The Effects of Stress on Mouse Prefrontal Cortex microRNA Expression

By

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Abstract

Chronic stress is associated with the onset of a variety of psychological disorders, including major depressive disorder (MDD). Not all who experience stress become depressed, however, and a genetic predisposition is characteristic of those that are susceptible. Psychological stress also has an impact on synaptic plasticity, affecting dendritic arborization and protein levels at the synapse. The current work examines changes in expression of microRNAs (miRNAs), a group of small, non-coding RNA molecules, following exposure to acute or chronic stress in two strains of mice with different genetic backgrounds that model susceptibility to stress-related disorders. miRNAs are post-transcriptional regulators of gene expression that inhibit effective protein synthesis via translational interference. miRNAs collectively target the mRNAs of the majority of protein-coding genes, including genes involved in synaptic plasticity, and receptors central to the neuroendocrine response such as the glucocorticoid receptor (GR). Given that stressors alter synaptic plasticity, we hypothesize that microRNAs may mediate stressor-induced changes. The current study examines expression changes of specific candidate microRNA genes in the prefrontal cortex (PFC) in response to either three weeks of chronic, unpredictable stress or brief exposure to an acute, social stressor in stressor-reactive BALB/c and stressor-resilient C57BL/6 mice. Real time-polymerase chain reaction (RT-PCR) was used to examine stress effects on the PFC, and a significant decrease in miR-132 expression was observed following acute stress. Illustrating miRNA changes that occur will contribute to uncovering the underlying molecular effects of stress and future studies should examine expression of miR-132 targets after psychological stress.

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List of Abbreviations

5-HIAA – 5-hydroxyindoleacetic acid
5-HT – serotonin
5-HT1A – serotonin 1A receptor
5-HTT – serotonin transporter
ACC – Animal Care Committee
ACTH - Adrenocorticotropic hormone
ADHD – attention-deficit/hyperactivity disorder
AP-1 – activating protein-1
AVP – arginine vasopressin
BDNF – brain-derived neurotrophic factor
BSA – bovine serum albumin
CB1 – cannabinoid receptor type 1
CCAC – Canadian Council on Animal Care
CeA – central amygdale
CG1 – cingulate cortex 1
CG2 – cingulate cortex 2
CRH – corticotrophin-releasing hormone
CRHR1 – corticotrophin-releasing hormone receptor 1
CSF – cerebrospinal fluid
Ct – cycle threshold
CUS – chronic unpredictable stress
DA – dopamine
Daam1 – disheveled associated activator of morphogenesis 1
DDT – dithiothreitol
DEPC – diethylpyrocarbonate
DSM-IV-TR – Diagnostic and Statistical Manual, 4th ed. Text Revised
EPM – elevated plus maze
Erk1/2 – extracellular-signal-regulated kinase 1/2
GABA – γ-aminobutyric acid
$GABA_AR$ – γ-aminobutyric acid type A receptor
GAD – generalized anxiety disorder
GILZ – glucocorticoid-induced leucine zipper
GOI – gene of interest
GR – glucocorticoid receptor
Gstp2 – glutathionine S-transferase, pi2
GxE – gene-environment interactions
HPA - hypothalamic-pituitary-adrenal
IL-6 – interleukin-6
Mapk1 – mitogen-activated protein kinase 1
MAP2 – microtubule-associated protein 2
MDD – major depressive disorder
MeCP2 – methyl CpG binding protein 2

miRNA – microRNA
miRNP - miRNA-RiboNucleoProtein complex
miTG – miRNA targeted genes
mPFC – medial prefrontal cortex
MR – mineralocorticoid receptor
MRE – miRNA recognition element
mRNA – messenger RNA
OF – modified open field
PFC – prefrontal cortex
PTSD – post-traumatic stress disorder
PPF – paired-pulse facilitation
PV – paraventricular thalamic nucleus
PVN – paraventricular nucleus
RT-PCR – real time-polymerase chain reaction
PCR – polymerase chain reaction
SDS – sodium dodecyl sulphate
SSC – saline-sodium citrate
SSRI – selective serotonin reuptake inhibitor
TH – tyrosine hydroxylase
UCMS – unpredictable, chronic mild stress
UTR – un-translated region
VTA – ventral tegmental area

1.0 Introduction

1.1 Stress and the HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis is central to the stress response in mammals. A stressor in the environment causes the paraventricular nucleus (PVN) to release corticotrophin-releasing hormone (CRH), which in turn triggers the release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH travels through the body to the adrenal cortices, from which the glucocorticoid hormones are released, including cortisol in humans and corticosterone in rodents. The HPA axis maintains homeostasis via negative feedback loops, in which cortisol binds to high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid receptor (GR) in the hypothalamus and pituitary gland, resulting in decreased CRH and ACTH release, and therefore reduced cortisol. Following exposure to chronic or repeated stressors, however, HPA homeostasis can be disrupted with pathological consequences. Indeed, disrupted homeostasis is a characteristic symptom of depression.

1.2 Depression

Major depressive disorder (MDD) is a pervasive mental illness which affects 16.2% of people in the United States at least once in their lifetime (Kessler et al., 2003). Clinical diagnosis is based upon the presence of single or recurrent major depressive episodes, which are defined by symptoms such as depressed mood, anhedonia (or loss of interest in daily activities), appetite changes, sleep changes, and motor activity changes (American Psychological Association [DSM-IV-TR], 2000). A risk of developing depression has been found when subjects

are exposed to constant adverse conditions such as poverty, absence of social support and chronic marital stress (Hammen, 2005). Hypersecretion of corticosteroids increases the risk of developing depression, and can also increase abdominal obesity, prevalence of cardiovascular problems and osteoporosis (Brown et al., 2004). HPA hyperactivity is also displayed in healthy, first-degree relatives of those with depression, suggesting that the increased activity of the HPA axis characterizes not only those afflicted with depression but also those at higher risk (Modell et al., 1998). Biologically, those with MDD have increased levels of cortisol in blood, urine and cerebrospinal fluid (CSF), an exaggerated cortisol response to ACTH, and enlarged pituitary and adrenal glands, indicating HPA dysregulation (Monteleone, 2001). This dysregulation has been attributed to "glucocorticoid resistance", or altered negative feedback inhibition by glucocorticoids (Juruena et al., 2006).

Depression is also associated with imbalances or deficiencies in neurotransmitters called monoamines, notably serotonin (5-HT), norepinephrine, and dopamine (DA). Certain antidepressant medications, such as the selective serotonin reuptake inhibitors (SSRIs) for example, target the serotonergic system and elevate levels of serotonin available to bind post-synaptic receptors. The mood stabilizing effects elicited by these drugs are delayed, however, and it is now being postulated that lasting changes in gene expression, and not solely neurotransmitter levels at the synaptic cleft, may be responsible for antidepressant effectiveness (Lee et al., 2010).

1.3 Gene-Environment Interactions

A paramount theory regarding psychological disorders today is the diathesis-stress model, which declares that behaviour is a consequence of an interaction between genetic

factors and the environment. In the current context, it maintains that certain individuals have genetic predispositions to the pathological effects of stress. Many disorders have been proposed to be a result of gene-environment interactions (GxE), including depressive disorders, attention-deficit/hyperactivity disorder (ADHD), schizophrenia, and substance abuse disorders. A landmark study in this realm was that of Caspi and colleagues, who reported that a polymorphism in the promoter region of the gene encoding the serotonin transporter (5-HTT) moderated the effects of stressful life events on the development of depression (Caspi et al., 2003). Individuals with one or two copies of the short allele of the 5-HTT promoter polymorphism displayed more diagnosable depression than those with no copies of the short allele. In other words, stressful life events lead to depression but only if an individual has a susceptible genotype. The Caspi finding has been reiterated by recent studies. For example, a 2009 study determined that first episode depression was linked to an interaction between polymorphisms in genes encoding for both brain-derived neurotrophic factor (BDNF) and 5-HTT and stressful life events (Bukh et al., 2009). In 2010, upon investigation of 346 youth with varying polymorphisms in the 5-HTT gene, chronic family stress reported at age 15 corresponded to higher Beck Depression Inventory-II scores at age 20 in individuals with one or two short alleles in comparison to those with no short alleles, while acute stress did not interact with genes to result in depression (Hammen et al., 2010). Other studies have uncovered potential gene-environment interactions underlying cortisol release. In a non-clinical sample of humans, a significant history of stressful life events and homozygosity of the 5-HTT short allele predicted elevated cortisol secretions in response to a public-speaking stressor – even though this sample did not include depressed individuals (Alexander et al., 2009). A similar study

examined cortisol responses to a public speaking task in which the three potential audiences were manipulated: a critical evaluative audience, a supportive evaluative audience or no audience. The polymorphism in the promoter region of the gene for 5-HT-T yielding two copies of the short allele was related to a greater cortisol response to social evaluation (Way & Taylor, 2010). Given the hypercortisolemia associated with clinical depression, it is of note that both elevated cortisol and depression are independently associated with polymorphisms within the serotonin transporter gene.

As another example of how genes and environment interact to result in pathology, polymorphisms in the genes for corticotrophin-releasing hormone receptor 1 (CRHR1) and FKBP5 are found to influence the incidence of developing depressive and post-traumatic stress symptoms when exposed to early life trauma (Gillespie et al., 2009). CRHR1 and depressive symptoms are also linked in that a haplotype in CRHR1 protected against development of symptoms in individuals exposed to childhood maltreatment (Polanczyk et al., 2009). In order to study gene-environment interactions in animal models, specifically how those with different genetic backgrounds react to environmental stress, inbred mice strains that are known to differ in their susceptibility to stress can be utilized.

1.4 An Animal Model to investigate Gene-Environment Interactions

BALB/c mice and C57BL/6 mouse strains are commonly used to study gene-environment interactions as both are genetically homogeneous within their strain, but vary genetically and phenotypically between strains (Tarantino et al., 2011). The finding that BALB/c mice display higher levels of anxiety than mice of many other strains has been well documented, and some authors have suggested that this strain, when compared with other commonly used inbred

strains such as C57BL/6 & DBA, may be a realistic model of anxiety due to abnormalities in various neurotransmitter systems (Belzung & Griebel, 2001). When comparing behaviours with other commonly used mouse strains (DBA/2J and CB6F1/6J), C57BL/6 are considered the least anxious while BALB/c are considered the most anxious, as evident by a battery of tests such as the open field test and the elevated zero maze test (Tang et al., 2002). A similar study, utilizing wild-type, BALB/c and C57BL/6 mice, found that BALB/c mice avoided risk areas and showed high risk assessment, while C57BL/6 showed less risk assessment and more explorative behaviours, in the concentric square field, a modified open field (OF) and an elevated plus maze (EPM) (Augustsson & Meyerson, 2004). A study employing a modified hole board test yielded similar results, with C57BL/6 displaying more locomotion and exploratory movement than BALB/c mice, behaviours which typically indicate that animals are comfortable in their environment (Ohl et al., 2001). Lastly, when investigating anxiety and depression-like behaviours, entries into the open arm of the EPM were increased in BALB/c mice exposed to unpredictable, chronic mild stress (UCMS) in comparison to controls. The UCMS-induced increase was not apparent in the C57BL/6 strain, suggesting BALB/c behaviour is more influenced by stress (Mineur et al., 2006).

With regards to physiological differences between strains, a 2010 study reported restraint-induced c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN), hippocampus, prefrontal cortex (PFC) and paraventricular thalamic nucleus (PV) of both the BALB/c and C57BL/6 strains, but with C57BL/6 mice having a greater increase in c-Fos expression in the cingulate cortex 1 (CG1) and cingulate cortex 2 (CG2) regions of the PFC than high anxiety BALB/c mice (O'Mahony et al., 2010). c-Fos expression is an indicator of neuronal

cell activity, as c-Fos is expressed when an action potential fires (VanElzakker et al., 2008). The finding that c-Fos was higher in C57BL/6 mice was surprising as c-Fos expression was expected to be more elevated in the stressor-susceptible BALB/c mice than in C57BL/6 mice, as BALB/c show an increased reactivity to stress in terms of behaviour. The same study supported other behavioural literature (Michalikova et al., 2010), with BALB/c mice displaying more anxiety behaviours, in this case in the light-dark box (evidenced by fewer transitions between light and dark side). At the neuroendocrine level, an elevated plasma corticosterone concentration was evident in BALB/cBy mice compared to C57BL/6By mice after a noise stressor and did not differ between strains at basal level (Roy et al., 2007).

