An Investigation into the Neurobiology of Treatment Response in patients with Major Depression: The Placebo Effect.

by

Magdalena Sikora

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Doctoral Committee:

Professor Jon-Kar Zubieta, Chair
Research Assistant Professor Marta Peciña Iturbe
Professor Rachael D. Seidler
Assistant Professor Srijan Sen
Assistant Sekhar Chandra Sripada

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ABSTRACT

Recent trends in clinical neuroscience have moved toward identifying neurobiological predictors of antidepressant treatment effects in order to improve overall treatment efficacy in Major Depression, a pervasive and debilitating disorder in which complete remission occurs for only one-third of treatment-seeking patients. However, predictors of placebo effects have largely been overlooked. This is not a small concern: substantial placebo response rates have been documented within antidepressant clinical trials. Hence, neuroimaging predictors of placebo responses may elucidate the neural pathways responsible for depression recovery. Moreover, these predictors may identify patients with a greater susceptibility to placebo effects; in turn, informing patient stratification in antidepressant clinical trials to better distinguish between drug-specific and placebo effects or augment prescribed treatments for patients in clinical settings.

This dissertation takes a network-based resting-state functional connectivity (rsFC) approach to investigate predictors of placebo and antidepressant responses with particular focus on the default-mode, salience, and executive networks. This approach allows for consistency with the inherent network organization of the brain and the network-based characterization of depression. Through this investigation, enhanced rsFC of the rostral anterior cingulate (rACC) within the salience network has emerged as a strong predictor of responses to placebo with antidepressant expectations. Furthermore, heightened rACC rsFC within the salience network manifests as a neurobiological pattern differentiating healthy subjects from depressed patients. Finally, in light of evidence that genetic variability within placebo-related pathways modulate

placebo treatment outcomes in depression as well as analgesia, where neural and molecular bases of placebo have been extensively mapped, the final chapter of this dissertation observed an effect of genetic polymorphisms within the prepronociceptin gene, an endogenous opioid precursor neuropeptide associated with nociception and depression, on analgesic placebo-induced μ-opioid activation within the rACC and other well-established, placebo-related regions. This effect further corresponded with placebo-associated stress responses and anxiety.

These findings enlighten our understanding of the neurobiology behind depression recovery through placebo effects and illustrate the importance of the rACC within antidepressant responses and healthy functioning. Finally, they contribute to a growing database of potential clinical neuroimaging and genetic markers of placebo responses which may substantially benefit therapeutic care in depression.

Chapter One

Introduction

A major goal in translational research is to improve treatment outcome and treatment efficacy for individual patients. This is particularly relevant for patients with Major Depressive Disorder (MDD), a pervasive and debilitating disorder which afflicts nearly one-fifth of the population at least once throughout a lifetime (1). The disorder predominately manifests with episodes of persistent negative mood (feeling down, sad, or hopeless) often concurrent with anhedonia (lack of feeling, or motivation for, pleasure), although the latter symptom may exist independently from a negative mood state and still be labeled as a depressive episode. Additional emotional, psychological and somatic symptoms also often occur. While the neurobiological underpinnings of such alterations from healthy functioning are still largely unknown, MDD is considered a network-based disorder (2), which targets specific brain regions (3, 4) and involves major neurotransmitter systems (5, 6), resulting in profound cognitive and affective dysfunction (7, 8).

Currently, no definitive factor has been identified to cause or induce MDD within a patient. Instead, a combination and interplay of genetic and environmental risk-factors appear to contribute to disease manifestation: specifically, genetic heritability (9), personality traits such as neuroticism (10), or traumatic experiences or circumstances (11). The complex nature of the

disease is further complicated by the lack of efficacy of standard first-line treatments for MDD: complete remission after antidepressant medication or psychotherapy occurs only in a third of all patients seeking treatment (12). In general, the response rate – defined as a clinically meaningful reduction in depressive symptoms from baseline (~50%) – to either modality for standard antidepressant treatment is around 60%. Still, response to treatment, compared to absolute remission from symptoms, is associated with continued symptoms of impairment, and greater risk for relapse, episode recurrence, and suicide (13). Unfortunately, nearly a fifth of depressed patients may not demonstrate substantial, if any, improvement even after multiple, often intensive treatments (14).

Translational neuroscience research has the potential to improve treatment outcome in MDD by investigating the neural basis of antidepressant treatment effects within patients who recover from depression. This recovery may stem from particular treatments such as antidepressant medication or psychotherapy, or it may result from non-specific treatment effects, such as the placebo effect, which derives from active neurobiological processes induced by expectations and therapeutic environmental cues (15). Overall, this research may benefit treatment efficacy for patients with MDD by elucidating the neurobiology involved in depression recovery and possibly specifying neurobiological targets for future drugs or other interventions. Moreover, this may also inform neurobiological predictors of treatment response to currently available treatments. Ultimately, using baseline markers to select optimal treatments for a given patient may amend treatment outcomes comprehensively: primarily, by reducing the duration until the onset of a treatment response. This response is often patient-specific, yet clinical antidepressant protocols are based on average outcomes from large-scale clinical datasets (16); hence, current practice often leads to experimentation with multiple drug or intervention types

until a patient finally achieves a clinically meaningful response. Furthermore, this contributes to a decreased likelihood of remission after each treatment attempt (14% and 13% after third and fourth attempts versus 37% and 31% after first and second phase of treatment, respectively) (12). Therefore, a better grasp of the biological markers associated with treatment response may enable an earlier achievement of response. Altogether, improvement in antidepressant treatment efficacy may result in substantially diminished costs of treating depression: The financial burden of MDD-induced disability and related treatment totaled $210 billion in the United States alone in 2010 (17). Nearly half of this was attributed to treatment for MDD and comorbid disorders like anxiety, largely due to prolonged antidepressant treatment duration and unsuccessful antidepressant drug clinical trials (18).

We propose that investigating the neurobiology of treatment effects in depression in order to uncover the associated mechanisms responsible for depression recovery will have a substantial impact on treatment efficacy for MDD. Insights gained will have the potential to inform future treatment targets within patients, either by predicting the ideal treatment modality for individual patients or developing novel methods with antidepressant effects. This personalized medicine approach is particularly important for diseases such as MDD, for which multiple etiophathological mechanisms are believed to produce a similar clinical phenotype (19). For instance, although effective, targeting specific regions known to function abnormally within the disease, such as the dorsolateral prefrontal cortex (dlPFC) (20, 21), as in transcranial magnetic stimulation (TMS) (22), or the subgenual anterior cingulate (sgACC) (23), as in deep brain stimulation (DBS) (24), does not result in absolute remission across all subjects. At the same time, diffuse treatments such as serotonin reuptake inhibitors (SSRIs) or electroconvulsive therapy (ECT) also do not provide consistent, reliable treatment effects across patients (14).

Therefore, mapping the neurobiology associated with general MDD recovery or with a particular antidepressant modality may identify neural mechanisms necessitating a successful treatment response. Concurrently, this may enhance our ability to stratify patients according to their biologically optimal treatments. Here, we describe important findings from studies investigating neurobiological antidepressant treatment effects and how they have contributed to our understanding of the neural basis of MDD. Moreover, these treatment effects also stem from non-specific effects of treatment administration, notably the placebo effect, which has consistently been implicated in antidepressant treatment and its pharmacological development (18, 25, 26). Thus, we describe the significance of the placebo response in treating patients with depression; specifically, the existing knowledge of the neural mechanisms associated with this clinically meaningful treatment effect.

Neurobiology of Treatment Response and Disease

Examining the neurobiology behind the antidepressant response has elucidated the major theory behind the disorder: that it materializes from a dysregulation of cortical and limbic networks (27). In a landmark study using positron emission tomography (PET) and measures of glucose metabolism in a group of depressed men receiving fluoxetine, a standard SSRI antidepressant, Mayberg et al. (28) demonstrated that after six weeks of administered treatment, response was associated with decreases in limbic-paralimbic and striatal regions – sgACC, anterior insula, thalamus, hippocampus, striatum – and increases in brainstem and cortical regions – prefrontal, anterior and posterior cingulate cortex, and the inferior parietal cortex. This pattern differentiated responders from non-responders. While both groups demonstrated identical glucose metabolism changes after one week of antidepressant treatment – brainstem and hippocampal increases; striatal and posterior cingulate cortex decreases – ultimately, non-

responders demonstrated a continuation of this pattern and an absence of sgACC metabolism decreases and concurrent prefrontal metabolism increases as observed within the responders (28). This initial brainstem and hippocampal change followed by eventual metabolic cortical changes is in line with antidepressant medication effects outlined in rodent models (29). Furthermore, this general finding of treatment-induced enhancement of cortical activity and diminished limbic activity is consistent across medications (30, 31) and has relevance across treatment modalities: A similar pattern of decreased sgACC metabolism and increased metabolism within frontal, executive regions like the dlPFC was also observed within depressed patients who were responsive to DBS of the sgACC (24). On the other hand, implementation of psychotherapy demonstrates an overlap, but also a unique set of changes in brain function compared with medication. Specifically, cognitive behavioral therapy (CBT) is associated with metabolic increases within the hippocampus and dorsal cingulate, yet with reductions within prefrontal dorsal, ventral, and medial regions (32). In another study, both CBT and medication induced a reduction in the dorsomedial and lateral orbital prefrontal cortex, however, decreases in glucose metabolism within a posterior portion of the sgACC was specific to venlafaxine, a serotonin norepinephrine reuptake inhibitor (33).

These findings have support within the literature of MDD neurobiology. Generally, the most consistent finding is decreased frontal lobe function, localized mostly within the dorsolateral, ventrolateral, and orbital prefrontal cortex (34). Opposite patterns have also been observed, which may speak to a separate disease etiology or possibly, compensatory mechanisms resulting from MDD-related deficits in functioning (27). Concurrently, brain regions which drive mood and interoceptive states such as the sgACC, anterior insula, and hippocampus, as well as regions associated with the monitoring of salient, mood-related stimuli such as the thalamus and

amygdala seem to show greater reactivity in MDD (23, 35-38). Particularly, the sgACC is an important region – consistently hyperactive in depression (23, 24) which subsequently, best characterizes treatment-resistant depression (33). Furthermore, normalization of its excessive activity is associated with response to first-line treatments like medication or from more intensive treatments such as DBS (24, 33, 39). Broadly, the sgACC is linked with regulating negative emotional states: its activity is associated with active induction of negative mood via retrieval of sad, autobiographical memories, or via pharmacology (40, 41), or via passive exposure to sad or unpleasant stimuli (42).

Midline cortical brain regions are critical for mediating the relationship between diminished frontal lobe functioning and enhanced limbic activity. Chiefly, these regions are responsible for incorporating self-referential value into the processing of incoming stimuli, regulating limbic-driven emotional and mood states, and relaying this information to executive cortical regions, as well as the reciprocal communication pathway. Included regions are the medial prefrontal cortex (mPFC), the dorsal (dACC) and rostral ACC (rACC). The rACC is a region of consequence in major depression, as successful, sustained antidepressant response is associated with hyper-metabolism in the region across treatments (31, 32); while diminished metabolism associates with non-response (43); and pretreatment rACC activity is a common indicator of eventual treatment response (44). It also mediates the interaction between the mPFC and the more primitive, limbic-driven amygdala during self-referential processing of negative stimuli; this latter pattern has been shown to be excessive in patients with depression relative to healthy subjects (45) and a classic characteristic of depressive neurobiology [reviewed (46)].

Placebo Effects in Major Depression

Remarkably, high and growing rates of placebo responses occur within MDD (47), substantially hindering development of novel therapeutics and predictors of treatment response. This failure to differentiate placebo from antidepressant responses has caused large pharmaceutical companies to reduce or discontinue research focused on antidepressant treatment (48). In light of this, several strategies have been implemented to improve sensitivity of treatment-specific effects in clinical trials, primarily by reducing the number of placebo responders that enter a clinical trial and thus, the overall placebo response rate: including the use of placebo lead-in phases or sequential parallel comparison designs (49). One major limitation of these approaches is that results are ultimately obtained from placebo non-responders, which may not be generalizable to all patients with depression. Moreover, placebo responders may require lower doses of antidepressant treatment or no active treatment, which would contribute to a comprehensive reduction of the overall cost (50) and risk (51) of using antidepressants. Furthermore, ratings scales used to measure both placebo and antidepressant effects are imprecise and highly influenced by other factors, such as regression to the mean or natural fluctuation of symptoms, making the evaluation of treatment effects more nebulous. As a consequence, placebo effects continue to be a great barrier to the development of novel and successful therapeutics.

In contrast, since the neurobiology of placebo analgesia was discovered 35 years ago (52), neuroscientists have made substantial headway in identifying the cognitive, neural and molecular bases of placebo effects (53-57) in the field of pain; inadvertently, describing important pathways involved in homeostatic, stress, and reward responses. By contrast, only two existing studies have investigated the neural basis of placebo effects as they relate to depression (58, 59).

Still, the findings have proven to be consequential: Mayberg's initial work describing glucose metabolism changes in response to antidepressant treatment also demonstrated a localized overlap in glucose metabolism changes within placebo *and* antidepressant responders. Namely, increases in cortical areas and decreases in limbic areas following six weeks of fluoxetine treatment. Increases in brainstem metabolism and decreases in the anterior insula, striatum and hippocampus were specific to drug responders (59). However, after one week of treatment, eventual drug and placebo responders demonstrated a unique, shared pattern of glucose metabolism increases within the ventral striatum and orbitofrontal cortex, regions implicated in selection and expectations of rewarding stimuli. This neural pattern is theorized to underlie beneficial anticipatory, non-specific effects of treatment which lend to the placebo and overall antidepressant response (60).

The convergence of regional changes in brain function across responders to placebo and antidepressant treatment is imperative. As previously mentioned, placebo effects are consistently apparent in antidepressant randomized clinical trials (61), however, they also emerge across alternative treatments such as CBT (62, 63) or sham ECT (64). More specifically, within antidepressant clinical trials, placebo response rates are variable and range from 30-45% compared to around 50% for pharmacological drugs. In the last 30 years, the rate of placebo responses has been steadily increasing, nearly 10% each decade (47, 65). While these high, variable rates of placebo response have hindered rates of successful drug clinical trials –nearly half of all trials of approved antidepressants fail to show statistically significant separation between drug and placebo arms (66) – they also point to the therapeutic currency of non-specific effects of treatment. Incredibly, the placebo response arises from the psychosocial context in

which treatments are administered and manifests from a set of active neurobiological processes created from a set of expectations and conditioning within the therapeutic environment (15).

This capacity to engage neurophysiological mechanisms as a result of cognitive and emotional factors from a particular treatment and its environmental context represents a domain with great promise to inform our understanding of the neurobiological processes involved in resiliency to illnesses, such as depression (67). Within these mechanisms, there is substantial inter-individual variability as reflected in the range of placebo response rates (47), antidepressant response rates (14), and the nature of each individual patient's characteristic treatment response – for instance, psychotherapy is more beneficial for some patients than medication (68, 69). This variability is further supported by distinct patterns of brain metabolism after SSRI treatment versus psychotherapy (32, 59). However, a convergence in resiliency mechanisms does exist: effects of placebo and antidepressant medications share patterns of brain activity changes, as described above (59). It is likely that an interaction between drug and placebo mechanisms exists and contributes to the complete antidepressant response. However, further work is essential to disentangle treatment-specific from non-specific effects to better define the neural basis of resiliency to depression, which manifests in a reduction of depressive symptoms. Moreover, biological predictors of placebo responses and the nature of their modulation within a clinical response may also further inform our understanding of the neurobiological cognitive machinery responsible for ameliorating depressive symptoms as well as its variable nature between patients.

Neurobiology of the Placebo Effect

As briefly outlined above, substantial progress has been made in the field of placebo analgesia in defining the neural circuitry involved in placebo responses. Many of the findings from this research correspond with placebo-associated brain regions and neurotransmission in

Parkinson's disease (56, 70). Circumspectly, the neural circuit described to form the basis of placebo responses includes the rACC, dorsolateral and orbitofrontal cortex, insula, nucleus accumbens (NAc), amygdala, thalamus and periaqueductal grey (PAG). Dopaminergic and opioid neurotransmission in these regions have been shown to modulate certain aspects of the placebo analgesic response including expectations (71), reward saliency of the placebo (55), and changes in experience of affective and pain intensity (72, 73).

Over three decades ago, the μ-opioid neurotransmitter system, implicated in homeostatic (74), stress and reward mechanisms (75), was linked to the placebo response from an observation that placebo analgesic effects were blocked by naloxone, an antagonist with high affinity for μ-opioid receptors (MORs) (52). Evidence for this connection was furthered in a pivotal study in which PET imaging of regional blood flow was compared between the analgesic effects from administration of a placebo with expectations of analgesia and a fast-acting opioid analgesic, remifentanil: a convergence of increased blood flow within the rACC and PAG was observed across placebo and opioid analgesic administration (57). Remarkably, the rACC, implicated in self-referential executive and affective cognition, projects directly to the PAG, a primary modulator of pain signaling (76).

From our laboratory, more direct evidence of placebo-induced μ-opioid neurotransmission has been found utilizing PET and a selective μ-opioid radiotracer [^{11}C] carfentanil in conjunction with a pain challenge and placebo administration with expectations of analgesic effects: Placebo administration was significantly associated with endogenous μ-opioid activation within the rACC, sgACC, anterior insula (aINS), and the NAc (73). This activation also corresponded with participants' self-reported reductions of the physical and emotional elements of pain. In contrast, observed placebo-induced dlPFC opioid activation was negatively

related to the expectations of potential analgesic effects. This latter relationship was further developed with the finding that increased opioid activation within the region corresponded with higher expectations, but not with actual analgesic effects (71). Therefore, the region appears to be involved with the anticipatory component of a placebo response, which has been substantiated within a functional Magnetic Resonance Imaging (fMRI)-based placebo paradigm (72). A subsequent study utilizing the same, but more controlled, pain and placebo challenge demonstrated placebo-induced opioid activation within similar regions: rACC, sgACC, aINS, and the NAc. Additional activation was also observed within the orbitofrontal cortex, posterior INS, thalamus, amygdala, and PAG. More specifically, analgesic expectations prior and during administration of placebo associated with the NAc, PAG, and amygdala, while activity within the rACC and NAc corresponded to the experience of pain intensity.

The cognitive aspect of placebo responses also informs our understanding of its associated neural pathways. Placebo responses have been hypothesized to materialize primarily from expectations of the treatment effect or, in contrast, from prior conditioned learning about the treatment's effects. However, extant studies have found evidence to refute both theories [reviewed (15)]. Instead, recent work in our laboratory has demonstrated evidence for a Bayesian learning framework in which both types of processes are aptly incorporated. Here, placebo responses emerge as a consequence of therapeutic expectations from verbal and environmental cues and eventual outcomes, also partially influenced by conditioned learning. A pain and placebo challenge documented a lack of relationship between placebo response and expectations of analgesic effects. Instead, placebo responses were associated with a prediction error: individuals with the largest discrepancy between their expectations of analgesic effects and their actual perceived experience (perceived outcome>expectations) demonstrated the greatest placebo

responses. Prediction error was also associated with placebo-induced opioid activation in established placebo analgesic brain regions (ACC, insula, amygdala, thalamus) (71).

Placebo Effect: Reward

Dopamine neurotransmission has also been implicated within placebo neurobiology (53, 55, 56, 70). In analgesia, PET imaging with the selective dopamine D2/D3 radiotracer, [^{11}C] raclopride has shown that placebo administration with expectations of analgesic effects is associated with dopamine activation within the mesolimbic dopamine terminal, specifically, the caudate, putamen and NAc. Moreover, placebo-induced NAc dopamine activation positively correlated with the magnitude of opioid activation in the same region, putamen, amygdala, anterior and posterior INS, and the rACC. This is consistent with the hypothesis that NAc dopamine activation initially detects the saliency and reward value of the placebo and triggers downstream opioidergic activation (53, 77). Furthermore, greater reward-related fMRI blood-oxygen-level-dependent (BOLD) activity within the NAc has been found to significantly associate with increases in placebo-induced dopamine activation in the same region and placebo analgesic responses (55).

Of interest, deactivation of opioid and dopamine neurotransmission within participants who experienced a nocebo response or a worsening in their nociceptive experience after placebo administration has also been documented (53).

Placebo Effect: Personality Prediction

Investigation into placebo-related personality traits may be an accessible avenue to substantially improve clinical practice and clinical trials by identifying patients more susceptible to placebo effects; however, further work is necessary to incorporate these traits as their

predictive nature is influenced by other factors within a treatment context (78). Still, work in our laboratory observed that subjects with high levels of ego-resiliency, altruism and straightforwardness and low levels of anger and hostility showed greater placebo responses along with greater placebo-induced μ-opioid activation within well-documented placebo-related regions: ACC, orbital frontal cortex, INS, NAc, amygdala and PAG (79).

Placebo Effect: Genetics and Prediction of Placebo Response

Genetic markers are major candidate predictors of placebo responses, as recent work describes that the predisposition to respond to placebo may be, in part, a stable heritable trait. Mounting evidence has described genetic variation to modify placebo-associated pathways and in turn, placebo effects in depression, as well as in pain and other disorders [reviewed (80)]. Therefore, genetic screening may be a valuable tool in predicting placebo responses in patients with MDD and for further investigation of the neurobiology behind the placebo response. Evidence from studies analyzing placebo treatment across disorders has uncovered associations between placebo responses and genetic variation within important pathways involving dopaminergic, opioid, cannabinoid and serotonergic synthesis and signaling (54, 81-83). Specifically, work from our laboratory has provided evidence of genetic variation within the μ-opioid receptor gene (*OPRM1*) to influence analgesic placebo-induced dopaminergic activation (82). Examination of genetic variation has also uncovered involvement of the endocannabinoid system in μ-opioid-mediated placebo analgesia. Participants homozygous for the *FAAH* Pro129 allele – a functional missense variant of the *fatty acid amid hydrolase (FAAH)* gene encoding the major endocannabinoid degrading enzyme, who exhibit lower levels of endocannabinoids due to increased FAAH activity, reported higher placebo analgesic responses and placebo-induced opioid activation within the prefrontal cortex, ACC, INS, hippocampus, NAc and thalamus (54).

Antagonism of the system has also been shown to block non-opioid mediated placebo responses (84).

Interestingly, genetic markers implicated in dopaminergic and serotonergic pathways have been implicated in placebo responses. The variation within the gene for the monoamine oxidase A enzyme (*MAO-A*), involved in MDD through its essential role in metabolizing serotonin and dopamine, major players in MDD neuropathophysiology (85, 86), demonstrates an association with antidepressant placebo treatment: Individuals with low-activity of MAO-A, and therefore, higher dopaminergic and serotonergic tone, showed significantly higher antidepressant placebo responses than their genetic counterparts with highly active MAO-A (87). Across disorders, genetic variation within the gene encoding the major dopamine metabolizing enzyme, catechol-O-methyltransferase (*COMT*) also modulates placebo effects in patients with irritable bowel syndrome: those carrying the 'met' allele, who show reduced enzymatic activity and hence, increased prefrontal dopamine levels, were placebo responders, unlike their genetic counterparts who were unresponsive to placebo treatment (81).

Placebo Neurobiology from Analgesia to Major Depression

Regardless of the major potential contributions which may result from the examination of placebo neurobiology, this type of research in patients with MDD is currently limited (59). Moreover, our recent work has demonstrated the significance of placebo responses in antidepressant treatment: reductions in depressive symptoms in response to one-week of placebo administration with expectations of antidepressant effects were associated with greater placebo-induced activation of μ-opioid neurotransmission in the sgACC, NAc, thalamus, and amygdala (88). Moreover, greater placebo-induced endogenous opioid release was associated with better response to subsequent administration of antidepressant medication. Not in the least, the

observed placebo-related opioid neurotransmission occurred in regions also involved in MDD disease pathology (46), and a network responsible for regulating stress and emotion (89, 90) and overlap with those involved in placebo analgesia neural circuitry (67). Overall, this supports a tight interplay between placebo and antidepressant treatment effects within a clinical response in patients with MDD.

Altogether, the placebo response is a complicated neurobiological process which recruits multiple cognitive and emotional processes across brain regions and neurotransmitter systems. Given the intimate nature of the opioid and dopamine systems described in the neural basis of placebo analgesia, placebo mechanisms are associated with pathways related to homeostatic responses to stressful stimuli, and saliency and evaluation of rewarding stimuli. Similar mechanisms are also believed to underlie a substantial portion of MDD pathophysiology: abnormal homeostatic functioning after exposure to or interaction with a stressful or fearful stimuli, generally, with emphasized negative saliency (14), as well as abnormal reward processing are believed to contribute to symptoms of apathy and anhedonia (91). Therefore, investigating placebo neurobiology as it relates to depression may inform and eventually capitalize upon the neurological resiliency mechanisms which combat depression or the general mechanisms responsible for addressing any major stressors on the organism (77). Moreover, greater detail regarding MDD placebo neurobiology and its predictors, whether they are brain patterns obtained from clinical neuroimaging or genetic markers, can provide critical insight toward the improvement of clinical practice by identifying patients more susceptible to placebo effects and toward enhancing our understanding of the neurobiology behind recovery from depression.

Future Directions for Major Depression Treatment: Predictors of Treatment-Specific and Non-Specific Effects

Investigation of structural and functional predictors of treatment response in MDD is a nascent field with vast potential to improve antidepressant treatment outcomes. Functionally, and most consistently, the aINS and rACC are strong predictors of treatment response. While the insula demonstrates some conflicting findings *(92-94),* there is strong evidence the region may act as a treatment selector between those of varying levels of invasiveness (95). The rACC is an important predictor of treatment response [reviewed (96)]; increased pretreatment activity across imaging modalities – PET measuring regional blood flow and glucose metabolism, electroencephalography (EEG), single-photon emission tomography (SPECT), and resting and task-based fMRI – in this region is significantly associated with better response to many types of antidepressant treatments like medications, sleep deprivation, and repetitive TMS. Specifically, responders to antidepressant medication demonstrate enhanced metabolism within the rACC in relation to healthy controls. Moreover, this pattern differentiates responders from non-responders, with the latter group exhibiting diminished metabolism within the rACC in comparison to healthy controls (44).

