anterior. Both clinical and EMG studies were performed by the same physician experienced in MND (EY).

Target muscles and EMG

We recorded EMG from the abductor pollicis brevis (APB) and tibialis anterior (TA) bilaterally, using surface electrodes (Conmed Cleartrace). EMG was displayed visually on a large (42″) ceiling-mounted monitor, and was simultaneously passed through mixers for audible broadcast of individual target muscles, so that even when the screen was 'frozen' to show a stimulus-triggered event, muscle activity could still be monitored. We also recorded EMG from wrist extensors and abductor hallucis muscles bilaterally, allowing us to monitor in real-time neighboring muscles for signs of 'late' post-TMS activity that might presage spreading cortical afterdischarge. Signals were digitized and stored for later analysis (Spike2; Cambridge Electronics).

Transcranial magnetic stimulation

A Magstim 'Pyramid' stimulator consisting of four separate Magstim 200 magnetic stimulators, interconnected with three Magstim BiStim devices, allowed delivery of as many as four pulses through the stimulating coil. For APB, a round coil placed at the vertex was used for selective stimulation of either the left- or right-side motor cortex, depending on what side of the coil was facing up. For TA a double-cone coil placed at the vertex was used. Pulses were monophasic, with current direction over the targeted motor cortex from posterior to anterior in all cases. The *hotspot* was established and a mark was placed on the scalp for consistent repositioning of the hand-held coil for each target muscle (Calancie et al. 2018).

Three types of TMS inputs were used: (1) single pulse; (2) dual pulse (*conditioning-test*; C-T) with either 3 ms (SICI) or 10 ms (ICF) between pulses; and (3) four pulse (*superconditioning-test*; SC-T). We examined 12 unique combinations of SC-T inputs, by varying the interpulse interval (IPI) between the three SC pulses (fixed at either 1, 3, or 6 ms), and by varying the interval between the last SC pulse and the test pulse (the *test* interval; either 1, 3, 10, or 25 ms). We usually applied five trials of the same combination for averaging, but reduced this to three or four trials when responses were *much* larger than those to single-pulse TMS and causing the subject considerable discomfort; this occurred only for certain TMSsc inputs. We acknowledge this is a relatively small number of trials per condition, but opted for this approach to allow us to examine more target muscles during each study session. For a given target muscle during a particular trial, all comparisons between single, dual and four-pulse inputs always used the same *test* pulse intensity. All adjustments of stimulus intensity and interpulse intervals were done manually by a second investigator (i.e., one person was holding the coil and triggering stimulus deliveries, the other was adjusting stimulator intensity and BiStim interpulse interval settings). The person holding the coil was charged with ensuring that TMS delivery occurred at a time when the target muscle was quiet, or as quiet as possible. If a subject had trouble 'quieting' a muscle, we found that slightly changing the angle of the exam chair, repositioning the test limb, or using a pillow to support the limb was often helpful in reducing or eliminating background activity. Note that throughout this report, stimulus intensities are stated in terms of the percentage of the maximum stimulator output.

Procedure

Subjects made a brief, maximal voluntary contraction (MVC; isometric) in each target muscle, as we have shown in persons with incomplete spinal cord injury a good relationship between MVC and isometric muscle force (Calancie et al. 2001). Thresholds to single-pulse TMS with the muscle at rest (RT_1) were established (Calancie et al. 2018). This value was used to define the intensity of the test pulse (set to $1.2 \bullet RT_1$) for all inputs, and the conditioning pulse (set to $0.8 \bullet RT_1$) for dual-pulse inputs. As reported by many other groups, some target muscles were found to be unresponsive to very strong (75–80%) single-pulse TMS. We rarely used intensities > 80% of maximum because they caused considerable discomfort, especially when using the leg coil.

Once we began testing SC inputs (roughly one-third way into our study), we also established the resting thresholds to three-pulse stimulation (RT_3; all three pulses of identical intensity), for each of three different IPI values (1, 3, and 6 ms). The intensity of the SC pulses was set to $0.75 \bullet RT_3$ for more than 90% of all trials; we initially used an SC intensity of $0.8 \bullet RT_3$ for MND subjects, but when we saw these conditioning pulses alone (i.e., without a follow-on test pulse) sometimes caused an MEP we lowered the intensity to $0.75 \bullet RT_3$ for all subsequent trials.

Responses to single-pulse TMS were collected with the muscle at rest (MEPr) and when actively contracting (MEPa). We also tested dual-pulse inputs for SICI and ICF. The order of this testing was randomized within and between subjects. Testing of TMSsc was usually done with the target muscle at rest. However, if a target muscle was unresponsive to single-pulse TMS, and if the subject could tolerate the use of high stimulus intensities, we tested SC inputs while the subject was actively contracting the target muscle, using a test pulse intensity of 75–80% of maximum. Of course these trials were excluded from measures of RT_1, MEPr, SICI, or ICF, but still provided data on MEP latency and (sometimes) MEPa.

The order of the 12 unique TMSsc patterns applied was varied randomly. At various times during delivery of these inputs, we interposed additional blocks of single-pulse (for MEPr) and dual-pulse (for both SICI and ICF) TMS inputs. All TMS inputs, regardless of the pattern used, were delivered no faster than once every 5–8 s; this variance was intended to minimize a subject from anticipating the next stimulus by 'tensing up'. Longer gaps of 12–20 s occurred between blocks when stimulus patterns (pulse intensities; interpulse intervals) were being adjusted.

Testing of the left- and right-side motor cortex hand area was done separately. However, TMS using the double-cone coil activates both leg motor cortices simultaneously. In the cases when the L and R TA thresholds were identical, we tested both muscles at the same time. Otherwise we tested left- and right-side TA independently. All subjects were tested in the same room, whose temperature was set to 75° F.

Analysis

EMG records were offset to '0' volts and rectified. Maximum voluntary contraction (RMS magnitude) was measured from a 1-s period of maximal interference pattern, defined by cursors. Evoked responses were averaged point-by-point for each block of stimuli applied. Cursors were used to define background (200 ms pre-stimulus) and post-stimulus periods of excitation and/or inhibition. Background activity was subtracted from the excitatory (or inhibitory) short latency response to arrive at a *net* response. The period from 0 ms to the beginning of any excitatory (or inhibitory) response was excluded, to avoid inclusion of stimulus artifact(s). Cortical response latency was measured from the time of the test pulse (i.e., disregarding conditioning pulses) to the MEP onset. All analysis was done manually, without any automation, computer algorithm, or *Spike2* script.

Each of the multiple MEPr values gathered when examining a given target muscle with various dual pulse and TMSsc inputs was averaged with other MEPr values, and this resultant magnitude was used for normalization of all other TMS input patterns. In the cases when a target muscle was unresponsive to strong (typically 80%) TMS, for the purposes of RT_1 statistical analysis a value 5% greater than the strongest stimulus intensity tested—and found to be ineffective for eliciting an MEP—was used.

Statistics

Generalized linear mixed model (GLMM) was used as a general framework to account for correlations of repeated measures from the same subject. The visit number was used as a fixed effect to evaluate the trend of each outcome measure of interest. A two-way repeated measures analysis of variance (ANOVA) was used to evaluate the effect of IPI and test intervals on MEPs to dual-pulse inputs (SICI and ICF), along with their interaction. Since both IPI and its interaction with test intervals were deemed non-significant in both control and ALS subjects, only test intervals along with disease status were included in the ANOVA models to evaluate the difference between controls and ALS subjects at each test interval value. Appropriate contrasts were used to evaluate the difference between MEP measures of SC-T groups and SICI or ICF responses. One-way repeated measures ANOVA analyses were used to assess the difference between the APB and TA muscles in terms of MEPr, MEPa, MVC, RT_1, latency, SICI, ICF, MEPa/MEPr, MEPa/MEP_{SC} and MEPsc/MVC, and to assess the difference of the stimulus input threshold needed for three-pulse trains at fixed interpulse intervals of 1, 3, and 6 ms, followed by paired *t* tests as necessary. Pairwise comparisons were adjusted by Tukey's method when applicable. The analysis used SAS PROC mixed with the restricted maximum likelihood method for model fitting and compound symmetry as the correlation structure for measures from the same subject.

Results

Subject characteristics

Table 1 summarizes MND subject demographics. While we endeavored to test each subject at approximately 3-month intervals, this sometimes did not happen. We did not encounter any unexpected adverse events associated with delivery of traditional or superconditioning TMS in any of our subjects, who all agreed to multiple test sessions using TMSsc.

