USING NEUROIMAGING AND OPTOGENETICS TO BETTER UNDERSTAND THE NEURAL CIRCUIT BASIS OF MAJOR DEPRESSION

by

DEBHA NARSINGH AMATYA

Under the joint supervision of

Dr. Karl Deisseroth, MD, Ph.D

Bioengineering, Stanford University

and

Dr. Manpreet Singh, MD, MS

Psychiatry and Behavioral Sciences, Stanford University

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Approved: Karl Deisseroth, MD, Ph.D.
Date 5/11/2014
Research Advisor from the Department of Bioengineering

Approved: Manpreet Singh, MD, MS.
Date 5/7/2014
Faculty Reader from the Department of Psychiatry and Behavioral Sciences

Approved: Karl Deisseroth, MD, Ph.D.
Date 5/11/2014
Chair for Undergraduate Education in the Department of Bioengineering

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I. Abstract

Depression is a leading global health problem that affects hundreds of millions of people. Though the psychiatric disorder has been known to exist for centuries, its treatment still has yet to be perfected. The inability to clearly define the neurobiological mechanism of depression results in the lack of precise tools for the diagnosis and treatment of depressed patients. Advances in neuroscience research are working towards redefining depression in the language of brain circuits by characterizing the disease's structural, functional, and electrochemical profile. In the pioneering work of Helen Mayberg, one promising brain region that has been shown to be metabolically overactive in depression and therapeutically receptive via deep brain stimulation is the subgenual cingulate cortex. In this thesis, I describe the use of graph theoretical analysis of structural imaging data from depressed humans and optogenetic fMRI in the rat to evaluate the changes in whole-brain connectivity associated with depression, especially with respect to the subgenual cingulate and its associated brain regions. In the graph analysis, it was found that the structural correlation brain network in depressed patients was inefficient and less densely connected than in healthy controls. However, some regions, such as the amygdala and ventral frontal cortex, were found to be hyperconnected. In the optogenetic fMRI study, ventromedial prefrontal cortex (the rodent analog to the subgenual cingulate gyrus) stimulation was able to drive behaviors associated with depression and alter the functional connectivity of the region to other areas in the brain, such as the anterior prefrontal cortices, striatum and amygdala. Together, these studies indicate structural and functional abnormalities of the circuitry between the frontal cortices and subcortical limbic system, a collection of brain regions that control emotion, behavior, and reward processing. The presented data supports and expands upon Helen Mayberg’s hypothesis of an overactive subgenual cingulate cortex in depression. Both graph analysis and optogenetic fMRI require further research and development, but they have the potential to be useful tools in the deciphering of the neural circuit basis of depression and in the creation of impactful clinical tools to help manage the debilitating disorder.
II. Background

*Depression, a global health problem:*

An estimated 350 million people suffer from depression, making it the most significant cause of disability around the world (1, 2). Contrary to popular belief, depression is more than a normal, short-lived bout of sadness. The diagnostic criteria for an episode of depression is defined by the daily occurrence of low mood, accompanied by five of the following symptoms: sleep dysfunction, loss of joy, guilt, fatigue, poor concentration, change in appetite, psychomotor agitation or retardation, and suicidality for at least two weeks (3). Those who suffer from depression typically experience multiple episodes in their lifetime and report that the disease significantly impairs their social, occupational, and educational functioning (3, 4). The widespread reach, growing prevalence, and uniquely debilitating effects of the disorder make it a critical global health problem. In spite of the severity of the problems associated with depression, there have been few resources allocated to advance understanding of the pathophysiology and treatment of this disorder. Even the wealthiest countries spend only 5% of their health budget on psychiatric illnesses, and the poorest countries allocate on average ten times less than that amount (5). Thus, most patients who suffer from depression have limited resources to manage their illness, in addition to other health and social factors such as depressive symptoms and the stigma associated with a psychiatric diagnosis that may be barriers to treatment.

![Prevalence of depression around the world](Image)(6).
Limitations in the current standard of care:

Patients seeking psychiatric care have a number of treatment options available to them. Psychotherapy, which involves the restructuring of negative thought patterns through counseling, is considered an effective treatment for mild and moderate depression. In fact, it has been shown that the combination of psychotherapy and prescription medication is the most efficacious way to treat depression in young adults (7). However, more serious cases of depression show limited response to psychotherapies (4). For these cases, pharmacological interventions may be an option in order to treat aberrant brain functions that are linked to depression (4). The use of these pharmacological agents has historically been based on the monoamine hypothesis, a theory developed in the 1960s that stated that depression is principally caused by imbalances in the monoamine neurotransmitters dopamine, serotonin, and norepinephrine (8).

One class of drugs used to treat depression, selective serotonin reuptake inhibitors (SSRIs), is believed to improve the symptoms of depression while mitigating side effects in patients (4). Unfortunately, in younger patients SSRIs have also been shown to increase the risk of suicide, which mandated the U.S. Food and Drug Administration (FDA) to require “black box” warning labels for such drugs (4, 9, and 10). Importantly, 20-50% of depressed patients show a limited response to current therapies, inclusive of psychotherapies and pharmacotherapies (11, 12). Thus, there are several factors that complicate the contemporary management of depression. Though SSRIs have demonstrated efficacy in many instances, the risk and perceived risk of using them can be troubling to doctors and patients that seek to manage the disorder.

