**TITLE:**

**Bilateral Assessment of the Corticospinal Pathways of the Ankle Muscles Using Navigated Transcranial Magnetic Stimulation**

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**KEYWORDS:**

Transcranial magnetic stimulation, corticospinal tracts, tibialis anterior, soleus, tonic voluntary activation, neurophysiology, corticomotor response, brain neuronavigation

**SUMMARY:**

The present protocol describes the simultaneous, bilateral assessment of the corticomotor response of the tibialis anterior and soleus during rest and tonic voluntary activation using a single pulse transcranial magnetic stimulation and neuronavigation system.

**ABSTRACT:**

Distal legmuscles receive neural input from motor cortical areas via the corticospinal tract, which is the main motor descending pathway in humans and can be assessed using transcranial magnetic stimulation (TMS). Given the role of distal legmuscles in upright postural and dynamic tasks, such as walking, a growing research interest in the assessment and modulation of the corticospinal tracts relative to the function of these muscles has emerged in the last decade. However, methodological parameters used in previous work have varied across studies making the interpretation of results from cross-sectional and longitudinal studies less robust. Therefore, use of a standardized TMS protocol specific to the assessment of legmuscles’ corticomotor response (CMR) will allow for direct comparison of results across studies and cohorts. The objective of this paper is to present a protocol that provides the flexibility to simultaneously assess the bilateral CMR of two main ankle antagonistic muscles, the tibialis anterior and soleus, using single pulse TMS with a neuronavigation system. The present protocol is applicable while the examined muscle is either fully relaxed or isometrically contracted at a defined percentage of maximum isometric voluntary contraction. Using each subject’s structural MRI with the neuronavigation system ensures accurate and precise positioning of the coil over the legcortical representations during assessment. Given the inconsistency in CMR derived measures, this protocol also describes a standardized calculation of these measures using automated algorithms. This protocol is not conducted during upright postural or dynamic tasks. However, this protocol can be used to assess bilaterally any pair of legmuscles, either antagonistic or synergistic, in both neurologically intact and impaired subjects.

**INTRODUCTION:**

The tibialis anterior (TA) and soleus (SOL) are ankle antagonistic muscles located in the anterior and posterior compartment of the lower leg, respectively. Both muscles are uniarticular, while the main function of TA and SOL is to dorsiflex and plantarflex the talocrural joint, respectively1. Furthermore, TA is more functional for long muscle excursions and less important for force production, whereas SOL is an antigravity muscle designed to generate high force with small excursion of the muscle2. Both muscles are especially relevant during upright postural and dynamic tasks (*e.g.,* walking)3,4. Regarding neural control, the motor neuron pools of both muscles receive neural drive from the brain via the motor descending pathways5,6, in addition to varying degrees of sensory drive.

The main motor descending pathway is the corticospinal tract, which originates from the primary and supplementary motor areas and terminates in the spinal motor-neuron pools7,8. In humans, the functional state of this tract (corticomotor response – CMR) can be feasibly assessed using transcranial magnetic stimulation (TMS), a non-invasive brain stimulation tool9,10. Since the introduction of TMS and given their functional significance during upright postural task and walking, CMR of TA and SOL have been assessed in various cohorts and tasks11-32.

In contrast to the assessment of CMR in upper-extremity muscles33, no universal TMS protocol has been established for the assessment of CMR in lower-extremity muscles. Due to the lack of an established protocol and the large methodological variability across the previous studies (*e.g.,* type of coil, use of neuronavigation, level of tonic activation, testing side and muscle, use and calculation of CMR measures, *etc.*), the interpretation of results across studies and cohorts can be cumbersome, complicated, and inaccurate. As the measures are functionally relevant in various motor tasks, an established TMS protocol specific to lower extremity CMR assessment will allow motor neuroscientists and rehabilitation scientists to systematically assess the CMR in these muscles across sessions and various cohorts.

Therefore, the objective of this protocol is to describe the bilateral assessment of TA and SOL CMR using single pulse TMS and neuronavigation system. In contrast to previous work, this protocol aims to maximize rigor of the experimental procedures, data acquisition, and data analysis by employing methodological factors that optimize the validity and duration of the experiment, and standardize the CMR assessment of these two lower extremity muscles. Given that the CMR of a muscle depends on whether the muscle is fully relaxed or is partially activated, this protocol describes how the TA and SOL CMR can be assessed during rest and tonic voluntary activation (TVA). The following sections will thoroughly describe the present protocol. Finally, representative data will be presented and discussed. The protocol described here is derived from that in Charalambous *et al.* 201832.

