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Video Article

# A Multimodal Imaging- and Stimulation-based Method of Evaluating Connectivity-related Brain Excitability in Patients with Epilepsy

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## Abstract

Resting-state functional connectivity MRI (rs-fcMRI) is a technique that identifies connectivity between different brain regions based on correlations over time in the blood-oxygenation level dependent signal. rs-fcMRI has been applied extensively to identify abnormalities in brain connectivity in different neurologic and psychiatric diseases. However, the relationship among rs-fcMRI connectivity abnormalities, brain electrophysiology and disease state is unknown, in part because the causal significance of alterations in functional connectivity in disease pathophysiology has not been established. Transcranial Magnetic Stimulation (TMS) is a technique that uses electromagnetic induction to noninvasively produce focal changes in cortical activity. When combined with electroencephalography (EEG), TMS can be used to assess the brain's response to external perturbations. Here we provide a protocol for combining rs-fcMRI, TMS and EEG to assess the physiologic significance of alterations in functional connectivity in patients with neuropsychiatric disease. We provide representative results from a previously published study in which rs-fcMRI was used to identify regions with abnormal connectivity in patients with epilepsy due to a malformation of cortical development, periventricular nodular heterotopia (PNH). Stimulation in patients with epilepsy resulted in abnormal TMS-evoked EEG activity relative to stimulation of the same sites in matched healthy control patients, with an abnormal increase in the late component of the TMS-evoked potential, consistent with cortical hyperexcitability. This abnormality was specific to regions with abnormal resting-state functional connectivity. Electrical source analysis in a subject with previously recorded seizures demonstrated that the origin of the abnormal TMS-evoked activity co-localized with the seizure-onset zone, suggesting the presence of an epileptogenic circuit. These results demonstrate how rs-fcMRI, TMS and EEG can be utilized together to identify and understand the physiological significance of abnormal brain connectivity in human diseases.

## Video Link

The video component of this article can be found at http://www.jove.com/video/53727/

## Introduction

Transcranial magnetic stimulation (TMS) is a means of noninvasively stimulating regions of cortex via electromagnetic induction. In TMS, a large but spatially restricted magnetic flux is used to induce an electrical field in a target cortical area, and thereby modulate the activity of the underlying neural tissue. TMS to motor cortex results in motor evoked potentials that can be measured peripherally via electromyography (EMG). When applied in pairs or triplets of pulses, TMS can be used to assess the activity of specific intracortical GABAergic and glutaminergic circuits[1-3], and thus assess the balance of excitation and inhibition *in vivo* in human patients. In epilepsy specifically, TMS studies have shown that cortical hyperexcitability is present in patients with epilepsy[4,5], and may normalize with successful anti-epileptic drug therapy and thus predict response to medication[6]. Furthermore, TMS measures of cortical excitability show intermediate values in patients with a single seizure[7] and in siblings of patients with both idiopathic generalized and acquired focal epilepsies[8]. These findings suggest that TMS measures of cortical excitability may allow us to identify endophenotypes for epilepsy. However, the sensitivity and specificity of these measures are limited, likely because TMS-EMG can only be assessed with stimulation of motor cortical circuits, and many patients with epilepsy have seizure foci outside the motor cortex.

Electroencephalography (EEG) provides an opportunity to directly measure the cerebral response to TMS, and can be used to assess cerebral reactivity across wide areas of neocortex. Studies integrating TMS with EEG (TMS-EEG) have shown that TMS produces waves of activity that reverberate throughout the cortex[9,10] and that are reproducible and reliable[11-13]. By evaluating the propagation of evoked activity in different behavioral states and in different tasks, TMS-EEG has been used to causally probe the dynamic effective connectivity of human brain networks[10,14-16]. TMS-EEG measures have shown significant abnormalities in diseases ranging from schizophrenia[17] to ADHD[18], and in

disorders of consciousness such as persistent vegetative state[19]. Furthermore, several groups have identified EEG correlates of the paired-pulse TMS-EMG metrics that are abnormal in patients with epilepsy[20,21]. Of particular relevance, previous studies have also suggested that abnormal stimulation-evoked EEG activity is seen in patients with epilepsy[22-25].

Another means of evaluating brain circuits is via resting-state functional connectivity MRI (rs-fcMRI), a technique that evaluates the correlations over time in the blood oxygenation level dependent (BOLD) signal from different brain regions[26]. Studies using rs-fcMRI have demonstrated that the human brain is organized into distinct networks of interacting regions[26-29], that neuropsychiatric diseases may occur within specific large-scale distributed neural networks identified by rs-fcMRI[30], and that the brain networks identified via rs-fcMRI are often abnormal in neuropsychiatric disease states[31,32]. In terms of potential clinical applications, rs-fcMRI has several advantages over conventional task-based fMRI application[33], including less reliance on subject cooperation and concern over variable performance. Consequently, there has recently been an explosion of studies exploring rs-fcMRI changes in different disease states. However, one of the limitations of rs-fcMRI is the difficulty in determining whether and how correlations (or anticorrelations) in the BOLD signal relate to the electrophysiological interactions that form the basis of neuronal communication. A related problem is that it is often unclear whether the rs-fcMRI changes seen in various disease states have physiologic significance. In particular with regards to epilepsy, it is unclear whether abnormalities in rs-fcMRI are due solely to interictal epileptiform transients, or exist independently of such electrophysiological abnormalities; simultaneous EEG-fMRI is needed to help evaluate between these possibilities[34].

