Title: Investigation of Cortico-limbic Networks and Their Dynamics in Major Depressive Disorder

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I. BACKGROUND AND SIGNIFICANCE

The neurobiology of Major Depressive Disorder (MDD) remains unclear. Functional neuroimaging approaches such as Positron Emission Tomography (PET) and functional MRI (fMRI) have delineated abnormalities in resting metabolism and task-based activation in this condition.[1-3,23,25,26] Affected cortico-limbic regions include those involved in: emotional processing [e.g., amydala and nucleus accumbens (NA)], visceromotor control and homeostasis (e.g., insula and hypothalamus) and emotional regulation [e.g., orbitofrontal (OFC) and dorsomedial (dmPFC) prefrontal cortex]. Consistent changes are also seen in the subgenual cingulate (sgACC, BA25) and in the dorsolateral prefrontal cortex (DLPFC), wherein PET studies have revealed hypermetabolism and hypometabolism[1-4], respectively.

The observation that dysfunctions are present in distributed cortico-limbic regions suggests that MDD symptoms might evolve from aberrations in distributed processing *between* the regions in question, in other words, at the level of neural networks. Building on this possibility, the use of network measures may provide valuable endophenotypes of psychiatric diseases such as MDD.[13] Consequently, there has been increasing interest in characterizing the functional architecture of neural networks in depression[1-7], especially those encompassing prefrontal and subcortical regions heavily implicated in its pathogenesis.

Functional connectivity MRI (fcMRI) relies upon correlations between spontaneous low frequency fluctuations in the blood oxygenation level-dependent (BOLD) signal to delineate intrinsic brain networks in the absence of explicit task demands.[27-29] fcMRI studies in normals have revealed topographically consistent patterns of functional connectivity among the aforementioned cortico-limbic regions. Using seed based analytic approaches, sgACC has been shown to be functionally correlated to several limbic regions including the OFC, amygdala and NA .[9,21,22,30] This functional connectivity strongly recapitulates structural projections to and from the sgACC in animal and human studies.[31-33] In addition, sgACC shares functional connectivity with portions of the default network (DN), specifically the dmPFC[9], the temporal pole and the posterior cingulate/precuneus (pCC).[21,22] Relevant to this study, sgACC is functionally correlated to a medial portion of the DLPFC (superior frontal gyrus, a part of the DN), but is anticorrelated to another portion of DLPFC (middle frontal gyrus).[21,22] These correlation patterns have been borne out within our own data (**Fig 1**).

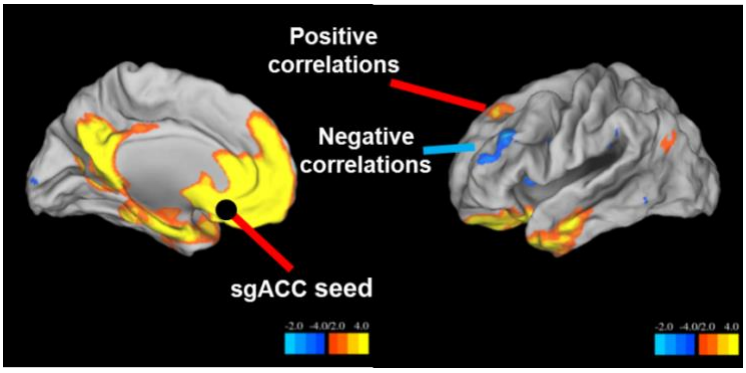

Fig 1. Voxel-wise functional connectivity maps derived from seeding the sgACC in 26 healthy subjects in our lab. In DLPFC, both positive (red/yellow) and negative (blue) correlations are observed.

In MDD, evidence of aberrant network activity was first described in the form of reduced deactivations in the DN during task execution.[34-35] With regards to functional connectivity, studies suggest that the sgACC and dmPFC[9] are excessively coupled to their distributed cortico-limbic partners. Resting-state fMRI data have also elaborated abnormal network metrics in MDD, including increased regional homogeneity, increased nodal centrality and connectivity degree, and decreased small-worldness.[36,37] Notably, these metrics were identified in similar cortico-limbic and default regions, and they correlated with disease severity.

Greicius and colleagues[8] employed Independent Component Analysis (ICA) to demonstrate that DN functional connectivity was significantly increased in the sgACC, medial thalamus and OFC in MDD patients, and that increased sgACC connectivity correlated with duration of depressive episodes. Two other fcMRI studies (one using seed-based fcMRI, and another using ICA) correlated rumination scores in MDD patients to increased sgACC functional connectivity.[10,11] In addition, Sheline et al. used seed-based approaches to demonstrate heightened connectivity between sgACC and dmPFC in MDD patients.[9]

However, despite efforts to capture local abnormalities with PET, and network abnormalities with fcMRI, we currently lack integrative models placing these cortico-limbic nodes into dynamic systems (i.e., as they are likely to exist in the *in vivo* brain). The notion of "network dynamics" is supported by novel computational modeling approaches[14,38] which have uncovered temporal changes in functional connectivity over the course of fcMRI scanning sessions.[39-40] Taken in this light, complex interplays between local nodal processing and functional couplings between network nodes are constantly at work. In this way, connectivity between network nodes is conceived as being in a "dynamic equilibrium," in which small conformational departures occur rapidly and continuously, but in which steady states of network connectivity are realized over long time scales.[12,13] In MDD, the threshold for depressive symptoms might be lowered when this network equilibrium is shifted- causing abnormally high processing through limbic regions. Alternatively, mechanisms which normally compensate for these shifts might be aberrant in MDD. Within this framework, one might expect that interventions which restore such dynamic inequities might offer therapeutic promise.

One such intervention is repetitive transcranial magnetic stimulation (rTMS). rTMS delivered to DLPFC is an established treatment for MDD, with its clinical efficacy supported by large randomized, sham controlled trials[15,16], and several meta-analyses.[41-43] Also, the Neurostar rTMS treatment (Neuronetics®) has been FDA approved for certain forms of medication-refractory depression. Broadly, the effects of rTMS are known to propagate, via trans-synaptic means, to distal but interconnected regions with spatial specificity.[44,45] This suggests that it exerts its anti-depressant effects through re-modeling of cortico-limbic networks. That rTMS to DLPFC impacts medial limbic structures is also supported by $H_2{}^{15}O$ PET studies in normals showing that rTMS to DLPFC changes regional cerebral blood flow (rCBF) in ipsilateral anterior cingulate cortex and in the sgACC.[46,47] Also, another protocol used PET to demonstrate that DLPFC stimulation modulates neurotransmitter function in these regions.[48] Nonetheless, in spite of its efficacy in MDD, and despite evidence that its effects are mediated through trans-synaptic influences on limbic structures, the effects of rTMS to DLPFC upon cortico-limbic functional connectivity have yet to be characterized.

