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TITLE:

Measuring and Manipulating Functionally Specific Neural Pathways in the Human Motor System with Transcranial Magnetic Stimulation

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SUMMARY:

This article describes new approaches to measure and strengthen functionally specific neural pathways with transcranial magnetic stimulation. These advanced noninvasive brain stimulation methodologies can provide new opportunities for the understanding of brain-behavior relations and development of new therapies to treat brain disorders.

ABSTRACT:

Understanding interactions between brain areas is important for the study of goal-directed behavior. Functional neuroimaging of brain connectivity has provided important insights into fundamental processes of the brain like cognition, learning, and motor control. However, this approach cannot provide causal evidence for the involvement of brain areas of interest. Transcranial magnetic stimulation (TMS) is a powerful, noninvasive tool for studying the human brain that can overcome this limitation by transiently modifying brain activity. Here, we highlight recent advances using a paired-pulse, dual-site TMS method with two coils that causally probes cortico-cortical interactions in the human motor system during different task contexts. Additionally, we describe a dual-site TMS protocol based on cortical paired associative stimulation (cPAS) that transiently enhances synaptic efficiency in two interconnected brain areas by applying repeated pairs of cortical stimuli with two coils. These methods can provide a better understanding of the mechanisms underlying cognitive-motor function as well as a new perspective on manipulating specific neural pathways in a targeted fashion to modulate brain circuits and improve behavior. This approach may prove to be an effective tool to develop more

sophisticated models of brain-behavior relations and improve diagnosis and treatment of many neurological and psychiatric disorders.

INTRODUCTION:

Noninvasive brain stimulation is a promising assessment tool and treatment for many neurological disorders, such as Parkinson's disease, Alzheimer's disease, and stroke[1–4]. There is accumulating evidence establishing the relationship between the behavioral manifestations of neurological diseases and abnormalities of cortical excitability, neuroplasticity, cortico-cortical and cortico-subcortical connectivity[5,6]. Therefore, basic knowledge about brain network dynamics and plasticity in neurological conditions can provide invaluable insight into disease diagnosis, progression, and response to therapy. Functional magnetic resonance imaging (*f*MRI) is a useful tool to understand the complex relations between brain and behavior in both healthy and diseased brain networks and has the potential to improve treatment based on a network perspective[7–9]. However, *f*MRI is correlational in nature and cannot provide a causal link between brain function and behavior, nor manipulate functional connectivity to restore abnormal neural circuits associated with behavioral impairments in patients[10–12]. Transcranial magnetic stimulation (TMS) can both causally measure and modulate human brain function and behavior in health and disease[3,13–15].

TMS is a safe, noninvasive method to stimulate the human brain[16,17] and can be used to induce and measure plasticity[18]. This method can advance our understanding of causal relationships between individual brain areas and behavior[10–12,19] and their specific functional interactions with other nodes of a brain network[20–23]. To date, most studies have focused on the human motor system, given that TMS to the hand area of the motor cortex (M1) can produce motor evoked potentials (MEPs) as physiological readouts for changes associated with motor behavior[24], allowing examination of different inhibitory and excitatory circuits at the system level in the human brain[25]. Recent advances using a conditioning test TMS approach with two coils show that it is possible to measure functional interactions between different cortical areas. In the motor system, dual-site TMS experiments show that inputs from cortical areas interconnected with M1 can change with task demands, age, or disease[14,26]. Seminal work by Ferbert and colleagues has found that applying a conditioning stimulus to M1 prior to a test stimulus of the other M1 can result in inhibition of the MEP amplitude, a phenomenon known as short interval interhemispheric inhibition (SIHI)[28]. A number of TMS studies using this approach have also shown that M1 is strongly interconnected with the contralateral M1, ventral premotor cortex (PMv), dorsal premotor cortex (PMd), supplementary motor area (SMA), pre-SMA, primary sensory cortex (S1), dorsolateral prefrontal cortex (DLPFC), and posterior parietal cortex (PPC) at rest[27–42]. Interestingly, the effect of stimulation from these cortical areas on motor cortical excitability are anatomically, temporally, and functionally specific to the ongoing brain activity during the preparation of a movement (state- and context-dependent[43–67,69]). However, very few studies using dual-site TMS have characterized patterns of functional cortico-cortical connectivity with motor and cognitive impairments in patients with brain disorders[70–72]. This affords opportunities to develop new methods for assessing and treating motor and cognitive disorders.

Using this technique, it also has been found that repeated pairs of cortical TMS applied to cortical

areas interconnected with M1 such as contralateral M1[68–70], PMv[76–78], SMA[71], and PPC[80–82] can induce changes in synaptic efficiency in specific neural pathways based on the Hebbian principle of associative plasticity[83–86] and enhance behavioral performance[72–74]. Still, few studies have used this approach to study circuit and plasticity dysfunction in neurological disorders[2,75–84,90–96]. It remains to be shown whether strengthening functionally specific neural pathways with TMS can restore activity in dysfunctional circuits, or whether the prospective strengthening of intact circuity can augment resilience[97] in brain networks supporting motor and cognitive function across the lifespan and in disease. The lack of fundamental understanding of the neural mechanisms underlying neurological disorders and effects of stimulation on interconnected dysfunctional brain networks limits current treatment.

Despite its capability, TMS has yet to become a standard part of the armamentarium of neuroscience and clinical tools for understanding brain-behavior relations, pathophysiology of brain disorders, and the effectiveness of treatment. Therefore, to realize its potential and support its large-scale application, standardizing TMS methods is important because it is more likely to increase the rigor of future TMS experiments and reproducibility across independent laboratories. This article outlines how TMS can be used to both measure and manipulate functional interactions. Here, we describe this technique in the motor system (e.g., parieto-motor pathway[44]) by measuring TMS-based output measures (e.g., MEPs), where the method is best understood. However, it is important to note that this protocol also can be adapted to target functional coupling of other subcortical[85], cerebellar[86,87], and cortical areas.[73,74,88] In addition, neuroimaging techniques such as EEG[89–91] and fMRI[92,93] can be used to assess the TMS-induced changes in activity and connectivity[26,94]. We conclude by proposing that the study of the functional involvement of circuit-level cortical connectivity with these TMS methods in both health and disease makes it possible to develop targeted diagnoses and innovative therapies based on more sophisticated network models of brain-behavior relations.

PROTOCOL:

The following three TMS methods are described below. First, two methods are described to measure cortico-cortical connectivity using dual-site transcranial magnetic stimulation (dsTMS) while participants are either 1) at rest (resting state) or 2) performing an object-directed reach-to-grasp movement (task-dependent). Second, a cortical paired associative stimulation (cPAS) method is described to modulate the interplay between two brain areas in a controlled manner by pairing cortical stimuli (e.g., posterior parietal and primary motor cortices) to strengthen functional specific neural pathways with TMS and induce changes in cortical excitability. A representative data set is provided for each method. All the methods described in this protocol were approved by the University of Michigan Institutional Review Board in accordance with the Declaration of Helsinki.