**PROTOCOL:**

All experimental procedures presented in this protocol have been approved by the local Institutional Review Board and are in accordance with the Declaration of Helsinki.

1. **Consent Process and Safety Questionnaires**
   1. Prior to any experiment, explain to each subject the aim of the study, the main experimental procedures, and any potential risk factors associated with participating in the study. After answering any questions or concerns that subjects may have, ask subjects to acknowledge the consent process and sign the informed consent form.
   2. Administer MRI34 and TMS35 safety-screening questionnaires to ensure subjects’ safety and qualification for both MRI and TMS testing. Exclude all subjects who don’t meet all safety criteria from both MRI and TMS assessments.
2. **MRI and Preparation of the Neuronavigation System**
   1. Administer the MRI assessment prior to TMS assessment32. Have subjects liein a supine position with a cushion placed under their knees to ensure a comfortable posture. Instruct subjects to keep still in the scanner.
      1. Provide ear protection to the subjects to attenuate the loud noise of the scanner. Preferentially use ear plugs over ear muffs due to the use of bilateral supratragic notch for subject-image registration in the neuronavigation system (see 5.2).
      2. Obtain high-resolution T-1 weighted anatomical brain images (minimum requirements: 1 mm slice thickness and full brain and cerebellar coverage), either as NFTI or DICOM files. Ensure that nose is fully included in the images due to the use of subject’s tip of the nose for subject-image registration in the neuronavigation system (see 5.2).
   2. Upload MRI files into a neuronavigation system. Co-register manually each subject’s MRI to anterior and posterior commissures, so the subject’s MRI can be mapped using the Montreal Neurological Institute atlas.
      1. Reconstruct the skin and full curvilinear brain model by adjusting the bounding box around the skull and brain tissue, respectively. Identify four anatomical landmarks (tip ofthe nose, nasion - bridge of the nose, and supratragic notch of the right and left ear) using the skin model (see **Figure 1A**).
      2. Place a rectangular grid over legmotor cortical area at each hemisphere (see **Figure 1B**). Position the centered row of the grid at the center and over the gyrus of the legmotor cortical area where the corticospinal tracts that innervate legmotor pools originate36. Position the medial column of the grid parallel and adjacent to the medial wall of the ipsilateral hemisphere.
      3. Use a cortex based approach in which error in orientation has a negligible effect on the stimulation site37 instead of using a scalp based target approach in which any error in orientation can alter the stimulation site. Use this grid to find the hot spot. For motor mapping, use larger grids either by adding more spots and/or increasing the distance between spots (*e.g.,* 10 mm).
3. **Subject Preparation and Placement**
   1. Measure the electrophysiological responses by single pulse TMS using a total of 4 surface EMG electrodes. For the preparation and placement of the electrodes, use published guidelines38,39 and complete placement while the subject is in a standing position.
      1. Prepare the area over which each electrode would be placed by shaving and lightly exfoliating any dead skin cells and oils using alcohol swabs.

CAUTION: For subjects on blood thinners (*e.g.* people post-stroke), use caution during skin preparation due to high risk of bleeding.

* + 1. Attach electrodes bilaterally on TA. While in the standing position, ask subjects to lift their toes upwards and then place the electrode at the upper third of the line between the head of the fibula and medial malleolus (*i.e.,* muscle belly immediately lateral to the tibial crest).
    2. Attach electrodes bilaterally on the lateral SOL. While in the standing position, ask the subject to perform heel raise and then place the electrode at the lower third of the line between the lateral femoral condyle and lateral malleolus.
    3. Attach the ground reference passive electrode either on the patella or lateral malleolus. Depending on the EMG acquisition unit, place the ground electrodes either bilaterally or unilaterally.
  1. Test the electrodes’ placement and quality of the signal.
     1. Test the electrodes’ placement (*e.g.,* for clear visually detectable EMG bursts) by asking the subject to either dorsiflex or plantarflex the ankle in an upright posture while displaying the raw EMG signal of all muscles tested on a computer screen. In the case of a misplaced electrode, remove and replace it until there is clear visually detectable EMG bursts with minimal background noise. An adequate signal to noise ratio is critical in detecting a motor response (> 50 µV).
     2. Test the quality of the signal (*e.g.,* for baseline noise) by discharging the TMS units for a few times while the TMS coil is held away from the seated subject and with the muscles at rest. Check that the baseline signal for each EMG channel is close to zero (*i.e.,* the peak-to-peak amplitude should be less than 50 µV and there is no baseline noise, such as 50 or 60 Hz power line hum). If baseline noise is present in a channel, remove the corresponding electrode and repeat the skin preparation procedures. If the noise is still present (*i.e.,* peak-to-peak amplitude > 50 µV), adjust the reference electrode’s position and replace the electrolyte gel .
  2. Secure all electrodes using light foam pre-wrap tape. Periodically throughout the experiment, check to ensure that electrodes are securely attached and that the signal has good quality.
  3. Seat the subject in a chair. To ensure consistent feet placement across subjects, secure both feet in walking boots (*i.e.,* ankle foot orthosis) that allow the ankle ROM to be adjusted to a specific position and provide resistance during TVA testing. Adjust both hip and knee angles to avoid subject discomfort. Instruct the subject to keep still throughout the experiment. Use a forehead rest attached to the chair to keep subjects still during TMS application, if available.