Preliminary data: My prior work reveals that rTMS combined with fcMRI offers the ability to probe the dynamics of cortico-limbic networks. In Eldaief et al.[14] we studied healthy young participants who underwent neuronavigated rTMS to the left posterior inferior parietal lobule (lpIPL) node of their DN. First, subjects had a baseline resting-state fcMRI scan to individually identify an lpIPL target for rTMS, based upon their own fcMRI data. After this baseline, subjects returned for two additional sessions during which they had resting state fcMRIs before, and after, rTMS to lpIPL. In one session rTMS was delivered with low frequency (1 Hz) stimulation, and in another with high frequency (20 Hz) stimulation. Stimulation parameters (e.g. number of pulses and stimulation intensity) were identical across sessions and stimulation durations were comparable. We used a stereotactic rTMS neuronavigation system to ensure that the same lpIPL target was reliably stimulated during both sessions. After 20Hz rTMS to lpIPL, functional correlations decreased between lpIPL and other cortical regions of the DN (e.g. medial prefrontal cortex, mPFC, and pCC). 20 Hz rTMS did not alter functional connectivity between these regions and the hippocampus (HF). However, 1Hz rTMS increased functional connectivity between pIPL and HF, but not between lpIPL and other cortical DN nodes (**Fig 2**). This study demonstrates that different rTMS regimens to the same DN node elaborated two different functional network configurations.

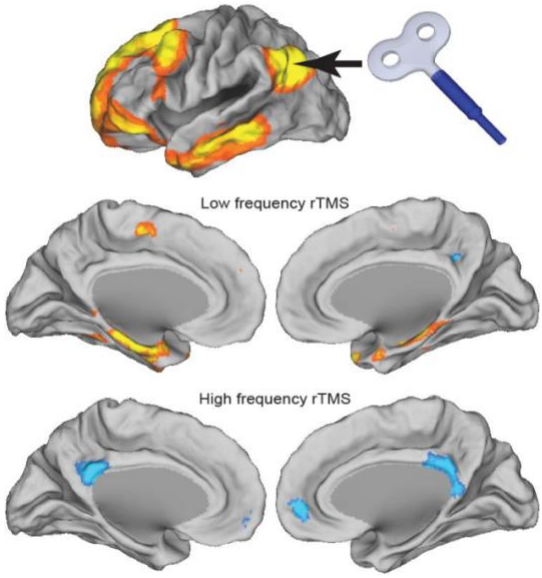

Fig 2. Functional connectivity maps derived from an lpIPL seed location at baseline (top). Voxelwise paired t-test maps representing **changes** in functional connectivity correlations are also shown after low (middle) and high (bottom) frequency rTMS. Connectivity decreases are shown in blue and increases in red/yellow.

More broadly, this study established the safety and feasibility of modulating intrinsic network activity with non-invasive brain stimulation. Dr. Pascual-Leone's (advisor) group has corroborated this by recently showing that active transcranial direct current stimulation (tDCS) to DLPFC decreases DN synchrony, but increases connectivity in an anticorrelated network.[49] Other research groups have published consistent results[50]: Keeser et al. showed that anodal stimulation of DLPFC with tDCS modulated connectivity in the DN[51]; Alon et al. found that excitatory stimulation of M1 with tDCS decreased intrinsic connectivity between bilateral M1.[52]

Nevertheless, further exploration of intrinsic network dynamics using rTMS and fcMRI would benefit from a greater understanding of the physiologic effects of rTMS upon the directly targeted brain region. In our preliminary study, connectivity changes may have been predominantly determined by stimulation dependent changes in excitability at lpIPL. While prior studies suggest that high and low frequency rTMS exert opposing effects on cortical excitability[53-55], we could not verify the magnitude, directionality or diffusivity of the stimulation at lpIPL. Another consideration is that tonic activity at lpIPL or at distal network nodes, might constrain the extent to which connectivity is modulated with rTMS.[56] This is especially germane to MDD, given the presence of baseline metabolic derangements at DLPFC and sgACC. An ideal way to circumvent this shortcoming is to simultaneously accrue information about changes in local nodal activity *and* changes in network connectivity.

This study will take advantage of a novel combined MRI-PET scanner[19,20] (the MRI-BrainPET camera) to *simultaneously* record BOLD fcMRI and ^{18}Flurodeoxyglucose (FDG)-PET data following rTMS to DLPFC, in both depressed patients and in healthy controls. We will specifically target regions of the DLPFC which share functional associations with the sgACC. The sgACC was chosen as the main limbic region of interest because of mounting evidence for the pivotal and integrative role it plays in MDD. For instance, sgACC activity is enhanced during acute sadness[57] and reduced after anti-depressant regimens.[58,59] Also, the sgACC is the target of deep brain stimulation (DBS) in treatment refractory MDD.[33,60] Network dynamics in MDD will be assessed by comparing identical stimulation regimens delivered to regions correlated, and anticorrelated, with sgACC, (analogous to how we previously used two different rTMS regimens to lpIPL[14]). Motivation for inclusion of the correlated region is self-evident based on our prior study.[14] Motivation for stimulating the anticorrelated region is twofold. First, sgACC anticorrelations are rather prominent in DLPFC.[21,22] Second, these tend to be more lateral, and thus anatomically more closely approximate the clinical stimulation target.[17,61,62] Primary outcomes of interest will be rTMS induced changes in regional cerebral metabolic rate of glucose uptake (rCMRglu) at the DLPFC stimulation sites and in sgACC; as well as rTMS induced changes in functional connectivity between sgACC-DLPFC. Secondary outcomes will involve rTMS induced connectivity changes between sgACC and three additional cortico-limbic nodes: dmPFC[9], NA[1-5] and amygdala.[30] Network dynamics will be assessed through observation of the interplay between local metabolic and network changes. While extant data[63] might be predictive of these dynamics (**Fig 3**), we expect that these will be revealed empirically.