Voluntary contraction, single-pulse, and dual-pulse TMS

Figure 1 includes findings from repeated evaluations of MVCs (top row), single-pulse TMS, and dual-pulse TMS (for SICI) in our MND subjects. Table 2 includes results from MND subjects and control subjects along with pairwise comparisons; those with significant differences are shaded. Also included in Table 2 are the limits to these

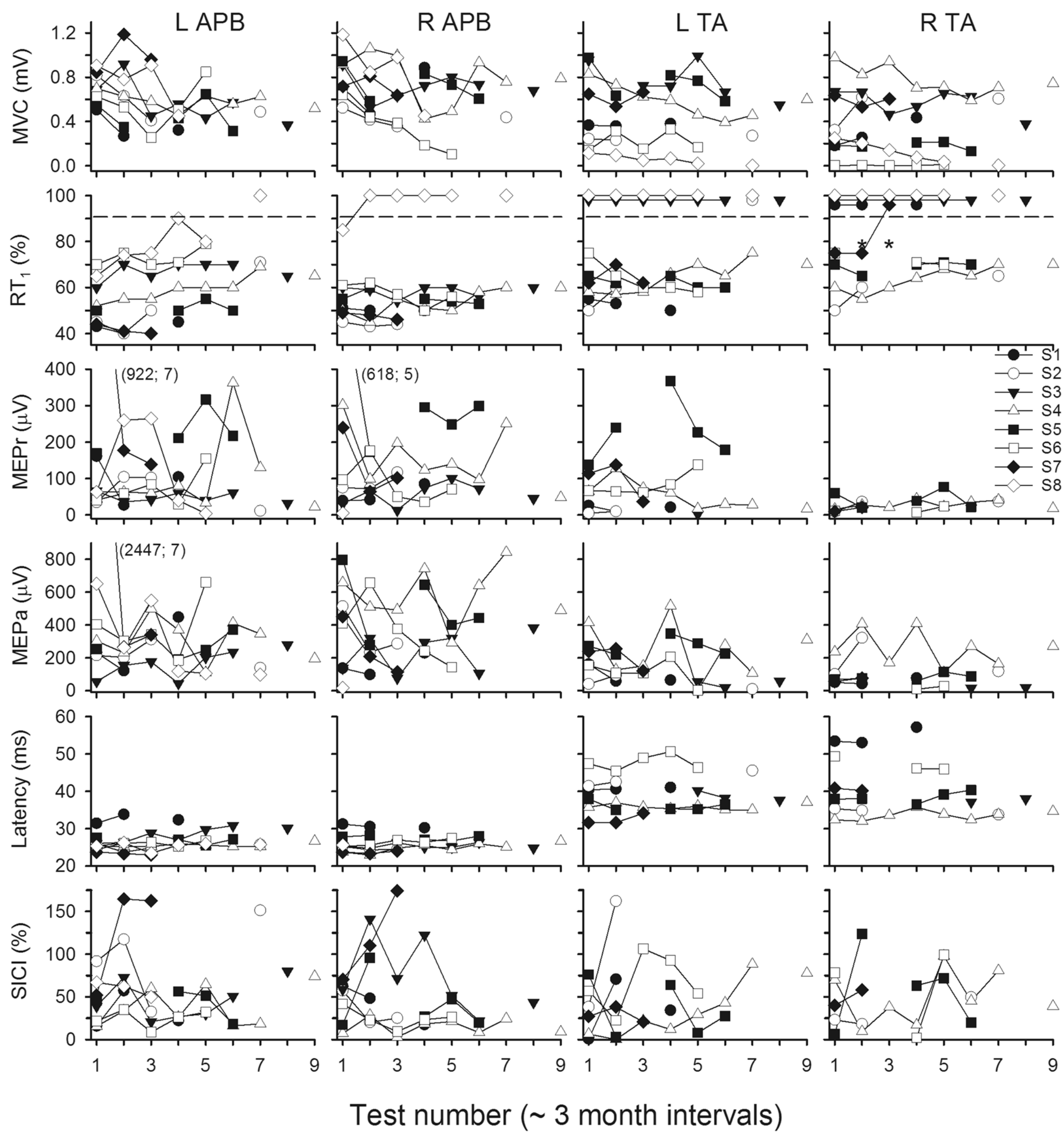


Fig. 1 Multiple individual data points from repeated examinations of all eight MND subjects for each of four target muscles as indicated. Legend for subject identifiers: row 3, far right. Approximate period from one examination to the next: 3 months. Top row: maximum voluntary contraction (MVC), showing EMG interference pattern (RMS) over a 1-s period of isometric contraction effort. Row 2: resting muscle threshold to single-pulse TMS (RT_1). If a target muscle did not respond at the highest intensity tested (usually 80% of stimulator maximum), data points were plotted above the dashed horizontal line; in these cases, there is usually no corresponding data point for that target muscle on that testing session for data in rows 3–5 (unless there *was* an MEP during active contraction). Row 3: magnitude of the MEP elicited with the muscle at rest, using a stimulus intensity 20% stronger than the measured RT_1 value. Several data points exceeded the chosen *y*-axis range; these points are indicated by an unconnected line, with the magnitude of the measured response, followed by the subject number, adjacent to that line. Row 4: magnitude of the MEP elicited with the muscle actively contracting (at ~ 10% of maximum), using a stimulus intensity 20% stronger than the measured RT_1. Row 5: minimum onset latency of the MEP to the TMS test pulse. Row 6: short-interval intracortical inhibition (SICI), normalized as a percentage of the MEP to single-pulse TMS at $1.2 \bullet RT_1$. For all panels, note that data points without connecting lines indicate a gap in consecutive test sessions. Another reason why a data point might not be evident within a given panel is that the target muscle was not examined on that test day, due to time constraints. Note that some points were shifted up or down by up to 2%, when they were obscuring other points with identical values, to enhance visibility

Table 2 Mean (± SD) values for certain contraction and TMS-specific measures for APB and TA target muscles from control (Calancie et al. 2018) and MND subjects

	Control		MND		Limits	
Measure	**APB**	**TA**	**APB**	**TA**	**APB**	**TA**
MVC (mV)	0.95 ± 0.26	0.51 ± 0.22	0.63 ± 0.23 p = 0.0009	0.43 ± 0.29 p = 0.36	< 0.5	< 0.3
RT_1 (% max)	46.6 ± 7.5	54.5 ± 9.0	59.8 ± 13.0 p = 0.0157	72.2 ± 11.5 p = 0.0018	≥ 60	≥ 65
RT_3 1 ms IPI	37.8 ± 6.9	44.5 ± 7.0	47.2 ± 9.1 p = .0024	52.9 ± 6.2 p = .0086	NA	NA
RT_3 3 ms IPI	37.6 ± 6.8	43.8 ± 7.5	47.9 ± 9.6 p = .0008	52.3 ± 6.5 p = .0076	NA	NA
RT_3 6 ms IPI	38.9 ± 6.6	41.7 ± 7.0	47.9 ± 8.5 p = .0035	49.2 ± 5.1 p = .0203	NA	NA
MEPr (µV)	190 ± 240	40 ± 40	127 ± 140 p = 0.56	61 ± 72 p = 0.46	≤ 20	≤ 10
MEPa (µV)	830 ± 650	270 ± 160	347 ± 310 p = 0.0232	160 ± 122 p = 0.0174	≤ 100	≤ 50
Latency (ms)	23.4 ± 1.4	32.4 ± 1.4	26.4 ± 2.4 p = 0.0014	38.8 ± 6.3 p = 0.0017	> 26.2	> 35.2
SICI (%)	49.5 ± 47.6	37.3 ± 33.5	53.0 ± 49.3 p = 0.50	47.5 ± 36.3 p = 0.32	> 70%	> 70%
ICF (%)	133.6 ± 64.8	253.1 ± 176.2	226.9 ± 161.1 p = 0.0210	236.4 ± 116.9 p = 0.67	< 100%	< 100%
MEPa:MEPr	9.41 ± 13.25	8.12 ± 3.92	4.25 ± 3.66 p = 0.19	5.20 ± 4.62 p = 0.0408	≤ 1.5	≤ 2.5
MEPa:MEPsc	2.64 ± 2.67	2.39 ± 1.56	1.06 ± 0.60 p = 0.0220	1.52 ± 1.53 p = 0.11	≤ 0.60	≤ 0.90

For trials in MND subjects in which a target muscle did not respond to strong TMS (i.e., data points lying above the horizontal lines in Fig. 1, row 2), a value 5% higher than the strongest stimulus we tested was used for calculations of RT_1. We usually limited stimulus intensity to 80% of the device maximum during establishment of thresholds, to avoid excessive subject discomfort. Shaded cells indicate a significant difference between this measure in MND subjects and its counterpart in control subjects. Also included are the values (i.e., 'Limits') beyond which measures are considered to be abnormal. Limits for RT_3 values of different IPIs were not established, as they are largely defined by RT_1 values

values *outside of which* measures were considered abnormal. Abnormal findings are summarized later in "Results".