Structural predictors of treatment response have also been identified. Reduced grey matter in the right hippocampus and the left dlPFC are both indicative of a poor response to antidepressant medication (97); whereas, data from nearly 200 patients with depression show that larger left hippocampal volume is predictive of a beneficial response to antidepressant medication (98). Thus, grey matter integrity within the hippocampus and dlPFC, two major regions involved within the pathology of MDD (99) is a potential biomarker of treatment response. Larger grey matter volume within the rACC and the insula has also been reported to

predict better treatment outcome with medication (100). Moreover, more substantive white matter integrity of the left cingulum bundle, or anterior cingulate-limbic white matter, is also significantly predictive of remission from antidepressant medication (101).

On the other hand, one of the most consistent predictors of non-response to standard treatments is heightened metabolic activity of the sgACC: this is valid for antidepressant drugs as well as psychotherapy (102, 103). This is of interest, as DBS within this region produces significant recovery in patients with treatment refractory MDD (24). Moreover, increased efficacy of rTMS has been associated with the extent to which the selected area for magnet placement, within the dlPFC, anti-correlates with the sgACC (104). Identification of predictors of non-response to treatment can assist in streamlining patients to more invasive treatments immediately after seeking treatment. This is with the hope that years of non-response to more standard treatments would be averted.

Alternative predictors may stem from personality traits or more stable, genetic variants. However, familial studies demonstrate that antidepressant treatment response is highly variable (105). Furthermore, genetic variants are known to interact with environmental factors or life events which predispose individuals to MDD and may influence responses to SSRIs (106, 107). While no consistent genetic marker exists in treatment response prediction, variations within the promoter region of *MAO-A* have been observed to significantly associate with antidepressant treatment response in females (108). In addition, genetic variations within the tryptophan hydroxylase 1 gene (*TPH1*), involved in serotonin synthesis (109), and brain-derived neurotrophic factor gene (*BDNF*), involved in behavioral antidepressant effects (110), have demonstrated a significant association with antidepressant treatment response [reviewed (111)].

Notably, the same functional *BNDF* 66Val/Met polymorphism exhibits an effect on dopamine activation in response to placebo administration with analgesic expectations (112).

Predictors of Non-Specific Treatment Effects: The Placebo Effect

Studies which have investigated predictors of antidepressant treatment response have implemented an open-label design which overlooks the contribution of the placebo effect within the antidepressant treatment response. This is of consequence since similar regions found to associate with the prediction of and involvement in the antidepressant treatment response – the rACC, insula, and dlPFC – have also been consistently described in the neurobiology of placebo analgesia. The rostral ACC demonstrates a pivotal, intricate role within the formation of placebo responses by recruiting and modulating subcortical anti-nociceptive regions, like the PAG, during placebo-induced analgesia (113). Moreover, in addition to fMRI BOLD activity, PET-based opioid and regional blood flow have all independently documented the region's critical involvement in placebo analgesic effects (57, 72, 73). Further emphasizing its capability to moderate such a comprehensive mechanism, the rACC is involved in regulating self-referential emotional, cognitive, and reward processes (45, 114, 115). In connection to MDD, this key placebo region often demonstrates aberrant activity within depressed patients, in isolation or within the context of rewarding or negative stimuli (91, 116, 117); not in the least, is its consistent emergence from several meta-analyses as a neural predictor of antidepressant treatment response (96, 97).

Moreover, the opioid system, intimately related to placebo analgesia, is an emerging target for antidepressant treatment (118). In parallel, the dopamine system has long been implicated in the pathophysiology and treatment of major depression. Major antidepressant medications influence dopamine neurotransmission and the system is believed to be a primary

driver of a subset of MDD etiologies (86). Abnormalities within the same reward circuitry implicated in placebo analgesia (55, 67) have also been described as a major facet of MDD neuropathophysiology [reviewed (119)]. Still, the neurobiology of placebo in depression has been largely discounted, despite the mounting convergence of evidence across neural pathways associated with placebo and MDD neurobiology. Overall, these findings illustrate the profound import placebo neurobiology may have in depression. The main objective of this dissertation is to investigate predictors of antidepressant treatment effects, primarily, predictors of placebo effects. Specifically, we are interested in clinical neuroimaging-based predictors because they provide insight into the neural basis of depression recovery as well as provide an objective, clinical marker, impermeable to patient or clinician biases or other confounds. Additionally, we are interested in clinical neuroimaging predictors under genetic influence to contribute to the growing 'placebome' [the group of genes, including their related pathways and molecules, hypothesized to affect an individual's response to placebo treatment (80)] and further uncovering the neural correlates of placebo responses.

Our primary focus on placebo responses is of relevance as current literature has largely ignored placebo effects inherent within overall antidepressant effects when examining predictors of treatment outcome. It is feasible that many predictors are capturing the placebo response or an interaction between the active treatment and the placebo. Differentiating treatment specific from non-specific effects may substantially inform future treatment targets and better define the neural basis of depression recovery. Moreover, identifying predictors of placebo response is critical to improve clinical practice and clinical trials for novel pharmacological agents: patients with greater likelihood of responding to placebo may benefit from lower dosages of medication, cognitive-based therapy, or enhanced patient-clinician interactions. They may also be ideal

candidates to investigate placebo and treatment-specific interactions. Alternatively, they may be separated from likely placebo non-responders to enable examination of drug effects in the absence of a placebo response, which would define drug effects in a specific subset of depressed patients.

While we are focused on clinical neuroimaging and related genetic markers, accumulation of comprehensive evidence for various treatment response predictors (functional + structural neuroimaging + genetic variants + personality traits + treatment and disease history) will enable neuroscience and psychiatry to ultimately obtain a relative footprint of treatment response affinity and specificity within a patient with MDD. This is all in the quest to substantially improve treatment efficacy for each individual patient.

Here, we employ a neurobiological perspective of network-based resting-state functional connectivity in order to investigate predictors of placebo effects in addition to overall antidepressant treatment effects. Although wide-spread and complex, MDD is generally described as a network-based disorder (2); therefore, our network-based approach to investigate neurobiological predictors of antidepressant treatment effects is fitting. Furthermore, recent work has demonstrated that the human brain is organized into wide-spread, cohesive, functionally connected networks (120-122). This finding has largely stemmed from research within a relatively recent field in neuroscience, resting-state functional connectivity (123), which measures the extent to which regions demonstrate a correlation between their fMRI BOLD signals in the absence of a task paradigm. Enhanced functional connectivity is thought to demonstrate more cohesive communication between regions or within a network. Importantly, examining brain function at rest may reduce variability across studies as well as obtain a better understanding of existing neural activity which contributes to the maintenance of MDD without

the presence of excessively salient stimuli (i.e. an fMRI task). Currently, resting-state functional connectivity is only beginning to emerge with conclusive MDD-related findings (23, 124, 125). However, as the methodology improves and becomes more standardized (126), functional connectivity may be an important tool in furthering our understanding of MDD neurobiology.

Chapter Two

In light of this, in the second chapter of this dissertation, we implement network-based resting-state functional connectivity to investigate predictors of antidepressant treatment effects within a randomized one-week placebo trial and within a subsequent ten-week, open-label antidepressant trial. Specifically, three networks will be investigated: the default-mode, salience, and executive networks because all three are believed to play a major role within MDD (127, 128). We will also investigate their relationship to depression severity. Finally, with the objective that these potential predictors may augment future clinical trials or treatment within a clinical setting, we applied machine learning algorithms to determine whether baseline, inherent functional connectivity may predict treatment effects at an individual subject level. Overall, we hypothesize that functional connectivity of the default-mode network will be positively associated with depression severity, based on prior literature (23, 129). Furthermore, regarding their implication in MDD and placebo analgesia, as described above, we hypothesize that functional connectivity of the rACC, dlPFC, and insula within their respective networks will predict response to one-week of placebo and ten-weeks of antidepressant administration.

Chapter Three

In the third chapter of this dissertation, we continue with our network-based resting-state functional connectivity approach in order to investigate neurobiological differences between a group of depressed subjects and healthy controls. This is with the intention that 1) potential

predictors of treatment effects may indicate neurobiological patterns that differ in depression from a healthy state since successful antidepressant response associates with healthier (i.e. less depressed) states of functioning. 2) Examination of neurobiological patterns which differentiate depression from healthy functioning may expand and detail potential treatment targets and our general understanding of the disorder. Finally, we also employ an alternative resting-state functional connectivity analytical method to highlight the contributions and restrictions of currently, yet still novel, available methods. We hypothesize that any resultant treatment effect predictors will show reduced functional connectivity within the group of depressed patients. Additionally, we hypothesize that default-mode network and salience network functional connectivity will be greater in depressed subjects, based on prior theories (127) [connectivity of the default-mode network has shown conflicting findings (125), whereas the salience network has not yet been explored], while the opposite pattern is expected within the executive network due to documentation of network hypo-connectivity within the disorder (130).

Chapter Four

Finally, in keeping with the substantial progress in mapping neural mechanisms responsible for placebo analgesic effects, we sought to capitalize on this advancement and demonstrate the potential of neuroimaging-genetic markers to modulate placebo analgesic responses and thus, further define its neurobiology. To do so, we selected the prepronociceptin (*PNOC*) gene – a neuropeptide precursor which has been associated with MDD (131), a member of the endogenous opioid family with expansive therapeutic potential (132), and extensively involved in nociception (133) – as a candidate genetic marker of placebo analgesic responses. To investigate this, we will examine the effect of *PNOC* polymorphisms on the activation of μ-opioid neurotransmission in response to placebo administration with expectations of analgesia in

the context of a pain challenge. Through these genetic variations, we will also explore placebo-associated stress responses and personality traits to further examine neurobiological correlates of the placebo effect.

Chapter Two

Investigating Predictors of Treatment Response in Major Depression

Major depressive disorder (MDD) has recently been conceptualized as a disorder with a network-based pathophysiology (2). Particularly, MDD manifests in cortico-limbic network dysregulation reflected by deficits in cognitive control and increased sensitivity of limbic networks (27, 130, 134, 135), which is thought to result in excessive and negatively-skewed focus on introspective processes, difficulty regulating emotions, and persistent difficulties in sustaining attention (7, 46). Regions involved in these networks have emerged as biological markers of response to antidepressant treatments (21, 58, 93, 95, 136, 137). Yet, the ability of these biomarkers to selectively distinguish drug-specific effects from other non-specific elements of the treatment response, such as the placebo effect, is still limited, with very few studies specifically addressing biomarkers of non-specific elements of antidepressant treatment response (59, 138). This is not a small concern, as placebo response rates in antidepressant clinical trials average 31-45% compared with response rates to antidepressants of ~50% and have increased at a rate of 7% per decade over the last 30 years (47, 65). Hence, further investigation is warranted in order to dissect the neural predictors of drug-specific and non-specific effects in MDD.

Of the major functionally connected networks identified within the brain's inherent organization (120, 122, 139), three have received special attention in MDD and the prediction of treatment response: the default-mode (DMN), salience (SN) and executive (EN) network (127,

140). The DMN, with key regions in the posterior cingulate, medial prefrontal, and bilateral parietal and temporal cortices (141), is associated with introspective cognition (142) and is abnormally downregulated in MDD (143). Elevated pretreatment activity of the rostral anterior cingulate (rACC), a region encompassed within the main anterior DMN node (142) has consistently been identified as a predictive marker of treatment response across imaging and treatment modalities (58, 136, 144, 145). It has been hypothesized that heightened rACC activity fosters better treatment outcome in patients with MDD by implementing adaptive self-reflection through its connectivity within the DMN (96). Moreover, antidepressant medication has been shown to decrease functional connectivity of the DMN (146).

The SN, anchored by the anterior insula (aINS) and dorsal anterior cingulate cortex (dACC), is enlisted during the integration of salient stimuli and interoceptive information to guide motivated behavior (147). In particular, the aINS is a hub of meta-awareness, affective, and interoceptive processing (148, 149) and has long been associated with MDD pathophysiology (38, 40, 129, 150, 151). A recent meta-analysis illustrates its activity as a neural predictive marker of MDD treatment response: generally, increased baseline insular activity has been associated with poor clinical response (97).

Finally, MDD is characterized by reduced functional connectivity of the EN (130) and hypoactivity of the network's key node: the dorsolateral prefrontal cortex (dlPFC) (152). The EN, which includes cohesive functional communication between the dlPFC and parietal cortex, is responsible for orienting to and engaging in attentive, goal-directed behavior (147); its dysfunction may contribute to a lack of control over heightened affective responses in MDD (127, 153). The dlPFC is also implicated in current MDD treatments and successful recovery

(24, 154, 155); while reduced grey matter volume in the region is an indicator of non-response to standard MDD treatments (21).

Nodes within these three networks have also been implicated in the neurobiological mechanisms of non-specific treatment effects in the field of placebo analgesia, where substantial headway has been made to identify the cognitive, neural, and molecular bases of the neurobiology of placebo effects (55-57, 73, 77, 156). These studies have demonstrated a key role of the rACC in the formation of placebo analgesia (57, 72, 113) potentially through its interactions with subcortical brain areas involved in endogenous opioid-mediated analgesic effects, such as the amygdala (157) and the periaqueductal gray (57), but also the INS (53, 73, 156). A number of neuroimaging studies have also reported placebo-associated changes in dlPFC function believed to be related to anticipatory mechanisms (71, 158, 159), consistent with the role of this region in cognitive executive function (160). In this regard, activity in EN-associated regions during pain anticipation was found to be predictive of the magnitude of placebo analgesia (161). Conversely, minimal information has been acquired as to the mechanisms implicated in antidepressant placebo effects, with notable exception of one investigation demonstrating an overlap in regions involved in placebo and medication effects (59) and our recent work describing the role of the opioid system in the formation of placebo responses in MDD (88).

Here, we take a network-based univariate and multivariate approach to predict responses to placebo and antidepressant treatment using resting-state functional connectivity (rsFC) with independent component analysis (ICA) (162), a data-driven approach that produces results within a framework of the brain's intrinsic connectivity networks and allows for identification of reliable, exploratory-based treatment response predictors. We investigated the relationship

between baseline rsFC of three networks (DMN, SN, left and right EN) and: 1) depression severity; 2) clinical response to one-week of placebo treatment with expectations of antidepressant effects; and 3) clinical response to ten-weeks of open-label antidepressant treatment.

First, among the three networks, only the DMN has been previously related to MDD duration (23) and maladaptive rumination (129); therefore, we hypothesized that increased baseline DMN rsFC would be associated with greater depression severity. Second, with respect to the prediction of treatment response, none of the connectivity networks have been specifically related to placebo or antidepressant medication responses in patients with MDD. However, central regions of the DMN, SN, and EN — specifically the rACC, INS, and dlPFC, respectively — have a key role in mechanisms implicated in antidepressant and placebo analgesic responses, as described above. Moreover, these networks are implicated within processes necessary for treatment responses: internal monitoring, saliency and higher-level cognition, respectively. Therefore, we hypothesized that increased baseline functional connectivity within central regions of the DMN, SN and EN would predict response to both one-week of placebo and ten-weeks of open-label antidepressant treatment. Finally, we applied multivariate relevance vector regression (RVR) to evaluate the hypothesis that baseline rsFC maps of the three networks would allow for prediction of placebo and antidepressant responses at an individual level.

METHODS

Subjects

Twenty-nine right-handed, un-medicated subjects with a DSM-V diagnosis of MDD were recruited via local advertisements [21 females; age: 18-59 (mean ± S.D.: 32 ± 13); Major Depressive Disorder: Predictors of antidepressant treatment response; PI: Dr. Jon-Kar Zubieta; R01 MH086858]. In addition to completing physical and neurological examinations, subjects were screened using the Mini International Neuropsychiatric Interview (163); inclusion criteria included a diagnosis of MDD, 17-item Hamilton Depression Rating Scale (HDRS) scores >12 and excluded suicidal ideation, comorbid conditions (medical, neurological or psychiatric, substance abuse or dependence), the use of psychotropic agents and pregnancy. Current anxiety disorder diagnoses (generalized anxiety, panic, agoraphobia, social phobia, and specific phobia) were included because of the shared risk factors between MDD and anxiety. Left-handed individuals and subjects who had used any centrally acting medications, nicotine, or recreational drugs within the past two months were excluded. Written informed consent was obtained in all cases. All of the procedures used were approved by the University of Michigan Investigational Review Board for Human Subject Use Research Committee. Data was collected and stored using Research Electronic Data Capture (REDCap) (164).

Authorized Deception Procedure

During the consent process, subjects were not informed of the purpose of the study (the study of placebo mechanisms), nor the manipulations in expectations that took place in the study by the labeling of placebos as active or inactive. To resolve this ethical dilemma, we followed the recommendations of Miller et al. (165) and Martin and Katz (2010) (166) by incorporating the following information into the consent form: “You should be aware that the investigators have

intentionally withheld certain aspects of the study. This is necessary to obtain valid results. However, an independent research committee has determined that this consent form describes the major risks or benefits of the study. The investigators will explain the withheld aspects of the study to you at the end of your participation." Upon completion of the study or if the subjects wished to discontinue the study at any point, subjects were debriefed about the study's purpose and the use of placebos.

Placebo Randomized Controlled Trial (RCT) and Antidepressant Open-Label Trial

During the first phase, subjects were randomized into either 1) one-week "active" placebo treatment (two pills/day), with expectations that the pill represented a potentially fast-acting antidepressant agent or 2) one-week placebo treatment described as an "inactive" control (two pills/day, vitamin C). After a three-day "washout" period of no pills, subjects crossed over into the alternative condition which they were not initially assigned. Subjects underwent a resting-state scanning session after each week of placebo (Figure 2.1).

Depression symptoms were assessed using the 16-item self-rated version of the Quick Inventory of Depressive Symptomatology (QIDS) (167) at the following time points: pre-randomization, baseline- and post- *active* and *inactive* conditions. The change in QIDS (ΔQIDS = $QIDS_{BASELINE}$ - $QIDS_{POST}$) was calculated for active and inactive treatment conditions, and the difference between conditions taken as an index of placebo response ($\Delta QIDS_{ACTIVE} - \Delta QIDS_{INACTIVE}$). Positive values reflected greater reductions in depressive symptoms as a result of "active" placebo administration.

Following the placebo phase and the two resting-state fMRI scan sessions, subjects received a ten-week open-label antidepressant trial with citalopram as an initial agent (starting at 20 mg/day and up to 40 mg/day in 45% of cases). In several cases, citalopram was not clinically

indicated and another antidepressant was given [sertraline (n=1), mirtazapine (n=1), fluoxetine (n=2), duloxetine (n=1), and bupropion (n=1)]. Participant's symptom changes were evaluated at the weeks 0, 2, 4, 8, and 10 using the QIDS. Antidepressant response was measured by the difference in QIDS between week 0 and 10 (ΔQIDS = Week 0 – Week 10).

Neuroimaging Methods

Resting State Functional MRI Acquisition and Prepreprocessing

After both the active and inactive placebo treatment (Figure 2.1), all subjects underwent an eight-minute resting state fMRI scan during which they were instructed to hold still in the scanner with eyes open.

Functional images were acquired on a 3-Tesla scanner (Philips Achieva, Best, Netherlands) using a single-shot echo planar imaging (EPI) sequence (39 slices, slice thickness = 3.5mm, TR=2000, TE=35ms, FA=90, FOV =20cm, 64 x 64 matrix). A high resolution structural image was obtained for anatomic normalization using a T1-weighted, gradient echo (MPRAGE) sequence (220 slices, slice thickness =1-mm, echo time = 4.6 msec, repetition time =9.8 msec, flip angle=8°, field of view = 240-mm^2).

Each resting state scan was preprocessed using FSL (http://www.fmrib.ox.ac.uk), SPM8 (www.fil.ion.ucl.ac.uk), and AFNI (afni.nimh.nih.gov/afni) at various stages. Preprocessing included slice time correction (SPM8), motion correction (SPM8), linear registration of resting state scan to the structural image using the boundary based registration algorithm in FSL's FLIRT (168-170), and nonlinear registration of structural images to MNI space with FSL's FNIRT (171, 172). The subject's scan was then normalized to MNI space with the field coefficients generated from FNIRT, resampled to 2 x 2 x 2 mm voxels, and smoothed with a 5-mm FWHM Gaussian kernel (SPM8).

Nuisance signal removal from voxel time series was performed on the preprocessed resting state scans using linear regression in AFNI's 3dTproject. The nuisance signals removed were linear/quadratic trends to account for scanner drift; the six rigid body realignment parameters and their first derivatives; and the top five principal components from both white matter (WM) and cerebral spinal fluid (CSF) BOLD time courses (173). The subject-specific WM and CSF masks used for principal component analysis were generated by segmenting the normalized structural image into tissue probability maps with FSL's FAST (174) (threshold at 0.99). To further reduce partial volume effects, the WM mask was twice eroded and the CSF mask was restricted to the ALVIN mask (175) of the ventricles.

Movement Analysis

To minimize the effect of motion, the instantaneous displacement between all volumes was calculated using the six motion parameters and any subject with a maximum movement greater than 3 mm was excluded. Frame displacement (176) was also calculated for each volume and if a scan had more than 60% volumes greater than 0.3mm frame displacement, the subject was removed. Eleven subjects were excluded from the analysis for a total of 29 subjects.

Group Independent Component Analysis and Intrinsic Network Selection

Independent component analysis (ICA) was conducted for all subjects' active and inactive scans using the Infomax algorithm within the Group ICA methodology in GIFT software (Medical Image Analysis Lab, University of New Mexico, Albuquerque, New Mexico; http://icatb.sourceforge.net/). ICA is a data-driven, source separation technique, that when applied to fMRI, separates the BOLD signals into statistically independent spatiotemporal components (177). Principle component analysis (PCA) was first utilized to reduce each subject's resting state fMRI data, after which, subjects' data were concatenated and further

reduced with PCA. ICA was then applied to reduce the complete data set into large-scale patterns of functional connectivity; a model order of 20 components was selected in order to isolate these major functional connectivity networks, as has been previously shown (178). The Infomax ICA algorithm was repeated 20 times in ICASSO to ensure the analysis converged to stable components. To identify subject-specific spatial maps and associated time courses which correspond to the group ICA components and avoid PCA bias from either scan condition, a spatiotemporal regression algorithm was applied (179, 180). During which, group-level spatial maps from group ICA results were used as spatial regressors in order to find the temporal dynamics associated with each map. In turn, these time courses were employed as a set of temporal regressors to find subject-specific maps (of the multi-subject spatial maps).

In resting state fMRI, the BOLD time series of intrinsic connectivity networks are composed of low frequency fluctuations (181); thus, components in which the power spectrum of its associated time course consisted of 50% high frequency signal (> 0.1 Hz) were considered as noise and removed from further analysis.

Resting State Functional Connectivity Networks

Briefly, 20 components were output through ICA utilizing the Infomax algorithm within the Group ICA methodology in GIFT software (Medical Image Analysis Lab, University of New Mexico, Albuquerque, New Mexico; http://icatb.sourceforge.net/). Of the resultant components, the networks of interest were selected using templates (for all networks, Figure 2.2) from the BrainMap (http://www.brainmap.org/icns) database, a comprehensive resting-state fMRI data source (182). To determine the component with the "best-fit" for each particular network, a linear-template matching procedure was performed on all 20 components, as described elsewhere (23). Briefly, for each network template: all 20 components were scored based on their best-fit

with the template by computing the average z-score of voxels falling outside the template in the component subtracted from the average z-score of voxels falling within the template. The component with the greatest value of this measure was identified as the network-of-interest: DMN, SN, right or left EN. For all networks, the component of interest had a best-fit score of at least two SD greater than the mean [network: best-fit score (mean ± SD); DMN: 15.7 (1.63 ± 3.58); SN: 5.2 (0.7±1.45); LEN: 12.0 (1.1 ± 2.7); REN: 4.6 (0.7 ±1.4)].

Data Analysis

All data analysis was performed using SPM8 (Welcome Department of Cognitive Neurology, University College, London, England) and Matlab (MathWorks, Natick, Massachusetts) software. One-sample t-tests and paired t-tests were used to analyze within-network functional connectivity within- and between-conditions, respectively. For each network, one-sample t-tests were performed: network-of-interest component from all 29 subjects during the 'inactive' (baseline) were entered into the analysis and ICA-assigned z-scores at each voxel were averaged across all subjects and compared to zero (Figure 2.2). Significance threshold was set at $p<0.05$ family-wise corrected (FWE).

For each network, a whole brain voxel-by-voxel regression analysis was performed between network functional connectivity (z-score of the weight on the ICA component of interest) at baseline with the following variables: depression severity (as measured by QIDS at pre-randomization); reductions in depression symptoms in response to placebo administration with expectations of antidepressant effects; and reductions in depression symptoms in response to ten weeks of antidepressant treatment. All analyses were restricted to voxels within those defined by the network-of-interest's one-sample t-test composed of corresponding network component maps

from all 29 subjects' inactive scans (masked at $p<0.05$-FWE corrected). Depression severity score and scan order were input as covariates in all analyses.

Significance threshold for *a priori* regions in all correlational analyses was held at a height of $p<0.005$ uncorrected; 3dClustSim of AFNI (http://afni.nimh.nih.gov) with 1000 Monte Carlo simulations including estimated smoothness and network-mask voxel size was used to compute the critical cluster size at $p<0.005$ (Bonferroni-corrected for number of networks and their corresponding main regions of interest in this investigation). All analyses were restricted to voxels within those defined by the network-of-interest one-sample t-test composed of all 29 subjects' inactive network components. To address differences in smoothness across the multiple correlation analyses performed for each network (for depression severity, placebo and antidepressant response): we selected the most stringent critical cluster size from all analyses within each network (Table 2.1: "baseline" for all analyses regarding inactive scan network functional connectivity; "placebo-induced Δ" for all analyses regarding network functional connectivity differences between 'inactive – active' scans). This value was used for all analyses regarding that specific network. Significance for all other peaks was held at $p<0.05$-FWE.

These data were extracted using MarsBar (183), for quantification of regional functional connectivity at baseline and placebo-induced changes, graphing, and determination of correlation coefficients (Pearson/Spearman correlations at $p<0.05$). Data are expressed as the mean ± one S.D., unless otherwise indicated.

Table 2.1: Critical cluster size for each functional connectivity network. Cluster sizes were calculated with 3dClustSim of AFNI and 1000 Monte Carlo Simulations for each network map (analyses examining baseline network functional connectivity and analyses examining placebo-induced changes in functional connectivity used different masks; thus, had different cluster sizes).