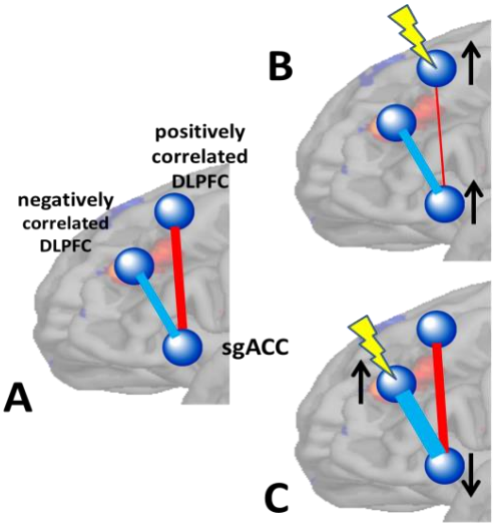

Fig 3. Schematic representation of how local and distributed changes induced by rTMS to DLPFC ***might*** interact. (A) No rTMS, (B) 20Hz rTMS to DLPFC positively correlated to sgACC and (C) 20Hz rTMS to DLPFC anticorrelated to sgACC. In this model, 20Hz rTMS increases rCMRglu at both DLPFC sites (up arrows)[53-55]. In B, this might decrease connectivity between DLPFC and sgACC (thinned red line) and increase rCMRglu in sgACC. In C, rTMS decreases rCMRglu in sgACC and increases anticorrelation between these regions (thickened blue line).

II. SPECIFIC AIMS

Aim 1: To assess rTMS induced changes in functional connectivity in cortico-limbic networks
Hypothesis 1.1: At baseline, MDD patients will have abnormally increased cortico-limbic connectivity.[8-11]
Hypothesis 1.2: rTMS to DLPFC will differentially modulate this connectivity, depending on whether positively or negatively correlated regions are targeted; and one of these targets will more robustly alter this connectivity.
Hypothesis 1.3: Connectivity changes will be more pronounced in MDD patients than in controls.
Aim 2: To assess cortico-limbic network dynamics in MDD by comparing rTMS induced changes in local glucose metabolism to rTMS induced changes in distributed connectivity
Hypothesis 2.1: 20Hz will comparably increase FDG uptake at both DLPFC stimulation sites in both groups.
Hypothesis 2.2: Stimulation of the two sites will differentially impact FDG uptake at sgACC, and will have a differential impact upon the strength of connectivity between sgACC and the stimulation site.
Hypothesis 2.3: The direction and magnitude of FDG changes at sgACC will predict the direction and magnitude of the associated changes in cortico-limbic connectivity.
Hypothesis 2.4: Local and distributed changes will be altered in MDD, reflecting aberrant network dynamics.
Hypothesis 2.5: Stimulation of a control site in parietal cortex or the cerebellum will not appreciably alter cortico-limbic connectivity or metabolism.

This work will better elucidate how intrinsic networks and their dynamics are altered in MDD. It will also assess how rTMS, or other neuromodulatory strategies, create the formation of more adaptive configurations. In turn, this will pollinate future trials that optimize MDD treatments through selective targeting of intrinsic networks.

III. SUBJECT SELECTION

A. *MDD patients:* **25 patients** (ages 18-50) with unipolar MDD (single episode or recurrent) will participate. We will specifically recruit unmedicated patients. At enrollment, patients must be medication free for a period consistent with four half-lives of the drug in question (e.g. 4 weeks for fluoxetine). Diagnoses of MDD will be made by each patient's referring physician, or, in the case that there is not a referring physician, will be made by the PI (a board certified psychiatrist) with DSM-IVR criteria using the Structured Clinical Interview (SCID).[65] Patients will also be required to score ≥18[7] on the 24 item version of the Hamilton Depression Rating Scale (HDRS).[24] All psychometric assessments will be performed by a trained technician. Patients with any of the following issues will be excluded: bipolar disorder, schizoaffective disorder, active suicidal ideation, or any history of psychosis. Given the high co-morbidity, concurrent anxiety disorders will be allowed, provided MDD is the major diagnostic consideration.
B. *Healthy subjects*: **25 healthy controls** (ages 18-50) will also be recruited. These subjects will be matched to MDD patients on the basis of age, gender and ethnicity. Controls must score ≤8 on the HDRS. These subjects must not meet criteria for any Axis I disorder (confirmed by DSM-IV criteria and by the SCID questionnaire), have no formal history of these, and must not be on any psychotropic medications.
C. *Exclusion Criteria (All Subjects)*
   1. A history of serious concurrent medical or neurological illness, especially any patient with a known space-occupying lesion in the brain/skull.
   2. Concurrent substance use, abuse or dependence.
   3. Contraindications to receive MRI scanning including, but not limited to the presence of metallic or ferromagnetic implants (e.g. pacemaker, defibrillator, vagal nerve stimulator, implantable pump, metallic tattoos and/or make-up, surgical aneurysm clips, etc.); or severe claustrophobia.
   4. Contraindications to undergo PET scanning, e.g. a history of receiving radiation exposure with PET within the past 12 months.
   5. Contraindications to receiving TMS, specifically a history of seizures/epilepsy in the subject or in a first-degree relative or history of unexplained syncope.
   6. Any subject who is pregnant (as assessed by a STAT quantitative serum hCG test) or currently lactating.

7. Any subject who has known metallic or ferromagnetic bioimplants, or who has a history of seizures or a first degree relative with seizures, or who has a history of significant claustrophobia.

D. *Sample Size Determination:* Sample size calculations for the rTMS/fMRI studies are based on Eldaief et al, as we are unaware of other studies that provide empirical estimates of effect size. The main outcome measure for the TMS/fMRI experiments will be a change (pre vs. post-rTMS) in correlation values (*z*) between sgACC and DLPFC. Our study of 17 subjects yielded an effect size of 0.15 for 20Hz rTMS induced changes between lpIPL and mPFC (SD=0.11). Based on this, sample sizes of 8 and 12 would have enough power (90%) to detect changes similar to those observed in our prior study at a significance level of 5% using an unpaired t-test or a paired t-test, respectively. With respect to PET, a comparable FDG-PET study[66] found an average change in rCMRglu of 5.3% (SD=2.77%) following high frequency rTMS. Based on this, sample sizes of 5 and 7 would be required (power, 90%; α, 0.05; unpaired and paired t-tests, respectively). However, because these effect sizes were all calculated from healthy controls, they might represent underestimates of the effect sizes needed for MDD patients. Therefore, we will conservatively recruit 25 subjects for each subject group.