These limitations highlight the importance of advancing our understanding of the pathophysiology of depression in order to develop more effective treatments. The majority of the neuroscience community now believes that the dated monoamine hypothesis, the basis of many current depression medications, is an incomplete picture of the disorder (8, 13, and 14). Even the process of diagnosis can be a frustratingly imprecise and subjective exercise. The diagnosis of depression, its various subtypes, and comorbidities like anxiety disorder, can often rely on the metrics derived from self-report questionnaires (15), rather than empirical or biological observations of depressive behaviors. A comprehensive assessment of depression includes a combination of self-report and clinically observed measures.
A new paradigm for understanding and treating:

In order to develop new and more effective treatments for depression, a new scientific paradigm for understanding the depressed brain must be discovered. Fortunately, it appears that the time for such a discovery is ripe; we are presently experiencing an intersection of medical need, technological advancement, and public interest in brain science. This is well illustrated by the announcement of the Brain Initiative by the White House in 2013. Reminiscent of the massive federal push behind the Human Genome Project, the Brain Initiative is currently a hundred million dollar project geared towards revolutionizing our understanding of the brain (16). Specifically, scientists are working to construct the brain connectome, a working map of the multitude of interconnected circuits in the brain. The hope is that the national publicity and federal resources of the Brain Initiative will accelerate tool development and mapping experiments necessary to develop the connectome, which would undoubtedly be a milestone in building a new understanding of the brain and its associated disorders (17). This new scientific understanding will subsequently pave the path for developing new diagnostic and therapeutic strategies that have the potential to revolutionize the treatment of psychiatric illnesses such as depression.
III. Introduction

The circuitry of depression:

As new research is conducted in depression, the language used to describe the disorder has shifted away from referring to it as a chemical imbalance and towards describing it as a neural circuit abnormality (11, 18, and 19). A brain circuit can be defined as a collection of neural components that communicate with one another through structural, chemical, and electrical cues in order to achieve a specific function or set of functions. Because a circuit is characterized by a dynamic set of structural, functional, and electrochemical properties, it is a more robust framework for describing the broad range of adaptive and maladaptive behaviors than are theories that focus on a single property or region of the brain (20). Thus, the key to understanding complex functions, such as behaviors, hinges on our ability to understand how brain regions communicate within their circuits, as well as how different circuits coordinate to share information or regulate one another.

In the late 1990s, Helen Mayberg published groundbreaking work that identified a key brain region that is involved in the “depressive circuit” called the subgenual cingulate cortex or Brodmann area 25 (BA25/CG25). Her work described the functional role of BA25 within the broader context of brain regions that she proposed it communicated with, rather than just focusing on the subgenual cingulate in isolation (21, 22). BA25 is located in the frontal cortex of the brain, below the corpus callosum. It plays a key role in regulating emotion by serving as a bridge between newer areas of the brain responsible for attention, rational thought, and cognition, and evolutionarily older parts of the brain that control basic functions, such as emotions and drives (23).
It was discovered that area 25 was overactive in depressed patients (21, 23). The onset of sadness in healthy subjects triggered metabolic activation of the subgenual cingulate, and recovery from depression triggered the deactivation of the same area (22).

Anatomy and function of area 25: The subgenual cingulate cortex connects dorsal regions of the brain responsible for executive functioning and cognition with ventral regions of the brain that manage emotions and drives (22).

The subgenual cingulate cortex is metabolically overactive in depression (22).
In 2005 Mayberg also published the first human study of deep brain stimulation (DBS) in depression. DBS involves the surgical implantation of an electrode that can modulate the activity of a target region. Mayberg’s stimulatory target by DBS was the subgenual cingulate of depressed patients who were unresponsive to conventional treatments. She hypothesized that chronic stimulation of area 25, the subgenual cingulate cortex, would gradually reduce the metabolic overactivity in the system and ameliorate depressive symptoms. Although the exact mechanism by which deep brain stimulation can inhibit and normalize metabolic overactivity is still unknown, this proved to be the case in 4 out of 6 patients in the initial study and 60% of a group of 20 patients in a subsequent yearlong study (11, 19). This unprecedented work was among the first to use a circuit level approach to target symptoms of depression in patients who failed traditional therapies. Such research not only sheds light on the mechanism of depressive pathophysiology, but it also presents new and tractable therapeutic targets for the treatment of patients with previously refractory cases of depression.

New technologies, new insights:

Helen Mayberg’s research was an important first step forward in mapping the neural circuitry of depression, but we are still far from a holistic picture of the disorder. In order to expand the understanding that the subgenual cingulate is metabolically overactive and an important target in depression, we need to map the brain structural and functional properties of depression. This will help to further develop our understanding of the neural circuitry underlying depression. Recent advances in neuroimaging and bioengineering have the potential to greatly improve our understanding of how the brain functions in depression. In this thesis, I will present two new research studies that focus on characterizing the neural circuitry associated with depression. The first of these studies utilized a graph analysis approach on human structural neuroimaging data to broadly explore anomalous network topology, inclusive of regions such as the subgenual cingulate cortex, in the depressed brain. The second study follows by causally stimulating the region analogous to the subgenual cingulate cortex in a rat model using optogenetics. We used functional magnetic resonance imaging (MRI) to observe how an optogenetically driven circuit abnormality affects brainwide circuit activity. Though the studies have many technical differences, both seek to understand the global, whole-brain response to circuit abnormalities at the regional level. In light of this, the graph analysis and optogenetic
fMRI approaches are complementary methods for understanding how broader depressive pathophysiology is related smaller scale abnormalities.

**Graph theoretical analysis of structural neuroimaging data:**

Since its introduction to neuroscience, magnetic resonance imaging has been a powerful tool for delineating both the structural and functional characteristics of the brain. Imaging has also been used to probe the abnormalities associated with mental illness, however, most techniques fail to capture the complex nature of the brain. Graph theoretical analysis is a new way of interpreting structural magnetic resonance imaging (MRI) data in order to compare the global network characteristics of healthy and sick brains (24, 25).

*Flowchart for graph theoretical analysis: Graphs can be created from a variety of recordings and analyzed with a common graph theoretical framework. (25)*
Graph theoretical analysis models the brain as a complex network. According to this analytic approach, a network has two key properties: nodes, which are the constituents of the network, and edges, which are the relations that connect nodes to one another. In our first study, the nodes are defined as anatomically defined brain regions of interest, such as the subgenual cingulate cortex, amygdala, or hippocampus. A structural correlation between two regions of interest is designated as an edge. Networks can be examined in a variety of ways; however three important metrics are small-worldness, clustering, and path length. A small-world network is a particularly efficient network that balances local interconnectedness with global integration (26). Therefore, this network is associated with a high degree of clustering in which neighboring nodes are all bound by edges for rapid communication. Often, many nodes will communicate with highly connected local hubs, which connect to other hubs from other parts of the brain. This unique efficiency in both local and global information exchange translates into minimized path lengths, which are typical distances involved in connecting one node to another in the network (24, 25, and 26).