The MVC for most subjects (both control and MND) across all measures was usually higher in APB than in TA muscles. MVC values were lower in most MND subjects compared to controls; subject #4 was a notable exception to this generalization, as only 3 of his 32 measures of MVC fell below the MVC limits (from Table 2). MVC values trended down over time in most subjects with MND ($p = 0.0152$ and 0.0288 for APB and TA, respectively). Subjects with particularly low MVC values always showed marked clinical weakness in that target muscle.

Resting thresholds to single-pulse TMS (RT_1) showed a wide range in MND subjects (Fig. 1, row 2), often lying within the normal range during early visits, but all subjects showed an abnormally high RT_1 value in at least one target muscle by the time of their final evaluation. For group comparisons, MND subjects showed significantly higher RT_1 values in both APB and TA compared to controls (Table 2). There was a trend towards increasing RT_1 values over time. This trend was significant in both APB ($p = 0.0040$) and TA ($p = 0.0090$). None of the MND subjects had an RT_1 value for any evaluation that was equal to or lower than the lowest seen in control subjects for either APB (lowest in controls was 35%; $n = 2$) or TA (lowest in controls was 40%; $n = 3$).

For every control and MND subject tested, RT_3 values were always lower, regardless of whether an IPI of 1, 3, or 6 ms was used, than that subject's RT_1 value. Table 2 includes comparisons of mean RT_3 values for each IPI; in all cases, the mean was higher in MND subjects compared to controls for both APB and TA. Within MND subjects, RT_3 values for 1 vs 3 ms, 1 vs 6 ms, and 3 vs 6 ms IPIs were not significantly different for APB ($p = 0.7839$, 0.7704, and 0.9997, respectively), while for TA the RT_3 for 6 ms IPIs was significantly lower than that for 1 ms ($p = 0.0104$) and 3 ms ($p = 0.0299$), whereas the RT_3 using 1 ms and 3 ms IPIs was not significantly different ($p = 0.8477$). All comparisons were made following Tukey–Kramer adjustment.

The magnitude of the MEP tested at rest (MEPr; Fig. 1 row 3) was highly variable within and between MND subjects, and there were multiple examples of MEPr magnitudes smaller than those considered normal. However, differences in MEPr between control and MND groups were not significant for either target muscle (Table 2). There were two instances of a resting MEP magnitude well above other MEPr values in the MND cohort (see Fig. 1 caption). However, several MEPr values from the APB of control subjects were larger still. MEPr values in MND subjects did not show any obvious trend over time ($p = 0.26$ and 0.91 for APB and TA, respectively).

MEP magnitudes were much larger in MND subjects when making an active contraction (MEPa; 4th row of Fig. 1). One single response (left APB; visit #1; subject #7) was much larger than all other MEPa values from MND subjects; that data point is off the scale in Fig. 1 (row 4). The magnitude of MEPa was significantly larger in control subjects for both APB and TA muscles compared to MND subjects (Table 2). There were some cases in which there *was* an MEP to strong stimulation when an MND subject was making a background contraction, but no response when tested at rest. In some MND subjects, there was a trend of MEPa magnitudes to decrease over time, but overall differences were not significant in either APB ($p = 0.32$) or TA ($p = 0.43$).

MEP onset latencies (2nd row from bottom of Fig. 1) showed little variability from trial to trial in MND subjects. Only one subject (#1; filled circle) had latencies that were abnormally prolonged in all four target muscles; not coincidentally, he was the one subject with an initial diagnosis of PLS. Differences in MEP latencies between control and MND subjects were significant in APB ($p = 0.0014$) and TA ($p = 0.0017$). There was no significant change in MEP latencies over time (APB: $p = 0.09$; TA: $p = 0.77$).

The bottom row of Fig. 1 shows normalized SICI values across all trials. There was no significant difference in measured SICI between control and MND subjects for either APB ($p = 0.51$) or TA ($p = 0.32$; Table 2). From the literature, we considered a SICI value > 70% to be abnormal (i.e., test MEP magnitude within 30% of unconditioned MEPr, or even *larger than* MEPr). Based on this cutoff, many individual SICI measurements from our MND subjects resulted in values in the normal range for both APB and TA muscles. Comparison of the total *incidence* of abnormal SICI values between control (12 of 49; 24.5%) and MND subjects (29 of 119; 24.4%) revealed no difference ($p = 0.95$).

The mean ICF from TA was not significantly different between control and MND subjects (Table 2; $p = 0.67$). However, ICF seen in APB muscles of control subjects was significantly smaller than that seen in MND subjects (Table 2; $p = 0.0210$). Trials in which ICF failed to show facilitation of the test MEP (i.e. ICF < 100%) were considered abnormal. The incidence of abnormal ICF values in control subjects (10 of 49; 20.4%) was actually *higher* than that in MND subjects (8 of 118; 6.8%). Based on the GLMM analysis that accounts for correlations of repeated measures from the same subject, this difference was significant ($p = 0.0312$).

Superconditioning TMS; resting target muscle

For our subjects in whom SICI or ICF appeared to be abnormal, what effect did delivery of additional conditioning pulses—a *superconditioning* input—have on the test MEP? For control subjects, these data are presented elsewhere (Calancie et al. 2018). In an MND subject (#7), Fig. 2 shows examples of MEPs from her left APB in response to five different TMS inputs. The MEPr to a single 48% test pulse (Fig. 2a) serves as the basis for subsequent comparisons. A dual-pulse input for SICI resulted in *facilitation* of the test MEP (Fig. 2b), whereas an ICF-specific input led to *attenuation* of the test MEP (Fig. 2c). Both response patterns are considered abnormal. However, in this subject application of a superconditioning input using 28% conditioning pulses—a conditioning intensity *weaker* than that used for dual-pulse inputs—and a 1-ms test interval resulted in strong inhibition of the test MEP (Fig. 2d). Finally, an SC input using a 10-ms test interval led to a large test MEP (Fig. 2e), approaching the EMG magnitude seen during this subject's MVC (968 µV; top row of Fig. 1). Note the test pulse intensity (48%) was identical across all conditions shown in Fig. 2. Thus, the normal responses of MEP inhibition and facilitation emerged in this subject when a brief *train* of conditioning pulses (i.e. a *superconditioning* input) preceded the test TMS pulse, compared to her abnormal responses to traditional dual-pulse TMS (i.e., when using a single-conditioning pulse).

We examined 12 unique combinations of SC-T intervals for their effect on the normalized test MEP. Figure 3a shows that for APB, a 1-ms test interval was optimal for inhibiting the test MEP, regardless of whether the interval between SC pulses was 1, 3, or 6 ms. A test interval of 3 ms had either no appreciable effect on the test MEP (using SC pulses of 1 and 3 ms IPI) or resulted in a net *facilitation* of the test MEP (SC pulses of 6 ms IPI). Longer test intervals (10 and 25 ms) caused a pronounced *facilitation* of the test MEP, with 10-ms test intervals having the greatest effect. The dashed horizontal lines in Fig. 3a show the mean inhibition and facilitation seen with dual-pulse inputs for SICI (lower line) and ICF (upper line) in these subjects.

The patterns of inhibition and facilitation seen in APB with TMSsc inputs were similar in the TA (Fig. 3b). Very short (1 ms) test intervals were best for causing inhibition of the test MEP (provided SC interpulse intervals were 1 or 3 ms), while test intervals of 10 ms were best for causing facilitation of the test MEP, on average. Test intervals of 25 ms were also good for causing facilitation in TA, but were not more effective, on average, compared to the dual-pulse ICF input (the same was true for APB).

