**JoVE58944 "Bilateral assessment of the corticospinal pathways of the ankle muscles using frameless stereotaxic transcranial magnetic stimulation,"**

**We thank the editor and the reviewers for their constructive and thoughtful comments, which we believe have resulted in a significantly improved manuscript. We have responded to editor’s and each reviewer’s comments below. Where appropriate, we refer to sections in the revised manuscript. All changes in the manuscript are indicated using track changes. We have highlighted the sections that will be recorded.**

**Response to Editor and Reviewers:   
  
*Editor:***  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Response**: **We thank the editor for this reminder.**

**Action**: **We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**Response: We thank the editor for this instruction.**

**Action: We have numbered correctly all sections.**

3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:  
In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details (e.g., lines 103-109, 138-145, 204-207, 216-220, 225-231, 234-248, 254-262, 291-297, 309-318, 330-368, etc.) about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

**Response: We apologize for any confusion.**

**Action: We have converted all the NOTES into steps.**

4. Lines 135: This step does not have sufficient details to replicate. Please provide more details.

**Response: We apologize for any missing information.**

**Action: We have elaborated on this step.***“2.2.1 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).”*

5. Line 211: It is unclear how this is done.

**Response: We apologize for the insufficient clarity of this step.**

**Action: We have added additional information to improve the clarity of this step.***“4.2 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.”*

6. Lines 251-262: Please break up into sub-steps.

**Response: Thank you for the suggestion.**

**Action: Per editor’s recommendation, we broke up this section into sub-steps.***“6.2.1 Find the suprathreshold intensity, which will be used during the 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 leg motor area36,42.  
6.2.2 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.   
6.2.3 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.”*

7. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

**Response: We thank the editor for this suggestion.**

**Action: We have revised the manuscript to combine short Protocol steps where feasible and appropriate.**

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

**Response: We thank the editor for this instruction.**

**Action: We have included single-line spaces between all paragraphs, headings, steps, etc.**

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that calculations are not appropriate for filming.

**Response: We thank editor for this instruction**

**Action: We have highlighted the appropriate sections.**

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Response: We thank editor for this instruction**

**Action: We highlighted complete sentences.**

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Response: We thank editor for this instruction**

**Action: We highlighted sections per editor’s instructions.**

12. Figures 3 and 4: Please include a space between all numbers and their corresponding units (260 µV, 34.6 ms, etc.).

**Response: We apologize for any confusion.**

**Action: We have added space between values and units.**

13. References: Please do not abbreviate journal titles.

**Response: We apologize for any confusion.**

**Action: We have corrected all journal titles.**

*Reviewer #1:*  
1. The strong recommendation to use a neuronavigation system is not well-supported, given the authors' comment that no study has yet determined whether neuronavigation improves accuracy of lower extremity TMS measures (line 507). It seems that the "true" hotspot could be identified without neuronavigation by selecting the location that produces MEPs with the shortest latency using the lowest stimulus intensity identified by PEST - reflecting direct activation of the hotspot, rather than oligosynaptic ipsilateral MEPs (which would produce longer latency) or current spread (which would require higher intensity). Given the costs of MRI and neuronavigation, it would be a pity to suggest that these are an absolute necessity, as this would discourage research in an area that already receives relatively little attention.

**Response: The reviewer raises an important point, and we apologize for the misunderstanding. It was not not our intention to strongly recommend that neuronavigation is required for the assessment of the lower extremity TMS measures. In the present manuscript, our goal was to present the experimental procedures of a protocol that a neuronavigation system can be used to improve the accuracy and precision of the stimulation of TA and SOL optimal spots. Numerous studies have examined the leg muscles CMR without using navigated TMS. Yet, using this protocol the CMR of both SOL and TA can be more accurately assessed.**

**Action: We have revised the last section of the fourth paragraph in the discussion to acknowledge the potential burden of using navigated TMS.   
*“****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, coil can be still precisely positioned over the stimulated site.****”***

2. Line 106: By "acknowledge the consent process", do you mean provide written informed consent?

**Response: Yes, we meant to say written informed consent.**

**Action: We have revised this line to improve clarity.   
*“****After answering any questions or concerns that subjects may have, ask subjects to acknowledge the consent process and sign the informed consent form.****”***

3. Line 160: It's easy to locate TA by palpation during dorsiflexion. By "tip of the fibula" do you mean head of the fibula?. And do you mean 1/3 from the top or the bottom of the imaginary line? It might be easier to recommend locating the muscle by palpation, immediately lateral to the tibial crest.

