

Assessing Primary Motor Cortex Excitability and Excitability Modulation by Pairing Transcranial Magnetic Stimulation with Electromyography

Daniel Rodrigues da Silva^{*,1}, André Delgado^{*,2,3}, Francisco Faro Viana^{1,4}, Carolina Seybert¹, Patrícia Pereira^{1,5}, João Borges^{6,7}, Gonçalo Cotovio^{1,8}, Albino J. Oliveira-Maia^{1,8}

¹ Champalimaud Research and Clinical Centre, Champalimaud Foundation ² Department of Psychiatry and Mental Health, ULS Loures-Odivelas, Hospital Beatriz Ângelo ³ Lisbon School of Medicine, University of Lisbon, FMUL ⁴ Institute for Systems and Robotics-Lisbon (LARSyS), Instituto Superior Técnico, Universidade de Lisboa ⁵ Portuguese Red Cross Health School ⁶ Faculty of Medicine, University of Porto, FMUP ⁷ Department of Psychiatry and Mental Health, Centro Hospitalar Universitário de São João, CHUSJ ⁸ NOVA Medical School, Faculdade de Ciências Médicas, NMS, FCM, Universidade NOVA de Lisboa

* These authors contributed equally

Corresponding Authors

Daniel Rodrigues da Silva

daniel.silva@research.fchampalimaud.org

Albino J. Oliveira-Maia

albino.maia@neuro.fchampalimaud.org

Citation

Rodrigues da Silva, D., Delgado, A., Faro Viana, F., Seybert, C., Pereira, P., Borges, J., Cotovio, G., Oliveira-Maia, A.J. Assessing Primary Motor Cortex Excitability and Excitability Modulation by Pairing Transcranial Magnetic Stimulation with Electromyography. *J. Vis. Exp.* (224), e68897, doi:10.3791/68897 (2025).

Date Published

October 7, 2025

DOI

10.3791/68897

URL

jove.com/video/68897

Abstract

We describe a protocol to assess motor cortex excitability and its modulation, through paired use of electromyography (EMG) and repetitive transcranial magnetic stimulation (TMS) of the primary motor cortex (M1). EMG recordings use a custom-built system, and surface electrodes are placed over the right first dorsal interosseous (FDI) muscle, with a ground electrode on the left elbow. TMS is performed using a magnetic stimulator with a figure-eight coil, guided by a neuronavigation System. The motor hotspot for the FDI is identified by systematically applying single TMS pulses while adjusting coil placement, and is used as the target for all TMS procedures. The resting motor threshold (rMT) is defined as the minimum intensity required to elicit motor evoked potentials (MEPs) ≥ 50 μ V in the EMG recording, in at least 5 out of 10 trials, while the active motor threshold (aMT) is the minimum intensity needed to evoke MEPs ≥ 200 μ V in at least 5 out of 10 pulses while the participant maintains 10-20% of their maximal voluntary muscle activation. Baseline cortical excitability is defined as the mean MEP amplitude obtained from 40 single TMS pulses applied at 120% rMT, with pulses delivered at random intervals (6-10 s). Motor cortex excitability modulation is achieved using an intermittent theta-burst stimulation (iTBS) protocol (50 Hz pulse triplets, 80% aMT, 5 Hz for 2 s on/8 s off, repeated 20 times; 600 pulses total). Excitability modulation is assessed through repetition of MEP amplitude measurements at four post-stimulation time points: immediate (T0), 10 min (T10), 20 min (T20), and 30 min (T30). This protocol ensures precise and reproducible

assessment of motor cortex excitability and captures the peak of the modulatory effects resulting from iTBS.

Introduction

Transcranial magnetic stimulation (TMS) is a non-invasive neurostimulation technique that induces a magnetic field capable of penetrating the scalp and skull, eliciting neuronal activity upon reaching cortical tissue¹. TMS enables *in vivo* assessment of central nervous system (CNS) neurophysiology in humans^{2,3,4}. When delivered in repeated trains (repetitive TMS, or rTMS) to specific cortical regions, TMS can induce changes in excitability of the cortical target that extend beyond the duration of stimulation, depending on the parameters of the protocol that was applied⁵. Single-pulse TMS is commonly employed to measure cortical excitability, and, when applied pre- and post-rTMS, allows for the quantification of cortical excitability modulation induced by TMS^{2,3,4}. The primary motor cortex (M1) has been one of the main targets for such protocols, as its functional output can be readily quantified through electromyographic (EMG) recordings^{6,7}. Compared to other neurophysiological and neuroimaging techniques, such as electroencephalography (EEG) and functional magnetic resonance imaging (fMRI), TMS-EMG offers more advantages for clinical implementation. EEG requires extensive electrode setup, careful artifact management, and sophisticated signal processing⁸, while fMRI involves higher costs, limited accessibility, and provides less temporal resolution⁹. TMS-EMG relies on relatively simple surface EMG recordings, with analysis centered on straightforward and objective metrics such as motor evoked potential (MEP) amplitude and latency. This simplicity in acquisition and processing, combined with shorter setup times and lower infrastructural demands, makes TMS-EMG particularly

suitable for translation into clinical practice, where rapid, reliable, and reproducible biomarkers are required¹⁰.

Measures of cortical excitability modulation are considered indirect indicators of neuroplasticity, defined as the brain's capacity to undergo functional and structural changes in response to intrinsic or extrinsic stimuli^{11,12}. Alterations in neuroplasticity have been implicated in various neuropsychiatric conditions, such as major depressive disorder (MDD)^{13,14,15,16}. Given the ability to probe neuroplastic mechanisms, *in vivo*, and practical applicability in clinical contexts, assessment of cortical excitability modulation derived from TMS-EMG has been studied as a potential biomarker for neuropsychiatric disorders^{17,18,19}. These measures may hold promise both as a diagnostic biomarker¹⁸ and as a predictor of treatment outcomes^{14,15}. Single- or paired-pulse TMS is normally delivered at intensities expressed as a percentage of the resting motor threshold (rMT), i.e., the minimum intensity needed to generate a motor response (rMT; commonly 110-130% rMT), with protocols often employing 20-40 pulses to reliably estimate corticospinal excitability and several hundred pulses for assessing modulation pre- and post-intervention, at different timepoints²⁰. Inter-pulse intervals (IPI) generally should be >4 s to minimize carry-over effects and ensure independence of successive MEPs²¹. When used for modulation of cortical excitability, protocols most often rely on low-frequency rTMS (rTMS; $\approx$ 1 Hz, 600-1800 pulses) or continuous theta-burst stimulation (cTBS; 3 pulses at 50 Hz, repeated every 200 ms, continuously for 40 s) to induce

inhibitory after-effects. For facilitation of cortical excitability, protocols most often rely on high-frequency rTMS (rTMS; ≥ 5 Hz, typically 5-20 Hz, 600-2000 pulses) or intermittent theta-burst stimulation (iTBS)²². iTBS typically consists of 600 pulses delivered as bursts of 3 stimuli at 50 Hz, repeated at 5 Hz for 2 s, with trains applied intermittently every 10 s, at intensities around 80% of the active motor threshold (aMT). This pattern has become the most widely adopted for both experimental and clinical purposes, due to its efficiency, shorter duration, and robust facilitation of cortical plasticity^{23,24}.