Glucocorticoid responsiveness also differs between the strains, as C56BL/6 mice expressed higher MR mRNA in the hippocampus and PFC and higher GR mRNA in the hippocampus in comparison to BALB/c mice. BALB/c mice, however, displayed significantly higher GR mRNA than C57BL/6 in the PFC. Perhaps most importantly, GR protein expression does not necessarily correlate with GR mRNA levels: BALB/c and C57BL/6 mice had similar GR mRNA levels in the amygdala, but GR protein expression was elevated in BALB/c mice and not C57BL/6. The authors suggest that pathways related to translation and protein stabilization may be different between the two strains (Brinks et al., 2007), suggesting different microRNA expression profiles between strains, which will be discussed later.

Stress also alters serotonin metabolism differently between strains. A metabolite of serotonin used as a marker of 5-HT turnover , 5-hydroxyindoleacetic acid (5-HIAA), increased in BALB/cBy in all brain regions tested (PVN, median eminence, hippocampus, amygdala, PFC), while 5-HIAA only increased in the PVN, amygdala, and PFC of C57BL/6By after an acute

stressor, suggesting that brain responses to stress differ between the strains and that cellular effects of stress within the hippocampus may differ between strains (Tannenbaum & Anisman, 2003). The Tannenbaum study also reported that neurochemical changes observed decreased upon repetitive stressors in C57BL/6 mice, while stressor-induced effects were maintained or exacerbated in chronically stressed BALB/cBy animals.

The neurotransmitter γ-aminobutyric acid (GABA) is also differentially affected by stress. GABA inhibits the HPA axis at the PVN via brain regions projecting to CRH-containing neurons (Cullinan et al., 2008). Higher concentrations of the GABA receptor subunits ($GABA_AR$) were observed in BALB/cByJ mice compared to C57BL/6ByJ after a 7 week chronic, intermittent variable stressor regimen (Poulter et al., 2010). Utilizing two genetically inbred strains will therefore prove very useful for examining gene-environment interactions, specifically how the effect of acute and chronic stress on genetic expression in the prefrontal cortex may differ depending on the genetic background of the individual.

1.5 Effects of Psychological Stress on the Brain

Studies of gene- environment interactions can focus on a range of outcomes from behavioural to molecular, and from whole organism to individual cells. However, studies that directly examine the impact of stressors on the mammalian brain have most commonly focused on four brain regions most closely associated with the pathological symptoms of depression, namely the paraventricular nucleus of the hypothalamus, the CA1 region of the hippocampus the central amygdala, and the prefrontal cortex.

1.5a Paraventricular Nucleus (PVN) of the Hypothalamus

Chronic stress has been shown to interfere with and modulate cells of the PVN. In rats, chronic stress affects PVN neurons by downregulation of molecules involved in HPA axis regulation. For example, endocannabinoids are molecules thought to modulate and contribute to the rapid feedback inhibition of the HPA axis by corticosteroids. Repetitive immobilization stress downregulated cannabinoid receptor type 1 (Cb1) in PVN neurons (Wamsteeker et al., 2010). Also, both acute and chronic restraint stress decreased GR mRNA in the PVN (Noguchi et al., 2010), which could result in decreased glucocorticoid negative feedback efficacy. PVN neurons are affected by psychological stress through another route as well, immune response activation. Interleukin-6 (IL-6) is a protein involved in inflammation which is found in higher concentrations in depressed individuals in comparison with controls (Dowlati et al., 2009). A recent study found that restraint stress upregulated Il-6 mRNA in the PVN of rats (Jankord et al., 2010). Therefore, the hypothalamic response to stress implicates cytokines in addition to traditional markers of the stress response. Footshock stress elevated c-Fos, a marker of neuronal activity, in the PVN of mice (Liu et al., 2009). Furthermore, stressor-induced changes in PVN activity are maintained over time, as administration of chronic variable stress to rats for one week resulted in altered c-Fos mRNA expression 30 days later when animals were exposed to a novel environment (Ostrander et al., 2009). Dopamine signalling may also be affected in the PVN following stress. Tyrosine hydroxylase (TH) is a rate-limiting enzyme involved in DA synthesis, and is upregulated in the PVN of rats exposed to immobilization stress (Kiss et al., 2008).

1.5b CA1 Region of the Hippocampus

The hippocampus is commonly associated with attention, navigation and long-term memory. Previous work identified effects of both acute and chronic social stress on the hippocampus. Mice exposed to acute social stress had elevated plasma, corticosterone and 5-HT levels in the hippocampus (Keeney et al., 2006). In a study on rats, three weeks of unpredictable stress reduced hyperpolarization responses to 5-HT in the CA1 region and suppressed synaptic plasticity (Joels et al., 2004). Additionally, expression of MR mRNA and protein occurs in abundance in the hippocampus of macaques, humans and rodents suggesting it as a region of the brain affected by stress (Sanchez et al., 2000; Klok et al., 2010; Reul & de Kloet, 1985). Early life stress, to which emotional disorders have been partially attributed (Briggs-Gowan et al., 2010), was found to mildly reduce GR and MR expression levels in the CA1 region of adolescent marmosets, indicating that effects of stress in development can have long-lasting effects similar to the mild-moderate reduction in hippocampal MR in depression patients (Arabadzisz et al., 2010). GR expression levels were also decreased in the CA1 region of adult squirrel monkeys exposed to social stress (Patel et al., 2008), and four weeks of psychosocial stress was found to decrease GR expression in the CA1 of tree shrews, but interestingly increase MR expression in that same region (Meyer et al., 2001). Further evidence for investigating the CA1 and its involvement in stress comes from the 2006 study that found daily restraint stress for three weeks results in a downregulation of glutathionine S-transferase, pi2 (Gstp2), a known defender against oxidative stress. This may result in increased oxidative damage leading to structural and functional damage of pyramidal cells in the CA1 (Ejchel-Cohen et al., 2006). Oxidative stress can damage cell structures and is associated with a variety of

diseases. The preceding study illustrates that chronic psychological stress decreases resilience to oxidative stress.

1.5c Central Amygdala (CeA)

The amygdala is consistently studied in stress research because of its role in the memory of emotional reactions. When analyzing the stress response, rats undergoing UCMS had elevated levels of CRH-containing neurons in their central amygdala (CeA) and PVN when compared with controls, with the authors suggesting that CRH systems in these regions may contribute to behaviours associated with depression which are often displayed during UCMS (Wang et al., 2010). To determine the direct impact of increased CeA CRH, CRH was overexpressed in rats via injection of a Lenti-CMV vector (Keen-Rhinehart et al., 2009). This resulted in increased expression of both CRH and arginine vasopressin peptide (Avp) in the PVN and decreased negative feedback on the HPA axis via glucocorticoids. These phenomena are both fundamental to the physiology of depression, and demonstrate that stress-induced increases in CeA CRH most likely elevate hypothalamic responsiveness to stressors by increased PVN neurosecretion of CRH. Psychosocial stress also results in changes to the CeA circuitry: chronic psychosocial stress in tree shrews led to a strongly reduced signal intensity of CRH-immunoreactive fibres in CeA (Kozicz et al., 2008). Given that glucocorticoid hypersecretion is characteristic of depression, rats were subcutaneously implanted with slow-releasing pellets: one group had implants that secreted corticosterone, while the other secreted a vehicle (cholesterol). CRH release in the CeA, triggered by an airpuff-stressor, was increased in the animals that received corticosterone pellets in comparison to the control rats receiving vehicle-secreting pellets (Merali et al., 2008), implying that chronic corticosterone exposure maximizes

the release of CRH in response to a stressor, resulting in dysregulation of the stress axis. As an example of how integral the amygdala is to an effective stress response, knockout of GRs in this region resulted in the prevention of conditioned fear behaviour which coincided with decreased in c-Fos and CRH expression (Kolber et al., 2008). Lastly, with regards to clinical human work, reduced amygdala volume is reported in unmedicated depressed individuals (Hamilton et al., 2008), as well as in patients treated with corticosterone (Brown et al., 2008). This draws a correlation between structural abnormalities within the amygdala and altered physiological functioning characteristic of mental disorders.

1.5d Prefrontal Cortex

The PFC is considered the most evolved brain region and is central to 'executive function', responsible for planning and decision making, moral dilemmas, personality expression and inhibition of inappropriate urges. Acute psychological stress can enhance glutamatergic transmission in prefrontal cortex and facilitate working memory (Yuen et al., 2009), while chronic unpredictable stress impairs cognitive flexibility in rodents via alterations in PFC neuronal morphology (Holmes & Wellman, 2009). Chronic stress also affects structure and function in the PFC. Rats exposed to a chronic stress paradigm had compromised working memory and behavioural flexibility, as well as decreased volume in layers I and II of the medial PFC (mPFC). This reduced volume did not correspond with a decreased neuron number, suggesting the lost volume corresponds to dendritic atrophy (Cerqueria et al., 2007). Consistent with dendritic atrophy, three weeks of daily six hour restraint stress decreased mean dendritic spine volume and surface area, as well as decreased the large:small spine ratio, in rat mPFC (Radley et al., 2008). The authors suggest these dendritic changes in the PFC may have

repercussions on function and receptor expression. In a similar study, apical dendritic length and dendritic branch points of neurons in the PFC both decreased in rats exposed to 21 days of chronic restraint stress (Bloss et al., 2010). It should be mentioned, however, that stress-induced dendritic atrophy is region specific, and that in other regions of the brain such as the basolateral amygdala, chronic stress has the opposite effect of enhancing dendritic arborization (Vyas et al., 2002), reflecting the disparate functions of the two regions.

In addition to dendrites and neurons being affected by stress, interference of neurotransmitter networks in the PFC also occurs. As mentioned previously, alterations in serotonin networks have been characteristic of depression, and a recent study found that chronic social defeat stress resulted in the downregulation of serotonin 1A receptor (5-Ht1a) mRNA in the PFC of defeated animals (Kieran et al., 2010). Interestingly, the same team found that chronic restraint stress resulted in upregulation of 5-Ht1a receptor mRNA, but found an unanticipated downregulation of actual protein expression (Iyo et al., 2009). The authors suggest there may be post-transcriptional mechanisms in place resulting in decreased protein abundance, leading to the idea of microRNA involvement, which will be discussed later. Another neurotransmitter network affected in the PFC is that of dopamine (DA). While the tuberoinfundibular dopamine pathway originating in the arcuate nucleus is involved in prolactin release and the nigrostriatal dopamine pathway is involved in Parkinson's Disease, the mesolimbic pathway is most relevant to mood-related disorders and stress. The mesolimbic pathway of DA begins in the ventral tegmental area(VTA) and travels through the amygdala, hippocampus and PFC, and is considered a reward pathway of the brain. Dysfunction of the mesolimbic pathway is predicted to be a contributing factor to the anhedonia and psychomotor

problems associated with depression (Stein, 2008). A tail pinch stressor increases the discharge rate of VTA neurons projecting to the PFC and a variety of stressors increase dopamine in the PFC (see Pezze & Feldon, 2004 for a review). Chronic stress also affects the dopaminergic system within the PFC: rats stressed chronically for four weeks displayed impaired working memory, a reduction in DA transmission and an accompanying increase in DA D1 receptor concentration. Upon introduction of a DA agonist, the working memory impairment was ameliorated (Mizoguchi et al., 2000). Taken together, the finding that psychological stress results in morphological and neurotransmission effects in the PFC suggests it as a prime area for potential changes in genetic expression. Due to time and cost, the current study will focus specifically on the PFC as it is a relatively homogeneous tissue in comparison to the hypothalamus and hippocampus, being composed of fewer distinct nuclei that may be affected differently by psychological stress. We expect the PFC to have fewer subregion-specific miRNA expression changes than other brain regions, which will make detection of miRNA expression changes in this region as a whole easier. For that reason, we will analyze solely the PFC in this study.

1.6 Stress-induced Gene Expression Changes in the PFC

Gene expression changes may result in altered PFC functioning, as modified protein expression has implications for synaptic plasticity, cellular structure and function, and nuclear receptor signaling. The PFC undergoes many changes in gene expression following exposure to stress. Acute restraint led to elevated mRNA levels of both Fos-related antigen2 (Fra2) and C-fos in the PFC of rats (Weinberg et al., 2007), both of which interact with activating protein-1 (AP-1) transcription factor complexes that regulate synaptic plasticity. Similarly, acute water-

immersion restraint stress upregulated glucocorticoid-induced leucine zipper (Gilz) mRNA in the mouse mPFC stress response. The role of Gilz in the brain has not yet been elucidated but Gilz mRNA is widely distributed throughout the brain, with increased expression levels in motor neurons suggesting a mediator role in the effects of stress-induced glucocorticoids on the motor system (Yachi et al., 2007).