A. *Source of Subjects/Recruitment Methods:* Healthy participants will be recruited via bulletin board announcements (please see advertising materials) placed throughout the Brigham and Women's hospital, Massachusetts General Hospital, MGH/Martinos Center, Harvard University Countway Library and Harvard School of Public Health, which will be first approved through the PHRC. There will also be two online advertisements, one through the website Craigslist and another through the Boston University job site "Quickie Jobs" (www.bu.edu/seo/studentjobservice/quickie-jobs). MDD patients will be recruited through all of these sources via a separate advertisement (please see advertisement materials). We will target individuals who are not currently undergoing treatment for their current major depressive episode. Prospective depressed subjects will be given a contact number and email in order to allow them to seek out more information about the study. They will then undergo a brief PHRC approved telephone survey/email survey (see attachments) with the PI to establish their eligibility. If they are considered eligible, they will be invited to participate in the first study visit where they will be consented in written form prior to undergoing study procedures. Other recruitment sources will for MDD patients will include individuals presenting to Partners sites for evaluation and management of depressive symptoms. Specifically, this will be comprised of three sources:

1. The BWH outpatient psychiatry department. In this case recruitment will be accomplished through a separate recruitment letter to subjects' primary psychiatric providers (please see separate attachment). The recruitment letter will be cosigned by the patient's primary psychiatrist and by the PI. The letter will include an "opt out" in which subjects will be permitted to call to opt out of study participation and not be contacted.
2. Patients of Dr. Eldaief (PI) who present to the Division of Cognitive and Behavioral Neurology with symptoms of major depression. In order to minimize coercion of potential subjects who are patients of the PI, patients will be offered a copy of the Consent Form and be instructed to call back if they are interested in participating. In addition, the PI will strongly recommend that they discuss their potential study participation with other health care providers (e.g. their primary care physician and psychiatric provider).
3. Patients of Dr. Dougherty (co-investigator) who present to the MGH Depression Clinical Research Program (DCRP) with symptoms of major depression.

In the latter two cases, the PI will strongly emphasize that study participation is voluntary and that subjects' decisions to not participate will in no way affect their care with said investigator or their future care at any Partners institution.

Once identified as potentially eligible, subjects will undergo the aforementioned telephone screening/email assessments with the PI to further delineate their eligibility.

IV. SUBJECT ENROLLMENT

A. Subjects will be randomized as to the order of the TMS sessions they undergo (there are two types of sessions in which one of two sites on the prefrontal cortex is stimulated). The order of these sessions will be counterbalanced across subjects. In other words, we will alternate between the two types of sessions and will have equal numbers of subjects having undergone a particular order of sessions. This will allow us to account for order effects.

Informed consent will be obtained by the principal investigator during the screening visit. Participants will be told that this is an MRI/PET study combined with TMS stimulation. The interviewer will describe the MR/PET procedures in depth, the TMS instruments in depth, and any risks previously described. These descriptions will include: how long the participant is expected to remain inside the magnet, how long the TMS sessions will be, and minor risks associated with exposure to radiation from the FDG ligand. Subjects will also be informed about the small space within the magnet and noises made by switching gradients. Subjects will also be told that the MRI procedure is NOT diagnostic, and that any abnormalities detected will be referred to a consulting radiologist for review. They will be told that the radiation they receive from PET scanning is not necessary for their medical care and is for research purposes only. In addition, the participant will be told how many experimental visits he/she is expected to complete and the duration and timing of these visits will be explicitly conveyed. Subjects will read the consent form without any time constraints. The subject will also be offered to take the consent form home and think about participation. Any and all questions will be answered to the best of the investigator's abilities. Every effort will be made to come up with clear, factual answers to all questions. The subject will be asked to summarize the procedure and recount it to the interviewer as a robust assessment of the subject's understanding of it.

Because there are not well validated versions of the questionnaires employed (e.g. the SCID and HDRS scales) in other languages, subjects who do not speak English will be excluded from study participation.

The subject will also be informed that, at any point, he/she may choose to terminate the study for any reason and that he/she has the option not to participate in the study. This includes being able to terminate the study during the experimental procedures proper. They will be informed that their refusal to participate in the study, or their wish to terminate it at some point, will have no effect on care and treatment received by them at any Partners hospital including the Brigham and Women's hospital and the Massachusetts General Hospital or any of their affiliated institutions, now, or at any time in the future. Should the subject indicate that he/she wishes to participate in the study, written informed consent will be obtained. All participants will be provided with copies of the signed consent form.

V. STUDY PROCEDURES

A. rTMS, PET and fMRI procedures:

fcMRI and PET will be acquired using an integrated Siemens 3.0 T whole-body Siemens MRI scanner fitted with a 3D dedicated BrainPET camera housed at the Athinoula A. Martinos Center for Biomedical Imaging at the MGH Charlestown Navy Yard. This scanner is capable of the simultaneous acquisition of PET and MRI data (**Fig 4**).

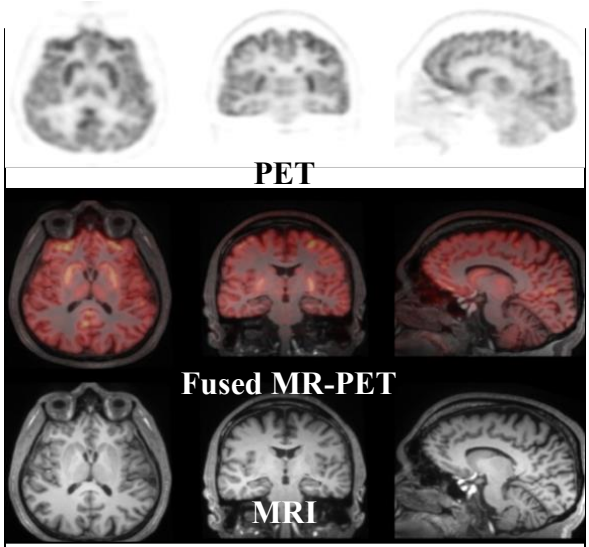

Fig 4: Simultaneous MR-PET data acquisition in a healthy subject using the MRI-BrainPET scanner. Axial, coronal and sagittal FDG-PET (upper), fused MR-PET (middle) and MR (lower) are shown.

fMRI procedures: Structural images will be acquired via a 3D-turbo field echo sequence which will generate high resolution 3D MPRAGE T1-weighted images. Functional images will be acquired using a T2*-weighted sequence. Functional data will be collected by using an asymmetric spin-echo, echo-planar sequence sensitive to BOLD contrast during three 6 minute functional runs while subjects rest and stare at a white fixation

dot. During this time, subjects will be instructed to lay as still as possible and "think about whatever they want."