However, clinical utility remains limited by unresolved methodological challenges. Efforts to address these challenges -- primarily by examining and improving the reliability of measurements -- have produced inconsistent findings^{25,26,27,28,29}. These inconsistencies can be attributed, at least in part, to substantial methodological pitfalls and heterogeneity across studies, including variability in rTMS parameters, where, for instance, pulse numbers range from 20 to 1800 pulses in low-frequency paradigms²² and from iTBS trains of 600 pulses to modified versions of 1200 pulses³⁰. Also, studies without neuronavigation have shown variability in MEP amplitude and lower modulatory effects of a rTMS protocol when compared to protocols with neuronavigation³¹. Sample sizes in published studies have also been relatively modest, often between 10 and 25 participants, limiting statistical power³². In addition, limited statistical approaches to evaluate reliability have been implemented, with some studies relying on measures other than the intraclass correlation coefficient (ICC)^{24,33}, which is generally regarded as the most appropriate index for test-retest reliability²⁶.

Protocols for cortical excitability acquisition and analysis with TMS-EMG paradigms may also contribute to additional variability. Specific methodological factors, such as the number of TMS pulses administered, may yield poor reliability, with estimates based on fewer than 20 MEPs, whereas averaging ≥ 30 MEPs provides a stable measure of corticospinal excitability. Similarly, the IPI have been shown to influence excitability with intervals of 2 s or less, inducing changes in cortical excitability due to cumulative effects of stimulation, whereas more conservative intervals of more than 4 s reduce these confounds²¹. EMG acquisition techniques^{34,35} can also influence measurement reliability, for instance, with the bipolar configuration offering improved spatial selectivity and reduced contamination from activity of nearby muscles relative to a monopolar configuration^{34,35}. Standardization of data acquisition and processing protocols is therefore essential to improve the assessment of cortical excitability modulation and to determine its stability -- a critical attribute of any reliable biomarker³⁶.

Hence, to pursue the use of cortical excitability modulation measures as candidate markers for neurobiological assessment and to establish their reliability for clinical application, we present a protocol resulting from a systematic approach grounded in the best available evidence in the field to minimize the potential impact of methodological heterogeneity and to ensure accurate and reproducible measurement of motor cortex excitability and its modulation^{37,38}. This included the implementation of appropriate EMG acquisition protocols³⁴, the use of neuronavigation systems³⁹, the collection of a sufficient number of MEPs²⁰, the employment of suitable IPI²¹, and comprehensive reporting of acquisition procedures and signal processing methods³⁴. Our results demonstrated improved signal stability when compared to the existing best practices in

the literature, thereby contributing to the methodological rigor necessary for the development of a robust neurophysiological biomarker.

Protocol

The procedures described herein were developed to collect the data reported by Seybert et al.³⁷ and Faro Viana et al.³⁸ All participants provided written informed consent prior to participation, explaining the purpose of the study, procedures, risks, and their right to withdraw at any time. The protocol was approved by the Ethics Committees of the Champalimaud Centre for the Unknown and conducted in accordance with the Declaration of Helsinki. Use of this protocol in other studies or locations should occur only after obtaining approval from the relevant local Ethics Committees and/or other competent authorities. A standardized safety checklist should be applied prior to TMS to minimize the risk of undesired side effects, using international guidelines^{40,41} as a reference framework that can be adapted to specific study settings. The protocol is designed for ~1 h 15 min duration (**Figure 1**).

1. Setup requirements and preparation

1. Ensure two technicians are available to optimally conduct the session, where one will apply TMS procedures and the other will provide support in additional steps.
2. Ensure the following equipment is available: a Neuronavigation system; a comfortable chair; an EMG device with the respective contacts and electrodes; a TMS stimulator with an 8-shaped coil, which allows for the input of iTBS, single-pulse protocols, and a connection with the neuronavigation and EMG system. Ensure one or two computers are available to allow for simultaneous observation of the EMG software and the neuronavigation software. Ensure the computer has a Bluetooth signal receptor to receive the EMG signal.
3. Ensure the following consumables are available: alcohol and gauze compresses; water sandpaper to clean the skin where the gel electrodes will be placed; a swimming cap for the participant; and earplugs for both the participant and the technicians.
4. Start setup preparation around 30 min before the participant arrives, to properly set up the neuronavigation system, the EMG board, and the TMS machine, as well as the software required for the physiological data acquisition.
5. Prepare the EMG system.
 1. Ensure the EMG board is fully charged - if applicable - and prepare the respective contacts - one as reference (unipolar - to be placed on the left elbow), and the other on the target muscle for registration (bipolar - to be placed on the right First Dorsal Interosseous). An additional cable is needed to sync the TMS pulse output to the EMG signal, connecting the TMS device to the EMG board.
 2. Plug the Medical-grade silver/silver chloride (Ag/AgCl) electrodes into the contacts (3 electrodes will be needed). Also, connect a cable from the TMS machine to the EMG board to output the trigger signal of each TMS pulse.
 3. For the in-house EMG device, strip the Velcro to a stable surface. Plug the contacts into the EMG device in the respective entry points.
 4. In one of the monitors/computers, open the BONSAI software⁴² and the respective EMG data acquisition script. Hit the **start** button, only when ready for data

acquisition (For further details, see **Supplementary File 1**, step 1).

6. Prepare the TMS device.

1. Define the excitability assessment protocol in the TMS device. This is composed of 40 single-pulses, at 120% rMT, with a random IPI of at least 6 s to avoid carry-over effects from previous stimulus.
2. Insert an iTBS protocol following the given stimulation parameters *a priori* in the device: 50 Hz pulse triplets, 80% aMT, 5 Hz for 2 s on/8 s off, repeated 20 times, 600 pulses total.
3. Attach paper strips in all parts of the TMS machine screen where maximum stimulation output (MSO - %) is visible so that the technician is blinded during rMT and aMT assessment (For further details, see **Supplementary File 1**, step 2).