Major Depressive Disorder (MDD) is characterized by abnormalities in neural structure, function, and connectivity in several brain regions. Although previous studies have already used graph analysis for the study of depression, these studies were limited by samples sizes, assessed individuals in the early stages depression, and evaluated only functional networks (27). For the first time, we aimed to apply graph theoretical analysis to structural gray matter networks in a large sample of adults with histories of chronic and recurrent MDD.

We hypothesized that, compared to healthy controls, MDD subjects would show (1) significantly different global and regional network topology; (2) a reduced contribution of hubs to the network; and (3) differences in which nodes were acting as hubs in the network, especially in relevant regions like the subgenual cingulate cortex, amygdala, hippocampus, and dorsolateral prefrontal cortex (24, 28).

**Optogenetic functional MRI:**

Another area of rapid growth that holds much promise for neuroscience and psychiatry is bioengineering. Optogenetics is a powerful new bioengineering technology that involves the
Selective introduction of light sensitive ion channels in the cell membranes of neurons in order to make them controllable by light delivered into the brain by fiber optics (29, 30). Optogenetics is a revolutionary tool for neuroscience because it allows for the casual exploration of circuit function with cellular specificity and millisecond temporal precision. When combined with imaging, optogenetic functional MRI (ofMRI) becomes a powerful tool for simultaneous causal neuronal control and whole-brain monitoring of circuit activity (31).

Cell type specificity with optogenetics: Traditional electrical stimulatory techniques perturb multiple cell types in a given volume. Optogenetics can selectively excite or inhibit specific cell types (27).

A challenge in neuroscience has been the causal manipulation of specific neural targets while maintaining an unbiased observation of whole-brain activity (27). OfMRI is a new solution to this challenge for its dual ability to toggle the activation of neural circuits and measure blood oxygenation level dependent (BOLD) activation across the brain. This new technique will be used to explore the brain circuits associated with depression in a rat model, specifically targeting the ventromedial prefrontal cortex, an analogous structure of the human subgenual cingulate cortex (32).

Anhedonia, which is the loss of pleasure or interest in activities previously valued as rewarding, and reduction in social interaction, a related symptom, are two common depression related behaviors that are studied in the rodent model. Experiments have shown that anhedonia can be rescued in model depressed rats by deep brain stimulation (DBS) inhibition of the ventromedial prefrontal cortex (vmPFC), which suggests that that it plays a key role in regulating...
the reward-seeking behavioral circuit (32). We will activate and explore these circuits by optogenetically elevating the activity of the vmPFC in rats, while monitoring patterns of brain activity using resting state fMRI. A stable step function opsin (SSFO) will be used for this experiment, because the small but sustained depolarization that it induces may best mimic the vmPFC activity expressed in clinically depressed humans (33). Behavioral testing will be used to investigate the impact of increased vmPFC activity on sucrose preference, a well-established behavioral test for anhedonia, and social interaction. Using fMRI, the whole-brain response to vmPFC stimulation will be observed and studied for downstream circuit activation. This dual optogenetic and fMRI circuit analysis of depressive symptoms in a rat model has not been performed before.

This study describes the circuitry involved in depression using two complimentary techniques simultaneously. It is hypothesized that (1) increased activity of pyramidal neurons in vmPFC is sufficient to cause anhedonia and reduced social interaction in rats; and (2) optogenetic activation of the rat vmPFC will generate changes in patterns of BOLD activity across the brain, which can be used to characterize circuit abnormalities associated with the anhedonic/socially withdrawn phenotype.
IV. Methods

Graph theoretical analysis:

Participants and MRI acquisition:

The major depressive disorder (MDD) group was comprised of 93 adults. The healthy control (HC) group was comprised of 151 adults. The two groups were not significantly different in their gender composition. Recruitment of subjects was performed at outpatient psychiatric clinics and in the community. Participants were assessed by the Structured Clinical Interview for DSM-IV to establish diagnostic group status and by the Beck Depression Inventory-II to assess symptom severity (34, 35). Participants were screened for all medical conditions that are known to affect cognition and ability to participate in MRI. 51% of the MDD group was on psychotropic medication at the time their MRI scan was performed. All of the collected participant medical information is summarized in Table 1. This study was approved by the Stanford University Institutional Review Board and was performed only after obtaining informed consent from all participants.
<table>
<thead>
<tr>
<th>Variable</th>
<th>MDD</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (n)</td>
<td>64</td>
<td>96</td>
</tr>
<tr>
<td>Male (n)</td>
<td>29</td>
<td>55</td>
</tr>
<tr>
<td>Mean Age (SD)a</td>
<td>38.59 (11.71)</td>
<td>33.08 (10.53)</td>
</tr>
<tr>
<td>Mean BDI-II Score (SD)a</td>
<td>30.28 (10.17)</td>
<td>2.07 (2.94)</td>
</tr>
<tr>
<td>Severity of Current Episode, n (%)</td>
<td>85</td>
<td>NA</td>
</tr>
<tr>
<td>Mild-moderate</td>
<td>49 (57.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Severe</td>
<td>36 (42.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Mean Age of Onset in Years (SD)</td>
<td>18.26 (10.31)</td>
<td>NA</td>
</tr>
<tr>
<td>Number of Depression Recurrences, n (%)</td>
<td>74</td>
<td>NA</td>
</tr>
<tr>
<td>Single episode - 0 recurrences</td>
<td>4 (5.5)</td>
<td>NA</td>
</tr>
<tr>
<td>One recurrence</td>
<td>10 (13.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Two recurrences</td>
<td>7 (9.4)</td>
<td>NA</td>
</tr>
<tr>
<td>3+ recurrences</td>
<td>53 (71.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Comorbidities, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysthymia (double depression)</td>
<td>7 (7.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Panic disorder</td>
<td>6 (6.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Social phobia</td>
<td>11 (11.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Obsessive-compulsive disorder</td>
<td>3 (3.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Posttraumatic stress disorder</td>
<td>9 (9.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Current Medications, n (%)</td>
<td>91</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0 medications</td>
<td>45 (49)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1 medication</td>
<td>24 (26)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2 medications</td>
<td>11 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3+ medications</td>
<td>11 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Number of Weeks Exposed to Medications, Mean (SD)</td>
<td>13.6 (29)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 2: Participant demographics: BDI-II, Beck Depression Inventory-II; HC, healthy control; MDD, major depressive disorder; NA, not applicable. a: p = .001 (24).

