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The role of miR-137 in schizophrenia

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THE ROLE OF MIR-137 IN SCHIZOPHRENIA

by

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DISSERTATION

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Dedication

For my grandfather,

Dr. Donald Plymale, who taught me the worth of hard work and the value of science.

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Abstract

The genetic basis of schizophrenia is still largely unknown. Recent evidence suggests that a microRNA (miRNA), miR-137 may be involved. The first large schizophrenia genome wide association (GWAS) study, published in 2011, identified a variant within the host gene of this miRNA (*MIR-137*) as the top association. Since then, further evidence for the potential influence of this miRNA within the disorder has accumulated. These studies used a variety of methods from GWAS to neuroimaging. However, few studies have evaluated the mechanism for the association of the *MIR-137* variant, nor how alterations in the miRNA may have downstream effects on its targets and the function of these targets within biological pathways. We hypothesized that the target genes of miR-137 are

involved in schizophrenia relevant pathways, such as those affecting neuronal development and plasticity, and that the *MIR-137* risk-associated variant may lead to altered regulation of such pathways. We predict that variants within the targets of the miRNA may further alter target regulation.

Here we completed the following three aims to determine the potential impact of dysregulation by this miRNA in schizophrenia. 1) We characterized the targets of the miRNA and the pathways over-representing these genes. 2) We determined the schizophrenia-risk association of these targets within these target enriched pathways. 3) We evaluated the influence of variants within target genes of an enriched pathway and the previously associated miR-137 host gene variant on structural gray matter.

Our results suggest that the targets of this miRNA are indeed involved in pathways relevant to neuronal development and plasticity and thus the development of schizophrenia. The target genes within these pathways contain variants associated with schizophrenia that may disrupt regulation by the miRNA. Variants within targets of the PKA signaling pathway coupled with the *MIR-137* variant may influence the development of gray matter within the occipital, temporal, and parietal lobe.

Overall, our studies further suggest that this miRNA is influential in schizophrenia and provide a map for future studies to determine the effects of dysregulation by miR-137 in the disorder.

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1. Introduction

1.1 Overall Significance

Schizophrenia is a severe mental disorder that greatly impacts the quality of life of those affected. Current medications have debilitating side effects and are ineffective in treating all associated symptoms. (Rummel-Kluge et al., 2012 and van Os, and Kapur, 2009) Heritability estimates suggest that the illness is highly heritable; however, the source of all estimated heritability remains unexplained (Toulopoulou et al., 2010). Attempts to determine these sources have demonstrated that genetic risk is likely conferred by many different genes each of modest contribution (Purcell et al., 2009). Revealing these modest risk contributor genes could uncover new drug targets that may lead to more effective treatments.

miRNA are a class of noncoding RNA molecules that post-transcriptionally regulate the expression of genes. Single miRNA molecules have the capacity to regulate the expression of hundreds of genes. A role for such regulators in the basis of schizophrenia is compelling given the polygenic evidence for the underlying genetic architecture.

The first large schizophrenia genome wide association study (GWAS) identified a single nucleotide polymorphism (SNP) within the host gene of miR-137 (*MIR-137*) as the top associated variant (Ripke et al., 2011). Subsequent larger GWAS have confirmed an association with this locus (Ripke et al., 2014). In vitro studies indicate that this miRNA influences several processes critical for proper neuronal development (Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010), suggesting that this host gene variant could affect these functions. Imaging genetics studies show that risk allele carriers

with schizophrenia have uniquely altered structural and functional MRI measures as compared to risk allele carriers that are healthy controls (Lett et al., 2013; Whalley et al., 2012). These findings, in agreement with the polygenic risk evidence (Purcell et al., 2009), suggest that additional genetic alterations may account for the distinct effect of this variant in patients. However, the mechanisms responsible for such findings are not known.

Alterations in genes regulated by miR-137 coupled with dysregulation by this miRNA may synergistically contribute to schizophrenia risk. Pathways highly enriched with such genes may be particularly sensitive to enhancement of risk and therefore may reveal the role of this miRNA in the disorder. Therefore determination of the possible target genes, pathways enriched with such genes, and the impact of such genes on schizophrenia risk and imaging measures may be instrumental in discovering the impact of this miRNA and reveal undiscovered sources of heritability.