1. Participant recruitment

1.1. Screen all participants for any contraindications to TMS[95–100] and magnetic resonance imaging (MRI) prior to recruitment. Recruit right-handed participants[101] for experiments investigating functional connectivity in the motor system.

1.2. Inform each participant about the study objectives, procedures, and risks approved by the local institutional review board. Obtain written consent before allowing the individual to participate in the study.

2. Electromyography (EMG) electrode placement

2.1. Instruct the participant to sit comfortably in the experimental chair with both arms supported in a relaxed position. Provide a chin rest for participants during TMS to keep head movement to a minimum during stimulation.

2.2. Clean the skin over the muscle of interest with a mild abrasive. Using a belly-tendon electrode arrangement, place one disposable Ag-AgCl electrode on the belly muscle and another on a bony landmark nearby for a reference site on both hands of the participant. Repeat this step for each muscle of interest.

2.3. Connect a ground electrode to the ulnar styloid process. It is important to inspect the level of surface contact of the electrodes with the skin throughout the duration of the experiment, because this precludes the impedance quality of the EMG signal. Placing tape over the surface electrode can improve the degree of contact with the skin surface.

NOTE: For reach-to-grasp actions common muscles studied are 1) the first dorsal interosseous (FDI), 2) abductor pollicis brevis (APB), and 3) abductor digiti minimi (ADM) muscles of the hand.

2.4. Connect surface electrodes with an EMG amplifier and a data acquisition system. Record and store the EMG signals from the amplifier to the data collection computer with EMG software for online monitoring and offline analysis of the EMG signal. Optionally, amplify the EMG signal 1,000x, and use a band-pass filter between 2 Hz and 2.5 kHz, digitized at 5 kHz by an analog-to-digital interface.

3. Localizing brain areas for targeted TMS

3.1. Method 1: Localizing without an MRI scan

3.1.1. Using the 10–20 EEG system mark C3, located approximately over the left primary motor cortex (M1), and P3, located approximately over a part of the angular gyrus in the left posterior parietal cortex (PPC), on the participant's scalp. Refer to methods previously described[102] for specific steps to localize brain areas with the 10–20 EEG system (see Figures 3 and 4 from Villamar et al.[102]).

3.1.2. Alternatively, an electroencephalography (EEG) head cap can be used to approximate the brain areas on the scalp. Place an appropriately sized EEG cap on the participant's head and align the Cz position on the cap with the marked Cz position on the participant's scalp. Mark C3 and P3 using the cap.

NOTE: Localization without an individual's MRI scan has the potential to be inaccurate[103]. Therefore, MRI-based neuronavigation is strongly recommended to increase the accuracy and reliability of targeting the TMS. This can potentially lead to less variability in the TMS-induced aftereffects.

3.2. Method 2: Using an MRI scan

3.2.1. Before the TMS session, obtain the participant's structural MRI (T1). Upload the scan to a neuronavigation system.

3.2.2. Create a three-dimensional reconstruction of the brain and skin overlay using the neuronavigation software. Place markers on the anatomical landmarks at the tip of the nose, nasion, inion, and the preauricular notches of both ears. Do not use the tragus as it can shift when ear plugs are inserted.

3.2.3. Locate the hand knob, the anatomical landmark that corresponds to M1[104], in the left precentral gyrus. Place a trajectory marker at this point with the neuronavigation system. This point should be aligned 45° from the midsagittal line and approximately perpendicular to the central sulcus. Record and name the anatomical landmark with the neuronavigation system (**Figure 1**).

3.2.4. Locate the nonmotor area of interest (e.g., over the anterior intraparietal sulcus area in PPC). Place a second trajectory marker over this anatomical landmark. Record and name the location with the neuronavigation system (**Figure 1**).

3.3. Perform coil and head registration with the tracking system

3.3.1. Calibrate both TMS coils with the calibration block separately using the neuronavigation system.

3.3.2. Place the head tracker securely on the participant's head so that the tracker is in view throughout the duration of the experiment.

3.3.3. Coregister the anatomical landmarks on the participant's head to the neuronavigation system. If an MRI was not obtained from the participant, use a template MRI from the Montreal Neurological Institute.

NOTE: It is important to not apply too much force with the pointer on the participant's skin to avoid discomfort and inaccuracies when performing registration. It may be valuable to check

regularly throughout the course of the experiment that the head tracker has not shifted. These procedures ensure precision when applying the TMS coil to a target area for stimulation during the experiment.

4. Localizing optimal TMS coil position and determining thresholds

NOTE: In this experiment, $Coil_{M1}$ refers to the coil used to deliver stimulation to M1, while $Coil_{Two}$ refers to the coil used to deliver stimulation to the other cortical area of interest (e.g., posterior parietal cortex). Thresholding over M1 must be determined for $Coil_{Two}$ to calculate the maximum stimulator output (MSO) used over nonmotor areas. Motor threshold values should be reported to allow for comparisons and reproducibility across experiments.

4.1. Localizing and thresholding with $Coil_{Two}$

4.1.1. Position the center of $Coil_{Two}$ over the target M1 location identified in the previous section to induce a posterior-anterior current direction in the brain.

4.1.2. To find the optimal location for activation of the target muscle, deliver pulses to M1 at 30% of the machine's MSO. Observe whether the delivered stimulation produces a muscle twitch and determine the amplitude of the motor evoked potential (MEP) recorded with the EMG electrodes from the muscle activity displayed by the data acquisition system.

4.1.3. If an MEP or a visible muscle twitch is not observed, continue to increase the stimulator output by 5% increments. The position, rotation, pitch, and yaw of the TMS coil may need to be adjusted to optimize the amplitude of the MEP. Repeat this until a response is observed.

4.1.4. Lower the intensity in a stepwise manner to the lowest intensity that produces at least 5 out of 10 MEP responses with an amplitude of ≥50 μV while the participant is at rest[97,98,105]. This is defined as the resting motor threshold (RMT).

4.1.5. Ensure for the duration of the thresholding session that both hands are in a resting position with both arms and hands supported with pillows.

4.1.6. Provide real-time visual or auditory feedback of muscle activity from EMG (e.g., on a monitor or speaker) throughout the session, especially if there is excessive muscle activity (e.g., older adult populations).