7. Prepare the ANT Neuro neuronavigation system for TMS.

1. Assemble the required hardware components. These include the infrared tracking camera, the participant's headband with reflective markers, a pointer tool, a calibration board (for the TMS coil), and a coil tracker (a circular device that attaches to the coil for real-time positional tracking).
2. Set the camera on the tripod and place it in a position such that it has a clear line of sight to all other neuronavigation components throughout the whole session.
3. Place the coil calibration tracker and board on the TMS coil.
4. Open the neuronavigation system software, insert the participant's ID, and calibrate the

neuronavigation components (For further details, see **Supplementary File 1**, step 3).

2. Preparing the participant

1. Once the participant arrives, assess the safety for application of TMS through the use of a questionnaire and/or structured interview based on published guidelines^{40,41}. Include items regarding the history of psychiatric and neurological disorders, loss of consciousness, hearing impairment, as well as the presence of metallic or magnetic implants, ongoing medication, and pregnancy status. Proceed with the protocol only if no safety concerns are raised.
2. Seat the participant in a comfortable chair where both forearms can be positioned in a resting position. Ensure the participant is at an appropriate angle for the coil to be positioned comfortably on their head.
3. Have the patient remove any eyeglasses, hair clips or pins, as well as any earrings, or other metallic and/or electronic devices. Provide earplugs, and ensure they are properly and stably positioned. Instruct the participant to remain awake, silent, and relaxed. Ensure that the participant is awake at all times to reduce, as much as possible, the fluctuations in the physiological signal.
4. To improve the quality of the electrode-skin interface and improve conductance of the electric signal, clean the skin over the FDI by gently scrubbing the area with water sandpaper followed by a quick rinse with alcohol embedded gauze³⁵. Repeat this procedure over the contralateral elbow. Once the skin is dry, place the ground electrode over the elbow to provide a zero-voltage reference point and the recording electrode on the contralateral hand over the FDI, with its center at

approximately 2.5 cm in the direction of the muscle tendon (see **Figure 2**).

5. Place a swimming cap on the participants' head to draw references for the location of the TMS stimulation site:
 1. Place a swimming cap on the participant's head with an anterior-posterior orientation. Ensure the cap is aligned with the participant's eyebrow line at the front and to the root of the helix of the ears on each side. Place it over the scalp, leaving the ears free and uncovered.
 2. Draw the medial sagittal line as well as the intertragus line on the cap to obtain the interception point on the vertex (**Figure 3A, B**).
 3. From the interception point, measure 5 cm to the left on the intertragus line and anteriorly on the sagittal line, to define 2 additional points (**Figure 3C, D**).
 4. Draw a diagonal line to connect the lateral point to the medial anterior point (**Figure 3E**).
 5. Starting at the lateral point, measure 2.5 cm in the antero-medial direction on the diagonal line to mark the initial estimate of the motor hotspot (**Figure 3F**).
 6. Mark 4 points in a radius of 0.5 cm around that initial estimate, to provide additional testing points for the motor hotspot. Mark the region considered for the motor hotspot (red line) (**Figure 3E, F**).
6. Set up the neuronavigation system for the participant.
 1. Place the elastic headband on the participant's head, ensuring that the marker strip faces the camera and is comfortable, as it must remain in place throughout the session.
 2. Use the pointer to define key anatomical landmarks on the participant's head (nasion, left and right

tragus, and head shape points), which will allow the system to align and track stimulation targets with respect to the participant's head anatomy (nasion, tragus, scalp landmarks and head shape), if using a standard MNI space and further aspects of the anatomy, including brain anatomy, if using individualized MRI scans. This ensures that the coil positioning is accurate with regard to individual anatomy during the TMS procedure (For further details, see **Supplementary File 1**, step 3).

3. TMS-EMG physiology assessment

1. Start running the BONSAI script⁴² for EMG data acquisition. During the recording, monitor the participant for signs of facial contraction, limb movement, inadvertent repositioning of the cap, or other behaviors that might impact the participant's physiology and physiological data acquisition.
2. Measure the Motor Hotspot.
 1. Place the TMS coil tangentially to the participant's scalp with the handle pointing posteriorly at a 45° angle relative to the midsagittal line and its center over the marked location in step 2.5 - this position will be used for all TMS procedures.
 2. Apply pulses at 30% of the MSO over the initial motor hotspot estimate.
 3. Increment the stimulation intensity in steps of 5-10% of MSO until a consistent motor response is observed.
 4. Apply single pulses at the initial estimate and at several locations surrounding it to determine the location that most consistently results in higher amplitude MEPs and, when possible, where the

visible contraction was limited to the FDI - this is the motor hotspot for the FDI (MH).

5. In the neuronavigation system software, select the pulse that determined the MH to guide further stimulation.
3. Establish the rMT.
 1. Use the neuronavigation system to guide the coil to the MH found at step 3.2 - the rMT is the minimum % MSO needed to elicit MEPs of at least 50 μ V in the contralateral FDI in at least 5 out of 10 TMS single-pulses delivered here, separated approximately by a 5-s interstimulus interval.
 2. Starting at the % MSO used to define the MH, reduce the intensity in steps of 2% until less than 5 out of 10 TMS single-pulses elicit a response of at least 50 μ V in the contralateral FDI.
 3. Increment MSO in 1% steps until at least 5 out of 10 TMS single-pulses elicit a response of at least 50 μ V in the contralateral FDI.
 4. Register the % MSO for the rMT.
4. Determine the maximum voluntary muscle activation.
 1. Instruct the patient to produce maximal voluntary muscle activation of the first dorsal interosseous (FDI) muscle through production of the shape of a circle by pushing their index fingernail against the belly of their thumb with maximum strength.
 2. Approximately 2 s after the participant initiates the muscle contraction, record the corresponding time point by pressing the space bar on the computer running the BONSAI script, skipping the initial ramp-up of activity and spikes resulting from electrode movement.

3. Repeat the process three times, with each contraction separated by a 1-min rest period to prevent fatigue and ensure recovery.
5. Establish the aMT.
 1. Use the neuronavigation system to guide the coil to the MH found at step 3.2 - the aMT is the minimum % MSO that produces MEPs of at least 200 μ V peak-to-peak amplitude in at least 5 out of 10 TMS single-pulses delivered here, during slight voluntary contraction.
 2. Ask the participant to maintain a submaximal contraction, between 10% to 20% of MVIC, using the EMG to monitor the ongoing contraction and provide real-time feedback (For further details, see **Supplementary File 1**, step 1).
 3. Starting at the % of MSO for the rMT, decrease the intensity in steps of 2% until MEPs of at least 200 μ V are produced in less than 5 out of 10 TMS single-pulses.
 4. Increment MSO in steps of 1% until MEPs of at least 200 μ V are produced in at least 5 out of 10 TMS single-pulses.
 5. Register the % MSO for the aMT.
6. Define baseline cortical excitability.