1.2 miRNA mediated regulation of gene expression

1.2.1 miRNA evolutionary significance

Despite the fact that non-coding DNA does not code for proteins, non-coding DNA makes up roughly 98.8% of the human genome (Amaral et al., 2008). Genomic comparisons across species indicate that the ratio of noncoding DNA to protein coding DNA is increasingly larger in more complex and higher order species, and humans appear to have the largest proportion of noncoding DNA (Mattick, 2004). This suggests that noncoding DNA may be responsible for many of the newly evolved processes and functions in higher order species and when altered may contribute to the diseases that

impact these processes. One particular class of noncoding RNA molecules coded by noncoding DNA, microRNA (miRNA) appears to increase with greater species complexity (Sempere et al., 2006). In fact many miRNAs were identified to be primate specific (Bentwich et al., 2005) and an estimated 1% of brain expressed miRNAs were found to be unique to humans when comparing chimps and humans (Wienholds, 2005). Furthermore expression and functional differences of even well conserved miRNAs across species has been identified, suggesting that specific manipulations of these molecules may in part drive the evolution of more complex functions in higher order organisms. (Ason et al., 2006; Niwa and Slack, 2007) Given that miRNA play a critical role in fine tuning higher complexity processes, alterations in their function may lead to disease states in which these processes are impaired.

1.2.2 miRNA biogenesis and post-transcriptional regulation

Discovered in 1993 in *Caenorhabditis elegans* (Lee et al., 1993), miRNAs are a small noncoding RNA molecules of approximately 22 nucleotides in length that post-transcriptionally regulate the expression of specific genes (Bartel and Chen, 2004). Individual miRNA molecules specify genes for regulation through complimentary binding of miRNAs to respective mRNA molecules (Bartel, 2009). miRNA influence and control a variety of processes, from controlling developmental gene expression in C. elegans (Lee et al., 1993), to rapid cellular response of environmental stimuli in neuronal plasticity (Olde Loohuis et al., 2012). Alterations in miRNA function are associated with a variety of diseases from cardiovascular illness, to neurodegenerative disorders, to cancer (Ruepp et al., 2010).

miRNAs are first transcribed in the nucleus, generally by RNA polymerase II, either from non-coding regions of the genome or less commonly within an intron of a protein coding gene. These latter transcripts of a less common origin termed mirtrons skip the first step of miRNA biogenesis, namely the processing of the primary transcript. Primary or pri-miRNAs, are cleaved within the nucleus by an enzyme complex called the microprocessor which contains the RNAse III enzyme Drosha and targeting protein DiGeorge syndrome critical region gene 8 (DCGR8). This processing leads to production of the secondary stage for these transcripts called pre-miRNA. In contrast, mirtrons are spliced out like a regular intron to form the pre-miRNA (Guo and Lu, 2010; Huang et al., 2014; Lin et al., 2006; Schwarz et al., 2003). At this stage, the transcript is exported to the cytoplasm via Exportin-5 for further processing to reach a final stage of maturity. In the cytoplasm these pre-miRNAs are targeted by a protein complex containing Dicer, the trans-activation response RNA binding protein (TRBP), and the protein activator of the interferon induced protein kinase (PACT). This protein complex cleaves the terminal loop of the pre-miRNA to produce a double stranded short RNA sequence. Either of these strands can act as the mature miRNA for subsequent post-transcriptional regulation and target different genes for regulation, but generally one strand is more commonly active. This phenomenon of strand bias is called strand asymmetry. Usually the strand with the least thermodynamically stable 5'end, most often derived from the 5' arm of the pre-miRNA stem loop, serves as the guide strand to guide mRNA silencing. The other strand called the passenger strand is later degraded. (Guo and Lu, 2010; Huang et al., 2014; Schwarz et al., 2003) The mature strand is taken up and bound by the microRNA induced Silencing Complex (miRISC) containing Argonaute 2 (Ago 2), which has

endonuclease activity and used by the complex to target complementary mRNA sequences and to repress subsequent protein expression. (Carthew and Sontheimer, 2009; Cuperus et al., 2011; Lindow and Gorodkin, 2007) In most instances, miRNAs lead to a reduction or silencing of subsequent protein expression, either via translational repression or mRNA degradation (Carthew and Sontheimer, 2009; Filipowicz et al., 2008).