4.1.7. Continuously ask participant about levels of comfort.

NOTE: It is important that all procedures described above are performed separately and repeated for each TMS coil to determine the specific parameters used in the experiment for the different sized coils (e.g., localizing optimal TMS coil position and determining stimulation intensities for motor thresholding). It also is important that the interval between the TMS pulses is >5 s to avoid inducing changes in cortical excitability.

4.2. Localizing and thresholding with $Coil_{M1}$

4.2.1. Repeat the steps described above to find the optimal stimulation location with the $Coil_{M1}$.

4.2.2. Determine the lowest stimulator intensity needed to generate MEPs of ≥1 mV in 5 of 10 trials in the target hand muscle when the muscle is completely relaxed. Mark and record the position of $Coil_{M1}$ using the neuronavigation system.

5. Dual-site TMS (resting state)

5.1. Use two figure-8 shaped coils (e.g., $Coil_{M1}$ and $Coil_{Two}$) connected to two individual TMS stimulators (e.g., two Magstim 200^2 units). Deliver the test stimuli (TS) over M1 with $Coil_{M1}$ (e.g., D70^2 figure-8 shaped coil, outside diameter of loop is 7 cm) and the conditioning stimuli (CS) to the other area of interest with $Coil_{Two.}$ (e.g., D50 Alpha B.I., outside diameter of each loop is 5 cm).

5.2. Determine the percentage of the MSO intensity for the conditioning stimulus (CS) for $Coil_{Two}$.

NOTE: The percentage of the MSO intensity is often between 70–140 of RMT and will depend on the specific parameters and objectives of the experiment (see Table 3 from Lafleur et al.[14]). For this experiment, the CS was set at 90% of RMT, similar to parameters used elsewhere[35,44,60].

5.3. For the test stimulus (TS), use the previously determined intensity that elicits MEP amplitudes of ~1 mV in the targeted quiescent hand muscle.

5.4. Set the precise interstimulus interval (ISI) between the CS and TS.

5.5. Use the supplied control software or external control via TTL pulses to control the ISI for the two pulses. The ISI often ranges from 4–20 ms (see Table 1 from Lafleur et al.[14]). For this experiment, the CS to PPC preceded the TS to M1 by an ISI of 5 ms.

5.6. Using a custom-made coding script, generate in random order the single-pulse TMS trials (TS alone) and paired-pulse TMS trials (CS-TS) at the specified ISI.

5.7. Position $Coil_{M1}$ over the left M1 and position $Coil_{Two}$ over the other area of interest.

5.8. Deliver the TS alone trials with $Coil_{M1}$. For the paired-pulse (CS-TS) trials, deliver the CS with $Coil_{Two}$ followed by the TS to $Coil_{M1}$ at the predetermined ISIs. This is illustrated in **Figure 2**. Repeat a minimum of 12 trials for each condition. Deliver the TS at least 1 s after the start of the trial to collect prestimulus EMG activity. Use a 4 s data acquisition sweep for each trial followed by a 1 s intertrial interval.

5.9. If necessary, adjust the TMS coil positions slightly to accommodate the placement of both coils over the selected targeted locations on the participant's head. Adjust and record the new location of $Coil_{M1}$ and $Coil_{Two}$ using the neuronavigation system accordingly.

5.10. Use the trigger button on the TMS machine for the supplied control software or the custom-made coding script from the external controller to deliver the programmed TMS pulses.

NOTE: For this experiment, a data acquisition system (e.g., CED Micro 1401) and software package (e.g., Signal version 7) were used to generate stimuli, capture data, control the external equipment, and run the analysis. The custom-made coding scripts used to run and analyze data from the experiments are available from the corresponding author.

6. Dual-site TMS (task context)

NOTE: Dual-site TMS also can be used to test whether functional connectivity at rest can be modulated by different task contexts.

6.1. Follow the same method described in the section above to examine functional interactions between different cortical areas interconnected to M1, but during the preparatory phase of a task that engages the network (e.g., during the action plan for a grasp).

6.2. Determine the time course and a cortical area of interest (e.g., PPC) to study functional interactions with M1 during the preparation of a complex movement plan (e.g., object-driven precision grip or whole-hand grasp[43–49,106]) for selective hand muscles.

6.3. Using a custom-made coding script, generate in random order the timing of TS alone trials and paired-pulse trials (CS-TS) at a given ISI after the 'GO' cue during the reaction time period (plan phase) such that the MEP recordings are collected before the movement initiation (premovement period) for the task.

6.4. Deliver single-pulse TMS (TS alone) or paired-pulse TMS (CS-TS) probes between 50 and 800 ms after the 'GO' cue[47,49] during the action plan of complex hand movements. See **Figure 3** for timing of an event-related trial for this experiment. The custom-made coding scripts used to run the timing of event-related trials are available from the corresponding author.

6.4.1. Before the testing session with TMS, have the participant perform the task for a minimum of 50 practice trials to establish a consistent reaction time. Encourage the participant to ask questions about the task to ensure reliable performance during the testing session with TMS.

6.4.2. Use the custom-made coding script to deliver all combinations of single-pulse TMS (TS alone) or paired-pulse TMS (CS-TS) and task (e.g., grasp a smaller top or grasp a larger bottom

object) during the reaction time period (plan phase) such that the MEP recordings are collected before actual movement initiation.

7. Cortical Paired Associative Stimulation (cPAS)

NOTE: This protocol involves delivering pairs of monophasic pulses to two different cortical areas over short periods to induce pathway-specific changes in synaptic strength between connections within the human brain. This approach is based on Hebbian principles of spike timing dependent plasticity[107–110]. Similar to dual-site TMS methods, cPAS is delivered with two TMS machines connected to two individual TMS coils over two different cortical areas (e.g., PPC and M1).

7.1. Using a custom-made coding script, generate 100 pairs of stimuli at 0.2 Hz (8.3 min duration each). For the experimental $cPAS_{Two \rightarrow M1}$ condition, deliver the first stimuli over the nonmotor area (e.g., PPC) with $Coil_{Two}$ with a specified pulse intensity (e.g., 90% RMT) for 5 ms before the second stimuli over M1 with $Coil_{M1}$ with a pulse intensity that elicits a MEP amplitude of ~1 mV in the targeted hand muscle.

7.2. It is important to control for: 1) directionality of the connectivity ($CTRL_{M1 \rightarrow Two}$); 2) timing ($CTRL_{ISI=500ms}$); and 3) stimulation site ($CTRL_{Control\ site \rightarrow M1}$) in separate sessions. For examples see[72,74,111,112]. The custom-made coding scripts for each cPAS condition are available from the corresponding author. The stimulation parameters (e.g., intensities and ISI) can be adjusted for different cortical areas. Refer to Table 2 from Lafleur et al.[14] for a summary of plasticity protocols.