	Analysis	Cluster Size Threshold (mm^3)
DMN	Baseline	864
	Placebo-Induced Δ (Inactive-Active)	936
SN	Baseline	928
	Placebo-Induced Δ (Inactive-Active)	1024
L EN	Baseline	784
	Placebo-Induced Δ (Inactive-Active)	928
R EN	Baseline	712
	Placebo-Induced Δ (Inactive-Active)	856

Multivariate Relevance Vector Regression (RVR) Analysis

We also investigated single-subject predictability of resting-state functional connectivity onto measured placebo and antidepressant medication responses using multivariate RVR as implemented in PRoNTo (http://www.mlnl.cs.ucl.ac.uk/pronto/) running under Matlab (Mathworks, 2012a release). RVR is detailed elsewhere (184). Briefly, it is a sparse kernel learning multivariate regression method formulated in Bayesian framework. The model weights are assigned a zero-mean Gaussian prior in which each weight is governed by its own hyperparameter. Iterative estimation from the fMRI data identifies the most probable values for these hyperparameters with sparseness achieved due to the posterior distributions of many of the weights peaking around zero. The voxels associated with non-zero weights are marked as relevance vectors, which can then be used to predict the target value (placebo-induced ΔQIDS) for a novel input vector (we first input salience network functional connectivity). RVR's major strength relative to other multivariate machine learning techniques [i.e. support vector machine (SVM), reviewed (185)] is that it computes quantitative prediction of a variable of interest

without a need for discrete categorical estimation (e.g. patients vs. controls) (186). Here, the subject's SN components were mean centered and input into the RVR analysis. An estimate for the model's predictability was calculated via leave-one-out cross validation, indexed using the Pearson correlation coefficient and mean squared error (MSE) between actual and the predicted placebo response measure (the same was performed for actual and predicted antidepressant treatment response). The significance of these metrics was determined through non-parametric permutation tests. In a single iteration, we randomly paired subjects' SN functional connectivity with subjects' placebo response values (and antidepressant response values, separately). Subsequently, we calculated the MSE and correlation for the random pairing. This was completed for 5000 iterations and built a distribution of MSE and correlation values from which p-values were calculated for accuracy of both, placebo and antidepressant response prediction.

RESULTS

Patient Characteristics

Twenty-nine participants with depression [QIDS (mean ± SD): 16.1 ± 4.1; Hamilton Rating Scale for Depression (HRSD-17): 23.7 ± 5.5] were enrolled in the study and completed the two-week randomized placebo trial, then entered the follow-up ten-week treatment with an open-label antidepressant (Figure 2.1). 79% of them (23/29) completed the entire open-label antidepressant treatment portion; those who dropped out before full completion were not significantly different in their depression severity scores and placebo responsiveness from those who completed the study (data not shown). To quantify changes in depression symptoms in response to one-week placebo administration with expectations of antidepressant effects, a single measure of placebo response was created by subtracting changes in depression severity between the two conditions: one-week "active" placebo treatment and one-week "inactive" placebo treatment [ΔQIDS = (QIDS baseline - post) *active* placebo – (QIDS baseline - post) *inactive* placebo]. In all analyses, positive values on this measure reflect a placebo response in the form of reductions in depression severity as a result of placebo administration with expectations of potential antidepressant effects.

Changes in QIDS were not significantly correlated with patient's age ($r = -0.13$; $p = 0.51$), nor depression severity at baseline ($r = 0.04$; $p = 0.83$); and there was no significant sex effect (Females: 2.7 ± 4.1; Males: - 0.5 ± 3.8; $p = 0.13$); there was also no significant effect on scan order (Active First: 3.4 ± 5.0; Inactive First: 0.7 ± 3.1; $p = 0.23$). However, scan order was input as a covariate in all analyses due to the importance of this measure in our study's experimental design. A sex effect was not seen in depression severity at baseline (Females: 16.1

± 3.9; Males: 16.1 ± 4.6; p = 0.99) nor antidepressant treatment response (Females: N = 19; 5.1 ± 3.9; Males: N = 4; 2.3 ± 6.6; p = 0.34).

Placebo- and Medication-induced Changes in Depression Symptoms

Reductions in depression symptoms after one week of the "active" placebo were significantly greater than after the "inactive" placebo treatment [ΔQIDS: 2.0 ± 3.4 for active; 0.17 ± 2.4 for inactive; F = 7.2, df = 1, p = 0.012], after controlling for the effect of scan order. The total placebo response measure ($\Delta QIDS_{ACTIVE} - \Delta QIDS_{INACTIVE}$) was highly variable, ranging from -8 to 11 (mean ± S.D.: 1.8 ± 4.2). Ten weeks of open-label antidepressant treatment produced a significant reduction in depression symptoms [QIDS at Week 0: 11.6 ± 4.3; at Week 10: 6.9 ± 3.9; t = 4.9, df = 22, p < 0.001].

Association of Baseline Resting Functional Connectivity with Pre-Randomization Depression Severity, Response to Placebo, and Response to Antidepressant Medication

Statistical component maps for each network (Figure 2.2) were examined against reductions in depression symptoms in response to one-week of placebo administration with expectations of antidepressant effects and in response to ten weeks of open-label antidepressant treatment. Increased baseline functional connectivity within the SN in the rACC was significantly associated with placebo response (significance held at p<0.005, > 928 mm^3); the association was also observed within the SN in the pINS and dlPFC at a trend level (<928 mm^3) [rACC: 0, 38, 4, 1784 mm^3; z-score= 4.35; r = 0.81; p<0.001; pINS: -42, -2, 2; 648 mm^3; z-score= 3.58; r = 0.60; p<0.001; dlPFC: -40, 34, 42; 544 mm^3; z-score= 3.61; r = 0.66; p<0.001; Figure 2.3]. This was not the case for the DMN, the left or right EN (*a priori*: p>0.005; other: p>0.05-FWE).

A linear regression analysis that included baseline functional connectivity of the rACC, pINS and dlPFC with the SN predicted 76% of the variance in placebo responsiveness (adjusted $R^2 = 0.76$), with rsFC in the rACC alone contributing 65% to the overall variance (adjusted $R^2 = 0.65$).

Increased baseline functional connectivity of the SN within the pINS, dlPFC, and rACC were also associated with reductions in depression symptoms in response to ten weeks of open-label antidepressant treatment although at a trend level (> 928 mm^3) [pINS: -40, -2, 2; 264 mm^3; z-score = 3.94; r = 0.68; p<0.001; dlPFC: -30, 44, 12; 152 mm^3; z-score = 3.59; r = 0.68; p<0.001; rACC: 0, 40, 8; 96 mm^3; r = 0.64; p=0.001; Figure 2.4]. Conversely, decreased baseline functional connectivity within the aINS of the SN was associated with the response to ten weeks of antidepressant treatment also at a trend level (> 928 mm^3) [aINS: 32, 14, -16; 352 mm^3; z-score = 4.07; r = -0.66; p<0.001]. No effects were found in the EN (*a priori*: p>0.005, mm^3; other: p>0.05-FWE).

Placebo-induced Changes in Network Functional Connectivity Predicts Response to Placebo and Antidepressant Medication

As expected, one-week of placebo administration with expectations of antidepressant effects did not result in significant changes within rsFC of the networks—as measured by the changes in rsFC from 'active' to 'inactive'—given high inter-individual variation in response to placebo (*a priori*: p>0.005; other: p>0.05-FWE-corr).

In order to account for the variability inherent in the placebo response measure, statistical component maps of rsFC changes in response to one-week of placebo administration were then examined against associated reductions in depression symptoms in response to one-week of

placebo administration and ten weeks of open-label antidepressant treatment. Placebo-induced rsFC reduction in the rACC within the SN was significantly associated with greater placebo response [rACC: 0, 36, 4; 928 mm^3; z-score = 4.47; r = 0.65; $p<0.001$], with the peak slightly below critical cluster size. No other significant effects were observed (*a priori*: $p>0.005$; other: $p>0.05$-FWE).

Multivariate Relevance Vector Regression (RVR): Single-Subject Level Prediction of Placebo Response and Treatment Response

Based on the results described above, we first applied RVR to rsFC of the entire SN in order to enable quantitative prediction of depression symptom reductions in response to placebo administration and in response to ten weeks of antidepressant treatment at the individual level. The same subjects were used as in the group-level analyses. RsFC of the whole SN was significantly predictive of placebo responses [correlation = 0.41; p-value = 0.018; mean sum of squares = 14.36; p-value = 0.019; (Figure 2.5)], with the greatest weights contributing to the prediction of placebo response located within the rACC. However, SN rsFC was not a significant predictor of response to open-label antidepressant treatment [correlation = 0.03; p-value = 0.34; mean sum of squares = 21.31; p-value = 0.36]. No significant effects were observed within the other networks.

Figure 2.1: Experimental Design. A) After screening, subjects are randomized into one of two conditions each lasting seven days: 1) Active: placebo administration with disclosure that it may provide antidepressant treatment effects; 2) Inactive: placebo administration with disclosure that it is an inactive agent. **B)** A three day washout occurs during which the patient receives no medication. **C)** Subjects cross-over to the alternative condition. **D)** *Resting-state fMRI scans are obtained immediately after each condition*. **E)** After full completion of the placebo trial, subjects undergo ten weeks of open-label antidepressant treatment. Depression measures are administered (* marks QIDS-16SR administration) at screening, pre- and post-active, pre- and post-inactive, and week 0, 2, 4, 8, 10 of the antidepressant trial.

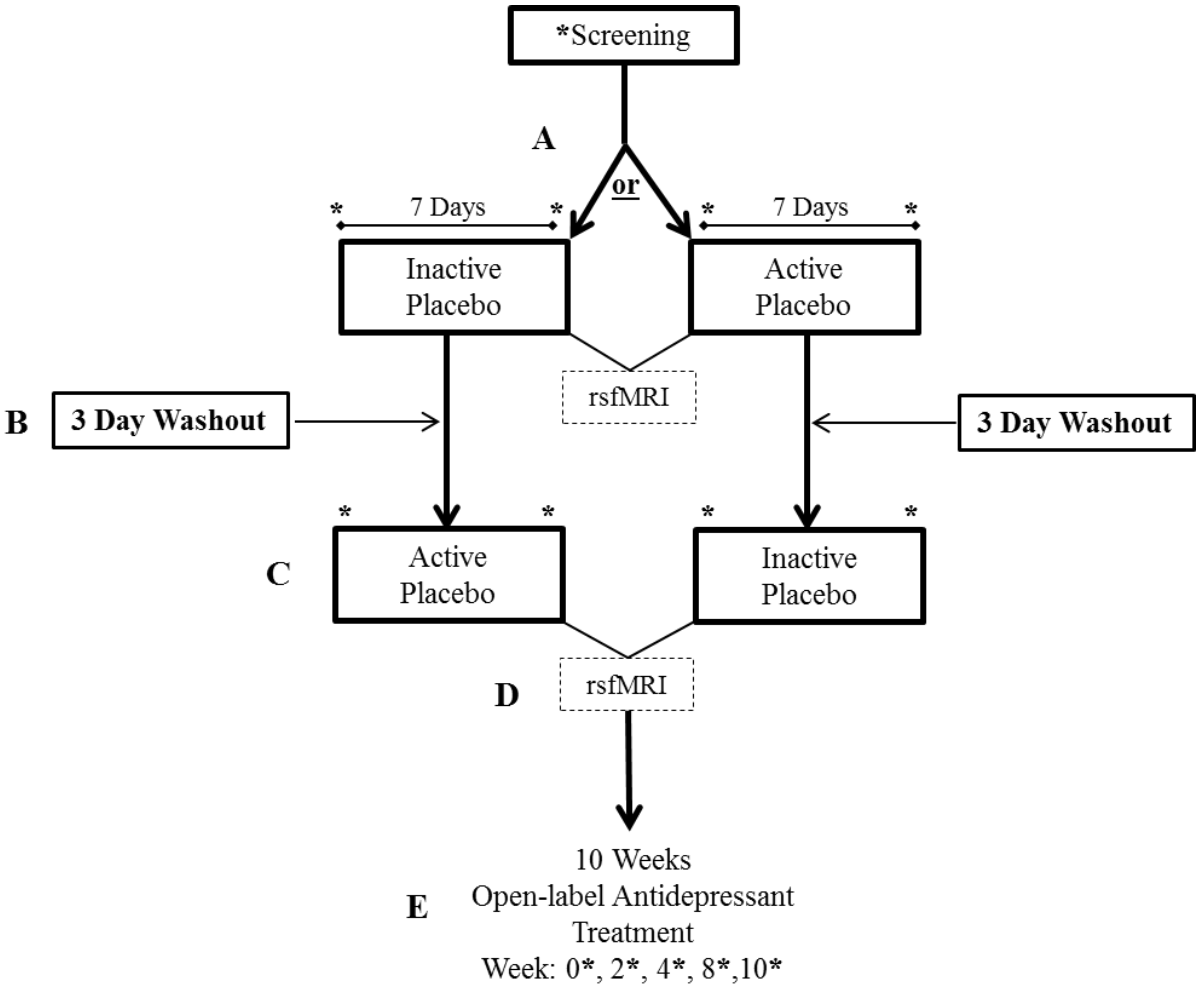


Figure 2.2: Functional Connectivity of Networks. One-sample t-tests including baseline (inactive condition) resting state fMRI scans for all 29 subjects for each ICA-component corresponding to the network: A) Default-mode, B) Salience, C) Left (left-side) and right (right-side) executive networks. The t-score bars are shown at the right; all images are displayed at a threshold of $p<0.05$ family-wise corrected. Images are shown in standard space of the Montreal Neurological Institute (MNI) template. N=29.

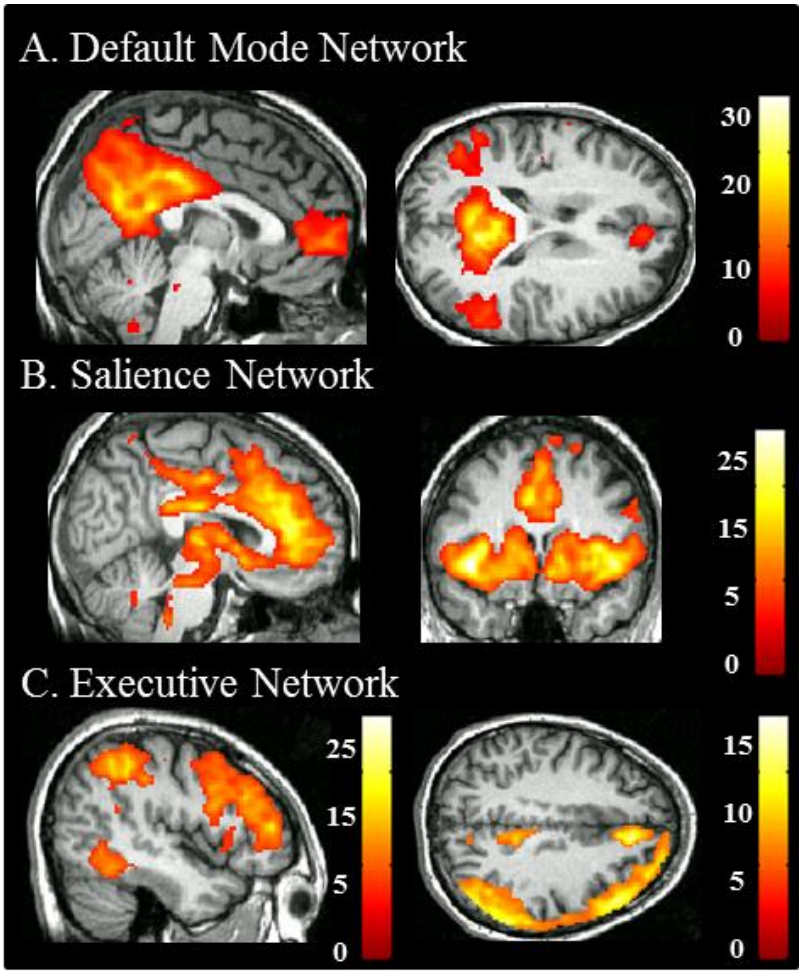


Figure 2.3: Baseline Functional Connectivity of the Salience Network Predicts Response to Placebo Administration. N=29. (Top left, bottom left, and bottom right): Voxel-by-voxel correlational analysis between baseline functional connectivity of the SN and decreases in depression symptoms in response to placebo administration. Clusters passing significance threshold are labeled. Image display is at $p<0.01$; t-score bar is shown in the bottom right. (Top Right): Association between reductions in depression symptoms in response to placebo administration and functional connectivity of rACC within the SN. Functional connectivity of this region predicted 65% of the variance in placebo responses.

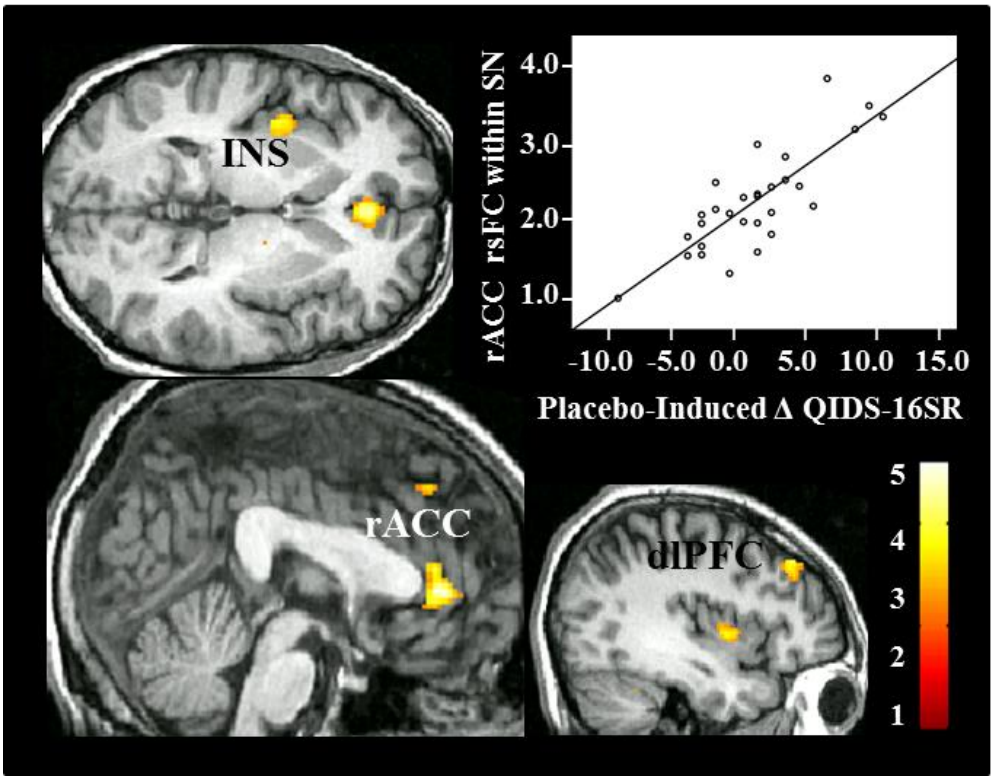


Figure 2.4: Salience Network Predicts Response to Ten Weeks of Antidepressant Treatment. N=23 A. Voxel-by-voxel correlational analysis between baseline functional connectivity of the SN (left) within the posterior INS (rACC and dlPFC not shown) and *decreases* in depression symptoms in response to ten weeks of antidepressant treatment. B. Voxel-by-voxel correlational analysis between baseline functional connectivity of anterior INS within the SN and *increases* in depression symptoms in response to antidepressant treatment. Image display at $p<0.01$; t-score bar for all images shown on bottom right.

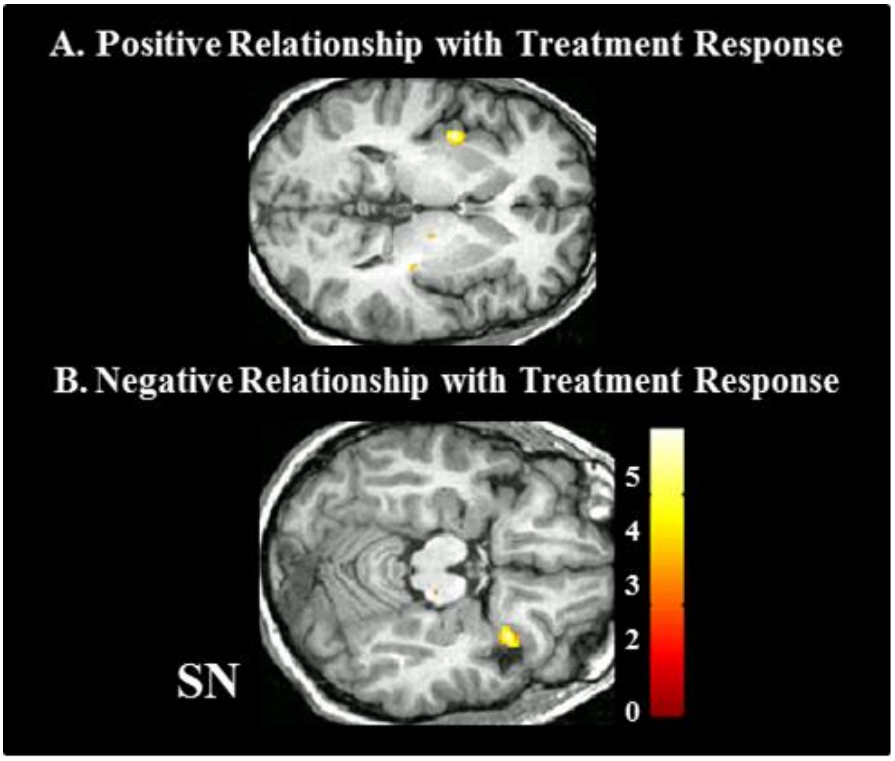
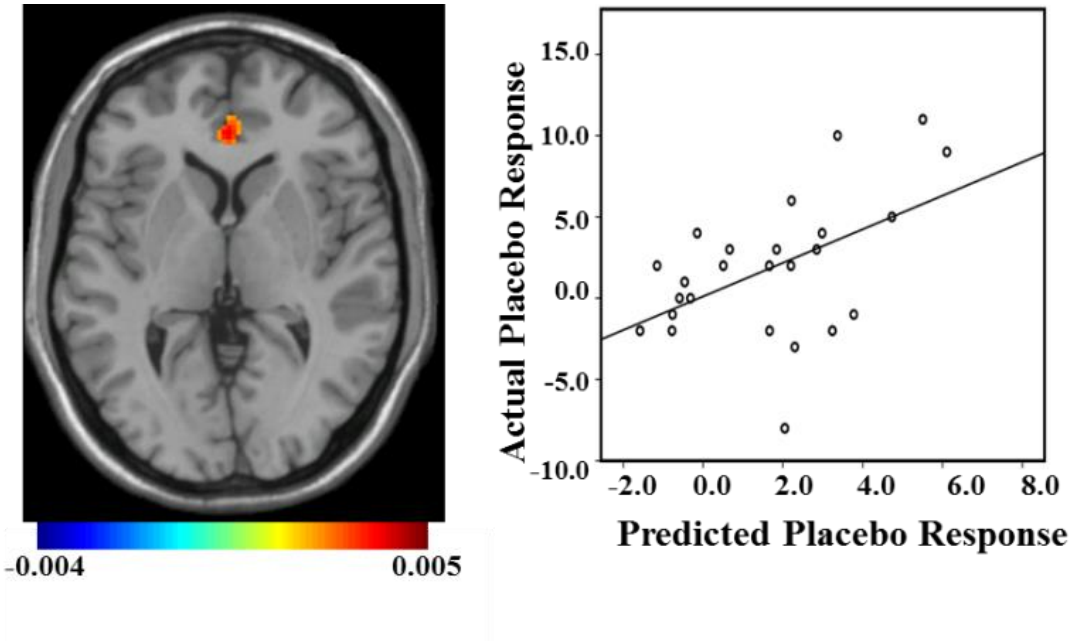


Figure 2.5: Multivariate Relevance Vector Regression Left: Mean predictor map with an arbitrary threshold of >50% of minimum and maximum voxel weight values. The map shows the relative contribution of each voxel to the regression function in relation to all other voxels. Color bar signifies weight value for all voxels. Right: Scatter plot showing the predicted placebo response value derived from each subject's baseline SN functional connectivity using RVR versus their actual placebo response *value* (r = 0.41; p-value = 0.018).



DISCUSSION

In this chapter, we illustrate the viability of network-based rsFC to identify potential imaging-based predictive markers of treatment response in MDD. We found that more cohesive functional connectivity of the SN, through connectivity of the rACC, pINS, and dlPFC predicted greater reductions in depressive symptoms following one-week of placebo administration. The same pattern of connectivity within the SN associated with a greater response to ten weeks of antidepressant treatment, albeit at a trend level; moreover, greater baseline functional connectivity of the aINS within the SN predicted poor response to antidepressant treatment. Additionally, clinical response to one-week of placebo treatment was associated with placebo-induced decreases in functional connectivity of the rACC in the SN. Finally, multivariate RVR demonstrated that SN functional connectivity predicts placebo responses at an individual level with statistically significant accuracy.

Enhanced baseline rsFC of the SN through the rACC, pINS, and the dlPFC predicted greater reductions in depression symptoms in response to placebo administration with expectations of antidepressant effects. Specifically, the SN regions predicted 76% of the variance in placebo responses; the rACC alone accounted for 65% of the variance. This finding potentially advances a more general understanding of the neurobiology of placebo effects across diseases: both opioidergic and BOLD activity within the rACC have been consistently implicated in placebo analgesia (57, 72, 113); the same has been reported within the pINS and dlPFC (72, 73), although with less consistency. Regardless, these findings evince a hypothesis that corresponding mechanisms of action are involved in the formation of placebo effects across disorders (77), with recent evidence in further support of this notion (88). Our data, to our knowledge, are also the first demonstration of SN involvement in MDD placebo effects and thus,

supplement the small existing body of work in the neurobiology of placebo in patients with MDD (59, 88). This is consistent with the role of the SN in saliency attribution: particularly, selecting and integrating biologically relevant stimuli with interoceptive states to guide input of attentional resources and behavior (187). In this case, the network was involved in attending to and incorporating a set of expectations within a therapeutic environment with internal states resulting in a reduction of depression symptoms. If the former hypothesis remains valid, SN rsFC may be the functional context of prior placebo analgesia-associated rACC findings as well as those of the pINS and dlPFC.