PET procedures:
Subjects will be instructed to fast and not to consume beverages, except for water, for at least 4 hours before the administration of FDG to decrease physiologic glucose levels and to reduce serum insulin levels near basal levels. While fasting, subjects should consume at least two to three 8-12oz glasses of water to ensure adequate hydration.

Subjects will also be instructed to avoid strenuous exercise for 24 hours before the FDG-PET to minimize uptake of the radiotracer in the muscles. A fingerstick will assess the blood glucose level before FDG administration to ensure it is below the specified limit (i.e. 200 mg/dl)

An intravenous catheter will be placed and will be flushed post-injection of FDG with 0.9% saline solution. Participants will receive an intravenous bolus of 5mCi (~185 MBq) of ^{18}Flurodeoxyglucose (FDG) by a trained technician (specifically Shirley Hsu or Grae Arabasz, both study staff). Injection will occur coincidentally to the onset of rTMS. Dynamic scanning will begin approximately 30 minutes after this so that dynamic changes in rCMRglu can be modeled[67] during the anticipated peak effects of rTMS. Emission data will be acquired over a 45 minute period and stored in list-mode format. Data will first be sorted in the line-of-response (LOR) space and motion correction will be applied using MRI-derived motion estimates for each individual frame. Next, data will be rebinned in the sinogram space, generating prompt and random coincidences sinograms.

rTMS procedures: rTMS will be administered with a Magstim Rapid System, using a 70 mm figure-of-eight air-cooled coil. For allsessions, 20Hz stimulation will be delivered at 110% of resting motor threshold via 75 trains of 1.2 second 20Hz rTMS (i.e., 24 pulses per train), each of which will be followed by inter-train pauses of 16.8 seconds for a total of 1800 pulses (22.5 min), for a total of 1800 pulses (22.5 min). These parameters are within recommended safety limits for rTMS[68], and replicate parameters used in Eldaief et al.[14] Similar rTMS durations have been shown in other paradigms to outlast the stimulation period by anywhere from 20 minutes[53,54] to up to 1 hour.[69] During rTMS, accurate targeting of DLPFC, or the control site, will be achieved with a frameless stereotactic optical tracking neuronavigation system (Brainsight™), which will permit real-time monitoring of coil placement and head position.[70]

B. Eligibility screening and baseline questionnaires:
Study procedures will first consist of completion of study questionnaires followed by experimental procedures. In order to ensure eligibility to participate, all subjects will complete a SCID and HDRS rating scale. These will confirm the existence/absence of major depression and the absence of comorbid psychiatric conditions. Other exclusionary criteria will be screened via non-structured clinical interviews. A subject's eligibility for safely receiving rTMS, PET and fMRI will be assessed by filling out the appropriate screening forms (see attachments). All of these procedures will be performed by Dr. Eldaief (PI) who is trained in administering these instruments and screening forms. Finally, women of childbearing age will undergo a STAT quantitative serum hcG to ensure that they are not pregnant.

C. Aim 1 and 2 Experimental Procedures

50 subjects (25 MDD patients and 25 matched controls) will each undergo three experimental visits. In addition, of these subjects a subset (no more than five) will be given the option to undergo a fourth experimental visit. During the first study visit, consent will be obtained. Following this, subjects will be assessed for eligibility by

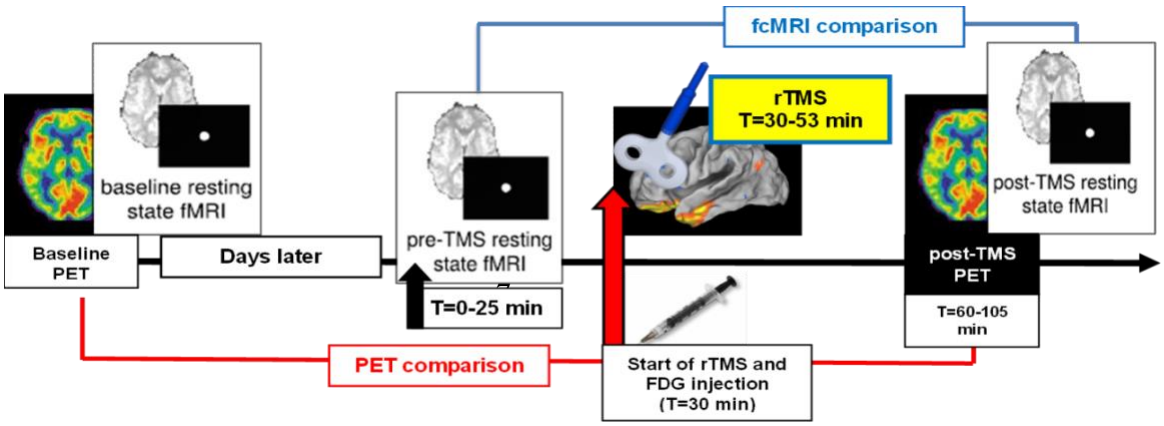

Fig 5. Experimental timeline. Baseline fMRI/PET done on visit 1. Subjects return days later for 2-3 more visits in which they have fMRIs before rTMS and fMRI/PET afterwards.

screening procedures mentioned above. If they are considered eligible, they will undergo a baseline scan during this visit.

