Anterior cingulate microRNA expression differences in mood disorder patients: contrasts with transcriptomic analyses in an animal model of depression

by

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Table of Contents

List of Tables

List of Figures

ABSTRACT

Mood disorders were initially recognized as a legitimate illness in Hippocrates' *Aphorisms.* Two of the most prevalent and debilitating mood disorders are major depressive disorder (MDD) and bipolar disorder (BP). While a number of studies have established a genetic component to these illnesses, the genomic architecture remains less than clear. Recently, microRNAs—small, non-coding RNAs that act as post-transcriptional regulators of gene expression—have demonstrated dysregulation in psychiatric disease state. Given these data, we hypothesized that miRNAs may exhibit mood-disorder-dependent differential expression in the anterior cingulate cortex (AnCg), a brain region heavily involved in the regulation of mood. In our first set of studies we employed qPCR analyses of 29 miRNAs implicated in psychiatric illness in AnCg of patients with MDD and BP versus controls. We validated a number of miRNAs as dysregulated in these illnesses and subsequently identified (and validated) several mRNA targets of miRNAs dysregulated in disease. Finally, we demonstrated alterations in the steady-state levels of two of these mRNA targets.

In a second set of studies we examined the impact of chronic stress on the transcriptomic network in medial prefrontal cortex (mPFC). Chronic stress is a major precipitant of neuropsychiatric illness and induces a number of physiological and genetic changes in a highly brain-region-dependent fashion. Due to this, we

hypothesized that chronic stress would have a significant impact on gene expression in mPFC, a brain region centrally involved in stress responses and regulating HPA axis activity. Furthermore, given that mPFC hypofunction is one of the most commonly observed stress-induced deficits, we hypothesized these gene changes would be consistent with mPFC hypofunction. By employing RNA sequencing and high-throughput qPCR analyses, we validated a number of mRNA and miRNA transcripts as stress-regulated. We finally constructed a biochemical “interactome” of stress-regulated mRNAs using bioinformatics tools and literature screens that is consistent with chronic-stress-induced glutamatergic hypofunction in mPFC. The work outlined in this thesis sheds light on transcriptomic events that occur in in human mood disorders or in animal models of chronic stress, which could be of clinical relevance in understanding the molecular architecture of mood disorders.

Chapter I: Introduction

Section 1: Depression – its history, descriptions and current impact

"Grief and fear, when lingering, promote melancholia."

-Hippocrates

History and background of depression

While depression has been recognized throughout much of human history, it was first classified as a distinct illness by the Greek physician Hippocrates in either the 4th or 5th century BC. In his *Aphorisms*, Hippocrates claimed depression arose from a preponderance of black bile based upon the four humors (the name of the illness, 'melancholia', translates literally to 'black bile'). While this characterization of a mental illness as having a physical basis was in many ways ahead of its time, one of its failures was in classifying depression as a single illness. "Depression," or a "low" mood, is an imprecise term and encompasses components of multiple different illnesses. Major depressive disorder, seasonal affective disorder and bipolar disorder, for instance, all present with "low" mood as a symptom but each presents with a unique symptomatic profile and is treated in different ways. For the purposes of this thesis I will be focusing on two major types of depression: major depressive (MDD) and bipolar (BP) disorder.

Definition of major depressive disorder

According to the DSM-5 (American Psychiatric, American Psychiatric, & Force, 2013), MDD is diagnosed when a patient has a collection of at least five of symptoms (listed below) that:

- Co-occur for a minimum of two weeks,
- Include **either** a low/"depressed" mood or anhedonia (reduced interest in nearly all activities),
- "Cause clinically significant distress or impairment in social, occupational, or other important areas of functioning," (American Psychiatric et al., 2013) and
- Is not better explained by either another medical condition or substance.

Symptoms indicative of MDD include:

1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feels sad, empty, hopeless) or observation made by others (e.g. appears tearful). (**Note:** In children or adolescents, can be irritable mood.)
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
3. Significant weight loss when not dieting or weight gain (e.g. a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.
4. Insomnia or hypersomnia every day.
5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
6. Fatigue or loss of energy nearly every day.

7. Feelings of worthlessness or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

Additionally MDD is often described in terms of “episodes”, or periods of time in which these symptoms are present. The duration of these episodes is highly variable, from as little as two weeks to multiple years in length, and their length may be inconsistent even within the same patient. Finally, while a diagnosis of MDD requires only a single episode in a patient’s life, it tends to be a recurrent illness with multiple depressive episodes occurring over the life of a patient.

Definition of bipolar disorder

Bipolar disorder (BP)—classically termed “manic-depressive disorder”—is another mood disorder that presents with major depressive episodes. The symptoms and diagnostic criteria are very similar to those for MDD and, in fact, BP may initially be mistaken for MDD due to this. A diagnosis of BP requires the presence of manic or hypomanic episodes--while MDD is often termed unipolar depression, a characteristic of BP is the oscillation between depressive and either manic or hypomanic episodes. The DSM-5 (American Psychiatric et al., 2013) defines a manic episode as follows:

1. A distinct period of abnormally and persistently elevated, expansive, or irritable mood and abnormally and persistently increased goal-directed activity or energy, lasting at least 1 week and present most of the day, nearly every day (or any duration if hospitalization is necessary).
2. During the period of mood disturbance and increased energy or activity, three (or more) of the following symptoms (four if the mood is only irritable) are present to a significant degree and represent a noticeable change from usual behavior:
    a. Inflated self-esteem or grandiosity.
    b. Decreased need for sleep (e.g. feels rested after only 3 hours of sleep).
    c. More talkative than usual or pressure to keep talking.
    d. Flights of ideas or subjective experience that thoughts are racing.
    e. Distractibility (i.e., attention too easily drawn to unimportant or irrelevant external stimuli), as reported or observed.
    f. Increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation (i.e., purposeless non-goal-directed activity).
    g. Excessive involvement in activities that have a high potential for painful consequences (e.g. engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments).

3. The mood disturbance is sufficiently severe to cause marked impairment in social or occupational functioning or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.

4. The episode is not attributable to the physiological effects of a substance (e.g. a drug of abuse, a medication, other treatment) or to another medical condition.

The diagnostic criteria between manic and hypomanic episodes are very similar, with the key distinctions that 1) elevated mood and energy must last for only 4 days (as compared to 1 week for a manic episode) and 2) the episode is not sufficiently severe to necessitate hospitalization or cause impairment. These distinctions are key, as BP is divided into two disorders--Bipolar I Disorder (which presents with depressive, hypomanic and manic episodes) and Bipolar II Disorder (which presents with depressive and hypomanic episodes only).

Prevalence and co-morbidity of MDD and BP

Although MDD and BP are clinically distinct and separate disorders, these illnesses share several unfortunate features. Chief amongst these is their high prevalence—in the USA, MDD and BP have a lifetime prevalence of 16.6% and 3.9%, respectively (Kessler et al., 2005). These illnesses are also often debilitating—while MDD and BP have a lower 12 month prevalence (6.7% and 2.7%, respectively), a high proportion of patients (30.4% and 44.8%, respectively) were classified as “serious” due to (but not limited to) the following: a suicide attempt with lethal intention, work disability and/or 30 or more days out of the year where the patient was completely unable to work and/or carry out normal function (Kessler et al., 2005).

It is important to note that, while a diagnosis of MDD or BP requires only an individual episode over the course of a patient’s life (a fact that is reflected in the statistics listed above), both MDD and BP have a strong likelihood of being recurrent. Those who

experience a single depressive episode have a >50% chance of experience additional episodes, while two depressive episodes raises the chance of experiencing another to 80% (Burcusa & Iacono, 2007). Similarly, patients diagnosed with BP are highly likely to experience a relapse (a recurrence of symptoms), with 40-50% experience a relapse within one year and between 70 and 90% experiencing relapse within five years (Gitlin, Swendsen, Heller, & Hammen, 1995; Lobban et al., 2010). Another potentially concerning observation is that the incidence of these disorders appears to be increasing over time (Kessler, Avenevoli, & Ries Merikangas, 2001; Kessler & Walters, 1998; Moreno et al., 2007), though this apparent trend may be explained by other factors such as greater recognition (and subsequent diagnoses) than in prior years.

In addition to their high prevalence, patients diagnosed with these disorders often demonstrate co-morbidity: that is, they are likely to have one (or more) illnesses or conditions that co-occur with these illnesses. These comorbidities span a wide range of maladies and include (but are not limited to) chronic pain, migraines, cardiovascular illness, metabolic disorders, diabetes and stroke (Arnow et al., 2006; Horrobin & Bennett, 1999; Krishnan et al., 2002; Merikangas & Stevens, 1997; Van der Kooy et al., 2007). It should be noted that, while these illnesses co-occur, whether they are causative (e.g. whether depression may cause stroke, or vice versa) is much less well understood.

Perhaps unsurprisingly, some of the most common conditions that present as comorbid with MDD and BP are other psychiatric illnesses. Both MDD and BP have high comorbidity with anxiety disorders (Hirschfeld, 2001; Keller, 2006). Patients with either of these disorders are significantly more likely to abuse drugs and/or alcohol as those

without illness (Cassidy, Ahearn, & Carroll, 2001; Grant, 1995; Olfson et al., 1997). Perhaps most alarming of all are the correlations between these disorders and suicide. While the average suicide rate of the general population has hovered around 0.01%, the suicide rate of patients with mood disorders is much higher: up to 15% for patients with MDD, and up to 20% for patients with BP. Over two-thirds of the 30,000 reported suicides in the USA in 1999 were due to depression (White House Conference on Mental Health, 1999). Combined, this data indicates a reduction in both lifespan and in quality of life for patients suffering from these disorders.

The economic and societal impact of depression

In addition to their great impact on the individual suffering from these illnesses, depression—here used to encompass both MDD and BP—represents a sizable public health concern. Currently, MDD (e.g. “unipolar” depression) represents a leading cause of illness with over 350 million people affected and is the leading cause of disability worldwide (reviewed in (Ferrari et al., 2013; Reddy, 2010). The incidence of depression is increasing over time while the average age of onset is decreasing (Hidaka, 2012) revealing a concerning trend in the potential societal and economic impacts of depression in the coming years (see Chapter I, Clinical Connections).

Given the prevalence and nature of these disorders, MDD and BP impose a rather large economic burden on society in the form of increased health care costs and reduced work productivity. Prior work indicates that, even after accounting for medical services due to non-depressive comorbid illness, patients presenting with depressive symptoms incur an increase of 50 to 75% in healthcare costs (Simon, VonKorff, & Barlow, 1995;

Unutzer et al., 1997). For BP patients this cost is projected to be even higher, with one study indicating an increase of 250% in healthcare costs over a sample 6-month time period (Simon & Unutzer, 1999). Strikingly, less than 10% of these increased costs were attributable to the direct treatment of depression (Unutzer et al., 1997), indicating a sizable drain on general medical services due to illness.

MDD and BP also heavily impact both work productivity and likelihood of employment: previous studies have indicated a 2- and 7-fold increase (in MDD and BP, respectively, compared to age- and sex-matched general outpatient controls) in working days missed due to illness, while patients with BP were 40% less likely to have paid employment (Akiskal et al., 2000; Kessler et al., 1999; Olfson et al., 1997; Ormel et al., 1994; Zwerling et al., 2002). Putting numbers to the combined costs of healthcare and lost productivity, it is estimated that depression (in all its forms) resulted in an annual cost of $83 billion dollars in 2000 the USA alone (Greenberg et al., 2003).

Finally, in addition to the economic burden these illnesses impose, these disorders come with a high societal cost. A remarkably high number of people with mental illness (both proportionally and in total numbers) are incarcerated, with incarcerated patients outnumbering those in hospitals three-to-one (Kramer, 2006). Unlike many other highly prevalent diseases, mood disorders still carry a strong social stigma—rather than being recognized as a legitimate illness, there is a pervasive belief that mental illness (depression in particular) represents a failure of character rather than a treatable disorder. Dovetailing with this, the majority of health insurance plans (at least in the USA) cover mental illness to a far lesser extent than non-mental illnesses, making

treatment difficult or expensive for a large proportion of patients with these illnesses ("Health Policy Brief: Mental Health Parity," 2014).

Current Treatments for MDD and BP

Due to their extremely high prevalence, personal impact and heavy economic cost, treating depressive illness is imperative. Treatments can be separated into two major groups, psychological (cognitobehavioral therapy, social rhythm therapy, etc.) and pharmacological treatments. While both psychotherapy and pharmacological approaches may be effective on their own, greater therapeutic efficacy is typically achieved with treatment regimens that incorporate both (Thase et al., 1997).

One of the longest-lasting hypotheses of depression, the monoaminergic hypothesis, was formulated in large part due the mechanisms of action of the first generations of antidepressants. Serotonergic reuptake inhibition (tricyclic antidepressants (TCA)) and inhibition of monoamine breakdown (monoamine oxidase inhibitors (MAO-I)) led to the notion that the underlying pathophysiological mechanism of mood disorders was a deficit of serotonin at the synapse. Even today, the majority of first-line pharmacological treatments for MDD still target the monoaminergic system. In addition to the TCA and MOA-I classes of antidepressants, selective serotonin reuptake inhibitors (SSRI), serotonin and norepinephrine reuptake inhibitors (SNRIs), and noradrenergic and specific serotonergic antidepressants (NaSSAs) are all used in the treatment of MDD.

In spite of their depressive symptomatic similarity, pharmacological treatment paradigms for MDD and BP differ greatly. This is, in large part, due to the fact that antidepressants may trigger either a manic or a hypomanic episode in a significant

fraction of BP patients (Henry, Sorbara, Lacoste, Gindre, & Leboyer, 2001; Preda, MacLean, Mazure, & Bowers, 2001; Yildiz & Sachs, 2003). In lieu of antidepressants, patients with BP are often treated with one or more mood stabilizers (such as lithium or valproic acid) or the glutamate-based anticonvulsant lamotrigine. In contrast to pharmacological treatment paradigms, however, psychological treatment options are fairly similar between the illnesses.

Additional parallels between treatment strategies for both MDD and BP can be drawn between treatment efficacy and pharmacological “lag”. A major difficulty in treating these illnesses is the rate of patients who do not respond to the initial pharmacological treatment plan: a large-scale study of depressive patients using monoamine-based antidepressants revealed that a minority (less than a third) of patients entered remission (Trivedi et al., 2006). Furthermore, multiple studies indicate that up to 50% of patients will not respond to the first treatment plan prescribed (a statistic that holds true for BP as well as MDD patients).

Another major difficulty in treating these illnesses is the “lag” time experienced between beginning medication and seeing therapeutic benefit. While the pharmacological mechanisms of both mood stabilizers and serotonin-based drugs occur extremely rapidly, it takes an average of six weeks for patients to experience therapeutic benefits. In addition to this initial lag, if the patient fails to respond to this initial medication there may a “wind-down” period where the patient is weaned off the medication to avoid withdrawal symptoms (particularly with serotonin-based medications). This represents another massive hurdle in treating these illnesses, as patient non-compliance increases

significantly following the therapeutic failure (or occurrence of undesirable side effects) of one or more pharmacological treatments (Demyttenaere, 2001).

While these medications are invaluable in the treatment of MDD and BP, they also underscore that our understanding of these disorders are far from complete. While we understand the pharmacological mechanisms of these compounds, how they exert their therapeutic effects is still unclear. Recent work in animal models has also revealed that our assumptions for the therapeutic mechanism of action in TCA antidepressants may be incomplete. mRNA and surface protein levels of *Adra2a* are increased in prefrontal cortex in both human depressive patients and animal chronic stress models, with increased signaling from these receptors thought to be a causal component of dysfunction in MDD (reviewed in (Cottingham & Wang, 2012)). The therapeutic effects of TCA are often attributed to increased norepinephrine. However, a study by Cottingham et al. involving desipramine (DI, a TCA-class antidepressant that inhibits serotonin and norepinephrine uptake) revealed that DI also induced the internalization of adrenergic receptors (Cottingham, Chen, Jiao, & Wang, 2011). Furthermore, they demonstrated that this receptor internalization—and not the physiological concentration of norepinephrine achieved following reuptake inhibition—was sufficient to induce *Adra2a* receptor internalization, consistent with prior work by Gilsbach et al. that showed elevated norepinephrine levels were insufficient to reduce *Adra2a* receptor levels (Gilsbach et al., 2006). Considering that many of our pharmacological treatments for depressive illness are focused on a relatively limited biological scope, and that we know comparatively little of the biological systems impacted by these illnesses,

understanding the molecular architecture of depressive illness would greatly aid us in developing new treatment strategies.

Section 2: Stress responses, glucocorticoid signaling and its impact on the CNS.

"Stress, in addition to being itself, can also be the cause of itself and the result of itself."

-Dr. Paul Rosch, Founder, American Institute of Stress

Definitions and purposes of stress

Much like the word "depression", "stress" is an extremely nebulous term whose definition depends heavily on context. Although he regretted his choice of the word "stress" to define this concept, a straightforward (if somewhat simplistic) definition was put forth by Dr. Hans Selye in 1936: "(Stress is) the non-specific response of the body to any demand for change." A more recent definition of stress characterizes it as, "…a real or interpreted threat to the physiological and psychological integrity of an individual that results in physiological and/or behavioral responses." (McEwen, 2000)These threats, termed "stressors", comprise a wide variety of physical (excess cold or heat, pain, etc.) and psychological (dangerous situations, fear, etc.) stimuli that threaten to disrupt an organism's internal stability in the face of changing external conditions. This process is termed "homeostasis" (Cannon, 1926) (from the Greek 'homeo' (similar/same) and 'stasis' (still).

A "stress response," then, is the reaction of one or more system(s) that act to minimize, mitigate and/or prevent deviations from this equilibrium due to one or more stressor(s). While this terminology is applicable at the cellular level, this thesis will focus primarily on

the physiological and genetic responses to stress at the organismal level (specifically, in the mouse (*Mus musculus*) and humans (*Homo sapiens*)).

Stress responses: the autonomic system, HPA axis and glucocorticoid release

There are two main intertwined systems that underlie the central stress response: the autonomic nervous system (ANS) (specifically, the sympatho-adrenomedullary axis) and the hypothalamic-pituitary-adrenocortical (HPA) axis (reviewed in (Ulrich-Lai & Herman, 2009)). The first of these systems, the ANS, has two major components: the sympathetic (responsible for the "fight-or-flight" response) and the parasympathetic (responsible for "rest-and-digest") divisions. Following the presentation of a stressor, the sympatho-adrenomedullary axis is engaged. Axonal projections from spinal cord indirectly innervate the cardiovascular system (amid other organs) and adrenal medulla. The brainstem modulates these pathways, leading to increased cardiovascular activity (heightened blood pressure and heart rate), decreased gut motility, and increased circulating levels of adrenaline (epinephrine) and noradrenaline (norepinephrine). This pathway simultaneously informs and engages so-called "higher order" components of the limbic system (including the medial prefrontal cortex (mPFC) (**Figure 1.1**) and anterior cingulate cortex (AnCg)) that regulate the duration and intensity of the ANS response. Of note is that this sympathetic response canonically has a short duration due to opposing effects of the parasympathetic division of the ANS.

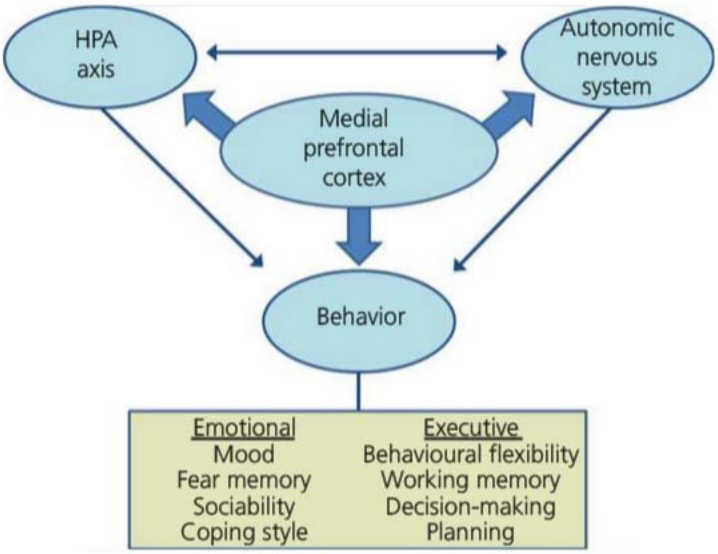

Figure 1.1: Linkages between mPFC, HPA axis, ANS and behavior. Reprinted with permission from (McKlveen, Myers, & Herman, 2015), Journal of Neuroendocrinology, under Wiley Publishing © 2015.

The HPA axis, in contrast to the ANS response, may be much longer in duration (which may be on the order of hours). The HPA axis does however, share several key points with the ANS response: it is quite rapid in its onset, and it directly innervates the paraventricular nucleus of the hypothalamus (PVN) which serves as the initiation point for the canonical CRH → ACTH → Glucocorticoid (GC) loop in the HPA axis response. After the initiation of the HPA axis response, the PVN secretes several stress-linked hormones including corticotropin releasing hormone (CRH). These hormones travel to the pituitary gland via the hypophyseal portal system, a network of blood vessels directly linking the hypothalamus to the pituitary. Following the arrival of these hormones, CRH stimulates the anterior pituitary to release adrenocotricotropic hormone (ACTH) into the general circulation. ACTH then acts upon the adrenal glands to stimulate the release of glucocorticoids, the main effectors of the HPA axis. The release of ACTH is fairly rapid, with glucocorticoids circulating throughout the body and reaching

peak circulating concentration within a matter of minutes following the stressor. Intriguingly, under conditions of moderate stress, glucocorticoids also serve to rapidly and efficiently terminate HPA axis activity via direct actions on the PVN, anterior pituitary and limbic system (particularly mPFC and anterior cingulate cortex (AnCg)) in a classic negative feedback loop.

As the primary effectors of stress responses, glucocorticoids trigger a large variety of responses that minimize the immediate impacts of the stressor and assist in the mounting of the stress response. Unlike the ANS response, however, the HPA axis is an adaptive response, capable of making changes in anticipation of immediate or future stressors. One of the key ways it accomplishes this is through the glucocorticoid-receptor-mediated regulation of gene expression, which will be covered in depth in the following section.

Impacts of stress on transcriptional control and gene expression

Glucocorticoids play a key role in gene expression across nearly every cell type in every major organ system and tissue (reviewed in (Chrousos, 2009)). In spite of the diverse array of processes and regulatory pathways they oversee, glucocorticoids share a basic mechanism of action when exerting their effects. Following their release, the vast majority of glucocorticoids are sequestered by glucocorticoid binding proteins in circulation. Only ~10% of the released glucocorticoids are unbound (“free”) and this form is able to easily permeate through the cell membrane where it binds to two major receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Following their binding to glucocorticoids, these receptors then translocate to the

nucleus where they bind to specific sequences of DNA (termed glucocorticoid response elements, or GREs) and mediate gene transcription.

While conceptually straightforward, a number of molecular and cellular phenomena add further layers of complexity and rigid regulatory control. The first is the relative binding affinity of these receptors, with MR notable for having a high binding affinity for its glucocorticoid and GR, in contrast, having a low binding affinity. These initial observations led to the notion that these receptors may play markedly different roles. This notion was later proven true, with MR currently thought to be predominantly responsive to processes involving low concentrations of glucocorticoids (e.g. endogenous glucocorticoid rhythmicity, initiation of the stress response, etc.) while GR is responsive to those involving relatively high concentrations (such as HPA axis output) (Rupprecht et al., 1993). Adding further complexities, heteromeric (e.g. GR/MR) receptor complexes may form as well, effectively adding a third “type” of glucocorticoid receptor with distinct pharmacological and functional properties.

Glucocorticoids, as well as affecting nearly every cell, have an extremely context-sensitive impact. In addition to the various receptor types, the specific anatomy--e.g. tissue, tissue sub-region and individual cell type--can have a profound effect on the expression (and complement) of genes subject to glucocorticoid regulation. In the CNS, for instance, MR is found primarily in the brainstem and hippocampus (HC), while GR is more broadly expressed throughout the brain (Patel et al., 2000). In addition, though GR mRNA tends to have an overall higher expression level than MR mRNA the relative enrichment is highly brain-region-dependent, leading to the observation that different stoichiometries of these receptors yields unique signatures of gene regulation (de Kloet,

Fitzsimons, Datson, Meijer, & Vreugdenhil, 2009). Glucocorticoids and their receptors may also have their activity either inhibited or potentiated by a variety of factors endemic to a given cell type or tissue. In parotid, colon and kidney the relatively high expression of 11β-hydroxysteroid dehydrogenase 2 (11βHSD2) catalyzes the conversion of glucocorticoids (which bind to both GR and MR) into aldosterone (which binds only to MR), severely limiting the impact of GR in these tissues (Edwards et al., 1988).

A variety of binding partners or other co-factors are also capable of modulating the transcriptional activity of glucocorticoid receptors (reviewed in (Rosenfeld & Glass, 2001) and (Zalachoras, Houtman, & Meijer, 2013)). Two specific examples, discussed further in Chapter 2, are the Nuclear Co-Activator 1 (*NCOA1*) and Nuclear Co-Repressor 2 (*NCOR2*) genes. Both genes are GR co-factors and are capable of not only altering the GR-mediated transcriptional response of individual genes (of particular note, CRH) via protein-protein interactions with GR but are also necessary for homeostatic control of the HPA axis. Glucocorticoid receptors may also exert regulatory control over gene transcription by regulating access of the transcriptional machinery to its target. A large variety of enzymes, collectively termed histone acetylases and histone deacetylases, catalyze the addition or removal of acetyl groups within nucleosomes (a nucleic acid organizational structure, consisting of DNA wrapped around several histone protein cores) which are, respectively, linked to an increase or a decrease in transcriptional activity (reviewed in (Zentner & Henikoff, 2013)). Not only is the transcriptional activity of GRs themselves affected by the acetylation status of histones,

but GRs are capable of recruiting (de)acetylases and thereby directly affect chromatin remodeling (Ito, Barnes, & Adcock, 2000).

Perhaps most intriguingly, stress has extremely far-reaching impacts on both gene expression and physiology in the brain. Chronic exposure to cortisol, for instance, induced the differential expression of over 1500 transcripts in fetal human brain aggregates (Salaria et al., 2006). Several hundred genes were identified as regulated by corticosterone in hippocampus at the mRNA level in rats (Datson, van der Perk, de Kloet, & Vreugdenhil, 2001), while mice implanted with a corticosterone pellet demonstrated changes in expression at the protein level in 150 genes across multiple brain regions (Skynner et al., 2006). Speaking to the wide variety of processes influenced by stress, these transcripts are linked to a wide variety of processes and systems within CNS—including neurogenesis, neurodevelopment, metabolism and vascular tone—in a highly brain region- and cell-type-specific fashion.

One striking effect of stress on gene expression is its impacts on processes underlying synaptic function. The fundamental unit of connectivity within the CNS, synapses and synaptic function represent the summation of a staggering variety of proteins from divergent biological, cellular and mechanistic processes. These include growth and neurotrophic factors (e.g. neuronal growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*)), signaling peptides (e.g. proenkephalin (*PENK*), vasopressin (*AVP*), adenylate cyclase activating polypeptide 1 (*Adcyap1*)) second messengers (cyclic adenosine monophosphate (cAMP), calcium (Ca2+), etc), and—of major relevance to this thesis—neurotransmitters. Intriguingly, stress has been demonstrated to have significant effects on much of the machinery underlying neurotransmission in the limbic

system, particularly within the PFC. Sustained stress (as well as depressive illness) has been observed to increase activity within the locus coeruleus (LC), resulting in not only local increased noradrenergic (NA) tone but increased sensitivity to NA in mPFC via upregulating NA receptor expression and sensitivity (specifically, *ADRA2A*) (Mana & Grace, 1997; Nakane, Shimizu, & Hori, 1994; Pavcovich, Cancela, Volosin, Molina, & Ramirez, 1990; Wilson et al., 1998). More recently, stress has been shown to significantly alter GABAergic neurotransmission in a mPFC (Perrine et al., 2014).

