Biological Psychiatry

Archival Report

Prospective Validation That Subgenual Connectivity Predicts Antidepressant Efficacy of Transcranial Magnetic Stimulation Sites

Anne Weigand, Andreas Horn, Ruth Caballero, Danielle Cooke, Adam P. Stern, Stephan F. Taylor, Daniel Press, Alvaro Pascual-Leone, and Michael D. Fox

ABSTRACT

BACKGROUND: The optimal target in the dorsolateral prefrontal cortex for treating depression with repetitive transcranial magnetic stimulation (rTMS) remains unknown. Better efficacy has been associated with stimulation sites that are 1) more anterior and lateral and 2) more functionally connected to the subgenual cingulate. Here we prospectively test whether these factors predict response in individual patients.

METHODS: A primary cohort (Boston, n = 25) with medication-refractory depression underwent conventional open-label rTMS to the left dorsolateral prefrontal cortex. A secondary cohort (Michigan, n = 16) underwent 4 weeks of sham followed by open-label rTMS for nonresponders (n = 12). In each patient, the location of the stimulation site was recorded with frameless stereotaxy. Connectivity between each patient's stimulation site and the subgenual cingulate was assessed using resting-state functional connectivity magnetic resonance imaging from a cohort of healthy subjects (n = 1000) and confirmed using connectivity from patients with depression (n = 38).

RESULTS: In our primary cohort, antidepressant efficacy was predicted by stimulation sites that were both more anterolateral (r = .51, $p < .01$) and more negatively correlated with the subgenual cingulate ($r = -.55$, $p < .005$). However, subgenual connectivity was the only independent predictor of response and the only factor to predict response to active ($r = -.52$, $p < .05$) but not sham rTMS in our secondary cohort.

CONCLUSIONS: This study provides prospective validation that functional connectivity between an individual's rTMS cortical target and the subgenual cingulate predicts antidepressant response. Implications for improving the cortical rTMS target for depression are discussed.

Keywords: Depression, Dorsolateral prefrontal cortex, Resting-state functional connectivity, Subgenual cingulate, TMS, Transcranial magnetic stimulation

https://doi.org/10.1016/j.biopsych.2017.10.028

Neuroimaging studies suggest that activity in the left dorsolateral prefrontal cortex (DLPFC) is decreased in patients with major depressive disorder and increases with antidepressant treatment (1–9). Consistent with these findings, repetitive transcranial magnetic stimulation (rTMS) applied over the left DLPFC for multiple weeks is an effective treatment for medication-resistant depression (10–13). However, the antidepressant efficacy of rTMS varies greatly across individuals (14–16).

Patient characteristics such as age, degree of treatment resistance, illness duration, and baseline neuroimaging findings likely contribute to this variability (17–22). However, these factors cannot be easily modified to improve TMS response. Factors that are modifiable include stimulation site, frequency, intensity, and the number of pulses (23). Of these, the location of stimulation site has received particular attention (24–27). Based on the first rTMS studies for depression (28,29) and subsequent randomized controlled trials (12,13), most clinics identify the left DLPFC stimulation site by identifying the site over the motor cortex that produces a finger twitch, then moving 5 to 6 cm anterior to this site along the scalp surface (24,28–31). Owing to individual differences in anatomy, this method leads to different patients' being stimulated at different brain locations and heterogeneity in clinical response (24,25,32,33). Other targeting methods have been proposed (31,33–36), but they are limited by the fact that the optimal stimulation site within the DLPFC remains unknown.

To this end, two properties of the stimulation site have been associated with improved antidepressant response. First, patients are more likely to respond if they are stimulated at sites more anterior and lateral within the DLPFC (24,25,33). This observation led to an equation to predict antidepressant response based on the anatomical coordinates of a patient's stimulation site (25). Second, patients are more likely to respond if stimulated at sites more functionally connected to the subgenual cingulate (27). This limbic region is thought to be hyperactive in depression, and decreases in subgenual cingulate activity have been associated with antidepressant

SEE COMMENTARY ON PAGE e1

 ISSN: 0006-3223

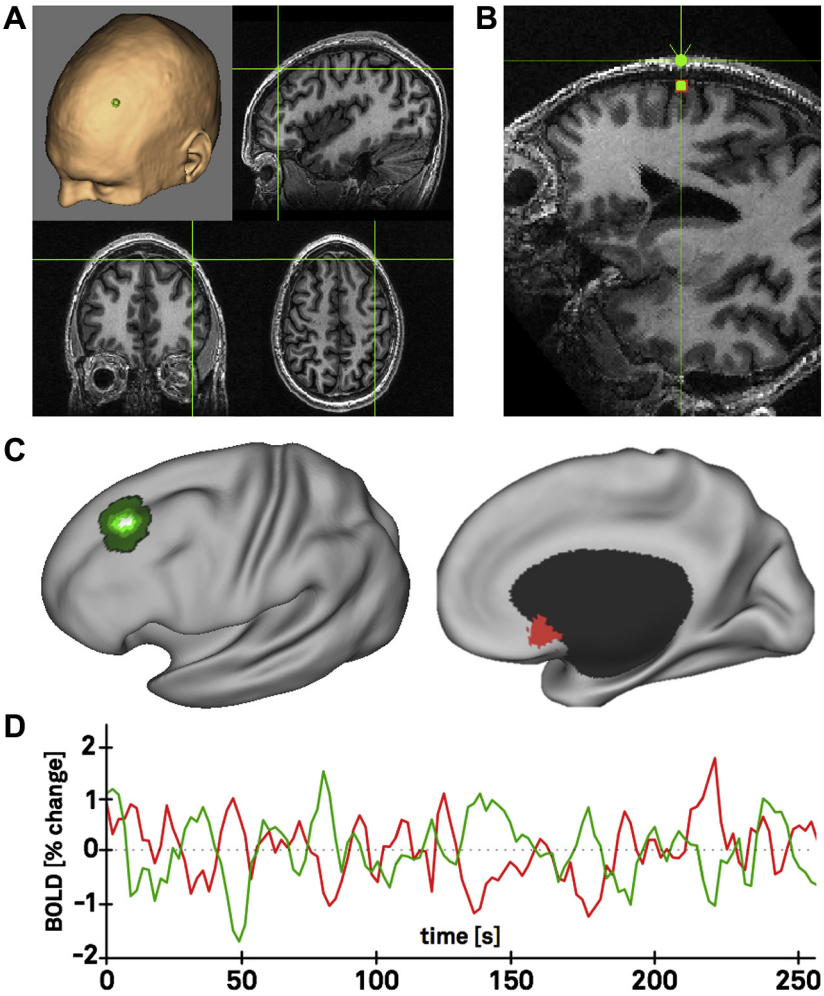

Figure 1. Analysis approach. **(A)** Each patient's transcranial magnetic stimulation site was recorded using their magnetic resonance imaging and a frameless neuronavigation system. **(B)** Stimulation sites on the surface of the scalp were projected to the nearest location on the brain surface using an automated algorithm. **(C)** Brain coordinates were transformed into standard atlas space and the volume of stimulated tissue was approximated using an existing transcranial magnetic stimulation model (green). Functional connectivity between the stimulation site (green) and the subgenual cingulate (red) was assessed using functional magnetic resonance imaging data from a large normative cohort of 1000 subjects. **(D)** Representative time courses from a single subject. BOLD, blood oxygen level–dependent.

response across a range of different therapies (1,4,9,37–42). Consistent with these findings, rTMS sites resulting in higher clinical efficacy are more negatively correlated (i.e., anticorrelated) with the subgenual cingulate based on resting-state functional connectivity (23). This functional relationship is present independent of whether one uses connectome data from normal subjects or patients with depression (27,43), although connectivity differences between groups have been reported (20,44–46).