MRI data was acquired at the Stanford University Lucas Center for Medical Imaging from three types of scanners: 1.5T GE Signa, 3T GE Signa, and 3T GE Discovery. Both groups contained equal proportions of each scanner type. In order to control for any scanner variance in our results, scanner type was used as a covariate in the analyses. The MRI images were acquired with the following parameters: spoiled gradient recall pulse sequences repetition time = 5.9 to
9.6, echo time = 1.1 to 3.4, flip angle = 11, 15, or 17, matrix = 256×256, field of view = 220 mm or 250 mm, voxel dimensions = .859 mm×.859 mm, and slice thickness = 1 mm to 1.8 mm.

Structural magnetic resonance imaging data preprocessing:

The Statistical Parametric Mapping 8 (SPM8) and Voxel-based Morphometry 8 (VBM8) toolboxes were used for MRI data preprocessing (Wellcome Department of Cognitive Neurology, London, United Kingdom). Segmentation was performed to extract gray matter tissue from white matter and cerebrospinal fluid. The gray matter segments were normalized to a template in Montreal Neurological Institute (MNI) space, using Template-O-Matic (36). Modulation was performed to correct shifts in gray matter volume ratios during normalization. Images were manually examined to ensure high quality. Nine HC and eight MDD images from the originally recruited sample were removed from the sample due to motion artifact distortion.

Region of interest extraction:

The Wake Forest University (WFU) PickAtlas Toolbox was used to divide the MRI images into 90 anatomically defined regions of interest (ROIs) (Appendix 1-1, 37). These regions were used to extract the gray matter volumes for each region from the normalized and modulated MRI images using the Region Extraction (REX) toolbox (http://web.mit.edu/swg/software.htm). Corrected gray matter volumes were acquired from the residuals of a covariate linear regression analysis with factors including age, gender, total brain volume, and scanner field strength (38).

Structural correlation networks creation:

A 90x90 matrix containing the Pearson correlation coefficient of the corrected gray matter ROI with one another was created (Figure 1). Each grid in this matrix represents the structural correlation of one ROI with another ROI across all participants (38). The diagonal elements in these matrices represent the correlation of a region with itself.
These correlation matrices were transformed into binary adjacency matrices by thresholding the correlation matrices at a value that ensured that no region was completely isolated. Therefore, the binary adjacency matrices represented a 90 node graph in which connections between nodes were represented as a value of 1. In order to test the sensitivity of the network to the thresholding value, analyses were performed at range of 12 thresholds, spanning from the 0.1 to 0.5 (38, 39). A threshold value of 0.1 corresponds to the minimum value required to ensure all nodes are connected in both networks.

**Global network analyses:**

First, the structural networks of the MDD and HC groups were analyzed for differences in their global features (Figure 5). Specifically, we analyzed three global metrics: small-worldness, clustering, and path length. The small-worldness (SW) metric is a function of clustering coefficient (C) and characteristic path length (L) relative to a random network. SW is calculated as $SW = (C/C_{null})/(L/L_{null})$. C, an indicator of node density, is a measure of the typical number of connections that exist between nodes. L, an indicator of network integration, is a measure of the typical shortest path length between all pairs of nodes in the network (25). $C_{null}$ and $L_{null}$ are calculated from the average of 20 random networks (26).
Regional network analyses:

Next, the MDD and HC groups were analyzed for network differences at a regional level (Figure 7 and 8). In each network, each node was examined for two metrics: betweenness and degree. Betweenness is the fraction of short paths in the network that involve the node. Regions with high betweenness are important to the network, because they typically connect other distant regions together (40). Degree is the number of paths, short or long, that involve the node, thus quantifying its network involvement. Hubs, which are highly connected information integrators in the network, are regions that have a degree higher than one standard deviation above the mean (25). These measurements were acquired with the minimum threshold required for a non-fragmented network.

Validating network comparisons between groups:

The statistical conclusions from the above analyses underwent a nonparametric permutation test in order to assess the sensitivity of the results to changes in the assignments of subjects to each group (41). 1000 new combinations of networks were created by randomly reassigning participants to different groups and repeating the graph creation and network analyses. The differences in network comparisons generated by these permutations were represented as percentiles in a permutation distribution (41, 42).

The global network measures (clustering, path length, and small-worldness) were plotted as functions of the threshold that was used to set network density (Figure 5). The relations of these metrics with network density in MDD and HC was explored through functional data analysis (FDA) (Figure 6). FDA is a sensitive method for comparing the shapes of the curves over a range of densities between groups (38, 43).

The Brain Connectivity Toolbox was used for network measure analysis, the Graph Analysis Toolbox was used for graph creation and comparisons, and the Brain Net Viewer (http://www.nitrc.org/projects/bnv/) was used to visualize the brain network results (44, 38).
Optogenetic Functional MRI:

*Animal preparation and targeting methods:*

18 juvenile, male, Sprague-Dawley rats were divided into 10 subject YFP control group and an 8 subject SSFO group. Represented in Figure 2, the fluorescently tagged opsin under the CamKII promoter (to target pyramidal neurons) was injected into the right vmPFC, a structure analogous to the subgenual cingulate cortex, with AAV5 vector, and a 300um optical fiber was surgically implanted at the same site (32, 45). The injection and stimulation site are shown in the sagittal view.

![CamKIIα SSFO YFP diagram](image)

**Figure 2 Stable step function opsin construct and injection site:** SSFO was tagged with YFP for posthumous histological study. Fiber site and injection site were both in the right vmPFC.