1.2.3 Interactions of miRNA and mRNA

miRNAs target mRNAs by binding to small sequences generally located in the 3' UTR of the mRNA. However, some miRNA target sequences located within the 5' UTR or coding sequence of a mRNA. The cognate miRNA binding sequence is referred to as the seed and most often consists of the first 2-8 nucleotides of the 5' UTR. (Filipowicz et al., 2008) These seed sequences within miRNA often perfectly match their cognate mRNA sequences. These cognate mRNA sequences appear to have had evolutionary pressure to be maintained and are often found to be conserved across many species. Other seed sites however appear to be recently evolved. Sequences adjacent to the seed-binding site or elsewhere in the 3'UTR of a miRNA called 3'-supplementary sites and 3'-compensatory sites may help to stabilize binding or compensate for imperfect seed binding respectively (Bartel, 2009). Research suggests that an adenine at position 1 of the targeted mRNA sequence may be involved in recognition of mRNA targets by the miRISC.

Multiple types of seed pairing sequences have been observed. The canonical or more frequently observed pairing consists of bound sequences of 7-8 nucleotides in length between the miRNA seed and the mRNA, as well as frequent pairing between the mRNA and the first nucleotide of the miRNA or the nucleotide directly after the seed sequence in

position 8. Less frequently, nucleotide parings of only 6 nucleotides in length or those of imperfect seed pairing with 3'-compensatory site pairings have been observed.(Bartel, 2009)

miRNA targets can be predicted using computer based algorithms or determined using experimental validation studies. A variety of miRNA target prediction algorithms are available with varied requirements based on seed site complementarity, binding thermodynamic stability, surrounding sequences, and conservation. Different algorithms also investigate different mRNA regions; some only evaluate the 3'UTR while others evaluate 5'UTRS and coding regions. (Lindow and Gorodkin, 2007) Experimental validation studies often examine the expression of potential targets in response to the miRNA of interest. New methods such as those of transcriptomics include: microarray profiling to test target level expression following transfection of miRNAs in cellular assays, RNA sequencing to detect changes in mRNA levels, high-throughput sequencing of RNA isolated by crosslinking immunopercipitation (HITS-CLIP) to find miRNA-mRNA-Ago complexes, and many more rapidly developing methods. In vitro assays include luciferase assays using transfections of plasmids containing the luciferase coding region with the 3' UTR of predicted targets along with precursors for the miRNA. In addition, studies using western blots of protein products of predicted targets, and animal studies of miRNA knockouts and over-expression are used to study the effect of specific miRNAs. Current annotations of experimentally validated targets are not very extensive, but with advancing target detection methodologies these annotations will soon be much more extensive. (Tarang and Weston, 2014)

1.2.4 Summary

miRNA are a class of noncoding RNAs that regulate the expression of specific genes. Each individual miRNA has the capacity to target hundreds of genes and thus can have great impact on a variety of biological processes. miRNA regulation alterations also appear to play a major role in a variety of diseases.

1.3 miRNA alterations in disease

Alterations in miRNA mediated gene regulation has been linked to nearly every variety of disease (Ruepp et al., 2010) including schizophrenia. These alterations may affect the biogenesis or function of the miRNA, or affect targeting of the miRNA to its respective targets (Sun et al., 2009). The following sections review current literature about miRNA alterations in phenotypes and diseases.

1.3.1 miRNA alterations

SNPs and copy number variations (CNVs) located within miRNA host genes or their regulatory regions can impact miRNA biogenesis and function and lead to subsequent alterations affecting phenotypes and diseases (Duan et al., 2009). There are several vulnerabilities associated with miRNA biogenesis that can be affected by SNPs and CNVs. Like protein coding genes, miRNA host gene transcription is regulated by a transcription factors, activators, or epigenetic alterations and accordingly any variant impacting regulation of transcription could have consequences on miRNA expression (Filipowicz et al., 2008; Slezak-Prochazka et al., 2010). miRNA biogenesis is often regulated by negative feedback loops either through auto-regulation by a miRNA itself or through double negative feedback loops by specific miRNA targets (Li and Carthew,