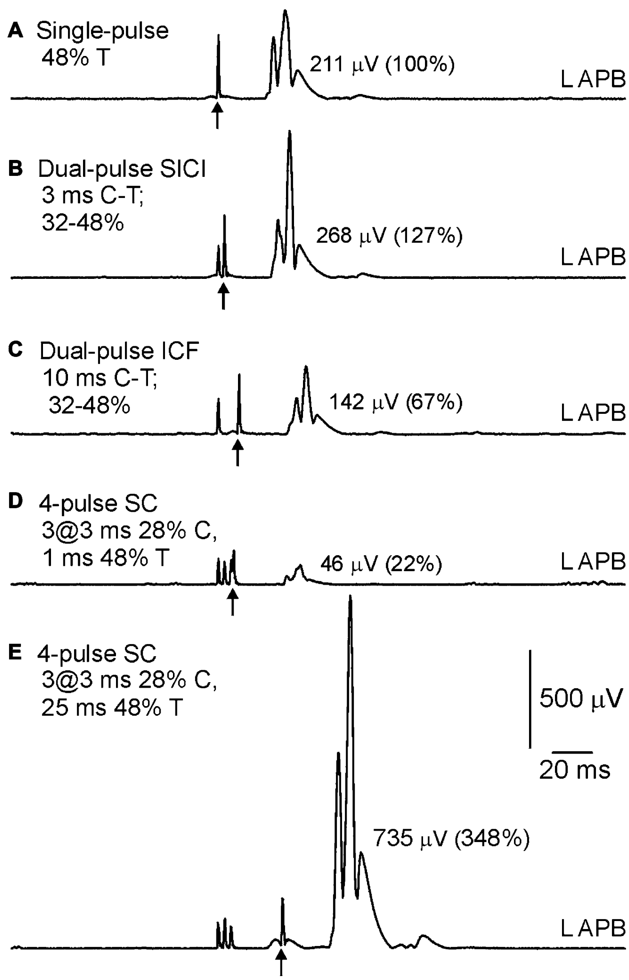


Fig. 2 Example of MEPs from subject #7, visit #3. **a** Average ($n = 5$) rectified EMG from left APB in response to single TMS pulses at 48% intensity (i.e., $RT_1 = 40\%$, conditioning and test intensities for dual-pulse inputs of 32% and 48%, respectively). Muscle was at rest at time of stimulation. This is the MEPr (unconditioned test response), with magnitude of 211 µV (note that because response magnitudes are based on RMS values between cursors, they are free of time units). All subsequent MEP magnitudes are listed and expressed as a percentage of this test response. In this and subsequent panels, the time of application of the 48% test pulse is indicated with a vertical arrow. **b** Average MEP to dual-pulse TMS for testing SICI, with conditioning-test interval (3 ms) and intensities (32 and 48%) as shown. **c** Average MEP to dual-pulse TMS for testing ICF. **d** Average MEP to the superconditioning pattern that returned the smallest response (46 µV) of the 12 patterns tested. Note the intensity of the superconditioning pulses (28%) is lower than that used in panels **b** and **c** for dual-pulse inputs, but the intensity of the test pulse (48%) is unchanged. **e** Average MEP to the SC input that caused the largest response. Time and magnitude calibration lines apply to all five panels

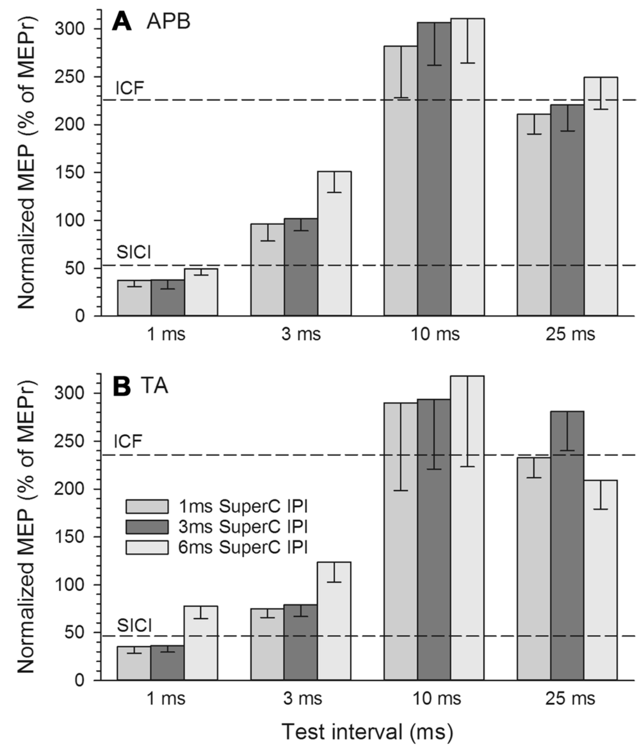


Fig. 3 Normalized average (− SE) MEP magnitude for each of the 12 superconditioning TMS patterns applied in MND subjects for APB (**a**) and TA (**b**) muscles. The average ICF and SICI magnitudes to dual-pulse TMS are indicated by dashed horizontal lines in each panel. Each group of three bars represents responses using test intervals of 1, 3, 10, or 25 ms, whereas the different shades of bars within each group represent different interpulse intervals (1, 3, or 6 ms) of the superconditioning pulses

The two-way repeated measures ANOVA assessing the impact of the superconditioning interval (1, 3, or 6 ms) and the test interval (1, 3, 10, or 25 ms) on the MEP magnitude suggested that in the TA muscles, the MEP magnitude was mainly determined by the test interval ($p < 0.0001$), and neither the interaction effect nor the effect of the SC interval was significant, with $p = 0.98$ and 0.25, respectively. A similar outcome was observed in the APB muscles, with the p values associated with the test interval, the SC interval, and their interaction, being < 0.0001, 0.35, and 0.22, respectively. Finally, we compared grouped findings with 1- and 3-ms test intervals (regardless of IPI) against SICI values, and findings with 10- and 25-ms test intervals (regardless of IPI) against ICF values. Only two such comparisons reached statistical significance: MEPs to 3-ms test intervals were found to be significantly *larger* than SICI values for both APB ($p = 0.0007$) and TA ($p = 0.0110$).

The significant effect of test interval and non-significant effect of SC interval (and its interaction with test intervals) on test MEP magnitude in both control (Calancie et al. 2018) and MND subjects caused us to restrict comparisons between control and MND subjects to only test intervals. Table 3 includes results of these pairwise comparisons; none were significantly different. Overall for both cohorts, 1-ms test intervals were most effective at causing

Table 3 Mean (± SD) values for normalized MEP magnitude averaged across the three superconditioning intervals (1, 3, and 6 ms) tested for APB and TA target muscles in control (Calancie et al. 2018) and MND subjects

Test interval	Control		MND	
	APB	TA	APB	TA
1 ms	30.5 ± 32.2	53.7 ± 62.1	41.6 ± 44.2 $p = 0.2196$	49.7 ± 45.6 $p = 0.9459$
3 ms	138.8 ± 116.7	137.5 ± 101.4	116.4 ± 105.0 $p = 0.3799$	92.5 ± 69.1 $p = 0.0596$
10 ms	232.5 ± 167.2	283.8 ± 269.8	299.8 ± 276.2 $p = 0.2775$	300.4 ± 391.6 $p = 0.3228$
25 ms	207.3 ± 166.4	247.1 ± 205.4	226.9 ± 157.4 $p = 0.6741$	240.9 ± 142.3 $p = 0.7767$

The p values for comparisons of each measure between control and MND subjects are also shown in the MND columns; none of the comparisons reached statistical significance. Instead, similar trends are seen in control and MND subjects in both muscle groups: 1-ms test intervals led to inhibition of the test MEP, while 10-ms test intervals were optimal for facilitation of the test MEP, on average

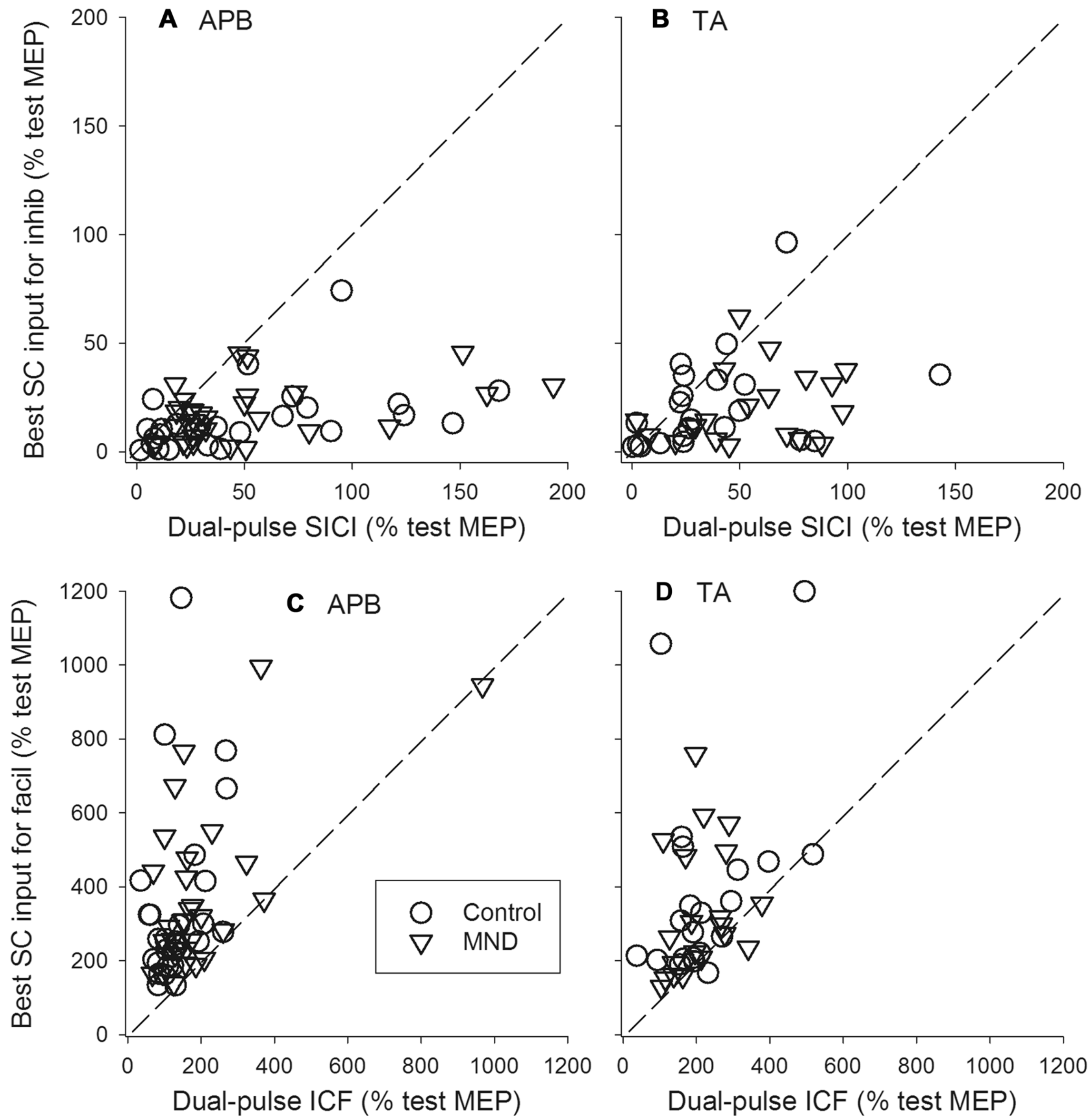
inhibition, and 10-ms test intervals were most effective at causing facilitation of the test MEP.