Posthumous histology was performed by brain slice acquisition and DAPI staining to observe YFP expression patterns near the vmPFC (Figure 9).
**Sucrose preference test:**

Sucrose preference testing was used to test anhedonic behavior (Figures 3 and 10). Non-depressed rodents typically prefer to drink sugar water over plain water (46). Over 12 days, both YFP control and SSFO rats were given the option of regular water and 1% sucrose water. Neither group received stimulation during the first two test days, both groups received stimulation during days 3-8, and neither group received stimulation days 9-12. Stimulation was delivered through 470nm blue light in a 5 second pulse at 6mW, and 4 x 5s stimulation bouts were administered over the 6 hour test period. Grams of sucrose and water consumed were measured over the test period. Sucrose preference was calculated by dividing grams of sucrose consumed over the sum of grams of sucrose and grams of regular water consumed.

![Sucrose preference test paradigm](image)

\[
sucrose \text{ preference} = \frac{\text{sucrose consumption (g)}}{\text{sucrose + water consumption}}
\]

**Figure 3 Sucrose preference test paradigm:** This was used to test changes in hedonic state due to vmPFC stimulation.

**Novel intruder test:**

In a preliminary investigation of social interaction, the novel intruder test was used to assess social behavior in YFP control (5 rats) and SSFO rats (4 rats) (Figure 11). Normally, rats will vigorously investigate intruder rats in their home cages, due to their social nature (33, 47).
Over a single day, rats were exposed to novel, male, juvenile intruders in three sessions: baseline, light and washout. In the baseline session, rats were given no stimulation. In the light session, rats were given a 5 second pulse of 470nm blue light. In the washout session, rats were given no light. During each session, social interaction (defined as sniffing, grooming, intentional contact, and fighting) was video monitored for 2 minutes. Sessions were separated by a two hour period of time. Video data was scored for social interaction by two blinded scorers and averaged.

Functional magnetic resonance imaging:

Two types of resting state scans were performed: 1) An "inactive" resting scan where no light was delivered to vmPFC, the rat simply rests in the scanner for the entire 5 minute scan 2) An "activated" scan in which a 5 s pulse of blue light was delivered to the vmPFC at the start of the scan, to increase the excitability of vmPFC for the duration of the 5 minute scan. Once the scan was complete, a 10 s pulse of yellow light was delivered to switch off the SSFO. The resting state and activated state fMRI scans were interleaved during a single fMRI scanning session (Figure 12, Figure 13). All scans were acquired on a 7T scanner using a spiral in-out pulse sequence. Rats were surgically implanted with an MRI-compatible head-fixation apparatus at the same time as they received their injection of the viral vector for SSFO and optical fiber implantation. Rats were trained in a 5-10 day habituation process for awake fMRI imaging.
Functional connectivity analysis was performed using Analysis of Functional Neuroimages (AFNI) (48). A seed region of interest, the vmPFC, was defined. The BOLD signal fluctuations over the course of the scans in the seed region were compared to the BOLD signal fluctuations across all other voxels of the brain, to identify and locate any changes in temporal correlation between the vmPFC and other brain regions during optical stimulation.
V. Results

Graph theoretical analysis:

Subject data:

The summary of participant demographics in Table 1 shows that the MDD and HC groups differed significantly in two ways. The MDD subjects were on average 5.51 years older than the HC subjects, and the MDD subjects on average scored 28.21 points higher on the Beck Depression Inventory-II than the HC group \((p<0.001)\). Although age across groups was different overall, there were no age differences among groups across the three scanners. Age was included as a covariate in the analysis.

Global network analyses:

The gray matter correlation matrices in Figure 1 were processed to create a structural connectivity graphs for both MDD and HC networks. The graphs allowed for the computation of the path length \((L)\), clustering coefficient \((C)\), and small-worldness \((SW)\). Throughout the range of tested densities, both networks exhibited a high clustering and similar path lengths relative to a random network. Therefore, networks in both groups behaved as small-world networks, balancing local connectedness with global integration. As network density increased, all metrics approach 1, meaning that they increasingly resembled random networks. The HC network was consistently a more small-world and highly clustered network over a range of network densities, as evidenced by Figure 5A and 5C. In contrast, Figure 5B shows that the MDD network had longer path lengths at intermediate network densities (0.2-0.3).
Figure 5 Global network measures over a range of network densities: A. At low network density, clustering is more evident in both networks in comparison to random networks. The HC network exhibits more clustering than the MDD network. B. At intermediate network density, the MDD network has longer characteristic path lengths. C. The HC network has a higher small-world index at the range of densities tested (24).
In order to statistically compare the global network measures of the two groups, the group differences over the thresholded ranges were calculated and plotted with the confidence intervals acquired from the non-parametric permutation analysis. This revealed that the group differences across all three metrics were statistically significant (Figure 6). Specifically, the MDD had significantly less clustering at low densities, significantly less small-worldness at intermediate densities, and significantly higher path lengths at intermediate densities.

Figure 6 Differential global network measures over a range of network densities: A. The MDD network has significantly less clustering than the HC network at densities below 0.2. B. Initially, the MDD network has significantly shorter path lengths than the HC network, but this trend reverses at intermediate densities. C. At 0.2-0.3, the MDD group has a significantly lower small-world index than the HC network (24).
FDA analysis of the global network measure curves confirmed some of the significant findings above. In the FDA analysis, clustering in the MDD network was significantly less than the clustering in the HC network (p = 0.01), but the other measures were not found to be significantly different. In summary, the analysis of global network differences revealed that the MDD network was significantly characterized by less clustering, longer path lengths, and a lower small-worldness index at some network densities. However, a more stringent FDA analysis revealed that the only significantly different network metric between the MDD and HC networks was clustering.

Regional network analyses:

Regional analyses of structural networks revealed differences in the MDD and HC groups that may mediate the global network findings already discussed (Figure 7, Figure 8). It was found that the MDD network had significantly higher nodal betweenness than the HC network in the R amygdale, R inferior frontal operculum, L medial orbitofrontal gyrus, and L middle temporal gyrus, and the MDD network had significantly lower betweenness in the L anterior cingulate, L superior orbitofrontal gyrus, L superior temporal gyrus, L superior temporal pole, L lingual gyrus, L fusiform gyrus, and R calcarine fissure. It was also found that the MDD network had significantly higher nodal degree than the HC network in the L supramarginal gyrus and R gyrus rectus, and the MDD network had significantly lower degree in the L posterior cingulate, L middle frontal, R superior frontal, L superior temporal pole, L putamen, and bilateral thalamus.
Figure 7 Regional betweenness and degree differences in MDD and HC nodes: Red regions correspond to nodes where the HC network had greater metric values than the MDD network. Blue regions correspond to nodes where the MDD network had greater metric values than the HC network. Heat map was constructed on ICBM152 surface, and metric values were taken at minimum network density (24).