7.3. Use the procedures described in previous sections to guide the precise location of the TMS coils.

7.4. Obtain baseline corticospinal measurements with $Coil_{M1}$ (e.g., ~24 MEPs).

7.5. Randomize the participants to one of four intervention groups: 1) $cPAS_{Two \rightarrow M1}$; 2) $CTRL_{M1 \rightarrow Two}$; 3) $CTRL_{ISI=500ms}$; 4) $CTRL_{Control\ site \rightarrow M1}$.

7.6. For this experiment only the experimental $cPAS_{Two \rightarrow M1}$ condition was tested and the PPC was used as the area of interest. When performing multiple sessions on the same participant, it is important that each experimental session is separated by at least 48 h in a randomized order to prevent crossover effects. It also is important to repeat sessions within each participant at the same time of day to control for alertness.

7.7. Use the custom-made coding script to deliver the specified cPAS condition.

7.8. Monitor the muscle activity of the other (left) hand during the experiment with EMG to ensure the hand is fully relaxed during the protocol.

7.9. Obtain corticospinal measurements with $Coil_{M1}$ (e.g., about 24 MEPs) at different times after cPAS (e.g., 0, 10, 20, 30, 40, 50, 60 min) to examine the time course of the TMS-induced effect on brain excitability.

NOTE: The experimental protocol used here is shown in **Figure 4.** Most studies to date have focused on the motor system because the MEP is a reliable outcome measure. However, behavioral measures[72–74] and functional connectivity strength with fMRI[92,93] and EEG[89,90] following TMS manipulation of associative plasticity can also be investigated. These methods can also be adopted for different cortical areas that do not include M1 as a cortical target.

8. Data processing and analysis

8.1. Visually inspect EMG data offline and discard any traces showing muscle activity in which the root mean square EMG activity in the muscles exceeded a background level of 10 μV during the 100 ms immediately before the TMS pulse to ensure the muscles were at rest[59,113].

8.2. Similarly, discard any trials with EMG activity that coincide with the TMS pulse during the movement preparation period (e.g., 800 ms window[47,49]) in dual-site TMS task context trials to exclude anticipatory responses.

8.3. For each MEP trial, measure the peak-to-peak amplitude between the minimum and maximum values in mV in the time window between 50 ms before and 100 ms after the TS[105].

8.4. Calculate the mean of the MEP amplitudes in millivolts from the TS alone trials and the paired-pulse (CS-TS) trials for each participant. Calculate the mean across all participants. Report these values.

8.5. Next, normalize the mean MEP amplitude from paired-pulse stimulation (CS-TS) trials from the unconditioned single-pulse (TS alone) trials for each participant and condition. Express the MEP amplitudes as a ratio to the baseline TS condition.

$$Normalized\ MEP\ amplitude\ (Ratio) = \frac{MEP\ amplitude\ (CS \rightarrow TS)}{MEP\ amplitude\ (TS\ alone)}$$

8.6. Calculate the mean across all participants. Report these values.

REPRESENTATIVE RESULTS:

Figure 5 shows the size of an exemplar MEP response elicited in the FDI muscle by TMS for an unconditioned test stimuli (TS alone to M1, blue trace) or conditioned stimuli from PPC (CS-TS, red trace) while the participant was at rest (top panel) or planning a goal-directed grasping action to an object (bottom panel). At rest, the PPC exerts an inhibitory influence on ipsilateral M1, as shown by the decrease in MEP amplitudes potentiated by a subthreshold CS delivered over PPC 5 ms before a suprathreshold TS over M1 (top panel). During the preparation of a grasp action, this net inhibitory drive at rest from PPC switched to facilitation (a release of inhibition). To

directly compare PPC-M1 interactions during rest versus task demands, the MEP amplitudes were normalized to TS alone trials for each condition and plotted as a ratio for MEP amplitude. The PPC-M1 interaction was facilitated from rest when planning an object-directed grasp (purple bars).

The top panel in **Figure 6** shows changes in MEP amplitudes during the administration of the cPAS protocol. MEP amplitudes induced by paired stimulation of PPC and M1 gradually increased over time during the stimulation protocol, suggesting plastic effects at the level of the parieto-motor connection, M1 corticospinal neurons, or both. The bottom panel of **Figure 6** shows changes in MEP amplitudes elicited in the resting FDI muscle by single-pulse TMS over M1 before and after the cPAS protocol. The size of the MEP amplitudes increased 10 min after the cPAS protocol, suggesting motor excitability aftereffects were induced after the administration of the repeated pairs of cortical stimuli over PPC and M1.

FIGURE AND TABLE LEGENDS:

Figure 1: Three-dimensional reconstruction of a typical participant's anatomical MRI with marked cortical sites over the primary motor cortex (M1, blue symbol) and posterior parietal cortex (PPC, red symbol) in the left hemisphere. Neuronavigation software for TMS was employed to target individually determined cortical areas with each figure-8 TMS coil.

Figure 2: Schematic representation of the dual-site, paired-pulse transcranial magnetic stimulation with two coils (dsTMS) used to probe functional interactions between the posterior parietal cortex (PPC) and primary motor cortex (M1) at rest (resting state). A CS was applied to the PPC to examine its effect on a subsequent suprathreshold TS to M1. Any change in the amplitude of the right-hand muscle response to TMS is measured with EMG. For this experiment, the CS intensity was 90% of RMT. The intensity of TS was adjusted to elicit a MEP of ~1 mV peak-to-peak in the relaxed FDI and ADM. The ISI between pulses was 5 ms.

Figure 3: The dsTMS approach used to probe functional interactions between PPC and M1 during a reach-to-grasp movement (task context). The illumination of an LED instructed the participant to plan one of two possible rightward hand actions on the target object: 1) grasp the smaller top cylinder or 2) grasp the larger bottom cylinder. TS alone or CS–TS at the specified ISI (e.g., 5 ms) was delivered 300 ms after the 'GO' cue (e.g., LED onset) during the reaction time period (plan phase) such that MEP recordings were collected before actual movement initiation (dotted black line).

Figure 4: Schematic of cortical paired associative stimulation protocol (cPAS) used to strengthen functionally specific neural pathways. The first stimulus was applied to the area of interest with $Coil_{Two}$ (e.g., PPC, red coil) 5 ms before the second stimulus was delivered to M1 (blue coil) with $Coil_{M1}$. The pairs of cortical stimuli were delivered at a frequency of 0.2 Hz (once every 5 s) and repeated for 100 trials (~8.3 min).