Gene expression changes are also observed after chronic stress. For example mRNA of M6a, a structural glycoprotein localized to axon membranes, was increased in the mPFC of rats after three weeks of daily restraint, suggesting that stress has an impact on the integrity of axons in the PFC (Cooper et al., 2009). The opposite was found in the hippocampus, with stress leading to a decrease in M6a levels, suggesting that M6a increase in PFC may be an adaptation to weakened synaptic input from the hippocampus. The gene coding for M6a, GMP6A, is associated with high levels of depression in a subgroup of schizophrenia patients (Boks et al., 2008) illustrating its involvement in stress-related mood disorders. With regards to synaptic transmission, the SNARE complex is involved in vesicle docking and is affected by chronic stress. Eight regulatory proteins that help make up the SNARE complex had upregulated mRNA following chronic restraint stress, suggesting that changes in gene expression of synaptic proteins may contribute to frontal cortex stress response (Muller et al., 2011). The upregulation of two SNARE proteins in particular, Vamp2 and syntaxin 1a, is thought to facilitate neurotransmitter release. Vamp2 in rat frontal cortex is upregulated by antidepressant treatment (Yamada et al., 2002), providing evidence that antidepressant and chronic stress administration do not always have opposite effects on synaptic proteins.

Lastly, chronic social stress affects gene expression as well. When exposed to social stress four times over 10 days, rats displayed increased levels of Bdnf mRNA and protein in the mPFC, reiterating the role of Bdnf in neural adaptations to stress (Fanous et al., 2010). This is in contrast to a wealth of studies (See Duman & Monteggia, 2006 for a review) that maintain BDNF is decreased after stress, though these studies predominantly focus on the hippocampus. PFC BDNF changes may not mirror those in the hippocampus. Also, Fanou et al (2010) found a significant elevation in Bdnf mRNA 2h after the last stress but not 28 days later, suggesting a transient elevation after stress. Intermittent social stress also impacts gene expression in squirrel monkeys, *Saimiri sciureus*. Social stress was achieved via separating cage-sharing dyads for three weeks and then reuniting individuals with an unfamiliar cage-mate for nine weeks. The separation/reuniting took place six times during the study, and monkeys in the stress condition displayed downregulation of candidate genes responsible for functions such as synaptic plasticity and nuclear receptor signaling, such as ubiquitin conjugation enzymes and ligases (Karssen et al., 2007). Tissue processing was performed three months after the last social stressor, and this may account for the downregulation of synaptic plasticity components not seen in the rodent SNARE complex study. These effects of stress on gene expression in the PFC may contribute to the molecular problems underlying pathophysiology of stress-related disorders. A wealth of studies has therefore revealed changes in protein-coding mRNA levels in the PFC in response to stressors. However, it is not always known whether these mRNA expression changes are the result of changes in transcription rate or in mRNA degradation. Furthermore, mRNA changes do not always correspond to changes in the levels of functional protein, due in part to post-transcriptional regulation of translation. MicroRNAs are a recently-

discovered class of genes that are involved in both the regulation of mRNA degradation, plus regulation of translation, yet have not been investigated extensively in the context of stressor response in the PFC. Therefore, this thesis will examine the impact of stress on microRNA expression in the PFC, which may reveal a role for microRNA in the previously discussed stress-induced protein changes in the brain.

1.7 microRNA

MicroRNAs (miRNAs) are a class of small (usually around 19-22 nucleotides), non-coding RNA molecules that act post-transcriptionally to alter protein expression. MicroRNAs target roughly 60% of mammalian mRNA molecules and surpass both kinases and phosphatases in number (1424 different miRNAs in humans according to miRBase release 17 in April 2011) (Kozomara & Griffiths-Jones, 2011), suggesting a significant role in moderating cellular functioning (Leung & Sharp, 2010). miRNAs may inhibit protein expression via interference of translation inhibition and elongation (Eulalio et al., 2008), recruiting deadenylating enzymes (Giraldez et al., 2006) or degradation of the target. They reduce protein expression via binding to the 3' un-translated region (UTR) of the corresponding mRNA molecule, resulting in mRNA destabilization (Bartel, 2009; Guo et al., 2010). The "seed region" of miRNA, located between nucleotides two and eight, is the sequence of nucleotides that must bind perfectly to the mRNA transcript. Partial base pairing occurs 3' to the seed region and stabilizes the miRNA-target interaction (Roshan et al., 2009). miRNAs associate with Argonaute proteins to form miRNA-RiboNucleoProtein complexes (miRNPs), with Argonaute proteins being responsible for cleavage of the target in certain cases. The binding of miRNPs to protein mRNA transcripts interferes with translation of the target. One miRNA molecule can complementarily bind to one

or many mRNA molecules, hence affecting expression levels of many different proteins. MicroRNA expression has been well documented in the murine central nervous system, with a study by Bak and colleagues profiling microRNA expression in 13 distinct brain regions under basal conditions, including the hypothalamus and hippocampus (Bak et al., 2008). Recently, artificial miRNA targets have been introduced into cells via viruses in order to study specific miRNA functions, as once the host miRNA are bound to the exogenous targets, their routine functions are inhibited and their role deduced (Brown & Naldini, 2009). miRNAs are involved in many cellular processes, such as apoptosis, metabolism and cell differentiation (Hunsberger et al., 2009), all of which are widespread processes in the brain.

1.8 MicroRNAs May Mediate the Effects of Stress on the PFC

Given the effects of psychological stress on the PFC and synaptic plasticity, miRNAs expected to be affected by stressor administration are as follows.

1.8a microRNA- 132

miRNA-132 has been implicated in the outgrowth of synaptic spines. A study using rat cortical neurons showed that miRNA-132 expression induced neurite outgrowth, while inhibition of microRNA-132 via transfection with an antisense 2'O-methyl RNA decreased the observed outgrowth, suggesting this microRNA as a regulator of neuronal morphology (Vo et al., 2005). miRNA-132 also affects glutamate receptor expression at the dendrites in cortical cells (Kawashima et al., 2010). BDNF administration increased miRNA-132 levels and transfection of exogenous miRNA-132 upregulated glutamate receptors, revealing the positive effect miRNA-132 has on postsynaptic protein levels. To examine the effect of glucocorticoids

on these BDNF-induced increases, the synthetic glucocorticoid dexamethasone was administered and decreased both miRNA-132 expression and postsynaptic protein levels. This displays the impact of stress hormones on miRNA expression, as well as highlights the potential neurotransmission effects of such disruptions. miRNA-132 also displays a similar dendrite-specific role in different cell types, as evident by a study on hippocampal neurons displaying miRNA-132 as being induced during a period of active synaptogenesis (Impey et al., 2010). This study knocked down the miRNA-132 target, p250GAP, and the increase in spine formation observed mimicked the effects of miRNA-132 transduction. The authors suggest miRNA-132 as a key player in activity-dependent structural and functional plasticity (Wayman et al., 2008), both being phenomena affected by psychological stress. Though the majority of studies on miRNA-132 were performed in vitro, a maternal separation stress study on rats revealed altered miRNA levels in the mPFC (Uchida et al., 2010). In comparison to controls, maternally separated rats had elevated levels of miRNA-132 14 days post-natal, suggesting miRNA-132 involvement in the synaptic activity of stressed animals.

1.8b microRNA-134

Like miRNA-132, miRNA-134 has also been implicated in dendritic protein synthesis. miRNA-134 is localized to the synapto-dendritic compartment of rat hippocampal neurons and decreases the size of dendritic spines (Schratt et al., 2006), essentially acting in opposition to miRNA-132. This finding was tested *in vivo*, with overexpression of miR-134 in cortical neurons of mouse brain resulting in decreased dendritic arborization (Christensen et al., 2010). miRNA-134 functions by inhibiting translation of Limk1, a protein kinase that controls spine development. When exposed to BDNF, miRNA-134 inhibition of Limk1 was ameliorated, adding

to the complexity of dendritic architecture regulation and reinforcing the neurotrophic role of BDNF. Recently miRNA-134 was shown to impair synaptic plasticity via decreases in dendritic spine density of hippocampal neurons but that the gene coding for the deacteylase protein Sirtuin-1, SIRT1, normally limits expression of miRNA-134. When SIRT1 was knocked down, unchecked miRNA-134 levels also resulted in a decrease in BDNF expression (Gao et al., 2010). The role of miRNA-134 is not entirely clear, however, as in contrast to the aforementioned studies, the expression of miRNA-134 (induced by upstream binding of the myocyte enhancing factor 2 (Mef2)) promoted dendritic outgrowth by inhibiting translation of the RNA-binding protein, Pumilio2 (Fiore et al., 2009). The authors acknowledge the dichotomy of miRNA-134 function (the decrease of dendritic spine size but increase in dendritic outgrowth) and suggest localization of miRNA-134 within different compartments of the cell, ie. Cell body vs. spines, may be responsible for the contradictory results. Lastly, after chronic restraint stress, miRNA-134 was downregulated in both the amygdala and hippocampus in rats (Meerson et al., 2010), changes which accompany altered synaptic structure and function in vitro.

1.8c microRNA-124

miRNA-124 is a brain-specific miRNA that is expressed at relatively high levels in many brain regions, such as the hippocampus, hypothalamus and frontal cortex. miRNA-124 plays a role in cellular morphology, in that overexpression in mouse P19 cells and primary cortical neurons results in neurite outgrowth while blocking miRNA-124 function delays neurite outgrowth (Yu et al., 2008). With regards to the negative feedback loops apparent in the HPA axis and potential implications in the stress response, miRNA-124a decreased GR protein levels in neuroscreen cells, a cell line displaying an accelerated response to nerve growth factor and

used to examine neurite outgrowth, and was found at adequately high levels in the PFC to alter the GR protein expression profile (Vreugdenhil et al., 2009). Lastly, *in vivo* studies have described an effect of stress on miRNA-124 expression in the brain. Acute restraint stress downregulated miRNA-124a-1 levels in the hippocampus (Meerson et al., 2010), a hypothesized result being decreased neurite outgrowth. The Uchida study examining maternal separation stress on miRNA expression also found elevated miRNA-124 levels in the mPFC of stressed mice at P14 (Uchida et al., 2010). Animal studies such as these reaffirm the hypothesis of miRNA-124 involvement during times of stress.

1.8d microRNA- 9

In contrast to the other miRNAs being discussed, justification for miRNA-9 comes primarily from *in vivo* studies. miRNA-9 was upregulated in frontal cortex of rats exposed to an acute restraint stressor (Rinaldi et al., 2010), whereas basal expression levels were substantially lower than in other brain regions. The authors suggest miRNAs as having a 'loose' control over protein synthesis in the frontal cortex and that miRNA expression changes may allow for synaptic efficacy changes through regulation of dendritic protein translation. In contrast to acute restraint stress, chronic restraint stress downregulated miRNA-9-1 in both the amygdala and hippocampus (Meerson et al., 2010). Finally, early life maternal separation stress increased miRNA-9 levels in the mPFC of rats at P14 (Uchida et al., 2010). The advantage of *in vivo* studies is the high validity of involvement in stress-induced PFC alterations, the disadvantage being a less clear picture of miRNA-9 function. Though miRNA-9 has been shown to be involved in embryonic development, via targeting various transcription factors and regulating neurogenesis (Shibata et al., 2011), less data has been obtained on miRNA-9 expression in adults.

1.8e microRNA-30a

miRNA-30a will be discussed due to its presence in human cortex and interference with BDNF expression. miRNA-30a is enriched in layer III pyramidal neurons, a site of BDNF synthesis, and binds to the 3'-UTR of BDNF mRNA. In cell cultures, overexpression of miRNA-30a resulted in a significant 30% decrease in BDNF protein levels (Mellios et al., 2008). Follow-up studies found that ablating BDNF from the cortex resulted in elevated miR-30a levels (Mellios et al., 2009), suggesting a feedback loop between BDNF expression and miR-30a levels. Increased BDNF in the brain is thought to be pivotal in the therapeutic effects of antidepressant effects and UCMS decreases BDNF protein level in the hippocampus of rats (Zhang et al., 2010).