NOTE: This phase aims to establish the participant's baseline MEP's amplitude, serving as a reference for subsequent comparisons.

 1. Use the neuronavigation system to accurately position the coil over the motor hotspot, as defined in step 3.2.
 2. Confirm that surface EMG electrodes are correctly placed over the target muscle (e.g., first dorsal

interosseous), and verify signal quality (minimal noise and clear MEPs)

3. Load the single-pulse stimulation protocol on the TMS device.
4. Insert the previously defined rMT and start the protocol (stimulation parameters described in step 1.6.1).
5. Assess the baseline cortical excitability by averaging the peak-to-peak amplitudes of 40 MEPs.

NOTE: The peak-to-peak amplitude is defined as the difference in voltage between the minimum and maximum points of each individual MEP waveform. For further analyses, MEP amplitudes may be normalized to baseline values to control for inter-individual variability, following procedures described in previous literature³².

7. Apply the cortical excitability modulation protocol .

NOTE: This step involves delivering an iTBS protocol to modulate cortical excitability .

1. Load the iTBS protocol on the TMS device.
2. Insert the previously defined aMT and start the protocol (stimulation parameters described in step 1.6.2).
3. Immediately after the end of the iTBS protocol, start a stopwatch to monitor post-stimulation intervals.

8. Assess cortical excitability modulation.

1. T0, i.e., immediately after the end of the cortical excitability modulation protocol reapply the single-pulse stimulation protocol using 120% rMT, as described in step 3.6.
2. Validate neuronavigation accuracy by placing the pointer on the nasion and verifying alignment in the

neuronavigation system software. Ensure the spatial deviation is < 5 mm. Record the value.

3. T10, T20, and T30, i.e., 10, 20, and 30 min after the cortical excitability modulation protocol - repeat the single-pulse stimulation protocol as in step 3.8.1. Revalidate neuronavigation accuracy as in step 3.8.2.
4. Upon completion of the final stimulation block, stop the BONSAI script. Finalize the neuronavigation session in the software. A "bin." file is generated from the bonsai data acquisition.
5. Assess cortical excitability at the post-modulation time points (T0, T10, T20, and T30) using the same procedure as baseline, i.e., by averaging the peak-to-peak amplitudes of 40 MEPs recorded at each time point. This allows for the comparison of excitability changes relative to baseline following the modulation protocol.
6. Calculation of cortical excitability modulation: Quantify the modulation of cortical excitability induced by the stimulation protocol by calculating the delta MEP (ΔMEP), defined as the difference between the mean amplitude of MEPs recorded at each post-intervention time point (T0, T10, T20, T30) and the mean amplitude of MEPs recorded at baseline, normalized by the mean amplitude of MEPs recorded at baseline. This measure allows assessment of the magnitude and duration of cortical excitability changes induced by the iTBS protocol.

$$\Delta MEP = \frac{MEP_{post-iTBS} - MEP_{pre-iTBS}}{MEP_{pre-iTBS}} \times 100$$

9. Question the participant about any discomfort and provide means for contact if any needs or questions may arise after the session.

Representative Results

This protocol was designed to provide a reliable and reproducible assessment of corticospinal excitability while remaining generalisable across different EMG systems and TMS setups. We opted for the conventional threshold-hunting procedure as it is widely validated, reproducible, and comparable across studies, while acknowledging that alternative methods are also well-established (e.g., threshold hunting methods based on parameter estimation by sequential testing)^{43,44}. Collecting a total of 40 single-pulse TMS stimuli ensures that enough MEPs are recorded for a reliable estimation of MEP amplitude, even when some pulses fail to elicit a response²⁰. Intervals between pulses are randomized with a minimum of 6 s to prevent carry-over effects from previous stimuli²¹ and to make the pulse sequence unpredictable, reducing anticipatory muscle pre-activation. Using a single-pulse TMS intensity of 120% rMT is a well-established standard that elicits measurable MEPs while capturing plasticity changes induced by iTBS consistently across participants. Offline EMG processing, including filtering, artifact removal, and monitoring of background muscle activity, ensures that measured MEPs reflect true corticospinal excitability rather than noise and are not influenced by muscle pre-activation (see **Supplementary File 1**, step 4 for details). While it is possible to acquire Post-iTBS MEPs for more than 30 min using this protocol, we opted to end the data acquisition at T30 post-iTBS, which is consistent with evidence that LTP-like plasticity resulting from this protocol predominantly occurs within this window³². Overall, the protocol emphasises reproducibility,

reliability, and conceptual generalisability, independent of specific devices or software.

All personnel involved in TMS sessions should be trained in institutional safety protocols to ensure participant well-being. During the session, if a participant reports mild symptoms such as lightheadedness, the session should be paused, the participant should be provided with water, and/or laid down with legs elevated, if needed. A nurse or physician should be contacted as appropriate. Headaches may occur during or shortly after the session. Participants should be informed that this is expected and may take simple over-the-counter analgesics, if needed. If headaches persist, the research team should be contacted. In the rare event of a seizure, the session must be immediately stopped, and emergency procedures must be initiated according to institutional protocols.

Data from 47 healthy volunteers were collected using the structured and standardized protocol described here. This methodology, designed to enhance the reliability of cortical excitability assessments, indeed yielded consistent results across two assessments conducted 6 weeks apart³⁷. A significant modulation of left motor cortex excitability, as assessed by the post-iTBS change of MEPs amplitude (Δ MEP) was observed at T0, T10, and T20 at the first assessment, whereas the effect at T30 did not reach significance (T0: $W = 259$, $p < 0.01$, $r = 0.42$; T10: $W = 320$, $p < 0.01$, $r = 0.51$; T20: $W = 291$, $p < 0.01$, $r = 0.46$; T30: $W = 190$, $p = 0.06$, $r = 0.32$), and at all time points 6 weeks later (T0: $W = 293$, $p < 0.01$, $r = 0.48$; T10: $W = 278$, $p < 0.01$, $r = 0.46$; T20: $W = 315$, $p < 0.01$, $r = 0.52$; T30: $W = 275$, $p < 0.01$, $r = 0.45$), indicating robust excitability modulation across time (**Figure 4A**). The stability of excitability modulation at T0 was further supported by an

ICC of 0.67 (95% CI = 0.31-0.85; $p < 0.01$), demonstrating moderate to good test-retest reliability at the earlier moments after iTBS (**Figure 4B**). Nonetheless, the temporal reliability of cortical excitability at T10, T20, and T30 was not observed, suggesting limitations of applying this protocol to assess plasticity-like phenomena at later time points. Moreover, key neurophysiological parameters, including aMT, rMT, and baseline MEP amplitude, remained stable throughout the study period, further underscoring the internal consistency

and procedural reliability of the protocol. Finally, the influence of potential confounding factors-including handedness, state anxiety, expectation regarding TMS effects, sex, and age-was systematically evaluated. No significant associations were found between these variables and cortical excitability modulation, supporting the robustness of the observed effects and of the protocol across individual characteristics, as reported in Seybert et al.³⁷.