1. **TVA Testing** 
   1. Determine bilaterally the maximum voluntary isometric contraction (MVIC) of each muscle. For each motion (*i.e.,* dorsiflexion and plantarflexion), instruct subjects to maximally contract the contralateral examined muscle (*e.g.,* right TA) 4 times (~5 s contractions separated by 60 s of rest) while subject is seated in the posture described above.
   2. Calculate the maximum muscle activity value during each MVIC (*i.e.,* the average within a 100 ms window centered around the maximum rectified and smoothed EMG) of the last three trials, the average of the three values, and the 15% and 5% of each muscle’s average MVIC.

CAUTION: A larger % MVIC can be used, but it may not be feasible in clinical cohorts (*e.g.,* people post-stroke).

1. **Registration in Neuronavigation System**
   1. Place the subject tracker, either a headband or glasses, with reflective markers on the subject’s head at the opposite side from the stimulated hemisphere so the tracker does not obstruct positioning of the coil during the stimulation of each grid spot.

CAUTION: In the case that a headband is used, ensure that it is snug on subject’s head, yet not overly tight because it may cause a headache after an extended period of time.

* 1. Verify the proper position of the motion capture camera by placing the subject tracker, the pointer, and the coil tracker in its capture volume space. Perform the subject-image registration by placing the tip of the pointer on the 4 anatomical landmaks (see **Figure 1A**).
  2. Once all anatomical landmarks are sampled, verify whether registration occurred accurately by placing the tip of the pointer on several spots over the subject’s skull (*i.e.,* validation stage). If the distance from the tip of the pointer to the reconstructed skin is less than 3 mm, proceed to TMS experiment; otherwise, repeat the subject-image registration until the desired error values are obtained. During the experiment, repeat registration if the subject tracker is accidentaly moved.

1. **TMS**
   1. Use the same methodological parameters during rest and TVA.
      1. Apply single pulse stimuli on the optimal site (*i.e.,* hot spot; see next paragraph for further details) of the examined muscle. Apply reach stimulus randomly every 5-10 s to avoid stimulus anticipation and to minimize the carry-over effects of the previous pulse to the subsequent one40.
      2. In case that two TMS units are simultaneously used, set the units at either the standard or simultaneous mode41. The standard mode applies a weaker pulse than a single unit, whereas the simultaneous mode applies a stronger pulse than a single unit. The use of either one could be based on the needs of the protocol and the total number of stimuli.
      3. Use a double cone coil to induce a posteroanterior intracranial current. If necessary, use the neuronavigation system to control the coil manually and correct its position in relation to the desired stimulated spot prior to each stimulus.
      4. Across sessions and subjects, randomize the order of the examined muscle and hemisphere. Always administer the TVA condition after the rest condition to avoid any interference with testing at rest (*e.g.,* fatigue of the descending pathways due to TVA testing).
   2. Determine bilaterally the hot spot of both muscles.
      1. Find the suprathreshold intensity, which will be used during hot spot hunting, by applying a single stimulus over the centered spot next to the interhemispheric fissure (see blue and red squares in **Figure 1B**). Use this spot because it is located at the locus of the legmotor area36,42.
      2. Start at low intensity (*e.g.,* 30% maximum stimulator output; MSO) and gradually increase the TMS intensity by 5% increments, until reaching the intensity that elicits a motor evoked potential (MEP) with a peak-to-peak amplitude greater than 50 µV in all contralateral examined muscles for 3 consecutive stimuli.
      3. Determine immediately after each stimulus whether a MEP has been elicited based on both the raw waveforms and peak-to-peak amplitudes (search window: 20-60 ms post-TMS onset) of all examined muscles.
      4. Apply one TMS pulse on each spot of the grid (total 36 stimuli). After the completion of the hot spot protocol, transfer the amplitude and latency values of each spot for all contralateral muscles in a spreadsheet and sort amplitude from high to low and latency from low to high. Identify the hot spot of contralateral TA and SOL as the location in the grid with the largest amplitude and the shortest latency43.