**Response: We thank reviewer for pointing this out.**

**Action: We have revised this step to improve clarity.***“3.1.2 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).”*

4. How far apart should the recording electrodes be placed on the skin?

**Response: We are using a single differential electrodes that the two discs housed in plastic case have distance of 20mm from each other.**

5. Line 185: What is "underwrap"?

**Response: We apologize for the confusion. It is a light foam wrap.**

**Action: We have revised this sentence to improve clarity.***“3.3 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.”*

6. The criterion of 50 microvolts peak to peak seems fairly generous for 'rest' EMG. It's common elsewhere to use a criterion of < 0.01mV RMS calculated over at least 100 ms.

**Response: We thank reviewer for this excellent methodological suggestion.**

**Action: We have added this suggestion as an alternative approach at the end of the third paragraph in the discussion.** *“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.”*

7. Line 190: Is 5 degrees from vertical noticeably reclined? This will seem essentially upright for many participants.

**Response: The reviewer is correct; it is 5 degrees from vertical position. 5 degrees takes some pressure off of the hamstring and helps those with balance limitations. Much easier to maintain a neutral pelvis in this position. We have used this position in both neurologically intact (see Charalambous et al J Electromyography Kines 2018) and impaired adults (ongoing studies), and we had no complains. However, we do agree with the reviewer that this position might be upright and uncomfortable for some participants.**

**Action: To avoid any confusion, we have deleted the instruction about the chair adjustment.***“3.4 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.”*

8. Line 204: Is the AFO expected to provide resistance against a maximal isometric dorsiflexion (or plantarflexion)? Is this sufficient and stable?

**Response: We thank the reviewer for this excellent question. Yes, the specific AFO used in this study can provide resistance during both motions with adequate stability. During dorsiflexion, subject pull the foot against two straps whereas for plantarflexion subject push against the bottom of the AFO which was placed either against the wall or the floor. Yes, the AFO we used was able to provide sufficient stability.**

**Action: To improve clarity on this matter, we have revised the corresponding section.***“3.4 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.”*

*Reviewer #2:*  
  
1. Major problem is that authors claim that proposing protocol can be used for neurophysiological evaluation of the descending motor pathways in neurologically intact and IMPAIRED subjects. However, results are presented for non-injured subjects only. They have not examined this protocol in the impaired subjects. In the neurologically impaired, for example subjects with spinal cord injury, it may not be feasible to use the similar protocol because of the diminished conduction through cortico-spinal tract and thus transmission to corresponding motoneurons and then to leg muscles. So, authors must be careful and restrict wording to healthy subjects, as they are reporting.

**Response: We thank reviewer for pointing this out. Though we present results from a neurologically intact subject and the protocol as written may imply that it can be used only in this population, we have used this protocol in individuals with neurologic impairments with success (between two dissertation projects and two funded NIH studies, totaling of approximately 240 TMS lower extremity sessions have been completed in individuals post-stroke). The manuscripts for these projects are currently work in progress.**

2. Minor problems - it must be clearly indicated parameters of stimulation protocol, such as TMS frequency, coil used, the range of % of max intensity for inducing threshold MEP, etc.

**Response: We thank the reviewer for these methodological questions. The TMS frequency was 0.25 Hz, the coil used was Magstim double cone coil (as listed in the material table), and the range of RMT was: SOL: left - 53 ± 13, right – 52 ± 11; TA: left – 52 ± 12; 48 ± 11 % MSO. This information and data are included in our recent paper (Charalambous et al J Electromyography Kines 2018).**

*Reviewer #3:*.  
  
1. The methods section states that "apply one TMS pulse on each spot of the grid (total 36 stimuli)". Given the known inter-trial variability of TMS-evoked MEP amplitudes, as well as additional variability caused by slight variations in coil orientation or location, the delivery of only 1 stimulus pulse at each grid site can be considered a disadvantage. Please provide your rationale for not delivering 3-5 pulses at each site. If this was to save experimental time, it should be clearly stated, with a caveat added regarding the limitation related to not delivering multiple pulses at each site (to enable the use of an averaged response for each site).