In addition to these systems, the glutamatergic system has also been widely studied in the context of stress, revealing not only regional but also stress-specific alterations in response to chronic stress. For instance, following chronic immobilization stress, Vyas et al. reported dendritic spine retraction and neuronal atrophy in the hippocampus (HC), contrasting with their observation of dendritic arborization in the amygdala (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002). In this same study, the authors observed no significant changes in hippocampus but observed *atrophy* in amygdalar bipolar neurons following a different stressor (chronic unpredictable stress), demonstrating a stressor-specific effect. Deficits in glutamate neurotransmission have also been observed in the mPFC. Yuen et al. demonstrated stress-induced reductions in glutamate-mediated neurotransmission, caused by both significant reductions in cell surface specific AMPA and NMDA receptor subunit expression as well as reductions in total protein expression (Yuen et al., 2012). Pointing to a possible mechanistic role underlying the physiological impacts of stress in the mPFC, neuronal atrophy, mEPSCs, spine loss and shrinkage in glutamatergic neurons (specifically, pyramidal cells) has also been observed following chronic stress in mPFC (Cook & Wellman, 2004; Radley, Arias, & Sawchenko, 2006;

Radley et al., 2004). Of further note, these changes appear to be brain-region-specific: while prior work has identified similar reductions in spine size in hippocampus, Yuen et al. identified no commensurate alterations in mEPSCs in other limbic structures (e.g. hippocampus) or in striatum (Yuen et al., 2012).

In summary, stress has far-reaching effects on gene expression. Its regulation of gene expression and physiological events is highly context-specific, dependent on not only the organ itself but also individual cell type(s). This regulation is particularly important to consider within the brain, where stress heavily influences synaptic physiology and function, potentially via modulation of neurotransmission across a variety of neurotransmitter systems within the mPFC. This is of key relevance given that a major goal of this thesis is to identify 1) the effect of chronic stress on gene expression changes at the RNA level in mPFC, and 2) elucidate the prospective cell type(s) these changes may be occurring in.

Finally, while this thesis has devoted substantial space to the impact of stress on gene expression, we have so far focused solely on protein-coding (e.g. mRNA) transcripts. Given the common transcriptional machinery employed by various RNA species, stress—via glucocorticoids—is capable of regulating a variety of non-coding transcripts. This includes microRNAs (miRNAs), the subject of the following section.

Section 3: Gene expression and the role of microRNAs.

"Certainly, microRNAs are important regulators inside of the cell."

-Dr. Craig C. Mello

Gene expression: a background

Central to the continued survival of a biological organism is the ability to maintain homeostasis. As previously described in Chapter 1.2, homeostasis is a central feature to the continued survival of an organism. This process occurs on not only the physiological and psychological levels previously described, but also on the molecular and cellular level within organisms. One key tool with which organisms maintain biochemical homeostasis is through the modulation of gene expression.

Gene expression can be defined as the flow of genetic information from DNA to a functional gene product (either RNA or protein). This process was described as the central dogma of molecular biology and can be summarized as follows: DNA is transcribed into RNA, which is subsequently translated into protein (Crick, 1970). Information may not flow from protein to protein, or from protein to nucleic acid. Implicit in this, however, is the notion that RNA may alter the flow of genetic information through

interactions with RNA—one example of which is the interaction of microRNAs (miRNAs) with mRNAs, discovered in the late 20th century (Lee, Feinbaum, & Ambros, 1993).

MicroRNAs: history, definition and mechanism of action

Originally discovered in 1993, miRNAs were initially thought to be a biological quirk of the nematode *C. elegans*—a single small RNA (the *lin-4* miRNA) was identified as capable of repressing a single target (the *lin-14* messenger RNA (mRNA)) (Lee et al., 1993). Seven years later, a second miRNA—termed *let-7*—was identified in *C. elegans* and found to be capable of repressing the *lin-41* mRNA (Reinhart et al., 2000). Shortly after this study, screens identified a large number of similar RNA species not only within *C. elegans* but also evolutionarily conserved orthologues of these RNAs in multiple species (including humans), indicating a much broader role than previously described (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001).

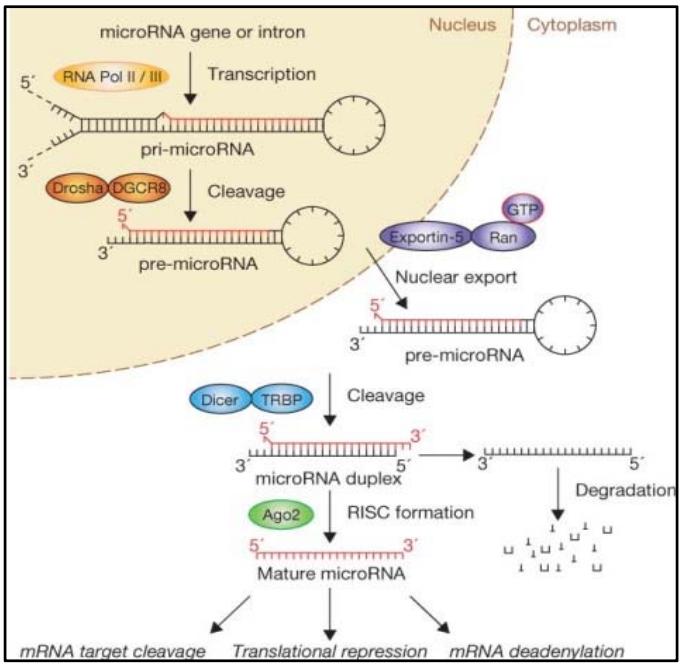

Figure 1.2: Overview of miRNA biogenesis. Following transcription by RNA polymerase II/III, miRNAs undergo a sequence of cleavages to generate the mature miRNA duplex. Reprinted by permission from Macmillan Publishers Ltd: Nat. Cell Biol. ((Winter, Jung, Keller, Gregory, & Diederichs, 2009)), ©2009.

So what are miRNAs? Collectively, the term ‘miRNA’ refers to any member of a family of endogenous small RNA molecules (18-22 nucleotides in length) that act as sequence-specific post-transcriptional regulators of gene expression. While miRNAs are subject to many of the same transcriptional controls as mRNAs, one key distinction is that miRNAs are non-coding transcripts (though they may be present in intronic regions of protein-coding mRNA transcripts (Zhu, Kalbfleisch, Brennan, & Li, 2009)). Also unlike other RNA species, miRNAs undergo a unique biogenesis (**Figure 1.2**) (reviewed in (Ha & Kim, 2014)). miRNAs are initially transcribed as a long (80 to several hundred nucleotides) primary miRNA transcript, termed a pri-miRNA, containing a stem-loop secondary structure (**Figure 1.2**). Prior to being exported from the nucleus the *DGCR8* enzyme cleaves the pri-miRNA, leaving only the stem-loop structure (now termed a pre-miRNA). Once in the cytoplasm, the pre-miRNA undergoes further processing by the *DICER1* enzyme (a component of the RNA-induced silencing complex (RISC)), which cleaves the “loop” structure and reduces the pre-miRNA to a 22-nt dsRNA. Following this, the “passenger” strand is usually degraded while the remaining strand of the dsRNA duplex is loaded into the RISC machinery and guided to the mRNA target. Following this, the mature miRNA will exert its effect by binding to the 3’ UTR of mRNA targets. While this binding occurs in a sequence-specific fashion, miRNA/mRNA interactions are not perfectly complementary (the exception being nucleotides 2-8, termed the “seed sequence”, which guides miRNAs within the RISC complex to their mRNA targets). As such, while identifying prospective miRNA/mRNA interactions can be accomplished using bioinformatics approaches (Agarwal, Bell, Nam, & Bartel, 2015), validating these interactions requires an empirical approach. miRNA interactions with

mRNAs subsequently impact gene expression via either degradation of mRNA transcripts or inhibition of protein translation (reviewed in (Eulalio, Huntzinger, & Izaurralde, 2008)). Of particular interest given this property is the “one-to-many” relationship miRNAs have with their targets: a single miRNA species may target anywhere from several dozen to several hundred individual mRNA targets (Selbach et al., 2008). Coupled with the observation that a majority of protein-coding mRNA transcripts are predicted to be subject to regulation by miRNAs and that a high degree of evolutionary conservation is observed in miRNAs expressed between species (Friedman, Farh, Burge, & Bartel, 2009), this evidence provides solid footing for the notion that miRNAs are important regulators of gene expression.

Expression of miRNAs within the central nervous system: biological roles within human disease and disease-like states

While relatively few miRNAs are uniquely expressed and/or enriched in the brain, a majority of miRNAs are expressed within brain tissue (Kosik & Krichevsky, 2005). miRNAs have been characterized as key mediators in a number of CNS processes and functions. Two seminal studies by Krichevsky et al. and Giraldez et al. revealed the widespread regulation of brain development by miRNAs and demonstrated their central role in regulating morphogenesis of the brain (Giraldez et al., 2005; Krichevsky, King, Donahue, Khrapko, & Kosik, 2003). Further work by Schratt et al. demonstrated the temporally sensitive pattern of expression of the brain-specific miR-134 in developing mouse brain (Schratt et al., 2006), while a growing body of work has revealed the role of

multiple miRNAs and miRNA clusters in regulating the development of specific brain structures (reviewed in (Petri, Malmevik, Fasching, Akerblom, & Jakobsson, 2014).

In addition to temporal regulation, miRNAs also exhibit several levels of spatial regulation. At the organ level, miRNAs may be enriched not only within the brain but localized to specific subregions and/or specific cell type(s). Both miR-124 and miR-219 are CNS-specific miRNAs that demonstrate extraordinarily robust expression. miR-124, however, exhibits a pan-neuronal expression while miR-219 is expressed specifically within oligodendrocytes (Jovicic et al., 2013). This type of spatial regulation extends further, with multiple miRNAs demonstrating enrichment and/or specificity to cells with particular neurochemical phenotype(s) (such as glutamatergic vs GABAergic interneurons) (He et al., 2012).

Consistent with this tight spatiotemporal control of miRNA expression, miRNAs play key roles in a staggering array of biological processes within the CNS including (but not limited to) development, cell fate and synaptic plasticity (reviewed in (Salta & De Strooper, 2012)) (**Figure 1.3**). *Dicer1* is a key component of the miRNA biogenesis pathway, with its knockout sufficient to ablate mature miRNA expression and result in embryonic lethality (Bernstein et al., 2003). While not embryonically lethal, ablation of miRNA expression via *Dicer1* deletion specifically in the CNS in mice resulted in low numbers of *Dicer1*-null embryos with no post-natal survivors (Kawase-Koga, Otaegi, & Sun, 2009). In the same study, mice rendered null for *Dicer1* specifically in the cerebral cortex were viable and survived until postnatal day 30 but exhibited gross defects in cortical morphology, cell proliferation and neuronal migration, demonstrating an indispensable role for miRNAs in CNS development and migration.

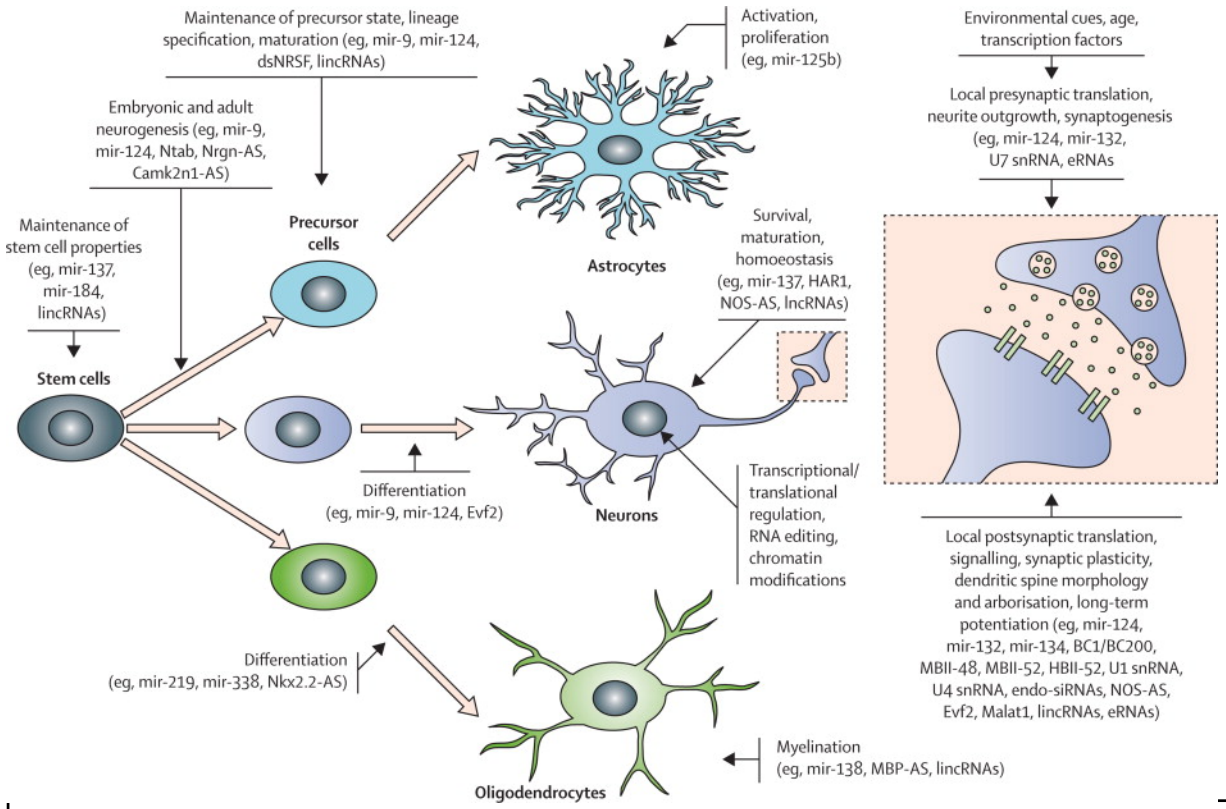

Figure 1.3: A sample subset of miRNA roles in the CNS. Reprinted from The Lancet, Volume 11, Issue 2, (Salta & De Strooper, 2012), Non-coding RNAs with essential roles in neurodegenerative disorders, p. 189-200, © 2012, with permission from Elsevier.

In addition to regulating development and neuronal migration, miRNAs are also key modulators of neuronal morphology and synaptic plasticity. miR-134 was identified as the first miRNA capable of regulating neuronal morphology via negatively regulating dendritic spine size and prospectively synaptic plasticity via BDNF interactions (Schratt et al., 2006). More recent work, however, has revealed a litany of miRNA regulatory events involved in altering synaptic plasticity (reviewed in (Aksoy-Aksel, Zampa, & Schratt, 2014; Bicker, Lackinger, Weiss, & Schratt, 2014)). An example of this is miR-132's interaction with *Mecp2*: miR-132 targets and represses *Mecp2* which, in turn, inhibits brain-derived neurotrophic factor (*Bdnf*) (Klein et al., 2007). As *Bdnf* is

necessary for adult synaptic plasticity and LTP (Andero, Choi, & Ressler, 2014), miR-132 is able to indirectly modulate changes in synaptic plasticity via modulation of *Bdnf* levels. Congruous with this role in regulating synaptic plasticity the expression, turnover and biogenesis of a subset of miRNAs has been shown to be tightly coupled to neuronal activity (Sim, Bakes, & Kaang, 2014), implying an important role for miRNAs in basal CNS function.

miRNAs are also capable of regulating a variety of behaviors across a variety of organisms. In the fruit fly *Drosophila melanogaster* ablation of miR-124 resulted in increased time spent climbing and a drastically reduced percentage of flight in males (C. Wang, Feng, Wan, Kong, & Yuan, 2014). Knockdown of one of several miRNAs in zebrafish is capable of inducing hyperactivity similar to that observed in zebrafish exposed to ethanol during larval development (Tal et al., 2012). One study of particular clinical interest was performed by Stark et al. employing a mouse model of the 22q11 microdeletion syndrome (Stark et al., 2008). This model replicates a chromosomal microdeletion carried by ~2% of patients with schizophrenia and disrupts expression of the *Dgcr8* gene, a key component of the miRNA biogenesis machinery (Bassett & Chow, 1999; Gregory et al., 2004). Mice carrying this deletion demonstrated not only structural and functional neural abnormalities but also impaired prepulse inhibition, a features that demonstrates clinical parallels in human schizophrenic patients (reviewed in (Larrauri & Schmajuk, 2006)).

Given the number of biological processes that miRNAs regulate, coupled with the capability of one miRNA species to regulate a large number of mRNA targets, it is perhaps unsurprising that a growing body of literature implicates miRNAs as both

effectors and signatures of diseases within the CNS. Likely due to the wide variety of cellular processes they oversee, disruptions in the miRNA regulatory network have been tightly linked with various forms of cancer and tumor suppression (reviewed in (Jansson & Lund, 2012)). In the CNS, miRNA “clusters” have been associated with a variety of illnesses including (but not limited to) epilepsy, stroke, Alzheimer’s, and Huntington’s disease (Cogswell et al., 2008; C. Liu, Zhao, Han, Li, & Li, 2015; Maciotta, Meregalli, & Torrente, 2013).

Of particular interest to this thesis, however, is the growing number of studies that have identified differential miRNA expression in human patients with a variety of neuropsychiatric illnesses. Seminal work in 2007 identified differential expression of miRNAs within the dorsolateral prefrontal cortex (DLPFC) in patients with schizophrenia (SZ) and schizoaffective disorder (Perkins et al., 2007). In the intervening years a growing body of literature has demonstrated differential miRNA expression across a range of mood and affective disorders including major depressive disorder (MDD), bipolar disorder (BP) and SZ (Bavamian et al., 2015; A. H. Kim et al., 2010; Miller et al., 2012; Moreau, Bruse, David-Rus, Buyske, & Brzustowicz, 2011). To date this body of work has largely focused on schizophrenic patients with an emphasis on examining miRNA expression within DLPFC and cerebellum. Absent from these studies, however, has been the anterior cingulate cortex (AnCg), a brain region centrally involved in the regulation of mood, affect and cognition (Drevets, Savitz, & Trimble, 2008; Ebert & Ebmeier, 1996; Mayberg et al., 1999; Posner & DiGirolamo, 1998). A growing body of literature has also demonstrated further connections between AnCg dysfunction and mood disorders, with altered AnCg activity differentiating MDD versus BP patients and

predicting successful therapeutic responses to pharmaceutical and cognitive treatments (Diler et al., 2014; Fujino et al., 2015; Mulert et al., 2007; Pizzagalli et al., 2001; Salvadore et al., 2009). Neuropsychiatric illnesses likely comprise, at least in part, the dysregulation of multiple brain regions, and an understanding of how each of these regions may be affected is key to understanding the whole. As such, a major goal of this thesis is to identify miRNAs differentially expressed within anterior cingulate cortex (AnCg) of patients with unipolar and bipolar depression and to characterize putative mRNA targets of these miRNAs.

Section 4: Clinical Connections -- Linking Chronic Stress, microRNAs and Depression

"Depression isn't a straightforward response to a bad situation; (it) just is, like the weather."

-Stephen Fry

As described in previous sections, MDD and BP are debilitating illnesses and each have an extremely high prevalence. While this background has focused on the diagnoses, prevalence and societal impact of these illnesses it has not yet touched on our current understanding of their respective causes and treatments. The purpose of this section will be to describe the etiology of MDD and BP, identify the linkages between chronic stress and these illnesses, and provide context for the work described within this thesis.

<u>Linkages between HPA axis dysfunction and mood and affective disorders</u>

As may be inferred from the litany of various symptoms that may (or may not) present within MDD and BP (see Chapter 1.1), mood disorders are extraordinarily heterogeneous. One of the major difficulties in developing diagnostic criteria has been that this heterogeneity extends from the symptomatic to the cellular, molecular and systems level. Due in large part to this, diagnoses based solely on biomarkers remain problematic.

While there is, as of the time of this writing, no definitive biomarker for mood disorders, a series of intriguing studies were published beginning in 1956. Several groups independently observed increased levels of plasma cortisol (hypercortisolemia) in patients presenting with anxiety, schizophrenia and major depression compared to controls (Basowitz et al., 1956; Bliss, Migeon, Branch, & Samuels, 1956; Board, Persky, & Hamburg, 1956; Board, Wadeson, & Persky, 1957), providing the first linkage of these illnesses to the HPA axis.

Although these studies were acute examinations of cortisol level (e.g. taking place over several hours), a later study examined chronic levels of cortisol in major depressive patients versus controls (Gibbons & Mc, 1962). The authors recorded chronic elevations in cortisol in a majority of depressed patients. More intriguingly, they observed two striking correlations: 1) plasma cortisol concentration tended to positively correlate with the severity of a patient's depressive symptoms, and 2) plasma cortisol concentration tended to *negatively* correlate with the likelihood a patient would improve. Combined, these early studies provided compelling evidence that the HPA axis—via cortisol—may play a role in mood disorders. This finding has since only been strengthened, as hypercortisolemia is one of the most consistently observed pathophysiology in both MDD and BP patients (Axelson et al., 1993).

Another key piece of evidence linking MDD and BP to HPA axis dysfunction is the dysregulation of the circadian clock (loosely defined as the collection of biochemical events that enable the ~24 hour rhythmicity observed in many biological processes) and its impact on these illnesses (reviewed in (Bunney et al., 2015)). Similar to the linkage between glucocorticoid levels and depressive symptoms, prior studies have established

1) a positive correlation between the degree of circadian dysfunction and depressive severity and 2) patient improvement correlating with resynchronization of the circadian clock (Bunney & Bunney, 2013; B. P. Hasler, Buysse, Kupfer, & Germain, 2010). The circadian clock is also responsible for the diurnal variation in circulating glucocorticoids: glucocorticoid concentrations peak during sleep and are highest at waking, with levels tapering off during the day and rising again during sleep.

This linkage between the circadian clock, HPA axis function and mood disorders is intriguing for several reasons. The first is that a diurnal rhythmicity in mood and mood disorders has been observed (Wirz-Justice, 2008). Additionally, while MDD patients maintain this glucocorticoid rhythmicity, the dynamic range of these oscillations is frequently smaller than those of control patients (remaining high throughout the day) and they are also more likely to exhibit temporal desynchronization of glucocorticoid levels (Holsboer, 2000; Scharnholz et al., 2010). Furthermore, dysregulation and/or differential expression of genes linked to the biochemical regulation of the circadian clock have been strongly associated with both MDD and BP (Edgar & McClung, 2013; Soreca, 2014). The disruption of rhythmic processes under the control of the circadian clock and/or glucocorticoids are also often observed in MDD and BP, particularly sleep disturbances.

Finally, the targeting of HPA axis dysregulation has been successfully employed in both the diagnosis and treatment of depression. The dexamethasone suppression test (DST) is one such example, whereby patients are administered dexamethasone and monitored for changes in plasma cortisol and ACTH. As dexamethasone binds to the glucocorticoid receptor this should (under normal conditions) mediate significant

reductions in both plasma cortisol and ACTH following administration. If these reductions are not observed, glucocorticoid receptor function is assumed to be blunted (potentially due to the physiological effects of persistently elevated glucocorticoid levels, explored in the following section). Furthermore, consistent with hypercortisolemia playing a presumptive causative role in depression, several studies have demonstrated therapeutic success via treatment with several HPA axis inhibitors. Examples of these have included GR receptor antagonists (prospectively blocking the negative impacts of high cortisol levels) and CRH receptor antagonists (presumptively blunting the CRH cascade) (Belanoff, Flores, Kalezhan, Sund, & Schatzberg, 2001; Holsboer & Ising, 2008). However, as these diagnostics and treatments have proven less consistent than ideal, research is ongoing.

In summary, the strong associations of HPA axis dysfunction and elevated glucocorticoid levels with mood disorders provides a compelling rationale for further examining their roles in the molecular architecture of MDD and BP.

Physiological and behavioral impacts of stress in the context of mood disorders

While the stress response was covered in an earlier section (see Chapter 1.2), it must be noted that this 'idealized' response (and subsequent rapid return to homeostasis) is more characteristic of short-term—or acute—stress. These demands, in isolation, are not harmful: while they add to the allostatic load (e.g. the natural "wearing out" of the body and its subsequent susceptibility to disease), their negative impact is miniscule compared to their adaptive benefits. Under conditions of prolonged and/or heavy stress, the allostatic load may become too great with the excessive release of glucocorticoids

becoming damaging to the brain. Multiple lines of evidence support this idea, with chronic stress (via hypercortisolemia) representing a prospective 'trigger' which contributes to the precipitation of depressive illnesses (Holsboer, 2000).

At present, however, there are several caveats in the current approaches to understanding chronic stress and its linkages to mood disorders. There is no clear consensus in the field at what point stress becomes "chronic." Researchers using animal models to examine chronic stress, for instance, may employ behavioral paradigms lasting anywhere from 10 days to 6 or more weeks. Further complicating the ability to translate findings between animal models of chronic stress and humans is the diverse array of behavioral paradigms (and their temporal duration) employed by researchers: chronic unpredictable/variable stress (CUS/CVS), chronic mild stress (CMS), chronic social defeat stress (CSDS), chronic restraint stress (CRS) and learned helplessness are all accepted models of chronic stress (Barlow, Morrison, & Sullivan, 1975; Herman, Adams, & Prewitt, 1995; Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991; Willner, Muscat, & Papp, 1992). However, these models may not only exhibit inter-model variability but also temporal variability: researchers employing the CUS model for 10 days observed an *increase* in local GABA concentrations in mPFC, while those employing CMS for a longer duration (6 weeks) observed a concomitant *decrease* (Perrine et al., 2014; Venzala, Garcia-Garcia, Elizalde, & Tordera, 2013). Finally, it is important to note that animal models do not directly represent "depression" but rather multiple physiological, behavioral and/or genetic deficits representing adaptive brain responses that resemble depression.

In spite of these limitations we have gained a great deal of insight regarding the physiological and behavioral impacts of chronic stress and their prospective role in the etiology of MDD and BP. These models of chronic stress all induce sustained elevations in circulating glucocorticoids. As stated in the prior section, hypercortisolemia (measured in circulating, cerebrospinal and urinary glucocorticoid levels) is one of the most commonly observed pathophysiologies in patients with MDD and BP. Keeping with this, both animal models of chronic stress and human MDD and BP patients often present with adrenal hyperplasia (e.g. increased size of the adrenal glands)—consistent with the notion of chronic-stress-induced hypercortisolemia.

Further consistent with increased glucocorticoid levels is the observation that multiple brain regions demonstrate altered glucocorticoid receptor expression both in response to chronic stress and in human MDD and BP patients. These observations are of note for several reasons. The first is that manipulating glucocorticoid receptor levels within notable stress-sensitive regions of brain (particularly forebrain) has been shown to precipitate depressive-like symptoms in mice (M. P. Boyle et al., 2005; Wei et al., 2004). Additionally, reductions of glucocorticoid receptor specifically in the forebrain resulted in not only depressive-like symptoms but also HPA axis dysregulation within mice, with these symptoms following a temporal profile similar to that of depression (M. P. Boyle et al., 2005). A 2012 study by Guidotti et al. not only observed these chronic-stress-induced reductions in glucocorticoid receptor expression, but demonstrated that antidepressant treatment was sufficient to normalize GR expression levels (Guidotti et al., 2013).

The hypothesis these data support, whereby prolonged elevations of glucocorticoids suppress glucocorticoid receptor expression, is a particularly attractive mechanistic explanation for depression. This is due in part to the observation that multiple brain regions exhibiting suppressed glucocorticoid receptor expression in chronic stress and depressive illness also exhibit morphological abnormalities. Chief amongst these is the PFC: MDD and BP patients both present with significant reductions in the steady-state levels of glucocorticoid receptor in PFC (Webster, Knable, O'Grady, Orthmann, & Weickert, 2002). While depressive patients tend to display reduced gray matter volume in multiple brain regions, the prefrontal cortex (and its multiple sub-regions) demonstrates the largest and most significant reductions observed. The anterior cingulate cortex (AnCg), for instance, demonstrates a ~10% reduction in volume depressive patients compared to controls, while the DLPFC demonstrates a ~25% reduction (Grieve, Korgaonkar, Koslow, Gordon, & Williams, 2013). These findings are also compelling due to the observation that alterations in mPFC and AnCg activity may be predictive of not only depressive illness but also response to pharmacological treatments (Fujino et al., 2015; Mulert et al., 2007). Consistent with these observations (e.g. reductions in total AnCg volume indicating reduced activity), deep brain stimulation targeting the cingulate cortex (resulting in increased phasic neuronal activity) resulted in a high proportion of patients with treatment-resistant depression experiencing antidepressant-like effects (~66%) (Mayberg et al., 2005).

Prior studies have also demonstrated chronic stress- and glucocorticoid-induced reductions in spine size and neuronal activity. Specifically, both chronic stress and artificially induced elevations in glucocorticoid levels resulted in neuronal atrophy, cell

loss, reduced dendritic arborization and dendritic spine size shrinkage in not only PFC but in other limbic structures (including hippocampus and nucleus accumbens) (Cook & Wellman, 2004; Radley et al., 2006; Radley et al., 2004). In contrast to these observations, other groups observed *increased* dendritic arborization and activity in the amygdala (Vyas et al., 2002) as well as increased activity in the locus coeruleus (Mana & Grace, 1997; Nakane et al., 1994; Pavcovich et al., 1990; Wilson et al., 1998). Intriguingly, these opposing effects are highly consistent with stress-induced mPFC hypofunction given that 1) the locus coeruleus is activated in stress and inhibits mPFC activity, and 2) the mPFC acts as a "brake" on amygdalar activity.