Mounting evidence from studies employing a variety of neuroimaging modalities and treatments indicate that increased pretreatment activity in the rACC is associated with better response to antidepressant treatment, as recently reviewed (96). More specifically, metabolism in the region has been observed to differentiate responders from non-responders to antidepressant medication (44). However, the open-label nature of these and prior studies (44, 136, 188, 189) makes it difficult to dissect drug-specific from non-specific treatment effects. Our study is an initial attempt in isolating predictors for different aspects of treatment effects, such as placebo. Here, in corroboration, we observe that functional connectivity of the rACC, INS, and dlPFC significantly predict placebo responses, with the greatest weight residing within the rACC. The same regions also predict responses to antidepressant treatment response although at a lower statistical threshold, which is suggestive of their relation to placebo effects inherent in treatment responses. In confirmation, a recent study using a RCT found that elevated pretreatment theta current density in the rACC, the major predictor of antidepressant treatment response, predicted treatment outcome in both the medicated and placebo group (138).

Our findings demonstrate a stronger association between SN rsFC with placebo response than with antidepressant treatment response. This may be explained by the smaller sample size of MDD patients who completed the ten weeks of antidepressant treatment and the greater variability inherent to the longevity of the measure. Yet, a feasible hypothesis states that rsFC of the rACC, INS, and dlPFC within the SN are more specific to placebo, or non-specific effects of treatment. However, it may be that this relationship is capturing an interaction between active treatment and placebo effects. Future work within a RCT with parallel drug and placebo arms is critical in elucidating these important details.

In all, these findings point to a major predictive role of the rACC within the SN in the response to placebo and the overall response to antidepressant treatment (antidepressant + placebo + other non-specific effects). Furthermore, we observed the normalization of heightened baseline rACC rsFC after placebo administration to be associated with greater placebo responses; this illustrates that modulation of the rACC may be an important element of placebo neurobiology. A relevant hypothesis may state that enhanced rACC rsFC within the SN may represent a greater capacity for integrating salient stimuli with adaptive cognitive-affective functioning, which is conducive to the manifestation of a placebo response. Normalization of heightened rACC activity has also been demonstrated in MDD responders to sleep deprivation, but absent in non-responders, unfortunately, a placebo-arm was not incorporated (190). Thus, malleability within the rACC may extend to necessitate a successful antidepressant treatment response or to placebo mechanisms inherent in the antidepressant response.

Similarly, baseline activity of the aINS has also been observed to be predictive of antidepressant response (97). However, its role as a biomarker is complex. Recent findings target this region as a marker for treatment *selection* (19): low pretreatment aINS metabolic activity

associates with poor response to rTMS (93, 191), but successful outcomes with psychotherapy (95). Conversely, greater baseline aINS activity was predictive of poor outcome regardless of treatment avenue (97, 137, 192); yet, a recent study reported opposing results (95). However, the authors later posited that greater aINS activity is indicative of a patient requiring a more intensive treatment than standard first-line options (19). In confirmation, we also describe a negative association between increased aINS functional connectivity within the SN and response to ten weeks of antidepressant treatment, but not one week of placebo treatment. It is possible that the aINS may play a greater role predicting drug-specific response to antidepressant treatments, while the rACC and more posterior regions of the insular cortex may contribute more to the prediction of non-specific responses, such as placebos. Interestingly, the single study to directly compare neurobiological changes in antidepressant and placebo responses found metabolic reductions in the aINS to be specific to antidepressant responders, whereas responses to both fluoxetine and placebo were associated with an overlapping increase in cortical brain regions, including the pINS (59). Alternatively, it could be hypothesized that baseline connectivity within the aINS predict, long-term, but not short-term, placebo responses. Still, this hypothesis would need to be further investigated in the context of an RCT.

Finally, we demonstrated that multivariate RVR application to baseline SN rsFC, with the greatest weight within the rACC, was significantly predictive of placebo responses at an individual-subject level, although this was not the case for antidepressant responses. The utilization of SVM, a similar method, has gained substantial attention in the last years as a predictive classifier in MDD with diagnostic and prognostic qualities (193-195). For example, in a sample of 37 depressed patients and 37 healthy controls, whole-brain structural MRI significantly classified nearly 90% of patients exhibiting clinical remission and nearly 70% of

those with an MDD diagnosis (196). Instead, we employed RVR to enable quantitative prediction without need for group classification, as recently described (186). The potential applicability of SN rsFC as an individual-subject predictor of placebo response will need to be further confirmed in larger samples; yet, this information could ultimately augment clinical treatment through identification of patients with a greater likelihood of responding to placebo treatment. They may benefit from lower dosages of antidepressant medication or cognitive interventions. This may also be relevant in the context of clinical trials to better distinguish patients with greater susceptibility to placebo effects, allowing for patient stratification and possibly, more detailed clarification of the effects of treatment components (specific versus non-specific).

A limitation of the current study was the small sample size (N = 29 for the placebo phase; N = 23 for the antidepressant phase). This is especially relevant to the RVR analysis, where independent confirmation is needed to establish whether SN rsFC is a predictor of placebo response at an individual level. In addition, the subsequent nature of the open-label treatment after the placebo phase was not optimal for dissecting drug and placebo-specific effects. Future RCTs with parallel placebo and drug arms will need to be conducted to better identify and separate predictors of treatment response. However, our data have the potential to inform future designs of antidepressant clinical trials and personalized medicine for MDD patients by identifying individuals with greater likelihood in responding to non-pharmacologically specific interventions and who may ultimately be selected for less intensive treatments, lower dosages of medication, or for enhanced patient-clinician interaction. Moreover, these findings inform the importance of SN resting-state functional connectivity in the neurobiology of MDD recovery.

Chapter Three

Functional Connectivity Dysfunction in Major Depression

A primary goal of neuroscience is to identify neural characteristics differentiating depressed subjects from healthy individuals. Eventually, a comprehensive understanding of aberrant functioning within MDD will lead to more targeted treatments or potentially, brain-based biomarkers of depression that will enable clinicians to address the disease in a more preemptive manner. Generally, neurobiological differences between healthy brain functioning and that of the depressed brain will expand our understanding of the neurobiology contributing to disease manifestation and maintenance. In a reciprocal manner, these disparities may be targets for new pharmacological treatments or the development of new predictors of antidepressant treatment response. However, to date, most of our understanding regarding neuropathophysiology in MDD stems from task-based functional MRI (fMRI) experimental paradigms. While essential to disentangle the mental processes impaired in the disorder, task-based induction of cognitive and emotional processes does not necessarily mimic resting brain functioning. The latter may be a more consistent or representative pattern of brain functioning which manifests in depressive symptomology and serves to maintain it.

Advances in resting-state fMRI have enabled non-invasive examination of inherent brain functioning (123, 197, 198). Influential work utilizing resting-state functional connectivity (rsFC) has uncovered that the brain's organizational structure is composed of dissociable large-

scale connectivity networks; each representing coherent brain activity sharing distinct temporal and spatial characteristics (121, 122). Development of independent component analysis (ICA) that is applicable to resting-state fMRI blood oxygen level-dependent (BOLD) signal has put forward an automated, data-driven methodology to decompose the BOLD signal into a set of independent spatiotemporal components (162), which are ultimately representative of previously identified, fundamental, intrinsic networks (120). In essence, this understanding has placed our knowledge of specific regions and their associated mechanisms within the framework of a cohesive network versus their isolated activity. Moreover, expansive effort into investigating connectivity networks and rsFC has revealed neurobiological abnormalities inherent in clinical populations; this includes MDD (21, 23, 124, 199).

Apropos, MDD has been conceptualized as a disorder with a network-based pathophysiology (2), as briefly described in the previous chapter. Particularly, MDD materializes from cortico-limbic network dysregulation with diminished frontal lobe functioning over increased limbic activation and sensitivity to negative valence (27). This results in a persistent processing of external and internal stimuli from a negatively skewed perspective (7). As described in Chapter One, of the major connectivity networks identified (139), three networks have distinct relevance in MDD: the default-mode (DMN), salience (SN) and executive network (EN) (127, 128). While in the first chapter, we described the role of these networks as potential predictors of treatment response; here, we will focus on the specific role of each of these networks in the neurobiology of MDD as their connectivity compares to healthy functioning.

Probably the most integral to MDD pathophysiology is the DMN, with key regions in the posterior cingulate cortex (PCC), medial prefrontal (mPFC), and bilateral parietal and temporal cortices (121, 141). The network is known to disengage during external, goal-directed tasks and

instead support internal, introspective cognition (142, 200). Excessive self-focus, inattention, and rumination in MDD is believed to manifest through an inability to down-regulate DMN connectivity (129, 143, 201). Additionally, its connectivity is thought to be heightened in MDD (124, 202), but this has been disputed (125), however, a recent meta-analysis describes enhanced connectivity within the anterior portions (mPFC and regional areas) of the DMN in MDD (203).

Conversely, the SN is involved in orienting to external, biologically relevant stimuli and detecting those with relative consequence and integrating interoceptive information with these stimuli in order to guide behavior (187, 204). Its main nodes, the anterior insula (aINS) and the dorsal anterior cingulate (dACC) (147, 205) are imperative in processes of emotion and cognition affected by major depression (206). Particularly, the INS, a hub of meta-awareness, affective, and interoceptive processing [reviewed (148)]; has long been associated with MDD pathophysiology (38, 40, 129, 150, 151). Consistently the region has been identified as a predictor of antidepressant treatment response. Moreover, modulation of its activity is also a major component within the antidepressant response (19). Due to its role in selecting and orienting to salient stimuli of interest, the salience network is likely a major player in MDD neuropathophysiology as the disease is characterized by excessive sensitivity to negative salient stimuli (46).

The main nodes of the EN, or the frontoparietal network, consist of the dlPFC and lateral parietal cortices. Mainly, it's responsible for directing and sustaining attention to an identified source of salience, in the context of goal-directed behavior (147). Hypo-activation of the dlPFC and thus, dysregulation of this network contributes to a lack of control over heightened affective responses in MDD and difficulties in sustaining attention to executive tasks (153). Moreover, the region is a specific target for repetitive transcranial magnetic stimulation (rTMS), an intensive,

yet effective antidepressant treatment (22) and normalization of dlPFC functioning is associated with antidepressant responses (24).

Despite the consistent description of MDD as a network-based disorder (2, 128), explicit investigation of large-scale network rsFC differences between healthy individuals and those with MDD is still limited, especially regarding the SN and EN in their entirety (207). Of the extant literature, most studies have employed seed-based functional connectivity to isolate large-scale networks. This relies on manual selection of seed coordinates and size; the resultant connectivity map is produced by the correlation of the BOLD times series from each voxel in the brain with the time series extracted from the selected seed region. Importantly, the location and size of the seed affects the specificity of the resultant connectivity network maps (208). Therefore, seed characteristics can substantially impact the nature of resting-state connectivity results from seed-based analyses. These analyses are also highly susceptible to noise and motion artifacts. Therefore, some findings may reflect similarities in noise signal instead of actual BOLD time series between regions.

Instead of seed-based analyses, a recent trend has emerged to capitalize on data-driven methods like ICA which are generally more impermeable to influences of experimentor bias (208, 209). As ICA decomposes the resting-state signal into statistically independent components, noise signals are more easily identified and discarded. However, ICA also has some methodological limitations: a "correct" model-order (component number) size is nonexistant which leaves its configuration up to the experimentor. However, large-scale investigation of massive rsfMRI datasets have developed recommendations of model-order size that are dependent on the experimentor's objectives (examination of large-scale networks vs. their subnetworks). Still, as a downside, a model-order which only isolates the large-scale

connectivity networks risks producing false null results due to low sensisitivy to group effects. An alternative method which bypassess this lack of sensitivity, as well as some of the limitations of seed-based analyses (i.e. experimentor selected seed coordinates) is a multivariate distance matrix regression (MDMR) based connectome-wide association study (CWAS) (210). This approach identifies specific voxels with functional connectivity patterns that significantly differentiate the diagnostic, or MDD, group from healthy subjects. A comprehensive description of this methodology is included in the *Methods* section.

Here, we sought to investigate the nature of these three large-scale connectivity networks: the DMN, SN, left and right ENs, between a group of depressed patients and age-matched healthy subjects. We utilized group ICA to isolate and compare these networks. We hypothesized that network-based connectivity would significantly differ between depressed and healthy control subjects and that these would inform our understanding of abnormalities in MDD brain functioning. In particular, in line with previous work (21, 23), we hypothesized that depressed patients would show enhanced connectivity within the anterior portions of the DMN would. Also, we expected increased rsFC of the SN to be observed in patients with MDD due to enhanced emotional reactivity associated with the disorder (127); moreover, in light of our findings from the previous chapter that increased connectivity within this network and the rostral anterior cingulate (rACC) predicts placebo and antidepressant treatment, we hypothesized that this pattern of "responsiveness" would be similar in the resting connectivity of healthy subjects. We also hypothesize to find hypo-connectivity within the EN in patients with MDD compared to healthy controls based on a previous meta-analysis of seed-based findings (130). Finally, to address some of the limitations imposed by ICA, we implemented an exploratory analysis to

investigate connectivity differences using MDMR-based CWAS to potentially target specific areas of functional connectivity differences between the MDD group and the healthy subjects.

METHODS

Subjects

Twenty-one right-handed, un-medicated subjects with a DSM-V diagnosis of MDD [15 females; age: 18-27 (mean ± S.D.: 22.9 ± 0.6)] and 21 age-matched healthy control subjects [14 females; age: 20-23 (mean ± S.D.: 21.7 ± 0.9)] were selected from two different studies [MDD: Predictors of Antidepressant Treatment Response; PI: Dr. Jon-Kar Zubieta; R01 MH086858; Controls: Michigan Characterization of Affective Neural Circuit Endophenotypes (M-ChANCE); PI: Dr. Brian Mickey]. For participants with MDD, in addition to completing physical and neurological examinations, subjects were screened using the Mini International Neuropsychiatric Interview (163); inclusion criteria included a diagnosis of MDD, 17-item Hamilton Depression Rating Scale (HDRS) scores >12 and excluded suicidal ideation, comorbid conditions (medical, neurological or psychiatric, substance abuse or dependence), the use of psychotropic agents and pregnancy. Current anxiety disorder diagnoses (generalized anxiety, panic, agoraphobia, social phobia, and specific phobia) were allowed because of the shared risk factors between MDD and anxiety. Left-handed individuals and subjects who had used any centrally acting medications (nicotine or recreational drugs) within the past two months were excluded. Written informed consent was obtained in all cases. All of the procedures used were approved by the University of Michigan Investigational Review Board for Human Subject Use Research Committee. Data was collected and stored using Research Electronic Data Capture [REDCap (164)]. Healthy controls were recruited from the M-ChANCE study, which aims to investigate neurobiological vulnerability to depression. Subjects enrolled were healthy adults, aged 18-23 years. Psychiatric disorders, neurological disorders, pregnancy, and inadequately managed general medical conditions were excluded. For neuroimaging, the use of centrally acting medications or recreational drugs within the past month, and alcohol or nicotine within the past 48 hours within

subjects were also excluded. All MDD and control subjects were scanned on the same fMRI machine (see *Neuroimaging* section) and completed the Patient Health Questionnaire (PHQ-9) for state depression severity measures (211).

Neuroimaging Methods

Resting State Functional MRI Acquisition and Preprocessing

All subjects underwent an eight-minute resting state fMRI scan during which they were instructed to hold still in the scanner with eyes open and staring at a cross on a screen.

Functional images were acquired on a 3-Tesla scanner (Philips Achieva, Best, Netherlands) using a single-shot echo planar imaging (EPI) sequence (39 slices, slice thickness = 3.5mm, TR=2000, TE=35ms, FA=90, FOV =20cm, 64 x 64 matrix). A high resolution structural image was obtained for anatomic normalization using a T1-weighted, gradient echo (MPRAGE) sequence (220 slices, slice thickness =1-mm, echo time = 4.6 msec, repetition time =9.8 msec, flip angle=8°, field of view = 240-mm^2).

Each resting state scan was preprocessed using FSL (http://www.fmrib.ox.ac.uk), SPM8 (www.fil.ion.ucl.ac.uk), and AFNI (afni.nimh.nih.gov/afni) at various stages. Preprocessing included slice time correction (SPM8), motion correction (SPM8), linear registration of resting state scan to the structural image using the boundary based registration algorithm in FSL's FLIRT (168-170), and nonlinear registration of structural images to MNI space with FSL's FNIRT (171, 172). The subject's scan was then normalized to MNI space with the field coefficients generated from FNIRT, resampled to 2x2x2 mm voxels, and smoothed with a 5-mm FWHM Gaussian kernel (SPM8).

Nuisance signal removal from voxel time series was performed on the preprocessed resting state scans using linear regression in AFNI's 3dTproject. For CWAS and seed-based

correlation analyses, bandpass filtering (0.009-0.1 HZ) was performed simultaneously with nuisance signal regression. The nuisance signals removed were linear/quadratic trends to account for scanner drift; the six rigid body realignment parameters and their first derivatives; and the top five principal components from both white matter (WM) and cerebral spinal fluid (CSF) BOLD time courses (173). The subject-specific WM and CSF masks used for principal component analysis were generated by segmenting the normalized structural image into tissue probability maps with FSL's FAST (174) (threshold at 0.99). To further reduce partial volume effects, the WM mask was twice eroded and the CSF mask was restricted to the ALVIN mask (175) of the ventricles.

Movement Analysis

To minimize the effect of motion, the instantaneous displacement between all volumes was calculated using the six motion parameters and any subject with a maximum movement greater than 3 mm was excluded. Frame displacement (176) was also calculated for each volume and if a scan had more than 60% volumes greater than 0.3mm frame displacement, the subject was removed. Due to movement, one control subject was excluded from the analysis for a total of 20 control subjects.

Group Independent Component Analysis and Intrinsic Network Selection

Independent component analysis (ICA) was conducted for all subjects' resting-state scans using the Infomax algorithm within the Group ICA methodology in GIFT software (Medical Image Analysis Lab, University of New Mexico, Albuquerque, New Mexico; http://icatb.sourceforge.net/). ICA is a data-driven, source separation technique, that when applied to fMRI, separates the BOLD signals into statistically independent spatiotemporal components (177). Principle component analysis (PCA) was first utilized to reduce each

subject's resting-state fMRI data, after which, subjects' data were concatenated and further reduced with PCA. ICA was then applied to reduce the complete data set into large-scale patterns of functional connectivity; a model order of 20 components was selected in order to isolate these major functional connectivity networks, as has been previously shown (178). The infomax ICA algorithm was repeated 20 times in ICASSO to ensure the analysis converged to stable components. To identify subject-specific spatial maps and associated time courses which correspond to the group ICA components and avoid PCA bias from either scan condition, a spatiotemporal regression algorithm was applied (179, 180). During which, group-level spatial maps from group ICA results were used as spatial regressors in order to find the temporal dynamics associated with each map. In turn, these time courses were employed as a set of temporal regressors to find subject-specific maps (of the multi-subject spatial maps).

In resting-state fMRI, the BOLD time series of intrinsic connectivity networks are composed of low frequency fluctuations (181); thus, components in which the power spectrum of its associated time course consisted of 50% high frequency signal (> 0.1 Hz) were considered as noise and removed from further analysis.

Resting State Functional Connectivity Networks: Template Matching and Filtering

20 components were output through ICA utilizing the Infomax algorithm within the Group ICA methodology in GIFT software (Medical Image Analysis Lab, University of New Mexico, Albuquerque, New Mexico; http://icatb.sourceforge.net/). Of the resultant components, the networks of interest were selected using templates (for all networks, see Figure 3.1) from the BrainMap (http://www.brainmap.org/icns) database: a comprehensive resting-state fMRI data source (182). To determine the component with the "best-fit" for each particular network, a linear-template matching procedure was performed on all 20 components, as described elsewhere

(23). Briefly, for each network template: all 20 components were scored based on their best-fit with the template by computing the average z-score of voxels falling outside the template in the component subtracted from the average z-score of voxels falling within the template. The component with the greatest value of this measure was identified as the network-of-interest: DMN, SN, right or left EN. For all networks, the component of interest had a best-fit score of at least two SD greater than the mean [network: best-fit score (mean ± SD); DMN: 23.1 (2.06 ± 5.14); SN: 9.4 (1.1 ± 2.14); LEN: 8.4 (0.9 ± 1.83); REN: 4.8 (0.67 ±1.31)].

Data Analysis

All data analysis was performed using SPM8 (Welcome Department of Cognitive Neurology, University College, London, England) and Matlab (MathWorks, Natick, Massachusetts) software. To characterize each network, one-sample t-tests were performed: network-of-interest component from all 21 depressed and 20 healthy subjects were entered into the analysis and ICA-assigned z-scores at each voxel were averaged across all subjects and compared to zero (Figure 3.1). Significance threshold was set at $p<0.05$ family-wise error corrected (FWE). These results were masked for subsequent analyses comparing network-functional connectivity across groups.

For each network, a two-sample t-test was performed between network functional connectivity (z-score of the weight on the ICA component of interest) across subject groups: ICA-assigned z-score at each voxel were averaged within each group and then compared across groups (MDD > Controls; MDD < Controls). All analyses were restricted to voxels within those defined by the network-of-interest's one-sample t-test composed of corresponding network component maps from all 41 subjects' resting-state scan (masked at $p<0.05$-FWE corrected).

Age was input as a covariate in all analyses along with subjects' mean frame displacement values (to correct for movement differences across groups).

Significance threshold was held at a height of $p<0.005$ uncorrected; using 3dClustSim of AFNI (http://afni.nimh.nih.gov) with 1000 Monte Carlo simulations including estimated smoothness and specific network mask's voxel size was used to compute the critical cluster size at $p<0.01$ (Bonferroni-corrected for number of networks in this investigation). All analyses were restricted to voxels within those defined by the network-of-interest one-sample t-test composed of all 41 subjects' network component (see Table 3.1 below for network-specific extent critical cluster size).

Table 3.1: Critical cluster size for each functional connectivity network. Cluster sizes were calculated with 3dClustSim of AFNI and 1000 Monte Carlo Simulations for each network map (as created by one-sample t-test described above).

Functional Connectivity Network	Critical Cluster Size (> mm^3)
Default Mode	840
Salience	920
Left Executive	880
Right Executive	760

Further ICA Reduction (Joint ICA)

As a post-hoc analysis in response to our group ICA results, we implemented joint ICA to further reduce and analyze the major large-scale networks of interest. In this regard, we had more control over network decompositions to ensure each large-scale network was separated into subnetworks in an equal manner. ICA was performed separately on each functional connectivity network of interest from GIFT using the Matlab-based Fusion ICA (FIT: http://icatb.siourceforge.net). FIT toolbox was designed primarily for joint ICA (jICA). The method was introduced and described here (212); jICA is a multivariate, multimodal technique

used to identify coupled networks spanning modalities that covary across subjects [reviewed (212)]. Here, only one modality is input. Under this framework, we are assuming networks themselves are a linear mixture of statistical independent components which is reasonable considering the selection of a low model order of 20 for GIFT analysis to isolate major functional connectivity networks. In ICA, all subjects' ICA-produced network components were arranged as a feature vector and concatenated to form a data matrix. Features, or each voxel's ICA-assigned z-score, were normalized by the square root of the mean of all squared extracted voxel values. Minimum Description Length criteria (212) was used to estimate the dimensionality of the feature matrix. PCA was used to reduce the dimensionality of the data down to the estimated components from the previous step. The Infomax algorithm (213) repeated 20 times in ICASSO to ensure stable components, was used to decompose the reduced feature-matrix to three maximally independent component images and subject specific mixing (loading) parameters. Difference in component expression between MDD and controls was examined through a two-sample t-test of subjects' loading parameters.

Connectome-Wide Association Study (CWAS)

To address the limitations of ICA and its potential to produce false null results, we employed an alternative methodology to examine functional connectivity between depressed and healthy populations: we incorporated multivariate distance matrix regression-based connectome-wide association study into our investigation. The human connectome comprises all neural connections throughout the brain and provides the basis for behavior and cognition. To uncover neural circuitry variation between clinically diagnosed MDD and healthy control populations, a CWAS was performed using the publicly available R software package *Connectir* (210). In this analytic technique, multivariate distance matrix regression (MDMR) is used to identify voxels

whose whole-brain connectivity patterns vary significantly between phenotypic variables; in this case, the phenotypic variable of interest is a clinical diagnosis (MDD vs healthy controls). MDMR itself is a powerful analytic tool that provides advantages over more traditional univariate techniques or mass-univariate techniques. Taking a multivariate framework, the simultaneous contribution of entire sets of functional connections to clinical diagnosis is evaluated reducing the scale of the multiple comparison problem which plagues mass-univariate analyses. Given human brain functioning is driven by networks rather than individual regions (122) simultaneous analysis of functional connections can reveal network relationships unseen in mass-univariate methods.

The CWAS analysis, as performed by *Connectir,* was completed in three steps (see (210) for more comprehensive methodology description). First, for each participant, the Pearson correlation of BOLD fMRI signal between each pair of voxels in the brain was computed, resulting in a *v* x *v* correlation matrix where *v* is the number of voxels. Computations were restricted to voxels included in all participants' scans and labeled as gray matter in the prior mask provided by FSL (threshold > 0.5). Second, for each voxel, the distance between connectivity patterns (i.e., each voxel's correlation with the rest of the brain) for every possible pairings of the participants in the dataset was calculated. The distance measure used was $\sqrt{2(1-r)}$, where *r* is the Pearson correlation. The result was an *n* x *n* matrix of distances among participants for each voxel where *n* is the number of participants. The third step used MDMR to test how well group differences explain the distances between participants obtained in the second step. Essentially, MDMR was used to test the differences of connectivity patterns between MDD and controls for each voxel. Given MDMR's regression like framework, movement was controlled for using mean FD. The result is a pseudo F-statistic for each voxel where significance

was determined using permutation testing. The null distribution was simulated 30,000 times by randomly permuting subject labels for the variable of interest and recalculating the pseudo F statistic. The p-value for the original pseudo F statistic was determined from this simulation. We controlled for multiple comparisons across voxels using Gaussian Random Field theory (voxel threshold of $Z > 1.65$, cluster size threshold $p < 0.05$). The voxels with the highest significant differences in connectivity between MDD and controls were used as the center for post-hoc seed-based analyses explained below.