These two observations regarding stimulation site have led to suggestions that the rTMS site for depression should be moved more anterolaterally within the DLPFC or to a site more anticorrelated with the subgenual cingulate (24–27,35,47). These suggestions are not mutually exclusive and, depending on the distribution of the stimulation sites, can be correlated (27). However, before either can be considered, these retrospective observations must be confirmed prospectively. Further, it remains unknown whether these factors are independent predictors, predictive of all or only some antidepressant symptoms, and specific to active (vs. sham) stimulation.

In the present study, we used neuronavigation to record the precise patient-specific location of stimulation across two independent cohorts undergoing rTMS for treatment of depression. The first cohort (Boston, $n = 25$) was prospectively collected specifically to test two existing hypotheses regarding the relationship between stimulation site and antidepressant response (25,27). A second cohort (Michigan, $n = 16$) was retrospectively analyzed to evaluate the relationship to sham stimulation.

METHODS AND MATERIALS

Full methodological details can be found in the Supplement. Briefly, data from two cohorts of patients with medication-resistant major depressive disorder treated with 4 to 7 weeks of daily rTMS applied over the left DLPFC were included. The primary cohort was prospectively enrolled and received conventional open-label rTMS at the Berenson-Allen Center at Beth Israel Deaconess Medical Center in Boston, Massachusetts. Outcome was measured using the Beck Depression Inventory-II (BDI). The secondary cohort was treated at the Department of Psychiatry at the University of Michigan, Ann Arbor, Michigan, and underwent 4 weeks of blinded, sham stimulation. Some of these patients then received open-label

active rTMS similar to our primary cohort. These patients were the sham arm of a larger study collected for a different purpose (NCT01900314), the results of which will be published separately. Outcome was assessed using the Montgomery–Åsberg Depression Rating Scale (MADRS).

In both cohorts, the precise location of individual stimulation sites was recorded using a frameless neuronavigation system (Figure 1). In the first cohort, the stimulation site was identified using the 5.5-cm rule per routine clinical practice at our center (30). Specifically, the site over the motor cortex that evokes a maximal finger twitch was identified, and then the coil was moved 5.5 cm anterior to this site as measured along the scalp surface. In the second cohort, the stimulation site was identified based on perfusion changes during a working memory task. Regions of interest reflecting the electric field induced by TMS at these sites were generated based on a previous model (26,43). Resting-state functional connectivity of each subject's stimulation site with the subgenual cingulate was assessed using a normative connectome dataset from 1000 healthy subjects (48). Because functional connectivity differences can be seen in depression (20,44–46), results were replicated using a connectome dataset from 38 patients with medication-refractory depression (43).

RESULTS

Patient and Treatment Characteristics

Our primary cohort included 25 right-handed patients with medication-resistant major depressive disorder (17 females) with a mean age of 54.8 years (SD = 9.9, range = 28–67 years of age; see Supplemental Figure S1 for details of patient flow). The mean BDI score at baseline was 38.6 (SD = 9.3) and significantly decreased to 21.2 (SD = 13.0) after the course of rTMS treatment ($t_{24} = 10.05$, $p < .0001$). Thirteen of 25 patients were identified as responders (defined as ≥50% reduction in BDI score). On average, the treatment consisted of 28.5 sessions (SD = 3.4), administered over a period of 4 to 7 weeks (mean = 6.0, SD = 0.8). There were no significant correlations between percent change in BDI scores and gender ($r_{pb} = .01$, $p = .96$), age ($r = -.05$, $p = .82$), number of treatment sessions ($r = -.11$, $p = .62$), applied TMS frequency ($r_{pb} = .16$, $p = .46$), or current medications, including antidepressants ($r = -.20$, $p = .34$), mood stabilizers ($r = -.07$, $p = .74$), antipsychotics ($r = .19$, $p = .38$), and stimulants ($r = -.13$, $p = .53$). Trendwise correlations were found between treatment outcome and baseline BDI scores ($r = -.34$, $p = .09$ with a better response for less severely depressed patients), TMS device ($r_{pb} = .37$, $p = .07$ with a better response for patients receiving treatment with the Magstim [Norrisville, NC] device) and benzodiazepines ($r = -.38$, $p = .06$ with a better response for patients taking this medication). However, none of these variables was a significant predictor of response when correcting for multiple comparisons or in a multivariate analysis (including gender, age, all medications, TMS device, and baseline BDI score).

Predicting Clinical Response Based on the Stimulation Site

The cortical stimulation site identified by the 5.5-cm technique (Boston cohort) was highly variable across different patients (Figure 2A). Average Montreal Neurological Institute (MNI) coordinates were x = −33 ± 7, y = 30 ± 9, and z = 50 ± 9. Repeated markings of the stimulation site within one of these patients suggests that within-subject variability of the stimulation site was low relative to intersubject variability (4.4 ± 1.4 mm vs. 12.7 ± 6.1 mm, T = 3.01, $p = .005$) (Supplemental Figure S2).

Based on a linear combination of the anatomical coordinates from each patient's stimulation site (25), the Herbsman equation significantly predicted antidepressant response in the Boston cohort ($r = .51$, $p < .01$, Figure 2B). As previously hypothesized (25), more anterior and lateral stimulation sites were more effective.

Functional connectivity between each patient's stimulation site and the subgenual cingulate was also a significant predictor of antidepressant response in the Boston cohort ($r = -.55$, $p < .005$) (Figure 3A, B). As previously hypothesized (27), more effective rTMS sites were more strongly anticorrelated with the subgenual cingulate. Repeating this analysis using connectome data from depression patients produced similar results ($r = -.51$, $p = .01$). Functional connectivity estimates from the normative (1000 healthy subjects) and depression (38 patients) connectomes were nearly identical ($R = .96$, $p < 10^{-14}$); thus, only the larger connectome was used for subsequent analyses. The peak subgenual anticorrelation within the left DLPFC (based on $n = 1000$) was located at x = −42, y = 44, z = 30 mm.

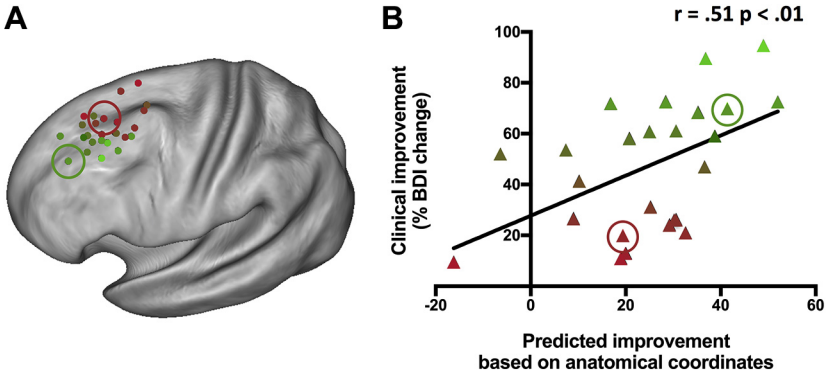

Figure 2. Anatomical location of the transcranial magnetic stimulation site predicts antidepressant response. **(A)** The location of the stimulation site in standard atlas space is shown for each patient in our primary cohort (Boston, $n = 25$), color coded from green to red based on clinical response. **(B)** Clinical improvement (percent change in the Beck Depression Inventory-II [BDI] score) was predicted based anatomical coordinates of each patient's stimulation site and a previously published equation (16). There was a significant correlation between measured and predicted clinical improvement ($r = .51$, $p < .01$).