As TMS can be used to produce transient or sustained changes in the activations of different cortical regions, TMS studies provide a means of causally assessing the significance of different resting-state fMRI connectivity patterns. One approach is to use rs-fcMRI to guide therapeutic stimulation efforts in different disease states; it could be expected that TMS targeted to regions that are functionally connected to areas known to be involved in different disease states is more likely to be therapeutically effective than TMS targeted to regions without such functional connectivity, and indeed several studies have found preliminary evidence for this[35,36]. Another approach would involve using TMS-EEG to causally assess the physiologic significance of different resting-state fcMRI patterns. Specifically, one can test the hypothesis that regions that show abnormal functional connectivity in a specific disease state should show a different response to stimulation in patients than in healthy subjects, and that these physiologic abnormalities are present specifically (or primarily) with stimulation of the abnormally connected region.

To illustrate the above, we provide an example of a recent study in which rs-fcMRI, TMS and EEG were combined to explore cortical hyperexcitability in patients with epilepsy due to the developmental brain abnormality periventricular nodular heterotopia (PNH)[37]. Patients with PNH present clinically with adolescent- or adult-onset epilepsy, reading disability, and normal intelligence, and have abnormal nodules of gray matter adjacent to the lateral ventricles on neuroimaging[38,39]. Previous studies have shown that these periventricular nodules of heterotopic gray matter are structurally and functionally connected to discrete foci in the neocortex[40,41], and that epileptic seizures may originate from neocortical regions, heterotopic gray matter, or both simultaneously[42], suggesting that epileptogenesis in these patients is a circuit phenomenon. By using resting-state fc-MRI to guide TMS-EEG, we demonstrated that patients with active epilepsy due to PNH have evidence of cortical hyperexcitability, and that this hyperexcitability appears to be limited to regions with abnormal functional connectivity to the deep nodules.

The protocol is conducted in two separate sessions. During the first session, structural and resting-state blood-oxygenation level-dependent (BOLD) contrast MRI sequences are acquired (for patients), or just structural MRI sequences (for the healthy controls). Between the first and second sessions, resting-state functional connectivity analysis is used to define the cortical targets for the patients, and the MNI coordinates for these targets are obtained. The equivalent cortical targets (based on MNI coordinates) are then identified for each healthy control subject. In the second session, the TMS-EEG data is obtained.

In the example given in this paper, functional-connectivity MRI analyses were performed using an in-house software toolbox and the MRI software[43,44]. Neuro-navigated TMS was performed with a transcranial magnetic stimulator with real-time MRI neuronavigation. EEG was recorded with a 60-channel TMS-compatible system, which utilizes a sample-and-hold circuit to avoid amplifier saturation by TMS. EEG data were analyzed using custom scripts and the EEGLAB toolbox[45] (version 12.0.2.4b) running in MATLAB R2012b.

## Protocol

The protocol described here was approved by the institutional review boards of the Beth Israel Deaconess Medical Center and the Massachusetts Institute of Technology.

# 1. Subject Selection

1. Patient selection for research protocol.
    1. Identify patients with active epilepsy (seizures within the past year) or a history of remote epilepsy (prior seizures, but with no seizures in the past five years either on or off medication) and periventricular nodular heterotopia on structural brain imaging.
    2. Exclude patients without any history of seizures. Also exclude patients with alternative possible etiologies for seizures (*e.g.,* a history of traumatic brain injury, stroke, meningoencephalitis) or with EEG findings consistent with an alternative diagnosis (*e.g.,* idiopathic generalized epilepsy, mesial temporal lobe epilepsy).
    3. Exclude patients with additional neurologic or psychiatric disease, or with any other unstable medical condition. Also exclude patients with a history of prior brain surgery, inability to tolerate MRI, recent illicit substance or heavy alcohol use, or a specific MRI[46] or TMS[47] contraindication.

2. Healthy control subject selection.
    1. For each PNH patient (in our prior published study[37], 8 patients, ages 20 - 43 years mean 30.25; 3 male, 5 female), identify an age- and gender-matched healthy control.

2. Exclude subjects with any ongoing neurologic or psychiatric disease or on psychoactive medications, any other unstable medical condition, a history of prior brain surgery, inability to tolerate MRI, illicit substance or heavy alcohol use, or any other specific MRI or TMS contraindication.

# 2. Generating the Stimulation Targets

1. Using a 3T MRI system, acquire high-resolution structural whole-brain slices using a T1-weighted sequence. Use the following acquisition parameters: 128 slices per slab, a 256 x 256 matrix, field of view (FOV) 256 mm, slice thickness 1.33 mm with 0.63 mm interslice gap, voxel size 1 x 1 x 1.33 mm$^3$, repetition time (TR) 2,530 msec, inversion time 1,100 msec, echo time (TE) 3.39 msec, flip angle 7°.
2. Using a 3T MRI system, acquire resting-state functional images using an echo-planar sequence sensitive to blood-oxygenation level-dependent (BOLD) contrast. While performing this scan instruct the patients to rest quietly with eyes open without performing any specific task. Use the following acquisition parameters: FOV 256 mm, voxel size 2.0 x 2.0 x 2.0 mm, TR 6,000 msec, TE 30 msec, flip angle 90°, acquisition time 6.4 min.
3. Using MRICroN software[44], identify each discrete region of nodular heterotopia (either each individual nodule or an inseparable contiguous cluster of nodules) [46]. Use the Pen tool to manually outline heterotopia regions of interest (ROIs), slice by slice in the axial plane on T1-weighted structural images.
4. Use the CONN functional connectivity software toolbox[48,49] to perform four sequential steps in resting-state functional data processing: Setup, Preprocessing, Analysis, and Results.
    1. For Setup, use menu choices to start a new project and enter basic experiment information. Load the functional images, realigned and co-registered to the anatomic images for each subject.
    2. Load the structural images. Load heterotopia ROI files created in step 2.3. Enter details of the experimental condition; since this is resting-state, enter a single condition with onset 0 seconds and duration equal to the complete duration of each session. The toolbox will extract the heterotopia ROI BOLD time-series. Inspect for possible inconsistencies.
    3. For Preprocessing, confounding sources of BOLD variation include respiratory-induced modulations of the main magnetic field and cardiac pulsations, as well as subject motion. Remove confounders via the integrated principal component-based method that analyzes time-series data from regions unlikely to be associated with neural activity, such as ventricles and large vessels, to identify physiological noise processes[50]. Preview the total variance explained by each of the possible confounding sources. Apply a band-pass frequency filter (0.01 Hz < f < 0.1 Hz) and Gaussian smoothing (6 mm full width at half-maximum).
    NOTE: The toolbox will by default identify sources of possible confounders, including BOLD signal from the white matter and cerebrospinal fluid and realignment parameters (subject motion).
    4. For Analysis and Results, identify the sources of interest as the heterotopia ROIs. Preview the connectivity measure of correlation (rather than regression), and display using threshold values for correlation coefficients.
        1. For each subject, create seed-to-voxel connectivity maps utilizing each discrete region of heterotopic gray matter as a seed ROI, demonstrating the correlation between the average BOLD signal time series of the ROI and every other brain voxel.
        2. Perform second-level analyses for between-subject or between-source contrasts (optional). Display the results using height (voxel-level) and extent (cluster-level) thresholds; uncorrected and false discovery rate-corrected p-values are shown.