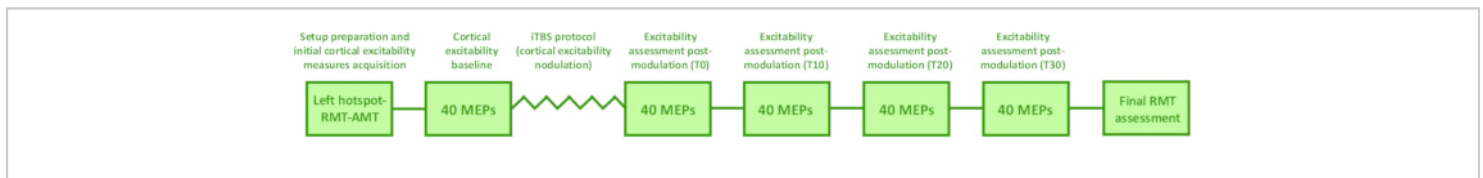


Figure 1: Left M1 excitability and its modulation assessment - experimental protocol overview. [Please click here to view a larger version of this figure.](#)

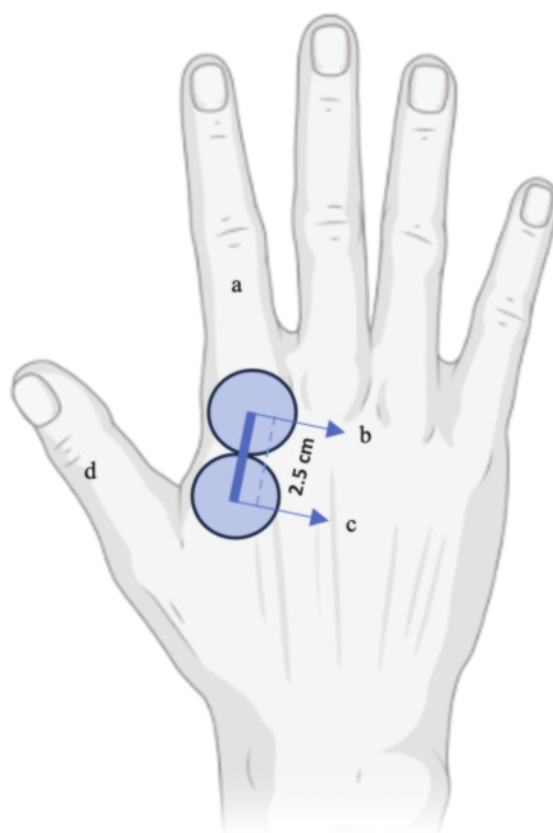


Figure 2: Electrode positioning for EMG recording over the First Dorsal Interosseous (FDI) muscle. (a) index finger; (b) tendon region; (c) FDI muscle belly; (d) thumb. The active electrode is placed on the belly of the FDI, and the second electrode is positioned about 2.5 cm distally toward the tendon. The reference electrode should be located on the opposite elbow. [Please click here to view a larger version of this figure.](#)

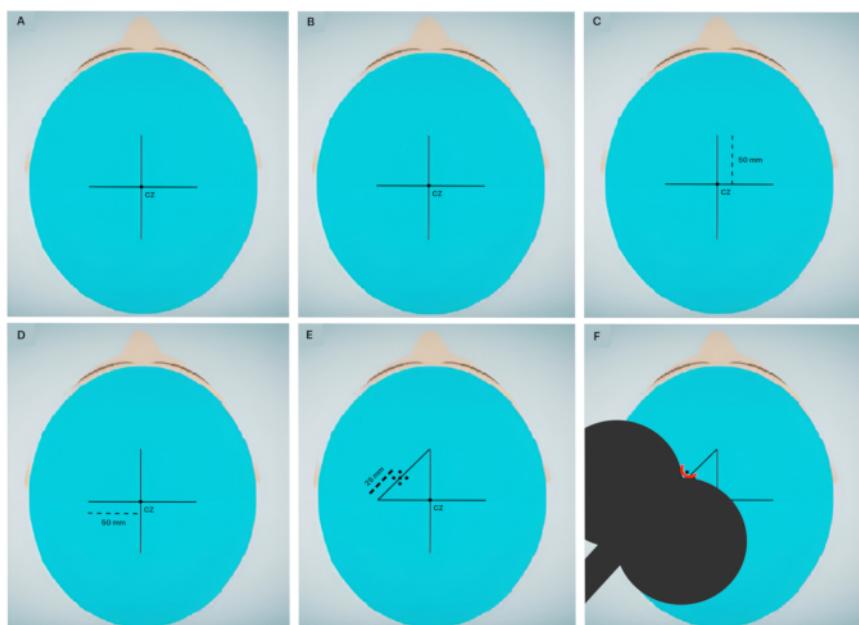


Figure 3: Cap marking procedure. (A,B) Identification of anatomical reference lines: the medial sagittal line and the intertragus line and determine the vertex; (C,D) From the vertex, two reference points are marked by measuring 5 cm laterally along the intertragus line (left) and 5 cm anteriorly along the sagittal line; (E) Construction of a diagonal line connecting the lateral point to the anterior point. Along the diagonal, a point is marked 2.5 cm in the antero-medial direction from the lateral point, representing the initial estimate of the motor hotspot. Refinement of the hotspot estimation by marking four additional points within a 0.5 cm radius of the initial estimate, creating a cluster of stimulation sites for motor threshold determination; (F) Establishment of the motor hotspot and respective marking on the cap (Red line). [Please click here to view a larger version of this figure.](#)