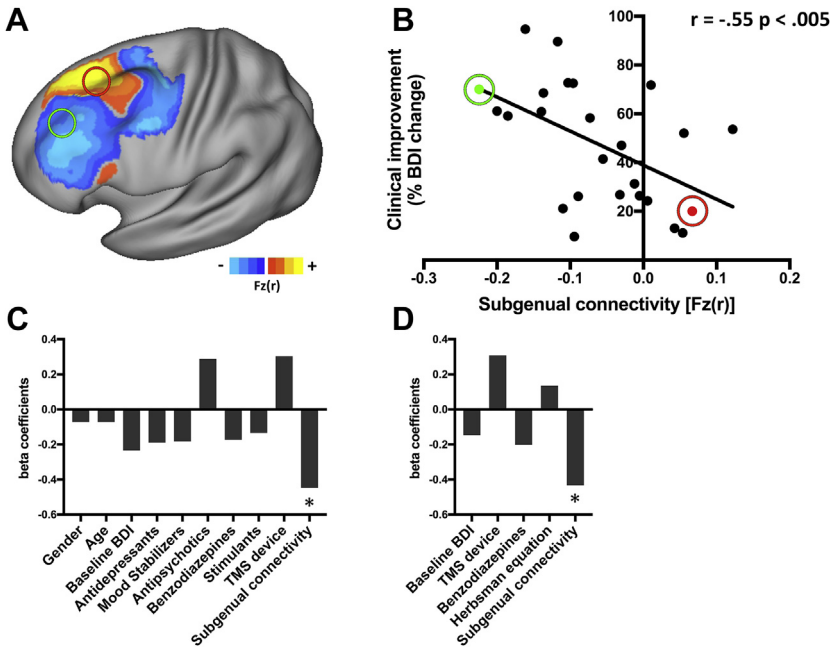

Figure 3. Functional connectivity between the transcranial magnetic stimulation (TMS) site and the subgenual cingulate is an independent predictor of antidepressant response. **(A)** The stimulation sites for an example responder (green) and nonresponder (red) are shown overlaid on a map of functional connectivity with the subgenual cingulate from 1000 healthy subjects, masked to highlight the dorsolateral prefrontal cortex. **(B)** Functional connectivity between each patient's stimulation site and subgenual cingulate was a significant predictor of antidepressant response ($r = -.55$, $p < .005$). Subgenual connectivity was a significant independent predictor of antidepressant response in multivariate analyses of **(C)** baseline clinical variables and **(D)** all variables with signs of predictive utility, including the coordinate-based Herbsman equation. BDI, Beck Depression Inventory-II.

Next, we tested whether these two factors (coordinates and connectivity) were independent predictors of antidepressant response in the Boston cohort. When combined in a multivariate analysis with baseline clinical variables (gender, age, all medications, TMS device, and baseline BDI scores), subgenual connectivity was a significant independent predictor of response ($\beta = -1.24$, $p < .02$) (Figure 3C). When this same analysis was run using the Herbsman equation, it just missed statistical significance ($\beta = .68$, $p = .054$). When all variables with signs of predictive utility in the present study were combined (subgenual connectivity, Herbsman equation, baseline BDI scores, TMS device, and benzodiazepines), subgenual connectivity was the only independent predictor of clinical response ($\beta = -1.17$, $p < .05$) (Figure 3D).

Symptom Specificity

To explore whether subgenual connectivity predicted improvement in all or only some antidepressant symptoms in the Boston cohort, we segmented BDI scores into cognitive, affective, and somatic symptoms based on an established 3-factor model (49). Subgenual connectivity was a significant predictor of improvement in cognitive ($r = -.58$, $p < .005$) and affective symptoms ($r = -.63$, $p < .001$), but not somatic symptoms ($r = -.15$, $p = .48$).

In an exploratory analysis of 21 individual symptoms described by items in the BDI, subgenual connectivity was associated with improvement in sadness, loss of pleasure, self-dislike, self-criticalness, suicidal thoughts, loss of interest, and worthlessness (Table 1). However, there was no association with improvement in irritability, appetite, or fatigue. Further, subgenual connectivity was associated with a lack of improvement in sleep symptoms and interest in sex. In other words, the more anticorrelated a patient's stimulation site was to the subgenual, the better the improvement in sadness but the worse the improvement in interest in sex.

Predicting Sham Stimulation

A limitation of the above analyses is that results could be driven by placebo rather than active rTMS. We therefore retrospectively examined data from a separate cohort in which 16 patients were treated with sham stimulation for 4 weeks (Figure 4A). There was no association between placebo response and subgenual connectivity ($r = .01$, $p_1 = .49$) (Figure 4B). Twelve of these 16 patients remained depressed after sham stimulation and subsequently received open-label active rTMS at the same stimulation site (Figure 4C). Antidepressant response to active stimulation was predicted by subgenual connectivity similar to our primary cohort ($r = -.52$, $p_1 < .05$) (Figure 4D).

Repeating this same analysis using the Herbsman equation returned different results. Surprisingly, the Herbsman equation (i.e., more anterolateral coil position) was associated with response to sham stimulation ($r = .53$, $p_1 < .05$) but not active stimulation ($r = -.09$, $p_1 = .39$).

This dataset also controlled for a second concern, which is that subgenual connectivity is only a predictor of response when using the 5-cm method to identify the stimulation site. This second cohort targeted stimulation based on perfusion changes during a working memory task, resulting in stimulation locations that were significantly more anterior, lateral, and inferior compared to those in our primary cohort (Michigan MNI coordinates $x = -33 \pm 4.7$, $y = 50 \pm 7.9$, $z = 30 \pm 9.4$ mm, all coordinates $p < .05$). Owing to this difference in targeting, this cohort also provided better separation between the Herbsman equation and subgenual connectivity, as the two variables were correlated in the Boston cohort ($r = -.67$, $p < .001$) but not in the Michigan cohort ($r = .08$, $p = .80$).