Figure 8 shows a map of the hubs that were uniquely found to exist in a single group. The HC network contained 20 hubs in the frontal and motor areas of the brain. The MDD network contained 16 hubs in the medial frontal and medial temporal areas of the brain.

Figure 8 Unique network hubs: The hubs are mapped on ICBM152 surfaces. The left hemisphere is depicted on the left and right hemisphere is depicted on the right. Green spheres correspond to HC specific hubs (R middle cingulate, R/L inferior frontal triangular gyrus, R/L middle frontal gyrus, R/L superior orbitofrontal gyrus, R superior frontal gyrus, R supplemental motor area, and L superior temporal pole). Blue spheres correspond to MDD specific hubs (L middle orbitofrontal gyrus, R/L fusiform, R/L gyrus rectus, and L middle temporal gyrus). The sphere size corresponds to the degree of the hub (24).
Optogenetic functional MRI:

Optogenetic methodology:

Histological and electrophysiological data demonstrated targeting of vmPFC pyramidal neurons. Targeted tissue was rendered more sensitive to depolarization upon blue light activation, which can be reversed by yellow light inactivation (Figure 9, scale bar 1 mm). Similar expression patterns in other histological preparations from subject rats further validated the optogenetic targeting.

Figure 9 Histological and electrophysiological validation: YFP expression was targeted to the expected site, the vmPFC. In vivo anesthetized electrophysiological multi-unit recording: blue light pulse resulted in a sustained bout of increased neuronal firing, which was extinguished by yellow light.
Behavioral data:

In the sucrose preference test, during 6 test days of stimulation, a small but statistically significant decline in sucrose preference occurred in the SSFO group only (Figure 10, two-way ANOVA, p < 0.01). Sucrose preference declines in the SSFO group starting on the first day of stimulation and reached a stable value. Sucrose preference of the SSFO group rapidly recovered to normal levels the day after stimulation ended. Sucrose preference of the YFP control group remained fairly constant throughout the entire 12 days. Sucrose preference of the SSFO group otherwise matched that of the YFP group before and after the light period.

![Figure 10 Stimulation diminished sucrose preference only in the SSFO group.](image)

In the preliminary novel intruder test, only the SSFO group experienced a significant decline in social behavior during light stimulation (two-way ANOVA, p < 0.05, Figure 11). Baseline and washout values of social interaction time were not significantly different between YFP control and SSFO groups. Normal social interaction levels in the SSFO rats appeared to be rescued after opsin inactivation.
Figure 11 Stimulation diminished social interaction only in the SSFO group.

Functional magnetic resonance imaging:

Figure 12 shows the resting state t-score BOLD signal map (p< 0.01) for whole-brain connectivity of an SSFO injected rat without optogenetic stimulation. The greatest functional connectivity existed at the target site itself.

Figure 12 Resting state functional connectivity paradigm and data - no light stimulation.
In the opsin activated paradigm (Figure 13), rats were given a 5 s pulse of stimulatory blue light and then scanned. This rat displays an altered pattern of connectivity across the brain, with enhanced correlation at the fiber site as well as downstream regions such as the ventral striatum, septal region, anterior cingulate, amygdala, anterior prefrontal cortex, and hippocampus.

Figure 13 Opsin activated resting state functional connectivity - light stimulation: The ROIs shown correspond to regions of functional connectivity associated with the stimulated vmPFC.
VI. Discussion

Graph theoretical analysis:

This study was the first large scale graph theoretical analysis of gray matter structural data comparing healthy and depressed human subjects. We showed that both depressed and healthy individuals had small-world networks that efficiently balanced local connectedness with global integration (25, 26). However, we also found significant topological differences in how these networks were organized, at both the global and regional measurements. At the global level, we found that the MDD network was significantly less clustered than the HC network in both a differential analysis and a functional data analysis. Regional analyses suggested that lower clustering may be mediated by local abnormalities in the portions of the prefrontal cortex, striatum, and medial temporal cortex (including the amygdala) – areas that are known to play important roles in executive functioning, reward processing, and emotion (19, 49, and 50). Therefore, our study suggests that the lack of structural correlations between these brain regions and their neighbors may weaken the overall brain network and play a role in the manifestation of the pathophysiology of major depression.

Global network topological abnormalities:

The primary finding in the global network analysis was that the MDD network was characterized by significantly reduced clustering, according to both differential and functional data analyses. A reduction in clustering corresponds to an inability of nodes to form the appropriate closely linked “neighborhoods” necessary for structural and functional organization (51). This reduced network strength may translate into the symptoms that are associated with depression, such as anhedonia, sadness, and introversion. This conclusion is supported by research that has found abnormalities in the emotional and cognitive neural circuitry of depressed brains (19, 52). However, the research presented here is different from the majority of research studies in depression because the global network measures were not derived from the analysis of a single or small group of brain regions. Most of these studies are not only limited in the scope of their analyses, but they sometimes also generate contradictory results, possibly
stemming from differences in illness-related factors such as symptom severity or comorbidities (24, 53).

By analyzing the more subtle structural correlational network abnormalities associated with depression, perhaps a more consistent and sensitive classifier for major depression can be constructed. Such a classifier would not only offer a net set of causes for depressive pathophysiology but also potential diagnostic criteria and therapeutic targets. One day, a psychiatrist may use the graph analysis of patient’s structural and functional MRI images to help clinch a disorder diagnosis, determine the cause of the disorder, and tailor therapy to optimally target the individual’s unique pathophysiology. Until then, the reliability and validity of structural correlation network abnormalities in depression must be further explored, as elaborated below.