1.8f Other microRNAs of Potential Interest

A variety of other miRNAs that will not be included in the candidate gene RT-PCR approach for our study are also relevant. miRNA-26a targets mRNA of MAP2, a microtubule-stabilizing protein, and inhibition of miRNA-26a resulted in increased MAP2 levels in hippocampal neurons (Kye et al., 2007). Acute restraint stress upregulated expression of miRNA-26a in the PFC of CD-1 mice (Rinaldi et al., 2010) which could lead to decreases in MAP2 levels. Another miRNA, miRNA-195, like the previously discussed miRNA-30a, inhibits BDNF expression and displays an inverse correlation with BDNF protein levels in the adult human PFC (Mellios et al., 2009; Mellios et al., 2008). microRNAs are also involved in neurotransmitter receptor signaling. NMDA is a neurotransmitter that acts as an agonist for the excitatory neurotransmitter, glutamate. UCMS increases NMDA receptors in the PFC, while decreases NMDA receptors in the hippocampus of Wistar-Kyoto rats (Lei & Tejani-Butt, 2010), indicating brain-region specific effects of stress. miRNA-219 targets the calcium/calmodulin-dependent

protein kinase II γ subunit (CaMKIIγ), a component of the NMDA receptor signaling cascade, and inhibition of miRNA-219 resulted in altered behavior characteristic of disrupted NMDA receptor transmission (Kocerha et al., 2009). Lastly, though not expressed in adequate levels for function in the PFC, miRNA-18a downregulates GR and may play an important role in stress hormone negative feedback loops in the PVN. *In vitro* studies show that miRNA-18 represses GR activity (Vreugdenhil et al., 2009) and 14 days of repeated restraint stress increased miRNA-18 levels in the PVN of Fischer 344 rats compared to Sprague-Dawley counterparts (Uchida et al., 2008). This corresponded to a decrease in GR protein levels but an unaffected GR mRNA expression profile. Future studies investigating the impact of stress on the PVN should examine this miRNA in particular.

1.9 Hypotheses

The overarching aim of this study is to elucidate miRNA expression changes occurring in the PFC following psychological stress. In this manner, our objective is to gain further insight into molecular changes that may underlie onset of mood disorders in humans. Given the effects of stress on cells of the PFC, the changes at the dendrites and the involvement of miRNAs in dendritic protein expression and elaboration, we predict: 1) that expression of specific miRNAs in the PFC will be altered in both chronically and acutely stressed animals, compared with controls, 2) that stressors will have a greater impact on miRNA expression patterns within mice that are genetically susceptible to stressors, compared with stressor resilient mice, 3) a difference in miRNA expression between animals that are exposed to stressors chronically and those only receiving exposure to a single stressor. More specifically, we predict that miRNAs impacted by an acute stressor will also be affected by chronic stressors, but to a greater extent.

Furthermore, chronic stressors are predicted to induce changes in miRNAs that were seemingly unaffected by acute stressors, and which may underlie pathological outcomes of chronic stress. To our knowledge this will be the first study to examine the effect of psychological stress on miRNA expression in these two strains.

1.10 Experimental Design

To examine the effects of psychological stress on miRNA expression in the mouse PFC, we will employ real-time polymerase chain reaction (RT-PCR). RT-PCR will be used in a candidate gene approach, in which we will investigate miRNA-132, miRNA-134, miRNA-124, miRNA-9, and miRNA-30a as outlined in section 1.7a based upon their roles in processes affected by stress. Our study involves three treatment groups: one control group which will not be exposed to any stressors, one group which will be exposed to one acute social stressor and one group which will be exposed to chronic unpredictable stress for 21 days. We will expose animals to both chronic unpredictable stress as well as social stress because these are conditions that most resemble the stressors preceding development of mood disorders in humans. The use of unpredictable stressors will also avoid the confound of habituation that may occur in studies repeating the same type of stress each day. Each treatment will be applied to two different mouse strains, yielding a 2 x 3 design. This will allow for comparisons of microRNA expression between stressor treatment groups, as well as display how stress may alter miRNA expression differently depending on strain.

2.0 Methods

2.1 Animals

Adult (10-12 weeks old) male C57BL/6 and BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and allowed a 14-day acclimatization period before treatment began. Animals were housed in pairs on a 12:12h light:dark schedule (lights on at 8 am) with *ad libitum* access to food and water. Nestlets were provided for environmental enrichment. All procedures were approved (P10-9) by Carleton University's Animal Care Committee (ACC) to ensure compliance with guidelines set by the Canadian Council on Animal Care (CCAC). Mice were randomly assigned to one of three treatment groups: control, chronic stress and acute stress. Our study was a 2x3 design, with two strains and three stressor groups, with each group having a sample size of 9. At the end of the study, due to one animal not surviving and others being excluded for various reasons, the sample sizes were as follows: Control C56BL/6: n = 6, Control BALB/c: n = 9, Acute C57BL/6: n = 7, Acute BALB/c: n = 7, Chronic C57BL6: n = 7, and Chronic BALB/c: n = 9. Weight results contain data for 6 animals per each group while corticosterone data include the following sample sizes per group: Control C56BL/6: n = 7, Control BALB/c: n = n = 8, Acute C57BL/6: n = 7, Acute BALB/c: n = 7, Chronic C57BL/6: n = 7 and Chronic BALB/c: n = 9. Animals were excluded based on the following three factors, in order of importance: 1) no aggressive encounter occurring during the acute stressor, 2) poor RNA Quality Index (RQI) and 3) single housing during the study.

2.2 Acute Social Stressor - Resident-Intruder Paradigm

Animals in both the acute and chronic stress conditions were exposed to a CD-1 bully mouse for 15 minutes on day 21 of the study. The CD-1 mouse bullies were retired breeders physically larger than the experimental mouse. The experimental mouse was added to the home cage of the bully mouse and the mice were allowed to interact. When the interaction became too violent, a mesh partition was introduced that allowed for olfactory and visual information exchange, but no physical altercation. Animals were routinely split up within the first minute of introduction, as the experimental mouse would often display a submissive pose, in which he stood motionless, upright on his hind legs. Only in one case did the bully mouse and the experimental mouse not become separated, as no fight occurred. This experimental mouse was excluded from the subsequent analysis. After 15 minutes, experimental mice were returned to their home cages and remained there for 90 minutes, followed by euthanasia.

2.3 Chronic Unpredictable Stress (CUS)

Animals in the chronic stress conditions were exposed to two different stressors daily, with one stressor occurring in the morning and the other in the afternoon. The stressors were administered on a variable and unpredictable schedule. The chronic stressor paradigm included the following ten stressors: restraint in a semicircular Plexiglas tube (4 cm diameter X 12 cm long), with tails taped to prevent mice turning (15 min); wet bedding, in which cage bedding was soaked with water (1 hr or 4 hrs); forced swim in water of 20°C within a plastic bucket of 30 cm diameter and 27 cm high (5 min); placement on the open arm of the elevated plus maze (5 min); placement in a tight fitting triangular baggie resulting in complete restraint (with a hole cut for the nose to facilitate breathing) (15 min); cage swap, in which each pair of mice was

moved to their neighbours' cage, left there for 4 hours, then returned home; exposure to dirty bedding from a breeding colony, which was introduced into their home cage (1 hr); tail pinch, during which a paper clip was fastened to the mouse's tail, with a folded gauze pad placed between skin and clip to avoid tissue damage (6 min); light stressor, during which lights in a holding room were manipulated to stay on overnight; and startle, where mice were restrained and exposed to bursts of noise ten times over five minutes, at random intervals, using Startle Reflex software (Version 4.43, Med Associates Inc., St. Albans, VT). On day 21, the CUS protocol ended with the acute social stressor.

2.4 Weight Measurement

Animals were weighed on the first day of the experimental period, and every third day until the day of decapitation. Weight gain was computed by subtracting weight of animal on day 1 from weight on day 21.

2.5 Decapitation and Tissue Processing

Mice were euthanized via live decapitation and brains were immediately removed and placed in a cooled acrylic matrix (Stoelting, Cat. #51382), after which the following dissections were performed: 1 mm Coronal slices were made using razor blades and micropunches (20 guage) of three brain regions, the CA1 of the hippocampus, the PVN of the hypothalamus and the CeA, were taken and placed in microcentrifuge tubes at -80°C. A v-shaped section of the PFC was dissected out and kept under identical conditions. Trunk blood was also collected for blood plasma corticosterone level processing.

2.6 Corticosterone Radioimmunoassay

Approximately 1 ml of trunk blood was collected in ethylenediaminetetraacetic acid-containing tubes which were then placed on wet ice until an eight minute centrifugation at 3600 rpm. The supernatant was then stored at -80°C until plasma corticosterone levels were measured using a standard radioimmunoassay kit for mice and rats (MP Biomedicals, Orangeburg, NY).

2.7 TRIzol RNA Extraction using PureLink Micro Kit Column

Tissue samples were added to a clean, sterile culture tube containing 1 ml of TRIzol reagent (Invitrogen) and then homogenized, using the PowerGen 125 (Fisher Scientific), for 15 seconds. 1 ml of the resulting tissue-TRIzol mixture was transferred to a labeled 1.5 ml microcentrifuge tube and incubated for 5 minutes at room temperature to allow for dissociation of nucleoprotein complexes. Next, 200 µl chloroform (Sigma) was added and the samples were shaken vigorously for 15 seconds. The samples were then incubated at room temperature for 2-3 minutes. After incubation, samples were centrifuged at 12,000 g for 15 minutes at 4°C. The upper, colourless RNA-containing phase was then transferred to a new sterile microcentrifuge tube. For each sample, an equal volume of 100% ethanol was added to the aqueous phase and vortexed.

The PureLink RNA Micro Kit User Guide (Invitrogen) protocol entitled "TRIzol Plus Total Transcriptome Isolation" was used for RNA extractions and steps were performed according to manufacturer's instructions. Upon the RNA elution step, 12-22 µl of water is recommended to flush out the RNA sample bound to the column. 14 µl of RNase-free water were added to the center of the column, to achieve the desired stock RNA concentration, and incubated for 1

minute. After centrifugation at 12,000 g for 1 minute, the column had absorbed 2 µl of water, and the resulting elute contained 12 µl of RNA sample. The final RNA sample was then stored at -80°C or kept on ice, for RNA quality analysis with the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and Experion Automated Electrophoresis Station (Bio-Rad).

2.8 RNA Quality Control

2 µl of 10X diluted RNA sample were analyzed with the NanoDrop 1000 spectrophotometer and readings of A260, A280, A260/A280 were recorded in order to quantify RNA sample contamination and concentration. An A260/A280 ratio greater than 1.8 designates the RNA sample as "pure". The mean A260/A280 ratio for RT-PCR samples was 1.95 (SD = .071). Each sample was then diluted again to a concentration between 0.5 ng/µl and 5 ng/µl in order to be accurately analyzed using Experion RNA High Sens Analysis Kits (Bio-Rad).

The Experion Automated Electrophoresis Station tests for RNA degradation by comparing the ratio of two peaks of two major ribosomal RNA fragments: the 28S and 18S fragments. In an undegraded sample, this ratio should be 2.0 and be constant between tissues, reflecting the composition of each ribosome. As larger RNA ribosomal molecules degrade more rapidly, this ratio will be reduced if degradation has taken place. The ratio contributes to the RQI, and a score of 5 is adequate for PCR. The mean RQI for Rt-PCR samples was 8.8 (SD = .44).

2.9 microRNA Real Time-Polymerase Chain Reaction (RT-PCR)

2.9a Overview

TaqMan® MicroRNA Assays (Applied Biosystems) were used when performing PCR. The two-step procedure of miRNA-specific reverse transcription followed by polymerase chain reaction (PCR) detected and quantified miRNAs present in the experimental samples.

Fluorescence emitted during polymerization was measured by the MyIQ ™ Single-Colour Real-Time PCR Detection System (Bio-Rad) and analyzed by Bio-Rad's iQ5 v2 software.