These physiological observations are also noteworthy for their prospective behavioral effects. Many maladaptive behavioral responses induced by chronic stress mirror those observed in patients with psychiatric illness. Anxiety, for instance, is not only one of the default emotional states of the stress response but is highly co-morbid with those suffering from depressive illnesses (Hirschfeld, 2001; Keller, 2006). Depressive illness is also often accompanied by increased emotionality and decreased control over emotions, which—together with anxiety—may be a behavioral result of the aforementioned amygdalar hyperactivity. This is consistent with the mPFC's role as a "brake" on amygdalar activity, particularly in light of the fact that the mPFC exerts higher-order (e.g. cognitive) control over dysphoria and general emotionality (Joormann & Gotlib, 2010). Combined, this would represent not only a chronic-stress-induced increase in emotionality (via amygdalar hyperfunction) but, due to stress-induced prefrontal cortical hypofunction, one would have reduced cognitive control over

emotional state (making it increasingly difficult to “think” one’s way out of the dysphoria experienced in depression).

Another behavioral impact of chronic stress that mirrors that observed in depressive patients is a reduction in the ability to feel pleasure (specifically, anhedonia). Stress exerts a measure of control over the reward system and reduces the ability to perceive pleasure during the stress response (Gold, 2015). One can see how this would be a beneficial adaptation: acutely reducing the ability to feel pleasure in a stressful situation would, in theory, minimize the likelihood of distraction and thus maximize the odds of an effective response. Chronic stress, however, impairs pleasure via inhibiting the NAc, the central component of the reward pathway. In addition to the previous observations of reduced NAc volume and activity in depressive patients, prior studies have identified direct innervation of the NAc by the mPFC and established it acts as a positive regulator of NAc activity (Murase, Grenhoff, Chouvet, Gonon, & Svensson, 1993). Given that anhedonia and a ‘blunting’ of the ability to feel pleasure is a central feature of depression, this provides further evidence of 1) the linkage of chronic stress to precipitation of depressive illness and 2) the mPFC—specifically, via prospective PFC hypofunction negatively influencing NAc activity—may be a central player in the mechanistic underpinnings of MDD and BP.

In summary, as many of the physiological and behavioral alterations observed in MDD and BP patients are mirrored in the effects of chronic stress, these data provide a compelling case for a causative role of chronic stress in these illnesses. Additionally, given their particularly stress-sensitive natures and linkages to many brain regions impacted by chronic stress and mental illness, the mPFC and AnCg have been cast as

areas of intense interest in understanding these conditions. Due in part to these observations, the work outlined in this thesis has focused on examining gene expression changes in AnCg (Chapter II) and mPFC (Chapter III) in human depressive patients and an animal model of stress, respectively.

The molecular architecture of MDD, BP and chronic stress: glutamate and GABA

Traditional genetic approaches to characterizing MDD and BP have been fraught with difficulty. For perspective, a recent genome-wide association study (GWS) employing 11,974 patients with bipolar disorder and 51,792 control patients yielded only two statistically significant single-nucleotide polymorphisms (SNPs): *CACNA1C* and *ODZ4* ("Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4," 2011). Similarly, another study employing 6,783 patients with MDD and 50,695 patients observed no statistically significant SNPs in MDD versus control patients (Ripke et al., 2013).

In spite of these findings, however, both MDD and BP have significant heritability, estimated at >40% for each disorder (Craddock & Jones, 1999; Lohoff, 2010) and indicating a strong genetic component. Similar to that observed in the physiology of these illnesses, the molecular and genetic architectures of these diseases are extraordinarily complex, involving a truly staggering array of prospective genes of interest. As such, Chapters II and III will focus on two major hypotheses of particular relevance to the work outlined in this thesis. It is important to note, however, that these hypotheses are not mutually exclusive (either of themselves or other hypotheses, such as the monoaminergic hypothesis of depression (reviewed in (Delgado, 2000)).

The Glutamatergic Hypothesis of Depression: Given the large body of literature identifying brain regions with differential activity in MDD, BP and chronic stress, a necessary next step was identifying components of the neural circuitry that may underlay these disruptions. Given that many brain regions present with either hyperactivity (e.g. amygdala, subgenual cingulate cortex, orbitofrontal cortex) or hypoactivity (PFC, hippocampus, nucleus accumbens) in stress and depressive illness, a logical starting point was examining excitatory and inhibitory components of the neural circuitry.

Excitatory neurotransmission in the CNS is largely mediated by glutamate. Glutamate is the most prevalent neurotransmitter in the brain, with an overwhelming proportion of neurons (>80% in some areas, notably neocortex) being glutamatergic (Orrego & Villanueva, 1993). Glutamatergic neurons also serve as the primary excitatory outputs for virtually all neurotransmitter systems (particularly monoaminergic neurotransmission) and brain regions. Glutamatergic pyramidal cells, for instance, are the major output of the PFC and receive heavy (inhibitory) monoaminergic innervation from the locus coeruleus. A current hypothesis is that this prefrontal cortical hypofunction observed following chronic stress and in depressive illness may be 1) causative in nature and 2) the result of impaired glutamatergic output.

Several lines of inquiry have yielded evidence that bolsters this hypothesis. As mentioned previously, chronic stress induces neuronal atrophy and dendritic spine retraction in the mPFC. A recent study identified that chronic stress significantly reduced glutamatergic synaptic transmission via reductions in surface levels of AMPA and NMDA glutamatergic receptors and that this effect was glucocorticoid receptor-

dependent (Yuen et al., 2012). These findings of reduced NMDA and AMPA receptor expression in mPFC have been replicated in human MDD patients, although data in BP patients is less consistent (Choudary et al., 2005; Feyissa, Chandran, Stockmeier, & Karolewicz, 2009). Evidence outlining glutamatergic hypofunction in mPFC is also in line with the aforementioned 1) increased post-synaptic expression of inhibitory adrenergic receptors (particularly *Adra2a*) in mPFC and 2) increased adrenergic and noradrenergic drive onto mPFC via stress-induced locus coeruleus hyperactivity. This body of literature is also consistent with evidence demonstrating reduced expression of genes responsible for glutamate reuptake and recycling in the mPFC of depressive patients (Choudary et al., 2005; Miguel-Hidalgo et al., 2010). Furthermore, specific optogenetic activation of layer V pyramidal cells in mPFC was sufficient to induce an antidepressant-like phenotype in mice subjected to a forced-swim test (Kumar et al., 2013)

Finally, reductions of glutamate (and glutamate metabolites) have been observed in mPFC and AnCg in MDD and following chronic stress (Auer et al., 2000; Chiba et al., 2012; G. Hasler et al., 2007; Mirza et al., 2004; Rosenberg et al., 2004). Once again, however, the data is less clear for BP, with various studies showing increases, decreases and no change in glutamate and its metabolites in PFC for various cohorts of BP patients (Bhagwagar et al., 2007; Davanzo et al., 2003; Michael et al., 2003; Ongur et al., 2008; Port, Unal, Mrazek, & Marcus, 2008).

Finally, a major strength of the glutamatergic hypothesis is the success observed in its use as a therapeutic target. In recent years, ketamine (an NMDA receptor antagonist) has come under intense investigation due to its effects as an extremely rapid and

efficacious antidepressant (Park, Niciu, & Zarate, 2015). Multiple other drugs targeting the glutamatergic system have also come under scrutiny for their prospective use in treating depression (reviewed in (Dutta, McKie, & Deakin, 2015)). Riluzole, a drug that acts to inhibit presynaptic glutamate release and facilitate its uptake, has exhibited antidepressant properties (Banasr et al., 2010). Lamotrigine, which acts to inhibit glutamate release, has also shown efficacy as an antidepressant specifically in BP patients (though not in MDD patients) (Amann, Born, Crespo, Pomarol-Clotet, & McKenna, 2011; Geddes, Calabrese, & Goodwin, 2009; Reid, Gitlin, & Altshuler, 2013). Given the dysregulation of glutamate signaling in MDD, BP and chronic stress (components of which are reviewed in **Figure 1.4**), and the antidepressant efficacy of agents targeting the glutamatergic system, glutamate may play an important role in the pathophysiology of depressive illness.

The GABAergic hypothesis of depression: In contrast to glutamate's role in the CNS, gamma aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the CNS. While representing a smaller total number of cells, GABAergic neurons make up a sizable minority of neurons (ranging between 20 and 40% of total neurons, depending on brain region) and are noted for their extreme diversity of neurochemical cell types (reviewed in (Rudy, Fishell, Lee, & Hjerling-Leffler, 2011)).

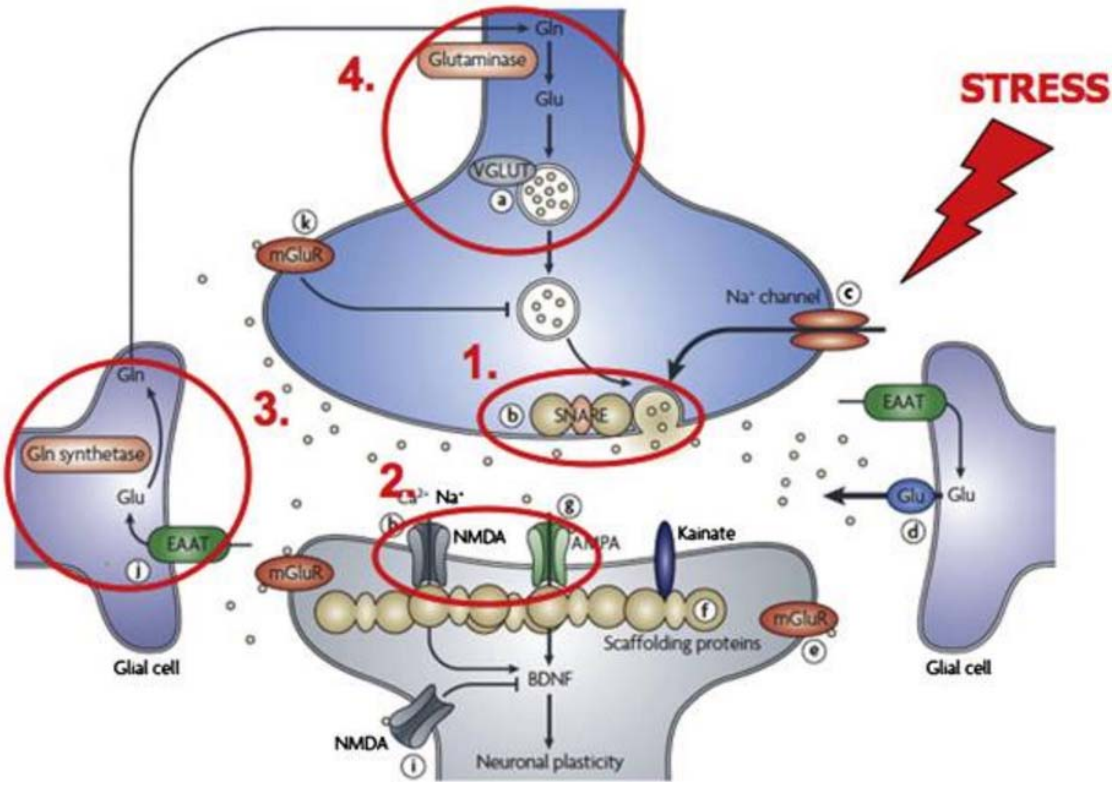

Figure 1.4: Schematic demonstrating components of glutamatergic neurotransmission impacted by stress. Red circles indicate components of the neural and glial machinery impacted by stress such as glutamate release (1), AMPA/NMDA glutamate receptors in postsynaptic cells (2), and glutamate clearance/recycling (3 and 4). Reprinted from Anxiety and Depression, Vol. 62, Issue 1, (Sanacora, Treccani, & Popoli, 2012), "Towards a glutamate hypothesis of depression: an emerging fronteir of neuropsychopharmacology for mood disorders," pp. 63-77, ©2012, with permission from Elsevier. Figure adapted from (and reprinted by permission from) Macmillan Publishers Ltd: Nat. Rev. Drug Discov., (Sanacora, Zarate, Krystal, & Manji, 2008), ©2008.

The GABAergic hypothesis of depression is a more recent one, but a growing body of evidence has placed it squarely alongside that of the glutamatergic hypothesis. In part this is due to the conceptual model of neural activity in the brain as a glutamatergic "push" with a GABAergic "pull" counteracting it. In the context of depressive illness, this raises the possibility that GABAergic hyperfunction (rather than, or in addition to, glutamatergic hypofunction) plays a mechanistic role in mPFC and AnCg hypofunction.

A growing body of literature has identified the differential expression of multiple ionotropic and metabotropic GABA receptor genes in both MDD and BP, particularly in the mPFC and AnCg (reviewed in (Luscher, Shen, & Sahir, 2011)). Additional studies have been performed on several of these genes using animal models with relevance to chronic stress and depression. Mice rendered heterozygously null for the gamma ionotropic GABA receptor subunit *Gabrg2* have been characterized as an animal model of depression, demonstrating HPA axis dysregulation as well as an anxious/depressive phenotype and exhibiting synaptic and dendritic defects (Ren et al., 2015). These findings are in line with prior anatomical data demonstrating that paraventricular nucleus of the hypothalamus (PVN)—a major component of the final neuronal pathway involved in the mammalian stress response—is regulated by GABAergic inhibition via the frontal cortex (Radley, Gosselink, & Sawchenko, 2009).

In addition to these studies, mouse knockout models of the metabotropic GABA receptors *GABBR1* and *GABBR2* exhibit an antidepressant-like phenotype (decreased immobility in the forced swim test), while rats subjected to chronic unpredictable stress demonstrate increased basal GABA release in PFC compared to non-stressed controls (Mombereau et al., 2004; Mombereau et al., 2005; Perrine et al., 2014). Finally, more recent electrophysiological data reveals a chronic-stress-induced increase in inhibitory tone on mPFC pyramidal cells (Herman, University of Cincinnati, unpublished).

Several studies have also revealed antidepressant effects in targeting the GABAergic system. Mice treated with *GABBR* antagonists have demonstrated an antidepressant- and anxiolytic-like phenotype (Mombereau et al., 2004). While canonically used in the treatment of anxiety disorders, the antidepressant efficacy of benzodiazepenes (BZ)--

which act as positive allosteric modulators of ionotropic GABA receptors--is still largely unclear, though at least one BZ (specifically, alprazolam) has been demonstrated to possess antidepressant effects (Warner, Peabody, Whiteford, & Hollister, 1988). These findings are of note due to the primary use of BZs in treating anxiety and the aforementioned strong comorbidity between anxiety and depressive illness.

The epigenetic architecture of MDD, BP and chronic stress: microRNAs

As previously described (see Section 1.3), miRNAs are noteworthy primarily for 1) the high proportion of mRNA transcripts they regulate and 2) the ability for one miRNA species to regulate anywhere from several dozen to several hundred individual mRNA targets. Given the significant genetic complexity of MDD, BP, and maladaptive responses to chronic stress, miRNAs are uniquely positioned to play a key role in the regulation of these conditions.

Perhaps the strongest evidence is that a growing body of literature has identified the dysregulation of numerous miRNAs across a variety of brain regions in not only MDD and BP but also in SZ and schizoaffective cohorts (Bavamian et al., 2015; A. H. Kim et al., 2010; Miller et al., 2012; Moreau et al., 2011). Intriguingly there appears to be a strong directional trend, with miRNAs dysregulated in patients with mental illnesses tending towards repression. Finally, in addition to mental illness, miRNA expression levels may be modulated by chronic and acute stress in a region specific manner (Haramati et al., 2011; Rinaldi et al., 2010; Shaltiel et al., 2013).

Prior work has also established miRNAs regulating key components of HPA axis activity and genes of relevance to the aforementioned hypotheses of depressive illness. miR-18

and miR-124, for instance, are able to negatively regulate the glucocorticoid receptor as well as the glucocorticoid-induced GILZ gene (Vreugdenhil et al., 2009). Additionally, inhibition of miR-124 in hippocampus induced antidepressant-like effects (Bahi, Chandrasekar, & Dreyer, 2014). A number of miRNAs were found to be regulated in rat hippocampus by chronic treatment with the mood stabilizers lithium and valproic acid (Zhou et al., 2009). One of these miRNAs, miR-34a, was also found to negatively regulate metabotropic glutamate receptor 7 (*GRM7*), a gene previously linked to BP (Zhou et al., 2009).

In addition to genetic linkages, miRNAs have been linked to behavioral alterations. A 2008 study by Uchida et al. identified that Fisher 344 rats, but not Sprague-Dawley rats, are susceptible to repeated restraint stress (Uchida et al., 2008). In this same study, the authors identified miRNA-mediated repression of the glucocorticoid receptor in PVN specifically in stress-susceptible Fisher 344 rats and postulated this behavioral difference could be due to miRNA overexpression (Uchida et al., 2008). Haramati et al. demonstrated the regulation of miR-34c by both acute and chronic stress regulation in amygdala and that overexpression conferred an anxiolytic-like phenotype (Haramati et al., 2011). Shaltiel et al. also demonstrated that miR-132—a miRNA strongly linked to schizophrenia (Miller et al., 2012)—was elevated in the hippocampus of mice subjected to predator-scent-induced anxiety and that modulation of miR-132 expression induced an anxiety-like phenotype via targeting of acetylcholinesterase (Shaltiel et al., 2013).

In summary, we know that miRNAs are dysregulated in chronic stress and mental illnesses across a variety of brain regions. Two points that are currently unclear, however, is 1) whether miRNAs are dysregulated in AnCg of MDD and/or BP cohorts

versus controls and 2) what miRNAs are regulated by chronic stress in mPFC. Investigating these points will be the major goals of chapters II and III, respectively.

Section 1.5: Hypothesis and Experimental Design

"A hypothesis is a novel suggestion that no one wants to believe. It's guilty until found effective." -Edward Teller

Due to the established links between chronic stress and mood disorders, shedding further light on the genetic architecture of each of these conditions may provide key insights into their pathophysiological mechanisms and prospective therapeutic targets. Given the strong linkage between these conditions, the aim of this thesis is to examine the following two hypotheses:

1. *MicroRNAs are dysregulated in various brain regions of patients with various mental illnesses, including MDD and BP. Given its central role in the regulation of mood and affect, miRNAs are likely to a) be differentially expressed in the AnCg of patients with MDD and BP and b) target genes of relevance to pathophysiological processes.*
2. *Chronic stress is a major precipitant of mental illness and is a powerful modulator of gene expression. Given the mPFC is a central regulator of stress responses (and is a particularly stress-sensitive brain region), chronic stress will result in widespread gene expression changes consistent with inhibited mPFC function.*

These hypotheses will be explored in Chapters II and III, respectively. I have elected to interrogate these hypotheses using the following methods:

Hypothesis 1

- Employ high-throughput quantitative real-time PCR (qPCR) assays to measure steady-state levels of miRNAs in AnCg of MDD and BP patient cohorts versus controls.
- Utilize *in silico* miRNA target prediction algorithms, molecular cloning and *in vitro* luciferase assay methodologies to identify and validate mRNAs of interest that are regulated by disease-linked miRNAs.
- Test whether steady-state levels of validated mRNAs change in MDD and/or BP cohorts versus controls using qPCR.

Hypothesis 2

- Following a 14-day chronic variable stress (CVS) paradigm that induces depressive-like behavior in mice, obtain total RNA samples from medial PFC (mPFC) of mice subjected to CVS or non-stressed controls.
- Use next-generation RNA sequencing to generate expression profiles within mPFC.
- Employ high-throughput qPCR assays to 1) validate next-generation sequencing data and 2) identify stress-regulated miRNAs.
- Utilize *in silico* miRNA target prediction algorithms, molecular cloning and *in vitro* luciferase assay methodologies to identify and validate mRNAs of interest that are regulated by stress-regulated miRNAs.

- Utilize laser-capture microdissection (LCM) to generate material from prelimbic and infralimbic subregions of mPFC from CVS and non-stressed control animals.
- Employ qPCR methodologies to identify subregion specificity of stress-regulated mRNAs.

Chapter I References

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Chapter II: The microRNA network is altered in anterior cingulate cortex of patients with unipolar and bipolar depression

A version of this chapter has been submitted to the *Journal of Psychiatric Research* and is awaiting a response.

ABSTRACT

MicroRNAs (miRNAs) are small, non-coding RNAs acting as post-transcriptional regulators of gene expression. Though implicated in multiple CNS disorders, miRNAs have not been examined in any psychiatric disease state in anterior cingulate cortex (AnCg), a brain region centrally involved in regulating mood. We performed qPCR analyses of 29 miRNAs previously implicated in psychiatric illness (major depressive disorder (MDD), bipolar disorder (BP) and/or schizophrenia (SZ)) in AnCg of patients with MDD and BP versus controls. *miR-132, miR-133a* and *miR-212* were differentially expressed in BP, *miR-184* in MDD and *miR-34a* in both MDD and BP. *In silico* target prediction algorithms identified putative targets of differentially expressed miRNAs. Nuclear Co-Activator 1 (*NCOA1*), Nuclear Co-Repressor 2 (*NCOR2*) and Phosphodiesterase 4B (*PDE4B*) were selected based upon predicted targeting by *miR-34a* (with *NCOR2* and *PDE4B* both targeted by *miR-184*) and published relevance to psychiatric illness. Luciferase assays identified *PDE4B* as a target of *miR-34a* and *miR-*

184, while *NCOA1* and *NCOR2* were targeted by *miR-34a* and *184*, respectively. qPCR analyses were performed to determine whether changes in miRNA levels correlated with mRNA levels of validated targets. *NCOA1* showed an inverse correlation with *miR-34a* in BP, while *NCOR2* demonstrated a positive correlation. In sum, this is the first study to 1) demonstrate miRNA changes in AnCg in psychiatric illness and 2) validate *miR-34a* as differentially expressed in CNS in MDD. These findings support a mechanistic role for miRNAs in the regulation of stress-responsive genes disrupted in psychiatric illness.

INTRODUCTION

Known as *melancholia* at the time of Hippocrates, 'depression' is a general term that encompasses a large number of mood disorders. Two of these particularly debilitating disorders—major depressive disorder (MDD, or unipolar depression) and bipolar disorder (BP; bipolar depression)—are also extremely common, with a lifetime prevalence of 16.6% and 3.9%, respectively (Kessler et al., 2005). Though a genetic component has been established (due in part to a high degree of heritability (Bierut et al., 1999; Lohoff, 2010; McGuffin et al., 2003; Smoller & Finn, 2003)), the genomic architecture of these disorders remains poorly understood.

In recent years, however, microRNAs (miRNAs)—small, 21-23 nt RNAs that canonically act as post-transcriptional regulators of gene expression—have become an increasing focus for understanding CNS processes. Greater than 40% of all protein-coding transcripts are predicted to be regulated by miRNAs (Tan et al., 2009; Xie et al., 2005). MiRNAs are also highly enriched within the CNS, with greater than two-thirds of

identified miRNAs expressed in brain (Bak et al., 2008; Cao, Yeo, Muotri, Kuwabara, & Gage, 2006; Sempere et al., 2004). MiRNAs are also key governors of CNS processes at both the cellular level (e.g. synaptic plasticity, neuronal differentiation and neuronal migration (Cui et al., 2012; Makeyev, Zhang, Carrasco, & Maniatis, 2007; Morgado et al., 2014; Schratt et al., 2006)) and the systems level, with miRNAs linked to the regulation of HPA axis glucocorticoid negative feedback and complex behaviors such as responses to both acute and chronic stress as well as mood and anxiety (Bahi et al., 2014; Haramati et al., 2011; Honda et al., 2013; Katsuura et al., 2012; Muinos-Gimeno et al., 2011; Vreugdenhil et al., 2009).

The role of miRNAs in the regulation of stress responses is of particular interest given that chronic stress is not only a precipitant of mood and affective disorders (Breslau & Davis, 1986; Ilgen & Hutchison, 2005) but HPA axis disruption is one of the most commonly observed pathophysiologies in MDD patients, with symptomatic severity correlating with extent of hypercortisolemia (Gibbons & Mc, 1962; Vythilingam et al., 2004). Intriguingly, a number of studies have directly demonstrated dysregulation of the miRNA regulatory network in patients with a variety of mood and affective disorders, with the vast majority focusing on schizophrenia (SZ) (Beveridge, Gardiner, Carroll, Tooney, & Cairns, 2010; Beveridge et al., 2008; A. H. Kim et al., 2010; Miller et al., 2012; Moreau et al., 2011; Perkins et al., 2007; Santarelli, Beveridge, Tooney, & Cairns, 2011; Shi et al., 2012; Smalheiser et al., 2014; Wan et al., 2015). Absent from these studies, however, has been analysis of the anterior cingulate cortex (AnCg), a brain region centrally involved in the regulation of mood, affect and cognition (Drevets et al., 2008; Ebert & Ebmeier, 1996; Mayberg et al., 1999; Posner & DiGirolamo, 1998).

Alterations in AnCg function have been increasingly linked to mood disorders with AnCg activity previously demonstrated to differentiate patients with unipolar versus bipolar depression (Diler et al., 2014) and also to predict successful pharmaceutical and cognitive treatment response (Fujino et al., 2015; Mulert et al., 2007; Pizzagalli et al., 2001; Salvadore et al., 2009). Further work has also established alterations in various systems within AnCg in MDD and BP disorders, including dysregulation in the fibroblast growth factor (FGF) system and clock genes (Bunney et al., 2015; Cheng et al., 2007; Evans et al., 2004).

In the present study we assessed miRNA expression in the AnCg of both MDD and BP patients compared to controls. As miRNAs exert their regulatory effects by targeting mRNA transcripts, we employed bioinformatics approaches to identify mRNA targets of miRNAs whose expression varied due to disease and validated several mRNAs as direct targets. Finally, we examined the steady-state levels of a subset of validated mRNA targets and identified two that vary as a function of affective disease.

MATERIALS AND METHODS

Postmortem brain tissue and RNA extraction

RNA samples derived from human post-mortem AnCg tissue were provided by the Pritzker Neuropsychiatric Research Consortium. The initial acquisition of tissue, microdissection of AnCg and subsequent RNA extraction that generated these samples is described in detail in (Evans et al., 2003). Briefly, brains were extracted during autopsy and sliced into coronal slabs approximately 0.75 cm thick. Slabs were then snap-frozen and stored at -80 degrees C until subsequent dissections. Anterior

cingulate cortex (AnCg, corresponding to Brodmann's Area 24) was identified and dissected from left hemisphere, with all dissections being performed with tissue slabs on dry ice. Following dissection, total RNA was extracted from each sample using TRIzol (Invitrogen).

Patient demographics and information—including gender, brain pH, post-mortem interval, medication history and agonal factor status—are listed in **Supplementary Table 2.1**. A total of 37 patients (n=8, BP; n=15, MDD; n=14, Control) were used for all miRNA and mRNA qPCR experiments. Patient diagnoses were based on criteria from the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, and were obtained from medical examiners, medical records and a family member. Patient samples were matched on two primary criteria, brain pH and agonal factor score. Given that our group has previously observed the significant impact of low brain pH on gene expression (Li et al., 2004) all patients were noted to have a brain pH $\geq$ 6.55 to mitigate gene expression variance. Additionally, as a prolonged agonal state (e.g. coma, multiple organ system failures, respiratory arrest, etc.) tended to be associated with lower brain pH, all patients included in this study had no agonal factors.

MicroRNA selection, reverse transcription and detection

29 MicroRNAs (**Supplementary Table 2**) were selected for qPCR analyses. These miRNAs were based upon several criteria including prior published association with psychiatric illness(es) at the time of miRNA selection, shared dysregulation between multiple psychiatric illnesses (e.g. SZ, BP and/or MDD), abundant expression and prior literature validating interactions with mRNAs previously implicated in mental illness

(outlined in **Supplementary Table 2**). Total human RNA (7.5 ng) was reverse transcribed with the High Capacity RNA to cDNA Kit (Applied Biosystems Inc., Carlsbad, CA) as per manufacturer's instructions using custom pooled RT primers corresponding to miRNAs selected for analysis. Following reverse transcription, first-strand cDNA was subjected to preamplification per manufacturer's instructions (Applied Biosystems Inc., Carlsbad, CA) using custom pooled preamplification primers. The resulting preamplified material was diluted 1:4 in 0.1x TE buffer before being subjected to qPCR. qPCR was performed using custom TaqMan Low-Density Array (TLDA) cards (Applied Biosystems Inc., Carlsbad, CA). Each TLDA card accommodated 4 biological samples and measured 29 miRNAs, as well as RNU48 as a control, in technical triplicate. RNU48 showed no significant variability between patient cohorts (CTRL, MDD and/or BP). qPCR reactions were run and measured on a ViiA7 thermocycler (Applied Biosystems Inc., Carlsbad, CA) using the following conditions: 2 minutes at 50 C, 10 minutes at 95 C (1 repeat); 15 seconds at 95 C, 1 minute at 60 C (40 repeats). Following detection, miRNAs were analyzed for differential expression using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). miRNAs with a raw p-value < 0.05 in this initial screen were subjected to additional validation via individual assay qPCR. miRNAs in these subsequent validations were considered significantly differentially expressed with a two-tailed Student's t-test result of $p<0.05$).