DISCUSSION

There are three important findings in the present study. First, stronger anticorrelation between a patient's cortical rTMS site

Table 1. Improvement in Only Some Depression Symptoms Is Related to Connectivity With the Subgenual Cingulate

Beck Depression Inventory-II Depression Symptom	Mean Improvement (Raw Scores) (All)	Correlation With Subgenual Connectivity (*r*) (All)	Correlation With Subgenual Connectivity (*r*) (No 0 Values)
Sadness	1.36	−.35	−.35
Pessimism	1.04	−.32	−.32
Past Failure	0.72	−.11	−.07
Loss of Pleasure	0.76	−.36	−.36
Guilty Feelings	0.80	−.21	−.20
Punishment Feelings	0.52	−.15	−.28
Self-dislike	1.00	−.44	−.44
Self-criticalness	0.96	−.34	−.33
Suicidal Thoughts or Wishes	0.36	−.44	−.57
Crying	1.08	−.29	−.25
Agitation	0.52	−.13	−.15
Loss of Interest	1.04	−.59	−.59
Indecisiveness	1.00	−.13	−.15
Worthlessness	0.76	−.37	−.37
Loss of Energy	1.00	−.18	−.18
Changes in Sleeping Pattern	0.44	.47	.41
Irritability	0.92	−.06	−.15
Changes in Appetite	0.52	−.07	−.10
Concentration Difficulty	0.76	−.38	−.38
Tiredness or Fatigue	0.96	.08	.08
Loss in Interest in Sex	0.96	.32	.55

Mean improvement (raw score change) for each question in the Beck Depression Inventory-II is shown along with the correlation between this improvement and subgenual connectivity to the stimulation site for all patients (all). Patients with 0 scores at baseline and after treatment were excluded in the last column.

and the subgenual cingulate predicts clinical outcome in individual patients. Second, subgenual connectivity is an independent predictor of clinical response and specific to active compared to sham stimulation. Third, subgenual connectivity predicts improvement in cognitive and affective but not somatic symptoms of depression. These findings each have implications for identifying the optimal rTMS site for treatment of patients with medication-resistant depression.

Predicting Response Based on Subgenual Connectivity

The strongest finding in the current study is confirmation that connectivity between the rTMS-targeted cortical site and the subgenual cingulate predicts antidepressant response. This hypothesis was originally based on retrospective analysis of existing data (27). Confirmation of this hypothesis in a prospective cohort specifically collected for this purpose is important, especially given the poor reproducibility of most neuroimaging findings (50–53) and the potential clinical implications. Post hoc analyses further suggest that subgenual connectivity is an independent predictor of response when combined with other variables, is not related to sham response, predicts improvement in only certain depressive symptoms, and predicts active rTMS response across different rTMS cohorts.

It is important to note that unlike our primary Boston cohort, the Michigan cohort was not specifically collected to test our hypothesis. Further, the Michigan cohort differed in many ways from the Boston cohort such that results are not directly comparable between cohorts (e.g., determining which cohort had a better TMS response). However, variance in outcome within a cohort, and the factors responsible for that variance, are comparable. For example, while the Michigan cohort cannot be used a sham control for the Boston cohort, we can conclude that subgenual connectivity within the Michigan cohort is unrelated to the response to sham stimulation. Further, the differences between cohorts is also an advantage with respect to generalizability of our results. It is remarkable that despite differences in center (Boston vs. Michigan), targeting method (5.5-cm vs. task activation), target distribution in the DLPFC (Figure 2A vs. Figure 4), outcome measure (BDI vs. Montgomery–Åsberg Depression Rating Scale), average duration of daily active rTMS (6 vs. 4 weeks), and preceding treatments (none vs. sham), subgenual connectivity predicts similar variance in clinical outcome during open-label TMS in both groups.

The current results add to accumulating data that connectivity to subcortical limbic regions plays a role in mediating the rTMS antidepressant response (12,20,26,27,43,54–56). In particular, the subgenual cingulate has been linked to sadness in normal subjects (3,57,58) and antidepressant response across a wide range of therapies (1,2,59–62).

Subgenual connectivity predicted rTMS-induced improvement in some but not all depression symptoms. The strong association with affective symptoms is expected given data implicating the subgenual cingulate in sadness and mood (3,57,58). The strong association with cognitive symptoms is less intuitive; however, the "cognitive" factor is less about cognitive function such as attention or working memory and more about negative thoughts such as guilt, pessimism, and self-dislike (49). Subgenual connectivity may identify the specific part of the DLPFC mediating such thoughts. Whether improvement in "cognitive" symptoms relates to any objective improvement in cognitive function remains unknown, because there is a known dissociation between the two (59).

Finally, subgenual connectivity was not related to improvement in somatic symptoms. This is not surprising, because these symptoms of depression are likely mediated by other brain systems (2,60,61). Interestingly, subgenual connectivity predicted less improvement in sexual interest, consistent with decreased libido observed in response to many selective serotonin reuptake inhibitors (62), which are also drugs that suppress subgenual activity (4,38).

Predicting Response Based on Anatomical Coordinates

The current study is the first to show that the Herbsman equation, based on more anterior and lateral stimulation coordinates, predicts antidepressant response in a prospective cohort (Boston cohort). An interesting question is why anterolateral coil position was predictive in our Boston cohort but failed to predict response in our Michigan cohort or when

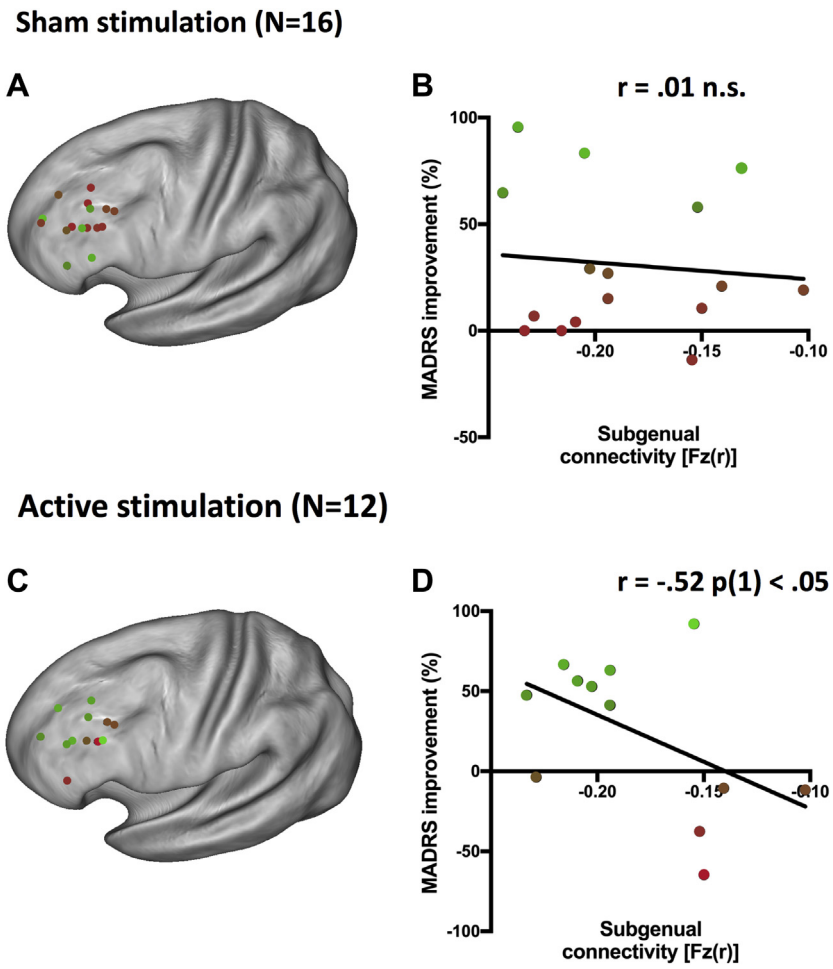

Figure 4. Subgenual connectivity predicts response to active but not sham stimulation. **(A)** The anatomical location of the stimulation site is shown for each patient in our secondary cohort (n = 16), all of whom received sham stimulation. **(B)** Subgenual connectivity did not predict sham repetitive transcranial magnetic stimulation response (r = .01, p_1 = .49). **(C)** A subset of these patients went on to get active stimulation at the same stimulation sites (n = 12). **(D)** Subgenual connectivity was a predictor of clinical response to active stimulation, matching results from our primary cohort ($r = -.52$, $p_1 < .05$). MADRS, Montgomery–Åsberg Depression Rating Scale. n.s., not significant.

previously applied to other TMS cohorts (24,27,63). The most likely explanation is that in our Boston cohort, there was a strong correlation between the Herbsman equation and subgenual connectivity. When the variance related to subgenual connectivity was controlled for in a linear model, the Herbsman equation was no longer predictive. In datasets using alternative methods to target rTMS such as task activation (Michigan cohort) or positron emission tomography (63), the correlation between the Herbsman equation and subgenual connectivity is reduced, and only subgenual connectivity remains predictive.