5. Use MRICroN software to manually outline two targets of interest, a connected target and a non-connected target, for TMS, using the Pen tool[43]. Using the "Overlay" function superimpose the functional connectivity maps created above onto the structural images for each subject.
    1. Ensure that the target region is a region of cortex that has significant functional connectivity to the gray matter heterotopia as described above. Ensure that the non-connected target is a similar size region that does not demonstrate significant functional connectivity to any heterotopia ROI, and is located at least 2.5 cm away from the connected target on the cortical surface to minimize the risk of neighborhood stimulation effects during TMS.
    2. Choose targets such that the likelihood of large TMS-induced artifacts is small[51]. Specifically, avoid selecting targets in the lateral temporal or frontopolar regions, as these are likely to produce large muscle contraction and/or eye movement artifacts that can obscure the early TMS-EEG signal[51]. Save the outlined targets as new target ROIs.
6. Determine the MNI coordinates for each target ROI in each subject. Then use these coordinates to identify the equivalent two target sites in each subject's matched healthy control subject.

# 3. TMS-EEG Experimental Setup

1. Upload structural scans (typically high-resolution T1-weighted 3D volumetric images) into the neuronavigation system.
2. Using the neuronavigation software, mark the desired targets on the images. Also mark external anatomic markers (the nasion, bilateral tragus) that will be used for coregistration and neuronavigation during the stimulation session. If using an EEG cap with rotatable electrodes and electrode wires, orient wires perpendicular to the long axis of the TMS coil[52].
3. Contact the subject prior to the experimental session to remind him or her not to use conditioners or other hair products (shampoo is acceptable) on the day of the TMS-EEG session, to avoid alcoholic drinks the evening prior to the TMS-EEG session, and to drink his or her usual daily caffeine consumption prior to the TMS session.

# 4. Experimental Session

1. Confirm that the subject passes TMS safety criteria, ideally via a structured questionnaire[53]. Confirm that the subject did not consume alcoholic beverages the prior night, did not drink significantly more or less than his or her usual daily caffeine consumption, did not consume over-the-counter sleep aids that alter cortical excitability (such as diphenhydramine) the prior night, and received a typical night's sleep (as sleep deprivation can increase cortical excitability[54]).

2. Ask the subject to sit in a comfortable chair.
3. Mount the EEG cap on the subject and prepare the electrodes.
    1. Measure the subject's head and select an EEG cap of appropriate size to help enable low electrode impedances.
    2. Thoroughly clean the skin underneath each electrode using a cotton-tip applicator and alcohol.
    3. Add conductive gel to each electrode. Do not add too much gel that it leaks between electrodes, as that may create a bridge and lead to common signal between different electrodes.
    4. If necessary, to ensure good contact between the scalp, the gel and the electrode, try pressing down on each electrode after adding the gel. To minimize charging artifacts, ensure that the gel does not spread outside the electrode holder. Homogeneously reduced the conductance levels to minimize recording artifacts.
    5. Place the reference and ground electrodes as far from the stimulation coil as possible to minimize the possibility of TMS-induced electrode artifact contaminating the entire recording. It is preferable to place these electrodes above bony structures, in presumably "inactive" zones with minimal cortical activity.
       NOTE: Even in studies for which the target locations are variable, frontopolar regions are unlikely to be selected as targets because TMS to these regions can result in large eye movements, contraction of the frontalis and facial muscles[51], and, frequently, scalp pain and headache; consequently, the TMS-EEG signal during stimulation of these regions is often obscured by large artifacts.
    6. Since these regions are thus unlikely to be chosen as targets for stimulation, use the forehead for placement of the reference and ground electrodes. Place them within a few centimeters of each other to minimize common mode noise.
       NOTE: In situations where all the stimulation targets are in one hemisphere, the contralateral mastoid would be another option.
    7. Check electrode impedances as follows; plug the EEG output cables into the "impedance" jack of the EEG recording system, then press the "measure impedances" button on the EEG system. Ensure that the electrode impedance is not greater than 5 kΩ.