In silico target prediction and vector construction

Putative mRNA targets of dysregulated miRNAs were identified based on predicted targeting in miRanda (Betel, Koppal, Agius, Sander, & Leslie, 2010) (August 2010

Release) and/or TargetScan (Lewis, Burge, & Bartel, 2005) (Release 6.2) *in silico* target prediction algorithms using default parameters. A gene ontology analysis of predicted targets of miR-34a was performed using DAVID Bioinformatics (Huang da, Sherman, & Lempicki, 2009a, 2009b) using default parameters (**Supplementary Table 3**). Following this, candidate mRNA targets were narrowed to those associated with terms in the top-scoring functional clusters. Targets were subsequently selected for validation based upon 1) a direct association (based upon prior literature) with neuropsychiatric illness and/or 2) function in a biological process implicated in the pathophysiology of mental illness (e.g. glucocorticoid signaling, synaptic plasticity, transcriptional regulation). Target genes were amplified via end-point PCR (**Supplementary Table 4**) and subsequently cloned into a previously described US2 plasmid expression vector encoding firefly luciferase driven by the human UBC promoter (US2-Luc) (Yu et al., 2008). Putative miRNA binding sites were identified via the aforementioned prediction algorithms and subsequently mutagenized using the QuikChange XL kit (Agilent Technologies, Santa Clara, CA) following manufacturer's protocol. Mutagenesis primers (**Supplementary Table 4**) were designed using the web-based QuikChange Primer Design software (Agilent Technologies, Santa Clara, CA).

Cell culture, transfection and luciferase assays

96-well plates were coated for 15 minutes in 0.1 mg/mL poly-L-lysine (ThermoFisher Scientific, Waltham, MA) and washed with 1X PBS to aid in cell adherence. HEK293 cells were plated at 70-90% confluence in 75 ul DMEM media (Invitrogen, Carlsbad, CA) containing Pen-Strep (Life Technologies, Carlsbad, CA) and 10% FBS

(ThermoFisher Scientific, Waltham, MA) per well. Cells were maintained at 37 C at 5% CO_2.

24 hours following plating HEK293 cells were transfected with 0.5 ul Lipofectamine 2000 (Invitrogen, Carlsbad, CA), 300 ng US2-Firefly Luciferase (US2-Luc) plasmid containing the 3' UTR of a gene of interest, 300 ng US2-Renilla Luciferase (US2-RL) plasmid and either 0.5 pmol miRNA mimic (MISSION MicroRNA Mimic) (Sigma-Aldrich, St. Louis, MO) (miRNA treated) or 1 ul sterile saline (vehicle treated) in 50 ul OPTI-MEM media (Life Technologies, Carlsbad, CA) per well (n=6 per treatment group). Cells were allowed to incubate for 24 hours before proceeding to luciferase assays. Immediately prior to luciferase assays, OPTI-MEM media was aspirated and replaced with 75 ul of DMEM media per well. Protein lysates were prepared by adding 75 □l of Dual-Glo Luciferase Assay Reagent (Promega, Madison, WI) per well and transferring the resulting lysate to a 96-well microassay plate (Sigma-Aldrich, St. Louis, MO). Luciferase assays were then performed following manufacturer's instructions (Dual-Glo® Luciferase Assay System (Promega, Madison, WI)). Luminescence was measured on a FluoStarOptima (BMG Labtech, Germany) with renilla luciferase serving as a transfection efficiency control.

Messenger RNA reverse transcription and qPCR detection

The same RNA samples used for the miRNA expression analyses were used for messenger RNA studies. Briefly, RNA samples (25 ng per sample) were converted to cDNA with random hexamer priming using SuperScript II Reverse Transcriptase

(Invitrogen, Carlsbad, CA) following manufacturer's instructions. The resulting cDNA was diluted 1:4 in sterile water before use in subsequent qPCR studies.

Individual TaqMan gene expression assays (Applied Biosystems Inc., Carlsbad, CA) were used to measure gene expression for *NCOA1* (Assay ID# HS00186661_m1), *NCOR2* (Assay ID# HS00196955_m1) and *PDE4B* (Assay ID# HS00963643_m1) with beta actin (Assay ID# HS99999903_m1) serving as a reference control. Messenger RNA qPCR detections were run on a ViiA7 thermocycler (Applied Biosystems Inc., Carlsbad, CA) using the previously described thermocycler conditions. All samples were run in technical triplicate and were analyzed for differential expression using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and were considered significant with a two-tailed Student's t-test result of $p<0.05$.

RESULTS

Differential expression of a subset of miRNAs in AnCg of patients with MDD or BP disorder.

Following qPCR detection 3 miRNAs—*miR-33a*, *miR-144* and *miR-431**—were excluded from analysis due to high variability among technical replicates and cycle threshold values >30. After exclusion, 26 miRNAs were examined for differential expression in BP and MDD cohorts versus controls (**Table 2.1**). Of these, 5 miRNAs—*miR-132*, *miR-133a* and *miR-212* in the BP cohort; *miR-184* in the MDD cohort, and *miR-34a* shared between cohorts—exhibited raw p-values < 0.05 (**Table 2.2**) (although none passed multiple correction testing, e.g. FDR<0.15).To validate the differential expression of this subset of five miRNAs we performed an additional round of qPCR

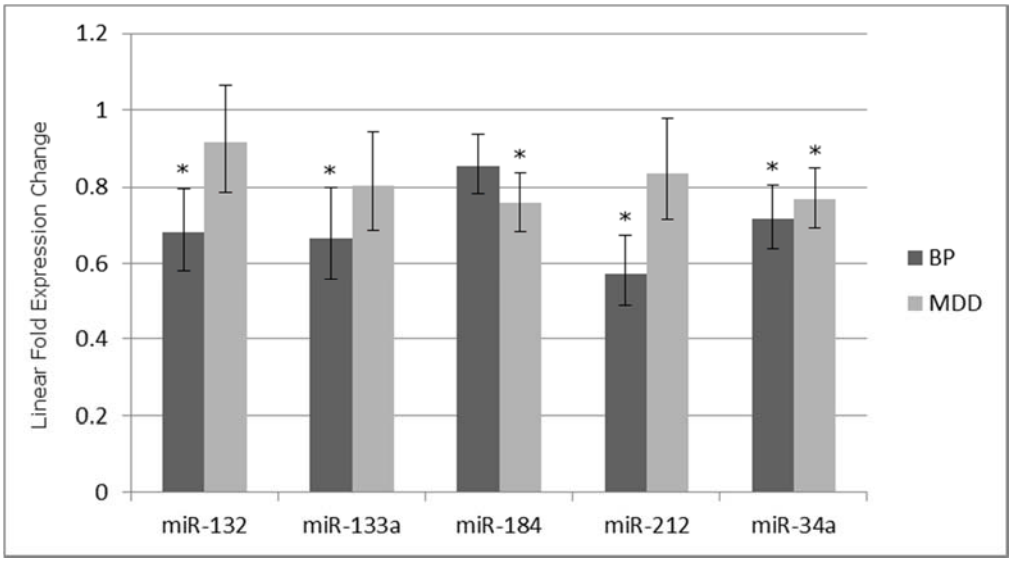

Figure 2.1: A subset of miRNAs validate as differentially expressed in AnCg of BP and/or MDD patients. Vertical axis represents linear fold change in BP or MDD cohorts versus control patients (BP: n=8; MDD: n=15; Control: n=14). Expression levels were measured using Taqman-chemistry-based qPCR using individual assays. Three miRNAs (*miR-132, 133a* and *212*) were dysregulated in BP, one (*miR-184*) was dysregulated in MDD and one miRNA—*miR-34a*, previously linked to schizophrenia—was dysregulated in both groups. All samples were run in technical triplicate with the snoRNA *RNU48* serving as a reference control. (*: $P < 0.05$, #: $P \leq 0.10$; error bars represent SEM.)

validations employing individual miRNA assays. Analysis of this subsequent round of qPCR data identified all 5 miRNAs as differentially expressed with consistent directionality, magnitude of fold-change and disease specificity to the initial screen (**Figure 2.1**).

Intriguingly, these miRNAs were dysregulated in a unidirectional fashion: all dysregulated miRNAs were repressed compared to control patients. While it did not achieve significance, *miR-195*—previously linked to regulation of brain-derived fneurotrophic factor (*BDNF*) (Mellios, Huang, Grigorenko, Rogaev, & Akbarian, 2008)—exhibited a strong trend towards repression in both BP and MDD cohorts (p=0.10 and p=0.09, respectively). We note that three of these miRNAs—*miR-132*, *miR-212* and *miR-34a*—have previously been shown to be dysregulated in PFC of SZ patients (A. H. Kim et al., 2010; Miller et al., 2012). These results suggest the shared dysregulation of

several miRNAs across several neuropsychiatric conditions, with *miR-34a* serving as a consistently dysregulated miRNA in MDD, BP and SZ. Given this, along with the large number of validated miR-34a targets previously linked to multiple mood and affective disorders (**Table 2**), we elected to focus primarily on miR-34a for subsequent analyses.

In silico target prediction analyses of dysregulated miRNAs

Following our validation of miR-34a's differential expression we performed a gene ontology analysis on the 655 putative *miR-34a* targets identified by the TargetScan algorithm (Lewis et al., 2005). Based upon the enriched terms in the two most significant GO clusters—specifically, 'Synapse' (Cluster 1) and 'Transcription Regulator Activity' (Cluster 2) (**Supplementary Table 3**)—we were able to identify 3 putative targets of *miR-34a* based upon our previously described selection criteria. Prior work has shown that two of these genes (*NCOA1* and *NCOR2*) modulate the transcriptional activity of the glucocorticoid receptor (van der Laan, Lachize, Vreugdenhil, de Kloet, & Meijer, 2008), while *PDE4B*—a genetic risk factor for mental illness (Fatemi et al., 2008; Millar et al., 2005; Numata et al., 2009; Pickard et al., 2007)—regulates cAMP signaling and is enriched at the synapse (Bradshaw et al., 2008; Millar et al., 2005). Further sequence analysis indicated that, in addition to putative *miR-34a* binding sites, both *NCOR2* and *PDE4B* possess putative *miR-184* binding sites, potentially indicating common regulatory targets for both miRNAs disrupted in MDD patients.

Target validations of dysregulated miRNAs

Following the identification of *NCOA1*, *NCOR2* and *PDE4B* as putative mRNA targets of dysregulated miRNAs, luciferase vectors containing their respective 3' UTRs were generated as previously described (Yu et al., 2008). HEK293 cells transfected with US2-Luc plasmids containing a wild-type 3' UTR from *PDE4B* (*PDE4B* WT 3' UTR) and either a miR-34a mimic or a miR-184 mimic yielded reduced luciferase values relative to control (**Fig. 2.2A**) (n=6 per treatment group). Similarly, HEK293 cells transfected with *NCOA1* or *NCOR2* WT 3' UTR yielded reduced luciferase activity when transfected with *miR-34a* or *miR-184* mimics, respectively (**Fig. 2.2b and 2.2c**). Though it exhibited a strong trend, treatment of HEK293 cells transfected with *NCOR2* WT 3' UTR and miR-34a did not result in a statistically significant repression of luciferase activity (**Fig. 2.2c**). The specificity of these mRNA/miRNA interactions were demonstrated when mutating predicted *miR-34a* or *miR-184* binding sites (34a-MUT or 184-MUT, respectively) was sufficient to relieve repression in the presence of miRNA mimics (**Fig. 2. 2a-c**). These findings demonstrate the direct regulation of these genes of interest by miRNAs dysregulated in BP and MDD patients.

qPCR of validated miRNA targets

As one way that miRNAs can exert their regulatory influence is via the degradation of mRNA transcripts, we employed qPCR methodologies to examine steady-state levels of our validated targets of *miR-34a* and/or *miR-184* (**Fig. 2.3**). Consistent with miRNAs' canonical role as negative regulators of gene expression, we observed a significant increase in *NCOA1* mRNA levels in the BP cohort but not the MDD cohort. In contrast,

we observed a significant *decrease* in *NCOR2* mRNA levels specific to the MDD cohort. Neither BP nor MDD cohorts showed significant alterations in *PDE4B* mRNA expression. Taken together, these results suggest the possibility that the reduction in *miR-34a* levels may influence steady-state levels of *NCOA1* in BP patients.

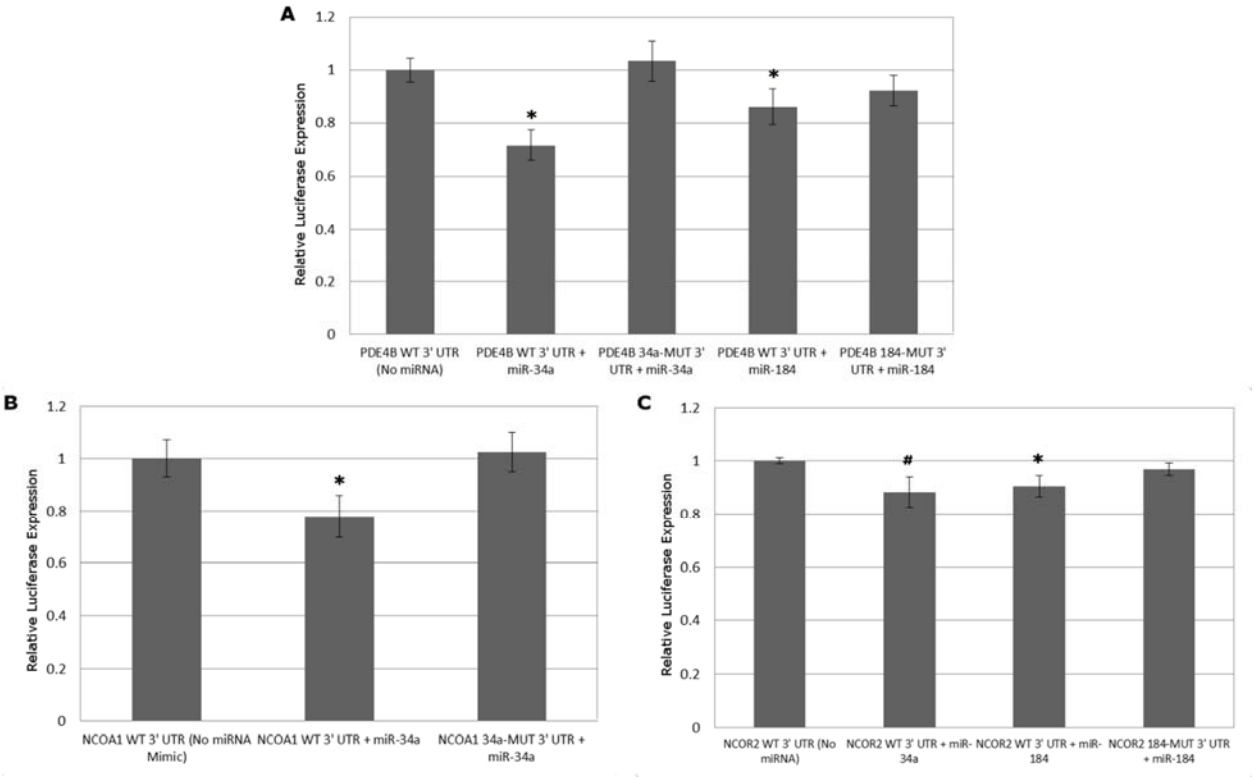

Figure 2.2: *PDE4B*, *NCOA1* and *NCOR2* are targets of miRNAs dysregulated in psychiatric illness. HEK293 cell cultures were plated and cotransfected with a miRNA mimic (either *miR-34a* or *miR-184*) and a firefly luciferase vector (Yu, Chung, Deo, Thompson, & Turner, 2008) containing either a wild-type 3' UTR (WT 3' UTR) or a 3' UTR mutagenized at a predicted miRNA binding site (34a-MUT or 184-MUT 3' UTR) (n=6/group). A vector encoding renilla luciferase was used to normalize luciferase activity. Significant reductions in luciferase activity were observed for *PDE4B* when cotransfected with either *miR-34a* or *miR-184* (**Fig. 2a**), *NCOA1* when cotransfected with *miR-34a* (**Fig. 2b**) and *NCOR2* when cotransfected with *miR-184* (**Fig. 2c**). Mutagenesis of the predicted binding sites was sufficient to relieve these 3' UTR constructs of miRNA-induced inhibition.Mutagenesis of sites in the *NCOR2* 3' UTR was not performed since this UTR showed no significant repression by *miR-34a*. (*: $P < 0.05$, #: $P \leq 0.10$; error bars represent SEM.)

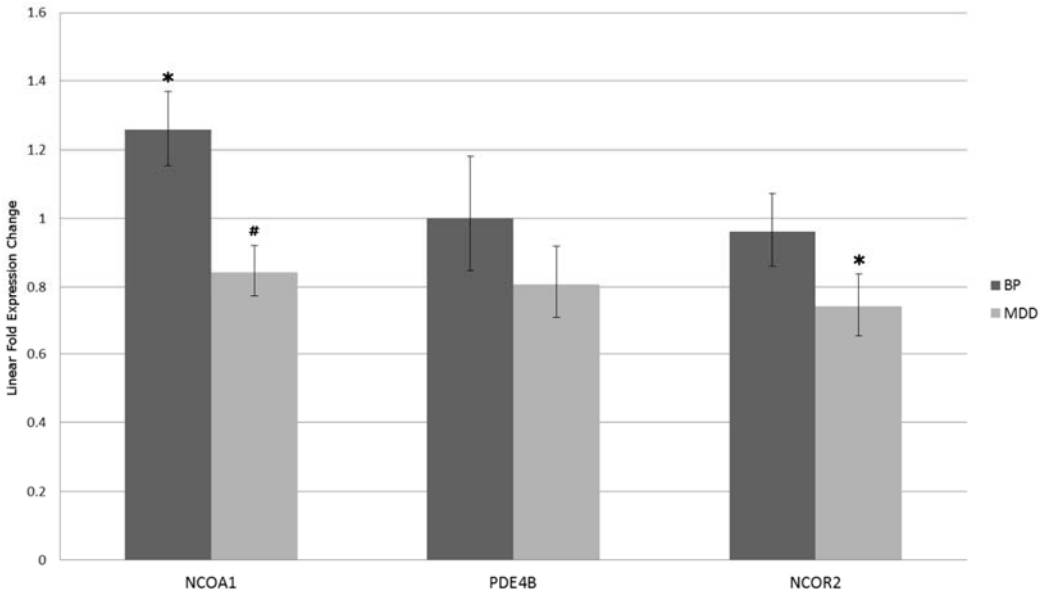

Figure 2.3: Steady-state mRNA levels of validated targets are altered in BP and MDD patients. The mRNA levels of *NCOA1* and *NCOR2* were dysregulated specifically in the BP and MDD cohorts, respectively. The same patient cohorts were used in both miRNA and mRNA expression analyses (BP: n=8; MDD: n=15 ; Control: n=14). Vertical axis represents linear fold change in BP or MDD patients versus controls. mRNA expression levels were analyzed using single-tube TaqMan qPCR assays specific to the genes of interest. Bars represent linear fold changes in BP and MDD cohorts compared to control patients with error bars representing SEM. (*: $P < 0.05$, #: $P < 0.10$)

DISCUSSION

Given their enrichment in the brain, their regulation of key CNS processes, their widespread regulation of protein-coding transcripts and their dysregulation in a number of illnesses, miRNAs are uniquely positioned to play a key role in the pathology of psychiatric illness. In this study we examined the expression of 26 miRNAs in the AnCg of MDD and BP patients versus controls. From this, we identified 5 miRNAs—3 in BP, 1 in MDD and 1 shared across both cohorts—that were differentially expressed in patients with psychiatric illness. Intriguingly, fold changes were unidirectional with all differentially expressed miRNAs reduced in MDD or BP versus controls. Additionally, we

examined a subset of putative targets for differentially expressed miRNAs, and were able to validate *NCOA1* as a target of *miR-34a*, *NCOR2* as a target of *miR-184* and *PDE4B* as a target of both *miR-34a* and *miR-184*. mRNA levels of *NCOA1* showed an inverse correlation with *miR-34a* in the BP cohort, while mRNA levels of *NCOR2* showed a positive correlation with *miR-34a* and *miR-184* in the MDD cohort (with *PDE4B* showing no expression change in either). While we note that it is possible that repression may occur at the level of translation rather than transcription (Wilczynska & Bushell, 2015), patient samples were not initially processed for protein examination; as such, we were unable to investigate this line of inquiry. While we recognize that the observed changes in miRNA expression levels are relatively modest, we feel it important to note that cooperative repression by a miRNA in combination with other miRNAs or RNA-binding proteins allows a non-linear relationship between miRNA expression and extent of mRNA target repression (Broderick, Salomon, Ryder, Aronin, & Zamore, 2011; Jacobsen, Wen, Marks, & Krogh, 2010; Mukherji et al., 2011), potentially allowing even minor changes in miRNA levels to act as ‘switches’ rather than simply fine-tuning gene expression.

These data add to our understanding of potential mechanisms underlying psychiatric disorders. While miRNA expression has been examined across a multitude of psychiatric illnesses, encompassing several brain regions, cell types and blood (Bavamian et al., 2015; Fan et al., 2014; A. H. Kim et al., 2010; Miller et al., 2012; Moreau et al., 2011; Sun et al., 2015; Walker et al., 2015), to our knowledge this is the first work to examine miRNAs dysregulated as a function of psychiatric illness in AnCg.

Additionally, comparing prior work examining miRNA dysregulation in the DLPFC—another brain region of intense interest in the pathology of mental illness—of BP and MDD patients to our results in AnCg, we see little overlap in the specific miRNA species dysregulated as a function of brain region. Potential explanations for this lack of overlap across studies include the usage of different cohorts of patients and the heterogeneity of neuropsychiatric illness. However, given prior work revealing that the complement of transcripts dysregulated in mental illness is highly dependent on brain region, the notion of region-specific patterns in miRNA disruption is consistent with current knowledge.

Of further note is that this specificity extends not only to miRNA species (e.g. *miR-34a* versus *miR-132*) but also to individual miRNA isoforms. In a prior study, *miR-133b* was differentially expressed in DLPFC of BP patients (A. H. Kim et al., 2010). In contrast, while *miR-133b* levels were not significantly different in our cohort of MDD and BP patients versus controls, *miR-133**a*** (encoded by a different gene than *miR-133b*) was significantly repressed in the AnCg of our cohort of BP patients (**Fig. 2.1**). As the mature forms of *miR-133a* and *b* differ in only one nucleotide (a U versus a G, respectively, at the final 3' residue), this single-nucleotide difference could represent a more subtle (but potentially significant) shift in target recognition between the two isoforms.

While *miR-132* and *miR-212* have been previously identified as differentially expressed in the DLPFC of SZ patients (A. H. Kim et al., 2010; Miller et al., 2012), we have identified these miRNAs—which are co-transcribed in the same primary transcript—as differentially expressed in a cohort of BP patients; an intriguing finding given that BP and SZ may share familial and genetic risk factors (Berrettini, 2003; D. Kim, Kim, Koo, Yun, & Won, 2015; Purcell et al., 2009; Shepherd et al., 2015), including miRNAs (A. H.

Kim et al., 2010; Miller et al., 2012; Walker et al., 2015). Additionally, as miR-34a expression levels are dysregulated in cohorts of both SZ and BP patients (in DLPFC and cerebellum, respectively) (Bavamian et al., 2015; A. H. Kim et al., 2010), our finding that *miR-34a* is significantly differentially expressed in a cohort of patients with MDD identifies *miR-34a* as the first miRNA to be differentially expressed in the CNS across 3 psychiatric illnesses—BP, MDD and SZ. Unfortunately, the lack of SZ patients in the present study precludes the possibility of identifying whether *miR-34a* is regulated across BP, MDD and SZ specifically in AnCg: an intriguing possibility given the postulated linkage between BP and SZ (Berrettini, 2003; Purcell et al., 2009) and several shared symptoms of BP and MDD.

Given prior evidence that suicide may be a strong factor in influencing miRNA expression (Smalheiser et al., 2014) we also performed miRNA expression analyses specifically in the suicide subgroups of our MDD and BP cohorts. Analyses of miRNA expression in the suicide subgroups of MDD and BP mirrored the directionality, magnitude of fold-change and disease specificity compared to entire patient populations (**data not shown**). Additional miRNA expression analyses using only non-suicide patients revealed strong trends in directionality of fold change and disease specificity identical to both suicide-subgroup and whole-group analyses, but these changes were not statistically significant. We note, however, that the total number of patients in the non-suicide groups are extremely small (n=2, BP; n=5, MDD) and, as such, we are unable to definitively conclude whether suicide defined a subgroup of BP or MDD subjects.

miR-34a and *miR-184*'s respective regulation of *NCOA1* and *NCOR2*—which, in turn, can alter the glucocorticoid receptor's (GR) transcriptional activity (Lachize et al., 2009; van der Laan et al., 2008)—is intriguing for several reasons. *NCOA1* has been identified as necessary for proper stress responses (Lachize et al., 2009; Winnay, Xu, O'Malley, & Hammer, 2006) while prior literature demonstrates both *NCOA1* and *NCOR2* modulate the GR-mediated transcription of corticotropin releasing hormone (*CRH*), a psychiatric risk factor linked to MDD pathology (van der Laan et al., 2008). Prior work has demonstrated the direct transcriptional repression of *miR-184* by the psychiatric risk factor methyl CpG binding protein 2 (MeCP2) (Nomura et al., 2008). Additionally, the *miR-34* family (including *miR-34a*) has previously been linked to stress (Haramati et al., 2011). Chronic stress is thought to act as a precipitating environmental factor for a host of mental illnesses and induces chronic elevations in circulating glucocorticoids (e.g. hypercortisolemia). Altered HPA axis activity—particularly hypercortisolemia—is one of the most consistently observed pathophysiologies in MDD patients (Gibbons & Mc, 1962) and is observed in both BP and SZ (Altamura, Boin, & Maes, 1999; Daban, Vieta, Mackin, & Young, 2005; Jakovljevic, Muck-Seler, Pivac, & Crncevic, 1998). As glucocorticoids exert powerful transcriptional effects through the GR (with GR mRNA level significantly reduced in MDD versus control patients in a prior study (Qi et al., 2013)) and *miR-34a* exerts influence over a GR cofactor, these findings suggest a role for *miR-34a* in the transcriptional response to stress.

The shared regulation of *PDE4B* by *miR-34a* and *miR-184* is also of note given *PDE4B*'s linkage to MDD, BP, SZ and anxiety (Fatemi et al., 2008; McGirr et al., 2015; Millar et al., 2005; Numata et al., 2009; Padmos et al., 2008; Pickard et al., 2007; Yuan

et al., 2011). In addition to PDE4B binding to and being regulated by the psychiatric risk factor *Disrupted in Schizophrenia 1* (*DISC1*) (Millar et al., 2005), pharmacological inhibitors of *PDE4B* activity have previously been tested as atypical antidepressants (Fleischhacker et al., 1992; Zeller, Stief, Pflug, & Sastre-y-Hernandez, 1984). While these drugs were never widely deployed (due primarily to negative side-effects at therapeutic dosage), the directionality suggested by this pharmacological treatment—i.e. that *increased PDE4B* is correlated with MDD—is substantiated by prior work demonstrating an increase in levels of PDE4B protein in cingulate cortex of MDD patients versus controls (Yuan et al., 2011). This prior work is also consistent with our current data, in which decreases in miR-34a and *miR-184* (**Fig. 2.1**)—which can negatively regulate *PDE4B* (**Fig. 2.2**)—should lead to an increase in *PDE4B* protein levels. While *PDE4B* mRNA levels were unchanged in either BP or MDD cohorts of the present study, it is important to note that miRNAs can exert their regulatory influence through translational inhibition as well as via mRNA degradation (reviewed in (Wilczynska & Bushell, 2015)).