CAUTION: If the largest amplitude and shortest latency are not at the same spot, define the hot spot using the largest amplitude.

* 1. Determine bilaterally each muscle’s resting motor threshold (RMT).
     1. Select the grid spot in the neuronavigation system that corresponds to the examined muscle’s hot spot.
     2. Use an adaptive threshold-hunting method for RMT determination of the examined muscles44. Set the initial intensity and step size at 45 and 6% MSO, respectively32. Run the RMT hunting twice for each muscle and use the average for the subsequent CMR assessment.
  2. Assess bilaterally TA and SOL CMR during rest.
     1. Select the grid spot in the neuronavigation system that corresponds to the examined muscle’s hot spot. Apply 10 single TMS pulses at 1.2 RMT of the examined muscle.
     2. Prior to each stimulus, instruct the subject to stay still and relax the examined muscles bilaterally and monitor the activity of all muscles using a real time visual feedback displaying on a computer screen. In case any muscle is active before or after TMS, discard that trial and apply an additional single pulse. Repeat until 10 waveforms for each contralateral examined muscle at rest have been collected.
  3. Assess bilaterally the TA and SOL CMR during TVA.
     1. Select the grid spot in the neuronavigation system that corresponds to the examined muscle’s hot spot.
     2. Ask subjects to contract the examined muscle at 15% ± 5% MVIC and apply 10 single TMS pulses at 1.2 RMT. Instruct subjects to keep the smoothed moving line (root mean square amplitude of 0.165 s) of the examined muscle, either TA or SOL, within the two horizontal cursors (MVIC range: 15% ± 5%) and sustain that contraction at that level for few seconds.
     3. When TA is the examined muscle, ask subjects to pull slightly up against the bootstraps on their contralateral leg(*i.e.,* the legwith the examined muscle contralateral to stimulated hemisphere). When SOL is the examined muscle, ask subjects to push slightly down against the boot on the contralateral leg.
     4. Monitor the muscle activity of the active examined muscle and the remaining resting muscles using a real time visual feedback display on a computer screen. Discard that stimulus and apply an additional single pulse again in case the examined muscle’s activity is either below or above the predetermined range or any other muscle is activated. Collect 10 trials while the examined muscle is activated at the predetermined range.

1. **Data Analysis**
   1. For all CMR measures except RMT, calculate the value of each measure from each MEP sweep (the total duration should be at least 500 ms with minimum 100 ms pre-stimulus duration) for all muscles and then average these 10 values to get a single value (*i.e.,* mean)32. Amplitude and cortical silent period (CSP) are proxy excitability measures of CMR, whereas latency is a proxy connectivity measure of CMR. For both rest and TVA, normalize latency relative to each subject’s height, as latency is influenced by distance to the examined muscle45.
   2. Calculate MEP amplitude and latency during rest.
      1. Calculate amplitude (µV) from the raw EMG as the largest difference between positive and negative peaks (*i.e.,* peak-to-peak) of the MEP. For these two particular muscles, search for peak-to-peak within a time window of 20-60 ms after TMS onset.

CAUTION: Though the MEP search window of 20-60 ms may work for neurologically intact subjects and people post-stroke, wider MEP search windows (*e.g.,* 20-75 ms) might be required for other neurological populations (*e.g.,* multiple sclerosis).