Figure 5: Exemplar MEP traces for an unconditioned test stimulus (TS alone, blue trace) or conditioned stimulus (CS-TS, red trace) for the resting state (top panel) and context-dependent

(bottom panel) condition. Bar graphs show the MEP amplitudes from the dsTMS protocol while the participant is at rest or performing a grasping task (action). When the participant was at rest (top panel), CS-TS (red bar) decreased the mean amplitude of MEPs (inhibition) compared to the unconditioned TS alone (blue bar). In contrast, when the participant planned the reach-to-grasp task (bottom panel), the mean MEP amplitude increased (facilitation) for CS-TS (red bar) trials compared to the TS alone (blue bar) trials. To directly compare the PPC-M1 interaction for rest versus action condition, the mean MEP amplitude elicited by paired-pulse stimulation (CS–TS) was normalized by calculating the ratio of the amplitude relative to the mean unconditioned MEP amplitude (TS alone). Purple bars represent the normalized MEP amplitude for each condition. Y = 1 indicates no effect of CS on M1 excitability (dotted black line), whereas ratios higher than 1 indicate increased M1 excitability and ratios lower than 1 indicate decreased M1 excitability because of conditioned stimuli (CS-TS). Error bars represent SEM.

Figure 6: MEPs during cPAS. Top panel shows that MEP amplitudes increased during the administration of cPAS. The bottom panel shows the effect of cPAS protocol on MEP amplitude. After the cPAS intervention (red bar) corticospinal excitability increased after 10 min (dark grey bar) compared to baseline (light grey bar), as assessed by MEPs in the quiescent hand muscles. The red bar represents the paired stimulation intervention, cPAS (100 pairs at 0.2 Hz, ~8.3 min). This suggests that modulating parieto-motor interactions with cPAS can induce transient changes in motor plasticity. Error bars represent SEM.

DISCUSSION:

The dual-site TMS method described here can be employed to investigate functional interactions between different cortical areas interconnected with the primary motor cortex while a participant is at rest or planning a goal-directed action. While brain imaging is correlative, basic knowledge from dual-site TMS methods can reveal causal brain-behavior relations associated with changes in cortico-cortical circuits. In addition, cortical paired associative stimulation with two TMS coils applied in areas interconnected with M1 can be employed to strengthen functionally specific connectivity for movement control and increase the efficiency of inducing plasticity. Taken together, these methods demonstrate that these TMS protocols can both measure and manipulate neural activity underlying information flow between brain areas in an anatomical-, task-, and time-dependent manner within the motor system. This affords opportunities to test different hypotheses related to the causal contribution of cortical areas to motor function.

In this light, the approach also can provide an essential foundation for understanding network connectivity at a systems-level in neurological and psychiatric patients with similar symptomology and enable its use as both a tool to diagnose and treat circuit dysfunction. Therefore, it is important for more studies to explore other cortical areas outside the motor system to test its generalizability across brain networks in both healthy and diseased brains. This is an important factor given that one cannot assume that the response to TMS in one brain region will produce the same physiological effect when applied to another region. It is also advantageous that these procedures can be extended to more complex movements, and other domains outside of movement such as cognition, perception, and mood. Indeed, several studies

using dual-site TMS and cPAS have begun to examine the effects and feasibility of study in the visual and cognitive systems[73,74,88]. Importantly, this will afford opportunities to develop a more sophisticated understanding of the neural underpinnings linking brain activity to motor, cognitive, and affective function. As a result, it is critical that a solid mechanistic knowledge about neural circuit dynamic in patient populations is investigated before determining the usefulness of applying these protocols in future clinical settings.

Although growing evidence suggests that TMS is a novel approach capable of characterizing synaptic dysfunction and plasticity in neurological and psychiatric disorders such as Parkinson's disease, Alzheimer's disease, and stroke, the clinical utility of these assessments needs to be established on a larger scale. Moreover, to date all work in patient populations has focused only on the functional circuits while the participants are at rest. It is vital that future studies with dual-site TMS consider state- and task-dependent effects, particularly when the patient is challenged, to fill knowledge gaps in understanding how altered brain dynamics contribute to specific motor, cognitive, and affective dysfunctions. Importantly, this setting allows for unprecedented opportunities to comprehensively study functional brain circuits and plasticity noninvasively by both recording and manipulating neural activity. This can eventually be translated to novel clinical therapies for brain disorders.

Awaiting these clinical advances, a critical first step is to increase the rigor and reproducibility of TMS experiments across independent laboratories by providing well-defined methodological procedures that are easily deployable and shareable. The following guidelines for the TMS procedures described above can help standardize the design, implementation, and conclusiveness of findings. First, stimulation parameters such as the intensity, duration, ISI, timing, coil position, and anatomical locations should be carefully documented and repeated in the same task context across multiple independent laboratories to encourage large-scale testing and application. Second, brain targets should be precisely defined based on clear anatomical and functional criteria that capture brain activity within brain circuits associated with behavior. Third, neuronavigation should be used to guide the TMS coil placement when targeting said brain circuits. It also is recommended that experiments be hypothesis-driven and use both a control task to ensure changes are related selectively to the task context and a control brain site outside the putative targeted network to rule out the nonspecific effect of stimulation. Fourth, to better inform the diagnostic accuracy and therapeutic effectiveness of these methods in future clinical settings, basic research will need to use a multimodal approach combining TMS measures and manipulations with neuroimaging and behavioral measures to better characterize the underlying pathological changes and effect of treatment. Fifth, variability of individual responses using dual-site TMS methods need to be reported because it could provide important information about how interventions can be optimized for different brain areas, leading to new treatments based on individual pathophysiological mechanisms. Finally, researchers need to be transparent when reporting findings by including negative results[42] and make data publicly available for interpretation to increase sample sizes and promote more efficient science. This comprehensive approach will increase rigor and reproducibility in both the collection and analysis of data that can guide future basic neuroscience and clinical studies. Ultimately, this will enable improvements in experimental design and optimize targeted therapies, thereby reducing

morbidity and impairments in neurological and psychiatric disorders.

