Role of Sustained Excitability of the Leg Motor Cortex after Transcranial Magnetic Stimulation in Associative Plasticity

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Abstract

Changes in the strength of corticospinal projections to muscles in the upper and lower limbs are induced in conscious humans following paired associative stimulation (PAS) to the motor cortex. We tested whether an intervention of PAS consisting of 90 low-frequency (0.1 Hz) stimuli to the common peroneal nerve combined with suprathreshold transcranial magnetic stimulation (TMS) produces specific changes to the motor evoked potentials (MEPs) in lower leg muscles if the afferent volley from peripheral stimulation is timed to arrive at the motor cortex after TMS-induced firing of corticospinal neurons. Unlike PAS in the hand, MEP facilitation in the leg was produced when sensory inputs arrived at the motor cortex over an estimated range of 15 to 90 ms after cortical stimulation. We examined if this broad range of facilitation occurred as a result of prolonged subthreshold excitability of the motor cortex following a single pulse of suprathreshold TMS so that coincident excitation from sensory inputs arriving many milliseconds after TMS can occur. We found that significant facilitation of MEP responses (> 200%) occurred when the motor cortex was conditioned with suprathreshold TMS tens of milliseconds earlier. Likewise, it was possible to induce strong MEP facilitation (85% at 60 min) when afferent inputs were directly paired with subthreshold TMS. We argue that in the leg motor cortex, facilitation of MEP responses from PAS occurred over a large range of interstimulus intervals due to the paired activation of sensory inputs with sustained, subthreshold activity of cortical neurons that follow a pulse of suprathreshold TMS.
**Introduction**

Increases in the excitability of the corticospinal tract (CST) can be induced in conscious human subjects by repeatedly pairing low-frequency stimulation of a peripheral nerve with activation of the primary motor cortex by transcranial magnetic stimulation (TMS) in an intervention termed ‘paired associative stimulation’ (PAS; Stefan et al. 2000). PAS-induced facilitation occurs when inputs from peripheral nerve and TMS arrive at the motor cortex nearly simultaneously, with the hypothesis being that both inputs activate a common neuron within the motor cortex (Stefan et al. 2002). The ensuing increase in motor evoked potentials (MEPs) from CST activation is thought to be mediated by increases in synaptic strength between coincidentally activated neurons via mechanisms related to associative long-term potentiation (LTP).

Several studies in both *in vitro* and *in vivo* systems have shown that modifications in the synaptic strength between coincidently activated neurons depend on the temporal ordering of pre- and postsynaptic activation (reviewed in Dan and Poo 2006). For example, in cultured hippocampal neurons, LTP is induced if postsynaptic firing generated by a strong input occurs immediately after the activation of an excitatory postsynaptic potential (EPSP) generated by a weak input (Bi and Poo 1998). In this situation, the EPSP generated by the weak input occurs during the action potential of the postsynaptic neuron which strongly depolarizes the postsynaptic membrane and facilitates the influx of calcium through N-methyl-D-aspartic acid (NMDA) receptors involved in LTP induction (reviewed in Linden 1999). In contrast, long-term depression (LTD) occurs if postsynaptic firing just precedes presynaptic activation where the EPSP generated by the weak input occurs during the afterhyperpolarization of the postsynaptic neuron, which may dampen NMDA receptor-mediated calcium influx to induce LTD (see also Dan and Poo 2004).
During PAS in humans, modifying the timing between TMS (strong input) and peripheral nerve stimulation (weak input) produces similar asymmetric changes in the excitability of the CST as described above for hippocampal neurons (Wolters et al. 2003; Ziemann et al. 2004; Mrachacz-Kersting et al. 2007). For example, MEP responses in hand muscles are increased when a TMS pulse that produces firing of CST neurons occurs at the same time as excitation of the motor cortex from median nerve stimulation (Stefan et al. 2000; Stefan et al. 2002). The weak input from afferent stimulation is proposed to be strengthened because it occurs during the period of strong depolarization of the postsynaptic neuron activated by TMS. Likewise, MEPs in the tibialis anterior (TA) muscle are increased when a TMS pulse is applied 45 to 55 ms after common peroneal (CP) nerve stimulation such that TMS-induced firing of CST neurons coincides with the afferent excitation at the motor cortex (Mrachacz-Kersting et al. 2007). In contrast, MEP responses are depressed when the stimulation order is reversed whereby TMS inputs are timed to arrive at the motor cortex immediately before afferent inputs so that theoretically, the weak afferent input is activated during the afterhyperpolarization of the postsynaptic neuron (Wolters et al. 2003; Ziemann et al. 2004; Mrachacz-Kersting et al. 2007). Outside of this approximately 20 ms interstimulus window, pairing TMS and peripheral nerve stimulation has no effect on CST excitability to upper extremities (Wolters et al. 2003, see also Dan and Poo 2006 for similar results in vitro).