2.9b Reverse Transcription

Reverse transcription was carried out in 15 µl volumes composed of 3 µl 5X RT primer, 5 µl RNA sample containing 1-10 ng total RNA (0.2-2 ng/µl) and 7 µl master mix, made up of 0.15 µl 100 mM dNTPs, 1 µl MultiScribe™ Reverse Transcriptase (50 U/µl), 1.5 µl 10X reverse transcription buffer, 0.19 µl RNase inhibitor (20U/µl), and 4.16 µl nuclease-free water. The resulting 15 µl reaction mix was incubated at 16°C for 30 mins, 42°C for 30 mins, and 85°C for 5 mins, using a MyCycler Thermal Cycler (Bio-Rad).

2.9c Polymerase Chain Reaction (PCR)

TaqMan probes were used to quantify amplification occurring during this step. TaqMan probes bind to the RT product cDNA. The probes consist of a fluorophore located proximal to a quencher, a substance known to inhibit fluorescence signals. The probes anneal to a region of DNA that is of interest, located in a region amplified by gene-specific primers. Once the exonuclease activity of Taq polymerase degrades the probe, and allows the fluorophore to become distant from the quencher, fluorescence is no longer inhibited and is detected by the PCR machine (Figure 1).

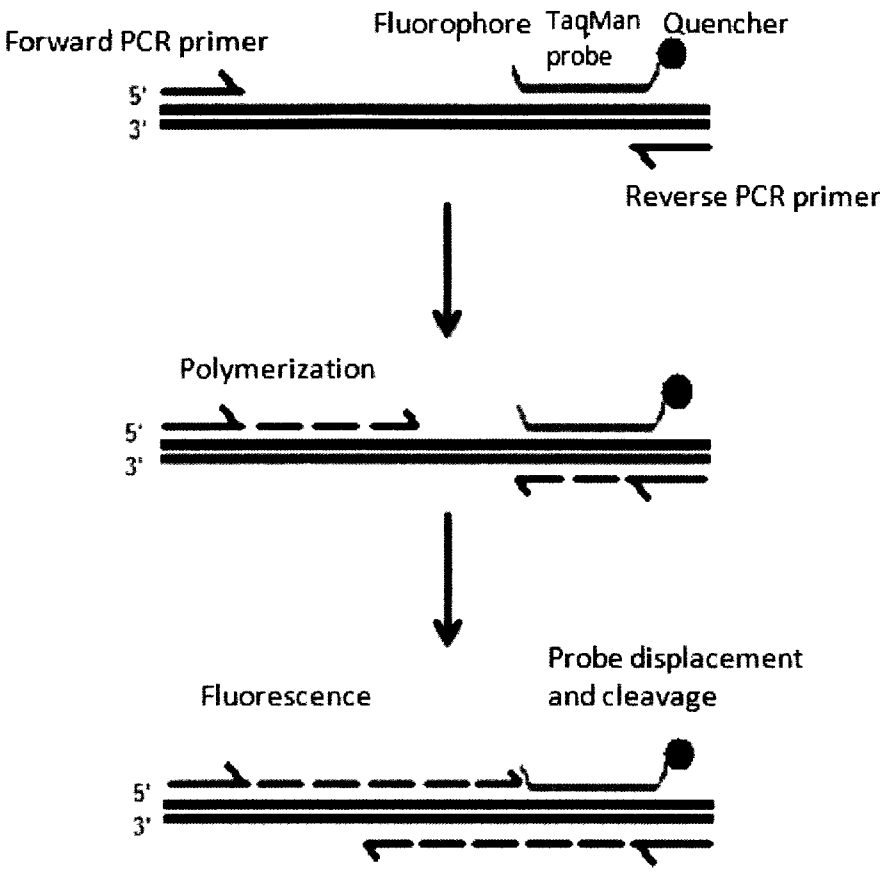

Figure 1. TaqMan probe fluorescence mechanism.

Each PCR reaction was 15 µl in volume and contained 0.75 µl TaqMan® Small RNA Assay (20X), 0.9975 µl RT reaction product, 7.5 µl TaqMan® Universal PCR Master Mix II (2X) and 5.7525 µl nuclease-free water. All reactions were run in triplicate. The PCR run parameters were set-up using iQ5 v2 software (Bio-Rad).

2.9d RT-PCR Data Analysis

The Livak method is an approach which compares cycle threshold (Ct) values and was used to analyze RT-PCR results. The cycle threshold is a standardized amount of PCR product that defines the end-point of the PCR reaction and is set during the exponential increase in

fluorescence that occurs during RT-PCR. The first step is normalizing to an internal reference gene. A difference between Ct values (ΔCt) is obtained by subtracting the Ct value of a known 'housekeeping' gene, a gene that does not fluctuate in expression as a result of treatment such as snoRNA-135, from the Ct of the GOI within a subject. The second comparison is between the individual ΔCt of the treatment subjects and the average ΔCt of the control group, yielding a product called ΔΔCt, which shows how different the differences between GOI and housekeeper are between the treatment groups and the control. To generate a fold-change relative to the control samples, cycle threshold data were exported to Excel for analysis by the Livak method equation of $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The base of two is used because each cycle of PCR is expected to double the amount of DNA present.

All screening of outliers within PCR results was done blind. If the majority of points in a single column were found to be outliers, the entire column would be excluded as pipetting error, due to errors using a multi-channel pipette. Also, for all analyses, the effects of outliers were minimized by utilizing the median of the triplicates ran for each sample. RT-PCR was ran on six animals per experimental group, however, based upon presence of outliers in housekeeping genes, animals were excluded and the following samples sizes were utilized in the analysis: Control C56BL/6J: n = 6, Control BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Acute BALB/cJ: n = 6, Chronic C57BL6J: n = 5, and Chronic BALB/cJ: n = 4.

2.10 Target prediction software for miRNA

DIANA-microT v.4 is an algorithm that, based on miRNA recognition elements (MREs), computes a miRNA targeted genes (miTG) score with a higher miTG score corresponding to a higher probability of correct prediction (Maragkasis et al., 2009). MREs are 7-, 8-, or 9-

nucleotide long sequences that consecutively base pair with the miRNA of interest. The overall miTG score is the weighted sum of the scores of all identified MREs on the 3'-UTR of the specific gene (Maragkasis et al., 2009). DIANA-microT v.3, the version preceding v.4, had slightly higher precision of around 66%, defined as the fraction of predicted targets that are actually downregulated, than other commonly used prediction softwares PicTar and TargetScan (Maragkasis et al., 2009b). To be accessed via miRBase (Kozomara & Griffiths-Jones, 2011), DIANA-microT v.4 was used to identify potential targets of miRNAs that were found to be significantly regulated by stress. We focused on predicted targets expressed in the brain that have, at minimum, a 0.650 miTG score and a known role at the synapse.

2.11 Statistical Analyses

To determine whether differences in blood corticosterone, weight gain, and $2^{-\Delta\Delta Ct}$ transformed gene expression results from PCR existed between experimental groups, 2x3 ANOVAs were ran for each dependent variable. Planned comparisons were carried out using a two-tailed t-test assuming unequal variances.

3.0 Results

3.1 Effect of stressors on weight gain

For both BALB/c and C57BL/6 mice, treatment groups were weight matched on day 1 (BALB/c: $F(2,15)=.679$, $p=.522$; C57BL/6: $F(2,15)=2.015$, $p=.168$). Weight gain determined at the end of the 21 day treatment period was significantly affected by both treatment ($F(2,30)=21.143$, $p<.001$) and strain ($F(1,30)=5.087$, $p<.05$), with a significant treatment x strain

interaction ($F(2,30)=4.807$, $p<.05$). There was significantly slower weight gain observed for chronically (BALB/c: M=1.25, SD=.419, $t(10) = -2.67$, $p<.05$ and C57BL/6: M=.815, SD=.523, $t(9) = -8.60$, $p<.001$) but not acutely (BALB/c: M=2.06, SD=.866, $t(8) = .275$, $p=.791$ and C57BL/6: M=2.85, SD=.885, $t(6) = -.405$, $p=.670$) stressed animals, compared to controls (BALB/c: M=1.95, SD=.492 and C57BL/6: M=3.00, SD=.339) (Figure 2).

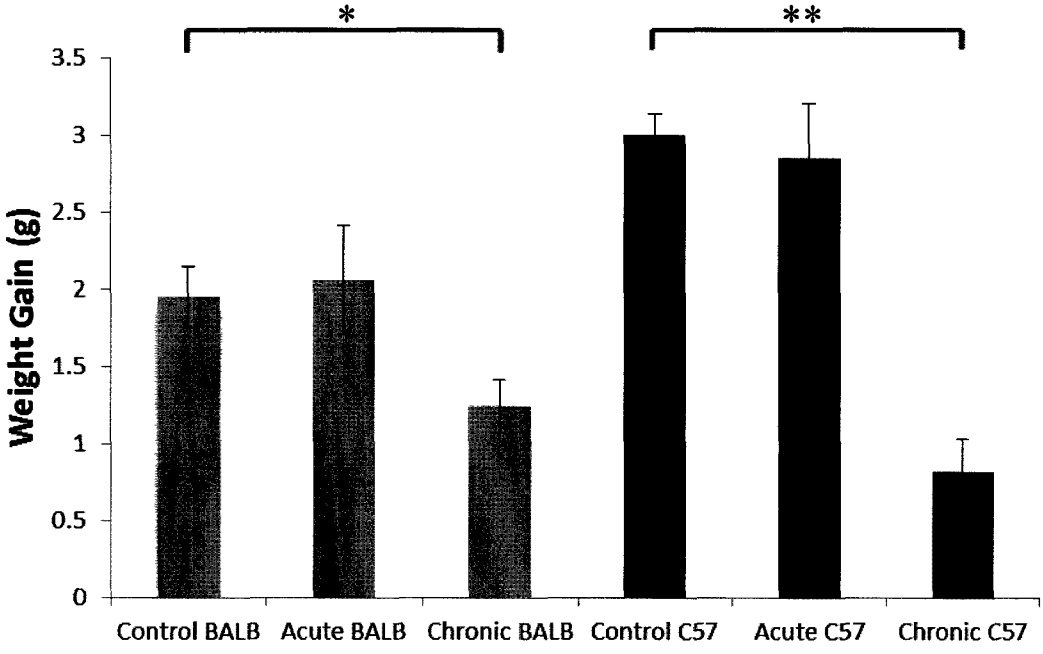

Figure 2. Weight gain by the end of the experimental period. Data are mean +/- standard error for all six experimental groups. * indicates $p<.05$ and ** signifies $p<.001$. n=6 for each group.

The weight changes in all study animals throughout the three week period is represented in Figure 3. The slower increase in weight in chronically stressed C57BL/6 mice throughout the study is indicated by a decreased slope.

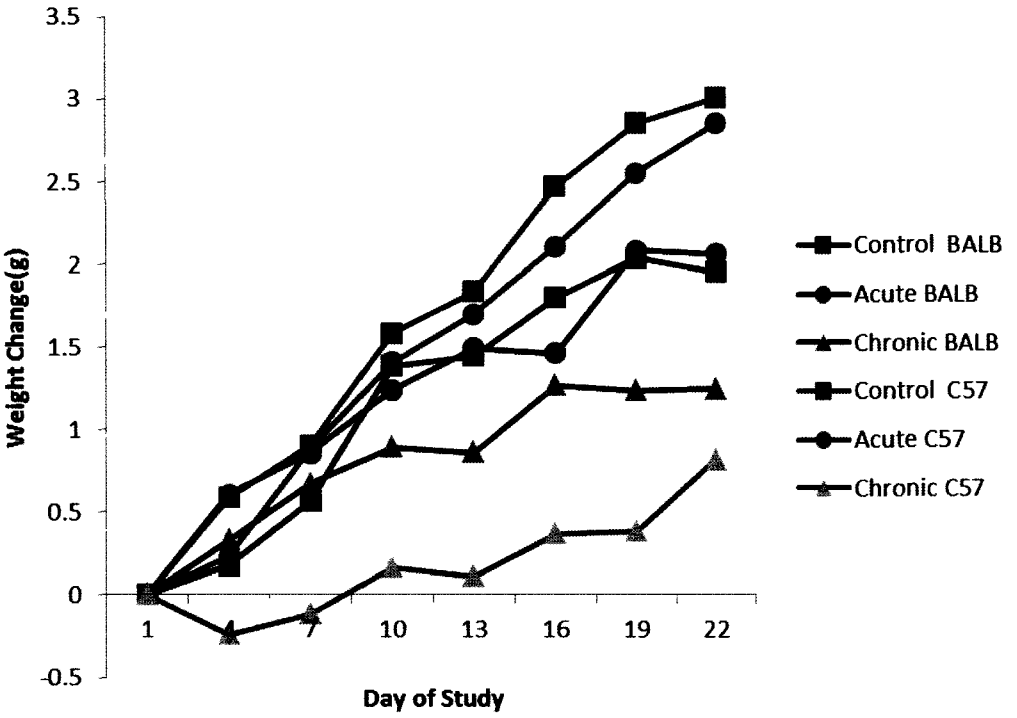

Figure 3. Weight change during the experimental period. Data are mean weight change from day one for every third day of the study. Black and red lines represent BALB/c and C57BL/6 animals, respectively. n=6 for each group.