2005). Therefore, SNPs or CNVs affecting the expression of a miRNA may have compounding consequences if the miRNA is self-regulating. Variants affecting the expression of regulatory target genes may also effect miRNA expression and thus may influence regulation of other target genes. Processing of the miRNA is also vulnerable to genetic variation. Terminal loop binding proteins, such as KSRP or Lin28 can inhibit or facilitate the actions of miRNA processing enzymes, Dicer and Drosha. Alterations in the pri-miRNA or pre-miRNA sequences can alter the binding of these loop binding proteins. (Filipowicz et al., 2008; Slezak-Prochazka et al., 2010). Variants in these sequences can also alter the way in which Dicer and Drosha cleave these premature miRNAs. This can lead to a shifted or altered seed sequence, alter which of the two strands is loaded into the miRISC complex, or change the processing rate by potentially altering premature miRNA stability (Sun et al., 2009) thus impacting regulation. Alterations in pri-miRNA and pre-miRNA sequences that are otherwise harmless could also theoretically lead to adenosine deaminase acting on RNA (ADAR) modifications that then subsequently lead to a shift in the seed sequences (Kawahara et al., 2007; Luciano, 2004)

Most examples of SNPs and CNVs impacting miRNA biogenesis and function have been best demonstrated so far in Cancer. One such example found a SNP associated with reduced lung cancer survival that caused a significant increase in the mature form of miR-196a2, but no alteration in the premature miRNA levels (Hu et al., 2008). Interestingly, the same genotype for the same SNP also appears to increase risk of congenital heart disease and increase levels of the mature form of miR-196a2 in cardiac tissue (Xu, et al., 2009). A few studies outside of cancer have also examined miRNA SNP consequences. One study identified ultra-rare variants associated with schizophrenia

that also appear to alter the biogenesis and function of a several miRNAs (Feng et al., 2009).

1.3.2 Target gene alterations

SNPs and CNVs altering miRNA target genes can also have impact on miRNA induced regulation. Due to the double-negative feedback loop between target genes and respective miRNAs, any expression changes in target genes through a variety of mechanisms (such as activators, repressors etc.) could affect the biogenesis of cognate miRNAs and secondarily cause downstream changes on additional target gene expression. Variants within target genes can also alter the targeting of miRNAs by altering target binding. There are a few cases where such a variant is associated with disease. One example includes a polymorphism in the seed site of the serotonin receptor 1B mRNA that disrupts regulation by miR-96 by reducing binding by the miRNA. This polymorphism is associated with conduct disorder phenotypes in humans (Jensen et al., 2008). It is also possible for variants to create new target genes by increasing the potential for miRNA binding. Such novel target genes created by SNPs have been associated with obesity related phenotypes (Richardson et al., 2011) and plasma triglyceride levels (Caussy et al., 2014).

1.3.2.1 Consequences of variants in seed site sequences

Clearly sequence changes within a target gene "seed site" in the region that binds to the seed of miRNA, could alter binding by the miRNA. The sequence alterations can either increase or decrease binding. There are two known cases of such alterations impacting diseases of the nervous system. A de novo chromosome 13 inversion associated with

Tourette's syndrome was found to cause a frameshift mutation in the binding sequence of SLITRK1 and increases binding strength for miR-189 (Abelson, 2005).A SNP identified as a risk factor for Parkinson's disease disrupts the miR-433 binding site within the target gene FGF20 and decreases binding. FGF20 expression was found to be increased with the risk genotype for this SNP (Wang et al., 2008).

1.3.2.2 Consequences of variants in 3' UTRs

Variants within 3'UTRs but outside of seed site sequences have been associated with phenotypes and diseases. A polymorphism near but not within the binding site for miR-24 within the target gene DHFR reduced repression of this target gene by the miRNA. The authors speculate that perhaps binding by the miRISC complex may be disrupted by this SNP and thereby reduce miRNA induced repression. (Mishra et al., 2007) It is also possible that SNPs within the 3' UTR but outside of the seed site of target mRNAs may alter binding availability of the mRNA by the miRNA due to secondary structure alterations of the mRNA (Day et al., 2014; Haas et al., 2012). Evidence suggests that polymorphisms associated with chronic Achilles tendinopathy and located within the 3'UTR of the miR-608 target COL5A1 alter the secondary structure of the target mRNA 3' UTR and thus may disrupt binding availability of the miRNA (Abrahams et al., 2013).