CWAS-Driven Post-Hoc Seed-based Connectivity

To determine the directionality and specific connections involved in the resultant CWAS effect, post-hoc seed-based correlation analysis was performed using 3mm spheres centered on voxels deemed significant by CWAS in the comparison across groups (MDD vs. control). Ten ROIs were identified in the comparison analysis (Table 3.3 for coordinate information). In a seed-based connectivity analysis, a spatially averaged BOLD time series is obtained from the ROI (Figure 3.7) and correlated with the BOLD time series of each voxel across the whole-brain. To create a seed-based functional connectivity map, the Pearson correlation coefficient between the extracted time series and each voxel in the subject's preprocessed resting-state scan is computed. A seed-based rsFC z-map for each ROI was created by application of a Fisher transformation to convert the r-scores to z-scores. This was performed on all resultant ROIs from CWAS analysis.

Seed-Based Connectivity: Data Analysis

For comprehensive ROI-specific seed-based functional connectivity z-maps, each subject's ROI-specific connectivity map was entered into a one-sample t-test analysis. Voxels were averaged across all subjects and compared to zero. Results were masked at $p<0.05$-FWE.

Two-sample t-tests on each ROI's seed-based functional connectivity z-map were performed across groups (in both directions: MDD>Controls; Controls>MDD). Age was input as a covariate along with each subjects' mean frame displacement value, to control for movement across groups. Results were restricted to voxels confined within the masked ($p<0.05$-FWE) one-sample t-test result.

Significance threshold was held at a height of $p<0.005$ uncorrected; 3dClustSim of AFNI (http://afni.nimh.nih.gov) with 1000 Monte Carlo simulations including estimated smoothness and the seed-based connectivity map mask's voxel size was used to compute the critical cluster size at $p<0.005$ (Bonferroni-corrected for number of ROIs in this investigation). All analyses were restricted to voxels within those defined by the network-of-interest one-sample t-test composed of all 41 subjects' ROI-specific seed-based functional connectivity z-maps (see table below for ROI-specific extent critical cluster size).

Table 3.2: Critical cluster sizes for each ROI-specific seed-based functional connectivity z-map. Cluster sizes were calculated with 3dClustSim of AFNI and 1000 Monte Carlo Simulations for each z-map (as created by one-sample t-test described above).

Region of Interest	Critical Cluster Size (> mm^3)
Anterior Insula	544
Left Subgenual Anterior Cingulate	520
Left Parahippocampus	522
Mid-Cingulate Cortex	642
Posterior Cingulate Cortex	509
Right Parahippocampus	604
Dorsal-Medial Prefrontal Cortex	486
Rostral Anterior Cingulate Cortex	474
Rostral-Dorsal Anterior Cingulate Cortex	544
Subgenual Anterior Cingulate Cortex	418

RESULTS

Subject Characteristics

The mean age did not differ significantly between groups (MDD: 22.9 ± 2.7; CTRLS: 21.6 ± 0.9; $t = 1.95$; $p = 0.06$); however, because there was a trend to significant difference, age was still input as a covariate for all analyses. Subjects in the depressed group scored significantly higher on their baseline PHQ-9 measure (MDD: 18.0 ± 4.5; CTRLS: 1.65 ± 1.7; $t = 15.3$; $p<0.001$). Although head movement in the scanner was not significantly different across groups (MDD: 0.24 ± 0.1; CTRLS: 0.21 ± 0.08; $t = -1.08$; $p = 0.28$), it was input as a covariate to account for any group differences in motion.

Network Functional Connectivity Group Differences

Resultant statistical component maps were compared between groups for each network-of-interest to examine between-group differences in network functional connectivity; however, results did not reach statistical significance.

Within-Network Functional Connectivity Group Differences (Joint ICA Results)

Due to the null findings from the group ICA, we employed joint ICA to further decompose each network in order to gain more sensitivity between groups. For each network, resultant statistical component maps were input into the joint ICA analyses and isolated into three statistically independent spatiotemporal components. Difference in component expression between MDD and controls was examined through a two-sample t-test of subject-specific loading parameters associated with each output component. SN (Figure 3.2) results demonstrated reduced weight within the bilateral rACC (Figure 3.2C) in MDD compared to healthy controls ($p = 0.035$). Left EN (Figure 3.3): controls demonstrated greater weight in the dlPFC and parietal

lobes (Figure 3.4C, $p = 0.006$). Loading parameters for all other components from each network did not differ significantly between groups ($p > 0.05$).

Connectome-Wide Analysis

To demonstrate an alternative method to investigate rsFC between groups which addresses some of the group ICA limitations, we employed MDMR-based CWAS to investigate whole-brain functional connectivity between depressed and healthy subjects. Fully preprocessed resting-state scans for each subject within each group was input into a CWAS analysis, functional connectivity for each voxel was computed for each subject. Functional connectivity of each voxel was compared with every possible pairing of all other subjects. MDMR produced an F-statistic map for each voxel in the whole-brain [(210) for in-depth exploration of methodology]: each voxel was tested to determine whether its functional connectivity patterns were more similar within-group than between controls and depressed subjects. Voxels with the highest statistical significant differences between-groups are shown in yellow (Figure 3.4); the coordinates of the voxel with the highest value serve as the center for ROI spheres used in post-hoc seed-based functional connectivity analysis as they suggest regions with the greatest whole-brain connectivity differences between depressed and healthy subjects. Resultant ROIs are listed in Table 3.3.

In order to examine the directionality and specificity of the CWAS results, resultant seed-based connectivity maps were compared between depressed subjects and controls (Figure 3.5) for all ten ROIs listed in Table 3.3. Seed-based connectivity two-sample t-tests were performed with age and movement input as covariates to control for any potential differences between groups. The results of the analysis are listed in Table 3.4 [and shown (Figure 3.5)], where only statistically significant peaks are described.

TABLES and FIGURES

Figure 3.1: Resultant Statistical Network Component Maps. One-sample t-tests composed of all 40 subjects' corresponding network component (DMN, SN, left and right EN, as labeled below). Each network is displayed at p<0.05-FWE.

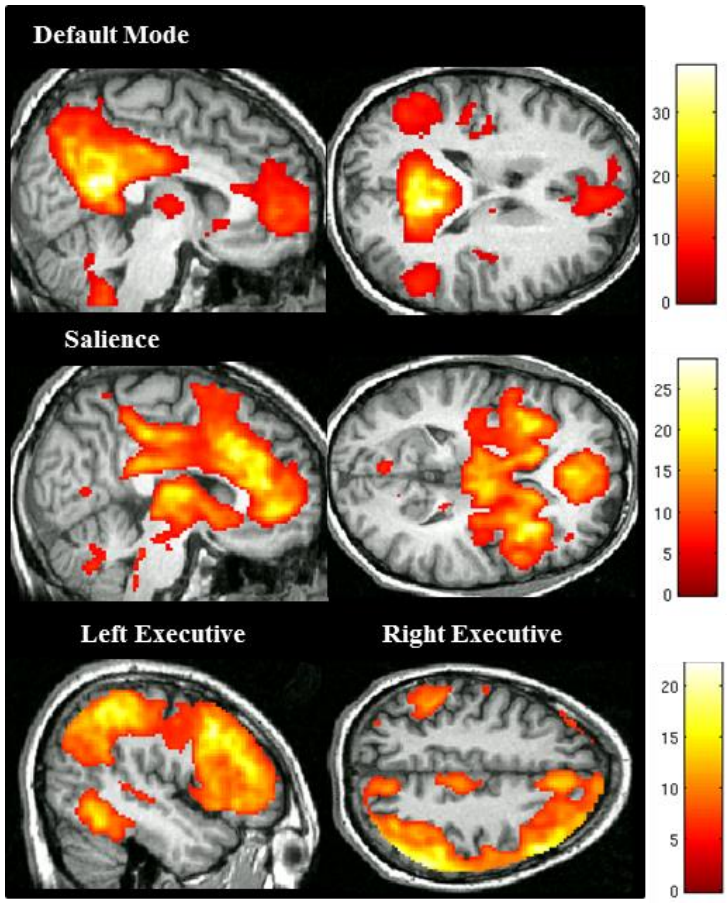
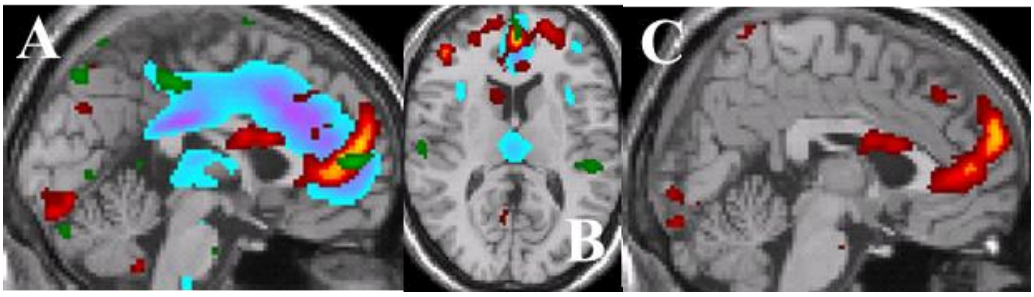
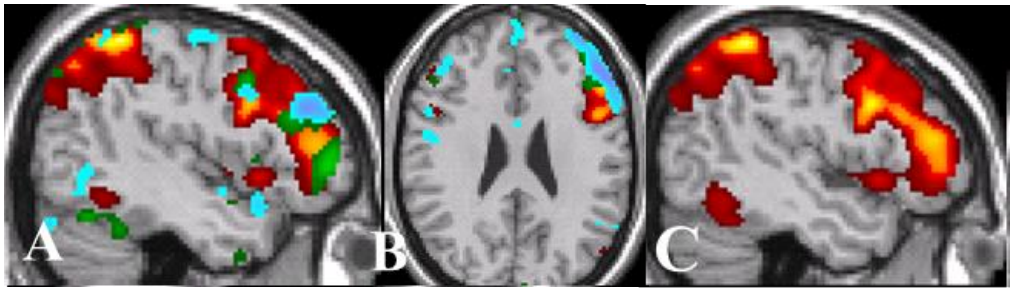


Figure 3.2: Salience Network Joint ICA Results. All subjects' salience network component from the group ICA output was input into joint ICA analysis and reduced to three within-network components [shown in A) and B), each component is displayed in a different color (green, cool, and hot)]. C) Shows the independent component with significantly greater loading parameters in control subjects than MDD subjects.



Cool	Component 1
Hot	Component 2*
Green	Component 3

Figure 3.3: Left Executive Network Joint ICA Results. All subjects' left executive network component from the group ICA output was input into joint ICA analysis and reduced to three within-network components [shown in A) and B), each component is shown in a different color (green, cool, and hot)]. C) Shows the independent component with significantly greater loading parameters in control subjects than MDD subjects [(C; p= 0.006)].



Cool	Component 1
Hot	Component 2*
Green	Component 3

Figure 3.4: Multivariate MDMR CWAS Results. Voxels with significantly different functional connectivity pattern between groups (MDD and controls). Voxels in yellow represent the highest statistically significant difference between diagnostic groups; voxel coordinates served as the center of 3mm radius ROI spheres for post-hoc seed-based functional connectivity analysis. Table 3.3 lists ROIs and their respective coordinates.

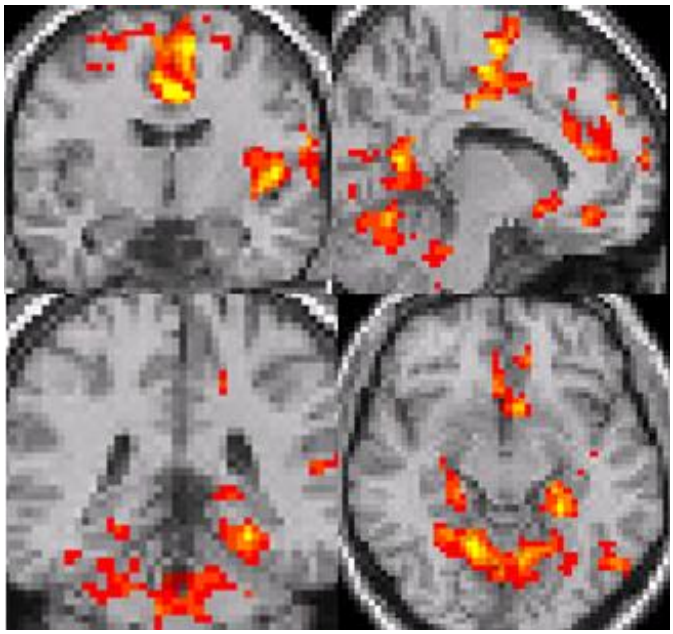


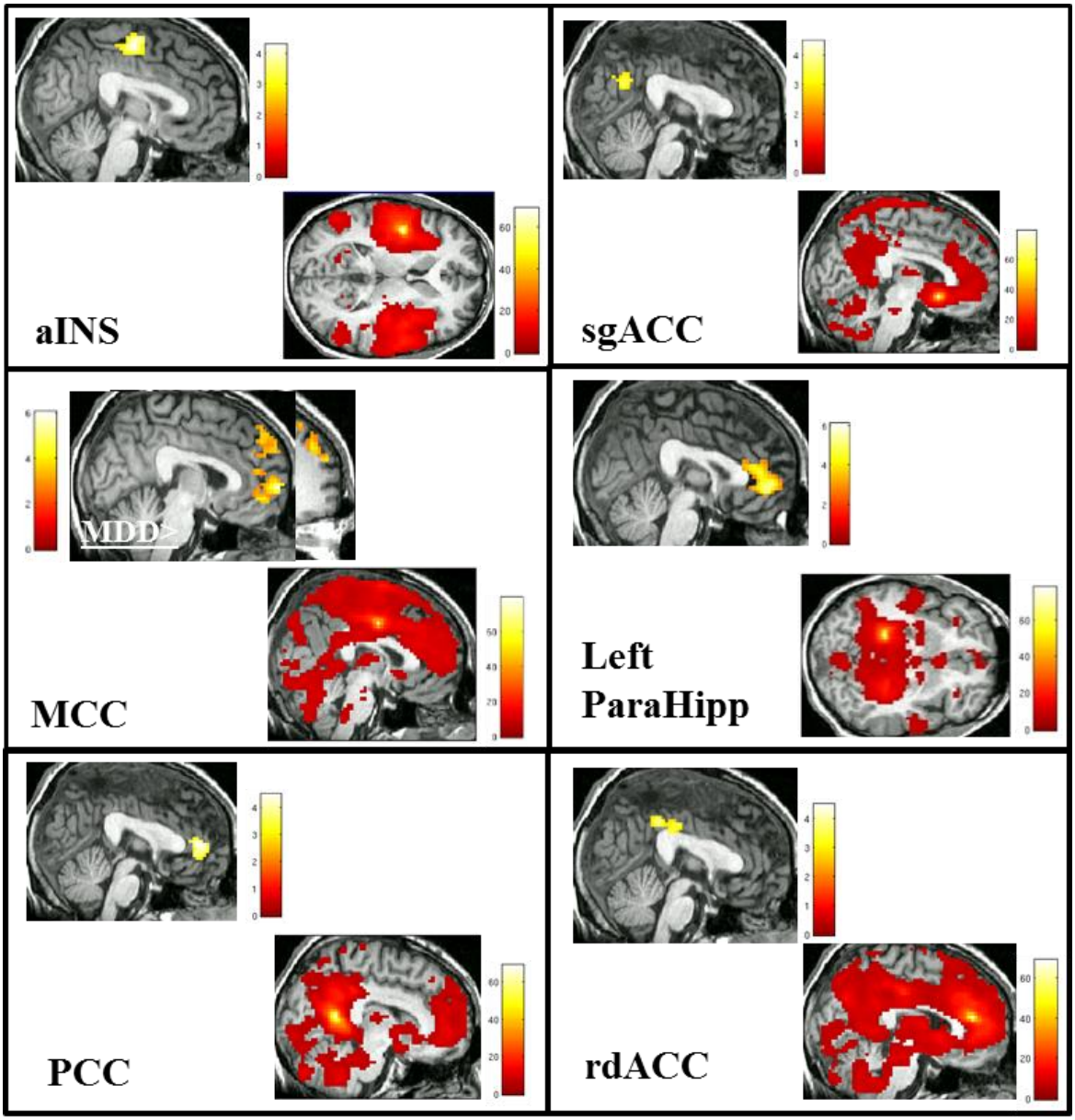
Table 3.3: Resultant ROIs from CWAS analysis. All ROIs were created using MarsBar and a 3mm radius centered on particular coordinate below.

Anatomical Label	**Coordinates**
Anterior Insula (aINS)	-45, -4, 7
Subgenual Cingulate	-6, 14, -11
Parahippocampus (Parahipp)	-24, -37, -11
Mid-Cingulate Cortex (MCC)	-3, -7, 40
Posterior Cingulate Cortex (PCC)	-9, -55, 13
Right Parahippocampus (Parahipp)	24, -25, -14
Dorsal medial Prefrontal Cortex (dmPFC)	-6, 50, 34
Rostral Anterior Cingulate (rACC)	-6, 38, -8
Rostral-dorsal Anterior Cingulate (rdACC)	-3, 38, -8
Subgenual Cingulate Cortex (sgACC)	3, 23, -8

Table 3.4: Seed-based Functional Connectivity Results. For each ROI seed-based functional connectivity z-map, the table below lists all significant peaks resultant from the two-sample t-test analysis between depressed patients and healthy control groups. The directionality of results are noted in the row each peak is listed [MDD greater than controls (MDD>); controls greater than MDD (CTRL>)]. All peaks pass p<0.005 height (uncorrected) and extent (corrected) threshold. See Methods for critical cluster size for each ROI. The following ROIs are not listed because the t-test results were null (subgenual anterior cingulate cortex (sgACC) and rostral (rACC). Abbreviations are as follows: anterior insula (aINS), parahippocamus (parahipp.), mid-cingulate cortex (MCC), posterior-cingulate cortex (PCC), dorsal medial prefrontal cortex (dmPFC), rostral-dorsal anterior cingulate (rdACC), somatosensory area (SMA), and medial PFC (mPFC).

ROI Seed		Region	Coordinates	mm^3	z-score
aINS	**MDD >**				
	CTRL >	SMA	3, -13, 58	896	3.85
sgACC	**MDD >**				
	CTRL >	PCC/Precun.	-6, -64, 28	1264	4.01
Left Parahipp.	**MDD >**				
	CTRL >	rACC	3, 38, 13	1944	4.19
MCC	**MDD >**	mPFC	-6, 62, 1	5968	5.03
		dmPFC	-18, 38, 49	5968	4.96
		cerebellum	30, -76, -35	1656	4.7
	CTRL >				
PCC	**MDD >**				
	CTRL >	rACC	-3, 38, 7	992	3.84
Right Parahipp.	**MDD >**				
	CTRL >	PCC	6, -49, -5	2320	5.07
dmPFC	**MDD >**				
	CTRL >				
rdACC	**MDD >**				
	CTRL >	PCC	-12, -49, 46	848	4.00

Figure 3.5: CWAS Seed-Based Analysis Results: Controls show greater functional connectivity. Ten ROIs of interest resulted from the CWAS analysis examining functional connectivity differences between MDD and control groups. Each block displays results from a single ROI (labelled); showing connectivity greater in healthy controls, unless otherwise indicated (MCC-seed). The bottom image shows a one-sample t-test of all subjects' seed-based functional connectivity z-map (at $p<0.05$-FWE). Top images show two-sample t-test of functional connectivity greater in controls, unless otherwise indicated (MCC-seed). Age was input as a covariate for all results. Display at $p<0.005$-uncorrected. All peaks pass height and extent significance. See Table 3.4 for peak information. Not shown ROIs: dmPFC, rACC, right parahippocampus, and sgACC.



DISCUSSION

While investigation of whole-brain, rsFC is still an emerging field, its application holds weight in uncovering brain regions – in the context of their respective connectivity networks or in relation to other regions of interest – involved in the manifestation and maintenance of major depressive symptoms. The objective of the present study was to follow-up on the findings obtained in Chapter One, from which we were interested whether predictors of treatment response were also essential markers of MDD neuopathophysiology, and further examine rsFC between a group of depressed individuals and a group of healthy counterparts. By employing two disparate methodologies in analyzing rsFC in order to address their respective limitations, we provide some information as to the nature of neural connectivity, in the context of intrinsic connectivity networks and in regard to more specific brain regions, which differentiates depressed from healthy individuals.

Using group ICA, we did not observe network-based connectivity differences between depressed and control subjects. However, upon separating each network into three subnetworks with joint ICA, in order to improve sensitivity, we found reduced rACC connectivity within the SN, the dlPFC and lateral parietal cortices within the EN of depressed patients compared to healthy controls. In a more targeted approach, MDMR-based CWAS identified specific regional functional connectivity patterns attributed to each group. Through this methodology, we isolated regions of interest possessing more cohesive connectivity maps within each phenotypic group. Of interest, depressed patients displayed significantly reduced functional connectivity between the PCC and rACC; the left parahippocampus and rACC; sgACC and PCC; and the right parahippocampus and PCC. In contrast, depressed subjects demonstrated greater functional connectivity between the MCC and dorsomedial prefrontal areas compared to healthy subjects.

We did not find significant group effects within the DMN, SN, nor either of the unilateral ENs. In contrast to our results, a seminal study observed enhanced functional connectivity between the sgACC and thalamus within the DMN in depressed subjects in relation to controls (23). It is possible our sample size did not have enough power to detect significant effects or differences in ICA model-order contributed to our null findings. While this ICA-based result has not been explicitly replicated, increased connectivity in more anterior portions of the DMN, such as the mPFC, has been documented in MDD (214). Still, studies utilizing ICA to examine network-based functional connectivity in MDD are limited. A recent meta-analysis investigating rsFC in MDD in relation to healthy subjects incorporated 36 studies, only eight of which used ICA. Generally, ICA reported increased connectivity in anterior regions within the DMN. The meta-analysis also reported changes in connectivity between the anterior and posterior regions of the network, such as the mPFC or sgACC and PCC, respectively. ICA studies mainly reported an anterior and posterior dissociation of the DMN. Interestingly, studies with anterior DMN seed regions confirmed this finding, while posterior DMN seed-based studies reported increased connectivity between anterior and posterior nodes [reviewed (203)].

Within-network connectivity of the SN and EN has not been frequently investigated in MDD. However, regarding the SN, one ICA-based study reported increased connectivity of the ACC with decreased connectivity within the bilateral aINS in depressed patients (207). In the same study, the EN was decomposed into three subnetworks due to their use of a high model-order. Of the subnetworks, the right angular gyrus within the left ventral EN and the postcentral gyrus within the right dorsal EN demonstrated increased connectivity in depressed patients. Generally, seed-based studies have confirmed decreased connectivity between the major nodes of the SN: INS and dACC (215). Although, increased connectivity between the INS and rACC

has also been reported (206). A seed-based meta-analysis demonstrated hypoconnectivity of the EN as a whole (130). In all, concrete network-based connectivity characteristics of depressed subjects warrants more comprehensive examination from larger datasets and standardized analytical procedures. Variations in model-orders through an empirical manner will provide insight into MDD-associated subnetwork functional connectivity that may also be more sensitive to connectivity differences between populations.

To honor our objective (investigate whether MDD exhibits particular functional connectivity abnormalities within intrinsic large-scale networks), we employed joint ICA to further reduce each of the main large-scale networks into statistically independent components due to our aforementioned null findings. Within the SN, functional connectivity within the rACC had significantly less weight within the SNs of depressed patients in comparison to healthy controls. Thus, the region appears to be more cohesive with the rest of the SN in healthy controls. The rACC is involved in self-referential, cognitive and affective processes (45); decision-making regarding reward attribution (216) and error detection (114); emotional conflict (115); and plays a significant role in MDD (44). Across neuro-imaging modalities and antidepressant treatments, enhanced pretreatment activity within the region is associated with a greater likelihood of successful treatment response. Moreover, we found more cohesive functional connectivity within this region and the SN to be a positive predictor of reductions in depressive symptoms in response to placebo administration with expectations of antidepressant effects as well as in response to antidepressant treatment, although the latter finding was observed at a lower statistical threshold. A possible hypothesis may be that MDD patients who possess greater integrity or malleability of the region—in the context of SN functional connectivity—may be equipped with the neural machinery responsible for or which contributes

to recovery from depressive symptoms. This hypothesis holds merit as the region is a central intersection between limbic and cognitive brain regions (27, 40) and its abnormal activity contributes to the manifestation of MDD (27); however, whether its general activity is heightened or diminished is inconclusive. Our findings place the rACC within the context of a well-documented, intrinsic connectivity network that may enhance our understanding of the role the region and its respective network plays in MDD. Further investigation is warranted in order to better detail the specific psychological functions attributed to the rACC within the SN in relation to MDD.

We also found that depressed subjects placed less weight within a component that included the majority of the left EN (lateral parietal cortex and dlPFC), further corroborating reported diminished connectivity within this network in MDD (130). This muted connectivity evinces the practice of rTMS on the network's main node, the dlPFC (152), as an effective MDD treatment for some patients [examination of parietal cortex rTMS as an effective antidepressant is inconclusive and limited (217)]. In most cases, stimulation of the left dlPFC produces a significant reduction of depressive symptoms, albeit not always full remission (218). Although the nature and degree of cognitive deficits in depression still warrants further investigation (219), these findings point to disparities in the integrity of the left EN's functional cohesion between depressed and controls: This may contribute to the belief that MDD manifests from inadequate regulation by EN-associated cortical, executive regions over heightened limbic activity (27).

In an effort to address the limitations of ICA-based examination of rsFC (i.e. poor sensitivity in uncovering group effects due to model-order), we employed MDMR-based CWAS to uncover specific functional connectivity differences between groups. Altogether, in depressed patients, we found a pattern of reduced reciprocal functional connectivity between the rACC and

PCC; between the PCC and sgACC; PCC and right parahippocampus; and the rACC and left parahippocampus. Again, these findings warrant more comprehensive investigation as to their psychological and behavioral attributes. Yet, that these regions demonstrate a more cohesive interaction (as measured by greater functional connectivity) in healthy subjects, illustrates that the quality of their functioning may be essential components in the fruition of healthy cognitive and affective states that may be malfunctioning in depressed patients. Furthermore, these regions may be potential targets to normalize network dysregulation in MDD.