Finally, we note that—in addition to the work presented here—several groups have validated additional targets of *miR-34a* that are either directly dysregulated or indirectly implicated in biological processes thought to be disrupted in psychiatric disease (**Table 2.2**). Given that mental illnesses are noted for their heterogeneity of causes, symptoms and treatments, it is noteworthy that *miR-34a*—representing the only shared miRNA dysregulated in MDD, BP and SZ—targets transcripts implicated in the pathology of all three of these illnesses (**Table 2.2**). Intriguingly, *miR-34a* has been linked to acute

responses to stress while another *miR-34* isoform—*miR-34c*—was differentially expressed in animal models of both acute and chronic stress (Haramati et al., 2011). As stress-induced HPA axis dysfunction is a common precipitating event for mood and affective disorders—and our work presented here identifies *miR-34a* as a regulator of *NCOA1*, itself previously identified as a key modulator of both stress responses and HPA axis activity (Lachize et al., 2009; Winnay et al., 2006)—these findings suggest *miR-34a* as an attractive target for further investigation.

In sum: due to their enrichment in the brain, regulation of key CNS processes and the fact one miRNA can regulate hundreds of mRNA targets, miRNAs are hypothesized to play a key role in mood and affective disorders. This current work sheds light on miRNA dysregulation in AnCg (a brain region central to the regulation of mood and cognition) as well as the mRNA targets of these dysregulated miRNAs, and identifies *miR-34a* in particular as a miRNA dysregulated across multiple psychiatric illnesses and across multiple cortical brain regions known to participate in affect and whose role in the molecular architecture of these disorders remains to be fully described.

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Bradley S Carter: early-stage technical feasibility, final revision of manuscript.

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Tables and Supplementary Information

Table 2.1: Initial screen of miRNAs in AnCg of MDD and BP patients versus controls.				
	BP versus Control		MDD versus Control	
microRNA	*Fold Change*	*P-Value*	*Fold Change*	*P-Value*
miR-124	0.83	0.21	0.92	0.69
miR-132	***0.79***	***0.05***	0.97	0.73
miR-133a	***0.71***	***0.04***	0.88	0.30
miR-133b	0.73	0.13	0.80	0.17
miR-139-5p	0.87	0.12	0.90	0.56
miR-145*	1.03	0.90	0.90	0.55
miR-148a	0.95	0.72	0.95	0.70
miR-154*	1.00	1.00	0.93	0.74
miR-181a	0.92	0.40	0.91	0.35
miR-181b	1.01	0.94	1.09	0.69
miR-184	0.93	0.44	***0.84***	***0.02***
miR-195	0.87	0.10	0.87	0.09
miR-212	***0.79***	***0.03***	1.03	0.77
miR-219	0.99	0.94	0.91	0.56
miR-23a	0.86	0.56	0.96	0.83
miR-23b	0.86	0.44	1.08	0.61
miR-342-3p	0.93	0.33	0.99	0.92
miR-346	1.01	0.97	0.90	0.41
miR-34a	***0.69***	***0.02***	***0.77***	***0.03***
miR-423-3p	0.88	0.60	0.94	0.77
miR-487a	0.77	0.11	0.83	0.39
miR-574-3p	1.01	0.96	0.94	0.60
miR-584	1.00	0.99	0.99	0.96
miR-7	0.77	0.25	0.94	0.72
miR-769-5p	0.85	0.32	0.98	0.90
miR-889	0.84	0.19	0.85	0.16

Table 2.2: Validated mRNA targets of *miR-34a* with linkage to neuropsychiatric illness. Gene names in **bold** represent novel mRNA targets.

Gene	Gene Description	Gene function	Association with Disease
GRM7	Metabotropic Glutamate Receptor 7	Synaptic transmission	GWAS (BP): (Fleischhacker et al. , 1992, Zhou et al. , 2009)
ANK3	Ankyrin G	Actin/spectrin adaptor protein	GWAS (BP)(Bavamian et al. , 2015)
CACNB3	Voltage-Dependent L-Type Clacium Channel Subunit Beta-3	Synaptic transmission	GWAS (BP)(Bavamian et al. , 2015)
VEGFA	Vascular Endothelial Growth Factor A	Growth factor signaling	Dysregulation of mRNA and protein levels, linkage analysis (MDD) (Berent et al. , 2014, Tsai et al. , 2009)
NCOA1	Nuclear Receptor Co-Activator 1	Modulation of gene transcription via glucocorticoid receptor	Dysregulation of mRNA levels (BP), modulation of stress responses and HPA axis activity. (Lachize et al. , 2009, Winnay et al. , 2006)
NCOR2	Nuclear Receptor Co-Repressor 2	Modulation of gene transcription via glucocorticoid receptor	Dysregulation of mRNA levels (MDD)
PDE4B	cAMP-specific 3',5'-cyclic Phosphodiesterase 4B	Regulates synaptic transmission via controlling cAMP levels	Dysregulation of protein levels (MDD), SNP (SZ and BP (Fatemi et al. , 2008, Pickard et al. , 2007, Yuan et al. , 2011)
SIRT1	Sirtuin 1	Histone deacetylase	Dysregulation of mRNA levels (BP, MDD)(Abe, 2011)

Supplementary Table 2.1: Demographics and information for patients used in this study may be accessed at:

https://umich.box.com/s/g395mdeivulfon1q99o7jt1i3y8nmjqu

Supplementary Table 2.2: List of miRNAs chosen for differential expression analyses and rationale may be accessed at:

https://umich.box.com/s/l8kusc748d2yn5g61105fqs5w7hi9aa7

Supplementary Table 2.3: Gene ontology analysis of *in silico* predicted targets of *miR-34a* may be accessed at:

https://umich.box.com/s/l73bay2m2f0zw3gbsma2mxk9y8tflu0w

Supplementary Table 2.4: Primer design and information for end-point PCR and site-directed mutagenesis of miR-34a targets may be accessed at:

https://umich.box.com/s/sq4tjk93xhoe2ftf9rcnmv6j978kkdky

Chapter III: A chronic variable stress paradigm induces large-scale transcriptomic alterations in medial prefrontal cortex

Following the completion of our prior miRNA studies (Chapter II), we recognized that studies in human samples are confounded by both the sheer diversity of clinical depressive phenotypes as well as individual patient variability (e.g. lifestyle, environmental and genetic variabilities). These conditions make it extraordinarily difficult to examine potential mechanistic hypotheses, a direction in which we wished to move towards. Given that animal models afford researchers the opportunity to more rigorously define experimental manipulations, and stress is often a major precipitating factor in the onset of depressive illness in humans, we elected to employ an animal model of chronic stress to examine stress-induced transcriptomic alterations in a brain-region-specific fashion.

ABSTRACT

Stress is a central feature that enables an organism's to adapt to a variety of changing conditions. While acute stress is generally perceived as beneficial, chronic stress is often maladaptive and induces a number of physiological and genetic changes in a highly brain-region-dependent fashion. One of these brain regions is the medial

prefrontal cortex (mPFC), an extremely stress-sensitive region which is centrally involved in stress responses and regulation of HPA axis activity. We performed RNA sequencing and high-throughput qPCR analyses to query the impact of chronic stress on transcriptomic networks in mPFC and validated a large number of mRNA and miRNA transcripts as chronic-stress-regulated. Using laser capture microdissection (LCM), I examined infralimbic and prelimbic expression patterns in control and chronic stress samples, revealing subregional stress regulation in a subset of RNAs. Finally, given that mPFC hypofunction and shrinkage is commonly observed following chronic stress, we constructed a biochemical “interactome” of stress-regulated mRNAs that is consistent with chronic-stress-induced reductions in glutamatergic function in mPFC. Combined, these findings support the wide-ranging impact of stress on the mPFC transcriptome and is consistent with a potential mechanism for stress-induced glutamatergic hypofunction in mPFC.

INTRODUCTION

One of the key challenges that organisms face is maintaining homeostasis in the face of various environmental challenges (collectively termed ‘stress’). The hypothalamic-pituitary-adrenal (HPA) axis--one of the primary systems underlying the central stress response--serves an indispensable role in the survival of an organism by orchestrating physiological adaptations in response to stress. While these adaptations are beneficial during periods of acute stress, extended and/or frequent activation of the HPA axis—such as that induced by chronic stress—is often maladaptive. Of particular note, chronic stress has been linked to the precipitation of a variety of psychiatric disorders such as

mood and anxiety disorders (Holsboer, 2000; Kendler, Karkowski, & Prescott, 1999; Kessler, 1997).

Chronic stress is also noteworthy due to its significant impact on neuronal physiology. A number of studies have identified robust decreases in volume, cell number, dendritic spine size, dendritic branching and neuronal activity in the medial prefrontal cortex (mPFC) following chronic stress (reviewed in (Arnsten, 2009)). The mPFC acts as a "brake" on amygdalar activity and, consistent with this, prior work has identified increased dendritic branching and neuronal activity in the amygdala following chronic stress (Morgan, Romanski, & LeDoux, 1993; Quirk, Likhtik, Pelletier, & Pare, 2003; Vyas et al., 2002). The mPFC also acts as an inhibitor of HPA axis activity, with direct stimulation of the prelimbic subdivision (PL) of mPFC sufficient to inhibit not only HPA axis activity but also responses to stress (Jones, Myers, & Herman, 2011).

In addition to the physiological effects of chronic stress, a number of biological systems (particularly glutamatergic, GABAergic and monoaminergic neurotransmission) have been shown to be subject to regulation by stress within mPFC. Stress is capable of heavily modulating gene expression across nearly every organ, tissue and cell type in the body (Chrousos, 2009). This is particularly true in the CNS, where a multitude of biological processes—including cell division/cycle control, neurogenesis, synaptic plasticity, neurotransmitter synthesis, microRNA (miRNA) expression and glial/neuronal function—are regulated not only by brain region but also by individual cell type (Bak et al., 2008; Salta & De Strooper, 2012; Ziats & Rennert, 2014).

These physiological and genetic observations are noteworthy in the context of relating mPFC dysfunction and maladaptive responses to chronic stress. The first is that the

mPFC exhibits a high vulnerability to stress, with morphological changes being observed in as little as 1-2 days in mPFC compared to >7 days in other brain regions (Brown, Henning, & Wellman, 2005; Izquierdo, Wellman, & Holmes, 2006). Furthermore, given that the primary output of the mPFC are excitatory glutamatergic pyramidal cells, an intriguing possibility is that this reduction in mPFC function is due to glutamatergic hypofunction. A growing body of prior work has provided support to this theory, with a number of studies revealing significant alterations of genes linked to glutamatergic neurotransmission, glutamate reuptake/recycling and direct reductions of glutamate and glutamate metabolites in the mPFC following chronic stress (Sanacora et al., 2012; Sanacora et al., 2008). This work is consistent with the observation that pyramidal neurons demonstrate significantly increased (inhibitory) adrenergic receptors in mPFC (particularly *Adra2a*, a gene with significant ties to chronic stress and depressive pathology (reviewed in (Cottingham & Wang, 2012)). Finally, mPFC pyramidal neurons receive heavy inhibition via serotonergic, epinephrinergic and norepinephrinergic input from the locus coeruleus (LC) (which is activated by chronic stress) (Mana & Grace, 1997; Pavcovich et al., 1990). Taken together, these data support the hypothesis that the mPFC hypofunction observed following chronic stress may be 1) causative of these maladaptive responses to chronic stress and the precipitation of depressive illness (via reducing the mPFC's ability to negatively regulate HPA axis activity) and 2) the result of impaired glutamatergic neurotransmission.

In the present study we have employed RNA sequencing and high-throughput qPCR methodologies to analyze gene expression (via RNA levels) in mPFC of mice subjected to a 14 day chronic unpredictable stress paradigm versus unstressed controls. Using

laser-capture microdissection methods we revealed the subregional specificity of a subset of these transcripts. Following the identification and validation of a large number of stress-regulated mRNA and miRNA transcripts we employed an *in silico* target prediction analysis of our data set. We subsequently identified several putative mRNA/miRNA interactions within our cohort of validated stress-regulated RNAs and proceeded to confirm these interactions. Finally, we generated an interactome comprised of stress-regulated RNAs that provides a mechanistic framework for the morphological changes observed following chronic stress.

MATERIALS AND METHODS

Animals

All animal experiments were performed with adult male C57BL/6 mice (10 weeks of age). With the exception of exposure to the CVS paradigm (see below), mice were group housed on a 12:12 light/dark cycle (6:00 AM to 6:00 PM) and were provided with *ad libitum* access to food and water. All animal procedures were reviewed and approved by the University of Cincinnati Animal Care and Use Committee.

CVS Paradigm

We opted to employ the CVS paradigm due to the fact that 1) it is a robust and well-characterized model of stress and 2) it induces a number of physiological, genetic and behavioral deficits (including HPA axis hyperactivity and anhedonia-like behavior) (Flak, Ostrander, Tasker, & Herman, 2009; Herman et al., 1995; Imaki, Nahan, Rivier, Sawchenko, & Vale, 1991). Two separate cohorts of mice were employed in the CVS

paradigm (n=12, Cohort A; n=10, Cohort B) with each cohort consisting of one group exposed to the CVS paradigm and one group of non-stressed controls (n=6/group, Cohort A; n=5/group, Cohort B).

Mice employed in the CVS were subjected to the paradigm as previously described (Herman et al., 1995). Briefly, mice were exposed to 14 days of CVS consisting of two stressors per day (one AM stressor, one PM stressor). Stressors are summarized in **Table 3.1**. The morning following the final afternoon stressor mice were euthanized via cervical dislocation and brain tissue was extracted.

Tissue Collection and RNA Extraction

Depending on experimental utility, tissue was collected in one of two ways.

Following cranial extraction, brains from mice in Cohort A were placed into a chilled, stainless steel mouse brain matrix. Using coordinates from the Paxinos neuroanatomical atlas, neural tissue containing medial prefrontal cortex (mPFC) was identified and coronal “slabs” were generated using pre-defined slice intervals in the brain matrix. These slabs were then placed on a chilled stainless steel block and mPFC was microdissected on the basis of defined landmarks, with the ventral border defined by the ventralmost extent of the foreceps minor, the dorsal border defined by the lateral deflection of the corpus callosum, and the lateral border by the corpus callosum. The dissections were designed to be limited to the infralimbic (IL) and prelimbic (PL) cortices. mPFC tissue was then placed in a 1.5 mL microfuge tube and snap-frozen in liquid nitrogen. RNA was subsequently isolated via a standard TRIzol extraction

following manufacturer's instructions (Life Technologies, Carlsbad, CA) and concentration was measured on a NanoDrop 1000 (Thermo Scientific).

Brains from mice in Cohort B were immediately flash-frozen in liquid nitrogen and subsequently cut into 14µm-thick sections on a Leica CM1850 Cryostat (Leica, Wetzlar, Germany). Sections containing mPFC were then prepared for laser-capture microdissection (LCM) via ethanol dehydration (2x 75% ethanol, 1x 95% ethanol, 2x 100% ethanol washes, 30 seconds each wash) and xylene treatment (2x xylene washes, 5 minutes each). IL and PL subdivisions of mPFC were separately laser captured using an ArcturusXT LCM System (Thermo Fisher Scientific, Waltham, MA). Following LCM, IL and PL RNA samples were generated using the RNAqueous Micro Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. Due to the relatively low yield of this method, RNA concentration was quantified using the Quant-IT RiboGreen kit (Thermo Fisher Scientific, Waltham, MA).

RNA Sequencing and Analysis

Following extraction, RNA samples from Cohort A were subjected to RNA sequencing. Library construction and sequencing were performed by the University of Michigan DNA Sequencing Core (Director: Robert Lyons, Ph.D.). Libraries were generated from 100 ng of total RNA per sample using the Illumina TruSeq Sample Prep v2 kit (Illumina, San Diego, CA) following manufacturer's instructions. Following library construction samples were clustered on a cBot (Illumina, San Diego, CA), loaded with 4 samples per lane onto a HiSeq flow cell (Illumina, San Diego, CA), and sequenced on a HiSeq 2000 (Illumina, San Diego, CA) using version 3 reagents according to manufacturer's

instructions. Reads were subsequently aligned and mapped to the mouse genome (UCSC version GRCm38/mm10, Dec. 2011) using TopHat and Bowtie. Individual reads with low transition, >3 mismatches or mapped >10 times to the genome were excluded from analysis, with an average read count of 34.3 million reads used in subsequent analyses (**Supplementary Table 3.1**). Read counts were generated using standard RPKM normalization (Mortazavi et al, 2008; Nat Methods) (**Supplementary Table 3.2**). Reads were then analyzed for differential expression between CVS and non-stressed control groups, with a minimum fold change of 1.3 and raw p-value < 0.05 used to identify candidates for qPCR validation analyses.

Selection and Validation of Stress-Regulated mRNAs

A gene ontology (GO) analysis was performed to narrow the list of prospective targets for validation. All transcripts identified as differentially expressed in the RNA sequencing results were used in this preliminary screen, and a list of top GO terms associated with CNS-specific phenomena was generated. Greater than 60 transcripts from these categories were selected for subsequent validation based upon several criteria including association with processes dysregulated in mPFC in chronic stress (such as spine size, synaptic plasticity, neurotransmission, etc), association with HPA axis function and response, prior association with neuropsychiatric illness and prior use as a therapeutic target in mood/affective disorders (**Supplementary Table 3.3**).

Total RNA samples from Cohort A (15 ng/sample) were reverse transcribed using the High Capacity cDNA Kit and TaqMan Custom RT pool primers (Life Technologies, Carlsbad, CA) corresponding to the mRNA targets selected for validation. First-strand

cDNA was then subjected to preamplification using TaqMan Preamp Master Mix and TaqMan Custom PreAmp pooled primers (Life Technologies, Carlsbad, CA) corresponding to the aforementioned mRNA targets. Both reverse transcription and preamplification were performed according to manufacturer's instructions. The resulting preamplified material was diluted 1:8 in 0.1x TE Buffer prior to qPCR detection.
qPCR was accomplished using custom TaqMan Low-Density Array (TLDA) cards (Life Technologies, Carlsbad, CA) capable of measuring 64 mRNA targets in technical triplicate. TLDA cards were run on a ViiA 7 (Life Technologies, Carlsbad, CA) using standard TaqMan thermocycler conditions: 2 minutes at 50 C, 10 minutes at 95 C (1 repeat); 15 seconds at 95 C, 1 minute at 60 C (40 repeats). Differential expression was assessed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) with beta actin used as a reference control. Significance was set at $p<0.05$ in a Student's two-tailed T-test.
For Cohort B, both RNA pools (PL and IL; 15 ng per sample) were separately reverse transcribed with the High Capacity cDNA Kit (Life Technologies, Carlsbad, CA) using standard random hexameric primers. TaqMan Individual mRNA Assays were used to measure steady-state RNA levels of putative stress-regulated transcripts in both IL- and PL-derived samples. qPCR, differential expression and statistical analysis were both performed as outlined above. Beta actin was used as a reference control.

High-Throughput Screen and Validation of Stress-Regulated microRNAs

Total RNA samples from Cohort A (15 ng/sample) were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) with TaqMan MegaPlex Rodent Pool A primers (Life Technologies, Carlsbad, CA). Reverse

transcribed material was subsequently preamplified using TaqMan Preamp Master Mix (Life Technologies, Carlsbad, CA) with TaqMan Megaplex Rodent Pool A Preamp primers (Life Technologies, Carlsbad, CA). >350 microRNAs were initially measured using Rodent Pool A TLDA cards. microRNAs identified as differentially expressed in this initial screen were then subjected to additional qPCR validation using more individual miRNA assays. Thermocycling, qPCR detection, and differential expression were assessed as outlined above. U6 was used as a reference control.

Prediction and Validation of mRNA Targets of Stress-Regulated microRNAs

Putative mRNA targets of stress-regulated miRNAs were identified using the TargetScan 7.0 and miRanda (August 2010 release) algorithms. *Rac1* was selected for validation due to 1) its shared targeting by several stress-regulated miRNAs and 2) prior evidence that characterized it as a direct binding partner of a our subset of validated stress-regulated RNAs. Following selection, *Rac1* 3’ UTR was amplified via end-point PCR and cloned into a previously described US2-Luciferase vector (Yu et al., 2008) using standard molecular cloning methods. Following vector construction 3' UTR luciferase constructs were transfected into HEK-293 cells along with MISSION miRNA mimic (Sigma-Aldrich, St. Louis, MO) and US2-Renilla Luciferase constructs as a transfection efficiency control. Following a 24-hour incubation, protein lysates were collected from transfected cells and luciferase assays performed using the Dual-Glo Luciferase Assay Reporter System (Promega, Madison, WI) following manufacturer's instructions. Luminescence was measured on a FluoStar Optima (BMG LabTech, Germany). To confirm miRNA binding specificity, putative miRNA binding sites were

mutated using the QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) and then subjected to a second luciferase assay as previously described.

RESULTS

Chronic stress induces widespread mRNA dysregulation in mPFC

Our initial RNA sequencing analysis identified >15,000 individual gene IDs within each RNA sample from Cohort A mPFC (**Supplementary Tables 3.1, 3.3**). Of these transcripts >2,000 RNAs were identified as significantly differentially expressed in mice from Cohort A subjected to CVS versus non-stressed controls. In order to narrow down this list for subsequent qPCR validation studies we performed a gene ontology analysis on a list of all differentially expressed transcripts using the GoTermFinder Gene Ontology tool (E. I. Boyle et al., 2004). Given that stress affects nearly every cell type, tissue and organ system in the body, we focused our search specifically on terms associated with CNS-specific processes (**Supplementary Table 3.4**). In conjunction with this approach, we employed additional literature screens to identify additional stress-regulated targets that were either previously linked to stress (such as *Rgs4*, a previously identified risk factor for schizophrenia (Rivero et al., 2013; Volk, Eggan, & Lewis, 2010)) or validated binding partners of genes identified in the initial GO analysis. We limited our validation phase to approximately 60 mRNA targets (with additional negative and housekeeping RNA controls) due to limits imposed by the custom design of the commercial platform we employed (Taqman Low Density Array (TLDA) cards (Applied Biosystems, Inc.)).

Due to the custom design of the TLDA platform, we limited our validation phase to ~60 mRNA targets for qPCR validation (**Supplementary Table 3.3**) according to our previously mentioned criteria. 61 of these targets were identified as differentially expressed in our RNA sequencing data, while 2 (Grin2b and Nr3c1) were chosen as negative controls. The final mRNA (beta actin, Bact) was selected as a reference control. Following qPCR analysis, statistical analysis of qPCR data confirmed the differential expression of 31 of these 60 mRNA transcripts ($p<0.05$) (**Figure 3.1**). In addition, a direct examination of linear fold changes revealed a robust positive correlation ($R^2 = 0.8646$) between samples analyzed by deep sequencing and qPCR methodologies, indicating strong reproducibility of RNA sequencing data (**Supplementary Figure 3.1**).

We note that a large number of validated transcripts have showed prior association with mood and affective disorders, particularly Disrupted in Schizophrenia 1 (*Disc1*), its well-characterized binding partner Traf2 and Nck-interacting Kinase (*Tnik*) and the adrenergic receptor *Adra2a* (Burette et al., 2015; Cottingham & Wang, 2012; Q. Wang et al., 2011). Furthermore, a number of our validated stress-regulated targets have also been targets for therapeutic interventions in a number of mental illnesses. These include the status of *Adra2a* as a target for monoaminergic therapies in mood disorders, inhibitors of the tachykinin receptor *Tacr1* being explored as atypical antidepressants and, more recently, inhibitors of glycine transporter *Slc6a9* (GlyT1) being examined as an avenue for atypical antipsychotics (Ratti et al., 2013; Singer, Dubroqua, & Yee, 2015). Finally, while changes in steady-state levels of mRNAs are not necessarily indicative of changes at the protein level, we note that the directionality of these

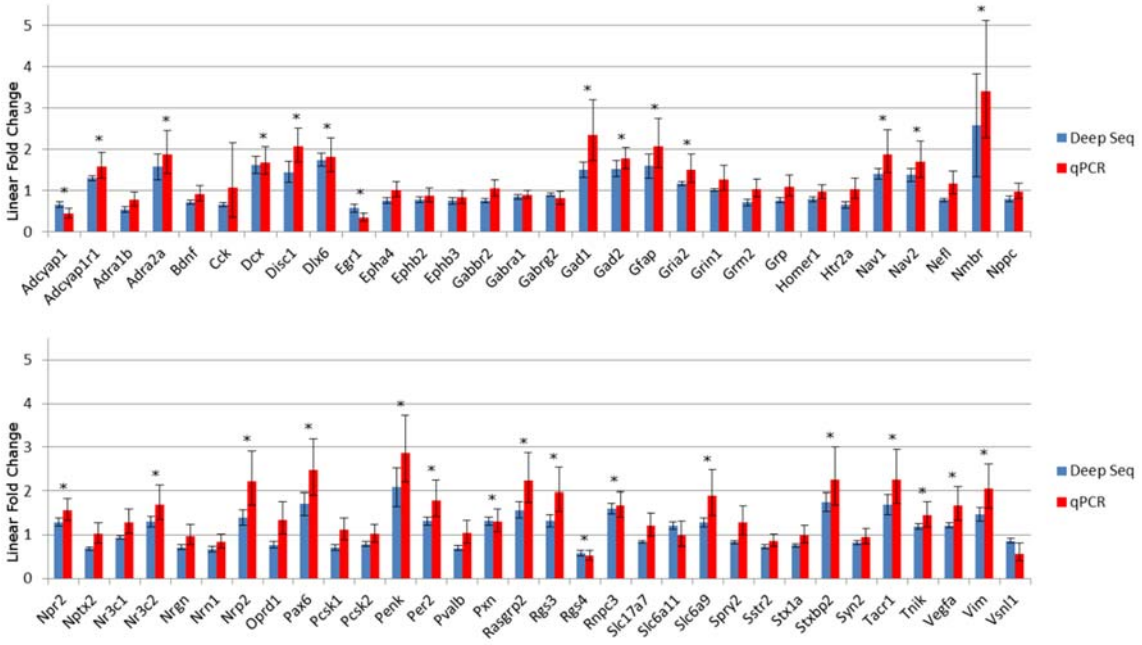

Figure 3.1: RNA sequencing and qPCR validation reveals widespread mRNA dysregulation in mPFC of CVS mice versus controls. Vertical axes in represent linear fold change of mRNA levels in CVS mice versus non-stressed controls (n=6/group; Cohort A) with blue and red bars representing fold changes assessed by RNA sequencing and TLDA-based qPCR, respectively. All transcripts shown were initially identified as putatively differentially expressed via RNA sequencing (linear fold change > 1.3, raw p-value < 0.05), while those labeled significant in this figure were validated by qPCR as significantly differentially expressed (*: p<0.05, #: p<0.10).

changes are consistent with the proposed mechanisms of therapeutic activity for these drugs (e.g. we observe increased GlyT1 in CVS, while inhibition of GlyT1 is proposed to have a therapeutic effect).

A subset of miRNAs are regulated by chronic stress in mPFC

Given the growing body of evidence that implicates both HPA axis activity and chronic stress regulation of a subset of miRNAs we also performed miRNA expression analyses on RNA samples from Cohort A mPFC. Of note, these samples were identical to those used to generate RNA sequencing and mRNA qPCR expression data.