More anterolateral coil position also failed to predict outcome in an earlier (and much larger) study using rTMS targeting similar to that used in our Boston cohort (24). Differences that may have contributed to the positive finding in our study include frameless stereotaxy to record the stimulation site, nonlinear transformation of magnetic resonance images into standard atlas space, 4 to 7 weeks of open-label rTMS in accordance with current clinical practice, and analysis of antidepressant response as a continuous rather than discrete variable.

An unexpected result in the current study is the finding that more anterolateral coil position (per the Herbsman equation) was associated with response to sham stimulation (Michigan cohort). This result highlights the fact that no similar association was seen between sham response and subgenual connectivity. However, why anterolateral coil position would lead to higher placebo response is unclear; this result should be interpreted with caution.

Other Predictive Factors

It should be noted that factors other than the location and connectivity of the stimulation are likely to play a role in TMS response (17–19). The only clinical variables with even trend-level predictive utility in the present study were baseline BDI score, TMS device, and benzodiazepines. However, none of these variables survived correction for multiple comparisons or was significant in our multivariate analysis. Further, some variables, such as TMS device, have been specifically evaluated in larger trials and found to not impact TMS response (64).

Individualized Versus Group Connectivity

It is worth highlighting that our study was based primarily on normative connectome data from a large cohort of healthy subjects, as was previous work on this topic, including the 2012 paper whose hypotheses this study was designed to

address (27,43). This is a major practical advantage, because magnetic resonance imaging–based connectivity data is not routinely acquired in TMS patients. If the stimulation site is recorded, our technique can be used to predict patient response. Further, normative connectome datasets are acquired with specialized magnetic resonance imaging hardware and cohort sizes in the thousands, leading to extremely robust connectivity estimates. Such normative connectome datasets have proven valuable in predicting stroke symptoms from patient-specific lesions (65–69) and clinical response from patient-specific deep brain stimulation sites (70), and we now show that it can predict clinical response from patient-specific TMS sites. Although differences in connectivity have been reported in patients with depression (1–9), using a disease-matched connectome had almost no effect on our results, and if anything, results were slightly better using the normative connectome. This is consistent with previous work from our group (65,70) and suggests that the signal-to-noise benefits of large normative connectomes may outweigh small differences in connectivity associated with a disease state.

An important question is how the results of the current study, based entirely on group connectome data, relate to previous work emphasizing the importance of individual differences in connectivity for identifying TMS targets (26,47,71). Single-subject connectivity estimates are inherently noisy compared to group connectome estimates, especially in brain regions with poor signal-to-noise ratio like the subgenual cingulate (26,71). Advanced processing strategies are required to generate reproducible individualized connectivity maps and TMS targets (26,72). Whether individualized connectivity with the stimulation site can improve on predictions of TMS response based on group connectome data is an important question for future work.

Clinical Implications

The current results provide the strongest evidence to date that subgenual connectivity with the stimulation site predicts antidepressant response, raising the question of whether this should change clinical practice. At a minimum, these results suggest that recording the location of the rTMS site with neuronavigation has prognostic value. If the planned stimulation site predicts a poor response, one could justify moving stimulation to a site predictive of a better response. Indeed, this strategy was used in one of the large randomized clinical trials of rTMS (12). When the 5-cm technique identified a site over the premotor cortex it was moved anterior and lateral (24). The current data suggest that subgenual connectivity, rather than anterolateral location, may better guide this correction.

Subgenual connectivity may also help inform the ongoing debate of how to best target rTMS. Although the 5-cm technique was used in the large randomized trials (10–13), other work has suggested using 5.5 or 6 cm (30,31). Recent consensus guidelines recommend targeting based on the electroencephalography F3 coordinate, which may better account for head size (31,33,34). It is possible to convert target MNI coordinates into scalp measurements that could allow for targeting of TMS without neuronavigation (73). However, the accuracy and utility of such an approach remains to be tested in patients.

MNI coordinates of our peak subgenual anticorrelation in the left DLPFC (x = −42, y = 44, z = 30), fall directly between two peak coordinates we reported previously (x = −44, y = 38, z = 34) and (x = −38, y = 44, z = 26) (27). The current coordinates should be considered an update of this previous work, because the current study used functional connectivity from 1000 subjects rather than 98. Whether intentionally targeting this coordinate improves antidepressant response remains unknown. A small randomized trial targeting rTMS to a site near our anticorrelation peak showed some benefit over the 5-cm approach but failed to meet its primary end point (35). To this end, it is important to note that the anticorrelation peak may not be best for all depression symptoms. The current data regarding which symptoms are most related to subgenual connectivity could prove useful in the design of a future clinical trial.

Limitations

There are several limitations to this study. First, although replication of an a priori hypothesis across two independent cohorts is strongly supportive, only one cohort was prospectively collected for this purpose, and both cohorts were relatively small (n = 25 and n = 16). Results will benefit from further replication in larger and ideally multicenter studies. Second, two different TMS devices and frequencies were used in our primary cohort. This does not significantly impact outcome in our current data or in previous literature (64,74,75), but it introduces heterogeneity that could potentially lead to false negatives. Similarly, there was significant heterogeneity between cohorts, precluding direct cross-cohort comparisons. However, as noted earlier, reproducibility within cohorts in the face of this heterogeneity is a major strength of the present study. Third, neuronavigation was only used to record the stimulation site during a single session for most patients in our primary cohort, raising questions regarding within-subject variability. However, repeated measurements in one of our patients (Supplemental Figure S2) and previous work on the reliability of neuronavigation (76,77) suggests that this variability is small relative to the variability across subjects. Further, any within-subject variability should bias us against the current findings. Fourth, our study was designed as a prospective test of a specific hypothesis, and thus focused on connectivity between the rTMS targeted cortical site and the subgenual cingulate. Connectivity to other brain regions may also be relevant to antidepressant response (20,54,55). Fifth, although we corrected for several clinical variables, we collected limited information on some factors that may influence TMS response, such as duration of the current depressive episode or genetic polymorphisms (17–19,78). Finally, and perhaps most importantly, this was a prospective observational trial, not a randomized controlled trial. Whether directly targeting the peak site of subgenual anticorrelation improves antidepressant response beyond conventional targeting remains to be tested.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by National Institutes of Health Grant Nos. R21 MH099196, K23 NS083741, R21 MH098174, and R01 MH113929, by Harvard Clinical and Translational Science Center/Harvard Catalyst National

Center for Advancing Translational Sciences, National Institutes of Health Grant No. UL1 RR025758, and by Clinical Sciences Translational Award (Michigan) UL1 TR000433. Additional assistance came from financial contributions from Harvard University and its affiliated academic health care centers, the Brain and Behavior Research Foundation, and by the Sidney R. Baer Jr. Foundation and the Nancy Lurie Marks Foundation. Research support was also provided by Neuronetics, which did not have a role in the design of the study. Work on this study was also supported by the resources provided by Shared Instrumentation Grants 1S10RR023043 and 1S10RR023401.