1.3.3 Summary

Variants can impact miRNA biogenesis, function, and targeting and individual examples have linked such variants to diseases and phenotypes. Variants may occur within miRNA host genes or within the targets of a miRNA. More specific examples of such variants

impacting diseases and phenotypes are likely to be identified soon. Alterations in miRNAs and their target genes may occur in schizophrenia.

1.4 Schizophrenia

1.4.1 Incidence and impact

Every year, roughly 15 out of 100,000 individuals develops schizophrenia. The average point prevalence of the disorder is 4.5 people out of every 1000. Lifetime risk for the development of the illness ranges from 0.3 to 2% and this risk is increased with urban childhood environment, migration, early social stress, maternal infection, cannabis use, early life infection, vitamin D deficiency, and male gender among other factors. The incidence of schizophrenia appears to be stable over time and appears to be neither increasing nor decreasing. (Tandon et al., 2008)

More than two-thirds of all cases are sporadic, meaning that most individuals are the first in their family to develop the disorder. However, familial prevalence greatly increases the risk of development. Monozygotic twin studies indicate that the risk of schizophrenia development is about 50% for a currently unaffected twin when the other is diagnosed. This risk is10-15% for non-twin siblings. (Tandon et al., 2008) These studies also provide measurements for heritability. Heritability of a disease is a measure of the proportion of disease risk that is due to genetic effects, either directly or indirectly through environmental interaction. The heritability of schizophrenia is estimated to be 81% (Sullivan PF, 2003). The genetic sources of such heritability remains largely unknown (van Os and Kapur, 2009).

Schizophrenia greatly impacts the wellbeing of those diagnosed, as well as caregivers and family (Hanzawa et al., 2013). Unfortunately current treatments are only partially effective (Blanchard et al., 2011) and are often associated with challenging side effects (Rummel-Kluge et al., 2012). Schizophrenia remains one of the costliest mental disorders and leads to additional secondary health detriments and early mortality of 12-15 years earlier than the average population, (Thornicroft et al., 2004 and van Os, and Kapur, 2009). Patients are largely unable to work, are limited in major daily activities, and have overall lower quality of life (Thornicroft et al., 2004). Lifetime suicide risk is 4.9% in patients (Palmer et al., 2005), which is significantly higher than the average population. This rate appears to have increased since the 1970s, although it may be more recently declining (Bushe et al., 2010). Overall schizophrenia is a very severe disorder with widespread detrimental impact.

1.4.2 Symptoms and onset

Schizophrenia has been described similarly as it is today for about one century (Tandon et al., 2008). Bleuler coined the term schizophrenia in 1911 to describe the "split-mind" like state of those affected (Moskowitz and Heim, 2011). Schizophrenia is a form of psychosis; however, there are many other forms of psychosis that differ from the illness in various ways. The illness is described by three types of symptoms: negative, positive, and cognitive. The negative symptoms refer to reduced motivation, reduced sociability, and decreased expressivity and speech. Positive symptoms are characterized by delusion and hallucinations. Cognitive symptoms often include memory impairment, executive functioning impairment and alterations in attention. (van Os and Kapur, 2009) Unlike other forms of psychosis, affective symptoms are generally less severe. Onset of the

illness typically occurs in early adulthood, although it can occur in childhood or late in life. Onset in males is often earlier than in females (Godar and Bortolato, 2014).

1.4.3 Genetic architecture

Determination of the genetic contributors underlying schizophrenia risk has been challenging due to several limiting factors. Diagnostic criteria are still evolving and show an overlap with several disorders, suggesting that some genetic mechanisms may be common to a particular symptom-type rather than disease. Indeed, risk loci identified in GWAS studies that may affect calcium signaling are common to autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia (Smoller et al., 2013). On the other hand, the heterogeneous presentation of symptoms in different subjects suggests that there may actually be several distinct forms of schizophrenia and each may have a unique genetic basis. It is possible that there are some shared underlying genetic commonalties across disorders that associate with a particular symptom-type and that predispose for development of a host of disorders, while other genetic causative factors may be specific to each disorder (Bertolino and Blasi, 2009). This suggests that studies to determine both types of genetic factors will be helpful.