**Regional network topological abnormalities:**

The depressed structural correlation network was also significantly altered in its topology at the regional level. We explored the 90 brain regions included in our analysis and examined each region’s nodal betweenness and degree. These metrics refer to the connection of the brain region with other nodes and its contribution to the network at large. Nodes with higher betweenness and degree play a more important role in the structural links that comprise the network (54). We found that the MDD network contained fewer instances than normal of these highly connected nodes, as well as fewer network hubs (Figure 7, Figure 8). The MDD network had 14 nodes that were of lower betweenness or degree than normal and had 4 fewer unique hubs than the HC network. These findings support research that implicates the loss of cortical connectivity in regions such as the dorsal prefrontal cortex and putamen, a subregion of the striatum, with major depressive pathophysiology (55, 19). The widespread deficit of regional connectivity in the MDD network may function to mediate the global loss of clustering in depression that was discussed above.

Interestingly, the depressed network also contained 6 nodes that had higher betweenness or degree than normal, notably in the amygdala and ventral prefrontal cortex. Helen Mayberg has already characterized how dysregulation of the connections between the frontal cortex and limbic
system play an important role in depression (21, 22). At present, the relation between metabolic overactivity and anomalies in structural correlation networks are unclear and unexplored. If structural network topology forms a basis for metabolic and functional abnormalities, overallocation of structural correlations in these nodes may provide a new way to understand depressive pathophysiology. However, more research into the functional, behavioral, and metabolic correlates of structural network abnormalities must be undertaken before such conclusions can be made.

**Limitations and future directions:**

The present study has three limitations that should be noted. First, the study was a cross-sectional analysis. An open area of investigation is the evolution of the depressive circuit over time, shedding light on how network abnormalities relate to disease severity. Second, three different scanner types were used to acquire the structural data. It is probable that this introduced some variance into the results. However, the proportion of each scanner was distributed equally across the MDD and HC groups, and scanner field strength was included as a covariate in the analysis mitigating the influence of this confound on the results. Third, we were limited in our ability to manipulate neural circuitry in vivo in human subjects to develop causal inferences about their functions. A complementary animal model study of depression could combine causal stimulation, neuroimaging, graph analysis, and ex vivo tissue analysis to uncover some of the biological basis behind network metrics.

As with any new tool, graph theoretical analysis should be further studied to determine if it can be applied to neuroimaging data to yield meaningful scientific and clinical results. Specifically, graph analysis should be applied in subject groups of varying depression severity, as well as in groups with different psychiatric disorders, in order to explore the sensitivity and specificity of the tool. Even if graph analysis proves to be a useful tool for classifying large groups of subjects, cost may limit its widespread application as a diagnostic tool for individual patients. However, continued development of this analytical method, along with others, should be pursued in order to ascertain whether these techniques do in fact have enough clinical predictive power to play some role in the treatment of the hundreds of millions of people that suffer from depression.
Optogenetic functional MRI:

The first study described in this thesis showed that subregions of the prefrontal cortex, striatum, and limbic system were associated with depressive pathophysiology. This effect may be mediated by high betweenness of the medial prefrontal cortical region in the depressed network (24). However, it is difficult to know whether this abnormal neural circuitry is playing a causal or compensatory role in the brain without the help of more powerful experimental techniques. The second study showed that optogenetic stimulation of excitatory neurons in the rat vmPFC promoted behavior characteristic of depressive symptoms and altered the brain wide patterns of functional connectivity with the vmPFC in areas relevant to depression.

Behavioral data:

Optogenetic stimulation of the vmPFC resulted in a significant anhedonic phenotype, which was demonstrated by a significant reduction in sucrose preference and reduced social interaction, in a preliminary study (Figure 10, Figure 11). These findings not only validate the first study’s findings that the region is psychiatrically relevant in depression, but they also demonstrate the causal nature of a hyperactive medial prefrontal cortex in driving behavior consistent with symptoms of depression. In addition, the behavioral data indicates that the deficits induced by the stimulation of the vmPFC were reversible. For both the sucrose preference test and the social interaction test, the animals were retested after sufficient time had passed to demonstrate that the previously stimulated rats could regain normal functioning. This supported Helen Mayberg’s hypothesis that therapeutic amelioration of vmPFC overactivity through DBS can help patients with refractory depression (11, 18). Whether this circuit abnormality is a general feature of major depression or a subset of the depression cases and symptoms is a question that must still be explored. Nevertheless, it appears that vmPFC overactivation does play a causal role in the circuitry involved in the depressive symptoms of anhedonia and social withdrawal.

Functional magnetic resonance imaging:

Functional imaging revealed a unique whole-brain pattern of activation when the vmPFC was stimulated, suggesting changes in circuit based connections between these areas (Figure 13).
Increased correlated activity was observed in the anterior regions of the prefrontal cortex (prelimbic cortex and medial orbital cortex), the ventral striatum, septal regions, and amygdala. The functional connectivity between these brain regions and the activated vmPFC suggests that they may communicate as part of a neural circuit that manifest the depression related behaviors described above.

These regions have all historically been included as members of the limbic system, a complex and important collection of brain regions that control emotion, motivation, and behavior (56, 57). Within the limbic system, these regions have specialized circuit functions. The prelimbic cortex communicates with the amygdala to manage a host of emotional responses, including, fear (58). It is possible that dysregulation of the normal communication of these regions in the depressed brain may contribute to the feelings of negativity and anxiety that are associated with the disorder (34). These findings are especially interesting given the fact the graph theoretical analysis presented before also found abnormal network topology in the frontal cortex, amygdala, and striatum. It is difficult to draw clear connections between hyperconnected structural correlations and functional connectivity; nevertheless, the studies together suggest that depressive pathophysiology is characterized by both structural and functional brain abnormalities, across a variety of analytical measures.
Other structures that were found to be correlated with the overactive vmPFC are related to the motivation and reward circuitry of the brain. The reward circuit, which has been extensively studied, contains many interconnections between the frontal cortex, amygdala, striatum, ventral tegmental area, and hippocampus, which can be glutamatergic, GABAergic, or dopaminergic in nature (19). Thus, the functional connectivity observed between these regions and the activated vmPFC may be understood as a dysregulation of the reward circuit at large. One area of the striatum in particular, the nucleus accumbens, is known to be an important modulator of hedonic states (19). Despite the fact that its connection to depression driving regions is not fully understood, the nucleus accumbens has already been explored as a therapeutic target in deep brain stimulation studies. One study showed that DBS applied to the nucleus accumbens was able to improve symptoms of depression in half of patients who were otherwise treatment resistant (60). Using ofMRI, we were able to show an increase in correlation of BOLD signal fluctuations between the vmPFC and the nucleus accumbens when we increased