* + 1. Calculate latency (ms) from the rectified EMG as the time between TMS onset and MEP onset (*i.e.,* the time when a rectified EMG trace first crosses a predetermined threshold - mean plus three standard deviations of the 100 ms pre-stimulus EMG)32,46.
  1. Calculate MEP amplitude, latency, and CSP during TVA.
     1. Calculate amplitude (µV) from the raw EMG as the largest difference between positive and negative peaks (*i.e.,* peak-to-peak) of the MEP. For these two particular muscles, search for peak-to-peak within a time window of 20-60 ms after TMS onset.
     2. Calculate latency (ms) from the rectified EMG as the time between TMS onset and MEP onset.
        1. Calculate the MEP onset differently in TVA than in rest. Calculate MEP onset and offset by finding the two time points that the rectified EMG trace crosses the predetermined threshold set to the level of 100 ms pre-stimulus mean EMG. Then, find the peaks that are at least greater than the mean of the pre-stimulus EMG plus three standard deviations and between those two time points. Then, search from the first peak to 50 data points (sampling rate of 5000 Hz) before that peak for the time that the rectified EMG trace first crosses the threshold of the mean pre-stimulus EMG. Define that time as the MEP onset32.
     3. Calculate CSP (ms) from the rectified EMG as the time between the MEP offset and EMG resumption (*i.e.,* absolute CSP: exclusion of MEP duration)47. Search from the last peak to 200 data points (sampling rate of 5000 Hz) after that peak for the time that the rectified EMG trace last crossed the threshold of the mean pre-stimulus EMG; define that time as the MEP offset. Then, calculate the resumption of baseline EMG, which is the time that the rectified EMG trace last crosses 25% of the mean pre-stimulus EMG32.

**REPRESENTATIVE RESULTS:**

**Figures 2-4** present data from a representative neurologically intact 31 year old male with height and weight of 178 cm and 83 kg, respectively.

**Figure 2** presents the bilateral hot spots and RMT of each ankle muscle. Using the spot located on the center of the legarea in each hemisphere (see squares in **Figure 1B**), the intensity of 45% MSO was bilaterally used for the hot spot hunting. The hot spot location for each muscle differed between hemispheres, yet as anticipated all four hot spots were located in the legmotor cortical areas. This finding indicates that TA and SOL may not share the same hot spot; therefore, CMR of each muscle should be assessed using each muscle’s hot spot as opposed to using the same hot spot for both muscles. Bilateral RMT was determined for each muscle, using an adaptive threshold-hunting method. The number of stimuli applied for RMT determination ranged from 6 to 22 stimuli. The difference between the two RMT values of each muscle ranged from 1% to 3% MSO. Combining these results suggests that using an adaptive threshold-hunting method can be an efficient approach to determine the RMT of an ankle muscle with low variability. Furthermore, all RMTs were lower than the intensity used for hot spot hunting (dashed line in **Figure 2**). This finding indicates that using the spot located on the legmotor area (see squares in **Figure 1B**) to determine a “true” suprathreshold intensity is feasible.

**Figure 3** presents the bilateral responses of TA and SOL when the hot spot of each muscle was stimulated during rest.For all bilateral stimulated hot spots, contralateral MEPs were elicited in both TA and SOL. However, the responses and latencies were always larger and shorter in TA than in SOL, respectively, regardless which muscle’s hot spot was stimulated. Ipsilateral responses were present mainly in TA and when the stimulated hot spot was proximal to interhemispheric fissure (see **Figure**: **2A** – TA hot spot, **2B** – both muscles hot spot). Conversely, ipsilateral responses were absent in both muscles when the stimulated side was further lateral from the interhemispheric fissure (>10 mm) (see **Figure 2A** – SOL hot spot). **Figure 4** presents the bilateral responses of TA and SOL when the hot spot of each muscle was stimulated during TVA. As in rest, contralateral MEPs were elicited in both TA and SOL for all bilateral stimulated sites during a 15% ± 5% MVIC. Only the examined muscle was activated; therefore, the remaining three muscles were at rest. CSP was present only in the examined activated muscle, both TA and SOL. As in rest, TMS over right TA and left SOL hot spots also elicited ipsilateral responses; those responses were present only in the ipsilateral TA (see **Figure 4A**,**D**). Conversely, TMS over the right SOL and left TA hot spots elicited only contralateral MEPs. Interestingly, late responses in contralateral SOL was present only when TA was activated; those responses were present bilaterally, occurred between 80-100 ms post-TMS, and had larger amplitudes than the MEPs (see † in **Figure 4A,C** ). These late responses with range 70-100 ms post-TMS have previously been reported to be prevalent in SOL only with TA TVA (0-40% MVIC)48,49.