To further complicate the search for the underlying genetic basis of schizophrenia, association studies suggests that the underlying architecture is highly polygenic, heterogeneous and muddled with environmental interactions, incomplete penetrance, epistasis, and pleiotropy (Bertolino and Blasi, 2009). In other words, it appears that genes may have different effects in different subjects, that many genes are playing a role, and

that these genes are interacting with one another and the environment. Consequently, the potential for a role of a miRNA, which clearly interact with many genes and respond quickly to environmental factors, in schizophrenia seems highly probable.

Common and rare variants both appear to play a role in schizophrenia (Sullivan et al., 2012). Given the potential for variants to disrupt miRNA regulation and have many downstream effects in multiple systems and pathways it is compelling to suggest that such alterations may occur in the underlying genetic basis of schizophrenia.

1.4.4 Summary

The genetic architecture of schizophrenia is very complex. Many genes appear to be involved and may have many interactive genetic and environmental partners. Further GWAS are necessary to examine the genetic basis of psychiatric symptom dimensions as a continuum across disorders, as well as those evaluating schizophrenia subtypes to tease out the genetic factors that are common and unique to specific subject groups.

1.5 miR-137 and schizophrenia

More and more studies are elucidating an important role for miRNA in psychiatric illness and schizophrenia. miRNAs are found to be differentially expressed in postmortem tissue and plasma of schizophrenia patients (Beveridge et al., 2009; Gardiner et al., 2011; Kim et al., 2010; Lai et al., 2011; Perkins et al., 2007; Santarelli et al., 2011). A deletion in chromosome 22, 22q11.2, is the strongest genetic factor for schizophrenia risk (Forstner et al., 2013). This deletion encompasses the DGCR8, of the microprocessor complex. A subsequent decrease in miRNA biogenesis may be responsible for the link between the deletion and schizophrenia risk. (Forstner et al., 2013). Alterations in the miRNA

processing protein Dicer and its gene *DICER1* are also associated with schizophrenia (Sanders et al., 2013) further suggesting that miRNA biogenesis may be altered in schizophrenia. Of all miRNA, miR-137 has an especially large level of evidence for involvement in the nervous system and its possible influence in schizophrenia. The following sections review the current evidence for the potential impact of miR-137 in schizophrenia.

1.5.1 miR-137 functional studies

miR-137 is expressed in brain tissue (Guella et al., 2013; Smrt et al., 2010; Sun et al., 2011) and localized to synaptosomes (Siegel et al., 2009). The miRNA appears to regulate neurogenesis and dendritic spine morphogenesis. Over-expression and antagonism studies suggest that a particular level of miR-137 expression is necessary for proper control of neuronal proliferation and differentiation of newborn neurons, in the hippocampus. development (Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010).

More recent studies have evaluated the functional role of the miRNA in the nervous system in a more pathway driven manner. One such study examined RNA expression changes following miR-137 over-expression and inhibition within a human neural progenitor cell line and found an overrepresentation of these genes in the neuronal differentiation pathway (Hill et al., 2014). These results confirm previous findings implicating a role for this miRNA in neurogenesis. Another similar study also evaluated pathways enriched with genes of altered expression following miR-137 over-expression in human neuronal stem cells. Apparent targets enriched pathways including those

involved with the major histocompatibility complex (MHC), synapse formation, and calcium channel signaling (Collins et al., 2014).

All of the above implicated pathways are relevant to schizophrenia. Neurogenesis is linked with cognition (Couillard-Despres et al., 2011). Consequently, neurogenesis alterations may mechanistically underlie the cognitive deficits associated with the disorder (O'Reilly et al., 2014; Toro and Deakin, 2007). Synapse formation (Yang et al., 2014), calcium signaling (Mukherjee et al., 2014 and Berridge, 2013), and the MHC (Mukherjee et al., 2014) have all been previously associated with schizophrenia. Overall, the functional studies of miR-137 demonstrate that the miRNA is an important regulator of the nervous system and may be involved in mechanisms expected to be altered in schizophrenia.

1.5.2 Genetic variant evidence

The first large scale schizophrenia GWAS identified 5 new common SNPs associated with the disorder (Ripke et al., 2011). This study utilized genotypic data from over 50,000 subjects all of European Ancestry. The top newly associated SNP was located in an intron of the host gene for miR-137. The other four newly associated SNPs were located in genes that are now verified targets of the miRNA including: CSMD1, C10orf26, CACNA1C, and TCF4 (Kwon et al., 2013). An analysis with 3,000 Han Chinese subjects confirmed schizophrenia-association of this miR-137 host gene SNP and the top SNPs from the CACNA1C gene (Guan et al., 2013). This further suggests that these SNPs may indeed play a role in the illness. Combining the data of this first

large scale GWAS with additional subjects, another SNP located near the miR-137 host gene was identified to be newly associated with the disease as well (Ripke et al., 2013).