Similar to PAS in humans, Jackson et al. (2006) showed that the connectivity between two populations of neurons in the primate motor cortex could be modified by synchronizing cortical activity at two distant sites in a manner consistent with Hebbian synaptic potentiation. By using
action potentials recorded from one site in the motor cortex to trigger electrical stimuli at distant site, repeatedly pairing the two events, over one day or more, produced a shift in the stimulus-evoked motor output of the recording site to resemble the motor output produced at the site of stimulation. In some cases, persistent changes in connectivity could be induced for more than one week when electrical stimuli were delivered up to 100 ms after neural firing. These findings are of interest as they challenge the notion that there exits a narrow temporal relationship governing synaptic plasticity between synchronized populations of neurons in the motor cortex of freely behaving animals. Likewise, preliminary reports in the leg have suggested that PAS in humans can increase MEP responses in the TA muscle when afferent inputs arrive at the motor cortex up to 30 ms after cortical stimulation (Mrachacz-Kersting et al. 2007; see also Perez et al. 2003). To further investigate these effects, we tested whether PAS-induced facilitation of the leg motor cortex can occur if afferent inputs arrive at the level of the motor cortex at long intervals after a pulse of TMS. Similar to PAS paradigms timed to provide synchronous excitation in cortex, we find that CST excitability to leg muscles was strongly enhanced when afferent inputs reached the motor cortex many milliseconds after cortical stimulation. Surprisingly, such facilitation occurred over a wide range of interstimulus intervals comparable to that observed in vivo (Jackson et al. 2006). In this paper, we examine if continued subthreshold activity in the leg motor cortex following a single pulse of suprathreshold TMS is sufficient to induce associative facilitation in the cortex when conditioned with afferent inputs from the lower leg.
Methods

Subjects

Twenty healthy volunteers (12 male and 8 female) with an average of 26 ± 6 years participated in the study for a total of 74 separate experimental sessions. All subjects gave their written informed consent, and the protocol was approved by the Human Ethics Research Board at the University of Alberta.

Recording and stimulation

EMG activity was recorded from the tibialis anterior (TA) and soleus (SOL) muscles on the examined leg (16 right and 4 left) and the contralateral TA using pairs of surface Ag-AgCl electrodes (Kendall LTP, Chicopee, MA). Electrodes were placed over the muscle belly 1.5 cm apart and parallel to the long axis of the muscle. EMG signals were amplified 1000 times and band-pass filtered between 10 and 1000 Hz (Octopus, Bortec Technologies, Calgary, AB). Raw EMG signals were digitized at 5 kHz using Axoscope hardware and software (DigiData 1200 Series, Axon Instrument, Union City, CA) and stored on a personal computer for off-line analysis. EMG activity from the TA muscle was also rectified, low-pass filtered at 0.3 Hz and transmitted to an oscilloscope so subjects could monitor their EMG activity during the experiment.

Electrical pulses of 1 ms in duration were delivered to the common peroneal (CP) nerve using a constant-current stimulator (DS7A, Digitimer Ltd., Hertfordshire, UK). Stimuli were delivered using a cathode (Kendall-LTP ES40076) placed over the CP nerve near the head of the fibula.
with a large anode (Axelgaard Manufacturing Co., Ltd.) placed on the medial side of the knee below the patella.

TMS was delivered using a Magstim 200 (The Magstim Co., Dyfed, UK) and a double-cone coil (P/N 9902-00: external wing diameter 110 mm) oriented in an antero-posterior direction. Paired-pulse TMS (described below) was performed using two Magstim 200 stimulators connected to a Bistim module (The Magstim Co.). The coil was placed over the leg area of the motor cortex with the handle pointing upwards and a few degrees from the mid-sagittal plane. The optimal stimulus site (usually located 1 cm lateral and 1 cm posterior to the vertex) was identified over the contralateral motor cortex using a suprathreshold stimulation intensity. The coil was suspended from an overhead gantry and stabilized on the head using two foam pads and a chin strap.

**Protocol for PAS intervention**

Subjects were seated comfortably in a chair with the examined leg bent at the knee and with the foot secured to a foot-plate. The intervention targeted the TA muscle at rest and delivered electrical stimuli to the CP nerve paired with TMS over the contralateral motor cortex. Stimulation of the CP nerve was delivered at 300% perceptual threshold (6 ± 2 mA) or 150% of motor threshold if the former was below motor threshold. TMS was delivered at an intensity that produced an MEP of approximately 0.3-0.6 mV in the relaxed TA (54 ± 7% maximum stimulator output: MSO). PAS consisted of 90 pairs of stimuli delivered at 0.1 Hz over 15 min (see also Kujirai et al. 2006).
**Effect of timing TMS in relation to CP nerve stimulation**

The influence of varying the timing between both stimuli applied during PAS on corticospinal excitability was investigated at six main interstimulus intervals (ISIs) and one control ISI (see below). Eight subjects were tested at each of the main ISIs and a total of eighteen subjects participated in this part of the study. The order of interventions at each ISI was pseudorandom within and across subjects, and at least three days elapsed between consecutive sessions in a single subject. Since conduction velocities in the nervous system vary between subjects, all ISIs were adjusted according to the latency of the MEP in the TA muscle (30 ± 2 ms). The time interval between the CP stimulus (weak inputs to cortex) and the TMS pulse (strong inputs to cortex) on average were equal to i) -40 ms (MEP latency minus 70 ms), ii) 0 ms (MEP latency minus 30 ms), iii) 20 ms (MEP latency minus 10 ms), iv) 35 ms (MEP latency plus 5 ms), v) 40 ms (MEP latency plus 10 ms), and vi) 60 ms (MEP latency plus 30 ms). In addition, five subjects were tested at the control ISI of -170 ms, where the interaction between TMS and peripheral nerve stimulation should be negligible (see Discussion).