Specifically, subjects will undergo simultaneous resting-state (fcMRI)/FDG-PET imaging *without* rTMS at this first visit. The baseline PET session will serve as a comparison for changes in rCMRglu following rTMS. Critically, strong evidence supports the test re-test reliability across FDG-PET scanning sessions separated in time, provided that scanning parameters remain identical.[71-74] Therefore, observed metabolic changes after rTMS will be attributable to the stimulation, and will be deemed unlikely to occur as a result of inherent variability across scanning sessions. Baseline fcMRI data will serve the purpose of creating functional connectivity maps from a sgACC seed. Similar to how lpIPL was designated in Eldaief et al.[14], these maps will be used to establish two cortical targets for rTMS in DLPFC: one which is positively correlated to sgACC, and another which is anticorrelated to sgACC[21,22] (**Fig 1**). After their baseline visit, subjects will return for the first of two rTMS sessions in which they will receive 20Hz stimulation to either the positively or negatively correlated left DLPFC region. They will then return for a third session in which the alternate region is stimulated (thus enabling a within-subject design). Subjects will be randomized to a given session order, and this order will be counterbalanced across subjects. It is expected that all three visits will be completed within two months from the time of enrollment. The experimental timeline for the rTMS sessions is depicted in **Fig 5**. Immediately before stimulation subjects will be rapidly scanned with resting-state fMRI *without* PET (for approx. 25 minutes). After this, they will fill out a brief (1 min) visual analog scale in which they rate sadness, anxiety, energy, concentration, happiness, anger and disgust on a scale of 0-10. They will then be taken into a room immediately adjacent to the scanner for rTMS (i.e. constituting "off-line" stimulation[75]). Subjects will be injected with FDG concurrent to the onset of rTMS. After rTMS they will again complete the mood analog scale. They will then be promptly returned to the scanner room for PET/fcMRI (within ~5 min) so as to acquire BOLD data as soon as possible after rTMS.[14] Dynamic PET acquisitions will correspondingly begin at this time (e.g. at approx. 30-35 min post FDG injection), and emission data will be collected for the next 45 min.

	Visit 1	Visit 2	Visit 3	Visit 4
Obtainment of Consent	X			
SCID questionnaire	X			
Hamilton Depression Rating Scale (HDRS)	X			
TMS screening form	X			
MRI screening form	X	X	X	X
STAT quantitative serum hcG	X	X**	X**	X**
TMS side effects questionnaire		X	X	X
Baseline fcMRI/FDG-PET without rTMS	X			
fMRI before and after rTMS; FDG-PET after rTMS		X	X	X

**Exact frequency of pregnancy testing will vary according to how far visits are apart

Table 1: Summary of study procedures by visit

VI. BIOSTATISTICAL ANALYSIS

fcMRI data: The main outcome measures of interest for fcMRI data will be the relative change in z-transformed correlation coefficients between two given cortico-limbic network regions of interest (ROIs), the difference between changes in these z-scores across conditions (e.g. correlated vs. anticorrelated stimulation), and this difference across subjects (e.g. MDD vs. controls). Six cortico-limbic regions of interest will be examined: DLPFC (the 2 stimulation sites), sgACC, dmPFC, NA and amygdala. Seed placement for sgACC will be based on MNI coordinates from other fcMRI studies.[21,22] Anatomical location of other seeds will be determined on an individualized basis as the points of maximal positive correlation with sgACC (or negative correlation in the case of one of the DLPFC sites). Seeds will be spherical and 8mm^3 in diameter. The primary correlation pair of

interest will be DLPFC-sgACC. Based on prior studies,[1-9,30] secondary correlation pairs of interest will be sgACC-dmPFC, sgACC-NA and sgACC-amygdala. fcMRI data will be analyzed using a combination of software packages (e.g., FMRIB Software Library, FSL, Freesurfer, SPM) and custom software developed in the Buckner (mentor) lab. These methods have been described in several other paradigms[27-29,76,77] and were employed in Eldaief et al.[14] We will apply a stringent in-house data quality control program to each dataset, described Yeo et al.,[78] to evaluate fMRI runs for low signal-to-noise and/or excessive head movement. Preprocessing will include global mean regression and temporal band-passing of data for signals >0.08 Hz. After preprocessing, region-to-region correlation strengths will be calculated with volumetric seed-based functional connectivity analyses: correlation maps will be produced by extracting the BOLD time course from a "seed" ROI in the brain, and then computing the Pearson's correlation coefficient (*r*) between that time course and the time course from all other brain voxels. Functional connectivity between two ROIs will be measured by *r* values, which will be transformed to the z distribution. Paired t-tests will directly compare changes in z scores before and after rTMS in a given individual, and two-sample paired t-tests will compare changes across conditions and across subjects.

FDG-PET data: MR-based motion estimates and a dual-echo ultrashort echo time MRI sequence (DUTE)[79] will be used for motion- and attenuation-correction of PET data, respectively. Other preprocessing steps will include image realignment, normalization to MNI space and smoothing. Weighted linear contrasts will then be used to identify changes in rCMRglu in the cortico-limbic regions of interest described above. Relative differences in normalized rCMRglc in these ROIs between the baseline PET scan and the post-rTMS PET scan within a given individual will be calculated on a voxel-by-voxel basis using a paired t-test. Two sample paired t-tests will compare rCMRglu changes across rTMS conditions, and across MDD patients and normals. The experimental PET and fcMRI data revealing local and distributed impacts of TMS will also allow for computational analyses which will be developed with the supervision and guidance of Dr. Deco (advisor).

Experimental Controls: To ensure that the effects of rTMS are specific to the cortico-limbic systems investigated, we will construct three extra-network seed regions: primary motor cortex (M1), primary auditory cortex (A1) and primary visual cortex (V1). A lack of rTMS induced changes in rCMRglu in these regions; and a lack of rTMS induced changes in inter-hemispheric functional connectivity between these regions, will corroborate network specific effects. To ensure that the effects of rTMS are not the result of inherent variability of connectivity between two fcMRI acquisitions separated in time, we will compare *pre*-rTMS fcMRI scans between the two stimulation sessions (correlated and anticorrelated). A lack of significant variability between these will argue that changes were caused by rTMS (identical controls were used in Eldaief et al.[14])
Rating scales: Paired t-tests will compare mood analog scale ratings before and after rTMS to assess the possibility that stimulation of cortico-limbic networks confers transient mood altering effects.