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Figure 1

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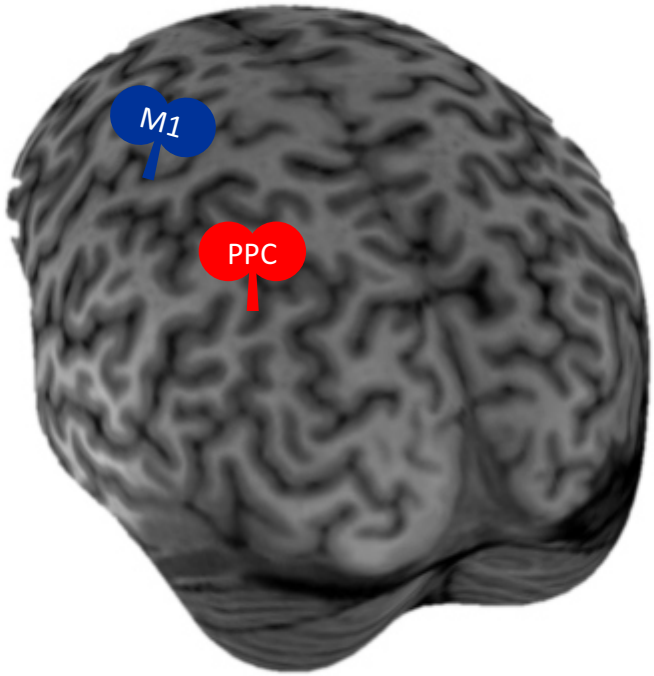

Figure 2

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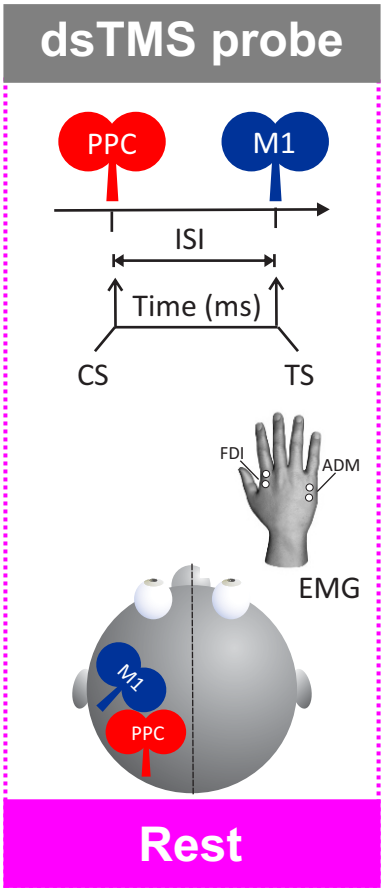

Figure 3

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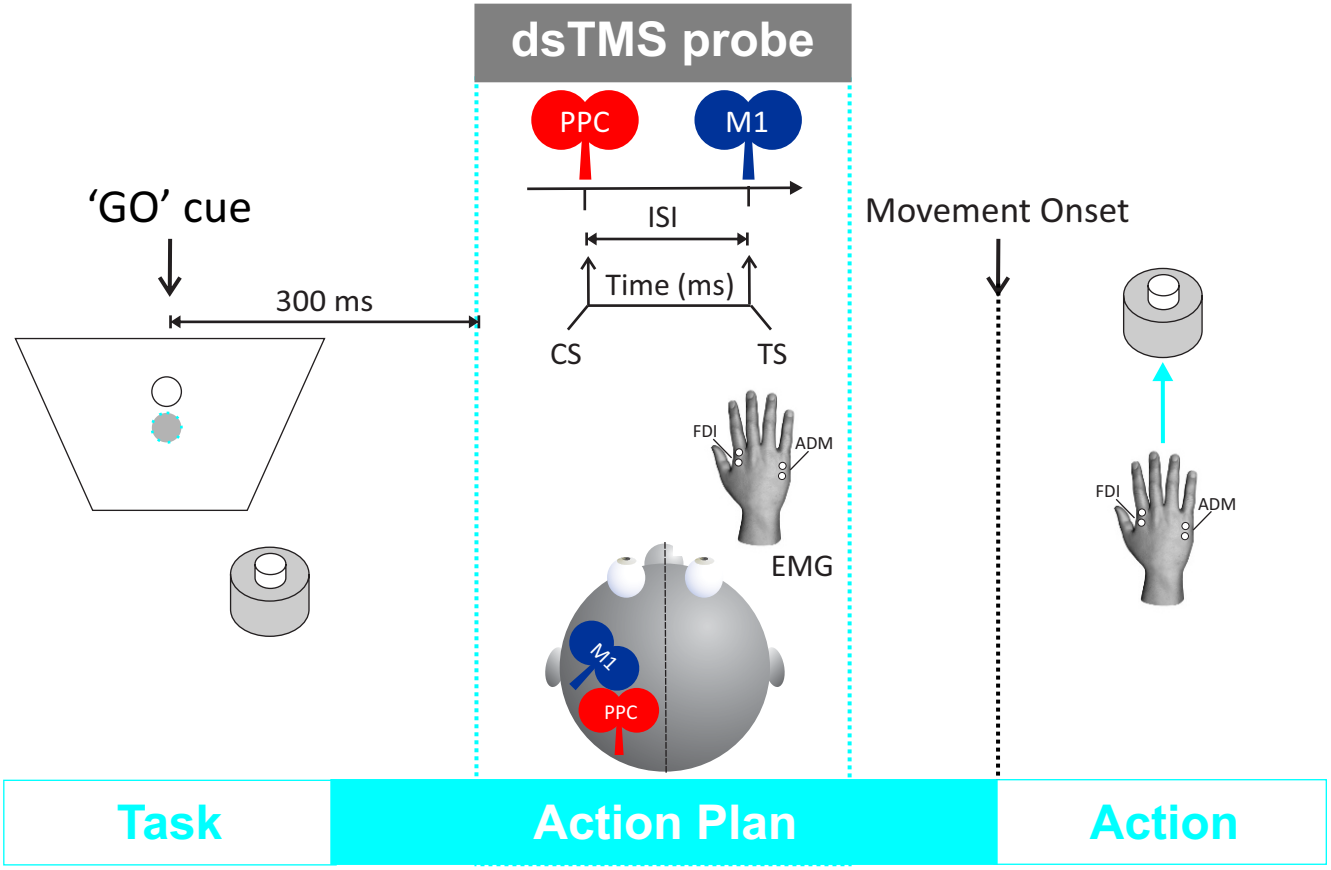