**Excitability of the CST**

CST excitability following PAS was investigated at rest and during voluntary contraction (active trials). MEPs at rest were recorded in blocks of twenty stimuli at the intensity used during PAS. Active MEPs were recorded in blocks of ten stimuli while subjects maintained a small contraction in TA corresponding to 10% of their maximum voluntary contraction (MVC). The stimulus intensity was set to elicit MEP responses of approximately 1.0 mV (43 ± 5% MSO) in the contracted TA. Two sets of resting MEPs and one set of active MEPs were recorded at
baseline, and single sets were recorded at 0, 10, 20, 30 and 60 min after PAS. Stimuli were
delivered every 8 s. The size of the MEP was measured as the peak-to-peak amplitude of the
non-rectified EMG response. MEPs were averaged and expressed as a percentage of baseline
values before PAS at each of the five time points listed above. During active trials, the level of
motor activity just preceding an MEP was evaluated from the mean value of the rectified EMG
recorded in a 50-ms period just before the stimulus. Average levels of motor activity evaluated
after PAS were within 1 SD of pre-PAS values, and up to 5% of the responses were discarded to
match EMG levels.

**Sustained excitability of the CST after suprathreshold TMS**

In five subjects, we investigated how long corticospinal projections to the TA muscle remain
excitable following a single pulse of suprathreshold TMS. To examine this, we evaluated the size
of the MEP response when conditioned by a prior suprathreshold TMS. The intensities of the
conditioning and test stimuli were set to produce an MEP response of approximately 0.3-0.6 mV
(similar to PAS) when given alone. ISIs of 20, 30, 50, 70, 80, 90 and 100 ms were tested. Five
paired stimuli were applied at each ISI in a pseudorandom order interleaved with ten
unconditioned test stimuli. Stimuli were delivered every 8 s. CST excitability was evaluated
from the peak-to-peak amplitude of the test MEP in the paired conditions and expressed as a
percentage of the unconditioned test (control) MEP.

**Subthreshold PAS**

To test whether PAS-induced facilitation of MEP responses occurred because of pairing of CP
inputs with cortical activity that was subthreshold to CST activation, we administered a modified
intervention of subthreshold-PAS in eight subjects using a protocol similar to that used in the hippocampus to produce heterosynaptic LTP *in vitro* (Huang et al. 2004). Subthreshold-PAS consisted of three CP stimuli paired with a single pulse of TMS over the contralateral motor cortex at 80% of the active motor threshold (AMT; 23 ± 4% MSO). AMT was defined as the lowest stimulus intensity that evoked an MEP with an amplitude > 50-100 μV in at least 3 of 6 consecutive stimuli during voluntary contraction. The three CP nerve stimuli (100 Hz; at 300% sensory threshold) were applied over a period of 15 to 35 ms before the TMS pulse. Subthreshold-PAS was administered at rest and consisted of 60 paired stimuli given at a rate of 0.2 Hz (lasting 5 min).

**Intracortical and spinal mechanisms**

In eight subjects, we investigated possible intracortical and spinal mechanisms responsible for PAS-induced facilitation using an ISI of 20 ms, where consistent facilitation of MEP responses occurred. Paired-pulse TMS was delivered using a conditioning stimulus of 95% AMT (33 ± 7% MSO). Test stimuli were given at an intensity that produced unconditioned MEPs of approximately 0.3-0.6 mV (57 ± 8% MSO). Short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) were evaluated using ISIs of 3 and 9 ms, respectively (Stokic et al. 1997). At each ISI, ten conditioned and ten unconditioned stimuli were delivered in a pseudorandom order. Stimuli were given every 8 s. ICF, SICI and MEPs were evaluated in the relaxed TA at baseline and 15 and 30 min after PAS. Since the size of the unconditioned MEP influences the excitability of intracortical circuits (Stefan et al. 2002), the intensity of the test stimulus was adjusted after the intervention (55 ± 8% MSO) to match the size of the
unconditioned MEP recorded before PAS. SICI and ICF were evaluated from the peak-to-peak amplitude of the conditioned MEP and expressed as a percentage of the unconditioned MEP.

Excitability of the H-reflex, the electrical analogue of the stretch reflex pathway, was investigated in the TA muscle. The stimulus intensity was adjusted to produce an H-reflex at around 50% of the maximal H-reflex amplitude during tonic dorsiflexion at 10% MVC. Thirty H-reflexes were evaluated before and 25 min after PAS. Stimulations were given every 2 s. The average size of the peak-to-peak H-reflex was measured with matched M-wave and background EMG.

**Statistical analysis**

Two-factor repeated measures ANOVAs (analysis of variance), treating the time of measurement and the motor state (rest or active) as within-subject factors, were used to compare the size of the MEP response after PAS at the different ISIs and for the single ISI used during subthreshold-PAS. When the interaction effect (time x state) was significant, the motor states were analyzed separately using one-way repeated measured ANOVAs (within-subject factor: time). A two-factor repeated measures ANOVA (within-subject factors: time, leg) was used to compare MEP responses in the stimulated and non-stimulated TA muscle. Post hoc $t$-tests (two-tailed) were used to evaluate the MEP response size at the different time-points compared to baseline. Post hoc testing was performed on the pooled data (rest and active) at ISIs showing a significant time effect with no interaction or state effects. The size of the MEP response following pairs of suprathreshold TMS and the effect of a 20 ms ISI on MEP, SICI, ICF and H-reflex responses
were all analysed using $t$-tests (two-tailed). The significance level was set at $p < 0.05$. All data are given as means $\pm$ SD.
Results