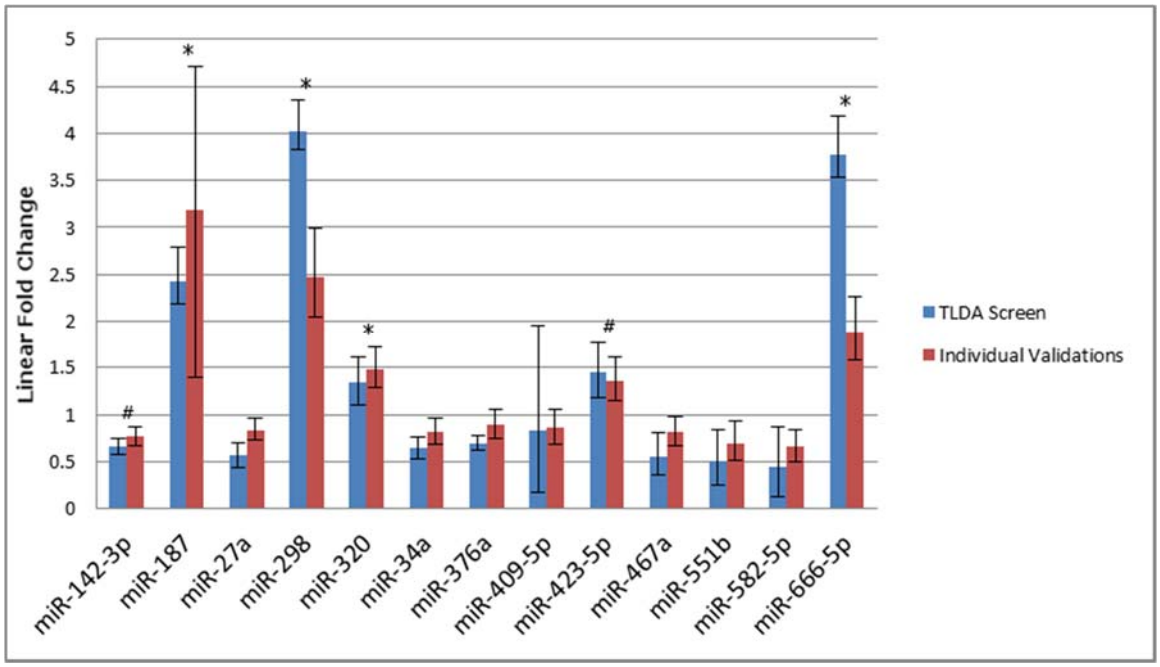

Figure 3.2: A high-throughput qPCR screen reveals altered steady-state levels of miRNAs in mPFC of CVS mice versus controls. Vertical axes in **Fig. 3.2** represent linear fold changes of miRNA levels in CVS mice versus non-stressed controls (n=6/group; Cohort A). Of the >500 miRNAs tested, 14 were identified as significant in our initial screen (blue bars). Of these, 4 (*miR-187*, *298*, *320* and *666-5p*) were validated as significantly differentially expressed with 2 additional miRNAs exhibiting a strong trend towards differential expression (*miR-142-3p*, p=0.07; *miR-423-5p*, p=0.09) following more stringent qPCR validation (red bars). (*: p<0.05; #: p<0.10)

Of the >500 miRNAs examined in our initial screen, 14 showed significant differential expression in Cohort A CVS animals compared to non-stressed controls (**Figure 3.2**). Due to technical limitations of this TLDA card format in high-throughput screens (specifically, each assay having only a single replicate per plate), we proceeded to perform individual assay qPCR validations in technical triplicate for all putative stress-regulated miRNAs identified in our initial screen (**Figure 3.2**). Of these, four miRNAs were validated as significantly differentially expressed: *miR-187-3p, miR-298-5p, miR-320-3p, and miR-666-5p*. Two additional miRNAs--*miR-142-3p* and *423-5p*--exhibited a strong trend towards repression ($p < 0.10$), but did not validate.

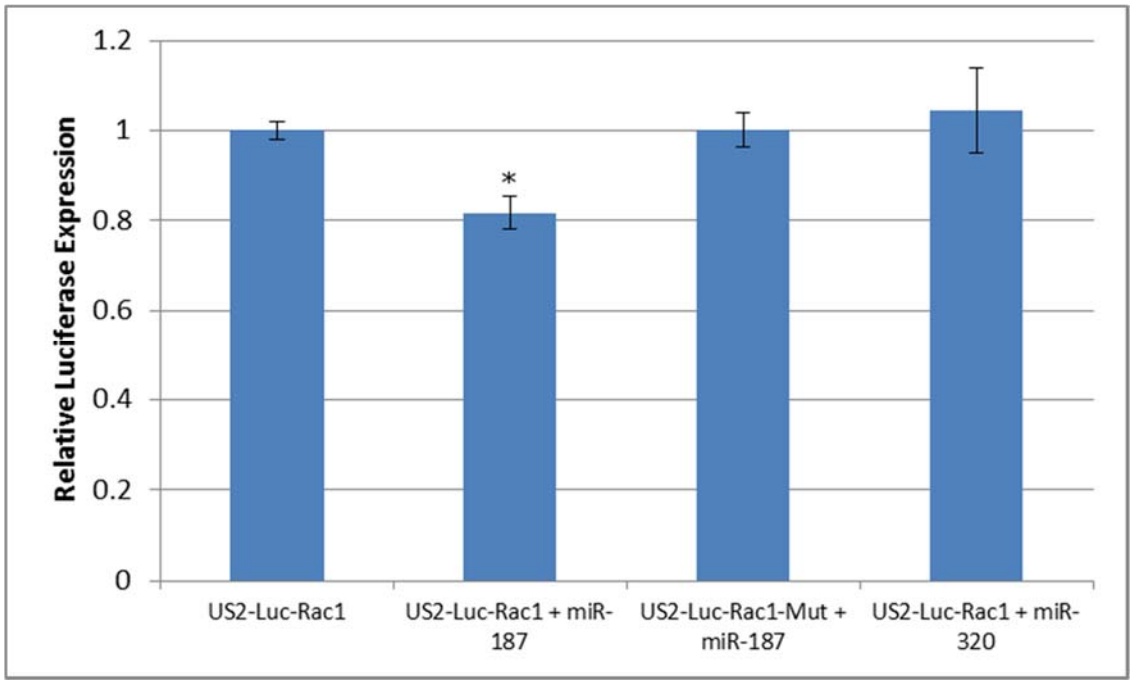

Figure 3.3: *Rac1* is targeted by the stress-regulated *miR-187* but not *miR-320*. *In silico* target prediction algorithms indicated miR-187 and miR-320 as putative regulators of *Rac1*. Following treatment with miRNA mimics, WT Rac1 3' UTR (US2-Luc-Rac1) demonstrated repression specifically upon miR-187 treatment. The subsequent mutagenesis of the putative miR-187 binding site (US2-Luc-Rac1-Mut) was sufficient to relieve this repression, indicating the specificity of this interaction. Vertical bars represent relative luciferase expression assayed by fluorescence. (*: $p<0.05$; error bars represent S.E.M.)

miR-187 targets *Rac1*, a downstream effector of *Disc1*

Following the validation of a number of stress-regulated miRNAs we sought to identify potential mRNA targets through *in silico* analyses. Based upon its targeting by multiple stress-regulated RNAs and role as a downstream effector of the stress-regulated *Disc1*, we identified *Rac1* as a putative mRNA target of interest. Firefly luciferase vectors containing WT and 3' UTR from *Rac1* (US2-Luc-Rac1) were generated as previously described and used in subsequent luciferase assays (Yu et al., 2008).

US2-Luc-Rac1 showed no significant change in luciferase activity when treated with the *miR-320* mimic (**Figure 3.3**). In contrast, US2-Luc-Rac1 demonstrated a significant decrease in luciferase activity when treated with *miR-187* miRNA mimic (**Figure 3.3**). Subsequent mutagenesis of the putative *miR-187* binding site in WT Rac1 3' UTR was

sufficient to relieve this inhibition, demonstrating the sequence dependency of this mRNA/miRNA interaction (**Figure 3.3**).

Characterization and prospective "interactome" for the impact of stress on PFC function

Our initial GO analysis identified multiple genes differentially expressed by stress that are implicated in the regulation of neuronal morphology, dendritic spine size and/or synaptic plasticity (**Supplementary Table 3.3**). Given this finding, and the identification that a number of our genes of interest are neuronally enriched, we explored potential links between stress-induced changes in gene expression and neuronal function. By deploying Ingenuity Pathway Analysis, STRING Analysis software and literature analyses, we identified a number of protein-protein interactions between a number of our genes of interest and genes in pathways linked to the regulation of spine size and synaptic transmission. Consistent with a neuron-centric hypothesis, prior work has identified not only the neuronal enrichment of a large subset of these transcripts but their enrichment within specific cell type(s). Of particular note, several of these transcripts--notably *Disc1* and *Tnik*, both risk factors for psychiatric illness—not only demonstrate enrichment at the glutamatergic synapse but also have empirically validated interactions at the protein level (Burette et al., 2015; Q. Wang et al., 2011) (**Figure 3.4**). Additionally, *Gad1* and *Gad2* are rate-limiting enzymes in the production of the GABA neurotransmitter (Asada et al., 1997; Kash et al., 1997) and, together with *Dlx6*, represent cell-type-specific markers for GABAergic cortical interneurons (Y. Wang et al., 2010).

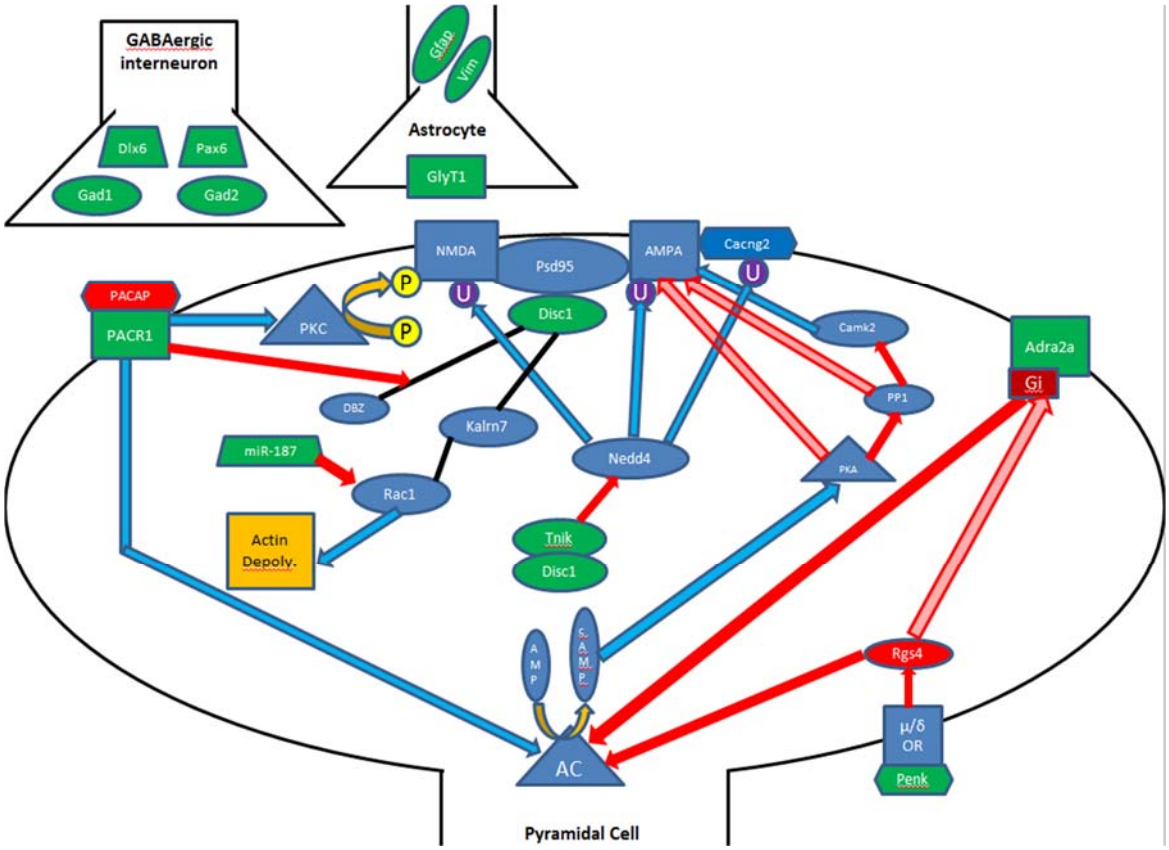

Figure 3.4: A mechanistic 'interactome' of transcripts altered by chronic stress in mPFC. Genes listed in green and red demonstrated increased and decreased expression, respectively, in mPFC of CVS mice versus non-stressed controls. Genes in blue did not change. Unidirectional arrows represent the action of one gene on another, with blue representing activation and red representing inhibition. Solid lines represent empirically validated protein-protein interactions, while faded lines represent indirect interactions. Yellow and purple circles represent phosphate and ubiquitin groups, respectively.

In addition to this presumptive neuronal network, two genes of note were identified as being glial-enriched. Of these, the *Adcyap1* receptor *Adcyap1r1* has been previously demonstrated to be expressed in astrocytes and regulates astrocytic glutamine metabolism (a key role in the biosynthesis of glutamate) (Figiel & Engele, 2000; Suzuki et al., 2003). The glycine transporter *Slc6a9* is also enriched in astrocytes and is noteworthy due to recent studies focusing on it as a prospective therapeutic target for novel antipsychotics (Aroeira, Sebastiao, & Valente, 2014; Singer et al., 2015). Combined, these data are consistent with 1) the convergence of multiple biological

pathways across various cell types onto mPFC pyramidal cells function and 2) their shared dysregulation following chronic stress in mPFC.

Subregion specificity of a subset of stress-regulated mRNAs

The mPFC features two primary subdivisions—specifically, the infralimbic (IL) and prelimbic (PL) subregions—that exert differential regulatory effects on in stress responses and to HPA axis activity (Jones et al., 2011; Radley et al., 2006; Sullivan & Gratton, 1999; Tavares, Correa, & Resstel, 2009). To examine the potential stress regulation of transcripts by subregion, we isolated RNA from both IL and PL of a second cohort of CVS mice by employing LCM methods. We subsequently performed qPCR analyses on a subset of stress regulated transcripts (*Adcyap1r1, Gad2, Rgs4* and *Tnik*) in an independent cohort of CVS and non-stressed control animals, with one transcript (*Nrgn*) serving as a negative control.

3 our 4 stress-regulated transcripts validated as differentially expressed in at least one subregion (**Figure 3.5)**. Consistent with our initial qPCR validation employing whole mPFC samples (Cohort A), *Nrgn* did not demonstrate differential expression in either subregion (**Figure 3.5**). Of note, 2 of our 3 validated transcripts--*Gad2* and *Tnik*--demonstrated differential expression specifically within the PL subregion, while *Rgs4* was significantly differentially expressed in both PL and IL subregions. Combined, this data demonstrates subregional specificity of the impact of chronic stress on gene expression and may contribute to the differential response of IL and PL in stress responses.

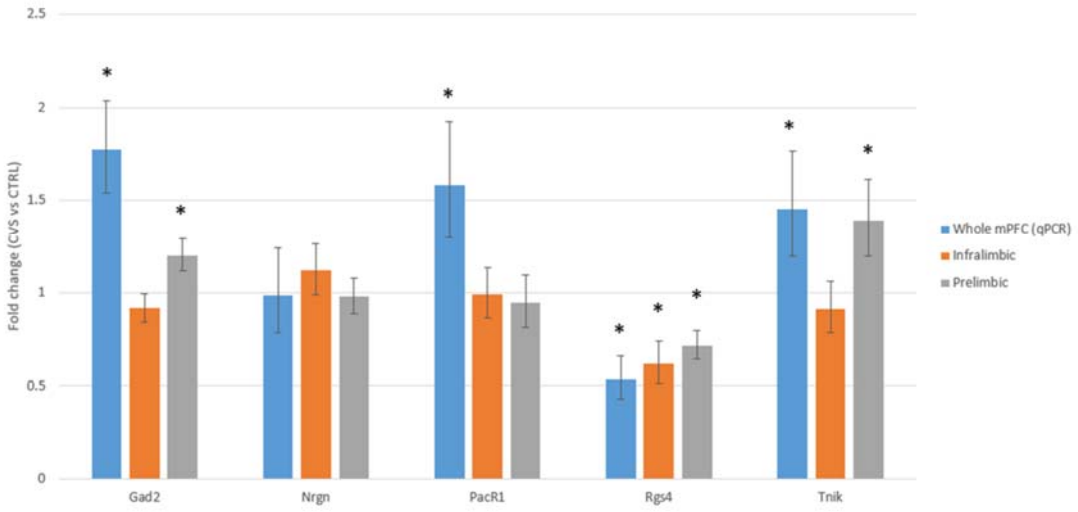

Figure 3.5: A subset of stress-regulated targets demonstrate subregional specificity within mPFC. Vertical axes represent linear fold change of mRNA levels in whole mPFC (Cohort A, n-6/group) or IL and PL subregions in CVS mice versus non-stressed controls (Cohort B; n=5/group). *Gad2*, *Rgs4* and *Tnik* all validated with directionality consistent with the first cohort of mice (Cohort A). While *Rgs4* was significantly differentially expressed in both IL and PL subregions, *Gad2* and *Tnik* demonstrated significant differential expression specifically within the PL subregion. (*: $p<0.05$)

DISCUSSION

Given the physiological impact of stress and its large-scale regulation of gene expression, understanding the molecular architecture of chronic stress may shed further light on the mechanistic underpinnings of its impact on neuronal physiology. This is particularly true in the mPFC, a brain region noted for its central regulation of mood and affect as well as its heavy implication in the pathophysiology of a host of mental illnesses (Arnsten, 2009; Cardinal, Parkinson, Hall, & Everitt, 2002; Phillips, Drevets, Rauch, & Lane, 2003). In our present study we performed RNA sequencing analysis and high-throughput qPCR screens using mPFC RNA samples to identify, respectively, stress-regulated mRNAs and miRNAs in CVS versus unstressed mouse controls (n=6/group, Cohort A). From this, we selected a subset of >60 mRNAs and 14 miRNAs

and subjected them to further qPCR validation, with 31 mRNAs and 4 miRNAs validating as differentially expressed. Additionally, we identified and validated an mRNA target--*Rac1*--as being regulated by a stress-sensitive miRNA, *miR-187*. We subsequently validated the differential expression of several mRNA transcripts in an independent cohort of CVS and control mice (Cohort B) and demonstrated the subregional specificity of a subset of these stress-regulated mRNAs. Finally, by combining these data sets, we generated a presumptive "interactome" of genes identified as differentially expressed in this current study.

Coupled with prior literature outlining specific gene functions and interactions, our data is consistent with a potential glutamate-centric mechanistic hypothesis underlying stress-induced mPFC hypofunction (**Figure 3.4**).

Disc1 has been identified as a prominent risk factor for a number of mental disorders and plays a key role in a number of CNS processes, including synaptic spine size (Blackwood et al., 2001; Brandon & Sawa, 2011; Farrell et al., 2015). Prior work by Hayashi-Takagi et al. has demonstrated that *Disc1* is also a direct **negative** regulator of glutamatergic spine size via interactions via a *Kalrn7*, *Rac1* and *Pak1* pathway (Hayashi-Takagi et al., 2010). In this same study, it was observed that *Rac1* levels positively correlate with spine size. While we did not observe altered steady-state levels of *Rac1* mRNA in our CVS animals, we did validate *Rac1* as a target of the stress-regulated miRNA miR-187 (**Figure 3.3**). Given the directionality of our observed changes (e.g. both *Disc1* and *miR-187* are increased in chronic stress and both negatively regulate *Rac1* function), our findings are consistent with a mechanistic role of

Disc1 and *miR-187* in chronic-stress-induced reductions in mPFC pyramidal cell spine size.

We also validated *Tnik*, a *Disc1* binding partner, as regulated by stress. A recent study demonstrated that *Disc1* and *Tnik* are direct binding partners, with *Disc1* inhibiting kinase activity of *Tnik* (Q. Wang et al., 2011). In this same study, researchers observed that this inhibition of *Tnik*'s kinase activity not only led to reductions in PSD95, GluR1 and stargazin protein levels, but also to reductions in AMPA mEPSCs in primary hippocampal neurons. An additional study identified *Tnik* as a binding partner, and negative regulator, of the E3 ubiquitin ligase *Nedd4* (Kawabe & Brose, 2010). Chronic-stress-induced reductions in surface levels of multiple AMPAR and NMDAR subunits in mPFC were found to occur via the ubiquitinylation/proteasomal pathway (Yuen et al., 2012). Intriguingly, these reductions in the expression of genes linked to glutamatergic neurotransmission are 1) dependent on *Nedd4* function (Yuen et al., 2012) and 2) would be expected to increase ubiquitinylation (and subsequent degradation) of glutamatergic receptors under conditions of reduced *Tnik* (due to loss of inhibitory control on *Nedd4*). While we acknowledge that both *Disc1* and *Tnik* validated as significantly increased in both Cohort A and Cohort B of CVS mice versus non-stressed controls, we also note that the magnitude of these fold changes (~1.5- and ~2-fold increase in *Tnik* and *Disc1*, respectively) are compatible with a proportionally greater inhibition of *Tnik* via *Disc1*. Combined, our data are consistent with reduced *Tnik* activity in chronic stress leading to a negative impact on glutamatergic neurotransmission in mPFC.

We also observed increased levels of the adrenergic α_{2A} receptor *Adra2a* in chronically stressed animals compared to controls (**Figure 3.1**). Both adrenergic and noradrenergic systems have been linked with chronic stress and mPFC function, with stress resulting in enhanced (nor)adrenergic transmission onto mPFC due to increased locus coeruleus activity (Nakane et al., 1994). *Adra2a* in particular is strongly implicated in depression and depressive-like behavior (reviewed in (Cottingham & Wang, 2012)), with human and animal studies showing robust increases in *Adra2a* mRNA expression, protein levels, *Adra2a* receptor sensitivity, and/or reductions in *Adra2a* receptor levels linked to antidepressant efficacy in animal models of chronic stress. Keeping with our hypothesis, *Adra2a* exerts a generally inhibitory influence via inhibition of neuronal function and negative regulation of adenylyl cyclase activity (Valdizan et al., 2010; M. Wang et al., 2007; Y. Wang et al., 2011; Wilson et al., 1998). Adding to this impact, the regulator of G-protein signaling *Rgs4* has previously been shown to negatively regulate *Adra2a* activity (Cavalli, Druey, & Milligan, 2000). Finally, as we demonstrated reductions in *Rgs4* mRNA in animals subjected to CVS, these findings are consistent with prolonged activation of *Adra2a*-mediated downstream signaling events—a hypothesis supported by previous observations of increased *Adra2a* receptor sensitivity in post-mortem human depressive studies (Gonzalez-Maeso, Rodriguez-Puertas, Meana, Garcia-Sevilla, & Guimon, 2002; Valdizan et al., 2010).

While our hypothesis focuses primarily on glutamatergic mPFC pyramidal cells, our findings also implicate both GABAergic interneurons and glia in stress-induced glutamatergic dysfunction. We observed significant increases in the GABAergic interneuronal marker *Dlx6* (Y. Wang et al., 2010), as well as both forms of the

GABAergic interneuronal marker glutamic acid decarboxylase (*Gad1* and *Gad2*). Intriguingly, these enzymes catalyze the rate-limiting step in the production of GABA, the primary inhibitory neurotransmitter in the CNS (Asada et al., 1997; Kash et al., 1997) and a study by Gilabert-Juan et al. has demonstrated not only elevations of mPFC *Gad1* in chronic stress but also increased dendritic complexity of *Gad1*-positive mPFC interneurons following chronic stress (Gilabert-Juan, Castillo-Gomez, Guirado, Molto, & Nacher, 2013), consistent with increased GABAergic inhibition following chronic stress. In addition to these findings, we also observed significant increases in both *Vim* and the astrocytic marker *Gfap*, consistent with a prior report indicating increased cortical *Gfap* protein levels following repeated restraint stress (Jang, Suh, Yoo, Lee, & Oh, 2008). We also validated a significant increase in the glial-enriched glycine transporter *Slc6a9* (GlyT1) (**Figure 3.2**). This finding is of note given that glycine acts as an important NMDAR cofactor and, together with glutamate, is necessary for NMDAR activation (Johnson & Ascher, 1987). Additionally, the directionality of this change is consistent with reduced NMDAR activity: increased GlyT1 would be consistent with increased clearance of glycine and reduced availability at the synapse, which would indicate reduced NMDAR activity. Combined, our findings are consistent with the convergence of multiple cell types in mPFC exhibiting a negative impact on glutamatergic transmission and mPFC activity following chronic stress.

Additionally, we have demonstrated the subregional specificity (e.g. PL vs IL) of stress-regulated transcripts. While *Rgs4* was significantly decreased in both PL and IL subregions, *Gad2* and *Tnik* both exhibited stress-induced increases specific to PL (**Figure 3.5**). We also note that PacR1 failed to validate in either PL or IL subregion,

suggesting the possibility of either 1) differential expression in another mPFC subregion (such as cingulate cortex) or 2) contamination of our initial whole-mPFC preparation by adjacent brain region(s). While the mPFC has an overall effect of inhibition on HPA axis activity, with general mPFC hypofunction potentially serving a causative role, prior work indicating subregional specificity of HPA axis control adds further complexity to our current understanding of stress responses. Previous studies have indicated the necessity of mPFC IL cortex in the initial HPA axis response (Ronzoni, Anton, Mora, Segovia, & Del Arco, 2016). Furthermore, inactivation of the IL cortex is sufficient to reduce both HPA axis activity and physiological responses to psychogenic stressors (Radley et al., 2006; Sullivan & Gratton, 1999; Tavares et al., 2009), indicating a positive correlation of IL activity and HPA axis activity. In contrast, the PL cortex appears to *inhibit* HPA axis activity in response to psychogenic—but not physical—threats (Jones et al., 2011). While additional studies will be needed to identify additional candidates and dissect the potential role of subregion-dependent genomic responses to stress, these findings are intriguing given 1) the PL specificity of several of our stress-regulated transcripts and 2) that specific reductions in PL activity, rather than IL, would be expected to underlie the stress-induced increased in HPA axis activity.

We acknowledge the potential limitations of interpreting our findings—specifically, that 1) all observations were performed in RNA samples rather than protein and 2) the extraordinary difficulty in accurately overlaying “whole brain region” transcriptomic data on specific components of the underlying neural circuitry. We note, however, that the proposed cell type(s) of interest and directionality of these changes—coupled with observations of protein functionality and validated interactions in the literature—are

extremely consistent with both prior findings and our proposed hypothesis.

In summary, given the significant effects of stress on gene networks and its impact on neuronal physiology, understanding the genetic impact of chronic stress will be instrumental in devising future treatments. Our current study illuminates the wide-ranging impact of chronic stress on mRNA and miRNA expression in the mPFC (a brain region central to the regulation of mood and affect). Additionally, these changes provide a mechanistic framework for how gene expression changes may underlie stress-induced glutamatergic mPFC hypofunction, a pathophysiology thought to be causative of several pathophysiologies observed in chronic stress and of which our knowledge of the underlying biology remains incomplete.

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Joshua A Azevedo: study design, data analysis.

Christopher L Cooke: data analysis and material generation.

Jessica M McKlveen: study design.

Fan Meng: bioinformatics analysis.

Manhong Dai: bioinformatics analysis.

James P Herman: study design, data analysis.

Robert C Thompson: study design, data analysis.

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TABLES AND SUPPLEMENTARY INFORMATION

Table 3.1: Schedule of stressors used for the CVS paradigm.		
Day	Timing	Stressor
1	AM	Elevated plus maze
	PM	Warm sim
2	AM	Orbital shaker
	PM	Cold room
3	AM	Cold room
	PM	Hypoxia and single housing (overnight)
4	AM	Orbital shaker
	PM	Warm swim
5	AM	Hypoxia
	PM	Cold room
6	AM	Orbital shaker
	PM	Warm swim and single housing (overnight)
7	AM	Cold room
	PM	Hypoxia
8	AM	Shaker
	PM	Warm swim and single housing (overnight)
9	AM	Hypoxia
	PM	Cold room and single housing (overnight)
10	AM	Warm swim
	PM	Hypoxia
11	AM	Cold room
	PM	Orbital shaker
12	AM	Hypoxia
	PM	Orbital shaker and single housing (overnight)
13	AM	Orbital shaker
	PM	Hypoxia
14	AM	Elevated plus maze
	PM	Warm swim
15	AM	Novel restraint

Supplementary Figure 3.1: qPCR validation correlates strongly with linear fold changes assessed by deep sequencing methodologies may be accessed at:

https://umich.box.com/s/7mhd9zb36u47819j9jrz3wmeifjt0deb

Supplementary Table 3.1: Summary of reads obtained from each sample in Cohort A may be accessed at:

https://umich.box.com/s/smfvj33k1msb2yi9l303fvsopezstvy1

Supplementary Table 3.2: Gene read counts from RNA sequencing of Cohort A may be accessed at: https://umich.box.com/s/y3lth7fqs6rmgrly0cclq2tiq2sopidu

Supplementary Table 3.3: Genes and Assay ID#'s for TaqMan Low-Density Array Cards may be accessed at:

https://umich.box.com/s/85k7e2etao54z53d4c869nxh4b7rr7y5

Supplementary Table 3.4: Gene Ontology Analysis of All Stress-Regulated Transcripts From RNA-Seq Data may be accessed at:

https://umich.box.com/s/q3las77ao8mhnwyax7tiliae0v0tdhus

Chapter IV: Analyzing transcriptomic alterations in a cell-type-dependent fashion: implementation of translating ribosomal affinity purification (TRAP) methods

"The brain is a monstrous, beautiful mess. Its billions of nerve cells--called neurons--lie in a tangled web that displays cognitive powers far exceeding any of the silicon machines we have built to mimic it."