Figure 14 Combining graph analysis and optogenetic fMRI findings: Red boxes mark regions where the human MDD structural correlation network had higher betweenness. The blue box marks where the MDD network had lower degree. The black arrows indicate where functional connectivity occurred during stimulation of the rat vmPFC (24, 59).
the excitability of vmPFC pyramidal neurons using SSFO, thereby characterizing a relationship between vmPFC and this target. This finding may help elucidate the pathological process underlying these symptoms and how best to target or approach them for developing new therapies.

Limitations and future directions:

This optogenetic fMRI study of the vmPFC’s role in depression can be improved in several ways. First, the behavioral tests used only capture a few symptoms associated with major depression. It should be investigated whether the vmPFC stimulation can also bring about anxiety, helplessness, and cognitive deficits associated with depression, such as inability to concentrate and indecisiveness, in the rat model (3). This would help to delineate the precise role of the vmPFC in depressive pathophysiology. Currently, we are expanding our behavioral testing to include important control tests including locomotion, and novel object interaction data. We have also begun to explore if clozapine, a commonly prescribed human antipsychotic drug often used in the context of the negative symptoms of schizophrenia (many of which are in common with depression, e.g. anhedonia and reduced social interaction), can rescue the social behavioral deficits induced by our stimulation paradigm. Second, the use of the rat model allowed for causal and controlled testing that would not have been possible in humans, but it also somewhat inhibits the extrapolation of results to the human brain. The rat brain structures do not exactly match those found in a human, and the depressive symptoms that effect human patients must be modeled by approximation in the rat e.g. social withdrawal versus reduced social investigation. The use of behavioral tests that are less biased towards the species of the subject could be used to improve confidence in the translation of basic research into human clinical relevance (61). Third, the functional imaging data presented in this thesis was acquired from a single animal. In this animal, activation of other brain regions in response to stimulated vmPFC, such as the hippocampus, were initially observed but subsequently not seen. Therefore, it is an active goal to acquire group data for the fMRI portion of this study in order to improve confidence in the findings.

The combination of causal stimulation and brain wide functional connectivity analysis is a powerful way to investigate brain circuits. Optogenetics and fMRI are complementary tools for
probing the brain, and they should be utilized together to systematically deconstruct the circuitry of depression. The optogenetic methods in this study only targeted the glutamatergic neurons on the vmPFC, however GABAergic and dopaminergic projections also play important roles in the studied areas of the brain (19, 62). A full analysis of this circuit must include a characterization of the various projections and correlated regions of interest. The question of whether pathological behavior is segregated to particular projections or the broader interaction of multiple circuit components will vastly improve our understanding of depression. The hope is that such a deep understanding of the circuitry underlying depression may improve our ability to diagnose and treat the disorder.
VII. Conclusions

The findings presented in this thesis increase our understanding of the structural and functional neural circuit bases of depression at both the regional and whole brain level. The first study revealed that the reduced clustering and efficiency of the depressed structural correlation network may be driven by the loss of connectivity of brain regions in some areas and the hyperconnectivity of regions in other areas, such as the regions in the frontal cortex, striatum, and the amygdala. These abnormalities may mirror Helen Mayberg’s research of metabolic overactivity in the subgenual cingulate cortex, and they suggest that alterations of normal structural topology in the limbic system of the brain may mediate depressive pathophysiology. This conclusion was explored from a causal and functional point of view in the second study using optogenetics and fMRI. Investigation of the behavioral and whole-brain response to stimulation in the vmPFC revealed that overactivation could drive pathological behavior and alter the pattern of functional connectivity to the region. The altered pattern was characterized by increased BOLD correlation with structures in limbic and reward circuits. These results offer new insights into the neural circuit mechanisms that underlie major depression and expand upon critical areas of research that will eventually improve diagnostic and therapeutic techniques for the many people that suffer from depression.
VIII. Appendix

Graph theoretical analysis:

Appendix 1-1: Regions of Interest

The included regions were the left and right ACC, anterior cingulate; AMYG, amygdala; ANG, angular gyrus; CALC, calcarine fissure; CN, caudate nucleus; CUN, cuneus; FG, fusiform gyrus; HIPP, hippocampus; HSHL, Heschl's gyrus; IFOp, inferior frontal gyrus, opercular part; IFOr, inferior frontal gyrus, orbital part; IFTr, inferior frontal gyrus, triangular part; INS, insula; IOG, inferior occipital gyrus; IPL, inferior parietal lobule; ITG, inferior temporal gyrus; L, left hemisphere; LNG, lingual gyrus; MCC, mid-cingulate; MedFOr, medial frontal gyrus, orbital part; MedSF, superior frontal gyrus, medial part; MFG, middle frontal gyrus; MFOr, middle frontal gyrus, orbital part; MOG, middle occipital gyrus; MTG, middle temporal gyrus; MTP, middle temporal pole; OFB, olfactory cortex; PCC, posterior cingulate; PCL, paracentral lobule; PCUN, precuneus; PHIP, parahippocampal gyrus; PLD, lenticular nucleus, pallidum; PoCG, postcentral gyrus; PrCG, precentral gyrus; PUT, putamen; REC, gyrus rectus; RLN, rolandic operculum; SFG, superior frontal gyrus; SFOr, superior frontal gyrus, orbital part; SMA, supplementary motor area; SMG, supramarginal gyrus; SOG, superior occipital gyrus; SPL, superior parietal lobule; STG, superior temporal gyrus; STP, superior temporal pole; THL, thalamus.
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