4. Prepare the EMG electrodes on the contralateral hand (use first dorsal interosseous or abductor pollicis brevis muscles; utilize the same muscle across subjects in a single study).
5. Give the subject earplugs to minimize risk of hearing loss and tinnitus.
   NOTE: Another option would be to utilize earphones playing white noise or colored noise (with spectral features matching those of the TMS click) throughout the recording process, at a volume sufficient to mask the auditory click produced by TMS; this would have the added benefit of minimizing the potential confound of TMS-induced auditory evoked potentials[10,55]. Of note, a thin layer of foam between the coil and scalp is also necessary to minimizing the auditory evoked potential.
6. Place the infrared detectors on the subject's head, ensuring that the detectors are placed in a way to minimize risk of movement during the experimental session.
7. Coregister the subject's head with the MRI images by identifying the location of the pre-selected external anatomic fiducial markers (section 3.2) on the subject using the pointer that is included with the neuronavigation equipment.
8. Familiarize the subject with stimulation by applying a pulse elsewhere (*e.g.*, the subject's arm), or by applying a low-intensity stimulation pulse (*e.g.*, 5% max stimulator output) to the scalp.
9. Determine the resting motor threshold (the minimum intensity that produces a motor-evoked potential at least 50 µV in size on 5/10 trials). One such method, the relative frequency method[56], is as follows.
    1. Determine the location of the subject's motor cortex on the hemisphere ipsilateral to the fMRI connectivity-based targets. When using neuronavigation, this is generally in the region of the "Omega" in the precentral gyrus. Angle the coil perpendicular to the gyrus, with the handle pointing occipitally.
    2. Begin stimulation at an intensity that is expected to be subthreshold (*e.g.*, 35% maximum stimulator output).
    3. Increase stimulation intensity in steps of 5% max stimulator output until TMS consistently evokes MEPs with amplitudes > 50 µV in each trial.
    4. Then decrease stimulation intensity in steps of 1% maximum stimulator output until less than 5 positive responses out of 10 are recorded.
       NOTE: This stimulation intensity plus 1 is defined as motor threshold. Alternatively, use adaptive threshold techniques[57] to identify motor threshold with fewer stimuli.

10. For stimulation of the target areas, set the TMS intensity to the desired value (*e.g.*, 120% resting motor threshold).
    NOTE: However, in cases where there are significant regional variations in scalp-cortex distance (*e.g.*, in patients with frontal lobe atrophy), such a technique may result in subthreshold stimulation. Alternatively, with appropriate neuronavigation systems capable of performing online estimations of the induced electric field, the intensity of the stimulation can also be set at a specific amplitude of the calculated induced electric field (in V/m) on the cortical surface[58].
11. Apply single pulses of TMS to each of the target regions using the neuronavigation software, with a variable interval between pulses to minimize cortical plasticity and subject expectancy effects (*e.g.*, every 4 - 6 sec, with an interval of at least 3 sec to avoid cumulative effects[59]). To maximize consistency, angle the coil perpendicularly to the long axis of the underlying gyrus, with the handle pointed posterolaterally.

# 5. EEG Data Pre-processing and Analysis

NOTE: TMS-EEG data usually contains large stimulation-related artifacts, particularly when stimulating away from the midline/vertex or with high stimulation intensities, and significant preprocessing may be necessary to obtain clean analyzable data. Independent Component Analysis (ICA) is one method that has been utilized for removal of TMS artifacts, and can be applied using publicly available toolboxes (*e.g.*, EEGLAB[45]) on the MATLAB platform. One validated approach[60] is as follows, describing the analysis of data collected using the Eximia EEG system:

1. Import the data into EEGLAB
    1. Click on "File", "Import data", "Using EEGLAB functions and plugins", "From EDF / EDF+ / GDF files (BIOSIG toolbox)".

2. Extract event times

1. Click "File", "Import event info", "From data channel". Fill in "Event channel" 1, "Preprocessing transform (data = 'X')" X > 0.1, "Transition length (1 = perfect edges) 0. Make sure "Delete event channel(s)?" and "Delete old events, if any?" checkboxes are checked.

3. Segment the data into epochs centered around the TMS pulse, from 1 sec before the pulse to 2 sec after. To do this, select "Tools", "Extract Epochs". If the TMS pulse is the only event type, "Time-locking event type(s)" field can be left blank. For "Epoch limits [start, end] in seconds" enter [-1 2].

4. Review EEG data visually (select "Plot", "Channel data (scroll)".) Remove bad channels (*e.g.,* channels with no signal, or with continuous excessive artifact). To do this, click "Edit", "Select data". In the "Channel range" field, enter the number(s) of the channel to be deleted (or click on the toggle box to the right and select the channels by name, then press "OK"), make sure the "on->remove these" checkbox is checked, and then press "Ok".

5. Set the potentials in all electrodes to zero from the time of the pulse until the EEG signal has returned to approximately one order of magnitude of the neural signal (*e.g.,* by cutting out data larger than 150 μV), or any later fixed time point (*e.g.*, 40 msec) to ensure that the large TMS artifacts do not distort the ICA separation.[61] This step will need to scripted in Matlab.

6. Perform an initial round of ICA, and remove the 1 - 2 components representing the large TMS-induced initial muscle activation.
   1. Run ICA using the FastICA method with the "symmetric approach" and the "tanh" contrast function using the following command line: "EEG = pop_runica(EEG,'icatype','fastica', 'approach', 'symm', 'g', 'tanh');".
   NOTE: Run ICA separately for each site, as the artifact produced by stimulation will vary as a function of stimulation site.
   2. Identify components consistent with TMS artifact by selecting "Tools", "Reject data using ICA", "Remove components by map". The topographic maps of all the ICA components will then be plotted. Click on the number for each component to plot component details (a larger map of the topographic distribution, the activity profile across trials, and the frequency spectrum).
   NOTE: The TMS pulse artifact components (typically 1 - 2) can be recognized by the dipolar topographic plot localized to the site of stimulation, the extremely large amplitude of the component activation immediately after the pulse, and the subsequent smooth exponential decay.
   3. Delete the artifactual components by selecting "Tools", "Remove components", and entering the relevant component numbers in the field for "Component(s) to remove from data". In the "Confirmation" box that pops up, press "Accept" after reviewing the ERPs that result after deletion of the selected component (press "Plot ERPs") and after reviewing single-trial effects (press "Plot single trials").
   NOTE: This step should be completed **prior** to filtering to minimize any filter artifacts from the TMS-induced muscle artifact, which can often be several millivolts.