We thank Antigoni Sinanis, Sara Pedersen, and Chester Pearlman for assistance with data collection, Elissa Wilker for statistical help, David Alsop for assistance with MRI acquisition, and the Brain Genomics Superstruct Project for contributing data.

APL serves on the scientific advisory boards for Starlab Neuroscience, Neuroelectrics, Constant Therapy, and Neosync, and is listed as an inventor on several issued and pending patents on the real-time integration of transcranial magnetic stimulation with electroencephalography and magnetic resonance imaging. MDF is listed as an inventor on submitted or pending patents using brain imaging to guide brain stimulation. AH received funding from Stiftung Charité, the Berlin Institute of Health, and the Professor Klaus Thiemann Foundation, as well as travel stipends from the Movement Disorders Society and Ipsen Pharma. SFT has received research support from Neuronetics and St. Jude Medical. All other authors report no biomedical financial interests or potential conflicts of interest.

The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic health care centers, the National Institutes of Health, the Brain and Behavior Research Foundation, or the Sidney R. Baer Jr. Foundation.

ARTICLE INFORMATION

From the Berenson-Allen Center for Noninvasive Brain Stimulation (AW, AH, RC, DC, APS, DP, AP-L, MDF) and the Division of Cognitive Neurology (APS, DP, AP-L), Department of Neurology, Harvard Medical School and Beth Israel Deaconess Medical Center, Boston; Department of Neurology (MDF), Massachusetts General Hospital, Boston; Athinoula A. Martinos Center for Biomedical Imaging (MDF), Massachusetts General Hospital, Charlestown, Massachusetts; Berlin School of Mind and Brain (AW), Humboldt-Universität zu Berlin, and the Department of Neurology (AH), Movement Disorder and Neuromodulation Unit, Charité – Universitätsmedizin, Berlin, Germany; Bioengineering and Telemedicine Center (RC), ETSI Telecomunicación, Universidad Politécnica de Madrid, and the Institut Guttmann (AP-L), Universitat Autonoma de Barcelona, Madrid, Spain; and the Department of Psychiatry (SFT), University of Michigan, Ann Arbor, Michigan.

AW and AH contributed equally to this work.

Address correspondence to Michael D. Fox, M.D., Ph.D., Beth Israel Deaconess Medical Center, Department of Neurology, 330 Brookline Ave, Boston, MA 02215; E-mail: foxmdphd@gmail.com.

Received Apr 20, 2017; revised Oct 7, 2017; accepted Oct 31, 2017.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2017.10.028.

REFERENCES

1. Mayberg HS, Lozano AM, Voon V, McNeely HE, Seminowicz D, Hamani C, *et al.* (2005): Deep brain stimulation for treatment-resistant depression. Neuron 45:651–660.
2. Brody AL, Saxena S, Mandelkern MA, Fairbanks LA, Ho ML, Baxter LR (2001): Brain metabolic changes associated with symptom factor improvement in major depressive disorder. Biol Psychiatry 50:171–178.
3. Mayberg HS, Liotti M, Brannan SK, McGinnis S, Mahurin RK, Jerabek PA, *et al.* (1999): Reciprocal limbic-cortical function and negative mood: Converging PET findings in depression and normal sadness. Am J Psychiatry 156:675–682.
4. Mayberg HS, Brannan SK, Tekell JL, Silva JA, Mahurin RK, McGinnis S, Jerabek PA (2000): Regional metabolic effects of fluoxetine in major depression: Serial changes and relationship to clinical response. Biol Psychiatry 48:830–843.
5. Biver F, Goldman S, Delvenne V, Luxen A, De Maertelaer V, Hubain P, *et al.* (1994): Frontal and parietal metabolic disturbances in unipolar depression. Biol Psychiatry 36:381–388.
6. Baxter LR, Schwartz JM, Phelps ME, Mazziotta JC, Guze BH, Selin CE, *et al.* (1989): Reduction of prefrontal cortex glucose metabolism common to three types of depression. Arch Gen Psychiatry 46:243–250.
7. Galynker II, Cai J, Ongseng F, Finestone H, Dutta E, Serseni D (1998): Hypofrontality and negative symptoms in major depressive disorder. J Nucl Med 39:608–612.
8. Fitzgerald PB, Oxley TJ, Laird AR, Kulkarni J, Egan GF, Daskalakis ZJ (2006): An analysis of functional neuroimaging studies of dorsolateral prefrontal cortical activity in depression. Psychiatry Res 148:33–45.
9. Koenigs M, Grafman J (2009): The functional neuroanatomy of depression: Distinct roles for ventromedial and dorsolateral prefrontal cortex. Behav Brain Res 201:239–243.
10. Carpenter LL, Janicak PG, Aaronson ST, Boyadjis T, Brock DG, Cook IA, *et al.* (2012): Transcranial magnetic stimulation (TMS) for major depression: A multisite, naturalistic, observational study of acute treatment outcomes in clinical practice. Depress Anxiety 29:587–596.
11. Connolly KR, Helmer A, Cristancho MA, Cristancho P, O'Reardon JP (2012): Effectiveness of transcranial magnetic stimulation in clinical practice post-FDA approval in the United States: Results observed with the first 100 consecutive cases of depression at an academic medical center. J Clin Psychiatry 73:e567–e573.
12. George MS, Lisanby SH, Avery D, McDonald WM, Durkalski V, Pavlicova M, *et al.* (2010): Daily left prefrontal transcranial magnetic stimulation therapy for major depressive disorder: A sham-controlled randomized trial. Arch Gen Psychiatry 67:507–516.
13. O'Reardon JP, Solvason HB, Janicak PG, Sampson S, Isenberg KE, Nahas Z, *et al.* (2007): Efficacy and safety of transcranial magnetic stimulation in the acute treatment of major depression: A multisite randomized controlled trial. Biol Psychiatry 62:1208–1216.
14. Loo CK, Mitchell PB (2005): A review of the efficacy of transcranial magnetic stimulation (TMS) treatment for depression, and current and future strategies to optimize efficacy. J Affect Disord 88:255–267.
15. Padberg F, George MS (2009): Repetitive transcranial magnetic stimulation of the prefrontal cortex in depression. Exp Neurol 219:2–13.
16. Slotema CW, Blom JD, Hoek HW, Sommer IE (2010): Should we expand the toolbox of psychiatric treatment methods to include repetitive transcranial magnetic stimulation (rTMS)? A meta-analysis of the efficacy of rTMS in psychiatric disorders. J Clin Psychiatry 71:873–884.
17. Brakemeier EL, Luborzewski A, Danker-Hopfe H, Kathmann N, Bajbouj M (2007): Positive predictors for antidepressive response to prefrontal repetitive transcranial magnetic stimulation (rTMS). J Psychiatr Res 41:395–403.
18. Fregni F, Marcolin MA, Myczkowski M, Amiaz R, Hasey G, Rumi DO, *et al.* (2006): Predictors of antidepressant response in clinical trials of transcranial magnetic stimulation. Int J Neuropsychopharmacol 9:641–654.
19. Lisanby SH, Husain MM, Rosenquist PB, Maixner D, Gutierrez R, Krystal A, *et al.* (2009): Daily left prefrontal repetitive transcranial magnetic stimulation in the acute treatment of major depression: Clinical predictors of outcome in a multisite, randomized controlled clinical trial. Neuropsychopharmacology 34:522–534.
20. Liston C, Chen AC, Zebley BD, Drysdale AT, Gordon R, Leuchter B, *et al.* (2014): Default mode network mechanisms of transcranial magnetic stimulation in depression. Biol Psychiatry 76:517–526.
21. Kimbrell TA, Little JT, Dunn RT, Frye MA, Greenberg BD, Wassermann EM, *et al.* (1999): Frequency dependence of antidepressant response to left prefrontal repetitive transcranial magnetic stimulation (rTMS) as a function of baseline cerebral glucose metabolism. Biol Psychiatry 46:1603–1613.
22. Paillère Martinot ML, Martinot JL, Ringuenet D, Galinowski A, Gallarda T, Bellivier F, *et al.* (2011): Baseline brain metabolism in resistant depression and response to transcranial magnetic stimulation. Neuropsychopharmacology 36:2710–2719.
23. Nahas Z, Teneback CC, Kozel A, Speer AM, DeBrux C, Molloy M, *et al.* (2001): Brain effects of TMS delivered over prefrontal cortex in depressed adults. J Neuropsychiatry Clin Neurosci 13:459–470.