Changes in corticospinal excitability

Corticospinal excitability projecting to TA was altered following PAS as a function of the ISI employed during the intervention. Figure 1A shows average traces of the resting MEP from one representative subject at the six main ISIs tested. In agreement with data published in the lower leg (Mrachacz-Kersting et al. 2007), MEP facilitation was not produced when PAS was delivered at an ISI of 60 ms, when the afferent volley from nerve stimulation arrived at the contralateral motor cortex ahead of the TMS pulse by approximately 10 ms (bottom trace in Fig. 1A). However, MEP facilitation was produced when afferent inputs arrived at the motor cortex after TMS-induced activation, at ISIs ranging from -40 to 40 ms (traces from top to bottom). At these ISIs, the afferent inputs are estimated to arrive at the motor cortex 90 to 10 ms after the TMS pulse, respectively. Such findings were consistent across subjects as shown for the group data in Figure 1B, which plots the average size of the MEP after PAS relative to baseline values at the various ISIs tested. Similar to the 60 ms ISI, MEP facilitation did not occur at an ISI of -170ms where afferent inputs are estimated to arrive at the motor cortex approximately 220 ms after the TMS pulse.

Insert Figure 1 near here

Time course of MEP changes after PAS

When MEP responses were facilitated at ISIs of -40 to 35 ms (solid symbols in Fig. 2A), MEPs began to increase immediately after the intervention (at time 0) and persisted for at least 60 min (last time point tested). MEP responses measured at rest typically increased during the first 20 min and remained elevated at the 60-min time point, ranging from 41 to 67% of pre-PAS values. Faster decreases occurred following PAS at an ISI of 40 ms, where MEP increases were less consistent.
(open circles in Fig. 2A). At PAS intervals where no visible MEP facilitation occurred (ISIs of -170 and 60 ms; open squares and triangles in Figs 2A), MEP responses remained close to pre-PAS values (at 100%) except for a transient decrease measured 0 min at the -170 ms ISI (n = 5).

Insert Figure 2 near here

When examining group data across the various ISIs (Fig. 2B-H), separate two-factor repeated measures ANOVAs (in both resting and active states) showed that peak-to-peak MEP amplitudes were significantly increased at ISIs of -40 ms (F(5,35) = 5.59, p < 0.001), 0 ms (F(5,35) = 4.92, p < 0.005), 20 ms (F(5,35) = 5.70, p < 0.001) and 35 ms (F(5,35) = 2.50, p < 0.05). These results indicate that corticospinal excitability was increased by PAS when the afferent volley reached the contralateral motor cortex an estimated 90, 50, 30 and 15 ms after the TMS pulse, respectively. The interaction effect (time x state) was significant at ISIs of -40 ms (F(5,35) = 2.67, p < 0.05) and 0 ms (F(5,35) = 4.77, p < 0.005), and MEP responses showed significant increases following PAS at ISIs -40 ms (F(5,35) = 4.30, p < 0.005) and 0 ms (F(5,35) = 4.42, p < 0.005) in the relaxed muscle, but not during tonic dorsiflexion (one-way ANOVAs: all p > 0.07). When MEPs were facilitated at ISIs of -40 to 35 ms, the majority of subjects demonstrated a 20% or greater increase in the resting MEP (data for each ISI is shown in Table 1). Changes in MEP responses were more variable at a 40 ms ISI, where the afferent volley is estimated to reach the contralateral cortex 10 ms after the TMS pulse, with only 3/8 subjects showing a 20% increase. The size of the MEP in the relaxed and contracted muscle was unchanged at ISIs of 60 and -170 ms, where the interaction between the afferent volley and cortical stimulation was likely minimal (see Discussion). Baseline MEPs were 0.5 ± 0.3 mV at rest and 1.1 ± 0.6 mV during voluntary contraction and were similar between the seven ISIs.
MEP responses in non-stimulated TA

TMS could evoke MEP responses in the non-stimulated TA muscle due to the adjacent position of the right and left leg motor cortices (data not shown). Appreciable MEP responses (> 0.1 mV) in the non-stimulated TA could be recorded at rest in fifteen experiments at facilitatory intervals of -40 to 35 ms. There was no significant interaction (time x leg) or differences in the MEP response between the simulated and non-stimulated leg. However, MEP responses in the non-stimulated TA were more variable compared to the stimulated muscle and were increased by 123 ± 59% versus 151 ± 40%, respectively.

Sustained excitability of the CST after suprathreshold TMS

The strength of corticospinal projections to lower leg muscles was increased after PAS protocols in which sensory inputs to the motor cortex arrived after inputs evoked from TMS (at ISIs from -40 to 35 ms). Thus, we examined if, after a single pulse of suprathreshold TMS, the motor cortex supplying the TA muscle remains excitable for an appreciable amount of time so that coincident excitation from sensory inputs arriving many milliseconds after TMS can occur. We evaluated the size of the MEP response when the motor cortex was conditioned by a prior suprathreshold TMS. As shown in Figure 3A for a single subject, the MEP response in the relaxed muscle could be facilitated by up to 80 ms following a conditioning TMS pulse. Interestingly, the maximum resting MEP response that could be evoked in this subject was 1.2 mV, while the conditioned MEP was increased to 2.6 mV, demonstrating a large degree of facilitation. MEP responses were consistently
and significantly facilitated in all subjects when the conditioning stimulus was applied 20 to 50 ms before the test stimulus (all \( p < 0.05 \)) showing that CST excitability is strongly enhanced for a considerable period of time after TMS-induced activation (Fig. 3C). To examine whether this MEP facilitation occurred as a result of increased excitability of segmental interneurons and/or motoneurons to the TA muscle, we also evaluated the size of the TA H-reflex when conditioned by suprathreshold TMS in the same representative subject. Since the H-reflex in the TA muscle is more readily evoked during background activity than at rest, the size of the conditioning MEP was matched during voluntary dorsiflexion. In contrast to MEP responses, the H-reflex was strongly depressed when the conditioning TMS pulse was applied 20 to 50 ms before the CP nerve stimulus (Fig. 3B) suggesting that the paired-TMS facilitation was likely supraspinal in origin.