VII. RISKS AND DISCOMFORTS

Risks attributable to MRI: There are no known or foreseeable risks associated with conventional MRI procedures except to those people who have electrically, magnetically or mechanically activated implants (such as cardiac pacemakers), or those who have cerebrovascular clips. Participants will therefore be screened very carefully (with a standardized MRI screening form-see attachment) to exclude the possibility that they have any such devices and/or implants, and will be excluded from participation in the event that they do. There are no known additional risks associated with functional MRI, and the functional MRI systems to be employed have been approved by the FDA and will be operated within the standards reviewed and accepted by the FDA. All studies will specifically adhere to these FDA approved safety levels for the Siemens system. These safety parameters include static magnetic field, time varying magnetic fields (dB/dt), specific absorption rate (SAR), and acoustic noise levels. Subjects will be informed about minimal risks of routine high magnetic field and non-ionizing RF radiation involved in MR imaging. Still, an MRI might be uncomfortable to a subject due to the risk of a) claustrophobia; b) boredom from lying still for an extended time or c) being perturbed by scanner noise.

Risks attributable to TMS: TMS has been used in a growing number of laboratories worldwide since 1984. Guidelines for the safe use of rTMS were published in 1993 by Pascual-Leone et al., and were updated in a recent paper by Rossi et al. 2009.[69] In this paper, parameters for the maximum repetitive TMS train duration,

frequency, and intensity in order to safely apply rTMS were described. The most worrisome side effect of TMS is that of a seizure, however, this is an extremely rare occurrence in subjects who do not have epilepsy, or who are not prone to seizures. To date, very few patients have had seizures associated with TMS reported in the literature, among the many thousands of normal subjects who have received it since 1984. In a review of published studies that applied rTMS to non-motor areas in healthy participants and patients between 1998 and 2003, only two seizures occurred among 3092 subjects exposed to rTMS. The precise risk ratio is uncertain, but the overall risk for this complication is thought to be less than 1/1,000 studies. TMS has also been rarely associated with pseudoseizures and syncope. More common risks associated with TMS, and measures taken to limit these, are presented in **Table 2**.

In terms of seizures, all rTMS sessions will be conducted by the PI (Dr. Eldaief) who is (1) a board certified neurologist, (2) has been trained in the safe and efficient administration of TMS (through a course offered at the Berenson-Allen Center for Non-Invasive Brain Stimulation at the Beth Israel Deaconess Medical Center) and (3) is trained in basic life support and in the recognition and treatment of convulsions, syncope and other medical/neurological emergencies. In addition, a fully equipped and regularly checked crash cart will be available at all sites where TMS stimulation will occur. This emergency equipment includes oxygen supply, IV line supplies, and emergency medications (e.g. benzodiazepines) in the event of a convulsion. Therefore, should any complication occur, rapid medical coverage for the subject will be provided, followed by Emergency Room care if appropriate. We will also monitor patients in detail during and after delivery of rTMS, using an approach drawn directly from suggested guidelines.[68] Notably, there is not expected to be an increase in the risk of seizures with stimulation of the control site. In fact, stimulation of the cerebellum is much *less* likley to result in seizure induction. Other measures to minimize risks from TMS are listed in **Table 2**.

Risk Associated with TMS	Measures taken to prevent/minimize this risk
20-40% of subjects experience MILD headaches or neck pain	Care will be taken to administer stimulation in a comfortable neck position and to avoid unnecessary stimulation of neck/scalp muscles.
TMS produces a loud clicking sound which may cause tinnitus or rarely short-term hearing loss	Subjects will be given earplugs, which, in animal and human studies, proven effective in preventing the risk of hearing disturbance due to TMS.
TMS could induce short-term changes in memory, attention and other cognitive functions	This is a theoretical risk, as none of the safety studies conducted has found such side effects.
Acute psychiatric effects such as mania and delusions have been described in patients with medically refractory depression or bipolar disorder who received rTMS. This seems to be a rare complication of rTMS (incidence <0.15% of patients).	Mania and delusions arising from TMS have not been described in purely schizophrenic cohorts, e.g. those without a comorbid affective disorder. Subjects will undergo a brief rTMS side effects questionnaire after rTMS to assess for these rare effects.
Concentration/memory deficits which are exceedingly scarce.	Subjects will undergo a brief questionnaire after every rTMS session to assess for these rare effects.

Table 2. Risks associated with TMS and measures to prevent them

Risks Attributable to PET:

a) *Venipuncture:* An intravenous catheter will be placed for this study to inject the FDG radioligand. FDG injection will be administered by a licensed nuclear medicine technologist. All safety procedures related to venipuncture (e.g. minimization of bleeding, bruising and infection) will be strictly adhered to and venipuncture will be carried out by an experienced technician. As such, subjects are expected to feel transient discomfort at the time of venipuncture. There is also the risk of slight bruising at the sight of venipuncture. Bleeding or infection risks (e.g. cellulitis) are possible following IV placement, but these are exceedingly rare.

b) *Radiation exposure:* The radiation exposure in this study will be small and there is no evidence that it represents a major health risk. We will follow the safety standards put forth by the Radioactive Drug

Research Committee (RDRC) and the Massachusetts General Hospital Radiation Safety Committee. As is the convention for receiving FDG-PET, it will not be the total number of scans a subject has, but the total amount of radiation exposure per year that will serve as the limiting factor. According to RDRC policy, adult research subjects should be limited to a total of 50 milliSieverts (mSv) of radiation dose per year. It is estimated that each FDG scan results in a whole body radiation exposure of 3.5mSv per scan. This would amount to a total of 10.5mSv of exposure per year for the majority of subjects (who will each undergo three FDG-PET scans) and 14mSV of exposure per year for subjects who agree to undergo an optional fourth study visit. Notably, exposure is limited to this amount because scanning will occur on an MRI-PET scanner, and not a CT-PET scanner. Careful monitoring of a subject's recent radiation exposures will occur, and subjects will be prohibited from participation if they have received radiation for any other study in the past 12 months. If subjects have participated in other research studies in the past 12 months that have involved radiation exposure, they will be asked to inform the investigators or study staff (by placing a check mark on the consent form verifying that they have or have not been exposed to other radiation in the past 12 months). If it is determined that the prior radiation exposure exceeds our current guidelines (i.e. 15 mSv/year) the subject will not be enrolled in this study.

We will use FDG purchased through commercial sources, specifically through PETNet and Cardinal Health. We will follow the safety standards approved by the Radiation Safety Committee for the use of radioligands. The IV injection will be administered either by a physician or by a trained technician. There is an extremely small risk of allergic reaction to FDG. Should there be an adverse event, Dr. Eldaief will be responsible for communicating with the IRB within the stipulated time frame.