24. Johnson KA, Baig M, Ramsey D, Lisanby SH, Avery D, McDonald WM, *et al.* (2013): Prefrontal rTMS for treating depression: Location and intensity results from the OPT-TMS multi-site clinical trial. Brain Stimul 6:108–117.
25. Herbsman T, Avery D, Ramsey D, Holtzheimer P, Wadjik C, Hardaway F, *et al.* (2009): More lateral and anterior prefrontal coil location is associated with better repetitive transcranial magnetic stimulation antidepressant response. Biol Psychiatry 66:509–515.
26. Fox MD, Liu H, Pascual-Leone A (2013): Identification of reproducible individualized targets for treatment of depression with TMS based on intrinsic connectivity. Neuroimage 66:151–160.
27. Fox MD, Buckner RL, White MP, Greicius MD, Pascual-Leone A (2012): Efficacy of transcranial magnetic stimulation targets for depression is related to intrinsic functional connectivity with the subgenual cingulate. Biol Psychiatry 72:595–603.
28. George MS, Wassermann EM, Williams WA, Callahan A, Ketter TA, Basser P, *et al.* (1995): Daily repetitive transcranial magnetic stimulation (rTMS) improves mood in depression. Neuroreport 6:1853–1856.
29. Pascual-Leone A, Rubio B, Pallardo F, Catala MD (1996): Rapid-rate transcranial magnetic stimulation of left dorsolateral prefrontal cortex in drug-resistant depression. Lancet 348:233–237.
30. Horvath JC, Mathews J, Demitrack MA, Pascual-Leone A (2010): The NeuroStar TMS device: Conducting the FDA approved protocol for treatment of depression. J Vis Exp 45:2345.
31. McClintock SM, Reti IM, Carpenter LL, McDonald WM, Dubin M, Taylor SF, *et al.* (2018): Consensus recommendations for the clinical application of repetitive transcranial magnetic stimulation (rTMS) in the treatment of depression. J Clin Psychiatry 79(1).
32. Herwig U, Padberg F, Unger J, Spitzer M, Schonfeldt-Lecuona C (2001): Transcranial magnetic stimulation in therapy studies: examination of the reliability of "standard" coil positioning by neuronavigation. Biol Psychiatry 50:58–61.
33. Herwig U, Satrapi P, Schonfeldt-Lecuona C (2003): Using the international 10-20 EEG system for positioning of transcranial magnetic stimulation. Brain Topogr 16:95–99.
34. Beam W, Borckardt JJ, Reeves ST, George MS (2009): An efficient and accurate new method for locating the F3 position for prefrontal TMS applications. Brain Stimul 2:50–54.
35. Fitzgerald PB, Maller JJ, Hoy KE, Thomson R, Daskalakis ZJ (2009): Exploring the optimal site for the localization of dorsolateral prefrontal cortex in brain stimulation experiments. Brain Stimul 2:234–237.
36. Rusjan PM, Barr MS, Farzan F, Arenovich T, Maller JJ, Fitzgerald PB, Daskalakis ZJ (2010): Optimal transcranial magnetic stimulation coil placement for targeting the dorsolateral prefrontal cortex using novel magnetic resonance image-guided neuronavigation. Hum Brain Mapp 31:1643–1652.
37. Dougherty DD, Weiss AP, Cosgrove GR, Alpert NM, Cassem EH, Nierenberg AA, *et al.* (2003): Cerebral metabolic correlates as potential predictors of response to anterior cingulotomy for treatment of major depression. J Neurosurg 99:1010–1017.
38. Goldapple K, Segal Z, Garson C, Lau M, Bieling P, Kennedy S, Mayberg H (2004): Modulation of cortical-limbic pathways in major depression: Treatment-specific effects of cognitive behavior therapy. Arch Gen Psychiatry 61:34–41.
39. Mottaghy FM, Keller CE, Gangitano M, Ly J, Thall M, Parker JA, Pascual-Leone A (2002): Correlation of cerebral blood flow and treatment effects of repetitive transcranial magnetic stimulation in depressed patients. Psychiatry Res 115:1–14.
40. Nobler MS, Oquendo MA, Kegeles LS, Malone KM, Campbell CC, Sackeim HA, Mann JJ (2001): Decreased regional brain metabolism after ect. Am J Psychiatry 158:305–308.
41. Malizia AL (1997): The frontal lobes and neurosurgery for psychiatric disorders. J Psychopharmacol (Oxford) 11:179–187.
42. Drevets WC, Savitz J, Trimble M (2008): The subgenual anterior cingulate cortex in mood disorders. CNS Spectr 13:663–681.
43. Fox MD, Buckner RL, Liu H, Chakravarty MM, Lozano AM, Pascual-Leone A (2014): Resting-state networks link invasive and noninvasive brain stimulation across diverse psychiatric and neurological diseases. Proc Natl Acad Sci U S A 111:E4367–E4375.
44. Greicius MD, Flores BH, Menon V, Glover GH, Solvason HB, Kenna H, *et al.* (2007): Resting-state functional connectivity in major depression: Abnormally increased contributions from subgenual cingulate cortex and thalamus. Biol Psychiatry 62:429–437.
45. Gudayol-Ferré E, Peró-Cebollero M, González-Garrido AA, Guàrdia-Olmos J (2015): Changes in brain connectivity related to the treatment of depression measured through fMRI: A systematic review. Front Hum Neurosci 9:1–17.
46. Brakowski J, Spinelli S, Dörig N, Bosch OG, Manoliu A, Holtforth MG, Seifritz E (2017): Resting state brain network function in major depression - Depression symptomatology, antidepressant treatment effects, future research. J Psychiatr Res 92:147–159.
47. Opitz A, Fox MD, Craddock RC, Colcombe S, Milham MP (2016): An integrated framework for targeting functional networks via transcranial magnetic stimulation. Neuroimage 127:86–96.
48. Yeo BTT, Krienen FM, Sepulcre J, Sabuncu MR, Lashkari D, Hollinshead M, *et al.* (2011): The organization of the human cerebral cortex estimated by intrinsic functional connectivity. J Neurophysiol 106:1125–1165.
49. Buckley TC, Parker JD, Heggie J (2001): A psychometric evaluation of the BDI-II in treatment-seeking substance abusers. J Subst Abuse Treat 20:197–204.
50. Boekel W, Wagenmakers EJ, Belay L, Verhagen J, Brown S, Forstmann BU (2015): A purely confirmatory replication study of structural brain-behavior correlations. Cortex 66:115–133.
51. Button KS, Ioannidis JP, Mokrysz C, Nosek BA, Flint J, Robinson ES, Munafo MR (2013): Power failure: Why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci 14:365–376.
52. Eklund A, Nichols TE, Knutsson H (2016): Cluster failure: Why fMRI inferences for spatial extent have inflated false-positive rates. Proc Natl Acad Sci 113:7900–7905.
53. Nichols TE, Das S, Eickhoff SB, Evans AC, Glatard T, Hanke M, *et al.* (2017): Best practices in data analysis and sharing in neuroimaging using MRI. Nat Neurosci 20:299–303.
54. Baeken C, Marinazzo D, Wu GR, Van Schuerbeek P, De Mey J, Marchetti I, *et al.* (2014): Accelerated HF-rTMS in treatment-resistant unipolar depression: Insights from subgenual anterior cingulate functional connectivity. World J Biol Psychiatry 15:286–297.
55. Salomons TV, Dunlop K, Kennedy SH, Flint A, Geraci J, Giacobbe P, Downar J (2014): Resting-state cortico-thalamic-striatal connectivity predicts response to dorsomedial prefrontal rTMS in major depressive disorder. Neuropsychopharmacology 39:488–498.
56. Wang JX, Rogers LM, Gross EZ, Ryals AJ, Dokucu ME, Brandstatt KL, *et al.* (2014): Targeted enhancement of cortical-hippocampal brain networks and associative memory. Science 345:1054–1057.
57. Phan KL, Wager T, Taylor SF, Liberzon I (2002): Functional neuroanatomy of emotion: A meta-analysis of emotion activation studies in PET and fMRI. Neuroimage 16:331–348.
58. Smith R, Fadok RA, Purcell M, Liu S, Stonnington C, Spetzler RF, Baxter LC (2011): Localizing sadness activation within the subgenual cingulate in individuals: A novel functional MRI paradigm for detecting individual differences in the neural circuitry underlying depression. Brain Imaging Behav 5:229–239.
59. Shilyansky C, Williams LM, Gyurak A, Harris A, Usherwood T, Etkin A (2016): Effect of antidepressant treatment on cognitive impairments associated with depression: A randomised longitudinal study. Lancet Psychiatry 3:425–435.
60. Cuthbert BN, Insel TR (2013): Toward the future of psychiatric diagnosis: the seven pillars of RDoC. BMC Med 11:126.
61. Woody ML, Gibb BE (2015): Integrating NIMH Research Domain Criteria (RDoC) into depression research. Curr Opin Psychol 4:6–12.
62. Serretti A, Chiesa A (2009): Treatment-emergent sexual dysfunction related to antidepressants: A meta-analysis. J Clin Psychopharmacol 29:259–266.
63. Paillere Martinot ML, Galinowski A, Ringuenet D, Gallarda T, Lefaucheur JP, Bellivier F, *et al.* (2010): Influence of prefrontal target region on the efficacy of repetitive transcranial magnetic