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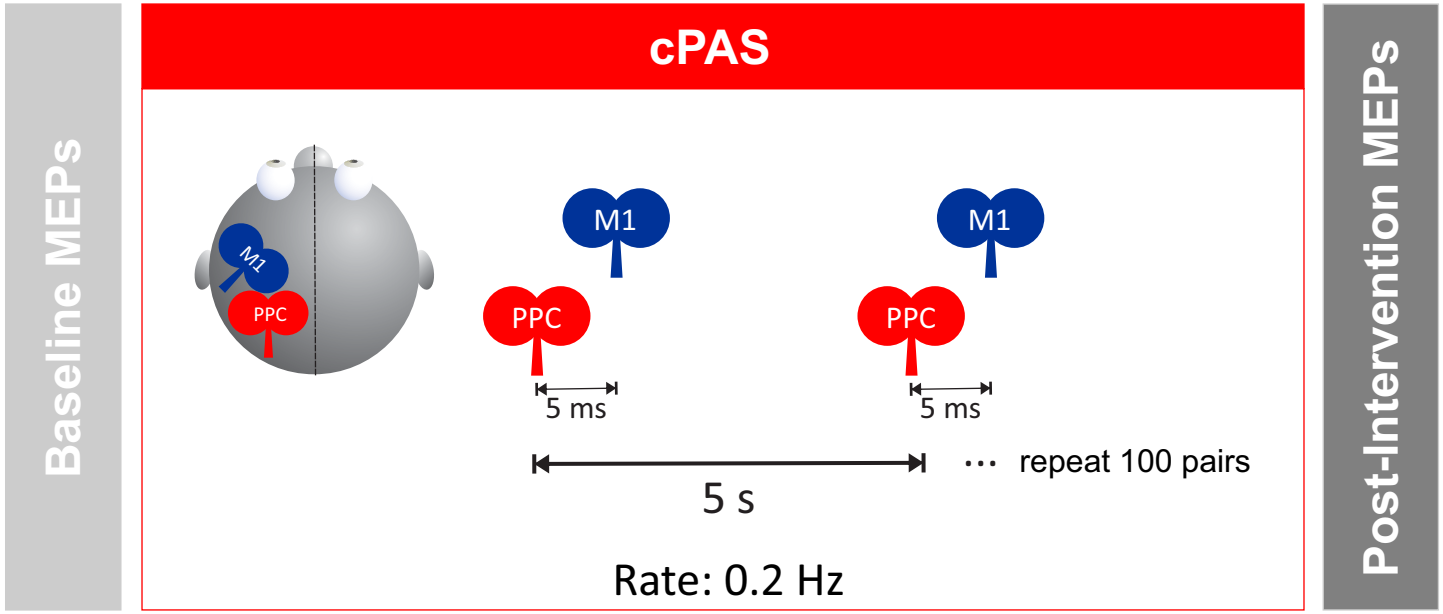


Figure 5

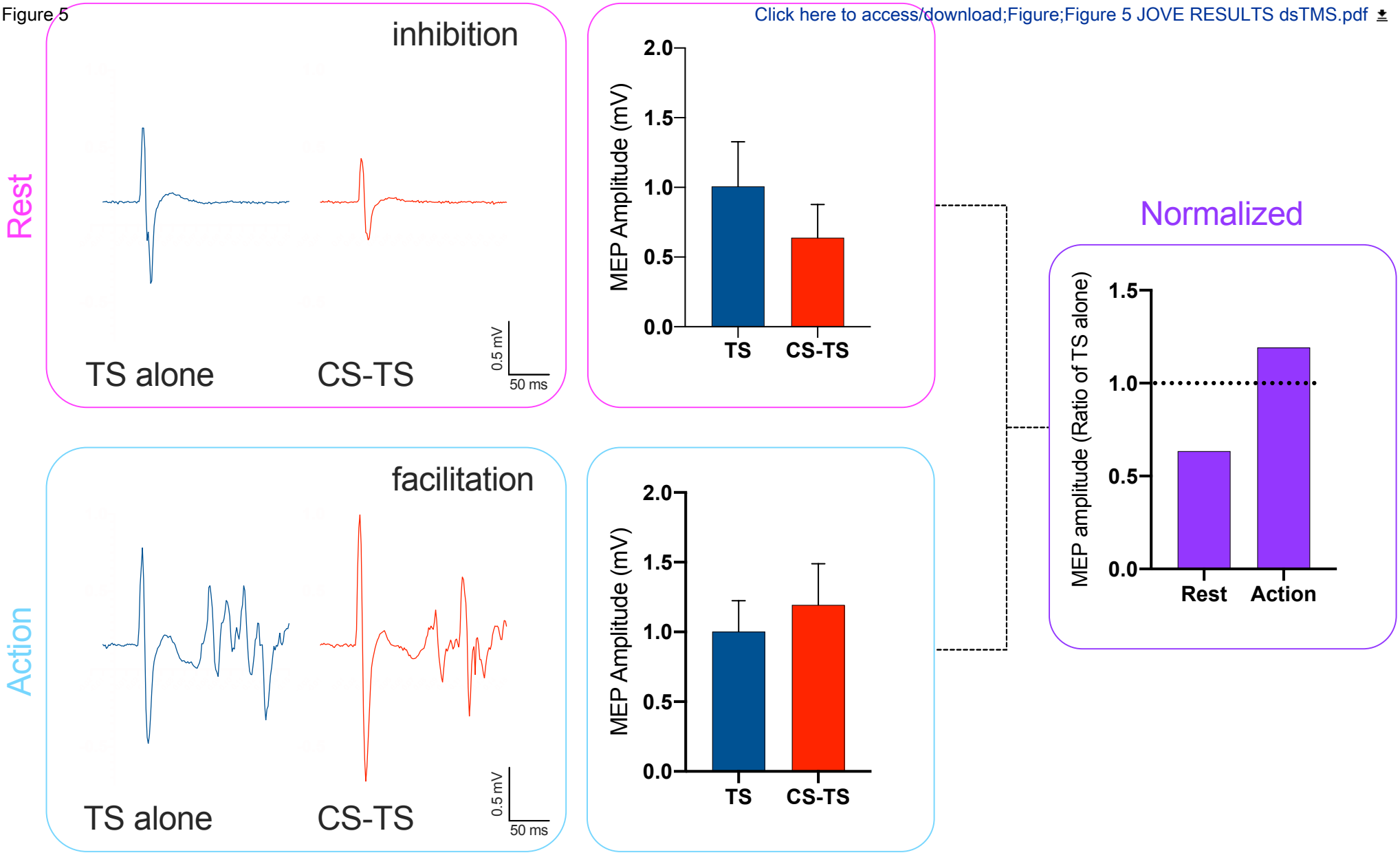
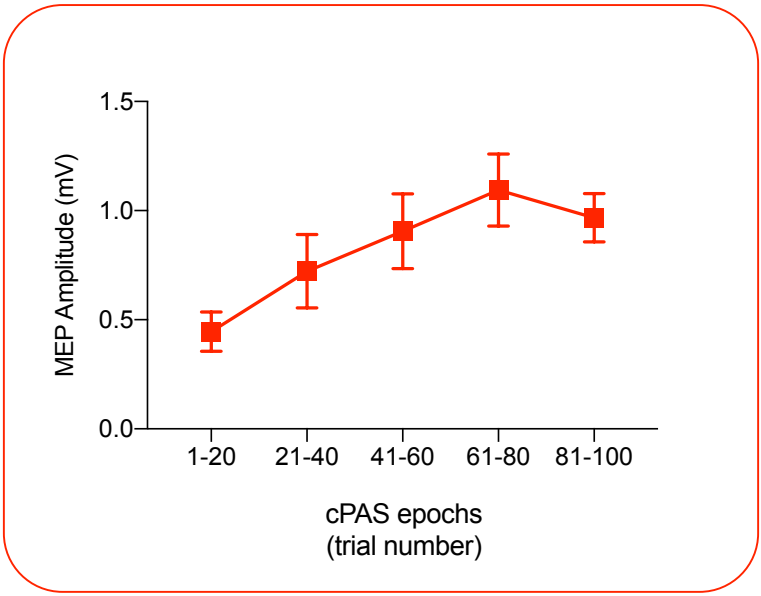
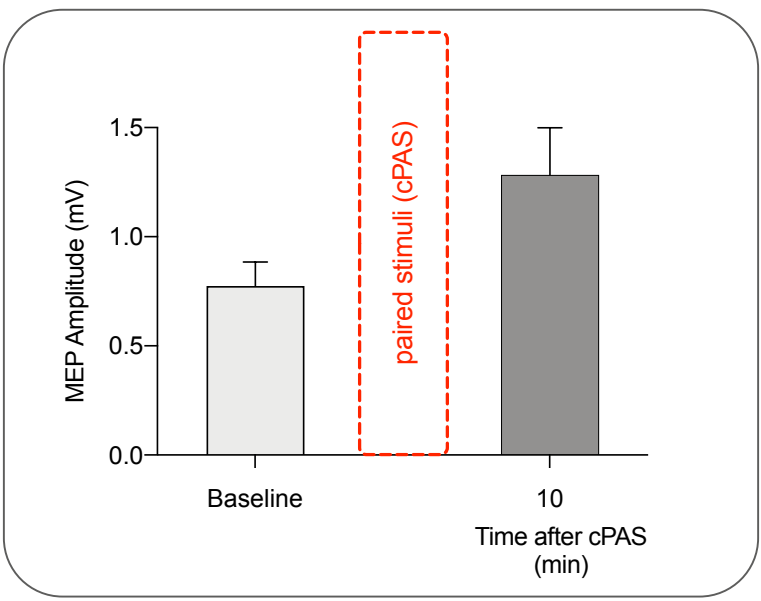