3.2 Blood Corticosterone Analysis

Analysis of blood corticosterone data found a significant effect of stressor treatment, $F(2,39)=8.29$, $p<.005$, but no significant effect of strain, $F(1,39)=.017$, $p=.898$ or interaction between treatment and strain, $F(2,39)=.925$, $p=.405$ (Figure 4). A significant increase in blood corticosterone levels was observed (Tukey post-hoc test, $p<.001$) in chronically stressed ($M=14.93$, $SD=8.45$), but not acutely stressed ($M=10.62$, $SD=7.17$), compared to control animals ($M=4.98$, $SD=1.90$). For BALB/c animals, significant elevations in blood corticosterone were found in both acutely ($M = 9.29$, $SD = 3.61$, $t(8) = 3.02$, $p<.05$) and chronically ($M = 16.6$, $SD = 8.87$, $t(9) = 3.01$, $p<.005$) stressed mice in comparison to controls ($M = 4.81$, $SD = 1.66$). Chronically stressed C57BL/6 mice had elevated levels ($M = 12.8$, $SD = 8.00$, $t(7) = 2.42$, $p<.05$) in

comparison to control animals (M = 5.17, SD = 2.27), but there was a lack of significant effect of acute stress (M = 12.0, SD = 9.71, $t(7) = 1.80$, $p=.115$) on blood corticosterone in the aforementioned strain. Numerically, corticosterone levels were similar between acutely and chronically stressed animals, but a lack of significance for the acute condition was probably due to the large variability within the acutely stressed animals.

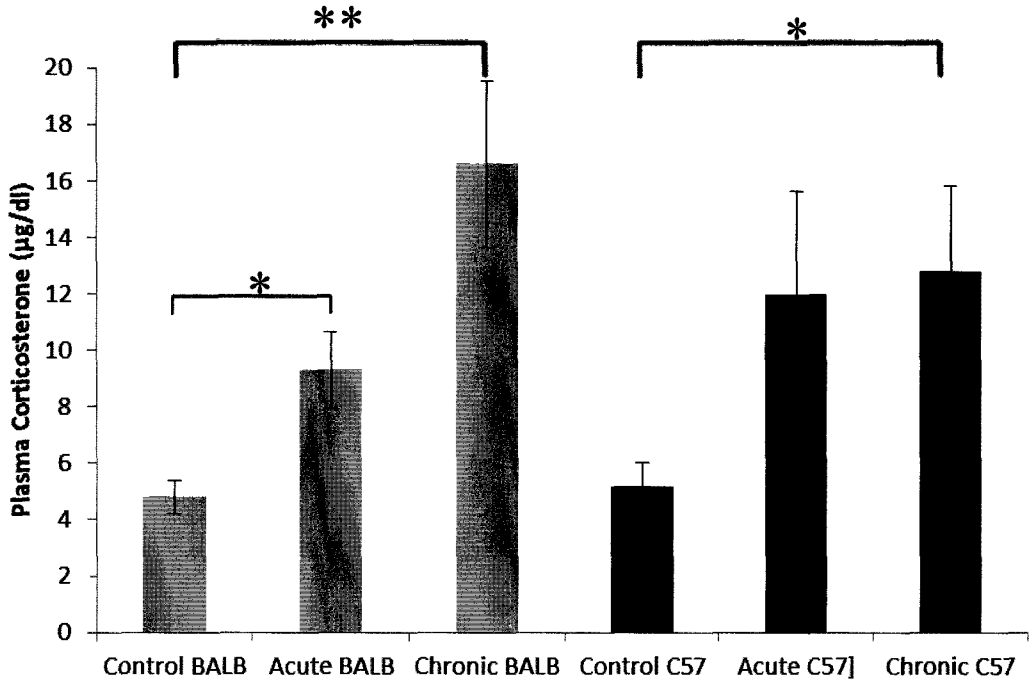

Figure 4. Blood corticosterone analysis results. Data are mean +/- standard error for all six experimental groups. * indicates significance compared to control mice, $p<.05$ while ** indicates $p<.005$. Sample sizes were as follows: Control BALB/c: n = 8, Acute BALB/c: n = 7, Chronic BALB/c: n = 9, Control C56BL/6: n = 7, Acute C57BL/6: n = 7, and Chronic C57BL/6: n = 7.

3.3 microRNA RT-PCR

3.3a microRNA-132

Analysis of microRNA-132 expression found a significant effect of treatment, $F(2,26) = 3.713$, $p<.05$, but no effect of strain, $F(1,26) = 2.565$, $p=.121$ or interaction, $F(2,26) = 2.337$,

p=.117 (Figure 5). A significant difference in miRNA-132 expression was found between acutely stressed animals and controls (Tukey post-hoc tests, p<.05). When the strains were examined individually through planned comparisons, none of the treatment groups differed significantly from controls (acutely stressed C57BL/6: $t(9) = 1.83$, p=.101 and chronically stressed BALB/c: $t(7) = 0.58$, p=.579), though there was a marginally significant 31% decrease in expression in chronically stressed C57BL/6 animals ($t(5) = 2.42$, p=.060) and 24% decrease in expression acutely stressed BALB/c animals ($t(8) = 1.90$, p=.094).

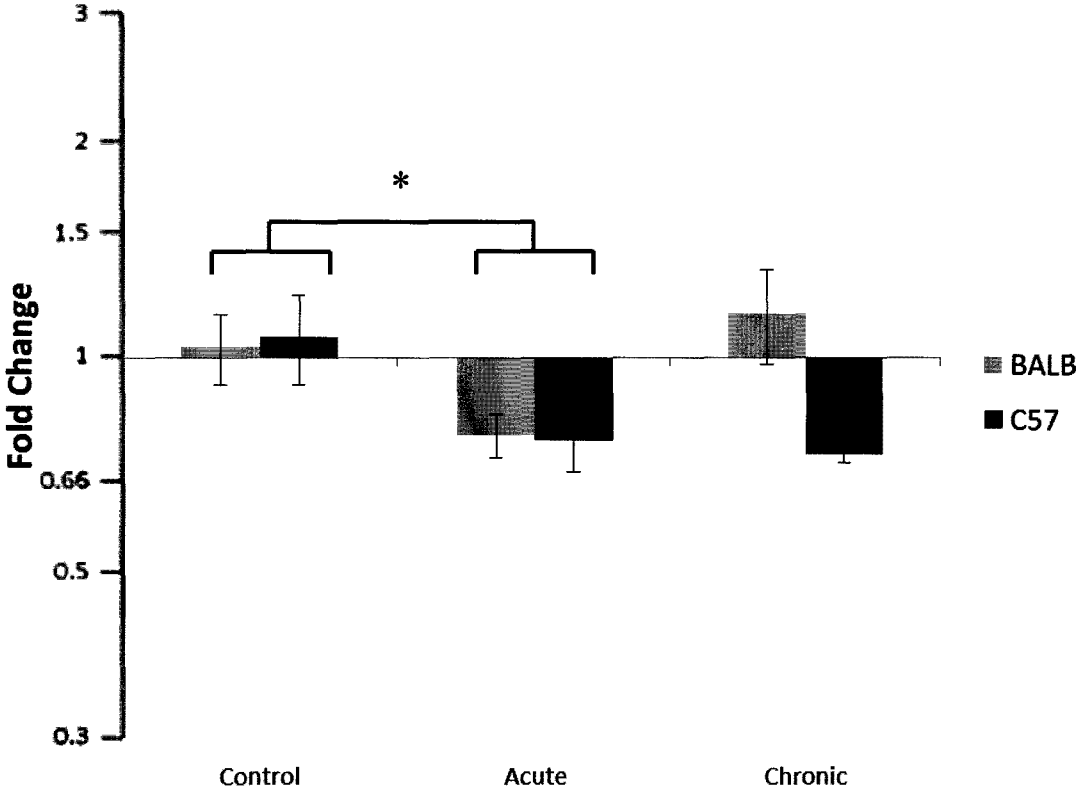

Figure 5. Gene expression profiles of miRNA-132 after stress. Data are mean fold change ± standard error. * indicates p<.05. Sample sizes were as follows: Control BALB/cJ: n = 6, Control C56BL/6J: n = 6, Acute BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Chronic BALB/cJ: n = 4, and Chronic C57BL6J: n = 5.

3.3b microRNA-134

The effect of strain was significant, $F(1,26) = 18.296$, $p<.001$, but there was no effect of treatment, $F(2,26) = .771$, $p=473$ (Figure 6) or interaction between strain and treatment, $F(2,26) = .248$, $p=.782$. Planned comparisons in BALB/c mice revealed a marginally significant effect of acute stress, which slightly decreased expression by 12% ($t(9) = 2.147$, $p=0.06$). Chronic stress had no effect on miRNA-134 expression, $t(3) = 0.289$, $p=0.792$. In C57BL/6 mice, no effect of acute, $t(7) = 0.134$, $p=.898$, or chronic stress, $t(9) = .770$, $p=0.461$, was observed.

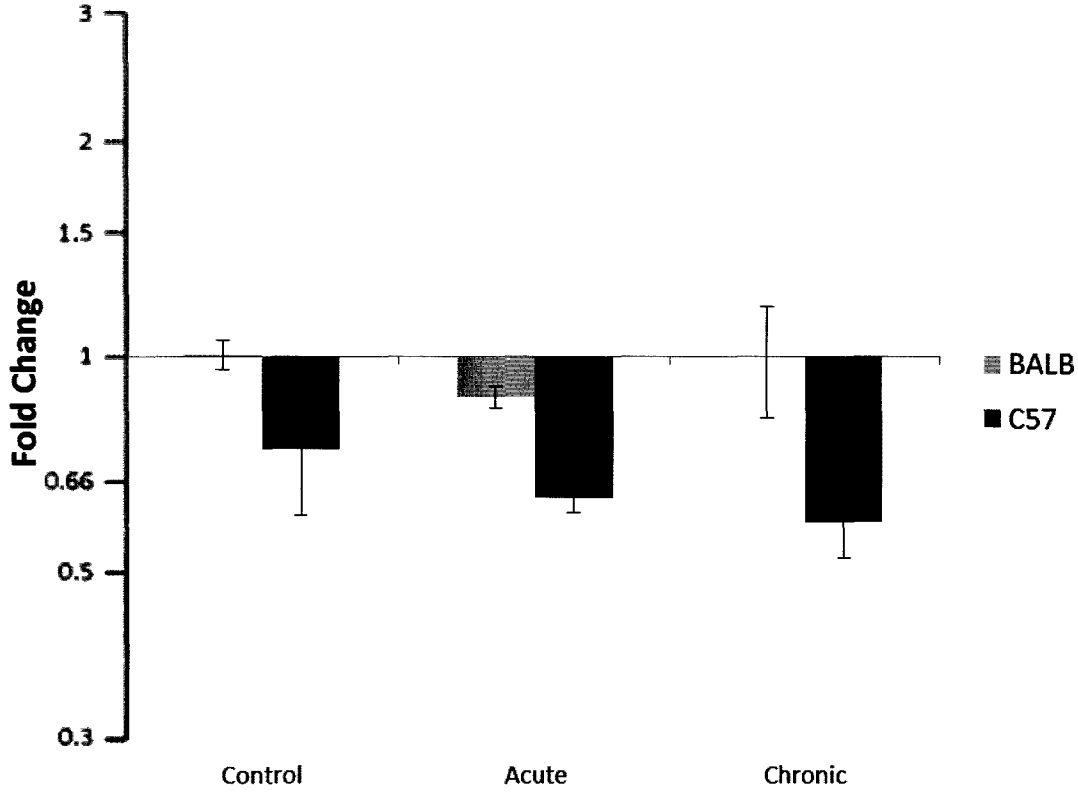

Figure 6. Gene expression profiles of miRNA-134 after stress. Data are mean fold change ± standard error. Sample sizes were as follows: Control BALB/cJ: n = 6, Control C56BL/6J: n = 6, Acute BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Chronic BALB/cJ: n = 4, and Chronic C57BL6J: n = 5.