A recent study identified two variants in the miR-137 host gene that occurred in higher frequency in patients with schizophrenia or bipolar disorder than controls in a cohort including just over 2,000 subjects. These variants reduced expression of the mature miRNA in a neuronal-like cell line (SH-SY5Y). Transcriptome analysis demonstrated that the reduced miR-137 expression disrupted regulation of many genes. Gene set enrichment analysis of these genes revealed that many of these deregulated genes are involved in glutamate signaling and synaptogenesis, particularly those of the protocadherin family. (Strazisar et al., 2014) Unfortunately this study did not analyze the effect of the GWAS implicated SNPs (Ripke et al., 2013, 2011) on miRNA expression, however it is possible that these SNPs may be merely tagging other variants that impact miR-137 biogenesis. One post mortem tissue study has however analyzed the influence of the first large GWAS identified miR-137 SNP (rs1625579) genotype on expression of the miRNA. This small study found a non-significant decrease in miR-137 expression within the dorsolateral prefrontal cortex (DLPFC) in both patient and control risk allele carriers and a significant decrease when evaluating controls only. Interestingly, expression of the target gene TCF4 appeared to be increased in risk allele carriers. (I. Guella et al., 2013).

1.5.3 Imaging genetics evidence

Many studies have evaluated the impact of the rs1625579 miR-137 host gene variant on imaging measures with varied results. In Lett et al., 2013, schizophrenia patients who

were carriers of the risk allele were found to have reduced white matter integrity, reduced hippocampal volume and increased lateral ventricle volume measures. No significant structural difference was identified between control subjects and schizophrenia subjects who were not carriers of the risk allele. These findings suggest that the impact of the risk SNP may be distinct in patients. This is in agreement with another study that analyzed only healthy subjects and found no effect of the genotype on white matter integrity measures of whole-brain fractional anisotropy or mean diffusivity (Kelly et al., 2014). Also suggestive of the genotype having a schizophrenia distinct effect, recent work in our lab demonstrates that the risk allele is associated with significantly increased mid-posterior corpus callosum volume in patients while trending toward a decrease in control carriers (Patel et al., unpublished observation). A functional magnetic resonance study (fMRI) also found distinct genotype effects on schizophrenia at risk subjects as compared to bipolar at risk subjects and healthy controls. Schizophrenia at risk subjects who were carriers of the risk allele showed increased activation of the amygdala and pre- and post-central gyrus during a sentence completion task as compared to non-carriers, while controls showed the opposite genotypic effect on activation. The bipolar at risk subjects had a control-like genotypic effect in the amygdala but no genotypic effect in the pre and post central gyrus. (Whalley et al., 2012) This further suggests that the risk genotype has specific effects in schizophrenia.

Other findings suggest that some effects of the genotype may be common to both schizophrenia and healthy control subjects. Within the Whalley et al., 2012 study, a main effect of genotype was identified across the three diagnostic groups, as greater activation during the sentence completion task was found in the posterior right medial frontal gyrus

in non-homozygotes of the risk allele (Whalley et al., 2012). In another fMRI study, higher (DLPFC) activation during the Sternberg Item Response Paradigm memory task was found in both control and patient risk allele carriers, though there was no genotypic effect on task performance. (van Erp et al., 2014) Hyperactivation of this region during working memory tasks is consistently found in schizophrenia and so the risk genotype may in part genetically underlie this associated phenotype. Healthy controls who were also risk allele homozygotes had significantly increased functional connectivity between the right amygdala and the cingulate gyrus and the right inferior frontal gyrus during a facial processing task (Mothersill et al., 2014). In this study, the healthy subjects who were homozygotes did not show increased activity in the amygdala, as was shown by Whalley et al., 2012, where only the schizophrenia homozygotes demonstrated increased activation. The extent to which the risk genotype alters connectivity in patients has not yet been examined. It is possible that connectivity is increased due to a common genotype effect in patients and controls and additional increased amygdala activity in homozygous patients may be due to a schizophrenia specific genotype effect. Finally, a resting state fMRI study on 290 healthy subjects found a genotype effect on DLPFC and hippocampal formation connectivity and working memory performance prediction (Liu et al., 2014).This has not been studied in schizophrenia patients so it is unclear if this genotypic effect is common to both or if there is more of an impact in patients. Further studies are required to determine the impact of this genotype on structure and functional measures. No studies have yet analyzed the impact of target SNPs in addition to the genotype on imaging measures.