Insert Figure 3 near here

Subthreshold PAS

As shown in Figure 1, MEP facilitation occurred in PAS protocols where CP inputs reached the motor cortex well after the discharge of corticospinal neurons from TMS. Because descending corticospinal volleys from the leg area of the motor cortex are recorded only up to 10 to 15 ms following suprathreshold TMS (Di Lazzaro et al. 2001), there should be little or no firing of corticospinal neurons at ISIs where afferent inputs reach the motor cortex 15 to 90 ms after a TMS pulse, i.e., at ISIs of 35 to -40 ms, respectively. Thus, it seems possible that facilitation of MEP responses at these intervals occurred when pairing CP inputs with cortical activity that was subthreshold to corticospinal activation. To examine if MEP facilitation in the TA muscle is possible with subthreshold TMS, we used a technique where subthreshold TMS was paired with a 10-ms train of CP nerve stimuli. This paradigm is similar to that used by Huang et al. (2004) to
produce LTP in hippocampal slice preparations. We used 60 sets of paired stimuli delivered at faster rate (0.2 Hz) to shorten the time of the intervention. In one subject, off-line analysis revealed that MEPs were too variable to establish a consistent baseline and were omitted from the analysis. In the remaining seven subjects, MEP responses gradually increased following subthreshold-PAS (individual subjects displayed in Fig. 4A). The interaction effect (time x state) was significant (F(5,30) = 8.93, p < 0.001; Fig 4B) and post hoc tests showed significant MEP facilitation both at rest (F(5,30) = 13.18, p < 0.001) and during tonic dorsiflexion (F(5,30) = 2.63, p < 0.05). At rest, MEPs plateaued 30 to 60 min after PAS and showed an average increase of 85 ± 42% at 60 min. In the contracted muscle, MEP responses increased immediately after subthreshold-PAS and were facilitated by 21 ± 16% over the 60-min period that followed the intervention. At baseline, MEPs recorded at rest and during voluntary contraction were 0.4 ± 0.2 mV and 1.1 ± 0.5 mV, respectively, and were similar to that used in the suprathreshold PAS protocols.

Insert Figure 4 near here

**Strength of intracortical and spinal circuits**

In a separate series of experiments, we evaluated the strength of intracortical and spinal circuits following PAS using an ISI of 20 ms (where the largest and most consistent facilitation occurred) to determine the loci of increased CST excitability (e.g. cortex or spinal cord). Despite significant increases in MEP responses 15 and 30 min after PAS (41 and 37% respectively, Fig 5A; n = 8), there were small but insignificant decreases in short-interval intracortical inhibition (SICI; Fig. 5B; paired t-test, all p > 0.08) and small but insignificant increases in intracortical facilitation (ICF; Fig 5C; paired t-test, all p > 0.2) after PAS. The size of the unconditioned MEP (0.4 ± 0.2 mV) used to evaluate SICI and ICF was unchanged throughout the experiments (paired t-test, all p > 0.9).
Similar to SICI and ICF, the size of the H-reflex was unchanged 25 min after PAS ($t$-test, $p = 0.4$; open bar in Fig. 5D).

*Insert Figure 5 near here*
Discussion

A conditioning intervention of low-frequency PAS delivered at rest can significantly increase the size of the MEP in lower leg muscles. Similar to the hand, MEP facilitation in the leg evolved rapidly over the first 10 min and persisted for many minutes after the intervention. Surprisingly, unlike PAS in the hand, facilitation in the leg was produced over ISIs when sensory inputs arrived at the motor cortex over an estimated range of 15 to 90 ms after TMS-induced firing of CST neurons. We argue that following a TMS pulse, the continued activity of the leg motor cortex that is subthreshold to CST neuron activation is sufficient to induce associative facilitation in the motor cortex when paired with afferent excitation from peripheral nerve stimulation. In fact, it was possible to induce strong MEP facilitation when sensory afferent inputs were directly paired with subthreshold TMS.

Effect of interstimulus interval on MEP facilitation

Similar to previous reports in the hand and leg, PAS in this study did not affect MEPs if the peripheral volley arrived at level of the motor cortex before the cortical stimulus at an ISI of 60 ms (Wolters et al. 2003; Mrachacz-Kersting et al. 2007). It is likely that excitation of the motor cortex from a CP nerve stimulus did not produce sustained periods of cortical activity. Thus, when using a 60 ms ISI, there was likely no coincident activation of the motor cortex from CP and TMS inputs and, hence, no PAS-induced changes in cortical excitability.