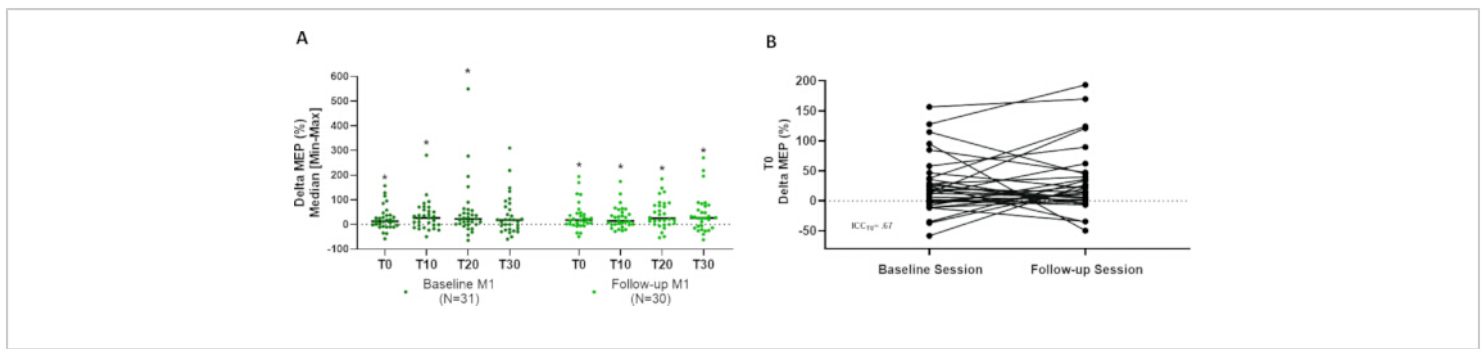


Figure 4: Representative results of the study. (A) Modulation of left motor cortex excitability following iTBS, reflected by changes in MEP amplitude (Δ MEP) at T0, T10, and T20 during baseline and follow-up session; (B) Test-retest reliability at T0 indicated by an intraclass correlation coefficient (ICC) of 0.67 (95% CI: 0.31-0.85; $p < 0.01$), supporting moderate to good consistency in excitability modulation. [Please click here to view a larger version of this figure.](#)

Supplementary File 1: Additional details of the protocol.

[Please click here to download this File.](#)

Discussion

Here, we present a comprehensive and rigorously structured protocol describing a methodology to assess cortical excitability and excitability modulation of the M1 using TMS combined with EMG. It describes, in detail, all necessary steps for the optimal conduction of the experimental session, including preparation of the experimental setup and calibration of the neuronavigation system, configuration of the TMS and EMG equipment, and standardized procedures to identify the MH, rMT, and aMT. These preparatory steps of the protocol are necessary for the final sequence of steps, including baseline assessment of cortical excitability, neuromodulation through application of iTBS and re-assessment of cortical excitability to quantify the resulting modulation.

A key strength of this methodology is its high degree of standardization and rigor, which addresses many of the limitations found in past studies. Past work in the field has

often suffered from methodological inconsistency, limited description of methods, and high intra- and inter-subject variability³⁵. Methodological variability includes the number of collected MEPs²⁰, IPI²¹, and EMG filtering methods that may have compromised the reproducibility of findings³⁴. The protocol described here avoids methodological pitfalls by incorporating neuronavigation to ensure precise coil placement³⁹, using validated EMG procedures³⁴, and adopting established best practices for stimulation and signal acquisition⁴⁵. Although the specific contribution of each of these procedures was not quantified, the collective application of these elements resulted in improved stability and reliability of the observed effects when compared with prior studies^{25,26,27,28,29,30}.

While methodological rigor and standardization are the foundation for reliable measurements, practical aspects of participant preparation and session management are some of the most important protocol steps that influence the quality of the data acquired. Proper skin cleaning for electrode placement, ensuring electrodes are placed adjacent and securely, and confirming that the neuronavigation headband

fits comfortably but firmly, are critical to minimizing session-to-session variability. Throughout the session, continuous monitoring of participant state -- including alertness, hand movement, and electrode stability -- is necessary to prevent artifacts and maintain high-quality recordings. When in the presence of issues acquiring TMS-EMG data, it is important to prompt adjustments to coil positioning or stimulation parameters. Regular verification of EMG signal quality is essential for addressing common technical issues, such as signal dropout, electrode detachment, or minor navigation drift. Subtle repositioning of the coil, ensuring stable electrode contact, or recalibrating the neuronavigation system can often restore signal quality and maintain consistency. It is also important to acknowledge that some fluctuations in signal or participant-related variability may be unavoidable despite best efforts. In such cases, rather than compromising data integrity, it may be preferable to pause the session, repeat critical measurements, or even reschedule to ensure that reliable and interpretable results are obtained.

The results obtained using this protocol showed that cortical excitability in the left M1 was significantly modulated after iTBS was applied. This modulation was similarly evident 6 weeks later, with the measure of excitability modulation revealing within-individual stability only when it was assessed immediately post-iTBS, suggesting a temporally-specific window of stability. Notably, excitability effects were not consistently maintained in later timepoints, a limitation possibly driven by increased variability over time^{25,27}. This observation reinforces the idea that the timing of cortical excitability measurements is critical, particularly when considering its potential role as a biomarker for diagnostic or predictive purposes. Indeed, despite the long-standing interest in using motor cortex excitability and cortex excitability modulation (Δ MEP) as

a clinical marker, its application has been limited by poor reliability and high variability. While some studies have reported moderate reproducibility, many others have found Δ MEP to lack the stability necessary for clinical use^{18,19}. The current protocol directly confronts this issue by providing a replicable framework to measure excitability with consistency. A robust TMS-EMG methodology also provides valuable insights into the underlying neurobiological mechanisms in both health and disease, facilitating reliable assessment of neuroplasticity-like phenomena in the human motor cortex. Specifically, iTBS is thought to engage Long-Term Potentiation-like processes, inducing NMDA receptor-dependent increases in synaptic efficacy, modulating inhibitory interneurons, and promoting cortical disinhibition, which collectively enhance MEP amplitudes post-stimulation²⁴. This interpretation is consistent with evidence from animal models. In rodents and non-human primates, TBS has been shown to induce long-lasting potentiation of corticospinal excitability and synaptic strength, demonstrating homologous plasticity-like mechanisms across species¹². Thus, the changes observed in MEPs following iTBS in humans likely reflect plasticity-related neural processes with strong translational grounding.

Beyond evaluating neuromodulatory protocols like iTBS, the standardized TMS-EMG methodology detailed here holds promise for diverse applications. In motor learning paradigms, changes in corticospinal excitability can serve as biomarkers for skill acquisition and neuroplasticity. For instance, studies have shown that visuomotor learning enhances corticospinal excitability, reflecting underlying neural adaptations⁴⁶. In stroke rehabilitation, assessment of corticospinal excitability can inform therapeutic strategies, with TMS neurofeedback used to upregulate excitability in the affected motor cortex, potentially aiding recovery⁴⁷. Pharmacologically,

TMS-EMG assessments can detect substance-induced changes of cortical excitability, providing insights into their CNS effects⁴⁸. Additionally, age-related differences in corticospinal excitability have been documented, with older adults often exhibiting reduced excitability, which may influence motor performance and learning⁴⁹. By extending the application of this standardized protocol to these areas, researchers can gain a more comprehensive understanding of corticospinal dynamics across different contexts and populations.