**Response: This is an excellent issue that the reviewer has pointed out. We agree with the reviewer that more stimuli per spot would be better, yet we chose a single stimulus per spot for two reasons. First, the scope of this protocol was not to map the representations of TA and SOL, which would otherwise be ideal to employ the multiple stimuli per spot approach and using the average response per site. Second, this protocol was developed to assess SOL and TA CMR before and after either biomechanical assessment or a single day/session intervention. Therefore, we intended to administer the TMS assessment within an optimized time duration.**

**Action: We have acknowledged the methodological consideration in the third paragraph of the discussion section.***“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, 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.”*

2. The methodological approach used here has several strengths. For example, the authors identify the hot spot of contralateral SOL and TA as the location in the grid with the largest amplitude and the shortest latency. The readers could benefit from inclusion of statements about whether other / previous studies have used both latency and amplitude to determine hotspot. If not, the innovation, pros, and cons of this methodological approach need to be emphasized.

**Response: We appreciate the reviewer’s comment. Though the majority of the studies define hot spot as the cortical location with the largest response (i.e., largest peak-to-peak amplitude), Rossini 1999 defines hot spot the “scalp position where the stimulus elicits the MEP of largest amplitude and minimal latency”. Furthermore, recent paper (Kalliomeni 2015) suggested that the latency should be also considered for motor mapping.**

**Action: We have corrected the references cited. We also acknowledged this methodological consideration in the fifth paragraph of the discussion.***“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 leg cortical representation, and is defined as the spot with the largest amplitude and shortest latency43.”*

3. In contrast to the number of pulses delivered at each site (1), the adaptive algorithm for determining the motor threshold was run twice for each muscle. This adds significantly to data-collection time. Please provide justification for this aspect of the approach.

**Response: We thank the reviewer for pointing this out. As others have previously reported (Silbert et al Clin Neurophys 2013), adaptive method is more efficient than the traditional relative frequency method even though both methods shared same accuracy. Therefore, we chose the adaptive method because it required fewer stimuli than the relative frequency method. Given that less than 20 stimuli required to assess the TA and SOL RMT (see Table 1 in Charalambous et al J Electromyography Kines 2018), we suggested running the RMT protocol twice to ensure accurate determination of the RMT. Each RMT determination takes less than 5 minutes. Also, pilot work demonstrated greater variance in lower extremity RMT, so we decided to perform the RMT determination procedure twice to reduce the effect of this variability.**

4. In the methods, authors state that "in case any contralateral examined muscles are active before or after TMS, discard that stimulus and apply an extra single pulse again". Please clarify if the trials were discarded in case the targeted contralateral muscle (e.g. TA for trials involving delivery of TMS to the TA hotspot) was active or ANY contralateral muscle. As currently stated, the sentence is confusing. This is important because this will help readers ascertain whether the authors controlled for activation of the agonist or target muscle or the antagonist muscle as well.

**Response: We thank the reviewer for pointing this out. For the rest condition, we expected all 4 muscles to be at rest, whereas for the TVA condition we expected only the target muscle to be activated. Therefore, if there was any activation during rest or TVA (any muscle other than the target muscle) that trial was discarded.**

**Action: We have revised the corresponding steps, the sections now read as follows:   
*“****6.4.2 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 that 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.”****“****6.5.4 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 that either 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.****”***

5. Please provide the rationale for using 10 waveforms instead of a greater number (some studies have recommended 15 to 30) for the collection of suprathreshold MEPs for each condition.

**Response: This is a great comment that the reviewer has pointed out. We reported 10 stimuli instead of higher number for two reasons. 1) 10 stimuli was number of stimuli used at the time that this protocol was developed (4-5 years ago), as well as those commonly reported previously. 2) The two papers that have showed that greater number of stimuli required for more accurate CMR assessment were published in 2016 and 2017.   
However we have acknowledged in the discussion section that more than ten stimuli should be considered.** *“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.”*

6. Similar to detailed methods provided for other aspects of the protocol, additional details regarding methods and anatomical landmarks used to determine the MRI site used for positioning the center of the rectangular grid would be valuable to readers for future replication. Also, how did the authors handle any variability in sulcus anatomy when locating the grid?

**Response: We thank the reviewer for this comment. The neuronavigation file of each subject was created by the same person who has extensive experience in cortical neuroanatomy. To ensure accurate grid placement across subjects the grid was placed based on two criteria. The middle row of the gird was placed perpendicular to the leg motor area while the medial column was placed parallel and adjacent to the medial wall of the hemisphere tested.**

**Action: We elaborated on this matter at section 2.5.***“2.2.2 Place a rectangular grid over leg motor cortical area at each hemisphere (see Figure 1B). Position the centered row of the grid at the center and over the gyrus of the leg motor cortical area where the corticospinal tracts that innervate leg motor pools originate36. Position the medial column of the grid parallel and adjacent to the medial wall of the ipsilateral hemisphere.”*

7. How was the window for determining peak to peak MEP (20 to 60 ms after TMS onset) determined? Is there a possibility that the window may need to be widened in patient populations (e.g. stroke, multiple sclerosis) beyond the 60-ms timeframe?