Both resting and TVA conditions were similar in that ipsilateral responses were elicited when certain hot spots were stimulated. The presence of ipsilateral responses could potentially be the result of stimulation of an oligosynaptic pathway (*e.g.,* cortico-reticulo-spinal tract) or the spread of the pulse’s current. An approach to distinguish between the two possible causes is to calculate the difference in latency between the contralateral and ipsilateral responses. Previous TMS studies have speculated that an ipsilateral response of > 3 ms delay relative to the contralateral response is an ipsilateral MEP (iMEP), and the potential pathway could be the cortico-reticulo-spinal tract (*i.e.,* oligosynaptic pathway)50-54. Conversely, any ipsilateral response with a shorter delay might be the result of the pulse’s current; therefore, such a response may not be an iMEP. During rest, ipsilateral responses had similar latencies as contralateral responses (see **Figure 3A**, C and D). Thus, these responses were most likely not iMEPs, but were likely elicited due to the spread of the pulse’s current applied adjacent to the interhemispheric fissure. When the right TA and the left SOL were activated during TVA, ipsilateral responses were only elicited in the TA and were delayed by > 3 ms compared to the contralateral response (see **Figure 4A,D**). These responses might be iMEPs, which may indicate stimulation of the cortico-reticulo-spinal tract. In summary, ipsilateral responses are common when the legmotor area is stimulated55; therefore, caution should be taken when these responses are interpreted as iMEPs.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Reconstructed Skin and Curvilinear Brain Models. (A)** A skin model with four anatomical landmarks (tip of the nose, nasion, and supratragic notch of the right and left ear) is used to calculate the subject-image registration during the assessment by placing the tip of a pointer on each landmark. **(B)** A 4 x 9 rectangular grid placed bilaterally over the legmotor cortical area. Squares denote the spots used to determine the suprathreshold intensity used for the hot spot hunting.

**Figure 2: Bilateral TA and SOL Hot Spots and RMT.** In both hemispheres, the star symbol denotes the hot spot of each muscle. Bar plots present the mean RMT of two assessments (open white circle) for each muscle, while the values below each circle denote the number of stimuli applied to determine the RMT using an adaptive threshold-hunting method. The dashed line indicates the intensity used for the hot spot hunting (45% MSO). **(A)** Hot spots and RMTs of the right/contralateral TA and SOL while TMS was applied over the left hemisphere. TA hot spot was over the legmotor area and proximal to the interhemispheric fissure whereas SOL hot spot was 10 mm lateral to TA hot spot. The number of stimuli used to determine TA and SOL RMT ranged 6-21 and 9-11, respectively. **(B)** Hot spots and RMTs of the left/contralateral TA and SOL while TMS was applied over the right hemisphere. As in the left hemisphere, TA hot spot was over the legmotor area and proximal to the interhemispheric fissure. SOL hot spot was 7.1 mm posterior-lateral to TA hot spot. The number of stimuli used to determine TA and SOL RMT were in the ranges 10-22 and 10-11, respectively.

**Figure 3: Bilateral TA and SOL CMR Assessment - Rest.** For the stimulation of each hot spot, the EMG of the bilateral resting TA and SOL were collected while the average waveform of each muscle is presented (total duration 500 ms; 100 ms pre-TMS). The √ and X symbols denote that MEP was either present (>50 µV) or absent (≤ 50 µV), respectively. In case of MEP’s presence, the values of the peak-to-peak amplitude (µV) and latency (ms) are presented. **(A)** Stimulation of the right/contralateral TA hot spot on left hemisphere. MEPs were elicited in both right/contralateral ankle muscles, with right TA having larger amplitude and shorter latency than right SOL. Given that the stimulated hot spot is located by the interhemispheric fissure and proximal to the legmotor area on left hemisphere, MEP on the left/ipsilateral ankle muscles was also elicited (only TA). **(B)** Stimulation of the right/contralateral SOL hot spot on left hemisphere. MEPs were elicited only on the right/contralateral ankle muscles; however TA had larger MEP amplitude and shorter latency than SOL. **(C)** Stimulation of the left/contralateral TA’s hot spot on right hemisphere. MEPs were elicited in both left/contralateral and right/ipsilateral ankle muscles with both TA having larger MEP amplitudes and shorter latencies than both SOL. This bilateral MEP elicitation is mainly due to the location of the stimulated hot spot and suprathreshold intensity. **(D)** Stimulation of the left/contralateral SOL hot spot on right hemisphere. MEPs were elicited in left/contralateral ankle muscles and right/ipsilateral TA.