7. Interpolate the missing data (during the zero-padded time period). This step will need to be done using a Matlab script.

8. Bandpass and/or notch filter the data (optional; or could be done at a later time point, *e.g.,* after second round of ICA artifact removal).
   NOTE: If the high-amplitude TMS-artifact has not been adequately removed, the temporal smoothing effect of a high-pass filter can lead to temporal dispersion of artifact. Furthermore, the passband rippling produced by low-pass filters can lead to prominent ringing artifact in the "clean" portion of the resulting filtered EEG signal.

9. Re-reference to Average reference (optional, or could be done at a later time point, *e.g.,* after interpolation of missing channels).

10. Remove individual epochs with large-amplitude artifacts, significant muscle activity, or other major artifacts.
    1. For semi-automated artifact rejection, select "Tools", "Reject data epochs", "Reject data (all methods)".
    2. Under "Find improbable data" enter 3.5 in the field for "Single-channel limit (std. dev.) and 3 in the field for "All channels limit (std. dev.)", then press the "Calculate" button immediately below. This identifies epochs that contain improbable data based on the distribution of values across data epochs.
    3. Under "Find abnormal distributions", enter 5 in the field for "Single-channel limit (std. dev.)" and 3 in the field for "All channels limit (std. dev.)", then press the "Calculate" button immediately below. This identifies epochs as containing artifacts based on the kurtosis of the data.
    4. To reject epochs with abnormally high or low values, under "Find abnormal values", enter 100 in the field for "Upper limit(s) (uV)" and -100 in the field for "Lower limit(s) (uV)" (although different limits may be needed in children, in whom EEG amplitudes are usually higher). Enter electrode numbers to apply voltage thresholding in the field marked "Electrode(s)"; to avoid rejecting all epochs with eye blinks, do not include frontopolar (and/or EOG) channels. Then press "Calc / Plot.".
    5. Scroll through marked epochs, and unmark epochs that do NOT contain artifacts by right-clicking on the epoch. Mark additional epochs that contain significant artifacts by left-clicking on the epoch. After confirming that all epochs containing artifacts are marked, click "UPDATE MARKS" button.
    6. To save which epochs are marked for deletion, click "Close (keep marks)" and then save dataset ("File", "Save current dataset as").
       1. To then delete the relevant epochs, select "Tools", "Reject data epochs", "Reject marked epochs". Click "Yes" on subsequent confirmation dialogue box. Save resulting dataset.

11. Perform a second round of ICA, and remove components corresponding to decay artifacts, blink artifacts, muscle artifacts, and electrode noise artifacts.
    NOTE: Removal of components consistent with auditory-evoked potentials may be considered, although these components may also contain neural evoked components directly related to the stimulation pulse (which also have peaks at similar time points). A better option that would minimize TMS-evoked potentials induced by the TMS "click", and thus eliminate the need for ICA-based removal, would be to perform noise masking as described in section 4.5 above, if tolerable by the subjects.
    1. Run ICA using the FastICA method with the "symmetric approach" and the "tanh" contrast function, as described in 5.6.1 above.
    2. Evaluate component properties as described in 5.6.2 above.
    3. Mark components consistent with residual TMS decay artifacts[62].
    NOTE: Identify this based on timing (maximal immediately after the pulse), morphology (a slow decay with overshoot, then slow recovery over tens to hundreds of milliseconds) and location (near the stimulation site). Also, ICA components can be organized in descending order of explained variance; as the TMS artifact is quite large, it is typically represented in the first components, and typically represent no more than 1 - 5 components.
    4. Using the ADJUST plugin[62] for EEGLAB, mark components consistent with blink artifacts.

NOTE: Identify this based on location (maximal frontopolar), timecourse (long periods with relatively minimal activity, followed by short periods of intense activation), spectra (high power at low frequencies) and morphology (triphasic).

5. Mark components consistent with muscle artifact[62].
NOTE: Identify this based on spectral features (significant power at beta frequencies and above), temporal distribution (very irregular / jagged), spatial distribution (maximal along the scalp periphery) and time-domain activity (spiky).

6. Mark components consistent with channel noise based on spatial distribution (isolated to 1 or 2 channels) and temporal distribution (often very peaky, high activity on few trials, or very slow large amplitude fluctuations) using the ADJUST plugin[62] for EEGLAB.

7. Remove marked components as in 5.6.4 above. Interpolate missing channels and perform subsequent analyses upon this data.
NOTE: Caution is required when interpolating channels. In particular, if a substantial proportion (*e.g.,* 10%) of channels or if adjacent channels are interpolated, the resulting dataset may be unreliable, particularly if the underlying brain activity has a high spatial frequency.

12. Load another dataset with all desired channels into EEGLAB. Then bring the dataset that you wish to perform the interpolation on to the foreground by selecting "Datasets", and then clicking on the relevant dataset.

13. Select "Tools", "Interpolate electrodes". In the resulting dataset, select "Use all channels from other dataset". For "Interpolation method", select "Spherical" and then press "Ok".

# 6. Assess for Evidence of Cortical Hyperexcitability

1. Calculate the global mean field potential (GMFP)[63] for each subject and stimulation site as a function of time, using the following equation:

$$GMFP(t) = \sqrt{\left[\sum_{i}^{K}\left(V_i(t) - V_{mean}(t)\right)^2\right]\Big/ K}$$

where $K$ is the number of electrodes, $V_i(t)$ is the voltage measured at electrode $i$ at time $t$, and $V_{mean}(t)$ is the mean voltage across electrodes at time $t$.