Mrachacz-Kersting et al. (2007) have demonstrated that PAS in the leg strengthens corticospinal connections to the TA muscle when inputs from CP nerve stimulation are timed to coincide with inputs from cortical activation. Although the arrival time of the afferent input in the human motor cortex is not directly established, the first negative potential (N40) over the sensory cortex following
peroneal nerve stimulation has an average latency of 42 to 47 ms (Shaw and Synek 1985; Mrachacz-Kersting et al. 2007). Since some central processing is also required to transmit the afferent input to the motor area (Nielsen et al. 1997; Petersen et al. 1998; Wolters et al. 2005), a 4-10 ms has been adopted to estimate the arrival time of the CP volley in the contralateral motor cortex (Mrachacz-Kersting et al. 2007). This indicates that a TMS pulse should be delivered 46-57 ms after CP nerve stimulation to condition the motor cortex with coincident peripheral and central inputs. Likewise, ISIs from 45 to 55 ms have been shown to facilitate CST projections to the TA muscle following 30 min of low-frequency (0.2 Hz) PAS. Although an ISI of 55 ms does not produce consistent increases in corticospinal excitability in all subjects, adjusting the ISI in each individual using somatosensory evoked potentials and adding a central processing delay of 6 ms has been shown to produce consistent MEP facilitation (Mrachacz-Kersting et al. 2007).

In this study, we demonstrated that MEP responses could also be facilitated at ISIs where the afferent excitation in the cortex was estimated to arrive over a range of 15 to 90 ms after the TMS pulse, assuming an average delay of 50 ms based on Mrachacz-Kersting et al. (2007). We propose that afferent inputs arriving tens of milliseconds after a TMS pulse still encountered subthreshold activity in the motor cortex so that coincident excitation of common neurons was possible. Evidence for prolonged activity of the cortex was supported by the paired-TMS responses. Following a single pulse of suprathreshold TMS, the strength of CST projections to lower leg muscles was strongly enhanced by over 200% for at least 50 ms after the cortical stimulus (and perhaps longer in pre-corticospinal neurons). Such sustained excitability is likely cortical in origin given that descending corticospinal volleys are increased (Nakamura et al. 1997; Di Lazzaro et al. 2002) and spinal excitability is depressed (Fuhr et al. 1991; Ziemann et al. 1993, see also Fig. 4B) for many tens of
milliseconds following a single pulse of suprathreshold TMS. This increase in cortical excitability may occur as a result of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor activation given the time course of AMPA and NMDA mediated EPSPs are around 100 ms (Clark et al. 1994; Edmonds et al. 1995; Kohn et al. 2002). By 200 ms after TMS, corticospinal activity likely returns to baseline values and cortical activation by CP and TMS inputs becomes relatively independent once again. This is supported by a lack of change in MEP responses when CP and TMS inputs to the motor cortex are separated by approximately 220 ms (at an ISI of -170 ms).

In the hand motor cortex, if inputs from peripheral nerve activation arrive at the motor cortex an estimated 5 to 15 ms after TMS, MEP responses are reduced (Wolters et al. 2003; Ziemann et al. 2004). The suppression of MEP amplitudes has been attributed to spike-dependent LTD mechanisms where the activation of weak (sensory) inputs occurs during the afterhyperpolarization of a common cortical neuron activated by a strong (TMS) input. Such time-dependent reduction in MEP responses has been reported in the leg if suprathreshold TMS is given 40 ms after CP nerve stimulation, again when inputs from peripheral nerve stimulation are estimated to arrive 10 ms after TMS (n = 5 subjects; Mrachacz-Kersting et al. 2007). In contrast to these results, our group data failed to show suppression of the MEP response at an ISI of 40 ms. As 3/8 subjects showed strong facilitation which washed out the smaller inhibition observed in 3/8 subjects, it is possible that we did not see an overall suppression of MEP responses because we did not test PAS at the correct ISI for each subject. Adjusting the ISI using somatosensory evoked potentials may be useful for targeting this inhibitory interval. Here, we cannot rule out that a narrow and distinct time-window may exist for each subject to induce PAS inhibition in the leg motor cortex at rest, which is in contrast to more consistent PAS-
induced inhibition that can be produced during walking (Stinear and Hornby 2005; Prior and Hornby 2006). In addition, facilitation of MEPs using a 40 ms ISI in some subjects may have occurred due to coincident activation of the somatosensory cortex by TMS and afferent inputs (Wolters et al. 2005) given the close proximity of the leg motor and sensory corticies.

**Facilitation of MEP responses from subthreshold PAS delivered at rest**

The idea that continued activity in the motor cortex after suprathreshold TMS is sufficient to enhance cortical excitability when paired with sensory inputs is supported by the finding that MEP responses were increased when sensory inputs were paired with subthreshold TMS. This facilitation may have also occurred as result of using the 10-ms train of CP nerve stimulation or a faster rate of stimulation (0.2 Hz). However, recent evidence in humans suggests that cortical inputs subthreshold to CST neuron firing are important for PAS-induced increases in cortical excitability. Subthreshold-PAS in the hand, applied at a rapid-rate (5 Hz: Quartarone et al. 2006) or at low frequency (0.1 Hz) using antero-posterior stimulation combined with background contraction (Kujirai et al. 2006) has been shown to facilitate MEP responses. Moreover, it has been argued that later indirect (I) waves play an important role in associative plasticity given that antero-posterior magnetic stimulation tends to activate cortical circuits that preferentially elicit I3 waves (Sakai et al. 1997). Here, sustained excitability of I3-related cortical circuitry activated by antero-posterior stimulation over the leg motor cortex may have been involved in facilitating cortical excitability when paired with afferent volleys from the periphery.
Site of origin of PAS-induced changes in MEP responses