**Figure 4. Bilateral TA and SOL CMR Assessment - TVA.** For the stimulation of each hot spot, the EMG of the bilateral TA and SOL were collected while the examined contralateral muscle was activated at 15 ± 5% MVIC. The average waveform of each muscle is presented (total duration 500 ms; 100 ms pre-TMS). The √ and X symbols denote that MEP was either present (>50 µV) or absent (≤50 µV), respectively. In case of MEP’s presence, the values of the peak-to-peak amplitude (µV), latency (ms), and CSP (ms) are presented. **(A)** Stimulation of the right/contralateral TA hot spot on left hemisphere. Right TA MEP was followed by CSP. MEP was elicited in contralateral/right SOL in which a late response (†) was also elicited (amplitude: 563 µV; latency: 82.8 ms). MEP was also elicited in left/ipsilateral TA, whose latency is delayed by 5.2 ms compared to the right/contralateral TA’s latency. **(B)** Stimulation of the right/contralateral SOL hot spot on left hemisphere. Right/contralateral SOL MEP was followed by CSP, and MEP was also elicited in contralateral/right TA. No left/ipsilateral MEPs were elicited. **(C)** Stimulation of the left/contralateral TA hot spot on right hemisphere. Left TA MEP was followed by CSP. MEP was elicited in left/contralateral SOL in which a late response (†) was also elicited (amplitude: 465 µV; latency: 96.3 ms). No MEPs were elicited in right/ipsilateral muscles. **(D)** Stimulation of the left/contralateral SOL hot spot on right hemisphere. Left SOL MEP was followed by CSP. MEPs were elicited in left/contralateral SOL and right/ipsilateral TA, whose latency is delayed by 4.7 ms compared to the left/contralateral TA’s latency. No MEP was elicited in right/ipsilateral SOL.

**DISCUSSION:**

Given the emerging interest in how the motor cortex contributes to the motor control of legmuscles during dynamic tasks in various cohorts, a standardized TMS protocol that describes the thorough assessment of these muscles is needed. Therefore, for the first time, the present protocol provides standardized methodological procedures on bilateral assessment of two ankle antagonistic muscles, SOL and TA, during two muscle states (rest and TVA) using a single pulse TMS with neuronavigation.

The findings described in the representative results section points out several critical steps which should be considered. First, CMR assessment of these muscles, as well as other legmuscles, should be conducted using a neuronavigation system in which each subject’s MRI should be used and each muscle’s hot spot should be determined. Neuronavigation can guide precise TMS stimulation over the target motor area, and when the subject’s MRI is used, the target motor area can be stimulated accurately56,57. Previous work examined the effects of using neuronavigation during a TMS assessment of upper extremity muscles58-60; findings from those studies were mixed. Yet, no study examined this effect for a lower extremity muscle. Given the location of the motor cortical areas of TA and SOL (*i.e.,* adjacent to the interhemispheric fissure at approximately 3-4 cm below the scalp surface)36,42,61, hunting for the “true” hot spot of each muscle using a grid placed on each subject’s anatomy increases the probability of feasibly eliciting an MEP in either muscle, especially in SOL. Using the same protocol presented here, we have recently shown that MEPs were successfully elicited in both TA and SOL in nearly all subjects (N = 21)32. The second critical step is the bilateral assessment of each muscle. In contrast to upper extremity motor areas, the two legmotor areas are adjacent to each other, and when a pulse is applied over one area the opposite area might be stimulated due to current spread. Therefore, any ipsilateral response in either muscle may indicate either the presence of an iMEP (a potential proxy of cortico-reticulo-spinal pathway)50 or just a direct stimulation of the opposite legmotor area. In the past, ipsilateral TA responses were reported, yet the stimulated site was based on anatomical landmark (10 and 15 mm posterior and lateral to vertex)62. Using this protocol, the hot spot of each muscle can be determined separately, and depending on the hot spot’s location either contralateral or bilateral responses can be elicited (see **Figure** **3** and **Figure 4**). Whether the bilateral response is a result of multiple descending pathways or just stimulation of a single pathway requires further investigation.