Possible risks associated with combined MRI-PET: There are no foreseeable additive risks from combining PET with MRI. The U.S. Food and Drug Administration (FDA) recently gave the first regulatory clearance of a hybrid PET/MRI scanner in the U.S. However, it should be noted that the specific combined PET/MRI machine that will be used as part of this study is not FDA approved.

Risks attributable to behavioral testing: The questionnaires employed as part of the study may cause some subjects to feel frustrated, bored or upset. It might also cause them to feel dysphoric as it may remind them of their clinical depressive symptoms. Subjects will be told that they may stop at any time during the testing.

Possible risks of delaying treatment in MDD subjects: MDD subjects may or may not have a treating psychiatric provider during their participation. If the subject does have a current provider, the PI will correspond directly with the subjects' psychiatric provider after seeking formal permission from the subject to do so (in the form of a signed medical release form to discuss his/her care supplied by said provider's office). This will allow the PI to (1) discuss maintenance strategies for the subject during their pre-treatment phase, (2) report worsening of the patient's mood to the provider and (3) receive reports from said provider about clinical deterioration. Maintenance strategies might include, for example, having MDD subjects undergo brief interpersonal therapy (IPT), either through the outpatient Psychiatry department at BWH or directly with their provider.

However, in some cases an MDD subject may refuse to have a psychiatric provider and refuse to undergo treatment for their depression (even after the risks of doing so are explained to them by the PI). That is, they may have decided to not pursue pharmacotherapy or psychotherapy to treat their depression. In these cases, treatment would not be "delayed," as the subject never intended it to be started. For these subjects, the PI (a board certified psychiatrist) will still monitor MDD subjects in the following ways to assess symptom worsening during their study participation. First, he will directly ask subjects at each of their three visits about possible symptom worsening. Secondly, and more objectively, this will be assessed by performing a HAMD questionnaire at each of the three study visits. Third the PI will provide brief patient education on Major Depression, specifically with respect to common symptomatology, theories as to the biological basis of the disorder and treatment options. Fourth, the PI and will encourage that they seek treatment in the form of psychotherapy during the study and with psychotherapy and/or pharmacotherapy after their study participation ends.

In addition, subjects with MDD who experience a 20 percent increase in their HAMD rating scores during the course of the study will be removed from the study and referred to a regular psychiatric clinician for the institution of prompt treatment. As mentioned, this will be assessed by performing a HAMD at each of the three study visits.

Depressed patients will be evaluated at each study visit with the Columbia Suicide Severity Rating Scale (CSSRS) as well as the HDRS. If subjects report active suicidal ideation (SI) on either scale they will be removed from the study. Of note, this course of action will be taken if a subject endorses active suicidal ideation at any visit, even if their HDRS score has not increased by 20% from their last visit. Suicide risk in these individuals will be further probed by examining their CSSRS responses. If the risk of suicide is deemed high and/or imminent (e.g., if the subject endorses frequent active SI, has intent and/or plan for self-injury) the subject will be immediately referred to the Emergency Department at MGH. In these cases of extreme concern, subjects will be taken to the ED with a securtiy escort and may have to be taken against their will. If the subject is determined to have SI but to not be an imminent risk for suicide, it will be suggested that they contact the Boston Emergency Services Team (BEST), which is jointly operated through MGH and Boston University and which is well equipped to provide acute care and to facilitate rapid referrals to psychiatric providers. Information on contacting the BEST team will be provided on the MDD resource sheet. Obviously, if the subject has an established psychiatric provider, they will be encouraged to contact that individual immediately as well.

VIII. POTENTIAL BENEFITS

It is not expected that individuals participating in this study will benefit directly from it, other than through financial compensation. Furthermore, MRI scans being used are NOT sufficiently clinically diagnostic to detect pathology. However, if a pathological finding is suspected, the subject will be referred for further neurological and/or radiological work-up.

This work will better elucidate how intrinsic networks and their dynamics are altered in MDD. It will also assess how rTMS, or other neuromodulatory strategies, create the formation of more adaptive configurations. In turn, this will pollinate future trials that optimize MDD treatments through selective targeting of intrinsic networks.

IX. MONITORING AND QUALITY ASSURANCE

The Principal Investigator (PI), Dr. Eldaief and his staff will have the responsibility for continually monitoring all aspects of the studies, including adverse events (AEs), and for reporting AEs according to the PHRC guidelines. Adverse effects will be collected from the start of the experimental protocol to the end of study participation. All adverse events, regardless of attribution to rTMS, to MRI scanning or to PET scanning or any other study associated procedure or questionnaire, will be collected and recorded using standard adverse event forms approved by the Partners IRB. Participants will be asked in an open-ended way about the presence of any such events. Also, a standard questionnaire for TMS-related adverse effects (see attachment) will be performed in the period after every rTMS session. Intensity of each adverse event will be graded as mild, moderate or severe. Events will be medically evaluated where appropriate, including testing and referral.

All study investigators attest that they have read the adverse event definitions provided by the PHRC and that they have undergone the required training in the protection of human subjects. The PI understands the responsibilities and reporting requirements for the PHRC. Adverse events and unanticipated problems will be reported to the IRB per PHRC reporting guidelines.

Descriptions of AEs and unanticipated events which are not AEs will include: detailed descriptions of the events themselves, descriptions of whether the event was expected or unexpected, descriptions of the severity and frequency of the events, determinations as to whether the event is related or possibly related to study procedures, determinations as to whether continuation in the study places subjects at an increased risk of harm, and whether changes to the research or other corrective actions are warranted.

To safeguard confidentiality and the privacy of protected health information, each study subject will be assigned a unique code number. A separate file linking the patient's name with study number and identifiers will be kept in a password-protected data file, accessible only by the study investigators. All study forms will be kept in secure locked file cabinets. The study investigators will assume full responsibility to maintain the confidentiality of all data. All study results will be presented only as statistical aggregates that will neither identify, nor permit identification of, individual subjects.

Dr. Eldaief, the principal investigator, will review the accuracy and completeness of the data and adherence to the PHRC approved protocol on a continuing basis. Guidelines regarding Data and Safety Monitoring Plans and Quality Assurance and Adverse Event Reporting Guidelines will be followed at all times.

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