As described above in the joint ICA results: the rACC manifests as an integral region in the amelioration of MDD symptoms. Consequently, this involves the region in processes such as regulating cognition and motivation, emotional decision-making, regulation of primitive, emotional responses, and self-referential cognition. Functionally, the region is also encompassed within the main anterior node of the DMN (142) and overlaps with the SN (220). The parahippocampus is a cortical, paralimbic region involved in processing of external spatial orientation, memory functions (221), emotional responses (222), and reward processing (223): it plays an important role in MDD. In particular, depression is associated with severe impairments in episodic memory (224) as well as a diminished capacity for positive memories (225), which contributes to the perpetual negative affective characteristic of the disorder (226). A recent study confirmed that MDD is associated with weaker memory for positive material caused by blunted activity from the dopaminergic midbrain and the medial temporal lobes, notably, the parahippocampus (227). Normally, the presence of rewarding stimuli activate these latter regions to enhance episodic memory encoding of positive experiences (228).

In the framework of the brain's intrinsic organization, the parahippocampus is responsible for mediating the connectivity between the DMN's main node, the PCC and the

hippocampus (229), a region mainly responsible for episodic memory processing and encoding (230). The hippocampus and parahippocampus are both subregions of the medial temporal lobe (MTL) memory system: another node of the DMN (231). Its central node, the PCC, has been shown to increase activity during auto-biographical memory retrieval, future planning, and mind wandering (232, 233). Moreover, upregulation of PCC activity has been observed after successful antidepressant treatment (24, 28).

Altogether, our results show that depressed subjects demonstrate diminished connectivity between these core DMN regions: the PCC, rACC, and parahippocampus. All of which point to a more cohesive DMN functioning within healthy resting connectivity [reviewed (142)]. While many in the field speculate that DMN connectivity is excessive in MDD (23, 124, 234), which may explain maladaptive ruminative tendencies plaguing depressed individuals (202), evidence is still contradictory and inconclusive (125, 235). Here, we show that depressed subjects exhibit less functionally connected, or less efficiently communicative DMN nodes. Alternatively, it may be the inability to regulate DMN activation that contributes to MDD, as has been demonstrated in MDD subjects during emotionally-valenced fMRI tasks (143).

Enhanced functional connectivity of the DMN was also corroborated by the finding that controls also displayed increased connectivity between the PCC and the sgACC, another node of the DMN. The sgACC is a central region involved in the regulation of negative mood states and extensively implicated in the pathophysiology of MDD (4). Physically, grey matter volume is significantly reduced within the sgACC in relation to controls; moreover, the effect is more pronounced with more persistent forms of MDD (i.e. chronic, multiple episodes) (236). Metabolically, the region is hyperactive during disease states and normalizes upon remission induced by antidepressant medications (4) and invasive deep brain stimulation of the region (24).

Although our current results cannot decipher the mechanisms associated with increased functional connectivity between the PCC and sgACC, it's possible a more cooperative relationship between the two regions may result in better regulation of affective and internal states.

Importantly, the rACC is also functionally connected within the SN. It's highly possible that increased connectivity between the region and the PCC or the parahippocampus demonstrates greater inter-network connectivity. Regardless, our results illustrate a heightened or more efficient connectivity within major nodes of large-scale networks in healthy subjects. These findings may augment the development of targeted interventions which ultimately normalize network activity in MDD. There is increasing evidence that psychopathologies, including MDD, manifest from an aberrant recruitment of the main networks we've investigated: DMN, SN, and EN (128). Interactions between these networks guide attentional shifts as well as regulation of certain cognitive domains which determine more dominant affective states. Specific to MDD, maladaptive rumination associates with DMN dominance over SN and EN activity (129). Our results may illustrate where connectivity dysfunction occurs in MDD in the form of network hubs. This could be brought into fruition through deep brain stimulation, or even antidepressant drugs designed for particular receptors which concentrate within these regions of interest, or other region-specific neural characteristics.

Depressed subjects did show stronger functional connectivity between the MCC and the dorsomedial PFC. Prior work did not find MCC connectivity differences between MDD and controls (237). With its projections to motor areas, the MCC is associated with response selection and skeletomotor function (238, 239). The region is also involved in processing the reward value of certain behavioral outcomes (240). As anhedonia is a hallmark attribute of MDD

and it's often seen as an issue in motivational aspects of reward as opposed to consummatory lack of pleasure (241), abnormal connectivity of the MCC, with its strong motor connections, with the more self-referential, prefrontal regions [also dysregulated in MDD (143)] may contribute to this symptom of MDD.

In all, our results inform our understanding of brain functioning in individuals diagnosed with MDD as it compares to currently healthy individuals. Although these findings do not attribute functional connectivity abnormalities to certain cognitive or affective processes, they do provide an initial framework of neural pathophysiology in MDD which may serve as a foundation for further investigation. This would necessitate a substantive sample size: patients could be grouped by symptomatology which would better position our understanding of the psychological and behavioral processes associated with neural abnormalities. Moreover, it is difficult to ascertain whether these abnormalities are an emerging phenotype due to the manifestation of depression or whether they are inherent neural abnormalities which give way to MDD.

Moreover, characteristic findings from these two separate approaches to analyze resting-state functional connectivity data highlight the benefits and deficiencies in each methodology: ICA allows for network-based examination of brain functions and thus, all results exist in the context of a large-scale network which mimics the network-based framework of actual brain functioning (122). However, correct model-order input into an ICA protocol is often nebulous. Empirical guidelines for selecting model-order sizes do exist and are based on whether the goal is to isolate major large-scale brain networks versus many smaller sub-networks (178). Yet, it still remains difficult to determine the optimal model-order for adequate analytical sensitivity,

consequently producing false null results. This lack of sensitivity may be why our group ICA results were not as significantly robust in comparison to CWAS.

CWAS, in contrast to ICA, guides analysis by highlighting regions which exhibit the greatest disparity in functional connectivity patterns between groups and thus, are prime target regions for seed-based functional connectivity (210). However, any significant results exist in relation to another region, instead of an entire network, although our results hint at network node abnormalities. Altogether, the results from the present study are two-fold: they illustrate functional connectivity abnormalities in MDD that instruct our understanding of MDD neuropathophysiology as well as the merits of two distinct methodologies for analyzing resting-state data, an emerging and valuable field within neuroscience research.

Chapter Four

Towards Imaging Genetic Markers of Placebo Effects in Major Depression: Insights from Prepronociceptin and Placebo Analgesia

High placebo response rates have been observed in Major Depressive Disorder (MDD) (47). Yet, investigation into the neurobiology of placebo effects as they relate to MDD has been limited (59, 88), despite the therapeutic capability of these effects (242) and their ability to confound antidepressant efficacy measurements within randomized clinical trials (RCTs) (243). This is a major factor contributing to the discontinuation of antidepressant drug development by major pharmaceutical companies (18). In contrast, the field of analgesia, also plagued by high placebo response rates in RCTs for chronic pain (244), has made substantial headway in uncovering the neurobiological mechanisms involved in placebo responses (52, 53, 57, 156, 245). From this work, extensive documentation has implicated the endogenous μ-opioid and the dopaminergic system in placebo analgesia neurobiology [reviewed (77)]. In further progressing these findings, recent evidence has shown the ability of genetic variations within pathways related to opioidergic and dopaminergic signaling, as well as endocannabinoid- and serotonergic-related pathways, to modify placebo effects in depression, as well as in pain and other disorders demonstrating inherent placebo responses (54, 81-83). These findings support the possibility of implementing genetic screening to better identify placebo responders and in effect, improving therapeutic care and RCT efficacy for MDD.

Growing evidence supports the notion that an individual's genetic makeup influences their predisposition to respond to placebo treatment [reviewed (80)]. For instance, the gene for the monoamine oxidase A enzyme (*MAO-A*) has been implicated in neuropsychiatric disorders such as MDD through its role in metabolizing monoamines, including serotonin and dopamine (246). When examining the association between *MAO-A* genetic variation with antidepressant placebo treatment, individuals with the genotype encoding for low-activity of *MAO-A*, and therefore, higher dopaminergic and serotonergic tone, had a significantly higher placebo response than those with the highly active *MAO-A* genotype (87). Relatedly, direct neural evidence of dopaminergic neurotransmission has been observed in the placebo effects of analgesia (53) and Parkinson's disease (56). Moreover, genetic variation within the μ-opioid receptor gene (*OPRM1*) modulates placebo-induced dopaminergic neurotransmission within the nucleus accumbens, a region central to reward saliency and placebo (53, 55). Across disorders, genetic variation within the gene encoding the major dopamine metabolizing enzyme, catechol-O-methyltransferase (*COMT*) also modulates placebo effects in patients with irritable bowel syndrome (81). In all, with the extensive number of genetic variations showing influence onto placebo-related neural pathways, genetic profiling of patients could potentially impact clinical trials and therapeutic treatment for a myriad of disorders, including MDD, by improving our ability to identify patients with greater susceptibility to placebo treatment.

A likely candidate genetic predictor of placebo effects in MDD and pain responses is the prepronociceptin gene (*PNOC)*. It transcribes prepronociceptin (ppN/OFQ) (247), the endogenous precursor protein to the neuropeptide, nociceptin/orphanin FQ (N/OFQ). PpN/OFQ, N/OFQ and N/OFQ's receptor (NOP) (248, 249) form the N/OFQ-NOP system within the opioid family (250, 251). This system plays a substantial role in the neurobiology of pain (252), anxiety,

stress, and emotional regulation (253), reward circuitry (254), mood (255), and learning (256). Animal models of depression have demonstrated stress-induced increases in N/OFQ-NOP mRNA expression in limbic regions (257) and strong antidepressant-like behavioral effects in response to NOP antagonism (258), comparable with those of common antidepressants such as fluoxetine (259). Moreover, elevated plasma levels of N/OFQ have been observed in women suffering from post-partum depression (255). In addition, a post-mortem examination found significantly decreased expression of N/OFQ in the dorsal anterior cingulate of suicide patients (260).

To examine the capability of *PNOC* polymorphisms to modulate or predict placebo responses, we investigated the association between *PNOC* variation and placebo-induced μ-opioid neurotransmission and participant-reported analgesic effects within a well-documented pain and placebo challenge (53, 54, 67, 73, 79, 112). The neurobiology of placebo analgesia has been extensively investigated [reviewed (67, 77)]; therefore, it provides a stable framework for examining the relationship between placebo treatment and novel genetic markers.

To further evince the likelihood that *PNOC* variability may modulate or predict placebo responses, the N/OFQ and μ-opioid system have a high homology – the relationship to placebo in the latter is supported by a large body of work (52, 57, 73, 82, 156): The *PNOC* gene shows structural similarity to endogenous opioid precursor genes (247) and NOP shares structural and functional machinery with the μ-opioid receptor (MOR) (251). There is also evidence of an interaction between the two systems: antagonism of N/OFQ can prevent the development of morphine tolerance in mice (261) and NOP activation enhances the effects of MOR agonists in non-human primates (262).

Moreover, the N/OFQ-NOP system is linked to nociception, anxiety and stress; pain and analgesia both induce stress-response mechanisms (263) and implicate anxiety (264). Non-human primate studies have consistently described anti-nociceptive effects of N/OFQ administration (133, 265). Across rodent models, administration of N/OFQ produces anxiolytic effects (266); while ppN/OFQ knockout mice show excessive anxiogenic behavior and impaired stress adaptation (267).

The present study sought to examine the effect of genetic variations within the *PNOC* gene on placebo-associated μ-opioid neurotransmission. Subjects underwent a sustained pain challenge with and without placebo administration (with analgesic expectations) using positron emission tomography (PET) with [^{11}C] carfentanil, a μ-opioid radiotracer designed to measure μ-opioid neurotransmission, as previously described (53). Based on previous work in our laboratory which demonstrated significant reductions in plasma cortisol levels during a pain challenge after placebo administration with analgesic expectations (79), we implemented an exploratory analysis on potential gene effects on stress-related measures during the pain and placebo challenge: subjects' plasma cortisol levels were measured during scanning. Furthermore, in light of evidence that N/OFQ system is intricately associated with anxiety-like responses in animal models (266, 267) and the role of anxiety in placebo responses (15), we sought to explore a potential linkage between variation within *PNOC* and anxiety trait.

We primarily hypothesize that *PNOC* variation will modulate placebo-induced μ-opioid neurotransmission and associated stress responses; additionally, that genetic variation within this gene will be linked to anxiety traits. If confirmed, we will provide evidence of the potential for *PNOC* genetic variation to modulate placebo-related neural pathways, all of which contributes to the growing 'placebome' (80). Finally, considering the antidepressant properties of the N/OFQ

system, this may be particularly applicable to placebo effects in depression, where predictors of placebo response are essential to better clinical treatment efficacy and pharmaceutical drug development.

METHODS

Subjects

Forty-nine, non-smoking volunteers [29 females; age: 19 - 40 (mean ± S.D: 26.08 ± 4.9)] participated in a pain-stress challenge with and without placebo administration as described previously (53). In addition to physical and neurological examinations, participants completed the non-patient version of the Structured Clinical Interview for DSM-IV (SCID-IV). Participants with current or pervious history of medical, neurological, or psychiatric illnesses (including substance abuse or alcohol intake of more than five drinks per week) were excluded. Protocols were approved by the University of Michigan Investigational Review Board and the Radioactive Drug Research Committee. Written informed consent was obtained from each participant.

Genotyping

Single nucleotide polymorphisms (SNPs) of the *PNOC* gene were genotyped in all subjects using the Illumina Golden Gate assay platform (San Diego, California), which employed the Addictions Array content of 130 genes (1350 SNPs) and 186 Ancestry Informative Markers (AIMs), and has been described elsewhere (268). Manual verification was completed for clustering and genotype calling for each locus; loci with call rates <90% were excluded. Using a minimum of 160 markers, AIMs scores were calculated through comparison to genotype data from the CEPH Diversity panel of similar derivation. Genotyping accuracy was confirmed by replicate genotyping of 10% of the total sample with a completion rate of >93% (mean 99.4%, median 100%) and replicates showed no errors at this loci. All genotyped subjects were assigned to two or, in one case, three groups (Table 4.1). Nine subjects were missing genotyping data for SNP rs1563945, so only 40 subjects were reported for that group.

Population stratification was evaluated as a potential confounder using European AIM scores (since the sample was predominantly Caucasian). To test for population stratification in the neuroimaging and personality traits data, we performed Spearman correlations between ethnic factor scores and changes in μ-opioid BP_{ND} or NEO anxiety scores, respectively. Thus, no confounding was present due to ethnic differences.

Experimental design

Subjects underwent two PET scanning sessions: one in the absence of ('pain' scan) and one ('pain + placebo' scan) in the presence of placebo administration (Figure 4.1). Each scan consisted of a control condition (0.9% isotonic saline, 5-25 minutes after start of scanning) and a painful condition (5% hypertonic saline, 45-65 min after start of scanning) delivered in the masseter muscle via needle. During the pain condition, a steady state of moderate muscle pain was maintained for 20 minutes after radiotracer administration by a computer-controlled delivery system through the infusion of medication-grade hypertonic saline solution (5%) into the left masseter muscle. Pain intensity was rated every 15 seconds from 0 (*no pain*) to 100 (*most intense pain imaginable*) using an electronic version of the 100-mm visual analog scale (VAS) and used by the computer controller to maintain constant pain in a manner comparable across subjects using a target of 40 VAS units, as previously described (269, 270). The same individual infusion profiles generated during the pain challenges were used for the studies with placebo administration ('pain + placebo' condition) (53).

During the 'pain + placebo' condition, subjects were given the following instructions before administration of the placebo: "We are studying the effect of a pain relief medication. This medication is thought to have analgesic effects through the activation of natural brain systems that suppress pain." The placebo condition consisted of the introduction of 1mL of 0.9%

isotonic saline into one of the intravenous ports every four minutes, with the volunteer being made aware of its occurrence. The placebo was introduced starting two minutes before the pain challenge and each injection lasted 15 seconds: subjects were aware that the study drug was to be administered because they were alerted by a computer-generated human voice recording, followed by a second-by-second count of the infusion timing (15 seconds). The VAS was administered as during the pain condition.

At the immediate completion of each challenge, 'pain' and 'pain + placebo,' subjects provided a qualitative measure of the experience by completing the McGill Pain Questionnaire (MPQ) (271), including an overall assessment of affect, sensory, intensity and unpleasantness aspects of pain. These measures along with the VAS scale scores served as a measure for psychophysical placebo response. Differences in values within these measures between conditions ('pain' - 'pain + placebo') were calculated to measure reported analgesic effects. Larger positive numbers on this calculated measure reflect greater reported placebo analgesic responses. Levels of expectancy and effectiveness of placebo were rated before and after the placebo administration, respectively, with the questions: a) expectancy: *'From 0 to 100 how effective do you think the treatment will be?'* and b) effectiveness: *'From 0 to 100 how effective do you think the treatment was?'* The internal affective state of the volunteers was rated with the Positive and Negative Affectivity Scale (PANAS) (272) and the Profile of Mood States (POMS) before and after the scanning session. The composite Total Mood Disturbance score (TMD) was used to evaluate the transient changes in negative mood (POMS-TMD) (273).

PET Data Acquisition and Preprocessing

Each subject underwent two 90-minute PET scans with [^{11}C] carfentanil, a μ-opioid receptor agonist radiotracer (274). For each scan, 15 ± 1 mCi (mean ± SD) of [^{11}C] carfentanil

was administered via an intravenous (antecubital) line. Radiotracer synthesis and image acquisition, coregistration, and reconstruction protocols were identical to those used in previous publications (53).

Three receptor-related measures were calculated: 1) μ-opioid BP_{ND} during the control condition (pain anticipation); 2) μ-opioid BP_{ND} during the pain challenge and 3) μ-opioid BP_{ND} during the 'pain + placebo' challenge (Figure 4.1). Activation of the μ-opioid system during 'pain' and 'pain + placebo' administration was assessed by calculating the μ-opioid BP_{ND} difference between control and pain conditions; and between 'pain' and 'pain + placebo' conditions, respectively; activation of the μ-opioid system during placebo administration with only the expectation of pain was assessed by μ-opioid BP_{ND} difference between the 'pain + placebo' and 'placebo pain anticipation' scan times. Reductions in the *in vivo* availability of μ-opioid receptors (i.e., binding potential [BP]) after an acute challenge are thought to reflect μ-opioid activation through competition between radiotracer and endogenous ligand for the receptor sites (275).

Statistical Analyses

A voxel-by-voxel mixed model of variance analysis was performed using SPM8 (Wellcome Department of Cognitive Neurology, University College, London) and Matlab software (MathWorks, Natick, Massachusetts). No global normalization was applied to the data, and therefore the calculations are based on absolute BP_{ND} values. A cortical mask which excluded the cerebellum but included regions with specific μ-opioid receptor binding potential (BP_{ND}> 0.1) was used for the PET data.

ANCOVA models were performed using genotypes for each independent SNP as the between-subject factor and four within-subject conditions (pain; pain + placebo condition; and

their corresponding control conditions); sex and scan order were input as covariates. The threshold for significance for neuroimaging analysis was set at peak-level of $p<0.05$ family-wise-error corrected to account for multiple comparisons of the voxel-based analysis. The stringency of this threshold was determined to account for independent SNP comparisons. These data were extracted for quantification of regional changes in BP_{ND}, plotting, examination of potential outliers and further statistical analyses using SPSS statistical Software (version 20.0; SPSS, Inc. Chicago, Illinois). Data are expressed as the mean ± 1 S.D., unless otherwise indicated.

Cortisol Collection and Analysis

Samples of blood were collected at a 10 minute interim over the course of each 90 minute scan. Only 34 subjects had complete blood collection that could be used for analysis. Cortisol was assayed using the automated Immulite 1000 chemiluminescent cortisol assay (Siemens Medical Solutions Diagnostic Division) with intra- and inter-assay variability of <5% and 7%–8%, respectively. Each pair of scans: 'pain with placebo' and 'pain without placebo' was conducted at the same time of day (starting at 8.30 a.m. or at 1.30 p.m.). Differences in plasma cortisol level between the 'pain + placebo' condition and the 'pain' condition were plotted (Figure 4.4: 'pain + placebo' – 'pain') for each 10 minute period between 0 and 90 minutes. For a sensitive measure of placebo-induced changes in cortisol plasma levels, area under the curve (AUC) was calculated with respect to increase (AUC_I) from placebo administration onset (time point 40-minute), as comprehensively described elsewhere (276). Briefly, for each subject, total AUC was calculated between scan time point 40- and 50-minutes; AUC with respect to the baseline (AUC_B) of this interim (the 40-minute time point) was calculated and subtracted from the total AUC values to compute AUC_I (AUC_I = total AUC – AUC_B). Therefore, decreases in

cortisol plasma levels after placebo administration are reflected by negative values within this measure.

Significant differences in placebo-induced changes in cortisol plasma levels between genotypes were examined via two-sample t-test using SPSS (SPSS for Macintosh, version 17; SPSS, Chicago, Illinois) with baseline cortisol plasma levels input as a covariate. Data are expressed as the mean ± 1 SD, unless indicated otherwise.

Trait Anxiety measures

We assessed subject trait anxiety using the anxiety facet of the neuroticism domain from the Revised NEO- Personality Inventory (277) and the 'pain' and 'pain + placebo' state and overall anxiety measures were also evaluated with the State Trait Anxiety Inventory (STAI) (278).

RESULTS

SNP-based analysis

Forty-nine healthy volunteers were genotyped for the seven *PNOC* SNPs on the array: rs2722897, rs1563945, rs7825480, rs2614095, rs351776, rs351784, rs4732850 (see Table 4.1 for allele distribution and demographic information). Linkage disequilibrium occurred between rs1563945 and rs7825480; and between rs2614095, rs351776, rs351784, and rs4732850 (Figure 4.2). Based on these linkage disequilibrium results, three independent analyses were considered for statistical purposes and thus, significance for behavioral data was established at a $p<0.017$ after Bonferroni correction. Still, to avoid the existence of multiple groups within each haplotype, independent SNP-based analyses were performed on the neuroimaging and behavioral data for each of the seven *PNOC* SNPs on the array (with neuroimaging data FWE-corrected for multiple comparisons).

PNOC rs1563945 effect on placebo-induced Δ *in* μ*-opioid* BP_{ND}

To investigate the effect of genetic variation within the *PNOC* gene with our available sample size, we performed seven SNP-based analyses on pain- and placebo-induced opioid activation maps. Among the seven SNPs in the Addiction Array, only rs1563945 showed a significant effect on placebo-, but not pain-induced opioid release. None of the other six *PNOC* SNPs had a significant effect on pain- or placebo-induced opioid activation; this included rs7825480 which is in linkage disequilibrium with rs1563945. Compared to their genetic counterparts, rs7825480 'G' carriers did demonstrate a placebo-induced increase in μ-opioid activation in the thalamus although at a lower statistical threshold [(MNI coordinates x, y, z, cluster size mm^3, Z-score, for all regions $p<0.05$ FWE-corr): 14, -5, 2, Z = 4.09; p = 0.3-FWE-corr].

Specifically, *PNOC* rs1563945 'A/A' homozygotes, compared to 'C' carriers, showed placebo-induced increases in μ-opioid activation in the following regions [MNI coordinates x,y,z, cluster size mm^3, Z score, for all regions $p<0.05$ FWE-corr (Figure 4.3)]: left rostral anterior cingulate cortex (rACC); -13, 55, 16; 184 mm^3; Z = 4.82; p = 0.019-FWE; mid-cingulate cortex (MCC); -5, -11, 38; 128 mm^3; Z = 4.93; p= 0.013-FWE; right caudate; 23, 19, 11; 144 mm^3; Z = 4.84; p= 0.018-FWE; left posterior insula (pINS); -38, -29, 20; 144 mm^3; Z = 4.7; p= 0.03-FWE; and right parietal lobule (PL); 58, -55, 19; 72 mm^3; Z = 4.93; p=0.013-FWE. A gene effect on placebo-induced μ-opioid activation was also observed at a lower threshold ($p<0.001$ uncorrected) in the right parahippocampus (16, -32, -8; 9,408 mm^3; Z= 4.52; $p<0.001$) and left posterior thalamus (-17, -30, 1; 9,032 mm^3; Z = 4.50; $p<0.001$). No significant effects were found in the opposite direction ('C' carriers > 'A/A').

During placebo administration with only expectation of imminent pain, an effect of *PNOC* rs1563945 ('A/A' > 'C' carriers) on μ-opioid activation in the left rACC (-12, 43, 21; 17,624 mm^3; Z= 4.52; $p<0.001$-uncorr.) and the right caudate (22, 23, 5; 10,688 mm^3; Z= 4.33; $p<0.001$-uncorr.) was also observed at a lower threshold. There was no significant effect on pain-induced Δ in μ-opioid BP_{ND}.

A quantification of genetic group differences in placebo-induced Δ in μ-opioid BP_{ND} is shown in Figure 4.3B; the same pattern was observed across all regions mentioned above.

PNOC rs1563945 effect on placebo-induced Δ in μ-opioid BP_{ND} and psychophysical placebo measures and affective states

In regions relevant to sensory and affective regulation of pain and placebo responses: placebo-induced regional activation of μ-opioid neurotransmission under *PNOC* rs1563945 influence was significantly correlated with psychophysical responses to placebo, as measured by

changes in MPQ Total, Sensory, Affect and Pain Intensity score after placebo administration (Figure 4.3A; see Table 4.2 for comprehensive results; PANAS and POMS were not included for clarity due to minimal significant findings). Placebo-induced changes in psychophysical placebo measures were calculated by 'pain' – 'pain + placebo' scores. Therefore, more positive values indicate greater reported placebo response (reduction in self-reported pain experience with placebo). In correlation results between opioid and psychophysical measures, positive correlations indicate greater placebo induced μ-opioid activation associates with greater changes (i.e. greater placebo response), in reported pain ratings.