3.3c microRNA-124

Like miRNA-134, the effect of strain was significant, $F(1,26) = 13.319$, $p<.005$, and the effect of treatment was not, $F(2,26) = 1.875$, $p=.173$ (Figure 7). The interaction of strain and treatment was also non-significant, $F(2,26) = .478$, $p=.626$. Planned comparisons revealed no effect of acute stress (BALB/c: $t(8) = -0.481$, $p=.643$ and C57BL/6: $t(9) = -0.798$, $p=0.445$) or chronic stress (BALB/c: $t(7) = 1.335$, $p=.224$ and C57BL/6: $t(8) = 0.328$, $p=0.751$) on miRNA-124 expression in either strain.

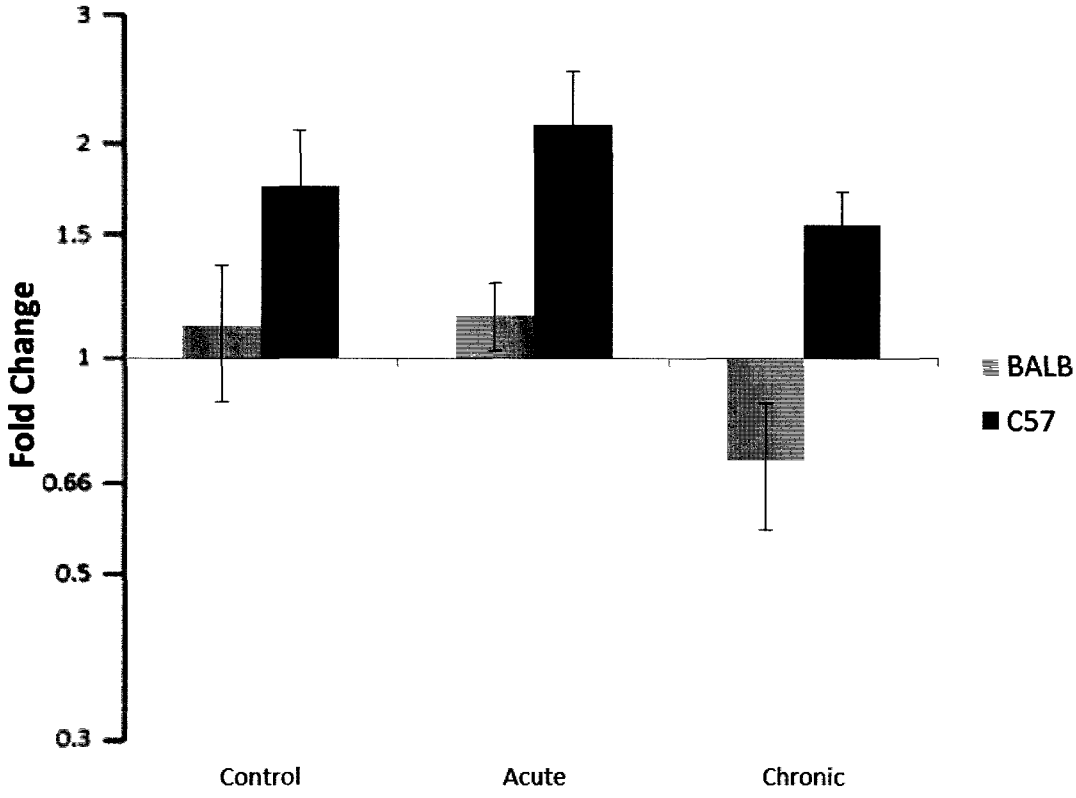

Figure 7. Gene expression profiles of miRNA-124 after stress. Data are mean fold change ± standard error. Sample sizes were as follows: Control BALB/cJ: n = 6, Control C56BL/6J: n = 6, Acute BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Chronic BALB/cJ: n = 4, and Chronic C57BL6J: n = 5.

3.3d microRNA-9

The effect of strain was significant, $F(1,26) = 16.334$, $p<.001$, and the effect of treatment was not, $F(2,26) = .450$, $p=.643$ (Figure 8). The interaction between strain and treatment was also non-significant, $F(2,26) = .248$, $p=.782$. The planned comparisons yielded no effect of acute stress (BALB/c: $t(9) = -0.381$, $p=0.712$ and C57BL/6: $t(8) = -0.715$, $p=0.495$) or chronic stress (BALB/c: $t(5)=-0.410$, $p=0.699$ and C57BL/6: $t(9) = 0.663$, $p=0.524$) on miRNA-9 expression in either strain.

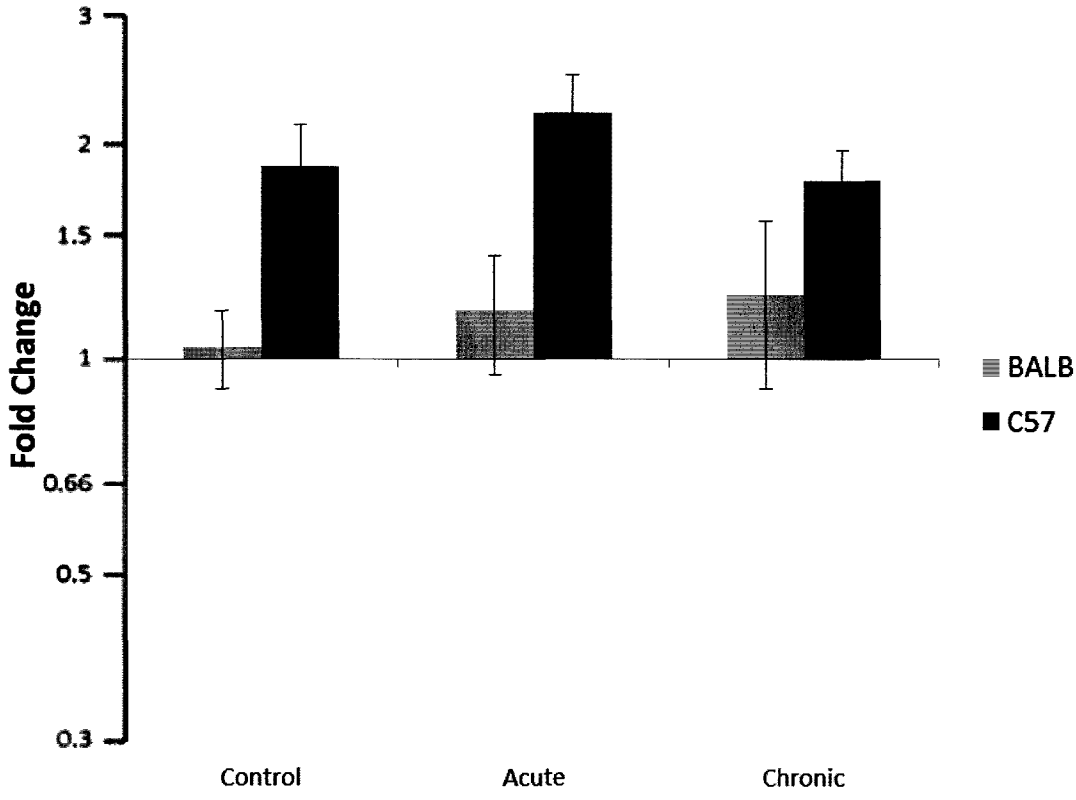

Figure 8. Gene expression profiles of miRNA-9 after stress. Data are mean fold change ± standard error. Sample sizes were as follows: Control BALB/cJ: n = 6, Control C56BL/6J: n = 6, Acute BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Chronic BALB/cJ: n = 4, and Chronic C57BL6J: n = 5.

3.3e microRNA-30a

There was no significant effect of strain, $F(1,26) = .254$, $p=.618$, or treatment, $F(2,26) = .180$, $p=.837$, on miRNA-30a expression (Figure 9). The interaction between strain and treatment was also non-significant, $F(2,26) = .165$, $p=.849$. T-tests found no effect of acute stress (BALB/c: $t(6) = 0.035$, $p=0.973$ and C57BL/6: $t(7) = 1.079$, $p=0.316$) or chronic stress (BALB/c: $t(6) = 0.060$, $p=0.954$ and C57BL/6: $t(8) = 0.384$, $p=0.711$) on miRNA-30a expression in either strain.

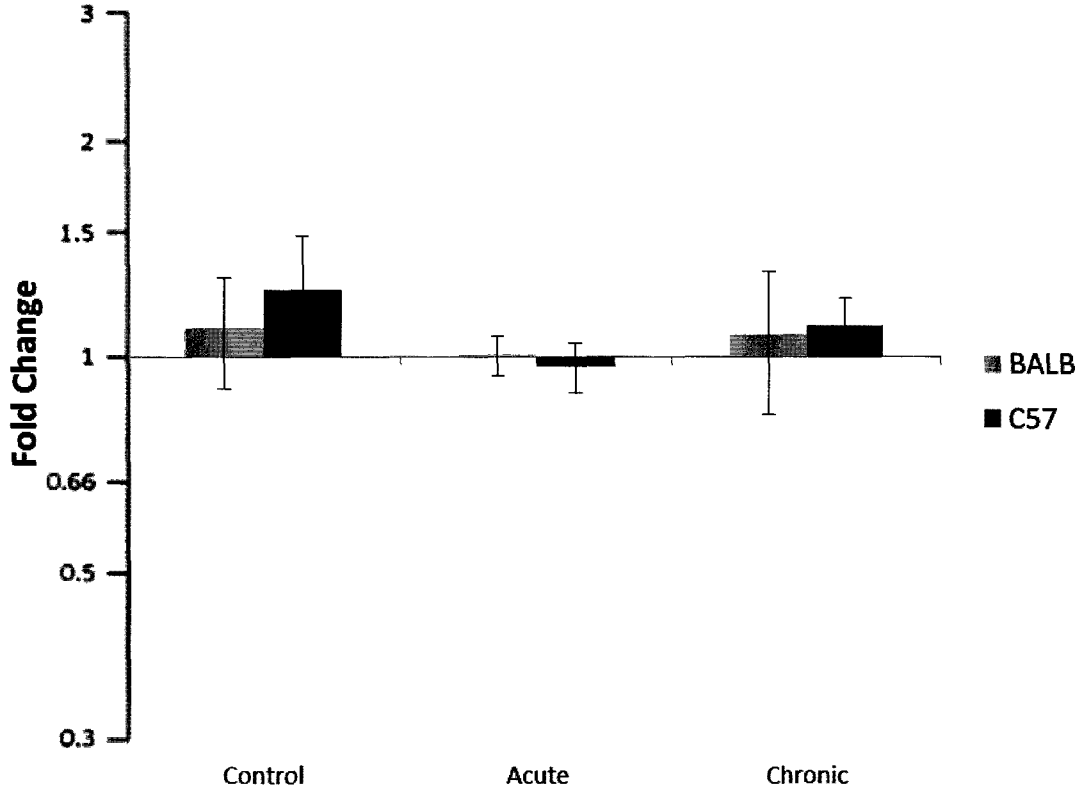

Figure 9. Gene expression profiles of miRNA-30a after stress. Data are mean fold change ± standard error. Sample sizes were as follows: Control BALB/cJ: n = 6, Control C56BL/6J: n = 6, Acute BALB/cJ: n = 6, Acute C57BL/6J: n = 5, Chronic BALB/cJ: n = 4, and Chronic C57BL6J: n = 5.

3.4 Predicted targets of miR-132

Using a miTG cut-off score of 0.650, which displayed the top 3% most likely genes to be targeted, 15 genes were predicted by DIANA-microT v.4 as being targeted by miR-132 (Table 1). Out of the 15 predictions, seven (Hbegf, Daam1, Shh, Paip2, Ep300, Mapk1, and Tjap1) were also predicted by TargetScan (235 targets total) and PicTar (156 targets total). Two genes in particular, Daam1 and Mapk1, have known roles in plasticity and stress-related processes in the brain.