The present protocol can be modified depending on the research design. While single pulse TMS is used in this protocol, paired pulse (test pulse is preceded by conditioning pulse)63,64 can also be used to assess the intracortical networks of these two ankle muscles. Similarly, after hot spot and RMT determination of each muscle, bilateral input-output curves of each muscle can be acquired to assess the relationship between TMS intensity (input) and the MEP amplitude (output). To assess the CMR of each muscle, 10 stimuli are applied on each hot spot during rest and TVA, yet recent reports have suggested that more than 10 stimuli should be used to assess reliably the CMR of a muscle65,66. Similarly, more than one stimulus per spot can be applied during the hot spot hunting (*e.g.,* 2-5 stimuli/spot) compared to a single stimulus per spot used in this protocol. By applying more than one stimulus per spot, the hot spot of each muscle might be more reliably determined. Recent study suggested that as few as two stimuli per spot might be sufficient for hot spot determination67. Furthermore, compared to the most widely used threshold hunting method, the relative frequency method68, which is based on the Rossini-Rothwell criterion69,70, the adaptive threshold-hunting method is used in the present protocol. Though the adaptive threshold-hunting method is more efficient (*i.e.,* fewer stimuli are required to determine RMT) than the relative frequency method, both methods share similar precision71. It is important to remember that all aforementioned modifications increases the total count of stimuli applied. Lastly, the current protocol used the criterion of less than 50 µV peak-to-peak amplitude to assess for baseline noise and for the “true” resting state. Discarding any EMG signal greater than 10 µV (root mean square calculated over 100 ms) is an alternative approach.

This protocol has few methodological considerations. First, the assessment of these two muscles is in a seated position, either during rest or TVA. As previously mentioned, both TA and SOL are crucially important during upright postural tasks and walking. Although previous studies have examined TA and SOL CMR during upright postural task14,72-76 and walking20,22,77-79, the assessment was only unilateral, and TMS was not guided by neuronavigation. Therefore, even if the present protocol is not used during these tasks, it can still provide a non-invasive window about the cortical drive of these two ankle muscles. Second, the active motor threshold (AMT) was not determined because there is not a well established methodology for that measure. Given that AMT is correlated with and is lower than RMT (~ 82%)80, MEP can be elicited during TVA even when using a suprathreshold intensity of RMT. Third, use of structural MRI of each subject with the neuronavigation system may not be feasible in all settings due to high cost of obtaining MRI and the neuronavigation system. However, certain neuronavigation systems including the one used in this protocol, can be used without subject’s MRI; but an average MRI is used. In this case, the coil can be still precisely positioned over the stimulated site.

While previous work has examined TA and SOL CMR during various tasks in different cohorts, no study used a standardized protocol that examined these two muscles bilaterally using neuronavigation with each subject’s MRI. Use of each subject’s structural MRI combined with a neuronavigation system promotes the accuracy and precision of stimulation of the motor cortical representations of both muscles. This is crucially important for the legmotor cortical areas. Also, given that the CMR of a muscle depends on whether the muscle is fully relaxed or is partially activated, this protocol describes how the TA and SOL CMR can be assessed during rest and TVA. Additionally, each hemisphere is stimulated while the bilateral CMR of each muscle is simultaneously assessed. Furthermore, rather than using the same hot spot for assessing a single muscle’s CMR, each muscle’s hot spot is determined using a standardized grid, which was laid over the legcortical representation, and is defined as the spot with the largest amplitude and shortest latency43. Though the relative frequency method is widely used to measure the motor threshold of a muscle68, this protocol uses an adaptive threshold-hunting method to reduce the experimental duration and total number of stimuli applied per session44. Finally, to reduce the duration of data analysis and to standardize the calculation of CMR measures, an automated data analysis methodology is used.

Future studies can use this protocol to further elucidate the cortical control of TA and SOL in both neurologically intact and impaired cohorts. One such application of the present protocol is the mapping of these two muscles. Though few studies examined the motor cortical area of TA81-84, only one study reported the motor cortical area of SOL from a single patient with focal cortical dysplasia85. A common characteristic that all these studies share is the use of the same neuronavigated TMS system, which is different from the system used in this protocol. However, this system is extremely expensive, and it is usually found in clinical settings such as hospitals. By modifying the present protocol, future studies can systematically investigate and establish normative data of cortical mapping measures for TA and SOL in neurologically intact adults. Such findings will establish which motor mapping measures should be used to specifically quantify the motor representations of each muscle. Another potential application of the present protocol is the assessment of these two muscles before and after a surgery or an intervention (behavioral: exercise; neurophysiological: repetitive TMS, transcranial direct current stimulation - TDCS) and during the recovery period in athletic or clinical cohorts. This will allow rehabilitation scientists to determine how a surgery or an intervention may alter the cortical drive of these two muscles.

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The authors have nothing to disclose.

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