It is clear from examination of the dashed lines in Fig. 3 that the effects of dual-pulse TMS inputs for either inhibition (SICI) or facilitation (ICF) were nearly comparable to, on average, the best of the 12 superconditioning inputs applied, for both target muscles. However, on a *trial-by-trial* basis (i.e., looking at individual data points, not averaged data) we found that the optimal SC input almost always caused deeper inhibition (usually with short test intervals) compared to the SICI input, and greater facilitation (usually with long-test intervals) compared to the ICF-specific input. For inhibition, Fig. 4a (for APB) and b (for TA) show the *smallest* normalized MEP of the 12 unique SC inputs tested during each test session plotted against the dual-pulse SICI response. The great majority of data points lie *below* the dashed lines of identity, indicating that the test MEP following the optimal (for inhibition) superconditioning input was *smaller than* the test MEP following the single-conditioning pulse (the test pulse intensity was the same for both inputs). This was true for both control (unfilled circle; 25 of 27 comparisons [93% incidence]) and MND subjects (unfilled down-point triangle; 27 of 31 comparisons [87% incidence; $p = 0.0002$]) in APB. In the TA, there were fewer examples of this pattern in control subjects (13 of 22 comparisons [59% incidence]), whereas MND subjects showed a high probability (18 of 20 comparisons [90% incidence; $p < 0.0001$]) that the optimal SC-T pattern for inhibition would cause a smaller test MEP in the TA than the SICI input pattern.

Figure 4c, d plot the normalized MEP seen for the single SC input of the 12 combinations that caused the greatest *facilitation* against the same test muscle's normalized ICF-specific MEP. In these comparisons, most data points lie *above* the lines of identity, indicating that the optimal superconditioning input caused a larger test MEP than was seen for the ICF input. This was the case for all 27 comparisons in the APB of control subjects (100% incidence) and for 90% of comparisons in MND subjects (28 of 31 comparisons; $p = 0.0001$). In the TA, this pattern showed an incidence of 86% (19 of 22 comparisons) and 75% (15 of 20 comparisons; $p = 0.0046$) in control and MND subjects, respectively. In summary, of 200 total comparisons in MND subjects across all 4 target muscles and for both inhibition and facilitation, there were only 28 exceptions to the pattern of the optimal superconditioning input exceeding the efficacy of a dual-pulse TMS input. Of these exceptions, almost all were clustered near the line of identity. In other words, although these *were* exceptions, the differences were almost always very small. While Fig. 4 illustrates data points drawn from just one of the 12 pairs of SC-specific inputs plotted against the corresponding dual-pulse input, in many cases multiple SC-specific inputs resulted in MEPs smaller than that to a SICI input (for inhibition) or an ICF input (for facilitation). This incidence is summarized in Fig. 4 legend.

Superconditioning TMS; active target muscle

It was not uncommon to find target muscles in our MND subjects that were unresponsive to single-pulse TMS, even when very high stimulus intensities were applied (Fig. 1, row 2). This was seen in 6 of 80 evaluations in APB (7.5% incidence), and 33 of 80 evaluations in TA (41.3%). In Fig. 2, we showed an example of how *abnormal* MEPs to traditional inputs were restored to normal response patterns following an SC input. What about if MEPs were absent entirely to single-pulse TMS inputs? During her third visit, subject #7 was still able to generate large EMG interference patterns during isometric voluntary contractions in both her TA muscles (Fig. 5a; time of contraction attempt indicated by horizontal dashed lines below EMG traces), despite her inability to walk independently. Following a strong single TMS pulse (75% intensity) when at rest, only her left TA showed an MEP (Fig. 5b). When contracting her right TA, the same stimulus intensity caused a larger left-side MEP (Fig. 5c),



but still no short latency MEP in her right TA. Rather, there was a small dip in EMG in the right TA, suggestive of a short latency inhibition (more on this later). Figure 5d shows the effect of a TMSsc input optimized for facilitation and delivered while the right TA was active. There was now a clear short latency MEP in her right TA. When the same TMSsc input was delivered with the subject *at rest* in her right TA, it was silent (not shown). Thus, the superconditioning input was clearly effective in causing an MEP in her right TA, but only when the muscle was actively contracting.

We could not elicit an MEP to single-pulse TMS at rest in 39 of the 160 target muscles tested over repeated trials of our 8 subjects. In 23 of these 39 'did not respond' trials, we also tested superconditioning TMS inputs. We saw an excitatory response—a short latency MEP—in 16 of these 23 trials, a *salvage rate* of almost 70%. In most of these cases, just as illustrated in Fig. 5, it was necessary for the subject to be making a contraction in the target muscle at the time of TMSsc delivery to elicit an MEP. Of the seven cases still with no MEP despite use of a superconditioning input and background contraction, six were seen in TA.

Superconditioning alone reveals inhibition

In many of the target muscles tested with three superconditioning pulses and a (stronger) follow-on test pulse, we also examined the effect of an SC train alone. For these trials, the intensity of the SC pulses was intended to be well below the target muscle's RT_3 value. Only 1 of the 47 trials of 3-pulse SC delivered with the target muscle *at rest* resulted in an MEP. Of the 24 trials when an SC train was delivered when the target muscle was *active*, an early and relatively large

◂**Fig. 4** Individual data points allowing direct comparison of the responses to TMSsc versus dual-pulse TMS for both control (unfilled circle) and MND subjects (unfilled down-pointing triangle). Top row: normalized MEP caused by the superconditioning input giving rise to the smallest MEP (i.e., optimized for inhibition) versus the response to the SICI-specific input (i.e., 3 ms C-T interval) for APB (**a**) and TA (**b**). Bottom row: normalized MEP caused by the superconditioning input giving rise to the largest MEP (i.e., optimized for facilitation) versus the response to the ICF-specific input (i.e., 10 ms C-T interval) for APB (**c**) and TA (**d**). In all panels, the dashed line of identity (with slope of '1') is drawn. For points lying below this line, the response to the SC input was smaller than the response to the dual-pulse input, whereas for points lying above this line, the response to the SC input was larger than the response to the dual-pulse input. For each pair of measures giving rise to a data point, the intensity of the TMS test pulse was the same. Only the smallest of the responses to SC-T inputs for inhibition is plotted in '**a**' and '**b**', but in many cases other combinations of SC-T inputs gave rise to responses that were also smaller than the SICI-specific input. Using a 1-ms test interval, this was seen in 19, 25, and 15 of the 31 comparisons with IPI values of 1, 3, and 6 ms, respectively. A 3-ms test interval resulted in smaller responses than the SICI-specific input in 8, 5, and 2 of the comparisons for IPI values of 1, 3, and 6 ms, respectively. Test intervals of 10 and 25 ms resulted in MEPs that were smaller than the SICI-specific input in only 7 of 62 comparisons across the 3 IPI values tested. In APB of MND subjects, larger responses to SC-T inputs compared to ICF-specific responses were seen with 10-ms test intervals in 20, 20, and 21 of 31 comparisons for IPIs of 1, 3, and 6 ms, respectively, while SC-T inputs using 25-ms test intervals led to larger responses than ICF-specific inputs in 17, 16, and 17 comparisons (for 1, 3, and 6 ms IPIs). A similar pattern was seen in TA for SC-T vs ICF-specific inputs: 10-ms test intervals led to relatively large MEPs in 9, 10, and 10 of 19 comparisons for IPIs of 1, 3, and 6 ms, respectively, while 25-ms test intervals enhanced the MEP over ICF-specific values in 11, 11, and 7 of 19 comparisons for IPIs of 1, 3, and 6 ms, respectively. In both APB and TA, test intervals of 3 ms were moderately successful at causing MEPs larger than those to ICF-specific inputs when the superconditioning IPI was 6 ms (11 of 31 comparisons for APB; 4 of 19 comparisons for TA). The same 3-ms test interval coupled with 1 and 3 ms IPIs was less successful at causing enhanced MEPs compared to the ICF-specific input (7 of 62 comparisons for APB; 2 of 38 comparisons for TA). Finally, test intervals of 1 ms rarely caused larger MEPs than those to ICF-specific inputs in APB (2 of 93 comparisons) and TA (0 of 57 comparisons)