In the hand, PAS-induced MEP facilitation is likely cortical in origin, given that MEPs are facilitated when evoked with TMS and not from TES (Stefan et al. 2000; Ridding and Uy 2003). The fact that we observed MEP facilitation with subthreshold-PAS using TMS at 80% of active motor threshold also points to a cortical origin of facilitation, given that there are no descending volleys evoked at rest with TMS below active motor threshold (Di Lazzaro et al. 1998) and likely no paired activation of corticospinal and peripheral inputs to the spinal cord. Furthermore, the excitability of the motoneuron pool, as measured using the F-wave and/or the H-reflex in the TA muscle (Mrachacz-Kersting et al. 2007) and the abductor pollicis brevis muscle (Stefan et al 2000; Wolters et al. 2003) are unchanged following PAS despite concomitant changes to the size of the MEP response (although see Meunier et al. 2007). In line with these results, we did not observe large changes in the H-reflex response (5% increase) despite a 40% facilitation of resting MEPs in the relaxed muscle and a 21% increase in the active MEP observed in a separate experiment (see Figure 2). Although we did not demonstrate that short-interval intracortical inhibition and intracortical facilitation were altered following PAS (similar to the hand), this does not necessarily mean that excitability changes did not occur at a cortical level. It is likely that cortical circuits recruited with short-interval intracortical inhibition, and potentially intracortical facilitation (Di Lazzaro et al. 2006) did not reflect changes in cortical excitability induced by PAS to the leg motor cortex.

Differences between the hand and leg areas of the motor cortex

In the primary motor hand area, there exists a precise and narrow (≈ 20 ms) temporal relationship between the arrival of afferent and cortical inputs resulting in PAS-induced facilitation and inhibition of MEP responses (Wolters et al. 2003). This finding is in contrast to the related structures of the leg,
which showed increases in MEPs over a wide range (≈ 80 ms) of PAS time intervals. However, it is unlikely that the broad temporal relationship between pairs of inputs are unique to the related structures of the lower leg as artificially pairing neural activity in the wrist area of the primate motor cortex at comparable intervals (≈ 100 ms) can increase connectivity between two distant sites (Jackson et al. 2006). Here we propose a few explanations why the leg, in comparison to the hand, exhibited PAS-induced facilitation over a large window of time intervals. First, the time course of MEP facilitation in the present study gradually increased following PAS and MEPs were more pronounced after 10-20 min. It is possible that MEP facilitation in the hand at ISIs where the afferent input arrived at the motor cortex tens of milliseconds after the TMS pulse was not observed by Wolters et al. (2003) because MEP responses were only collected immediately after PAS. Secondly, afferent inputs from the lower leg may be stronger in modifying cortical excitability as electrical stimulation of leg afferents modifies CST excitability of the leg motor cortex more rapidly compared to the related structures of the hand. For instance, at least 1.5 hours of repetitive electrical stimulation of the ulnar nerve is necessary to produce consistent increases to the size of the MEP in the abductor digiti minimi and first dorsal interosseous muscles (Ridding et al. 2000), whereas a similar stimulation of the CP nerve over a shorter 30-min period produces persistent increases (up to 60 min) to the size of the MEP in the TA muscle (Khaslavskaya et al. 2002; Knash et al. 2003). Thirdly, because TMS preferentially activates horizontally oriented interneurons in the outer layers of the cortex, which trans-synaptically activate corticospinal neurons (Day et al. 1987, 1989), the mechanism of cortical activation may provide an additional explanation for these differences. In the hand, PAS-induced changes in CST excitability are linked to the direction of TMS currents because subthreshold antero-posterior stimuli have been shown to induce MEP facilitation in the first dorsal interosseous muscle, whereas similar posterior-anterior currents have no significant effect (Kujirai et
al. 2006). Geometry of a double-cone versus a figure-of-eight coil alters inputs to CST neurons and likewise alters descending corticospinal volleys that comprise the MEP (Terao et al. 2000). A double-cone coil may preferentially promote PAS-induced facilitation at ISIs where the afferent excitation reaches the motor cortex after the stronger TMS pulse. In line with these findings it may be worthwhile examining the effects of coil size and geometry on PAS-induced changes in cortical excitability. Likewise, it would be useful to investigate the temporal relationship between the arrival of afferent and cortical inputs resulting in PAS-induced facilitation and inhibition of MEP responses in hand muscles by measuring MEP responses at later time-points after PAS.

Clinical implications

The potential to increase the strength of corticospinal connections to leg muscles by PAS makes this technique a possible therapeutic tool. Previous studies have shown that the connectivity of CST neurons is crucial for functional motor recovery after subcortical insult (Thomas and Gorassini 2005; Ward et al. 2006). Future studies using peripheral and/or CNS stimulation prior to rehabilitative training, as shown using transcranial direct current stimulation (Hummel et al. 2005) and repetitive TMS (Kim et al. 2006), shows potential for improving functional recovery of leg muscles after injury to the CNS when facilitation of cortical circuitry is required.
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Figure Legends

Figure 1. Changes in MEP response after PAS
A, Traces show the average MEP response collected in the relaxed TA muscle in one representative subject before (Pre) and after (Post) PAS at ISIs of -40 to 60 ms (top to bottom in ascending order). B, Graph shows the group resting MEP collected at the seven ISIs. Group means were calculated from the average MEP in each subject recorded after PAS (0 to 60 min post). The ordinate in (B) shows the size of the MEP as a percentage of the baseline value and the abscissa shows the ISI between the CP nerve stimulation and the TMS pulse. Shaded area in (B) indicates the estimated coincident arrival of both inputs in the contralateral motor cortex (Mrachacz-Kersting et al. 2007). Data are from eight subjects at the main ISIs (-40 to 60 ms) and five subjects at an ISI of -170 ms. Statistical analysis comparing the different ISIs was not performed as data at each ISI was obtained from groups of overlapping but different subjects.