Thus, we propose that this protocol offers a valuable foundation for future research aiming to establish reliable biomarkers of cortical plasticity. In particular, studies should seek to replicate these effects in larger and more diverse populations, including clinical groups. Future research should assess whether cortical excitability measured immediately after iTBS can consistently distinguish between healthy and clinical profiles, to understand diagnostic sensitivity, as well as prognostic properties, for example, in the prediction of treatment response. In conclusion, the methodology described here represents a significant advance in the standardization of cortical excitability assessment. By mitigating common sources of variability and emphasizing precision in experimental design, this protocol provides a robust and reproducible approach to studying cortical plasticity-like phenomena and its potential clinical applications.

Disclosures

AJO-M is recipient of a grant from Schuhfried GmbH for norming and validation of cognitive tests, and was national coordinator for Portugal of trials of psilocybin therapy for treatment-resistant depression, sponsored by Compass Pathways, Ltd (EudraCT number 2017-003288-36

and 2020-001348-25), and of esketamine for treatment-resistant depression, sponsored by Janssen-Cilag, Ltd (EudraCT NUMBER: 2019-002992-33); has received payment, honoraria, or support for attending meetings and participating in advisory boards from MSD, Neurolite AG, Janssen Pharmaceuticals, Angelini Pharma, and the European Monitoring Centre for Drugs and Drug Addiction; has received consultancy fees from Bioprojet Pharma and NaturalX Health Ventures (all outside the submitted work); is Vice President of the Portuguese Society for Psychiatry and Mental Health; is head of the Psychiatry Working Group for the National Board of Medical Examination at the Portuguese Medical Association and Portuguese Ministry of Health; is President of the Ethics Committee for the Public Institute for Addictive Behaviors and Dependence; and is President of the Scientific Council of the Portuguese Obsessive Compulsive Disorder Foundation. The remaining authors declare that they have no potential conflicts of interest involving this work, including relevant financial activities outside the submitted work and any other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing what is written. None of these agencies had a role in the design and conduct of the study, in the collection, management, analysis, and interpretation of the data, in the preparation, review, or approval of the manuscript, nor in the decision to submit the manuscript.

Acknowledgments

The authors gratefully acknowledge the help of the Scientific Hardware and Software Development Platform at Champalimaud Foundation for providing the resources and technical support necessary for this work. DRS (2023.05754.BD) and FFV (2024.00723.BD) are funded by doctoral scholarships from Fundação para a Ciência e

Tecnologia. GC is supported by a 2023 NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation. AJO-M is supported by a Starting Grant from the European Research Council (grant agreement no. 950357), and by the PsyPal project (grant agreement no. 875358), both funded by the European Union's Horizon 2020 Research and Innovation Programme. GC and AJO-M are supported by a Proof-of-Concept Grant from the European Research Council (grant agreement no. 101158262). The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of any of the funding agencies.

References

1. Hallett, M. Transcranial magnetic stimulation: a primer. *Neuron*. **55** (2), 187-199 (2007).
2. Badawy, R. A. B., Loetscher, T., Macdonell, R. A. L., Brodtmann, A. Cortical excitability and neurology: insights into the pathophysiology. *Funct Neurol*. **27** (3), 131-145 (2012).
3. Maeda, F., Keenan, J. P., Tormos, J. M., Topka, H., Pascual-Leone, A. Interindividual variability of the modulatory effects of repetitive transcranial magnetic stimulation on cortical excitability. *Exp Brain Res*. **133** (4), 425-430 (2000).
4. Polanía, R., Nitsche, M. A., Ruff, C. C. Studying and modifying brain function with non-invasive brain stimulation. *Nat Neurosci*. **21** (2), 174-187 (2018).
5. Peinemann, A. et al. Long-lasting increase in corticospinal excitability after 1800 pulses of subthreshold 5 Hz repetitive TMS to the primary motor cortex. *Clin Neurophysiol*. **115** (7), 1519-1526 (2004).
6. Groppa, S. et al. A practical guide to diagnostic transcranial magnetic stimulation: report of an IFCN committee. *Clin Neurophysiol*. **123** (5), 858-882 (2012).
7. Pascual-Leone, A., Tarazona, F., Keenan, J., Tormos, J. M., Hamilton, R., Catala, M. D. Transcranial magnetic stimulation and neuroplasticity. *Neuropsychologia*. **37** (2), 207-217 (1998).
8. Luck, S. J. *An Introduction to the Event-Related Potential Technique*. MIT Press, Cambridge (2014).
9. Logothetis, N. K. What we can do and what we cannot do with fMRI. *Nature*. **453** (7197), 869-878 (2008).
10. Farina, D., Merletti, R. *Surface Electromyography: Physiology, Engineering and Applications*. IEEE Press, Wiley, Hoboken (2016).
11. Feldman, D. E. Synaptic mechanisms for plasticity in neocortex. *Annu Rev Neurosci*. **32** (1), 33-55 (2009).
12. Cirillo, G. et al. Neurobiological after-effects of non-invasive brain stimulation. *Brain Stimul*. **10** (1), 1-18 (2017).
13. Castricum, J., Birkenhager, T. K., Kushner, S. A., Elgersma, Y., Tulen, J. H. M. Cortical inhibition and plasticity in major depressive disorder. *Front Psychiatry*. **13**, 777422 (2022).
14. Hinchman, C. A. et al. Corticomotor plasticity as a predictor of response to high frequency transcranial magnetic stimulation treatment for major depressive disorder. *J Affect Disord*. **303**, 114-122 (2022).
15. Oliveira-Maia, A. J. et al. Modulation of motor cortex excitability predicts antidepressant response to prefrontal cortex repetitive transcranial magnetic stimulation. *Brain Stimul*. **10** (4), 787-794 (2017).