Gene Name	Official Symbol	miTG Score	Also Predicted By	
			TargetScan	PicTar
Heparin-binding EGF-like growth factor	Hbegf	0.820	Yes	Yes
Dishevelled associated activator of morphogenesis 1	Daam1	0.759	Yes	Yes
Nucleus accumbens associated 2, BEN and BTB (POZ) domain containing	Nacc2	0.750	No	No
AT rich interactive domain 2 (ARID, RFX-like)	Arid2	0.722	No	Yes
RIKEN cDNA C130039O16 gene	C130039O16Rik	0.716	No	No
Zinc finger protein 644	Zfp644	0.713	Yes	No
MYC binding protein 2	Mycbp2	0.712	Yes	No
Sonic hedgehog	Shh	0.711	Yes	Yes
Neuro-oncological ventral antigen 1	Nova1	0.682	No	No
Polyadenylate-binding protein-interacting protein 2	Paip2	0.661	Yes	Yes
Jumonji, AT rich interactive domain 1A (Rbp2 like)	Jarid1a	0.659	No	No
RAR-related orphan receptor beta	Rorb	0.659	No	No
E1A binding protein p300	Ep300	0.658	Yes	Yes
Mitogen-activated protein kinase 1	Mapk1	0.656	Yes	Yes
Tight junction associated protein 1	Tjap1	0.653	Yes	Yes

*Adapted from microT v.4 (Maragkasis et al., 2009)

Table 1. Predicted targets of miR-132 ranked according to miTG score. DIANA microT v.4 was employed and a miTG score cut-off of 0.650 observed.

4.0 Discussion

The focus of the present study was on gene expression changes in the PFC induced by psychological stress. However, to evaluate the efficacy of the stressors, we first began by examining the peripheral effects of stress on the body. We found that chronic unpredictable stress resulted in reduced weight gain for both strains during the 21-day experimental period. This is consistent with previous studies suggesting that our experimental design was adequate in stressing the animals. For example, three weeks of unpredictable, chronic mild stress reduced weight gain in BALB/c mice (Farley et al., 2010), Sprague-Dawley rats (Wang et al., 2010), and CD-1 mice (Willner et al., 1996). Similarly, a seven week UCMS study using BALB/c mice decreased weight gain, but only after five weeks of stress (Surget et al., 2009). While stressor-induced blunted weight gain has been described often (Reber et al., 2006; Krishnan et al., 2007), the effect of stress on weight change depends on both strain and stressor type. For example, chronic social stress involving exposure to a bully mouse for three minutes a day for 10 days resulted in a significant weight gain for C57BL/6 mice, though the same study reported weight loss for socially stressed BALB/c mice (Savignac et al., 2011).

Stressor efficacy was also evaluated through corticosterone levels, measured following euthanasia 90 minutes after the end of the final stressor. Chronically and acutely stressed BALB/c mice displayed significantly higher levels than their respective controls. Significantly elevated levels were also found in chronically stressed C57BL/6 mice, together demonstrating that both acute and chronic stressor paradigms were effective. Under control conditions, strains did not differ in their corticosterone levels, consistent with previous literature (Nesher et al., 2011). Typically, chronic stressors, including footshock, social and restraint, result in

higher corsticosterone levels in BALB/c mice than in C57BL/6 mice (Shanks et al., 1990; Savignac et al., 2011; Browne et al., 2011), though our results suggest no difference between strains. Like the effect of stress on weight gain, it appears that the duration and type of stress may dictate the corticosterone response. Also, the above chronic stressor studies employed daily repetition of the same stress, and it is possible that habituation to stress may be resulting in decreased corticosterone in C57BL/6 in those studies, but not in our study which consisted of unpredictable stress. While no clear differences between strains were seen in weight gain or corticosterone levels, the stressor paradigms were nonetheless effective as determined by comparison with control animals, enabling examination of the impact of stressors on miRNA expression in the PFC.

To date, *in vivo* studies investigating miRNA expression changes after psychological stress have focused on the hippocampus and amygdala and to our knowledge, ours is the first to examine expression of miRNA-132 in the PFC after CUS. Our results displayed a significant decrease in expression levels after acute stress across both strains combined ($p<.05$), with BALB/c mice displaying a 24% decrease in expression after acute stress and C57BL/6 mice displaying a 28% decrease after acute stress. Work has previously focused on chronic exposure, and while we found no significant effect of chronic stress, there was a marginally significant 31% decrease in miR-132 levels. Using cell culture, miR-132 expression was shown to be upregulated by BDNF administration (Remenyi et al., 2010). Similarly, *in vitro* chronic pre-treatment with glucocorticoids, both for 48 or 72 hours, decreased BDNF-induced miR-132 expression and inhibited BDNF-dependent increases in postsynaptic protein levels (Kawashima et al., 2010). In the chronically stressed C57BL/6 mice of our study, the increased corticosterone

and corresponding marginally significant 31% decrease in levels of miR-132 are congruent with the Kawashima finding that glucocorticoids decrease miR-132 expression.

miRNA-132 was investigated because it has been suggested as a regulator of neuronal morphology, as expression leads to neurite outgrowth, while inhibition decreases observed outgrowth (Vo et al., 2005). Decreased miR-132 levels, via deletion of the miR-132 locus using Cre-Lox recombination, resulted in decreased dendritic length, arborization and spine density of neurons in the hippocampus (Magill et al., 2010). Given that chronic stress has been shown to decrease apical dendrite branches number and length in the mPFC (Cook & Wellman, 2004; Radley et al., 2004), these data coincide with the marginally significant 31% downregulation of miRNA-132 we found in C57BL/6 mice after stress. In contrast to our findings, miR-132 levels were upregulated in the hippocampus of rats after chronic immobilization stress (Meerson et al., 2010), suggesting that the stressor type and brain region studied may dictate miR-132 expression levels.

In *in vivo* work using transgenic mouse lines, miR-132 overexpression resulted in increased spine density and decreased methyl CpG binding protein 2 (MeCP2) levels in hippocampal neurons, as well as behaviourally impaired novel object recognition memory (Hansen et al., 2010). MeCP2 can act as a transcription factor, as binding of MeCP2 to the promoter of BDNF decreases BDNF expression (Chen et al., 2003), and when MeCP2 is knocked out, elevated BDNF levels and increased spatial memory are observed (Li et al., 2011), a finding suggesting that miR-132 may affect BDNF levels via alterations in MeCP2. *In vivo* studies confirmed that decreased miR-132 correlates with increased MeCP2 protein, but unaffected MeCP2 mRNA in mouse cortex (Lusardi et al., 2010). With regards to electrophysiological

experiments, miR-132 overexpression in cultured hippocampal mouse neurons led to paired-pulse facilitation (PPF) (Lambert et al., 2010), so the downregulation of miR-132 observed in our current study may impair PPF, which is consistent with the decrease in PPF in rat hippocampus following an acute stressor (Cazakoff & Howland, 2010). Taken together, the literature suggests that lowered miR-132 levels may contribute to, via interactions with BDNF, decreased synaptic plasticity and dendritic arborization which are trademark effects of chronic stress on the brain.

Potential targets of miR-132, found using software programs designed to predict targets based on complementarity to miRNA binding regions (Lewis et al., 2005; Betel et al., 2008; Maragkasis et al., 2009), include disheveled associated activator of morphogenesis 1 (Daam1) and mitogen-activated protein kinase 1 (Mapk1). These genes are expressed in the brain, and have known roles in processes affected by stress. Daam1 is expressed in dendrites and at the synapse, and is thought to effect morphology via interactions with the structural protein actin (Matusek et al., 2008). To date, no research has investigated expression of Daam1 in the PFC. However, overexpression of Daam1 in CA1 neurons of the hippocampus revealed a significant decrease in spine density but increase in spine length (Salomon et al., 2008). The decreased miR-132 in our study would therefore be predicted to increase Daam1 levels and decrease spine density.

Mapk pathway signaling is involved in many types of synaptic plasticity and memory (Sweatt, 2001). Specifically, Mapk1, also known as extracellular-signal-regulated kinase 1/2 (Erk1/2), is an enzyme involved in various cellular processes, such as dendritic mRNA transport (Nam et al., 2008). Acute footshock reduced and chronic footshock increased the number of Erk1/2 positive dendrites in the mPFC of rats (Kuipers et al., 2003), the latter finding which is

consistent with the marginally significant miR-132 decrease in chronically stressed C57BL/6 animals of our study. Also, adding to the complexity of miR-132-BDNF-Mapk/Erk interactions, U0126, an inhibitor of the Mapk/Erk pathway, suppressed BDNF-increased miR-132 levels in rat cerebral cortex cell culture (Kawashima et al., 2010), suggesting that Mapk/Erk is required for interactions between miR-132 and BDNF. Future studies could examine the impact of under- and over-expression of miR-132 on mRNA transcripts and protein levels of Daam1 and Mapk1.

Our results did not show an effect of stress on miRNA-9 expression levels. This does not support previous studies displaying an almost three-fold increase in miR-9 in frontal cortex of CD-1 mice following a two-hour acute restraint session (Rinaldi et al., 2010) and downregulation after one session or 14 days of 4-hour restraint stress daily (Meerson et al., 2010), though the latter study investigated the amygdala and hippocampus of rats. Likewise, miR-124 was not affected in our study, though the Meerson study found a significant downregulation in the hippocampus following acute stress (Meerson et al., 2010). The discrepancy between our study and the Meerson and Rinaldi studies may be due to the model organism used, the types of stressors administered and the length in days of the chronic stressor administration, all variables which differed between the previous studies and ours.

There are many follow-up studies based on the current research that should be performed. With regards to methodology, UCMS and CUS are very commonly used, but social stress is another area currently receiving much attention, with many studies using chronic social defeat stress as a model for depression while examining neurotrophic, neuroendocrine and behavioural alterations (Gomez-Lazaro et al., 2011; Covington et al., 2010; Wagner et al., 2011). Future studies should examine levels of the miRNAs investigated in our current study

after social defeat stress instead of CUS, primarily because physiological effects such as corticosterone levels and weight change differ between the two paradigms (Savignac et al., 2011), and determine whether there is a different effect on miRNA expression depending on the nature of the stress experienced. Using microarrays or candidate gene RT-PCR, other stressor paradigms such as prenatal stress (Pascual et al., 2010) and early post-natal stress (Peleg-Raibstein & Feldon, 2011) can be used, which may clarify the effects that early environmental stressors can have on gene expression and nervous system development, a current topic of interest in miRNA research (Gao, 2010; Eda et al., 2011). Also, the PVN, hippocampus and CeA are all brain regions known to be affected by stress and future studies should focus on these regions, as in many cases the justification for choosing our candidate genes came from studies examining those regions (ie. miR-134 expression in the hippocampus, (Schratt et al., 2006)). Though the five miRNAs examined here are good starting points, our approach is clearly not exhaustive. The candidate gene approach is a first step, however, and future studies on psychological stress should employ high-throughput gene analysis techniques such as miRNA microarrays.

First, to ensure our miR-132 finding was not a false positive, replication studies using larger sample sizes should be performed to increase confidence in our result. Also, utilization of locked nucleic acid-*in situ* hybridization experiments could reveal spatial expression patterns of miRNAs not detectable using RT-PCR (Obernosterer et al., 2007), and lead to new hypotheses of specific miRNA function. Subsequently, based on our discussion of miRNA involvement in dendritic branching and synaptic plasticity, a logical follow-up step would be to conduct immunohistochemistry studies on PFC tissue from stressed animals to determine whether or

not the miRNA changes we have observed, measured using RT-PCR, are consistent with expected morphological changes at the synapse. Up- and down-regulation of miR-132 in cell culture, via Cre-Lox recombination for example, could also be used to first examine the effects of altered miR-132 expression on BDNF mRNA expression levels, illustrated via RT-PCR or in-situ hybridization, and then second, examine effects on BDNF protein levels using Western Blotting. Also, our current study did not address any implications of CUS, or apparent gene expression changes, on behavior or cognition. Though no studies to our knowledge have examined miRNA-knockdown and behavior, experiments that knock-out miR-132, via aforementioned Cre-Lox recombination (Magill et al., 2010), could be used to see what effect alterations of miRNA-132 levels have on anxiety-related behaviours, measured by time spent in the open arm of the EPM and visits to the center of the OF (Post et al., 2011).

Lastly, our study examined the effect of psychological stress on gene expression in order to elucidate potential changes underlying affective disorders such as depression, disorders often preceded by chronic stress. Our study, however, focused solely on male animals and given the increased incidence of depression in women (Naninck et al., 2011; Grigoriadis & Robinson, 2007), and the fact that the HPA axis is influenced by sex hormones (Marques et al., 2009), studies comparing gene expression levels after stress between both genders should be performed.

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