2. Segment the data into "early" time periods when TMS-evoked activity is normally present in healthy individuals (*e.g.,* 100 - 225 msec), and late time periods, when abnormal delayed activity may be seen in patients with epilepsy (*e.g.,* 225 - 700 msec). Calculate the area under the curve (AUC) of the GMFP (AUC-GMFP) during each time period.
NOTE: Since the absolute magnitude of the evoked response can vary widely between individuals because of factors independent of cortical physiology (*e.g.,* skull thickness, scalp-cortex distance, individual brain anatomy) that may nonetheless vary between groups (*e.g.,* because patients with epilepsy may be on antiepileptic medications), raw amplitudes are of limited utility in evaluating the TMS-evoked potentials. To isolate whether patients with epilepsy have abnormally increased TMS-evoked activity, normalize the magnitude of the AUC-GMFP during later time periods by the magnitude of the AUC-GMFP during the "early" time periods.

3. Compare the normalized AUC-GMFP for each epilepsy patient to that obtained by stimulation of the same region in that patient's matched healthy control. A larger value (ratio > 1) in the epilepsy patient indicates that the epilepsy patient has increased excitability.

# 7. Source Estimation of Evoked Electrical Activity

1. Reconstruct the cortical surface for the subject using the Freesurfer[64] package.
   1. Run command "Generate FreeSurfer Output". Run command "Generate Surfaces". Run command "Create Source Space". Import digitized electrode locations from the neuronavigation software and align electrodes using MNE Analyze software (MNE Version 2.7.0)[65,66]; if individual electrode locations are not available, data from a subject with a similar approximate head size may suffice.
   2. Run command "mne_analyze". Click on "File", "Load digitizer data" (.fif). Click on "File", "Load Surface". Select path to MRI Freesurfer Reconstruction data.
   3. Click on "View", "Show Viewer". Click on "Adjust", "Coordinate Alignment". Click on "LAP". Click on LAP location in "Viewer" window. Repeat for "Nasion" and "RAP".
   4. Click on "Align Using Fiducials". Click on "X","Y","Z" field arrows to manually adjust coordinate alignment. Click on "Save Default" in "Coordinate Alignment" window to save -trans file.

2. Determine the forward solution using an appropriate method (*e.g.,* boundary-element modeling as implemented in the MNE[65,66] software). To do this, run command "MNE Do Forward Solution".

3. Identify the time points of the peaks in the GMFP for source analysis. To do this, run command "MNE_Browse_Raw" for .fif file.
   1. Click on "Adjust", Filter" to make filter changes. Click on "Adjust", "Scales" to make scale changes. Click on "Adjust", "Selection" to change montage selection.
   2. Click on time point in raw voltage data. Click on "Windows", "Show Annotator". Click on "mark" to code selected time point with corresponding number and comment. Overwrite comment field when applicable.
   3. In average field, enter annotation number. Click on "Average". Click on "Windows", "Manage Averages". Click on "Save As" and name .fif file.

4. Using the mean (across trials) evoked potential at the relevant time points, calculate the current source solution using an appropriate inverse operator (*e.g.,* minimum norm estimation as implemented in the MNE software). To do this, run command "MNE Inverse Operator".

5. Apply a voltage threshold to the resulting images to identify the source region of the evoked peaks.
   1. Click on "Windows", "Start MNE Analyze". Click on "File", "Open". Select time-point average .fif file in "Files" field. Select inverse .fif file in "Inverse Operator" field.
   2. Click on "File", "Load Surface". Select path to MRI Reconstruction data. Select "Pial" in "Available Surfaces" field.
   3. Click on "Adjust", Estimates" in "MNE Analyze" window. To adjust scale, left click in "Value Histogram" field to select threshold value distribution. Click histogram to adjust colormap thresholds.

4. Click on "img" in "MNE Analyze" field. Select ".tif", "Save".

## Representative Results

Resting-state functional connectivity fMRI can be used to identify regions of cortex that demonstrate high functional connectivity with the heterotopic periventricular gray matter nodules (**Figure 1**), and control regions without such connectivity. To determine whether such abnormal functional connectivity has physiologic significance, the cortical region with correlated resting-state activity can be chosen as the "connected" target sites for neuronavigated TMS, and the evoked EEG results compared to the EEG potentials produced by stimulation of a control non-connected target in the same patients. Furthermore, the same regions can be targeted in healthy control subjects (**Figure 2**) to determine whether the abnormal functional connectivity seen in the PNH patients has pathophysiologic significance for the patients' clinical epilepsy syndrome. Specifically, the presence of cortical hyperexcitability can be assessed on the individual patient level by determining the normalized area-under-the-curve of the Global Mean Field Potential, and then evaluating whether this value is larger for the epilepsy patient than his or her matched control (**Figure 2**). Source localization of the abnormal late peaks in the TMS-evoked potentials in patients with epilepsy can identify the brain regions from which the abnormal activity arises, and may spatially co-localize with the patient's seizure focus (**Figure 3**).
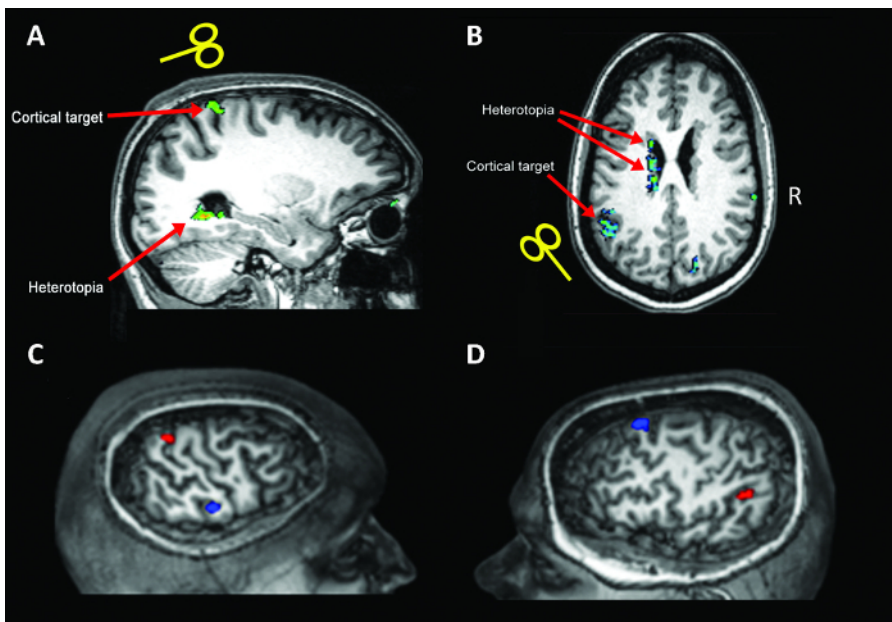