MEP (RMS magnitude ≥ 40 μV above background) was seen 4 times. Unrecognized at the time of testing, this indicates that the intensity of the SC pulses was stronger than we had wanted. A very small MEP (10–20 μV above background) was seen in another five trials. The two remaining patterns involved variations of pure inhibition. In seven cases, inhibition was noted early (at an onset latency comparable to a typical MEP, had one been present), with a magnitude 15–40 μV below background. Finally, we saw eight examples of no early effect of any kind and a late inhibition of 35 μV or more below background, beginning roughly at the time an MEP would end. The lower trace of Fig. 5c shows that even single-pulse TMS caused brief inhibition of the right TA muscle—note the 'dip' in activity beginning at a latency roughly equal to that of the left TA MEP onset, in the absence of any short latency MEP in the R TA. To summarize, a pure inhibition of ongoing voluntary contraction caused by a train of weak SC TMS pulses was seen in 15 of 24 trials (63%) in our MND subjects. Had we used even weaker SC pulses (e.g., ~65% of RT_3), this incidence might have been higher still, because of fewer excitatory responses.

Defining a score of target muscle abnormality in MND

The best two electrophysiologic indicators of abnormal central motor function in our MND subjects were a particularly low MVC score, and a resting muscle that was unresponsive to strong single-pulse TMS. MEPa and response latency also showed significant abnormalities in MND subjects (Table 2). The *ratio* between several other single pulse and TMSsc measures listed in Table 2 led to additional significant differences between control and MND subjects in one or both target muscles. The mean MEPa:MEPr ratio in control subjects was higher compared to MND subjects, a difference that was significant in TA ($p = 0.0408$), but not APB ($p = 0.19$). Nevertheless, MND subjects typically showed less overall facilitation of the test MEP during active contractions compared to controls. This diminished MEP facilitation in MND subjects was also evident when comparing the MEPa:MEP_{SC} ratio between these cohorts. With a value only slightly higher than '1.0', this ratio in the APB of MND subjects (1.057; Table 2) was significantly smaller than that in control subjects (2.644; $p = 0.0220$). A similar trend was seen in the TA, but the difference failed to reach significance ($p = 0.11$; Table 2).

There were six measures for contraction and TMS responses that could help distinguish between control and MND subjects, based on GLMM analysis resulting in significant differences between cohorts in one or both target muscles. These measures were MVC, RT_1, MEPa, MEPa:MEPr, MEPa:MEP_{SC}, and latency. To generate a composite score from these factors, we assigned a value of '1' for a given target muscle and study session to any of these six parameters that was outside the 'Limits' defined in Table 2. A target muscle that did not respond to strong single-pulse TMS at rest scored a value of '2', since by not responding to resting single-pulse TMS we could not make determinations of either RT_1 or MEPa:MEPr. With this scheme, the highest possible 'Abnormal' score in a test session would be '24' ('6' in each of 4 target muscles).

Using this metric, our 15 control subjects had composite 'Abnormal' scores of '0' ($n = 6$), '1' ($n = 4$), '2' ($n = 4$), and '3' ($n = 1$). That there can even be 'abnormal' responses amongst these six parameters in controls reflects the high MEP variability that is evident in *all* subjects, not just persons with neuromuscular disease. Compared to controls, most of our MND subjects had many more abnormal findings, with composite scores ranging from '1' to '12' at the

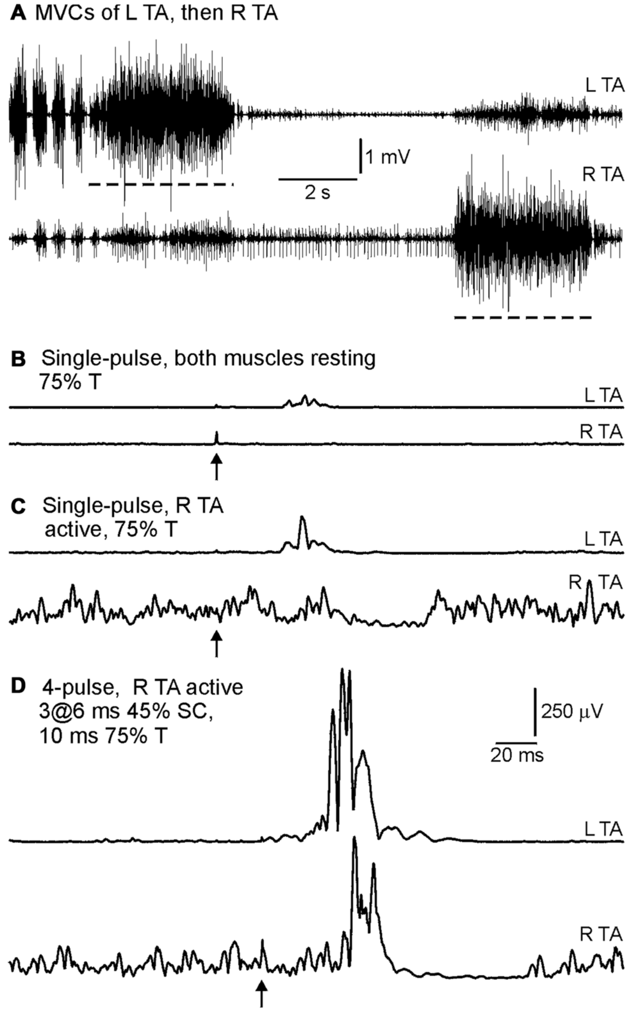


Fig. 5 Examples from subject #7, visit 3. **a** EMG interference pattern from left and right TA during maximum voluntary contractions (MVC). Time of sustained contraction in response to verbal cue indicated by dashed horizontal lines under each record. **b** Average rectified EMG (average of 5 trials) from single TMS test pulse (at vertical arrow) at stimulus intensity of 75% with both muscles at rest. The leg coil simultaneously stimulated left- and right-side motor cortex leg areas. There was a small MEP in her left TA, and no MEP in her right TA. **c** MEPs to the same (75%) stimulus intensity as in '**b**', but now the subject was contracting her right TA at the time of stimulation. The MEP from her left TA was facilitated, but in her right TA there was no short latency period of excitation, although there was a clear diminution (i.e., inhibition) of ongoing EMG for a period of time after the stimulus delivery. **d** MEPs to the same (75%) stimulation test pulse, preceded by 3 superconditioning pulses (45% intensity; 6 ms between pulses), with a 10-ms interval between the last of the superconditioning pulses and the test pulse. As in '**c**', the subject was contracting her right TA at the time of stimulation. There was a large MEP from the left TA, and now a small but well-defined short latency MEP from her right TA. Note that the latency of the left TA response was much shorter than that from the R TA. It is likely that the left TA was recruited at a low level in response to the train of superconditioning pulses alone, and then gave a strong response to the test pulse, whereas the R TA needed the much stronger test pulse (delivered 10 ms later) to cause recruitment of its lower motor neurons. The large difference in RT_1 values between these two muscles explains why both muscles were not tested simultaneously (left TA $RT_1 = 60\%$; right TA $RT_1 > 80\%$ [i.e., it did not respond to 80% single-pulse TMS, and it was not tested with anything stronger]). Note the horizontal (time) and vertical (EMG magnitude) calibration bars for '**a**' are different from those for '**b**–**d**'

initial visit, and climbing to a range of '4' to '21' at the final visit. With few exceptions, 'Abnormal' scores were higher in TA versus APB muscles. No MND subject had a composite 'Abnormal' score of '0' for any evaluation, but subject #4 was not far off through his first three evaluations (composite scores of '1', '2', and '1'), despite the fact that his bulbar motor function was already severely impacted at this time. Even at his final evaluation, he showed a total 'Abnormal' score of only '4'. Clearly this subject's disease burden was largely sparing of motor circuits governing his limb function.