PNOC rs1563945 effect on Δ in pain and affect ratings after pain and after placebo administration

We found no effect of rs1563945 on reported pain experiences: as measured by the MPQ, average VAS scores, volume of hypertonic saline to achieve average target pain ratings or the ratio between VAS ratings and volume infused to achieve target ratings. Instead, we observed a significant effect of *PNOC* rs1563945 ('A/A' > 'C' carriers) on behavioral placebo response as measured by the MPQ Intensity ratings ('pain' – 'pain + placebo'): (AA: 10.2 ± 14.9; C Carriers: - 4.1 ± 16.7; $F = 8.0$, $p = 0.007$); the same was observed with average VAS ratings in the first ten minutes: (AA: 1.1 ± 1.4; C Carriers: .26 ± 1.1; $F = 4.3$, $p = 0.045$) at a trend level. Similarly, at a trend level, 'A/A' homozygotes self-reported lower expectations of placebo effectiveness (AA: 40.2 ± 26.4, C Carriers: 58.4 ± 24.9; $F = 4.6$, $p = 0.039$); however, not for observed placebo effectiveness (AA: 83.63 ± 24.8, C Carriers: 81.8 ± 27.8; $F = 0.018$, $p = 0.8$). A trending gene effect was also observed on placebo-induced changes in pain ratings within MPQ Total (AA: 4 ± 8.7, C Carriers: -0.75 ± 8.6; $F = 0.64$, $p = 0.09$), Sensory (AA: 2.37 ± 5.7, C Carriers: - 1.0 ± 5.7; $F = 0.036$, $p = 0.078$) and Affect (AA: 1.45 ± 2.8, C Carriers: - 0.12 ± 2.4; $F = 0.38$, $p = 0.072$).

Of the other six *PNOC* SNPs, only rs351776 showed a significant effect on behavioral placebo as measured by MPQ Intensity (AA: 10 ± 21.4; AC: -3 ± 11.7; CC: 11.7 ± 8.3; F = 5.2, p = 0.009), Average VAS (0-10 minutes) (AA: 1.3 ± 1.4; AC: .06 ± 1.3; CC: .97 ± .9; F = 4.8, p = 0.013), and at a trend level, with MPQ Affect (AA: 1.07 ± 3.5; AC: -0.24 ± 1.9; CC: 2.8 ± 2.9; F = 4.3, p = 0.019). It also had a significant effect on pain ratings: MPQ Total (AA: 17.9 ± 8.3; AC: 21.8 ± 11.3; CC: 31.6 ± 10.9, F = 5.2, p = 0.009) and MPQ Sensory (AA: 11.2 ± 5.8; AC: 13.7 ± 6.4; CC: 21 ± 4.1, F = 8.0. p = 0.001).

None of the *PNOC* SNPs had a significant effect on pain- or placebo-induced changes in positive and negative affect as measured with the PANAS and POMS.

PNOC rs1563945 effects on pain and placebo-induced changes in cortisol plasma levels

Based on previous findings demonstrating placebo-induced reductions in cortisol levels (79) and our current findings showing an effect of *PNOC* rs1563945 on placebo-induced μ-opioid activation, we examined the effect of rs1563945 on plasma cortisol level changes after placebo administration. There was a significant gene effect on placebo-induced stress responses as measured through cortisol plasma levels. Compared to 'C' carriers, 'A/A' homozygotes showed a decrease in cortisol plasma levels in response to placebo administration (Figure 4.4). AUC_I analysis of cortisol levels in response to placebo administration (minute 40 to 50 of scan time) resulted in a significant difference between genotypes (AA: -13.6 ± 32; C carriers: 4.6 ± 19; F = 4.7, p = 0.039). Here, negative values illustrate reduction in cortisol at the onset of placebo administration. This stress-response measure was also significantly, negatively correlated with placebo-induced Δ in μ-opioid BP_{ND} in the rACC and caudate (Table 4.2; Figure 4.4); therefore, greater placebo-induced opioid activation is associated with greater decreases in plasma cortisol levels.

PNOC SNP effects on STAI and NEO Anxiety Measures

We found that placebo-induced Δ in μ-opioid BP_{ND} in the rACC was significantly negatively correlated with anxiety [rACC: STAI r = - 0.43, p = 0.005; NEO r = - 0.37, p = 0.02 (Table 4.2)]. Therefore, we explored a potential link between *PNOC* and anxiety trait, however the size of our sample limits our ability to form substantiated generalizations about *PNOC* SNP effects on personality traits. Still, from an exploratory interest, we find that *PNOC* rs1563945 showed a trending effect on trait anxiety as measured by the Anxiety NEO: 'A/A' homozygotes showed lower levels of anxiety trait, compared to 'C' carriers. However, rs7825480 did not show an effect on trait anxiety. Among all the other *PNOC* SNPs, rs2614095 and rs4732850, which are in linkage disequilibrium, showed a significant effect on trait anxiety score as measured by the STAI. *PNOC* rs351776 and rs351784 (Figure 4.2), also in linkage disequilibrium with rs2614095 and rs4732850, showed an effect on STAI score, although at a trend level. The means and significance values are shown in Table 4.3 along with SNP effects on Anxiety NEO.

State anxiety measures were taken during 'pain with' and 'pain without' placebo administration: again, only rs1563945 showed a gene effect on state STAI during pain (AA: 30.4 ± 4.9; CC: 39.7 ± 5.6; F = 13.8, p = 0.002) and placebo-induced reductions in state anxiety (AA: -3.6 ± 5.9; C Carriers: 1.44 ± 4.6; F = 3.9, p = 0.06) at a trend level. However, STAI state measures were only available for 18 subjects (9 'A/A').

Figure 4.1: Experimental Design All subjects underwent two 90 minute PET scans after [^{11}C] carfentanil injection. Each scan consisted of a control condition (0.9% isotonic saline, 5-25 minutes after start of scanning) and a painful condition (5% hypertonic saline, 45-65 min after start of scanning) delivered in the masseter muscle via needle. Pain intensity was rated every 15 seconds from 0 (*no pain*) to 100 (*most intense pain imaginable*) using VAS; pain maintained at a level of 40 VAS units via computer-controlled infusion. Placebo was introduced with 1 ml of 0.9% isotonic saline solution every four minutes, starting two minutes prior to pain anticipation and pain challenges.

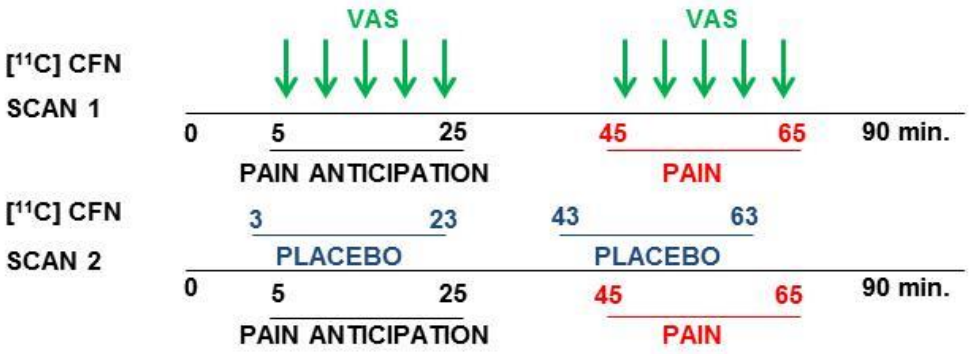


Figure 4.2: Linkage Disequilibrium of the Seven *PNOC* SNPs included in the analysis. The haplotypes match those identified in the HapMap Project.

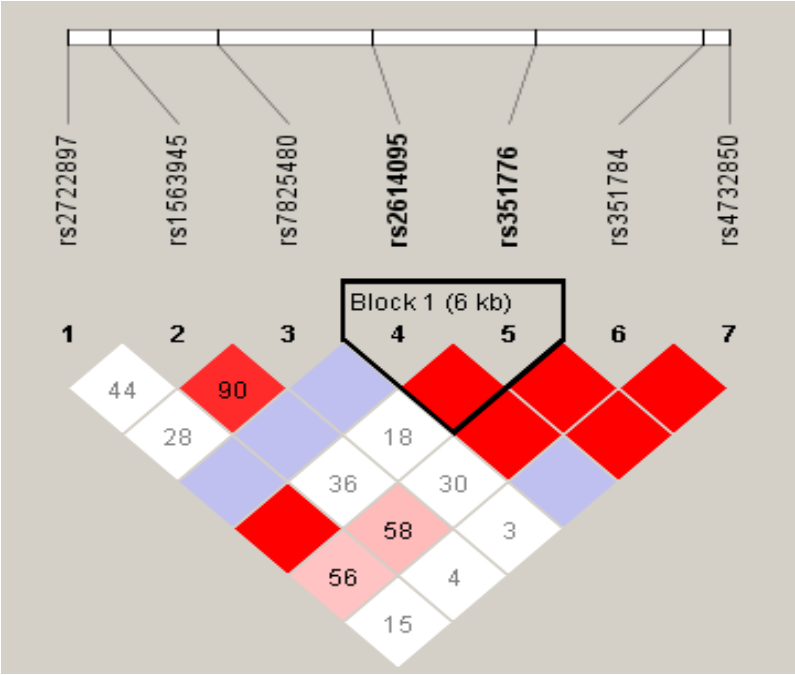


Table 4.1: Demographic and Hardy-Weinberg Equilibrium Data for *PNOC* SNPs
SNP-based comparison of age and European AIM demographics; H-W results. Sex (M/F = 20/29) and ethnicity (Caucasian/Total = 38/49) distribution were identical for all SNPs (except rs1563945: 18M/22F; 29 Caucasian/Total) and thus, were excluded from table data. There were no significant differences with respect to sex between genotype groups within each SNP group. (*p<0.05, **p<0.01, ***p<0.001).

	Demographics		***Hardy-Weinberg***	
***PNOC* SNP**	**Age**	**European AIM**	**Chi²**	**p value**
rs2722897			.27	.6
GG (34)	26.2 ± 4.8	.68 ± .4		
A Carriers (15)	24.2 ± 4.7	.64 ± .4		
rs1563945			< .001	.98
AA (24)	25.7 ± 4.3	.7 ± .4		
C Carriers (16)	26.6 ± 5.5	.5 ± .4		
rs7825480			.39	.5
AA (32)	25.9 ± 4.3	.68 ± .4		
G Carriers (17)	26.4 ± 6	.64 ± .4		
rs2614095			.36	.55
GG (22)	26. 2 ± 6	.54 ± .4*		
A Carriers (27)	25.9 ± 4	.77 ± .3*		
rs351776			.06	.8
AA (15)	25.4 ± 5	.53 ± .4		
AC (25)	26.2 ± 5	.68 ± .4		
CC (9)	26.7 ± 3	.84 ± .2		
rs351784			3.5	.06
GG (29)	26.6 ± 5.6	.8 ± .3**		
C Carriers (20)	25.3 ± 3.5	.4 ± .4 **		
rs4732850			.03	.86
GG (37)	26.4 ± 5	.72 ± .4		
A Carriers (12)	25.6 ± 4	.49 ± .4		

Note: All analyses of rs2614095 and rs351784 were completed with European AIM as a covariate

Table 4.2: Association between *PNOC* rs1563945 placebo-induced Δ in μ-opioid BP_{ND} with psychophysical placebo measures, anxiety, and plasma cortisol levels. μ-opioid activation in regions under *PNOC* rs1563945 influence was correlated with behavioral placebo measures: MPQ Total, Affect, Sensory, Unpleasantness, Intensity, and Average VAS (positive correlations signify greater opioid activation relates to greater reported placebo analgesic effects); trait anxiety measures: STAI and NEO Anxiety; and plasma cortisol levels. Cortisol levels were measured as the difference between cortisol plasma levels at time points: 40 minutes to 50 minutes at 'placebo + pain' from 'pain' condition. (**p<0.017, ***p<0.001).

	rACC	MCC	Caudate	Posterior Insula	Parietal Lobule
	r	r	r	r	r
Placebo-Induced Δ in Pain Rating					
Total McGill	.4**	.02	.4**	.3	.3
McGill Affect	.4	.1	.4**	.3	.4**
McGill Sensory	.4**	.1	.4	.3	.3
McGill Pain Unpleasantness	.2	.1	.2	.1	.3
McGill Pain Intensity	.3	.2	.3	.2	.3
Avg VAS (0 - 20 min.)	.3	.1	.4**	.2	.35
Avg VAS (0 to 10 min.)	.3	.2	.4	.3	.3
Trait Anxiety Measure					
STAI Trait	-.4**	-.2	-.05	-.4	-.2
Anxiety NEO	-.4	-.3	-.1	-.3	-.2
Placebo-induced Δ in Cortisol Plasma Level					
AUC 40 to 50 min.	-.4*	.05	-.7***	-.3	-.5**

Table 4.3: PNOC SNPs and Trait Anxiety

All *PNOC* SNP effects on STAI Trait and NEO Anxiety scores. Total subject number for each genotype given accordingly. (*p<0.017, **p<0.001).

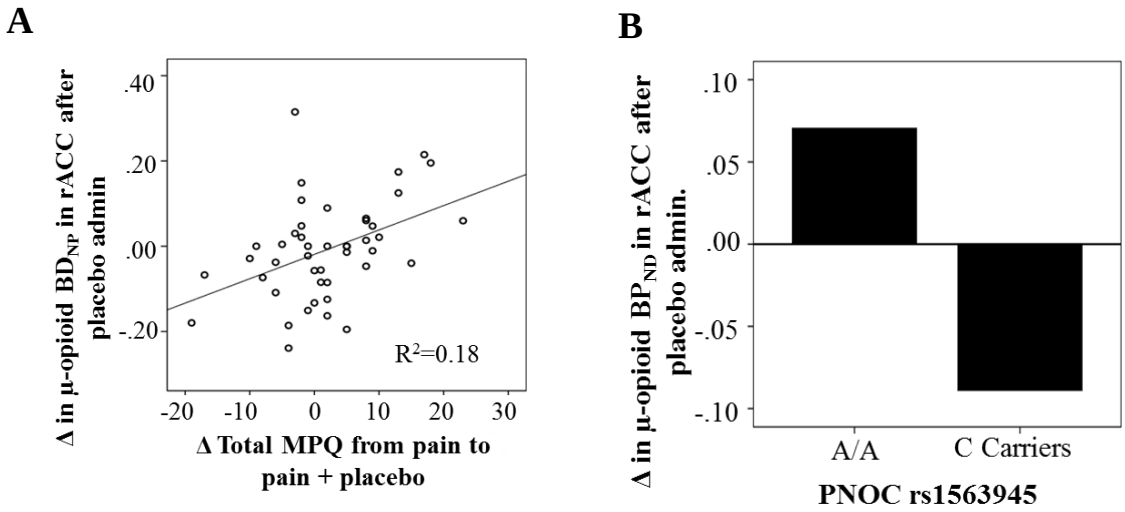
	Trait Anxiety Measure						
PNOC SNP (N)	**STAI Trait**			**Anxiety NEO**			
	Mean	**F**	**Sig.**	**N**	**Mean**	**F**	**Sig.**
rs2722897							
GG (34)	37.7 ± 10	4.5	.04	33	14.6 ± 5.9	.3	.6
A Carriers (15)	31.6 ± 5.5			15	13.7 ± 5		
rs1563945							
AA (24)	33.1 ± 7.4	1.28	.26	24	12.75 ± 4.3	3.8	.057
C Carriers (16)	36.3 ± 10.2			15	15.9 ± 5.7		
rs7825480							
AA (32)	35.6 ± 9	0.053	.8	32	13.7 ± 5	.9	.35
G Carriers (17)	36.3 ± 10			16	15.4 ± 6.6		
rs2614095							
GG (22)	32 ± 7	7.1	.01*	21	12.9 ± 5.7	3.4	.072
A Carriers (27)	38.9 ± 10			27	15.4 ± 5.5		
rs351776							
AA (15)	31.6 ± 5			15	11.4 ± 4.6		
AC (25)	36 ± 9.7	4	.025	24	15.7 ± 5.8	3	.057
CC (9)	42.3 ± 11.5			9	15.4 ± 5.4		
rs351784							
GG (29)	38.4 ± 10	6.6	.013*	28	15.1 ± 6	3.1	.083
C Carriers (20)	32.1 ± 7			20	13.2 ± 5		
rs4732850							
GG (37)	37.9 ± 9.7*	8	.007*	36	15 ± 6	1.5	.22
A Carriers (12)	29.5 ± 5*			12	12.6 ± 4		

Figure 4.3: *PNOC* rs1563945 effect on placebo-induced μ-opioid system activation

A) Association between placebo-induced Δ in μ-opioid BP_{ND} with placebo response (measured by Total MPQ score: pain – 'pain + placebo' conditions (r = 0.43; p = 0.004).

B) Quantification of placebo-induced Δ in μ-opioid BP_{ND} in the rACC (pain – 'pain + placebo') conditions in each rs1563945 genetic group. An identical pattern was observed across all significant regions.

C) Regions of greater μ-opioid release during placebo administration in A/A homozygotes compared to C carriers. Image display at p<0.005 uncorrected.



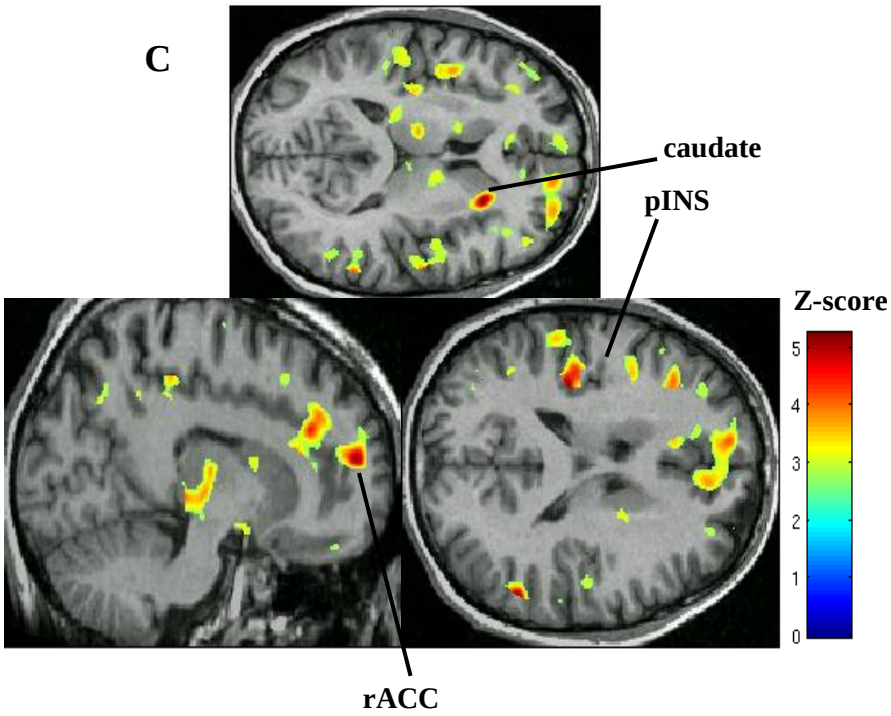
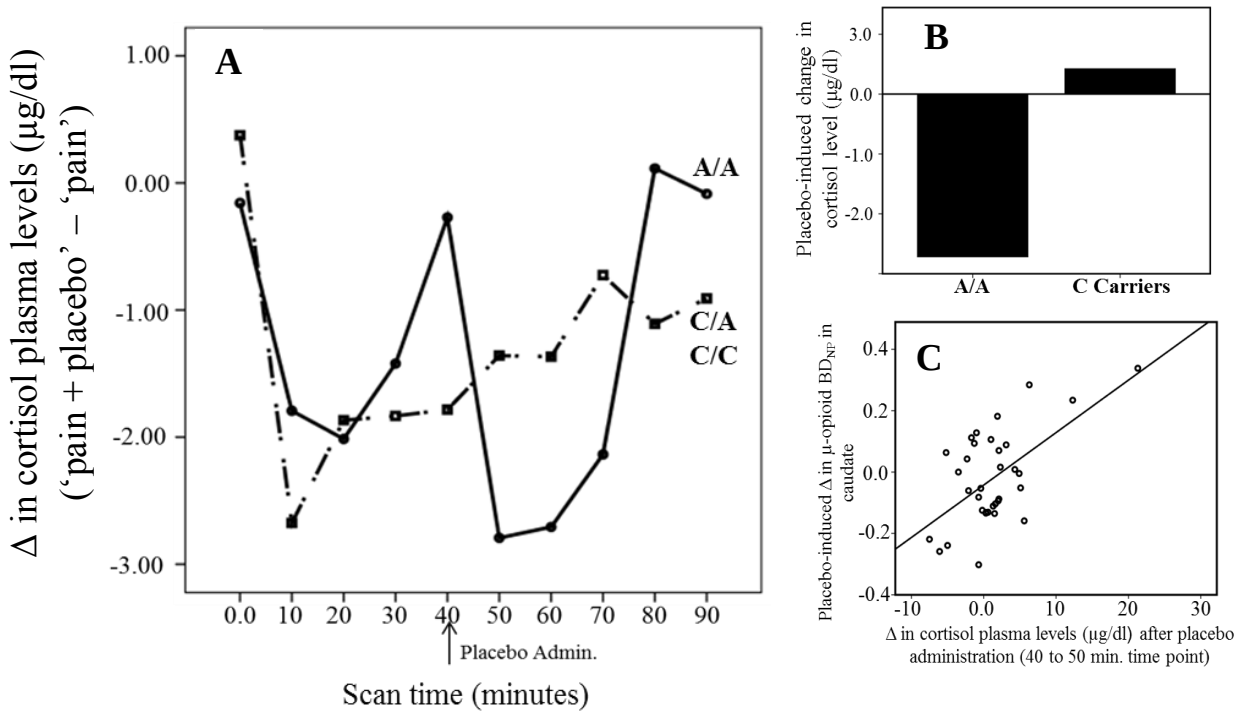


Figure 4.4: *PNOC* rs1563945 effect on placebo-induced Δ cortisol plasma levels
Plotted: **A**. Placebo-induced changes in cortisol plasma levels (µg/dl) [plotted: 'pain + placebo' – 'pain'] for each genotype group (**solid**: 'A/A' homozygotes; **dotted**: 'C' carriers). Placebo administration began at minute 42. **B**. Quantification of changes in cortisol plasma levels in response to placebo administration (40 to 50 minute time point). Negative values demonstrate reductions in cortisol plasma levels in response to placebo ('pain + placebo' – 'pain'). **C**. Association between placebo-induced reductions in µ-opioid BP_{ND} ('pain' –'pain + placebo') in the caudate with changes in cortisol plasma levels ('pain' - 'pain + placebo') conditions during the timeframe of placebo administration (50 minutes – 40 minutes after the start of scan time; r = 0.6; p<0.001). Here, more positive values demonstrate greater placebo-induced reductions in cortisol levels at the 50 minute time point versus at 40 minutes.



DISCUSSION

The present study examined the effects of *PNOC* polymorphisms on placebo-induced μ-opioid neurotransmission and plasma cortisol levels. Among the seven *PNOC* SNPs examined, rs1563945 had a significant effect on μ-opioid activation in response to placebo administration in several brain regions: the rACC, MCC, pINS and caudate. *PNOC* rs1563945 'A/A' homozygotes showed greater placebo-induced μ-opioid activation and self-reported psychophysical placebo responses, yet lower expectations of placebo effectiveness compared to their 'C' carrier counterparts; an identical gene effect, albeit at a lower statistical threshold, was also observed within the rACC during placebo administration with expectations of pain, but prior to actual infusion. A significant difference between genotypes in their stress response was also apparent through contrasting patterns of placebo-related changes in plasma cortisol levels: 'C' carriers demonstrated an increase in plasma cortisol levels, whereas cortisol levels of 'A/A' homozygote's declined. In connection, placebo-induced cortisol fluctuations were significantly associated with placebo-induced μ-opioid system activation in the rACC and caudate. Placebo-induced μ-opioid activation within these same regions was also significantly, negatively correlated with anxiety trait. Finally, an association between the *PNOC* gene and trait anxiety was observed within multiple SNPs of the same haplotype (rs2614095 and rs4732850), while rs1563945 demonstrated a weaker relationship.

Although our investigation would be aided by a NOP radiotracer [which is currently unavailable, but in development (279)], we provide some insight about the involvement of this opiate member in placebo analgesia through the examination of *PNOC* gene effects on μ-opioid activation in response to placebo administration. The μ-opioid system's key role in establishing placebo analgesic effects has been well-documented (53, 57, 73). Specifically, we found that

PNOC rs1563945 ‘A/A’ homozygotes showed substantially greater placebo-induced μ-opioid neurotransmission than their complementary group of ‘C’ carriers within the follow regions: the rACC, caudate, pINS, MCC and parietal lobule. In actuality and in contrast to their genetic counterparts, ‘C’ carriers displayed increases in μ-opioid BP_{ND} within these regions, signifying a reduction in opioid neurotransmission. While rs1563945 is a non-functional SNP, this disparity between genotypes highlights *PNOC's* influence, and by deduction, its neuropeptide products and associated receptors, on placebo-associated neural mechanisms. Of further interest, mounting evidence has described activity, including μ-opioid neurotransmission, within the rACC as an integral region in the formation of placebo analgesia (57, 72, 73, 113, 156). Moreover, involvement of the caudate, pINS, and MCC have also been documented in placebo neurobiology in the context of pain and major depression (53, 280). Our results suggest that ppN/OFQ and its family of products and receptors possess a level of influence on molecular mechanisms within placebo-associated brain regions.

We also observed that μ-opioid activation after placebo administration was significantly, negatively correlated with participants' trait anxiety levels. More specifically, we found that *PNOC* rs1563945 ‘A/A’ homozygotes, in addition to greater μ-opioid release in response to placebo, demonstrated lower levels of anxiety, albeit at trend levels. However, we found a significant gene effect of two other SNPs: rs2614095 and rs4732850 [both members of another haplotype (Figure 4.2)] on trait anxiety. While it is difficult to make substantial conclusions from our results due to our subject sample size, these findings hint at a potential relationship between the human N/OFQ-NOP system and anxiety as has been found in animal models: anxiolytic effects have been observed with low doses of N/OFQ administration (253) and non-peptide NOP agonists (266, 281). Yet, opposing findings have also been described with intracerebroventricular

N/OFQ administration (282). Importantly, our results suggest a contributory role of the N/OFQ-NOP system in modulating μ-opioid-based mechanisms associated with placebo analgesia and anxiety. Anxiety appears to be an important factor in placebo responses: research investigating personality predictors of placebo analgesia found low trait anxiety levels to predict better placebo responses (283) and reductions in anxiety are involved in the formation of placebo responses (284). Moreover, a functional MRI study found an association between placebo responses and reduced activation within anxiety-related brain regions (285).