-William F. Allman

INTRODUCTION

In prior chapters this thesis posited a cell-type-specific hypothesis for the prospective transcriptomic events underlying a major chronic stress-induced physiological change in mPFC. Our current body of knowledge supports this hypothesis, with a number of prior studies establishing the enrichment of specific transcripts in cell type(s) of interest (Cahoy et al., 2008; Gong et al., 2003). However, these studies (as well as nearly all similar studies at the time of writing) are fundamentally constrained by an inability to overlay changes in gene expression onto specific components of the underlying neural circuitry. While a number of genes are commonly considered “markers” of either CNS cell type (e.g. GFAP for astrocytes, MAP2 for neurons) or subtype (e.g. parvalbumin and *5HT3AR*, for distinct subsets of GABAergic interneurons (Markram et al., 2004; Rudy et al., 2011)), these markers represent a tiny minority of

transcripts expressed in the CNS. Rather, the majority of transcripts are expressed in more than one cell type ranging from reasonably specific (e.g. *Gad2*, which labels a majority of GABAergic interneurons) to the generic (such as beta actin). From a practical standpoint this adds a further layer of complexity to understanding the biological context of gene expression changes. For instance, a gene that is implicated in altered neuronal function would generate very different hypotheses on its prospective mechanism of action if it were expressed specifically in neurons as opposed to glia.

A major limiting factor in linking changes in gene expression to specific cell type(s) has been the lack of available methodologies. While a number of methodologies have been developed and/or adapted to enable more precise collection of relevant samples (e.g. laser-capture microdissection (LCM), cell sorting, cell and tissue culture methods), samples obtained via these methods are likely to be mechanically, temporally and/or physically removed from their *in vivo* environment which are likely to alter transcriptomic networks, potentially obfuscating genomic responses to various insults. As such, it would be ideal to identify and implement further approaches to minimize these variables.

Quite recently Doyle and Heiman et al. developed a novel method, termed Translating Ribosome Affinity Purification (TRAP) to address this problem (Doyle et al., 2008; Heiman et al., 2008). The authors engineered a transgenic mouse model that expressed an eGFP-tagged L10a ribosomal subunit downstream of a floxed stop codon, enabling the expression of this construct specifically in Cre-recombinase-positive cells. As L10a is a ribosomal subunit and forms part of the translation complex that binds to actively-translated transcripts, this approach enables the immunopurification (and

subsequent isolation) of actively translated mRNAs from genetically targeted cell types. By combining these TRAP methods with traditional neuroanatomical dissections, one can more readily associate transcriptomic changes to cells of a defined neurochemical phenotype (specified by Cre driver) and, hence, components of the neural circuitry within brain.

While the research outlined in this thesis has focused primarily on neuronal cell types, our laboratory has published several papers focused on the glucocorticoid regulation of transcriptomic changes in astrocytes both *in vivo* and *in vitro* (Carter, Hamilton, & Thompson, 2013; Carter, Meng, & Thompson, 2012). At the time we were unable to perform a full transcriptomic analysis of astrocytic gene expression changes *in vivo*. As TRAP 1) represented an opportunity to expand on this prior work, 2) would be deployable to my own neural-centric hypotheses, and 3) was consistent with funding opportunities at this time, our laboratory elected to focus our seminal efforts on deploying the TRAP methodology in astrocytes. Though astrocytes have received comparatively little attention, prior work has implicated them as an important set piece in understanding brain function in both health and disease. Astrocytes represent a large proportion of CNS cells and are more numerous than neurons, with ~1.4 astrocytes per neuron in human cortex (Bass, Hess, Pope, & Thalheimer, 1971; Nedergaard, Ransom, & Goldman, 2003). Astrocytes make extensive connections (averaging ~10,000 neuronal connections for each astrocyte) and play key roles in neuronal health, neurotransmitter recycling/biosynthesis and may also be involved in ‘glial signaling’ via glutamate release (Schousboe, Scafidi, Bak, Waagepetersen, & McKenna, 2014).

There is also growing evidence that astrocytes are involved in the pathophysiology of chronic stress and depression. Recent work in our laboratory demonstrated that chronic glucocorticoid treatment induced widespread dysregulation of gene expression in astrocytes both *in vitro* and *in vivo* (Carter et al., 2013; Carter et al., 2012). In addition, a number of studies have revealed significant reductions in astrocyte cell density and glial fibrillary acidic protein (*Gfap*, an astrocytic marker) across numerous brain regions implicated in neuropsychiatric illness, including prefrontal cortex and hippocampus. A number of studies (including the work outlined in this thesis) has demonstrated the chronic-stress-induced dysregulation of glial-specific transcripts in chronic glucocorticoid treatment and/or chronic stress. Perhaps most convincingly, injections of glial-specific toxins into mPFC in animal models induced depressive-like behavior that was not observed when neuron-specific toxins were used (Banasr & Duman, 2008).

In summary, astrocytes are strongly associated with multiple chronic stress and depressive pathologies and represent an intriguing target. While prior studies have been unable to directly link stress-induced gene expression changes to a specific cell type(s), the recently developed TRAP methodology enables us to obtain biological samples substantially enriched for a targeted cell type of interest. This focus of the present chapter will be to demonstrate the successful implementation of the TRAP method into our laboratory and describe preliminary data of the successful targeting of cells of a specific neurochemical phenotype (e.g. *Gfap* versus *Gad2*). Furthermore, we also describe the generation of preliminary gene expression data derived from mPFC astrocytes in mice subjected to a 14-day CVS paradigm versus non-stressed controls.

MATERIALS AND METHODS

Animals

All preliminary experiments were performed with adult male C57BL/6 mice between 2 and 10 months of age. All CVS animal experiments were performed with adult male $Rosa26^{L10a\text{-}eGFP}$ mice carrying Cre recombinase under the control of either the *Gfap* (astrocytes; $Gfap^{cre}$) or *Gad2* (GABAergic interneuron; $Gad2^{cre}$) promoter. Due to a deleterious phenotype arising from homozygous $Gfap^{cre}$, all mice used in the CVS paradigm (n=12, CVS; n=12, non-stressed controls) possessed a genotype of $Rosa26^{L10a\text{-}eGFP/L10a\text{-}eGFP}$; $Gfap^{cre/+}$.

With the exception of the 14-day CVS paradigm (see below), all animals were group housed with *ad libitum* access to food and water. With the exception of several CVS stressors (see below), all animals were kept on a 12:12 light:dark cycle (6:00 AM to 6:00 PM). All animal procedures were reviewed and approved by the University of Michigan Animal Care and Use Office (UCUCA).

CVS Paradigm

Due to its robust and well-characterized nature, as well as our prior success in deploying it (Chapter III), we opted to employ a slightly modified version of the 14-day CVS paradigm previously described (Herman et al., 1995). Mice were divided into two groups, with one group subjected to the CVS paradigm and one group acting as non-stressed controls (n=12/group). Mice in the CVS group were exposed to two stressors per day (one AM stressor, one PM stressor) which included cold swim (10 minutes at 18 degrees C), warm swim (20 minutes at 31 degrees C), vibrational stress (cages placed

on a plate shaker at maximum speed for 15 minutes), restraint stress (1 hour restraint), solitary housing (overnight duration), removal of bedding (overnight duration), and skipping a dark cycle (lights on overnight). The morning following the final PM stressor, mice were rapidly euthanized via cervical dislocation and brain tissue removed (see ***Tissue Collection – CVS Animals*** below).

Tissue Collection - Preliminary Samples

During preliminary testing of TRAP methodologies, mice were euthanized via rapid decapitation following isoflurane anesthetization. Given the exploratory nature of these efforts we employed less precise anatomical dissections than in our subsequent CVS studies (see below). Brains were removed and placed on a chilled stainless steel block and 70 mg of brain tissue (consisting primarily of neocortex) was excised. These samples were then placed into a pre-chilled 2 mL glass and Teflon homogenizer containing 1 mL ice-cold lysis buffer (20 mM HEPES KOH (pH 7.4), 150 mM KCl, 10 mM $MgCl_2$) containing the equivalent of one-tenth of a cOmplete EDTA-free protease inhibitor tablet (Roche), 0.5 mM DTT, 100 ug/mL cycloheximide and 10 ul each RNAsin (Promega Inc, Madison, WI) and Superasin (Thermo Fisher Scientific, Waltham, MA). Samples were then subjected to TRAP purification (see below).

Tissue Collection - CVS Animals

For CVS and non-stressed control samples, following euthanasia brains were placed into a stainless steel mouse brain matrix that had been pre-chilled on ice. Using a mouse neuroanatomical atlas (Paxinos and Franklin), approximate rostral and caudal

boundaries of mPFC cortex were identified on dorsal surface of the mouse brain. Razor blades were used at these boundaries to generate a coronal “slab” containing whole mPFC. These slabs were then placed on a chilled stainless steel block and further dissected via the removal of dorsal (to prelimbic cortex), ventral (to infralimbic cortex), and lateral (to mPFC) areas. mPFCs were then placed in a pre-chilled 2 mL glass and Teflon homogenizer 1 mL ice-cold lysis buffer (20 mM HEPES KOH (pH 7.4), 150 mM KCl, 10 mM $MgCl_2$) containing equivalent of $1/10^{th}$ one cOmplete EDTA-free protease inhibitor tablet (Roche), 0.5 mM DTT, 100 ug/mL cycloheximide and 10 ul each RNAsin (Promega Inc, Madison, WI) and Superasin (Thermo Fisher Scientific, Waltham, MA). Samples were then homogenized with a Teflon pestle (being careful not to introduce air bubbles) and subjected to TRAP purification. Due to input material requirements, mPFC dissections were “pooled” across mice (n=3 mice/sample), resulting in an effective sample size of 4 per group (n=4, CVS; n=4, control) before proceeding to TRAP purification.

Translating Ribosome Affinity Purification (TRAP)

TRAP methodologies were performed as previously described (Doyle et al, 2008). Affinity matrix was prepared by washing Dynabeads (Streptavidin MyOne T1 Dynabeads, Thermo Fisher Scientific, Waltham, MA) with 1x PBS before incubating with 1x PBS containing biotinylated Protein L for 35 minutes at room temperature. Beads were then collected on a magnet and washed 5 times with 1x PBS containing 3% (by weight) IgG-free bovine serum albumin (BSA) (Jackson ImmunoResearch). Following washes in PBS containing BSA, beads were incubated for 1 hour at room temperature

in 1 mL low salt buffer (20 mM HEPES KOH (pH 7.4), 150 mM KCl, 10 mM $MgCl_2$, 1% NP-40 containing equivalent of $1/10^{th}$ one cOmplete EDTA-free protease inhibitor tablet (Roche), 0.5 mM DTT, 100 ug/mL cycloheximide, 10 ul each RNAsin and Superasin) and 100 ug of eGFP antibody (50 ug each HtzGFP-19C8 and HtzGFP-19F7, Sloan Kettering MACF). After incubation, beads were collected on a magnet and washed 3 times with low salt buffer before being resuspended in the same.

Following tissue homogenization, an aliquot (10% of total homogenized tissue sample) was collected as a whole-tissue control (hereafter referred to as “Total”) and frozen at -80 degrees C. Lysates were subsequently cleared by centrifugation (10 minutes at 2,000 x *g* at 4 degrees C). Supernatant was then transferred to a new pre-chilled 1.5 mL Eppendorf tube and 1/9th sample volumes of 10% NP-40 and 300 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) added. Supernatant was then mixed by gentle inversion, incubated on ice for 5 minutes, and centrifuged (10 minutes at 20,000 x *g* at 4 degrees C). Final supernatant was then combined with affinity matrix and allowed to rotate end-over-end for at least 16 hours at 4 degrees C. Following binding, beads were collected on a magnet and supernatant (hereafter defined as “Depleted sample”) removed to a pre-chilled tube. Beads were then washed 4 times with high salt buffer (20 mM HEPES KOH (7.4), 350 mM KCl, 10 mM $MgCl_2$ containing equivalent of $1/10^{th}$ one cOmplete EDTA-free protease inhibitor tablet (Roche), 0.5 mM DTT, 100 ug/mL cycloheximide, 10 ul each RNAsin and Superasin). After the final wash, high salt buffer was removed and beads were allowed to warm to room temperature. 100 ul of lysis solution (RNAqueous Micro Kit,Thermo Fisher Scientific, Waltham, MA) was added to beads and incubated for 10 minutes at room temperature. Beads were subsequently

collected on a magnet, and the supernatant further purified using the RNAqueous Micro Kit. Immunopurified RNA yield and purity was then assayed on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA).

Reverse Transcription and qPCR Detection

Total RNA (15 ng/sample) was reverse transcribed using SuperScript II (Life Technologies, Carlsbad, CA) with random hexameric priming following manufacturer's instructions. To assay the relative enrichment of immunopurified material, we selected two main markers of neurochemical phenotype: glial fibrillary acidic protein (*Gfap*, a predominantly astrocyte-expressed marker) and glutamic acid decarboxylase 2 (*Gad2*, expressed exclusively within GABAergic interneurons in the CNS). qPCR analyses were subsequently performed using ABsolute Blue qPCR master mix (Thermo Fisher Scientific, Waltham, MA) on a ViiA 7 thermocycler (Life Technologies, Carlsbad, CA). Due to the potential bias for housekeeping genes of interest by individual cell type versus whole brain, fold enrichment was assayed by directly comparing CT values of genes of interest between total lysate and immunopurified material.

Next-Generation RNA Sequencing and Analysis

Immunopurified material was subsequently subjected to next-generation sequencing. Library construction and sequencing were performed by the University of Michigan DNA Sequencing Core (Director: Robert Lyons, Ph.D.). Libraries were generated from 100 ng of immunopurified RNA per sample using the Illumina TruSeq Sample Prep v2 kit (Illumina, San Diego, CA) following manufacturer's instructions. Following library

construction samples were clustered on a cBot (Illumina, San Diego, CA), loaded with 4 samples per lane onto a HiSeq flow cell (Illumina, San Diego, CA), and sequenced on a HiSeq 2000 (Illumina, San Diego, CA) using version 3 reagents according to manufacturer's instructions.

Reads were subsequently aligned and mapped to the mouse genome (UCSC version GRCm38/mm10, Dec. 2011) using TopHat and Bowtie. Individual reads with low transition, >3 mismatches or mapped >10 times to the genome were excluded from analysis. Read counts were generated using standard RPKM normalization (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008).

Immunohistochemistry

To verify the specific expression of the $Rosa26^{L10a\text{-}eGFP}$ construct in our targeted cell type(s) of interest, brains were collected for immunohistochemical staining from $Rosa26^{L10a\text{-}eGFP/L10a\text{-}eGFP}$; $Gfap^{cre/+}$ mice. Mice were deeply anesthetized with sodium pentobarbital then subjected to thoracotamy and transcardial perfusion with 4% paraformaldehyde. Brains were subsequently removed and further fixed for 12 hours in 4% paraformaldehyde at 4C. Following this post-fix treatment, 70 um thick brain sections were cut on a Leica VT 1000 S vibratome and tissue sections collected. Sections containing the mPFC were washed 3x with 1x PBS and blocked for 1 hour in blocking solution (1x PBS containing 3% normal goat serum (Invitrogen) and 0.3% Triton X-100 (Invitrogen) detergent) in 6 well microtiter dishes. Sections were then washed in 1x PBS and incubated overnight at 4 degrees C in blocking solution containing a 1:10,000 dilution of eGFP antibody (AbCam ab13970, AbCam, Cambridge,

United Kingdom) and a 1:1,000 dilution of *Gfap* antibody (Astro6, Thermo Fisher Scientific, Waltham, MA). After incubation, sections were washed 3x in 1x PBS and blocked for 1 hour in block solution containing a 1:1,000 dilution of Alexa Fluor488 (AbCam ab150169, AbCam, Cambridge, United Kingdom) and a 1:1,000 dilution of AlexaFluor 647 secondary (AbCam ab150079, AbCam, Cambridge, United Kingdom). Following incubation, sections were washed in 1x PBS and subsequently mounted onto SuperFrost microscope slides (ThermoFisher Scientific). Sections were imaged on an Olympus Fluoview FV 1000 confocal microscope.

RESULTS

Neuroanatomical expression of L10a-eGFP is dependent on cre recombinase

To ensure our mouse model expressed the L10a-eGFP reporter in our cell type of interest (as defined by the Cre driver), we performed dual fluorescent immunohistochemistry with antibodies against eGFP and *Gfap* in $Rosa26^{L10a\text{-}eGFP}$; $Gfap^{cre/+}$ and $Rosa26^{L10a\text{-}eGFP}$ mice. We subsequently examined mPFC (our area of interest) for colocalization of our markers and observed significant overlap in $Rosa26^{L10a\text{-}eGFP}$; $Gfap^{cre}$ (**Figure 4.1**), indicating the specific expression of eGFP in *Gfap*-positive cells. While we observed significant *Gfap* signal in $Rosa26^{L10a\text{-}eGFP}$ (e.g. wild-type) mice we observed no eGFP signal (data not shown), indicating the cre-dependency of eGFP expression.

TRAP purification of RNA is dependent on Cre recombinase

To test both the quantity of immunopurified RNA obtained, as well as further ensure the cre-dependency of L10a-eGFP expression, we initially performed immunoprecipitations on three different genotypes of mice: Rosa26$^{L10a\text{-}eGFP}$; Gfap$^{cre/+}$, Rosa26$^{L10a\text{-}eGFP}$;Gad2$^{cre/+}$, and Rosa26$^{L10a\text{-}eGFP}$ mice. All mice yielded total RNA in line with expectations (with a general rule of thumb indicating a yield of 1 ug of RNA for each mg of wet brain tissue) (**Table 4.1**). While material immunopurified from Rosa26$^{L10a\text{-}eGFP}$ mice yielded extremely low quantities of RNA (7.4 ng total, or ~0.1 ng RNA per 1 mg of wet tissue), mice expressing cre produced significantly greater quantities of RNA with

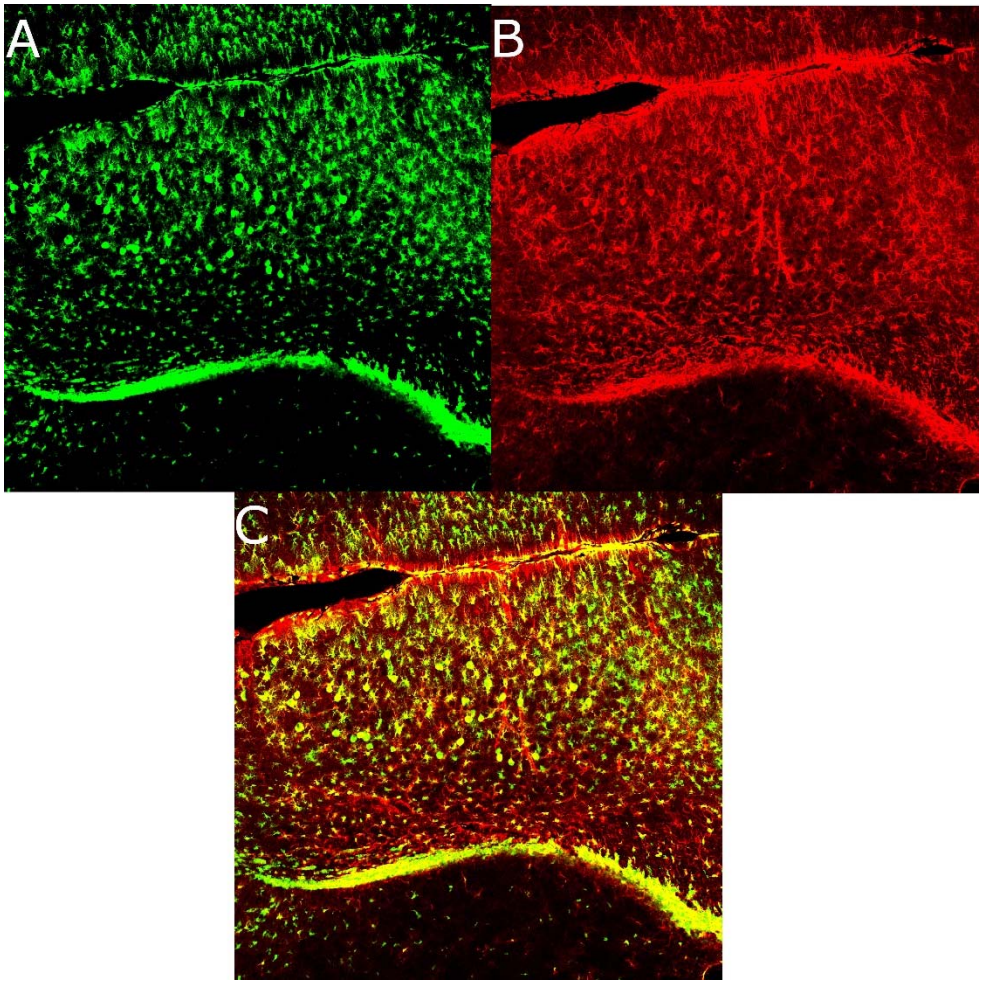

Figure 4.1: Co-localization of eGFP within astrocytes in Rosa26$^{L10a\text{-}eGFP}$; Gfap$^{cre/+}$ mouse. Images represent eGFP signal(**A**), *Gfap* signal (**B**) signal, and merged signal (**C**).

Mouse Genotype	Tissue Input Mass (mg)	Total Input RNA Mass (ng)	Recovered RNA Mass (ng)
Rosa26(eGFP:L10a) (WT Mouse)	70	61335	7.4
Gad2-cre;Rosa26(eGFP:L10a)	70	69650	762
Gfap-cre;Rosa26(eGFP:L10a)	70	68040	540

Table 4.1: RNA yield from TRAP immunopurification is Cre recombinase-dependent. Total RNA yield was genotype-independent and followed expected yield (e.g. 1 µg total RNA per 1 mg wet tissue). In contrast immunopurified RNA yield was Cre-dependent, with both Gad2$^{cre/+}$ and Gfap$^{cre/+}$ yielding a >70-fold increase in recovered material.

Rosa26$^{L10a\text{-}eGFP}$; Gad2$^{cre/+}$ and Rosa26$^{L10a\text{-}eGFP}$; Gfap$^{cre/+}$ mice yielding 762 ng and 540 ng of immunopurified RNA, respectively (**Table 4.1**). Collectively, these data indicate 1) background levels of immunopurified RNA in mice negative for Cre recombinase and 2) >50-fold increases in immunopurified RNA from mice positive for Cre recombinase.

qPCR analysis reveals Cre-driver-specific enrichment of RNA in immunopurified cell types

To test whether the immunopurified RNA samples obtained were consistent with the cre-driver utilized, we measured the expression of *Gad2* and *Gfap* and mRNAs in total RNA versus immunopurified material in both Rosa26$^{L10a\text{-}eGFP}$;Gad2$^{cre/+}$ and Rosa26$^{L10a\text{-}eGFP}$;Gfap$^{cre/+}$. Consistent with genotyping results, Rosa26$^{L10a\text{-}eGFP}$;Gfap^{cre} demonstrated an immunopurified enrichment (~4 fold) in *Gfap* expression compared to total RNA while *Gad2* expression was extremely depleted (<20% that of total RNA) (**Figure 4.2**). Additionally, qPCR analysis of another astrocyte-enriched mRNA (*Aldh1l1*) demonstrated nearly identical cell-type enrichment data as *Gfap* in Rosa26$^{L10a\text{-}eGFP}$;Gfap$^{cre/+}$ mice (data not shown). Similarly, Rosa26$^{L10a\text{-}eGFP}$;Gad2^{cre} exhibited a ~4-fold enrichment in *Gad2* expression from immunopurified material compared to total

RNA, while *Gfap* expression was only ~4% that of total RNA. These data are consistent with a cre-driver-specific expression of L10a-eGFP in, and subsequent sample enrichment for, our targeted cell types of interest.

Preliminary analysis of RNA sequencing results demonstrates robust expression of astrocyte-specific genes

After demonstrating the technical feasibility of the TRAP methodology I proceeded to subject 12 $Rosa26^{L10a\text{-}eGFP}$; $Gfap^{cre}$ mice to the CVS paradigm. An additional 12 $Rosa26^{L10a\text{-}eGFP}$; $Gfap^{cre}$ mice served as non-stressed controls. CVS treatments occurred as previously described (see Chapter III). Following CVS treatments, mPFC dissections were subsequently "pooled" before processing (3 mouse mPFC samples per pool), giving us an effective sample size of n=4/group. Two of these pooled samples (one CVS, one control) yielded quantities of RNA below that ideally useful for next generation sequencing. As a result, we submitted a total of 6 samples to the UM DNA sequencing core for RNAseq library construction and sequencing (n=3/group). Sequencing analysis identified >14,000 individual gene IDs in each sample. Consistent with our expectations, a number of genes previously confirmed to be enriched in astrocytes—particularly glutamine ligase (*GluL*), *Gfap*, and the glial excitatory amino acid transporter 2 (*Slc1a2*)—were among the most robustly expressed of any transcripts identified (**Table 4.2**). Collectively, this data demonstrates a significant enrichment for astrocyte-specific transcripts in our immunopurified material and paves the way for the

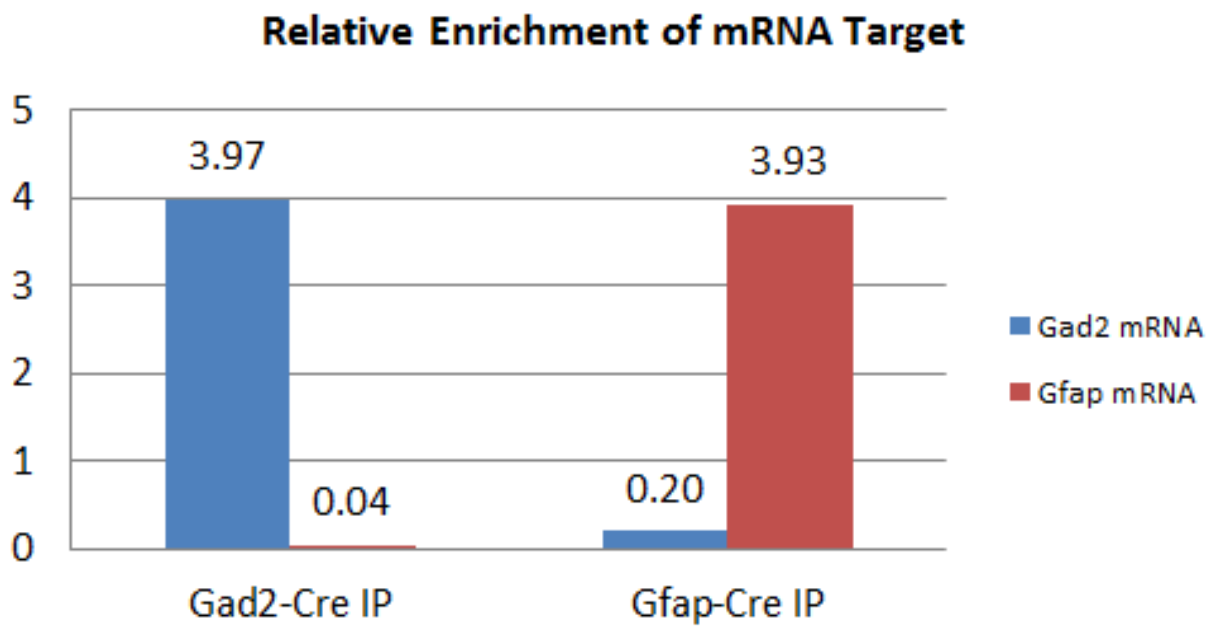

Figure 4.2: Relative proportions of *Gfap* and *Gad2* mRNA in immunopurified material versus total RNA are Cre recombinase-dependent. Both *Gad2* and *Gfap* mRNA levels were enriched ~4-fold in $Gad2^{cre/+}$ and $Gfap^{cre/+}$ animals, respectively (Y-axis represents linear fold enrichment in immunopurified material versus total RNA). In contrast, *Gad2* mRNA levels were depleted in $Gfap^{cre/+}$ mice, while *Gfap* levels were highly depleted in $Gad2^{cre/+}$ (both in immunopurified material versus total RNA).

identification and analysis of gene expression differences specifically within astrocytes.