Figure 2. MEP facilitation as a function of the intervention ISI
A, The time course of MEP responses collected at rest. ISIs showing significant MEP facilitation in (A) are shown in gray. The ordinate shows the size of the MEP as a percentage of baseline values and the abscissa shows the time at which measurements were taken (0, 10, 20, 30 and 60 min after PAS). B to H, Bar graphs show the peak-to-peak amplitude of the MEP response collected in the relaxed (rest: gray bars) and contracted (active: white bars) TA muscle following PAS at ISIs of -170 to 60 ms. There was a significant interaction effect (time x state) at ISIs of -40 ms (F(5,35) = 2.67, p < 0.05) and 0 ms (F(5,35) = 4.77, p < 0.005) and post hoc analysis showed significant facilitation at rest. There was a significant time effect at ISIs of 20 ms (F(5,35) = 5.70, p < 0.001) and 35 ms (F(5,35) = 2.50, p < 0.05) with no interaction or state effects. At these ISIs, post hoc testing at each time-point was performed on the pooled data. Asterisks indicate time-points showing significant changes in comparison to baseline (*p < 0.05, **p < 0.01, ***p < 0.005).
Figure 3. Effect of suprathreshold TMS on CST excitability

Raw sweeps in one representative subject show the conditioning effect of activating the CST using a single pulse of suprathreshold TMS on the TMS-induced MEP (A) and the H-reflex (B) in the TA muscle. MEPs were strongly facilitated (> 200%) at ISIs of 20 to 50 ms in (A), whilst the H-reflex was strongly depressed in (B). The expected time-period of the H-reflex response is marked below each sweep (horizontal line). C, Bar graph shows the size of the test MEP response when conditioned by suprathreshold TMS. The ordinate shows the size of the conditioned MEP response, as a percentage of the unconditioned control MEP response. The abscissa shows the ISI between conditioning and test stimuli. Data are from five subjects. Asterisks indicate significant differences compared to the control MEP (*p < 0.05).

Figure 4. MEP facilitation after subthreshold-PAS

A, Individual subject data showing MEP increases in the relaxed TA muscle following subthreshold-PAS. B, Bar graph showing group MEP data collected at rest (gray bars) and during voluntary contraction (active: white bars). The ordinate shows the size of the MEP response expressed as a percentage of the baseline MEP. The abscissa shows the time at which measurements were taken (0, 10, 20, 30 and 60 min after subthreshold-PAS). Data are from seven subjects. The interaction effect (time x state) was significant (F(5,30) = 8.93, p < 0.001) and post hoc testing showed significant MEP facilitation at rest (F(5,30) = 13.18, p < 0.001) and during background contraction (F(5,30) = 2.63, p < 0.05). Asterisks indicate significant differences compared to baseline values (*p < 0.05, **p < 0.005).
Figure 5. Effect of PAS on MEP, SICI, ICF and H-reflex
Bar graphs show the effect of PAS at an ISI of 20 ms on the size of the MEP response (A), SICI (B), ICF(C) and H-reflex (D) recorded in the TA muscle. The ordinate in (A) shows the size of the MEP response as a percentage of the MEP before the intervention. The ordinate in (B-C) shows the size of the conditioned MEP as a percentage of the unconditioned response. The abscissa in (A-C) shows the time at which measurements were taken. The ordinate in (D) shows the size of the response recorded 25 min after PAS as a percentage of the response size before the intervention, and the abscissa shows the amplitude of the H-reflex (clear column), M-wave (gray column) and the level of background EMG (black column). Data are from eight subjects. Asterisks indicate significant differences compared to baseline values (*p < 0.05, ***p < 0.005).
<table>
<thead>
<tr>
<th>ISI (ms)</th>
<th>Average change in MEP (%)</th>
<th>Range (%)</th>
<th>Subjects showing $\geq 20%$ increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-170</td>
<td>-1 ± 20</td>
<td>-22 to 25</td>
<td>1/5</td>
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<tr>
<td>-40</td>
<td>57 ± 48</td>
<td>7 to 159</td>
<td>7/8</td>
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<tr>
<td>0</td>
<td>42 ± 23</td>
<td>-2 to 68</td>
<td>7/8</td>
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<tr>
<td>20</td>
<td>48 ± 32</td>
<td>14 to 107</td>
<td>6/8</td>
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<tr>
<td>35</td>
<td>38 ± 38</td>
<td>-18 to 75</td>
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</tr>
<tr>
<td>40</td>
<td>34 ± 54</td>
<td>-18 to 134</td>
<td>3/8</td>
</tr>
<tr>
<td>60</td>
<td>6 ± 14</td>
<td>-13 to 27</td>
<td>2/8</td>
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</tbody>
</table>

**Table 1. Effect of PAS at different ISIs on the size of the MEP response**

The mean MEP was evaluated in each subject from the responses collected in the relaxed TA muscle 0 to 60 min after PAS.
Fig. 1
Fig. 2
Fig. 3
**Fig. 4**

**A**

Size of MEP (% of baseline) vs. Time (min)

**B**

Comparison of MEP size between Rest and Active conditions. 

*Significant difference from baseline.

**Significantly different from control condition.**
Fig. 5