Regarding stress responses to placebo analgesic administration, we observed that 'A/A' homozygotes demonstrated a placebo-induced drop in plasma cortisol levels. This pattern was significantly different from their 'C' carrier counterparts who showed a placebo-induced increase. Furthermore, plasma cortisol reductions in response to placebo administration were significantly associated with placebo-induced μ-opioid neurotransmission, confirming previous findings (79). Previous experimentation has observed that μ-opioid receptors are involved in the neurobiology of stress (74), stress-induced analgesia (286), and placebo analgesia (73) which is a stress-related mechanism (67). However, the direct relation between cortisol [the output of the stress response regulator, the hypothalamic-pituitary-adrenal axis (HPA) (287)] and molecular mechanisms of placebo analgesia is limited (79) and often this link has been disputed (288, 289). Here, we find significantly different patterns of placebo-induced plasma cortisol in placebo responders ('A/A' homozygotes) and non-responders ('C' carriers). These results strengthen existing evidence that placebo analgesia manifests through stress reduction (52) by opioid modulation of the HPA axis (290, 291). On the other hand, nocebo, or increases in pain, responses are related to increases in stress and anxiety: a study found benzodiazepine administration, a common anti-anxiety agent, to block nocebo-induced HPA hyperactivity and

hyperalgesia (292). Similarly, this pattern was replicated here: 'A/A' homozygotes – generally the responders – along with placebo-induced reductions in plasma cortisol levels, demonstrated placebo-induced anxiety relief, whereas their genetic counterparts – the non-responders – showed a placebo-induced increase in cortisol and anxiety. The anxiety findings were observed at lower statistical threshold, however this could be due to a diminished sample size for state anxiety measures.

Animal studies have also revealed ppN/OFQ and N/OFQ to have a large, yet complicated role in stress regulation and anxiety (132): in comparison to wild-type mice, ppN/OFQ knockout mice display an anxiogenic phenotype in reaction to environmentally-induced stress (293). The same effects are observed in N/OFQ-specific knockouts (267). Furthermore, NOP agonists induce anxiolytic behavior across rodent species (266, 294). However, contradictory evidence is also extant: N/OFQ administration increased anxiogenic behavior in rats (295), confirmed by induced anxiolytic behavior after NOP antagonism (296). Of interest, anxiety-related effects of N/OFQ appear to stem from an interaction with corticotrophin release factor (CRF), a major modulator of stress responses (297): N/OFQ infusion prior to CRF blocked the anxiogenic effects of CRF in rats (298). While the exact pathways and definitive effects of the N/OFQ-NOP system in relation to anxiety and stress are still elusive, the existence of its involvement in anxiety- and stress-associated mechanisms is indisputable (299). Our findings suggest a role in humans as well, which requires further and extensive probing.

Importantly, our results add to the growing literature understanding the genetic underpinnings of placebo effects: the placebome (80). To our knowledge, this is the first study to unearth a connection between genetic polymorphisms within the N/OFQ opiate family and

placebo, stress-related mechanisms, and anxiety in humans. These mechanisms are intertwined as anxiogenic behavior is elicited in response to stressful (threatening) situations (300), such as pro-nociceptive stimuli. Moreover, placebo analgesia is viewed as a mechanism utilized in stressful situations to combat perceived stress (i.e. a pain challenge) and is associated with reductions in anxiety (301). It is difficult to speculate on the molecular pathways contributing to our results. However, the implication of N/OFQ opiate family in placebo, anxiety and stress is sufficient to address the need for further investigation. This may also provide insight into the functionality between and within placebo, anxiety and stress; ultimately, contributing to a more comprehensive understanding of placebo-related pathways and the nature of their genetic influences.

In particular, examination of *PNOC* polymorphisms has provided insight into the physiological and psychological patterns associated with placebo responses: individuals of a specific genotype (rs1563945 A/A) exhibited greater μ-opioid neurotransmission in response to placebo and self-reported analgesic effects, which was accompanied by reductions in plasma cortisol levels, and lower expectations of placebo effectiveness. While the complementary genotype demonstrated a lack of reported analgesic effects and diminished placebo-induced μ-opioid neurotransmission. This was accompanied by increased plasma cortisol levels and expectations of placebo effectiveness. Moreover, insight can be gained by acquiring additional evidence to prior hypotheses: For instance, a recent study in our laboratory posited the discrepancy between expected and observed outcomes in regard to placebo effectiveness as a cognitive mechanism underlying placebo responses (71); here, there were no significant differences between observed placebo effectiveness across genotypes, yet rs1563945 ‘A/A’ homozygotes had lower expectations of placebo analgesic efficacy.

Further investigation into the genetic effects of *PNOC* polymorphisms on placebo-associated pathways is warranted, especially in MDD. Our findings support the potential of this gene to mark individuals likely to respond to placebo treatment. This is particularly relevant in the context of the high placebo response rates in MDD (47); supported by evidence of the N/OFQ system's role in mood and observed antidepressant properties (132, 258). Predicting placebo responders has valuable clinical application through augmenting the effort to differentiate drug-specific and placebo-specific effects and to better target neural circuitry associated with recovery for future treatments. Moreover, identifying placebo responders may enhance personalization of treatment, therefore improving treatment efficacy and concurrently, reducing treatment duration and costs (19, 161, 302). Altogether, these findings are suggestive of the potential role of the N/OFQ system's role in depression placebo neurobiology; particularly since corresponding placebo mechanisms are believed to be engaged across disease states like pain and depression (77, 88).

Chapter Five

Conclusions and Future Directions

Investigation into the neural basis of placebo responses in major depression has been largely unexplored. Yet, the mechanisms associated with this psychobiological phenomenon have critical potential in elucidating neural pathways implicated in resiliency to diseases like depression. These mechanisms contribute to the recovery from depression; therefore, their investigation may be an essential component in the overall research to improve treatment for the disease (67). One potential aspect of this study is to examine neurobiological predictors of placebo and antidepressant treatment responses in order to clarify the neurobiology associated with positive treatment outcomes and in order to better categorize patients on their susceptibility to respond to placebo or particular antidepressant treatments. Comprehensively, the antidepressant treatment response is composed of the 'actual treatment effect' + 'placebo effect' + other non-specific effects, and because of this, clinical antidepressant trial outcomes are often inconclusive in separating drug and placebo effects (18). The placebo response is also present in clinical settings of antidepressant treatment. Therefore, predictors of interest are those which are specific to antidepressant treatment and those which are specific to placebo responses; their investigation serves to inform mechanisms specific to treatments and those specific to resiliency mechanisms against depression symptoms. This type of knowledge may further improve future treatment targets as well as stratify patients in clinical settings and clinical trials by their

likelihood of responding to placebo or particular treatments. Identifying likely placebo responders may signify that they may benefit from less intensive treatment in the form of smaller medication dosages or more comprehensive patient-clinician interaction. Elements of the placebo response may be capitalized upon to relieve patient suffering and improve overall treatment experiences and efficacy (242).

Predictors of Antidepressant Treatment Effects

Here, we investigated predictors of placebo responses after one-week of placebo administration with expectations of antidepressant effects and predictors of antidepressant responses after ten week of an open-label antidepressant treatment. To do so, we employed a network-based resting-state functional connectivity perspective in order to capitalize on the inherent network-based organization of the human brain and the accessibility of the methodology – free from experimental task paradigm constraints. Specifically, we focused on the default-mode (DMN), salience (SN), and executive (EN) networks within our investigation. We found that increased cohesion within the SN in the rostral anterior cingulate (rACC) was significantly predictive of greater reductions in depressive symptoms in response to one-week of placebo administration with expectations of antidepressant effects. Greater connectivity within the posterior insula (pINS) and dorsolateral prefrontal cortex (dlPFC) within the SN also contributed to this finding, however at lower statistical thresholds. The same pattern of enhanced SN connectivity within the pINS, dlPFC, and rACC also predicted greater reductions in depressive symptoms after ten weeks of open-label antidepressant treatment response; moreover, anterior insula (aINS) functional connectivity within the network demonstrated a relationship in an opposing direction.

Of interest, the positive predictive relationship between the rACC, dlPFC, and pINS and antidepressant responses were observed at a lower statistical threshold compared to their relationship with placebo responses. This may be indicative of these regions specifically predicting only the portion corresponding to the placebo response inherent within the total antidepressant response. Alternatively, SN connectivity may be predicting the interaction between active drug and placebo effects. However, further work within the context of a randomized clinical trial (RCT) employing parallel drug and placebo arms, and is of a substantial sample size, is essential to corroborate these findings and relevant hypotheses.

Remarkably, the two regions within the SN which were most prominent in the prediction of treatment responses were the rACC and INS. Existing literature describing MDD treatment neurobiology consistently identifies these two regions as treatment response predictors [reviewed (19, 96, 97)]. However, these findings have emerged by examination of isolated regional activity or in relation to an experimental task. In contrast, our work provides a comprehensive, intrinsic framework for the regions: functional connectivity within the SN during a resting state. This further implicates the network within the neurobiology of placebo responses as well as a component of the antidepressant treatment response, none of which has ever been demonstrated.

Furthermore, we provide evidence of the rACC's capability to significantly predict reductions in depressive symptoms in response to placebo administration with expectations of antidepressant effects. Pretreatment activity within the region is the main predictor of treatment response across imaging modalities and across antidepressant treatments (44, 136, 188, 303); moreover , it is a substantive indicator of sustained treatment response (27). Therefore, our finding that it predicts placebo responses may place prior work in new light: the region may be predicting non-specific effects of treatment or possibly, an interaction between treatment-specific

and placebo effects. In all, these findings begin to outline the primary neuronal component involved within a successful reduction in depression symptoms. The intricate role of the rACC in placebo analgesia neurobiology (57, 72, 113), further evinces that the rACC is a key element in resiliency mechanisms, or in homeostatic responses to stressors such as a depressive episode or experience of pain.

Our findings regarding the insula are also of interest. Primarily, we found a dissociation between the anterior and posterior insular regions in their predictive capabilities. The aINS was observed to exclusively relate to antidepressant treatment response; specifically, decreased connectivity within the region was related to greater reductions in depression symptoms after ten weeks of open-label antidepressant treatment. This predictive pattern has been demonstrated in prior work, notably in a comprehensive meta-analysis of antidepressant treatment response predictors (97). However, alternative patterns of enhanced aINS metabolism have also been predictive of a better response to antidepressant medication (95); still, the region is theorized to be a strong marker of treatment selection. Particularly, lower levels of activity signify better response to cognitive therapy whereas higher levels are conducive to a patient requiring more intensive treatment (19). In contrast, the pINS was predictive of both placebo and antidepressant treatment responses. Interestingly, the one study to examine placebo neurobiology in MDD found aINS metabolism changes to associate specifically with antidepressant medication response, whereas increases in pINS glucose metabolism was associated with metabolism changes in both placebo and antidepressant responders (59). More posterior regions of the insula may be involved in shorter-term responses, mostly produced by placebo effects, while the aINS may be implicated in more long-term, sustained antidepressant treatment responses. While both areas are known to have cognitive functions, the pINS has more connections with areas in the

sensorimotor cortex and is involved with internal and external sensory perceptions while the aINS is better associated with autonomic, emotional, and higher cognitive processes (149). While both regions of the insula may be necessary for placebo and antidepressant responses, the aINS may have more executive and regulatory power to obtain longer-term, sustained antidepressant outcome. Correspondingly, treatment-induced modulation of the aINS is a pervasive marker of clinical outcomes (304).

Neurobiology of Major Depression

Following our investigation into treatment response predictors, a network-based resting-state functional connectivity approach was employed to investigate neurobiological differences between depressed subjects and healthy functioning controls. Although group ICA produced null results, a further decomposition of the three investigated networks demonstrated diminished connectivity of the rACC within the SN in depressed patients compared to healthy subjects. Additionally, a major subset of the left EN also demonstrated decreased connectivity within the depressed group. Overall this speaks to greater cohesion within the rACC of the SN and the left EN in healthy functioning individuals compared to patients with depression. Furthermore, through a connectome approach, functional connectivity between major nodes of the DMN – rACC, posterior cingulate cortex (PCC), and the parahippocampus, as well as the subgenual anterior cingulate (sgACC) – was significantly reduced within depressed subjects.

Altogether, these observations contribute to the understanding of neurobiological abnormalities attributed to MDD. Further examination is warranted to examine the psychological processes associated with these neural patterns; however, the emergence of increased rACC connectivity within the SN is of consequence. As described above, the region is intricately linked to antidepressant treatment responses and their success. Therefore, by demonstrating an

association between this rACC-SN pattern and healthy functioning individuals, these findings further progress the hypothesis that rACC is a central component to healthy cognitive-affective processing and contributes to the neural basis of MDD recovery. These observations also point to potential treatment targets for MDD. Notably, the major regions we observed to have greater functional connectivity in healthy controls have all been described as major nodes of the DMN, a network whose main responsibility is to monitor internal states of self (305). Aberrant downregulation and connectivity within the network has been described in MDD (23, 143). While the nature of its functional connectivity compared to controls is in debate (125), primarily, the network is believed to be dysregulated in MDD, mostly in relation to the engagement and disengagement of the SN and EN. The three networks are part of a triple network model: deficits in access, upregulation and deregulation of the three large-scale neural networks are hypothesized to serve as the major basis for affective and cognitive dysfunction in depression, as well as other psychopathologies (128).

While extensive investigation into the nature of inter-network relationships is still needed to define their implication in MDD manifestation, our work points to potential nodes as targets for ultimate network regulation or normalization. Deep brain stimulation of the sgACC, also a hub of the DMN and identified as a major node within an emotional and mood regulatory network (14), is effective in treating patients with treatment-resistant depression (24). Alternatively, the rACC, PCC or parahippocampus may be potential treatment targets. Although possibly not anatomically ideal for surgical stimulation, their activity may be modulated with regional receptor-specific medications or stimulation through improved transcranial measures such as electroencephalography (EEG) or transcranial magnetic stimulation (TMS).

Alternatively, a neurofeedback task may be employed to engage and strengthen these regions within depressed patients.

Finally, we investigated neuroimaging-genetic markers of placebo analgesia through the effects of genetic variations within the prepronociceptin gene (*PNOC*) – a gene with implications in MDD (132) – on placebo-induced μ-opioid neurotransmission and plasma cortisol levels. We also examined the relationship with levels of trait anxiety. The neurobiology of placebo analgesia has been extensively mapped so we sought to capitalize on this advancement to find potential clinical neuroimaging markers which may ultimately inform MDD placebo neurobiology. Moreover, our work also elucidated neural correlates of placebo neurobiology and not in the least, contributed to the development of the placebome (80). Research expanding the placebome serves to uncover genetic influences on placebo-associated pathways in order to identify candidate genetic markers for their eventual employment within clinical settings for a better assessment of likely placebo responders or for antidepressant clinical trials. Moreover, these markers may further uncover novel placebo-associated mechanisms; for instance, more details regarding aspects of neurotransmitter signaling or specific involvement of previously unexamined systems, such as the endocannabinoid system (54).

Here, we identified a single nucleotide polymorphism with significant effect on placebo-induced μ-opioid neurotransmission: individuals homozygous for the 'A' allele demonstrated greater μ-opioid activation – within the rACC, pINS, and caudate which are established placebo-related regions (77) – and greater reductions in self-reported pain measures than their 'C' carrier counterparts. Furthermore, the 'A' homozygotes' increased perception of analgesic effects and associated μ-opioid-based neurobiological response was significantly related to decreases in plasma cortisol levels and, although at a trend, associated with lower levels of

anxiety. These findings contribute to a general understanding of placebo responses: well-documented activation of the μ-opioid system, reductions in cortisol plasma levels, and lower levels of anxiety. The 'C' carriers confirmed these relationships as they demonstrated reduced μ-opioid activation, increases in placebo-induced plasma cortisol levels, and higher levels of anxiety. This also contributes to defining the neural basis of nocebo responses, or worsening in symptoms following placebo administration with expectations of therapeutic effects.

These results inform potential predictors of placebo responses in the form of genetic markers and anxiety traits. Their relation to the placebo response is corroborated by neural correlates in the form of μ-opioid activation and plasma cortisol decreases. These findings in placebo analgesia can be extended to investigate neurobiological mechanisms and correlates of placebo responses in MDD. Overlap in mechanisms may point to general resiliency mechanisms to homeostatic stressors such as pain or a depressive episode. Moreover, these and disease-specific placebo mechanisms may point to potential targets for future treatments. These findings also point to the potential of *PNOC* as a genetic predictor of placebo responses in MDD, especially since prepronociceptin and members within its opioid family are associated with depression (255). As in this chapter, a similar design with administration of a placebo with described antidepressant properties may uncover the acute neural mechanisms responsible for reductions in depressive symptoms, or depression recovery as well as investigate potential predictors of placebo response. Initial work in this regard has been described (88), yet further investigation is merited.

Rostral Anterior Cingulate

Emergent from our findings is the significance of the rACC within the neurobiology of placebo responses as they relate to MDD, healthy functioning, and placebo-induced opioid neurotransmission under genetic influences. Firstly, we found that functional connectivity of the region within the SN to be significantly predictive of reductions in depressive symptoms in response to one week of placebo administration with expectations of antidepressant effects. The region was also predictive of the response to ten weeks of antidepressant treatment, although at a lower statistical threshold. This weaker association may stem from its predictive nature to be specific to placebo responses inherent within the overall antidepressant treatment response or a drug-placebo interaction (however, it may also be due to the long-term nature of the antidepressant response measure or the reduced sample size in our study). Moreover, rACC functional connectivity within the SN was significantly reduced within a group of depressed patients compared to healthy subjects. This overlap in more cohesive rACC functional connectivity within the SN predicting reductions in depressive symptoms as well as being characteristic of a group of healthy functioning individuals is indicative of the potential significance the region has in a successful antidepressant response. More generally, the region's role within the SN may underlie the neural basis of the employment of adaptive neural processing within depressed patients which better matches the healthier affective-cognitive functioning of healthy individuals. Similar findings have been observed when examining pretreatment glucose metabolism in depressed patients: in comparison to healthy controls, rACC hyper-metabolism differentiated eventual antidepressant medication responders from the non-responders who demonstrated hypo-metabolism in the region (44). Moreover, normalization of heightened rACC activity upon a successful response to treatment has been observed within

patients with MDD undergoing sleep deprivation (190). We demonstrated a similar pattern of equilibration of heightened rACC connectivity within the SN to associate with greater reductions in depressive symptoms in response to placebo treatment.

Anatomically, the rACC lies within a cross-section between dorsal higher-order cognitive regions involved in executive, goal-directed cognition and ventral lower-order limbic regions involved in more primitive emotional responses such as anxiety and fear (27, 46); therefore, the primary responsibility of the rACC is to modulate and regulate top-down and bottom-up communication (306). Furthermore, the region is believed to inadequately modulate these communication pathways, which are already abnormal, in depression (44). Moreover, the region is also well-suited to foster antidepressant functioning as it demonstrates a key role within self-referential processing of emotional stimuli (45), evaluation of emotional significance of events (307) and resolving emotional conflicts (115), as well as cognitive-based processes including decision-making related to reward and error evaluation (216). In all, this enables the ultimate expression of affect and goal-directed behavior through integration and regulation of salient affective and cognitive information (96). Taken altogether, it's possible that the comprehensive functioning of the rACC contributes to antidepressant responses through strengthened regulation of a classic characteristic of MDD: irregular and heightened reactivity to negative stimuli within the internal and external environments. It may also serve to better recruit the executive, inhibitive functioning of upper-order cognitive regions. Nevertheless, more comprehensive investigation is warranted to determine the absolute psychological cognitive-affective processing occurring within the region prior to antidepressant treatment and during the engagement of a successful antidepressant response.

Although not reported, we also found increased functional connectivity within the rACC in the DMN to associate with better response to ten weeks of antidepressant treatment, albeit at a lower statistical threshold. This is in line with hypothesis that the role of the rACC within this network, which is associated with introspective processes forming the internal world of the self (305), may underlie a major portion of the neurobiology within a maintained anti-depressive state (96). On the other hand, the region's role within the SN may attribute to more immediate antidepressant responses, such as the placebo response. This is in line with SN functioning: the network is involved in selecting and orienting to relevant, salient stimuli and incorporating this information with interoceptive states to guide behavior (147). Therefore, placebo responses may materialize from the incorporation of expectations and contextual cues from the therapeutic environment via the self-referential and saliency-dependent processes of the rACC within the SN. While connectivity between the rACC and the DMN may engage more intimately with maintained antidepressant responses through formulating and sustaining an updated internal environment free from major depressive episodes. Possibly, some of the effects of the initial SN-driven antidepressant response may be translated to or acquired by the DMN functioning for continuation of the antidepressant response. However, more comprehensive research utilizing resting-state functional connectivity within a long-term RCT with parallel placebo and antidepressant arms will better illustrate the viability of this hypothesis.

Lastly, our findings highlight the central role of the rACC within neural pathways implicated in resiliency from homeostatic stressors such as a depressive episode or pain. Within these chapters, we found that functional connectivity in the region within the SN is associated with predicting reductions in depressive symptoms and in juxtaposition, is more cohesive in healthy individuals compared to depressed patients. Additionally, we found a gene effect on

placebo-induced opioid neurotransmission within the region that is related to placebo analgesic effects and related-stress responses. Moreover, the associated gene, *PNOC*, has antidepressant therapeutic potential (258). Altogether, this illustrates the ability of the region to foster general healthy functioning and resiliency across disorders. In addition, these findings place the SN in the context of placebo neurobiology, which may be the inherent framework for rACC mechanisms observed in placebo analgesia as well. Broadly, these findings advocate for future research into the particular psychological mechanisms involving the rACC that contributes to adaptive cognition and emotion regulation as well as further work into the region's communication within wide-spread, intrinsic networks such as the SN and DMN.

Future directions for treatment of Major Depression

Our results serve to inform the neurobiology of recovery from MDD. Potential therapeutic targets for MDD may result from these findings: the development and implementation of therapies which are based on enhancing the recruitment and engagement of rACC functioning or treatments which specifically target the region *in vivo* via its cytoarchitectural characteristics. Possibly, this could be implemented through the development of antidepressant pharmaceutical drugs which exert their effect through specific receptors (or sub-receptors) highly localized within the rACC. For instance, upregulating a class of serotonin 5-HT receptors within the region is hypothesized to strengthen inhibition of excessive limbic activity in regions like the amygdala (308).

Our findings also inform clinical neuroimaging predictors of antidepressant treatment response which may be implemented within the context of a clinical setting. By examining patients' SN resting-state functional connectivity or through genetic screening, clinicians may recognize patients with greater susceptibility to placebo effects. Consequently, clinicians may

prescribe these patients lower dosages of currently available antidepressant medications or recommend a more cognitive-based approach to treatment. However, the hypothesis that greater likelihood of placebo responsiveness translates into better response to psychotherapy will have to be evaluated. Still, enhanced interaction with the clinician and time-spent within a therapeutic environment may influence antidepressant responses in these patients. Recent data show that three quarters of placebo responders remain well even after a period of twelve weeks into the continuation of placebo treatment (61), which suggests that placebo effects are not transitory. Moreover, as placebo rates vary between antidepressant clinical trials (18), it is believed that the trials with larger placebo responses are more therapeutic and thus, may provide a framework in examining beneficial environmental influences on placebo responses. Insights from these studies may be implemented in patient-clinician protocols within actual clinical settings. For instance, increases in required doctor visits has shown to improve depressive symptom reduction (309). Additionally, interaction with clinicians demonstrating an optimistic and warm demeanor as compared to those who are more pessimistic and less friendly significantly improved patients' clinical responses (310). Moreover, physical attributes of the treatment like pill color, dosing regimen and even sophistication of the treatment procedure can influence clinical placebo outcomes (311).

Within antidepressant clinical trials, our findings may augment the stratification of patients through the potential clinical neuroimaging predictor of enhanced rACC connectivity within the SN or genetic markers such as *PNOC* polymorphisms. This is especially relevant in the former marker as we found it capable of predicting placebo antidepressant responses with significant accuracy at an individual level. Those individuals demonstrating a greater likelihood of placebo response may be stratified to better investigate isolated placebo effects or

comprehensive interaction between active treatment effects and placebo. Moreover, those with little likelihood of placebo response may uncover more drug-specific antidepressant effects; however, those patients may have alternative neurobiological processing that is conducive to a nocebo effect, or worsening in symptoms, which will also have an interaction with active treatment effects. Regardless, expanding clinical trials to investigate placebo, nocebo and drug effects as well as the inter-relationships between these processes will provide a more comprehensive evaluation of antidepressant effects for a widespread and variable sample of depressed patients. This will better match the highly inter-individual variations within the total population of depressed patients who are seeking treatment and therefore, contribute to the development of more effective antidepressant drugs.

Concluding Remarks

The neural basis of the placebo response as it relates to MDD is an important avenue in progressing our understanding of the neurobiology behind the amelioration of depressive symptoms. Identifying the neural markers and players within these antidepressant responses and non-responses may enhance our ability to predict treatment responses and consequently, to better tailor antidepressant treatment protocols for each patient. Furthermore, uncovering these placebo-associated resiliency mechanisms against depression may also serve to expand the scope of potential targets in the development of efficacious antidepressant treatments. Potential clinical neuroimaging markers, such as rACC resting-state functional connectivity within the SN or genetic markers within the placebome, may substantially improve clinical practice by recognizing patients with high susceptibility to placebo effects to modulate their treatments. Subsequently, this may be implemented within clinical antidepressant drug trials to better differentiate between specific active pharmacological effects and placebo effects. Moreover,

these predictive markers cannot be confounded by factors such as regression to the mean or the natural course of illness which often challenge antidepressant efficacy measurements. Finally, within a more comprehensive picture, further defining the neural, molecular and cognitive bases of placebo neurobiology and the corresponding predictors of placebo responses informs our understanding of the neurobiological networks and related emotional-cognitive processes which combat stress and illness and enable a return to healthy, homeostatic states. Findings of this nature ultimately have comprehensive applicability within general and depression-related clinical care.

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