We did not subject these composite scores between control and MND subjects to statistical analysis, in part because of numerous 'holes' in our MND data set (as a reminder, we did not begin using TMSsc inputs until part-way through the study), and in part because we are not prepared to conclude that the TMSsc parameters as presented herein are *optimal* for testing persons with MND through all stages of disease, and across all phenotypes.

Discussion

The results of this study were mixed. First, we did not see any direct evidence of cortical hyperexcitability in any of our MND subjects. Second, superconditioning TMS showed certain advantages to single- and dual-pulse TMS for examining central motor function in MND.

Cortical hyperexcitability: increased excitation?

Taken literally, cortical hyperexcitability in MND should include any or all of: (1) lower thresholds to TMS with the target muscle at rest; (2) enlarged MEP amplitudes; or (3) signs of cortical afterdischarge or spread.

We found that RT_1 was significantly higher in MND subjects compared to controls for both upper and lower limb target muscles. The RT_1 values we obtained in APB of our control subjects agree very well with other reports in the literature in which a similar approach was used (same stimulator; same coil; same or comparable target muscle) (Hufnagel et al. 1990; Furby et al. 1992; Mills and Nithi 1997b; Triggs et al. 1999), suggesting our methodology was sound.

There are reports that persons with ALS show an elevation of resting thresholds (Mills 1995; Triggs et al. 1999; Di Lazzaro et al. 2006; Munneke et al. 2013), especially during the latter stages of the disease (Eisen et al. 1993; Mills 2003; Attarian et al. 2009; Floyd et al. 2009). Our findings are consistent with these studies. However, a small number of publications have been cited repeatedly for exceptions to this finding. A lowered threshold to elicit MEPs in some persons with ALS was reported by Eisen et al. but the difference was not significant, and was seen in only 3 of their

40 subjects (Eisen et al. 1993). Mills and Nithi wrote that RT_1 was significantly lower in persons with ALS of recent onset (Mills and Nithi 1997a), but 21 of their 30 subjects showed fasciculations in target muscles at the time of testing. In fact these authors wrote the lower thresholds "… might be a manifestation of a phase of hyperexcitability of either cortical *or spinal* motoneurons prior to their ultimate demise" (emphasis added) (Mills and Nithi 1997a). We suggest that fasciculations, and their threshold-lowering effect could account for a portion of the disagreement about TMS thresholds and ALS (Caramia et al. 1991; Mills 1995; Kohara et al. 1996).

One group has reported the same (Vucic et al. 2011) or lower TMS thresholds in all ALS subjects (Geevasinga et al. 2014) or a subset with limb-onset disease (Vucic and Kiernan 2006; Vucic et al. 2008) compared to control subjects. However, in these studies anywhere from 0 to 23% of the ALS population was found to be unresponsive to the strongest TMS intensities tested. Such reports are common (Uozumi et al. 1991; Eisen et al. 1993; Urban et al. 2001), with an incidence from 16 (Attarian et al. 2009) to 55% (Triggs et al. 1999). Unresponsive cases should be considered extreme examples of 'high' thresholds for eliciting responses, of course, and would raise the average threshold for ALS subjects considerably if, for example, values related to the maximum stimulation intensities attempted were used in these calculations, as we did in the present study.

What about MEP amplitude? We found no significant difference in MEPr between control and MND subjects, and significantly smaller MEPa values in both APB and TA in our MND subjects. There are multiple reports of smaller MEP amplitudes in subjects with ALS (Uozumi et al. 1991; Eisen et al. 1993; Ziemann et al. 1997), whereas convincing evidence for *larger* MEPs in persons with ALS—an outcome that might be predicted in cases of cortical hyperexcitability—is lacking. Two reports of enhanced MEPs in ALS were based on single-motor unit histograms, in both cases comprising only a small proportion of the entire sample (Awiszus and Feistner 1995; Eisen et al. 1996). In one group with multiple reports of enlarged MEP amplitudes in ALS subjects, these were for responses normalized to a subject's compound muscle action potential following supramaximal peripheral nerve stimulation (Vucic et al. 2008, 2009, 2011; Geevasinga et al. 2014). In the two reports from this group in which *absolute* MEP amplitudes were described, these were smaller than those from controls (Vucic and Kiernan 2006; Vucic et al. 2008).

Finally, we did not see any example of 'late' excitation in a target or neighboring muscle following any pattern of TMS, including TMSsc inputs optimized for facilitation, in MND subjects.

In summary, we did not see any evidence for *increased excitability* of the motor cortex in MND subjects. We believe this finding is consistent with a number of other groups who have addressed this topic.

Cortical hyperexcitability: decreased inhibition?

Based on dual-pulse TMS measures of SICI, there are multiple reports of reduced cortical inhibition in ALS (Yokota et al. 1996; Ziemann et al. 1997; Caramia et al. 2000; Stefan et al. 2001). This finding, coupled with descriptions of postmortem cortical atrophy (Nihei et al. 1993; Sasaki and Iwata 2000), has led to the hypothesis that loss of inhibitory cortical interneurons is a consequence of *excitotoxicity* associated with excessive glutamate accumulation (Eisen et al. 1993, 1996; Yokota et al. 1996; Stefan et al. 2001). Accordingly, the term 'hyperexcitability' in this sense refers to the mechanism of neuronal loss, not a literal change in the excitability of upper motor neurons (Caramia et al. 1991; Awiszus and Feistner 1995; Mills 1995; Ziemann et al. 1997).

In our study, the overall incidence of 'abnormal' SICI values was not statistically different between control and MND subjects for either target muscle. In our hands at least, measures of SICI within our MND cohort did not, in and of themselves, provide any insight into their clinical status. Several studies from Ugawa et al. also found no difference in SICI between control subjects and persons with ALS (Hanajima et al. 1996; Groiss et al. 2017).

A number of publications from one group have described reduced (or absent) SICI in persons with ALS (Vucic and Kiernan 2006; Vucic et al. 2009; Geevasinga et al. 2014). Rather than using a traditional dual-pulse approach in which the MEP amplitude to the conditioned test pulse is compared to that from the test pulse alone, this group uses the *threshold-tracking* method (Awiszus et al. 1999; Fisher et al. 2002), precluding direct comparisons between that method and our approach. However, the effect of a short-interval conditioning pulse on a follow-on TMS test pulse reflects a combination of both inhibitory and excitatory influences on upper motor neurons (Ni and Chen 2008), and the balance can be easily tipped in one direction or the other with just slight changes in conditioning pulse intensity (Fisher et al. 2002; Butefisch et al. 2003; Peurala et al. 2008). A detailed description of the threshold-tracking method for cortical stimulation in control subjects established a test stimulus intensity as that needed to evoke an MEP of 0.2 mV (Fisher et al. 2002). This value lay on the steepest part of the target muscle's gain curve (see their Fig. 1b), hence would be the most sensitive to perturbations in output. However, in applying this approach to ALS patients (Vucic and Kiernan 2006), there appeared to be no attempt made to establish a new gain curve, despite the high probability of there being unusually high thresholds within at least a portion of this cohort.

The conditioning pulse intensities (70% of 'resting motor threshold') used in studies from this group (Vucic and Kiernan 2006) were relatively strong compared to those used in the present study. This is because our criteria for what constituted a positive MEP for defining RT_1 allowed for a much smaller response (10–20 µV) compared to their 200 µV (Vucic and Kiernan 2006). Using a relatively strong—but still subthreshold—conditioning pulse, the response to a test pulse of still higher intensity will also be relatively strong, since two excitatory and close-spaced cortical stimuli can be very effective at eliciting an MEP via temporal summation at the level of the spinal cord. Under these circumstances, the intensity of the test pulse needed to maintain a fixed response amplitude would be lower, giving the *appearance* of lower cortical inhibition caused by the conditioning pulse. A corollary to this mechanism has been termed *short-interval intracortical facilitation* (SICF) (Yokota et al. 1996; Ziemann et al. 1997).

This explanation also applies to dual-pulse TMS, and may account—at least in part—for other reports of reduced SICI in persons with ALS (Yokota et al. 1996; Stefan et al. 2001). Our argument is not original: Peurala et al. wrote "SICF may contribute to apparently reduced SICI in patients with neurological or psychiatric disorders", and "As a consequence, a reduction in SICI cannot be meaningfully interpreted as decreased inhibition or increased facilitation, if tested at only one S1 intensity" (in which 'S1' refers to the conditioning pulse) (Peurala et al. 2008).

Mechanism of superconditioning TMS

The single subthreshold conditioning pulse in the traditional C-T paradigm used for SICI and ICF is believed to act upon cortical interneurons 'upstream' from their upper motor neuron (UMN) targets (Kujirai et al. 1993; Ziemann et al. 1996; Di Lazzaro et al. 1998; Hanajima et al. 1998). In response to an SC train acting selectively—because of the very weak intensities used—upon these cortical interneurons, we believe that *temporal summation* at the synapse between interneuron and UMN is occurring. Therefore, compared to the effect of dual-pulse TMS (i.e., a single-conditioning pulse), the response to a train of 3 SC pulses was often more pronounced in both inhibition and facilitation.