stimulation in patients with medication-resistant depression: A [(18)F]-fluorodeoxyglucose PET and MRI study. Int J Neuropsychopharmacol 13:45–59.

64. Oliveira-Maia AJ, Garcia-Guarniz AL, Sinanis A, Pascual-Leone A, Press D (2016): Comparative efficacy of repetitive transcranial magnetic stimulation for treatment of depression using 2 different stimulation devices: A retrospective open-label study. J Clin Psychiatry 77:e743–e744.
65. Boes AD, Prasad S, Liu H, Liu Q, Pascual-Leone A, Caviness VS Jr, Fox MD (2015): Network localization of neurological symptoms from focal brain lesions. Brain 138:3061–3075.
66. Darby RR, Laganiere S, Pascual-Leone A, Prasad S, Fox MD (2016): Finding the imposter: Brain connectivity of lesions causing delusional misidentifications. Brain 140:497–507.
67. Fasano A, Laganiere SE, Lam S, Fox MD (2017): Lesions causing freezing of gait localize to a cerebellar functional network. Ann Neurol 81:129–141.
68. Fischer DB, Boes AD, Demertzi A, Evrard HC, Laureys S, Edlow BL, *et al.* (2016): A human brain network derived from coma-causing brainstem lesions. Neurology 87:2427–2434.
69. Laganiere S, Boes AD, Fox MD (2016): Network localization of hemichorea-hemiballismus. Neurology 86:2187–2195.
70. Horn A, Reich M, Vorwerk J, Li N, Wenzel G, Fang Q, *et al.* (2017): Connectivity predicts deep brain stimulation outcome in Parkinson disease. Ann Neurol 82:67–78.
71. Mueller S, Wang D, Fox MD, Yeo BTT, Sepulcre J, Sabuncu MR, *et al.* (2013): Individual variability in functional connectivity architecture of the human brain. Neuron 77:586–595.
72. Wang D, Buckner RL, Fox MD, Holt DJ, Holmes AJ, Stoecklein S, *et al.* (2015): Parcellating cortical functional networks in individuals. Nat Neurosci 18:1853–1860.
73. Mir-Moghtadaei A, Caballero R, Fried P, Fox MD, Lee K, Giacobbe P, *et al.* (2015): Concordance between beamF3 and MRI-neuronavigated target sites for repetitive transcranial magnetic stimulation of the left dorsolateral prefrontal cortex. Brain Stimul 8:965–973.
74. DeBlasio KL, Tendler A (2012): A retrospective chart review of 10 Hz versus 20 Hz repetitive transcranial magnetic stimulation for depression. SAGE Open 2: 10.1177/2158244012470109.
75. Shajahan PM, Glabus MF, Steele JD, Doris AB, Anderson K, Jenkins JA, *et al.* (2002): Left dorso-lateral repetitive transcranial magnetic stimulation affects cortical excitability and functional connectivity, but does not impair cognition in major depression. Prog Neuropsychopharmacol Biol Psychiatry 26:945–954.
76. Gugino LD, Romero JR, Aglio L, Titone D, Ramirez M, Pascual-Leone A, *et al.* (2001): Transcranial magnetic stimulation coregistered with MRI: A comparison of a guided versus blind stimulation technique and its effect on evoked compound muscle action potentials. Clin Neurophysiol 112:1781–1792.
77. Sparing R, Buelte D, Meister IG, Pauš T, Fink GR (2008): Transcranial magnetic stimulation and the challenge of coil placement: A comparison of conventional and stereotaxic neuronavigational strategies. Hum Brain Mapp 29:82–96.
78. Cheeran B, Talelli P, Mori F, Koch G, Suppa A, Edwards M, *et al.* (2008): A common polymorphism in the brain-derived neurotrophic factor gene (BDNF) modulates human cortical plasticity and the response to rTMS. J Physiol 586:5717–5725.