Figure 6



Corticospinal excitability



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Alpha B.I. D50 coil (coated)	Magstim		50mm coil
BrainSight 2.0 Software	Rogue Research		Neuronavigation software
BrainSight frameless Stereotactic System	Rogue Research		Neuronavigation equiptment
D70² Coil	Magstim		70mm coil
Discovery MR750	General Electric		3.0T MRI machine
Disposable Earplugs	3M		Foam earplugs
ECG Electrodes 30mm x 24mm	Coviden-Kendall	H124SG	Disposable electrodes
Four Channel Isolated Amplifier	Intronix Technologies Corporation	2024F	EMG amplifier
gGAMMAcap	g.tec Medical Engineering		EEG head cap
Micro1401-3	Cambridge Electronic Design		Scientific data recorder and processing machine
Nuprep Skin Prep Gel	Weaver and Company		Skin prep abrasive gel
Signal v.7	Cambridge Electronic Design		Data acquisition and analysis software
The Magstim BiStim²	Magstim		Transcranial magnetic stimulator (two 200² units)

Response to Reviewers.

We thank the reviewers for her/his constructive feedback. All revisions are highlighted in the revised version of the manuscript. Please see below for a point-by-point response to all concerns.

Reviewers' comments

1. Short interval inter hemispheric inhibition (SIHI) should be discussed in the introduction as this is a prototypical well-established dsTMS protocol. (Ferbert et al., 1992; Daskalakis et al., 2002), see also Stefanou et al JNeurosci for EEG state dependence of such TS vs CS-TS measures.

We thank the reviewer for pointing out this oversight. We have attached the following text to the manuscript (line 70-73).

Seminal work by Ferbert and colleagues has found that applying a conditioning stimulus to M1 prior to a test stimulus of the other M1 can result in inhibition of the MEP amplitude, a phenomenon known as short interval interhemispheric inhibition (SIHI).[43-44]

2. For probing connection strength using the CS-TS vs TS method with MEPs as a readout, this only applies to directional networks that terminate in the primary motor cortex. This limitation (and possible ways around it, e.g. with TMS evoked EEG potentials) should be more clearly discussed.

Again, we thank the reviewer for bringing this important concern to our attention. We have attached the following text to the manuscript (line 104-107).

Here, we describe this technique in the motor system (e.g., parieto-motor pathway,[44]) by measuring TMS-based output measures (e.g., MEPs), where it is best understood. However, it is important to note that this protocol also can be adapted to target functional coupling of other subcortical[85], cerebellar[86,87], and cortical areas[73,74,88]. In addition, neuroimaging techniques such as EEG[89-91] and fMRI[92,93] can be used to assess the TMS-induced changes in activity and connectivity [94,95]. We conclude by proposing that the study of the functional involvement of circuit-level cortical connectivity with these TMS methods in both health and disease opens up the possibility to develop targeted diagnosis and innovative therapies based on more sophisticated network models of brain-behavior relations.

3. 3.1. localising individual Brian areas without an MRI scan is potentially highly inaccurate and inadvisable. It should be stated that the MRI based neuronavigation is by far preferred.

We agree with the reviewer. We have attached the following text to the manuscript (line 176-179).

NOTE Localizing without an individual's MRI scan has the potential to be inaccurate.[104] Therefore, MRI-based neuronavigation is strongly recommended to increase the accuracy and reliability of targeted TMS. This can potentially lead to less variability in the TMS-induced aftereffects.

4. 3.2. "Method 3" -> there is no method 2 described

We changed to Method 2: Using an MRI scan

5. "parieto-frontal network" is mentioned as a keyword, but this method does not directly apply to networks completely outside of the motor system and so this keyword is misleading

We removed the key work.

6. "spike-timing dependent plasticity" is mentioned as a keyword but it is not discussed how this relates to the study. I would recommend using the keyword "paired associative stimulation" instead.

We added the key paired associative stimulation, as suggested by the reviewer.

7. Maybe emphasize that your elaborate approach might be even more accurate if highly accurate navigated TMS is used.

We agree with the reviewer. We have attached the following text to the manuscript (line 176-179).

NOTE Localizing without an individual's MRI scan has the potential to be inaccurate.[104] Therefore, MRI-based neuronavigation is strongly recommended to increase the accuracy and reliability of targeted TMS. This can potentially lead to less variability in the TMS-induced aftereffects.

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Author(s):	Elana R. Goldenkoff1 , Amir Mashni1, , Katherine J. Michon1,, Hannah Lavis1, Michael Vesia1,*

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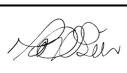
Signature:	
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