**Figure 1. Resting-state Functional Connectivity and TMS Targets. (A, B)** Regions with significant correlations in functional activation (blue/green) to the resting-state BOLD signal in the heterotopic nodules in two patients with periventricular nodular heterotopia and epilepsy. **(C, D)** The connected target site (red) and the non-connected target site (blue) in these two patients. (Modified with permission from Shafi *et al.*, 2015[37]). Please click here to view a larger version of this figure.
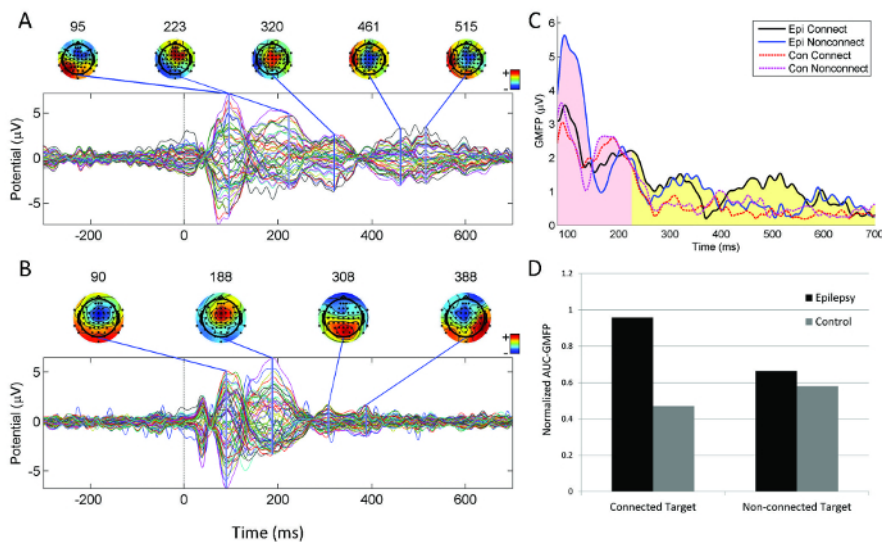
**Figure 2. TMS-evoked Potentials and Global Mean Field Potentials. (A)** The TMS-evoked potential produced by stimulation of the connected target in a patient with PNH and epilepsy. **(B)** The TMS-evoked potential produced by stimulation of the same region in the above patient's matched healthy control subject. **(C)** The global mean field potential (GMFP) produced by stimulation of the connected and non-connected targets for this patient and his matched control. **(D)** The normalized area-under-the-curve of the GMFP produced by stimulation of the connected and non-connected targets for this subject pair. Please click here to view a larger version of this figure.
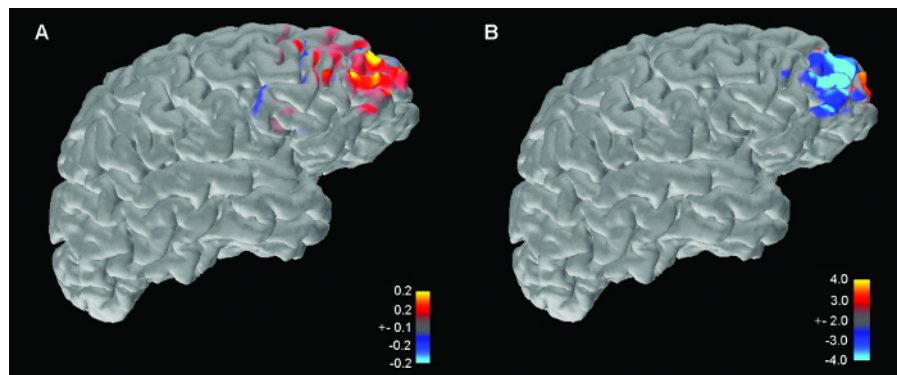


**Figure 3. Source Localization of TMS-evoked Activity and Seizure Onsets. (A)** Electrical source imaging results of a late TMS-evoked peak in a patient with epilepsy; scale is the estimated currents multiplied by $10^{-11}$. **(B)** Electrical source imaging results of a previously captured seizure onset in that same patient. (Modified with permission from Shafi et al, 2015[37]) Please click here to view a larger version of this figure.