**Response: This is an excellent question. Based on a work done in our lab in both neurologically intact and stroke-impaired subjects with varying height, we found that the latency of these two muscles was never less than 24 ms or greater than 50 ms. Therefore, we suggest the search window for MEP to be between 20 and 60 ms. Nevertheless, we agree with the reviewer that neurological populations other than that we have considered, may have longer latencies outside of this window. We have incorporated this into the text.**

**Action: We have added a CAUTION line in 7.2 section.   
*“****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).”*

8. The following finding is somewhat confusing and merits more detailed discussion and presentation in the paper: "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; conversely, TMS over the right SOL and left TA hot spots elicited only contralateral MEPs."  
The results and figures do not provide data to support this finding at a group level (only individual subject data are shown related to this point) or using statistics. Was this phenomena or observation consistent across all participants? Is there a potential methodological explanation for this finding? Does this relate to limb dominance? Please comment or clarify. If the finding is indeed variable, perhaps the emphasis on it can be reduced pending further confirmation using a larger sample study and support with statistical analysis?

**Response: This is an excellent observation. In the results section we present data from a neurologically intact subject to show the feasibility of the protocol. We recently published a paper which used this protocol and presents bilateral TA and SOL data from 21 healthy subjects. We are currently working on manuscripts that used the same protocol in stroke patients. We did observe similar results in other subjects as well. The main rationale for this ipsilateral response is due to the location of the stimulated site. Given the size of the double cone coil and the location of the leg motor area, it is highly expected that ipsilateral responses might be elicited. Based on our data, it is unclear whether this finding is related to leg dominance.**   
  
9. The discussion/interpretation regarding ipsilateral MEPS (iMEPs) being ascribed to an oligo-synaptic pathway needs more clarification and justification with references. Could iMEPs be caused by activation of uncrossed corticospinal tract fibers too instead of brain-stem-mediated pathways?

**Response: We thank the reviewer for pointing this out. It is possible that the uncrossed monosynaptic motor pathways might be stimulated. However, the ipsilateral latency should not be delayed and the amplitude should be similar to the contralateral MEP. Given the location of the motor leg area and the size of the double cone coil, any ipsilateral response in the lower extremity might be most likely due to the stimulation of the opposite hemisphere (i.e., stimulation of the crossed motor pathways of the opposite hemisphere). On the other hand, if the ipsilateral response is delayed and has much smaller amplitude than the contraletarel MEP, then this response might be due to oligosynaptic pathways. Definitely, future studies should further investigate this matter using the present protocol.**

**Action: To improve the clarity on this matter, we have revised the last section of the discussion’s second paragraph.***“The second critical step is the bilateral assessment of each muscle. In contrast to upper extremity motor areas, the two leg motor 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 leg motor 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 Figures 3 and 4). Whether the bilateral response is a result of multiple descending pathways or just stimulation of a single pathway requires further investigation.”*

10. Additional data, summary descriptive statistics, and figures would be beneficial in the results section. For instance, was the hotspot location determined using MEP latency and MEP amplitude identical or was there a disparity in the location based on these 2 criteria for certain participants?

**Response: We thank reviewer for this comment. Group data (descriptive statistics, reliability, etc.) have been presented in our recent paper (Charalambous et al J Electromyography Kines 2018). In this manuscript we chose to present representative data from a neurologically intact subject to demonstrate the pros and cons of this protocol. In the event that the largest amplitude and shortest latency is not at the same spot, then the spot with the largest amplitude should be defined as hot spot.**

**Action: We have added a CAUTION line in section 6.2.4.***“CAUTION: In an occasion that the largest amplitude and shortest latency are not at the same spot, define hot spot using the largest amplitude.”*

11. Participant characteristics and demographics would be useful.

**Response: Thank you for the suggestion. Information on participant characteristics and demographics has been reported in our recent paper – Charalambous et al J Electromyography Kines 2018.**

12. The average values of MEP amplitudes, latencies, and silent period for TA and Soleus are not provided in the results or figures. While the paper focuses on methodology, these data would serve as a useful reference for future studies using similar methodology in the same or other populations. Citations or references of these results from other papers from the authors' lab would also be useful.

**Response: Thank you for the suggestion. This data can be found in our recent paper – Charalambous et al J Electromyography Kines 2018.**