DISCUSSION

Chronic stress induces a number of physiological alterations and profoundly impacts gene expression. Given the cellular heterogeneity of the brain, understanding the specific impact of chronic stress on gene expression within specific cell types may shed light on the mechanism by which chronic stress induces its maladaptive effects while providing unique insights into how chronic stress impacts specific neural circuits. In the present chapter I present data implementing TRAP methodology, a technique that enables the analysis of gene expression that leverages cre-dependent cell-type driver mice. Further, I have generated preliminary data indicating the successful deployment

of this TRAP methodology to examine stress-induced gene expression changes within *Gfap*-positive cells (e.g. astrocytes) derived from mPFC, positioning me to expand this TRAP methodology to other cell types of interest (such as pyramidal glutamatergic cells and various GABAergic interneurons in mPFC, identified as cell types of interest in earlier chapters). While these initial results are promising there are several limitations to bear in mind going forward. The first is that relatively small sample size used in my RNA sequencing (CVS versus control, n=3/group) may negatively impact our ability to validate prospective genes of interest. Furthermore, while the TRAP methodology is a vast improvement on prior methods, it still exhibits collection artifacts (evidenced by the presence, albeit depleted, of *Gad2* and *Gfap* mRNA in mice expressing *Gfap-cre* and *Gad2-cre*, respectively). It is possible, however, that these artifacts may be further minimized by additional optimization within our laboratory. Efforts to improve yield and minimize potential artifacts in the TRAP methodology are ongoing.

In spite of these limitations, these preliminary findings represent a significant step forward in understanding the transcriptional dynamics of chronic stress within a specific cell type of interest. For example, this preliminary data identifies multiple mRNAs as downregulated following chronic stress but it currently remains unknown whether these mRNA changes are unique to astrocytes *in vivo*. In an attempt to address this question, future studies could compare and contrast my astrocyte gene expression to previously published TRAP results from both astrocytes as well as neurons to identify mRNAs whose expression changes due to chronic stress but are enriched in astrocytes

Gene ID	Expression Rank
Rn18s-rs5	1
Gm12896	2
Glul	3
Gm12895	4
Fam107a	5
Aldoc	6
Slc1a2	7
Ckb	8
Lars2	9
Gm15564	10
Actb	11
Ndrg2	12
Kalrn	13
Ubc	14
Gfap	15

Table 4.2: Rank-ordering of sequencing data reveals astrocyte-enriched transcripts in immunopurified material. Astrocyte-enriched transcripts are amongst the most robustly expressed in material immunopurified from $Rosa26^{L10a\text{-}eGFP/L10a\text{-}eGFP}$;$Gfap^{cre/+}$ mice.

compared to neurons. Such analyses could tentatively identify candidate RNAs that, upon *in situ* hybridization analyses, would reveal both enrichment in astrocytes and stress-dependent expression differences. Future work based on these questions, as well as others raised in this thesis, will make extensive use of these TRAP methods (see Chapter V). Combined with data generated from this present work, these studies are expected to shed light on gene expression within several major cell types in mPFC.

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Chapter V: Thesis Summary and Conclusions

The major goals of the research conducted in this dissertation were to shed light on how the miRNA regulatory network may be altered in AnCg of BP and MDD patients compared to controls and to characterize the impact of chronic stress on transcriptomic networks of mPFC in an animal CVS model. In addition, I have shown preliminary evidence demonstrating the successful deployment of a previously described methodology (e.g. TRAP) that enables analyses of the transcriptomic impact of chronic stress in a cell-type-dependent fashion. The purpose of this final chapter is to summarize the major findings of this work, examine questions raised by these findings and to explore potential next steps for the work described.

Summary of the Major Findings of this Dissertation

An overarching aim of this thesis has been to identify transcriptomic links to depression and chronic stress. The introductory chapter of this thesis began with an overview of bipolar (BP) and major (MDD) depressive illness (Chapter 1.1) and included a section devoted specifically to miRNAs within psychiatric illness (Chapter 1.4). Both BP and MDD are highly prevalent with significant heritability (Craddock & Jones, 1999; Kessler et al., 2005), but our understanding of their underlying genetics is still limited. In recent

years, miRNAs have been strongly implicated in neuropsychiatric illnesses due to their CNS enrichment, direct dysregulation in multiple neuropsychiatric illnesses and the capability of one miRNA to target multiple mRNA species (Selbach et al., 2008). Unlike my study, few prior studies had examined the dysregulation of miRNAs in MDD. Furthermore, the majority of studies examining miRNAs in psychiatric illness within the brain focused on the dorsolateral prefrontal cortex and cerebellum (Bavamian et al., 2015; A. H. Kim et al., 2010; Miller et al., 2012; Moreau et al., 2011; Perkins et al., 2007). Prior to the studies outlined in this thesis no work had examined the miRNA network within the anterior cingulate cortex, a brain region centrally involved in the regulation of mood, emotion and affect (Drevets et al., 2008; Ebert & Ebmeier, 1996; Mayberg et al., 1999; Posner & DiGirolamo, 1998). Given the relevance of both AnCg and miRNAs to mental illness I wanted to query whether miRNAs might be dysregulated in AnCg in BP and/or MDD patients and, furthermore, what mRNA targets they may regulate. As such, I formulated and tested the following hypothesis:

MicroRNAs are dysregulated in various brain regions of patients with various mental illnesses, including MDD and BP. Given its central role in the regulation of mood and affect, miRNAs are likely to a) be differentially expressed in the AnCg of patients with MDD and BP and b) target genes of relevance to pathophysiological processes.

In order to test this hypothesis I measured miRNA expression levels in AnCg from a cohort of BP, MDD and control patients (Chapter II). I initially selected 29 miRNAs based upon prior published association with psychiatric illness, prior literature indicating targeting of mRNAs previously implicated in mental illness, shared dysregulation across multiple psychiatric illnesses and/or robust expression. Following qPCR analysis of

these miRNA transcripts I identified 5 miRNAs with raw p-values < 0.05 (though none passed multiple correction testing, e.g. FDR < 0.15). *miR-132, miR-133a* and *miR-212* were differentially expressed specifically in BP vs control patients, *miR-184* was differentially expressed specifically in MDD vs control, while *miR-34a* was differentially expressed in both illnesses. To minimize the possibility of false positives I performed further qPCR validations with individual miRNA-specific qPCR assays and validated the differential expression of these miRNAs with directionality, magnitude of fold change, and disease specificity mirroring those observed in the initial screen.

Given that miRNAs are thought to largely exert their biological effects via the regulation of target mRNAs I next sought to query potential mRNA targets of miRNAs dysregulated in disease. A particular emphasis was placed on *miR-34a* given its shared dysregulation across both BP and MDD cohorts. I subsequently performed gene ontology and clustering analyses of all *in silico* predicted mRNA targets of *miR-34a*. Based on the two highest associations provided from this analysis—'Synapse' (Cluster 1) and 'Transcription Regulator Activity' (Cluster 2) (see **Supp. Table 2.3,** p. 94)—three mRNA candidates were chosen for subsequent validations: *PDE4B, NCOA1* and *NCOR2*. Prior work has shown that *PDE4B* is a genetic risk factor for mental illness (Fatemi et al., 2008; Millar et al., 2005; Numata et al., 2009; Pickard et al., 2007), and the protein product is enriched at the synapse (Bradshaw et al., 2008; Millar et al., 2005). *NCOA1* and *NCOR2* have previously been demonstrated to be glucocorticoid receptor co-factors and modulate its transcriptional activity (van der Laan et al., 2008). For example, *NCOA1* is necessary for regulating stress-induced corticotropin releasing hormone (a

major component of the HPA axis stress response, see Chapter I) (Lachize et al., 2009). Additionally, mice not expressing *NCOA1* exhibited increased HPA axis sensitivity (Winnay et al., 2006). Finally, both *NCOR2* and *PDE4B* were predicted to be targeted by *miR-184*, a miRNA differentially expressed in the MDD cohort as compared to controls.

To further query whether these mRNA/miRNA interactions occur directly, I constructed reporter constructs consisting of firefly luciferase upstream of the 3' UTR of these genes of interest (*NCOA1, NCOR2,* and *PDE4B*) and transfected them into HEK293 cells with either miRNA mimics or saline solution (negative control). Constructs that showed initial repression of luciferase activity upon treatment with miRNA mimic compared to saline were then mutagenized at their prospective miRNA binding site(s) and employed in luciferase assays to assess the sequence specific dependency of miRNA/mRNA interactions. From these analyses I validated *NCOA1* as regulated by *miR-34a*, *NCOR2* as regulated by *miR-184*, and *PDE4B* as regulated by both *miR-34a* and *miR-184* (see **Figure 2.2**, Chapter II). Combined, these data indicated the convergence of miRNAs dysregulated in illness on both an individual gene (e.g. *PDE4B*) and on a biological process linked to both chronic stress and depression (e.g. glucocorticoid signaling, via *NCOA1* and *NCOR2*).

As I identified several miRNAs that varied in MDD and/or BP cohorts compared to control patients, and in combination with prior research suggesting that miRNAs act primarily via modulation of mRNA levels and/or translational repression, I sought to determine if the steady-state levels of these validated mRNA targets were altered in

MDD and BP patient cohorts. Employing the same cohort of samples used in my miRNA expression analyses, I examined the steady-state levels of *NCOA1, NCOR2* and *PDE4B* mRNAs using TaqMan-based qPCR. I observed a significant *decrease* in *NCOR2* mRNA levels specifically in our MDD cohort, whereas I observed no significant change in steady-state *PDE4B* mRNA levels in either BP or MDD patients. In contrast, and consistent with canonical miRNA-induced repression of gene expression, I observed a significant *increase* in *NCOA1* mRNA expression specific to the BP cohort. While I am encouraged by these miRNA and mRNA findings, my work is not without caveats. Though I have validated several miRNA/mRNA interactions, there is no present evidence demonstrating co-expression of miRNAs dysregulated in disease with their validated mRNA targets. Future human neuroanatomical studies will be required to examine the cellular localization of these microRNAs and their mRNA targets. Additionally, while I have demonstrated the differential expression of several mRNA transcripts, I do not have protein expression data within this cohort of patients (though prior literature has demonstrated increased *PDE4B* protein levels in the cingulate cortex of MDD patients (Yuan et al., 2011). As such, while future studies will be needed to address both of these limitations, the results of the present study are consistent with the miRNA regulation of several transcripts implicated in neuropsychiatric illness.

To summarize: a majority of miRNAs are expressed within brain regulate key CNS processes, have a “one-to-many” relationship with their mRNA targets, and are hypothesized to play a key role in mood and affective disorders. While we note several caveats in our findings, the work performed in this thesis sheds light on miRNA dysregulation in AnCg (a brain region centrally involved in mood and cognition) as well

as mRNA targets of these dysregulated miRNAs. Additionally, this work identifies *miR-34a* as a miRNA of particular interest given 1) its dysregulation across multiple psychiatric illnesses, 2) its dysregulation in multiple cortical regions linked to psychiatric illness, and 3) its regulation of a large number of mRNA targets implicated in these disorders.

As these initial human studies progressed I recognized that studies in human samples are often confounded by both the diversity of clinical depressive phenotypes and individual (e.g. patient-to-patient) variability. As we wished to move towards examining more mechanistic hypotheses, and this variability makes probing mechanistic questions difficult, we elected to employ an animal model of chronic stress to examine stress-induced transcriptomic alterations in a brain-region-specific fashion. Chronic stress is a major precipitating factor for neuropsychiatric illness. Chronic stress also induces persistent hypercortisolemia (increased circulating glucocorticoids) and, as glucocorticoids are powerful mediators of gene transcription, influences widespread changes in gene expression. Additionally, there is a growing consensus in the field that human depression, as well as depressive-like behavior in animal models of chronic stress, may represent the summation of (mal)adaptive responses to chronic stress. One such maladaptive response is reduced activity and function in the mPFC, a brain region central to regulating HPA axis activity, stress responses and emotionality (Arnsten, 2009; Joormann & Gotlib, 2010). Given that gene expression changes likely underlie maladaptive responses to chronic stress (including mPFC hypofunction) I wished to test the following hypothesis:

Chronic stress is a major precipitant of mental illness and is a powerful modulator of gene expression. Given the mPFC is a central regulator of stress responses (and is a particularly stress-sensitive brain region), chronic stress will result in widespread gene expression changes consistent with inhibited mPFC function.

To test this hypothesis I employed RNA sequencing analyses of RNA samples generated from mPFC of mice subjected to a chronic variable stress protocol as well as non-stressed controls (Chapter III). I identified >15,000 unique gene IDs per sample, with a substantial number (~1,800) of these identified as putatively differentially regulated in CVS mice versus non-stressed controls (linear fold-change > 1.3, raw p-value < 0.05). I subsequently performed gene ontology and clustering analyses of differentially expressed transcripts and focused on CNS-specific categories (specifically, 'Neuron development,' 'Neuron projection development,' and, 'Synaptic Transmission'). Based on these criteria I chose ~60 mRNA targets to validate using high-throughput qPCR methodologies and, following expression analyses, validated 31 targets as differentially expressed in CVS versus control animals. Additionally, given growing evidence that miRNAs play a role in regulating HPA axis activity, stress responses, mood and anxiety (Bahi et al., 2014; Haramati et al., 2011; Honda et al., 2013; Katsuura et al., 2012; Muinos-Gimeno et al., 2011; Vreugdenhil et al., 2009), I examined miRNA expression in the mPFC of this same cohort of mice. I performed an initial screen of >300 miRNAs using high-throughput qPCR methodologies and identified 14 miRNAs as putatively differentially expressed (see **Figure 3.2**, Chapter III). Following more stringent validations employing individual assays, 4 miRNAs validated as regulated by stress:

miR-187, *298*, *320* and *666-5p (*see **Figure 3.2**, Chapter III*)*. Combined, these data indicate the dysregulation of a large number of mRNA and microRNA transcripts as a function of chronic stress with many validated transcripts linked to neuronal processes. Given the large number of transcripts that validated as stress-regulated, I next sought to contextualize these changes within the impact of chronic stress on mPFC physiology. Prior findings have demonstrated a large number of neuronal- and synaptic related pathologies, with the glutamatergic system gaining increasing prominence as a key player in maladaptive responses to stress (Sanacora et al., 2012). By employing bioinformatics pathway analysis tools and literature syntheses I characterized a prospective 'interactome' of our stress-regulated mRNA transcripts with an eye towards their impact on mPFC function (see **Figure 3.4**, Chapter III). From this analysis, the oncogene *Rac1* (a regulator of glutamatergic spine size and a downstream effector of the stress-regulated psychiatric risk factor *Disc1* (Hayashi-Takagi et al., 2010)) emerged as a putative target of the stress-regulated *miR-187*. I subsequently validated this miRNA/mRNA interaction in vitro and integrated it as a component of our mechanistic hypothesis

While the results of these transcriptomic and "interactomic" studies are intriguing, I urge caution and note that a number of future studies (**see below**) will be necessary to further assess the prospective cellular, biological and functional impact(s) of stress-induced transcriptomic alterations. Specifically, I note that these changes were observed at the RNA level and—as protein levels may not correlate with RNA levels—commensurate alterations at the protein level may not be observed. Furthermore I note that, even if protein levels were observed to change commensurate with my hypothesis,

further experiments would be required to determine whether these were accompanied by a functional response. In spite of these caveats, I note that this “interactome” is consistent with both my findings and current literature. Taken together, my hypothesis presents a starting point for examining the underlying genomic mechanisms that may influence hypofunction in mPFC in a cell-type-dependent fashion.

Finally, prior literature has established that two mPFC subregions—the IL and PL subdivisions—have previously been linked to regulation of stress responses and HPA axis activity (Jones et al., 2011; Radley et al., 2006; Sullivan & Gratton, 1999; Tavares et al., 2009). Of particular note, IL and PL cortices appear to have roughly opposite effects on HPA axis function: IL cortical activity appears to be necessary for the initiation of HPA axis responses, while stimulation of PL cortex inhibits HPA axis response specifically to psychogenic (but not physical) insults (Jones et al., 2011). Given these prior observations I sought to identify whether a subset of my validated mRNA transcripts demonstrated subregional-specific differential expression. To address this question, I performed laser-capture microdissection of IL and PL subregions in an independent cohort of mice subjected to 14 days of CVS and non-stressed control mice. Following qPCR expression analyses of 4 examined transcripts, 3 validated (*Gad2, Rgs4* and *Tnik*) with directionality and magnitude of fold expression consistent with the initial cohort. While *Rgs4* demonstrated differential expression in both PL and IL, *Gad2* and *Tnik* were differentially expressed specifically within PL cortex. While *Adcyap1r1* did not validate in either brain region it is possible that it may 1) be regulated in another brain region or 2) its initial significance was due to contamination of mPFC by unrelated brain regions. While the number of transcripts examined is relatively small, these

findings do indicate the subregion-specific dysregulation of several stress-regulated transcripts and are consistent with differential impacts of chronic stress on IL and PL regions.

Combined, these data indicate widespread gene expression changes in mPFC following chronic stress. Additionally, integrating these findings with bioinformatics analyses and prior literature indicates these changes may be part of a mechanistic framework for how gene expression changes could underlie stress-induced glutamatergic mPFC hypofunction (Chapter III). Finally, this work provides the first large-scale analysis of the transcriptional impacts of chronic stress in mPFC as well as the first empirical evidence of differential gene expression by subregion in a mouse CVS model.

Further Questions Prompted By This Work and Future Directions

The findings obtained from this dissertation research shed further light on transcriptomic alterations in both human neuropsychiatric illness and animal models of chronic stress. This thesis research hypothesizes a cell-type-specific basis for a subset of stress-regulated transcripts. Finally, this work outlines preliminary work employing an immunopurification model that will enable the acquisition of relevant biological samples from targeted cell type(s) of interest that is expected to be instrumental in assessing the cell-type-specific stress-regulated mPFC model hypothesized. While our group's focus on overlaying stress-induced gene expression changes on specific components of the neural circuitry has had preliminary success, it raises a number of intriguing questions that will need to be addressed by future studies. A subset of prospective future directions are discussed below:

What component(s) of the neural circuitry (e.g. cell type) drive dysregulation of miRNAs with altered expression in disease and disease-like states?

As we have noted elsewhere the brain is extremely heterogeneous in both function and in its multitude of cell types. mRNA expression can vary widely from one brain region to another and this heterogeneity is known to extend to specific cell types. While cell-type "markers" are commonly employed to identify a specific cell (sub)type, identifying the relative expression of RNA transcripts not confined to a single cell type has proven more challenging. Similarly, a recent article demonstrated the enrichment of a large number of miRNAs by neuronal cell type using a technique similar to the TRAP methodology for miRNAs (He et al., 2012). While results in this thesis implicate specific miRNAs as regulators of a subset of miRNA targets, these studies were conducted in an *in vitro* model system (e.g. HEK293 cells) engineered to express both miRNAs and mRNA targets together. At present, we lack *in vivo* evidence attesting to the co-expression of miRNAs with their validated mRNA targets (a necessary prerequisite for these interactions to impact cellular physiology).

A potential way to address this would be to employ dual *in situ* hybridization (ISH) approaches using radioactively tagged nucleic acid probes directed against miRNAs of interest and colorimetric nucleic acid probes directed against mRNA targets. These methods have the advantage of providing relative quantification of their targets via silver-grain counting (e.g. autoradiographic) methods and would provide strong evidence of co-expression within the same cell type. Similar methods have been previously employed (though only via colocalization of mRNAs, not mRNAs and

miRNAs) (Curran & Watson, 1995), although several technical considerations compromise an effort to deploy this method (specifically, the relatively low signal strength and half-life of the gamma-P33 labeled probes combined with the relative lack of abundance of our targeted miRNAs). To address these concerns I attempted to leverage *in vitro* transcription (IVT) methods and S^{35}-labeled UTP to generate our radioactive nucleic acid probes for miRNAs (which would, in theory, give us greater signal strength (e.g. greater specific activity) with a much longer half-life). This method, however, proved less than satisfactory: while I was able to demonstrate similar expression profiles for a number of well-characterized miRNAs using our IVT method, negative controls consistently exhibited extremely high and non-specific background signal. Despite multiple attempts to optimize this protocol I was unable to reduce nonspecific signal to an acceptable level.

Another way I might approach this question is to use fluorescent *in situ* hybridization (FISH) methodologies, with one probe directed against the miRNA of interest and the other directed against the mRNA target. Subsequent imaging analyses would be sufficient to identify whether the miRNA/mRNA pair colocalize and similar methods have been successfully employed in prior work (Deo, Yu, Chung, Tippens, & Turner, 2006; Wu, Piccini, & Singh, 2014). While these methods do not provide the quantification abilities of radioactive ISH approaches they do provide an effective method to query the neuroanatomy of miRNA expression and assay whether miRNAs of interest and their mRNA targets overlap. This would enable us to make stronger hypothesis on the particular function of an individual miRNA and avoid potential “false positive” interactions when attempting to ascertain biologically relevant miRNA functions (e.g.

when a miRNA may regulate an mRNA target *in vitro*, but this interaction is not biologically applicable).

An additional way we might query the specific cell type(s) driving miRNA dysregulation in disease- and disease-like states is to employ the miRNA tagging and affinity purification (miRAP) methodology established by He et al. (He et al., 2012). By employing mice carrying a cre-inducible argonaute 2 construct (*Ago2*, a core protein in the RNA-induced silencing complex (J. Liu et al., 2004)) fused to a *Myc* and eGFP tag, this methodology enables the immunopurification of miRNAs from specific cell types (specified by Cre driver). These studies leverage our laboratory's current expertise with RNA immunopurification methodologies and would serve to complement both the aforementioned *in situ* hybridization studies and TRAP methodologies.

How does chronic stress impact gene expression in neuronal (as opposed to glial) cell types?

As mentioned in Chapter I, stress affects gene expression in nearly every organ system, tissue and cell type. This is particularly true in the CNS, where significant morphological and physiological changes have been observed across the brain in both neurons and glia following both chronic stress and glucocorticoid treatment. Prior work in our laboratory has demonstrated both the *in vitro* and *in vivo* regulation of multiple astrocyte-enriched transcripts by chronic glucocorticoid treatment (Carter et al., 2013; Carter et al., 2012). Furthermore, preliminary data performed as part of this dissertation (Chapter IV) has indicated the stress regulation of numerous mRNA transcripts specifically within astrocytes that were NOT identified as stress regulated in our whole-

mPFC data. Together, this data indicates a cell-type-dependent impact of chronic stress on gene expression. This observation, then, prompts a major question: how does chronic stress impact gene expression in neuronal cell types?

One possible way to examine this would be to employ the previously described TRAP methodology ((Doyle et al., 2008; Heiman et al., 2008); see Chapter IV) using mice that express Cre recombinase under the control of neuron-specific promoters. We would be particularly interested in examining the transcriptomic impact of chronic stress in mPFC within the context of glutamatergic pyramidal cells (labeled by *Camk2a*) and GABAergic interneurons (labeled predominantly by *Gad2*) as these cell types have 1) been implicated in both maladaptive responses to chronic stress and human neuropsychiatric illness and 2) represent the predominant drivers of excitatory and inhibitory tone within mPFC. Furthermore, this approach would allow us to further test our 'glutamate-centric' hypothesis for the transcriptomic changes we observed in whole-mPFC analyses (see Chapter 3). These experiments would also provide us with unbiased gene expression analyses of basal expression and stress-regulation from each cell type (astrocyte, glutamatergic neuron and GABAergic neuron) of the canonical "tripartite synapse" within mPFC. We note that recent work by Tasic et al. focused on single-cell transcriptomics and has indicated there are likely many different glutamatergic neurons (Tasic et al., 2016) in addition to the established heterogeneity of GABAergic interneurons (Rudy et al., 2011). As such, whether all—or only a subset—of these neuronal subtypes regulate mPFC function in stress remains in open question. In spite of these challenges, however, these proposed studies would provide a potentially valuable 'atlas' of gene

expression changes at the RNA level that could guide future functional and mechanistic studies.

What are the functional consequences of stimulation and/or inhibition of specific cell types within the mPFC?

The overarching theme of this dissertation has been to examine large-scale transcriptomic events occurring in either human neuropsychiatric disorders or animal models of chronic stress. In the course of this latter aim we have described our rationale for investigating the mPFC and the potential relevance of its hypofunction following chronic stress. Missing from this work, however, has been the functional assessment of the impact of mPFC hypofunction on depressive-like behavior.

Several prior studies have suggested that this may be a profitable line of inquiry. A high-impact paper published in 2005 from Mayberg et al. (Mayberg et al., 2005) revealed that deep brain stimulation of subgenual cingulate cortex yielded therapeutic responses for treatment-resistant depression, indicating that modulation of activity in a specific brain region may be sufficient to relieve many symptoms of depression. A recent study by Covington et al. observed the reduction of multiple immediate early genes (IEGs) following chronic social defeat stress (CSDS) and demonstrated that optogenetic stimulation of whole mPFC was sufficient to not only restore expression of these IEGs but also induce a robust antidepressant-like effect in mice subjected to CSDS (Covington et al., 2010). A more recent study by Kumar et al. revealed that the specific optogenetic stimulation of layer-V PL pyramidal cells was sufficient to induce an antidepressant-like phenotype in mice subjected to a forced swim test as well as having

anxiolytic effects (though no antidepressant-like effects of optogenetic stimulation of PL pyramidal cells following CSDS were observed) (Kumar et al., 2013).

A logical extension of this published work, as well as my own, would be to employ the CVS paradigm with optogenetic stimulation specifically within pyramidal neurons (not only layer-V) in PL and IL subregions. This approach would allow me to assess whether activation of either subregion following chronic stress is sufficient to induce an antidepressant-like phenotype. An alternate (although potentially less clear) approach may be to perform optogenetic stimulation concurrent with the CVS paradigm, the rationale being to identify whether preventing the acquisition of mPFC hypofunction via optogenetic stimulation is sufficient to prevent the occurrence of CVS-induced depressive like behavior. Another way to approach this question would be to employ optogenetic inhibition of PL and IL function within mPFC. By using halorhodopsin, rather than channelrhodopsin, I would be able to induce mPFC hypofunction in the absence of stress and assay whether mPFC hypofunction is sufficient to induce (or render mice more susceptible to developing) depressive-like behavior.

A major benefit to these approaches is that they are theoretically capable of generating both transcriptomic and functional data from the same cohort of mice. It would be possible to generate mice carrying 1) Cre recombinase under the control of the *Camk2a* promoter and 2) the Cre-inducible L10a-eGFP ribosomal fusion protein, restricting the expression of this construct to pyramidal cells. We would then be able to inject these same mice with a virus expressing a Cre-inducible channelrhodopsin injected into PL or IL. Following optogenetic stimulation (or inhibition), we could then perform behavioral assays and subsequently identify transcripts regulated by stimulation/inhibition

specifically from mPFC subregion. While we note the need for feasibility studies (particularly to ensure that targeting a specific cell type in a specific subregion with several genetic manipulations does not affect baseline behavior), we have begun implementing optogenetics methodologies into our laboratory in pursuit of these questions. At present, we are currently collecting preliminary data on the co-expression of eGFP and mCherry-tagged channelrhodopsin in mPFC of $Rosa26^{L10a\text{-}eGFP/L10a\text{-}eGFP}$; $Gad2^{cre/cre}$ mice in preparation for undertaking of these experiments. While currently speculative, these findings may have clinical relevance for human depressive patients given the strong shared pathologies (e.g. HPA axis dysfunction, glutamatergic dysfunction and mPFC hypofunction) between chronic stress and mood disorders and may enable the identification of prospective therapeutic targets.

Synopsis of Dissertation Findings

- 3 miRNAs in a bipolar disorder (BP) cohort (*miR-132, miR-133a* and *miR-212*), 1 in a major depressive disorder (MDD) cohort (*miR-184*) and 1 in both BP and MDD cohorts (*miR-34a*) are significantly repressed compared to control patients.
- Several mRNAs linked to psychiatric illness are regulated by these microRNAs in an *in vitro* assay: *miR-34a* regulates *NCOA1*, *miR-184* regulates *NCOR2*, while both *miR-184* and *miR-34a* regulate *PDE4B*.
- The steady state levels of *NCOA1* and *NCOR2* mRNA are altered in BP and MDD cohorts, respectively.
- Exposure to the chronic variable stress (CVS) paradigm results in the differential expression of a large number of mRNA transcripts (assayed by RNA sequencing).

- Identified 4 miRNA and >30 mRNA transcripts as CVS-regulated with a subset of mRNA transcripts being regulated in a prelimbic cortex (PL)-specific manner.
- Identified *Rac1* as regulated by the stress-regulated *miR-187*.
- Generated a mechanistic hypothesis/“interactome” of CVS-regulated transcripts consistent with glutamatergic hypofunction in mPFC (based on bioinformatics analyses and literature analysis).
- Performed feasibility analysis of using translating ribosomal affinity purification (TRAP) methods to immunopurify RNA specifically from astrocytes and GABAergic interneurons.
- Employed TRAP methods to immunopurify RNA specifically from mPFC astrocytes in mice subjected to CVS versus non-stressed controls.

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