Further evidence for this conclusion can be seen when comparing RT_1 values to thresholds to a three-pulse high frequency train (i.e., RT_3). In our study, RT_3 was always considerably lower than RT_1 (by as much as 40%, in some individuals). Accordingly, the intensities of SC pulses were always lower than those used for dual-pulse inputs, yet their effectiveness was usually enhanced, a classic sign of temporal summation.

As already reported in control subjects (Calancie et al. 2018) and now confirmed in persons with MND, a test interval of 1 ms was often very effective at reducing the amplitude of the test MEP, when that interval was preceded by a subthreshold train of superconditioning pulses. Some investigators have argued that this is not inhibition occurring at the UMN at all, but rather transient refractoriness of some UMN axons to the second TMS pulse following their depolarization and discharge to the first pulse (Kujirai et al. 1993; Fisher et al. 2002; Peurala et al. 2008). In other words, UMN axons are simply unable to follow these very high instantaneous rates of stimulation. However, there are multiple lines of evidence from our own study, and from others that suggests the reduced MEP we report with 1-ms test intervals is a synaptically mediated effect, at least in part. First, we found the stimulus intensity needed to demonstrate this effect was consistently much lower than that minimum intensity needed to elicit an MEP to a three-pulse train (i.e. RT_3), arguing that such weak intensities are acting on the more superficially placed cortical interneurons alone, and are truly subthreshold for UMN axons. Second, there is ample evidence from intraoperative neuromonitoring studies using transcranial *electrical* stimulation that pulse trains with IPIs as short as 1 ms can elicit MEPs in target limb muscles (Calancie 2017), even in the deeply anesthetized state when cortical activity is diminished. Finally, TMS studies in awake subjects using dual- or four-pulse inputs that are suprathreshold for MEPs and have IPIs of 1 ms are very effective at eliciting motor responses, indicating that UMN axons *are* capable of following such high instantaneous rates (Bawa and Calancie 2004; Van den Bos et al. 2018).

This leads to the question: why did not TMSsc inputs using 3-ms test intervals cause inhibition of the test MEP, as we and many other groups have seen with dual-pulse TMS, yet 1-ms test intervals *did*? We do not have a satisfactory answer to this surprising finding, other than to point out that the interactions between subthreshold and suprathreshold inputs are known to be complex, even for dual-pulse inputs (Fisher et al. 2002; Ni and Chen 2008; Peurala et al. 2008). That the same should be true for four-pulse inputs, especially when the interpulse intervals are not uniform, almost goes without saying. It is worth examining the *one* series of four-pulse TMS inputs from the Ugawa lab's study of *triad-conditioned facilitation* (TCF) in persons with ALS that was identical to our TMSsc inputs described herein: three subthreshold conditioning pulses with a 3-ms IPI followed 3 ms later by a suprathreshold test pulse. In our case, as shown in our Fig. 3a, this condition led to a test MEP of ~ 102% (i.e., neither inhibition nor facilitation, on average), while from the Ugawa study their Fig. 2a showed an MEP (from the FDI) of ~ 105% (Groiss et al. 2017). These nearly identical findings from two independent labs using four-pulse TMS to pursue very different questions indicate the observed lack of inhibition with a 3-ms test interval preceded by three conditioning pulses is robust, and reproducible.

In a few of our MND subjects in which target muscles failed to show an MEP, even in response to strong TMSsc inputs optimized for facilitation, we saw that superimposition onto the actively contracting target muscle of superconditioning pulses alone—without a follow-on test pulse—briefly *suppressed* the ongoing EMG. We interpret this finding to indicate that cortical inhibitory interneurons were still functional in these MND subjects. In conjunction with our other results, this finding provides evidence of a predominant loss of the excitatory component of the interneuronal circuitry within the motor cortex in these subjects, while their inhibitory circuitry showed relative preservation. Using their 'TCF' input, the Ugawa reached the same conclusions of preserved SICI (hence inhibitory cortical function) in ALS subjects, with disruption of the '25 Hz' facilitation being the only significant difference they found in these subjects (Groiss et al. 2017). We recognize that our conclusions about the relative preservation of inhibitory and loss of excitatory circuitry in MND is at odds with much of the current MND literature, and will require confirmation within a larger sample size.

Advantages of superconditioning TMS

We found in the majority of direct comparisons that the best SC input for causing either inhibition or facilitation was superior to the effect of corresponding dual-pulse inputs. In MND subjects, a further advantage was seen with target muscles that were unresponsive to strong single-pulse TMS, but responded to SC inputs optimized for causing facilitation. Using traditional dual-pulse TMS, all these target muscles would be lost to quantitative evaluation of central motor conduction. Thus, using TMSsc inputs can *extend* our ability to quantitatively assess central motor function for a longer period of time in persons with MND.

Even when MEPs to single-pulse TMS *were* present, some muscle thresholds were very high, causing subjects considerable discomfort. Although not systematically examined, we found that when preceded by SC pulses, it was possible to lower the test pulse intensity and still elicit well-defined MEPs. As a quick test for an excitatory response, we suggest the following protocol: a 2-ms IPI for SC pulses, a 10-ms test pulse interval, SC pulse intensity of 0.7 RT_3 (the true resting threshold, not the intensity needed to elicit an arbitrary response magnitude), and test pulse intensity of 1.1 RT_1. Inhibition can be tested with the identical protocol, other than the test interval: a 1 ms test interval is recommended. These recommendations—subject to revision as we complete further testing—call for weaker test pulse intensities (the test pulse causes greatest discomfort), and much weaker superconditioning pulses, than typical dual-pulse protocols. This has important practical consequences, as the burden of repeated evaluations during clinical trials—and the associated risk of subject dropout—would be lessened if the discomfort associated with TMS evaluations could be lowered.

Like many neurological disorders, ALS is not restricted to just one region of the body, yet the great majority of TMS-based measures in ALS have been restricted to upper limb muscles. We believe an improved TMS-based protocol in suspected ALS should include testing of both upper and lower limb muscles bilaterally. And, the use of superconditioning TMS would make inclusion of leg muscles in this evaluation better tolerated by most subjects, due to lower test stimulus intensities.

Limitations of superconditioning TMS

Despite the demonstrated advantages of superconditioning TMS for eliciting MEPs, it was also clear that there were limits to its effectiveness. Certain target muscles in our MND subjects could not be driven to an excitatory response, even when using TMSsc optimized for facilitation, a strong test pulse, and with a background contraction. It is likely that in these instances, pathology associated with excitatory motor circuitry was too advanced to support a response to this novel TMS method.

Another limitation of our protocol—while not directly attributable to the superconducting approach itself—is evident from subject #4. Spanning our first three evaluations, his composite 'Abnormal' score was little different from that of some control subjects, yet his bulbar function had declined markedly by this third evaluation. This finding tells us that a more sensitive TMS-based screening tool would add in 1–2 bulbar-innervated muscles. These muscles *do* respond to TMS (Dubach et al. 2004; Fischer et al. 2005; Paradiso et al. 2005), although facilitation via a background contraction might be necessary (Urban et al. 2001).

Implications of superconditioning TMS in MND, and future studies

Delays exceeding 18 months between the onset of symptoms and arrival of a formal diagnosis of ALS have been reported (Swash and Ingram 1988), leading to calls for earlier and more reliable biomarkers for this disease (Swash 1998; de Carvalho et al. 2008). Methods for quantifying upper motor neuron involvement in cases of suspected ALS, in particular, have been lacking. Our findings, and results from multiple other groups, argue that no single electrophysiologic measure—be it SICI or something else—can fulfill this role.

With further development the 'Abnormal' index described herein may help address this need. It includes quantitative measures of voluntary innervation ('MVC'), a true threshold to elicit an MEP at rest (RT_1), and measures that capture the apparent limited effectiveness of an active contraction to facilitate the MEP compared to the resting condition (‘MEPa:MEPr’) or the superconditioned MEP (‘MEPa:MEP_{SC}’) (Triggs et al. 1999). Collectively, these measures incorporate muscle, neuromuscular, lower motor neuron, upper motor neuron, and cortical interneuronal (excitatory and inhibitory) contributions to central motor function. It is not unreasonable to expect that a disease with such diverse phenotypes as ALS will require a multi-factor assessment tool to help with early diagnosis and to follow disease progression. Of course, a complete quantitative evaluation of the entire neuraxis in MND should include detailed measures of lower motor neuron integrity, such as compound muscle action potential and/or MUNE measures; the absence of such measures reflects a further limitation of the present study.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval All the procedures performed were in accordance with the ethical standards of Upstate Medical University and with the 1964 Helsinki Declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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