## Discussion

Resting-state functional connectivity MRI has been used to identify network connectivity in the human brain, and to identify alterations of connectivity that occur in different disease states[26,31,32]. However, as fMRI functional connectivity is based on identifying correlations in the BOLD signal, and as blood oxygenation changes have a non-trivial relationship with underlying neural activity, the causal significance and physiological relevance of these fMRI connectivity findings is unclear. TMS enables spatially and temporally targeted manipulations of brain activity in specific cortical regions; when combined with EEG, TMS can be used to assess the brain's response to stimulation across different brain regions. Consequently, TMS-EEG can be applied to regions with altered fMRI functional connectivity to assess whether the observed alterations in connectivity have a physiologic correlate that might relate to the underlying disease pathophysiology.

This article presents a protocol using connectivity-guided TMS-EEG to assess cortical excitability in patients with epilepsy due to a malformation of brain development, periventricular nodular heterotopia, which is associated with the development of abnormal functional connectivity networks[37]. This protocol is used to demonstrate that patients with active epilepsy have cortical hyperexcitability that is specific to the regions with altered resting-state fMRI functional connectivity, and that hyperexcitability can be assessed on an individual subject level. In a patient with seizures previously captured on EEG, the abnormal late TMS-evoked activity is seen in the same region (distant from the stimulation site) from which the patient's seizures originate, suggesting that the region of abnormal functional connectivity is indeed part of the seizure-generating network.

There are a number of critical steps to successful completion of this protocol. Technical expertise with resting-state fMRI data collection, high-quality resting-state data, and experience with rs-fcMRI data processing and analysis techniques are essential for accurate determination of connectivity-based targets. Another important constraint in designing and executing TMS-EEG studies is the need for TMS-compatible EEG equipment; furthermore, for studies where precise targeting is critical, neuronavigation equipment is also necessary. Another limitation is that

TMS often generates substantial EEG artifacts, particularly when stimulating over the frontopolar and lateral temporal regions, and therefore it may be difficult to obtain high-quality data when the stimulation target is located in these regions. The data collection and EEG recording process also needs to be optimized to minimize artifacts in the EEG signal, and experiments should ideally be run by persons familiar with EEG data so that artifacts that do arise (*e.g.*, poor impedance as conducting paste dries) can be rapidly identified and minimized. One important step involves demonstrating the effects of eye blinks, muscle contraction and movement on the EEG to the research subject, as this can be critical in helping the subject to understand and minimize these types of artifacts.

Another important consideration is the minimization of biological artifacts that may limit interpretation of the results. One particularly important such biological artifact is the auditory evoked-potential produced by the TMS coil "click", which is known to contribute to the magnitude of the TMS-evoked potential, particularly at 100 and 180 milliseconds[55,67,68] when the TMS-evoked potential is also typically maximal. One method that has been shown to minimize the TMS auditory evoked potential is noise masking via the use of white or colored noise, with the addition of a thin layer of foam between the coil and scalp[10,55]. In the absence of such noise masking, differences in the TMS auditory-evoked potential could potentially contribute to differences in the evoked activity between subject groups (although not the observed differences in the evoked potentials between different sites within subjects.)

Finally, even if care is taken to optimize the recording, significant preprocessing is often necessary to recover clean data for analysis. Fortunately, validated methods for removing artifacts from TMS-EEG recordings have been published[60]; however, even with these techniques, recovery of very early signals (< 15 msec) can be very difficult or unreliable. An additional challenge is that EEG data is high-dimensional and complex, and therefore a clear prior hypothesis is often necessary to extract meaningful information. Furthermore, because TMS effects and EEG signals can vary significantly between subjects because of a wide range of non-cerebral factors that are difficult or impossible to control (*e.g.*, skull thickness, skull-cortex distance, concomitant medications, quality of sleep the night prior), outcome measures that are less reliant on the raw magnitude of evoked responses are likely to be more informative or meaningful.

Although technically challenging, the integration of rs-fcMRI, TMS and EEG together in one experiment enables tests of a wide array of hypotheses regarding the significance of specific connectivity findings on cortical physiology. In disease states, these technologies can be integrated together to assess the relationship between fMRI network connectivity changes, pathophysiologic alterations in cortical excitability and evoked brain activity, and disease expression. Notably, this protocol can be used to investigate cortical physiology via common outcome measures even when the focus of abnormal connectivity (and thus the stimulated region) differs from one subject to another, providing an output that may be meaningful at the individual subject level, and opening up the possibility of a personalized approach to investigation and ultimately treatment.

The protocol described in this study could also be expanded to assess specific features of cortical physiology in different subject groups. For example, a number of recent studies have suggested that the N45 component of the TMS-evoked EEG response represents activity of GABA-A receptors[69], whereas the N100 component of the TMS-evoked EEG response is a measure of GABA-B mediated inhibition[21,69]. Paired-pulse TMS-EEG with a long-interval intracortical inhibition protocol provides another measure of GABAergic activity, and has been shown to be altered in frontal regions in patients with schizophrenia relative to controls[70]. Thus, the above protocol could be modified to specifically address questions regarding GABAergic activity in regions with altered functional connectivity. Additionally, source localization of the peaks in the TMS-evoked potential may identify distant regions that are engaged by stimulation, and thus help inform which of the functional connections identified by conventional resting-state fMRI are capable of causally transmitting evoked activity. For situations in which the key network hubs are deep, rs-fcMRI can also be used to identify cortical targets that are accessible to stimulation, and thereby enable modulation of specific brain networks involved in normal behavior and in disease states[35,36,71]. In such cases, the techniques described in this study can be used to assess local and distributed single-pulse TMS-evoked activity before and after a repetitive plasticity protocol, to determine whether the plasticity protocol has indeed changed cortical excitability locally, and/or network excitability distally.

In summary, the integration of rs-fcMRI, TMS and EEG enables the exploration of how brain connectivity influences cortical physiology and behavior in human subjects. Moreover, these techniques can also be combined to assess how alterations in connectivity are related to pathophysiology in disease states, as illustrated in the protocol described above.

## Disclosures

## Acknowledgements

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