16. Pittenger, C., Duman, R. S. Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*. **33** (1), 88-109 (2008).
17. Player, M. J. et al. Neuroplasticity in depressed individuals compared with healthy controls. *Neuropsychopharmacology*. **38** (11), 2101-2108 (2013).
18. Vignaud, P., Damasceno, C., Poulet, E., Brunelin, J. Impaired modulation of corticospinal excitability in drug-free patients with major depressive disorder: a theta-burst stimulation study. *Front Hum Neurosci*. **13**, 72 (2019).
19. Bajwa, S. et al. Impaired interhemispheric interactions in patients with major depression. *J Nerv Ment Dis*. **196** (9), 671-677 (2008).
20. Goldsworthy, M. R., Hordacre, B., Ridding, M. C. Minimum number of trials required for within- and between-session reliability of TMS measures of corticospinal excitability. *Neuroscience*. **320**, 205-209 (2016).
21. Pellicciari, M. C., Miniussi, C., Ferrari, C., Koch, G., Bortoletto, M. Ongoing cumulative effects of single TMS pulses on corticospinal excitability: an intra- and inter-block investigation. *Clin Neurophysiol*. **127** (1), 621-628 (2016).
22. Fitzgerald, P. B., Fountain, S., Daskalakis, Z. J. A comprehensive review of the effects of rTMS on motor cortical excitability and inhibition. *Clin Neurophysiol*. **117** (12), 2584-2596 (2006).
23. Blumberger, D. M. et al. Effectiveness of theta burst versus high-frequency repetitive transcranial magnetic stimulation in patients with depression (THREE-D): a randomised non-inferiority trial. *Lancet*. **391** (10131), 1683-1692 (2018).
24. Huang, Y. Z. et al. Effect of physiological activity on an NMDA-dependent form of cortical plasticity in human. *Cereb Cortex*. **18** (3), 563-570 (2008).
25. Hinder, M. R. et al. Inter- and intraindividual variability following intermittent theta burst stimulation: implications for rehabilitation and recovery. *Brain Stimul*. **7** (3), 365-371 (2014).
26. Corp, D. T. et al. Large-scale analysis of interindividual variability in theta-burst stimulation data: results from the 'Big TMS Data Collaboration'. *Brain Stimul*. **13** (6), 1476-1488 (2020).
27. Jannati, A. et al. Test-retest reliability of the effects of continuous theta-burst stimulation. *Front Neurosci*. **13**, 447 (2019).
28. Schilberg, L., Schuhmann, T., Sack, A. T. Interindividual variability and intraindividual reliability of intermittent theta burst stimulation-induced neuroplasticity mechanisms in the healthy brain. *J Cogn Neurosci*. **29** (6), 1022-1032 (2017).
29. Vallence, A. M. et al. Inter- and intra-subject variability of motor cortex plasticity following continuous theta-burst stimulation. *Neuroscience*. **304**, 266-278 (2015).
30. Vernet, M. et al. Reproducibility of the effects of theta burst stimulation on motor cortical plasticity in healthy participants. *Clin Neurophysiol*. **125** (2), 320-326 (2014).
31. Chung, S. W., Hill, A. T., Rogasch, N. C., Hoy, K. E., Fitzgerald, P. B. Use of theta-burst stimulation in changing excitability of motor cortex: a systematic review and meta-analysis. *Neurosci Biobehav Rev*. **63**, 43-64 (2016).

32. Chang, W. H. et al. Optimal number of pulses as outcome measures of neuronavigated transcranial magnetic stimulation. *Clin Neurophysiol.* **127** (8), 2892-2897 (2016).
33. Fried, P. J. et al. Reproducibility of single-pulse, paired-pulse, and intermittent theta-burst TMS measures in healthy aging, type-2 diabetes, and Alzheimer's disease. *Front Aging Neurosci.* **9**, 263 (2017).
34. De Luca, C. *Electromyography*. In *Encyclopedia of Medical Devices and Instrumentation*. John Wiley & Sons, Hoboken (2006).
35. Mohr, M. et al. Intermuscular coherence between surface EMG signals is higher for monopolar compared to bipolar electrode configurations. *Front Physiol.* **9**, 566 (2018).
36. Parmigiani, S. et al. Reliability and validity of transcranial magnetic stimulation-electroencephalography biomarkers. *Biol Psychiatry Cogn Neurosci Neuroimaging.* **8** (8), 805-814 (2023).
37. Seybert, C. et al. Replicability of motor cortex-excitability modulation by intermittent theta burst stimulation. *Clin Neurophysiol.* **152**, 22-33 (2023).
38. Faro Viana, F. et al. Reducing motor evoked potential amplitude variability through normalization. *Front Psychiatry.* **15**, 1279072 (2024).
39. Herwig, U. et al. Transcranial magnetic stimulation in therapy studies: examination of the reliability of "standard" coil positioning by neuronavigation. *Biol Psychiatry.* **50** (1), 58-65 (2001).
40. Rossi, S., Hallett, M., Rossini, P. M., Pascual-Leone, A. Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. *Clin Neurophysiol.* **120** (12), 2008-2039 (2009).
41. Rossi, S. et al. Safety and recommendations for TMS use in healthy subjects and patient populations, with updates on training, ethical and regulatory issues: expert guidelines. *Clin Neurophysiol.* **132** (1), 269-306 (2021).
42. Lopes, G. et al. Bonsai: an event-based framework for processing and controlling data streams. *Front Neuroinform.* **9**, 7 (2015).
43. Dissanayaka, T., Zoghi, M., Farrell, M., Egan, G., Jaberzadeh, S. Comparison of Rossini-Rothwell and adaptive threshold-hunting methods on the stability of TMS induced motor evoked potentials amplitudes. *J Neurosci Res.* **96** (11), 1758-1765 (2018).
44. Silbert, B. I., Patterson, H. I., Pevcic, D. D., Windnagel, K. A., Thickbroom, G. W. A comparison of relative-frequency and threshold-hunting methods to determine stimulus intensity in transcranial magnetic stimulation. *Clin Neurophysiol.* **124** (4), 708-712 (2013).
45. Rossini, P. M. et al. Non-invasive electrical and magnetic stimulation of the brain, spinal cord, roots and peripheral nerves: basic principles and procedures for routine clinical and research application: an updated report from an IFCN committee. *Clin Neurophysiol.* **126** (6), 1071-1107 (2015).
46. Bagce, H. F., Saleh, S., Adamovich, S. V., Krakauer, J. W., Tunik, E. Corticospinal excitability is enhanced after visuomotor adaptation and depends on learning rather than performance or error. *J Neurophysiol.* **109** (4), 1097-1106 (2013).
47. Liang, W. D. et al. Upregulating excitability of corticospinal pathways in stroke patients using TMS

neurofeedback: a pilot study. *Neuroimage Clin.* **28**, 102465 (2020).

48. Ziemann, U. Pharmacotranscranial magnetic stimulation studies of motor excitability. *Handb Clin Neurol.* **116**, 387-397 (2013).
49. Cuypers, K. et al. Age-related differences in corticospinal excitability during a choice reaction time task. *Age (Dordr).* **35** (5), 1705-1719 (2013).