1.5.4 Summary

Increasing evidence suggests that miRNAs are involved in psychiatric illness and schizophrenia. There is substantial evidence that miR-137 in particular may be involved, from functional in vitro studies, to genetic association studies, to imaging genetics studies. This warrants further examination as to the role of this miRNA and its targets in schizophrenia.

2. Rationale, Hypothesis, and Specific Aims

2.1 Research rationale

The genetic basis of schizophrenia is largely unknown and current treatments are largely inadequate in treating negative and cognitive symptoms. Increased understanding for the genetic basis may allow for the discovery of more effective treatments. The first large schizophrenia GWAS with a large number of participants, identified a SNP within the host gene of miR-137 (Ripke et al., 2011). Studies suggest that proper miRNA expression is critical for many neurodevelopmental processes(Guella et al., 2013; Smrt et al., 2010; Sun et al., 2011). Imaging genetics studies of the identified host gene variant found alterations in structural and functional MRI measures (van Erp et al., 2013; Lett et al., 2013; Whalley et al., 2012). Postmortem tissue analysis suggests that expression of the miRNA may be altered in carriers of the risk variant (Guella et al., 2013). miRNAs have the capacity to regulate the expression of hundreds of genes. (Bartel, 2009). Thus, miRNAs have the potential to play extensive roles in polygenic disorders, and schizophrenia is a polygenic disorder (Purcell et al., 2009).

Given the functional role of the miRNA, the findings in the studies of the implicated variant, and the compelling capacity for miRNA regulation alterations in polygenic disorders, evaluation for the role for miR-137 in schizophrenia is warranted. Only limited studies have analyzed the impact of the target genes of this miRNA in schizophrenia. Disease associations with dysregulated miRNA-mRNA interactions due to target gene polymorphisms have been identified (Jensen et al., 2008; Richardson et al., 2011; Wang et al., 2008). Pathways highly enriched with miR-137 targets may be especially vulnerable to dysregulation by the miRNA as multiple genes tightly linked in cascading

functions may be disrupted. Evaluation of such pathways may assist in uncovering the biological mechanism for the risk association of the implicated *MIR137* variant and eventually assist in uncovering new drug targets.

2.2 Hypothesis

miR-137 target genes play a role in the pathophysiology of schizophrenia by disrupting target enriched pathways involved in neuronal development and plasticity.

2.3 Specific aims

2.3.1 Specific aim 1

Characterize through bioinformatics the putative and validated miR-137 target genes and the potential significance of these genes in schizophrenia:

1) Determine the putative and experimentally validated target genes of miR-137 and evaluate for inclusion of schizophrenia-associated genes

2) Evaluate and compare the distribution of miR-137 target genes across the genome to genomic regions with schizophrenia associated CNVs.

3) Evaluate the temporal expression pattern for miR-137 target genes to determine if such genes have a unique expression pattern that might mirror time points of development, schizophrenia onset, or deterioration.

4) Evaluate target genes using ingenuity pathway analysis (IPA) for significant canonical pathways and networks to determine the possible impact that these targets if regulation by miR-137 was altered.

2.3.2 Specific aim 2

Evaluate miR-137 target gene contribution to schizophrenia risk:

1) Determine schizophrenia-risk association for lists of predicted and validated target

2) Determine schizophrenia-risk association of pathway-specific target gene sets for pathways vulnerable to dysregulation by miR-137

2.3.3 Specific aim 3

Evaluate miR-137 target gene contribution to structural gray matter alterations in schizophrenia:

1) Determine effects of risk associated polymorphisms within target genes on structural gray matter

2) Evaluate interaction effects of the *MIR137* variant and target gene variant on structural gray matter

3. Potential Impact of miR-137 and Its Targets in Schizophrenia

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3.1 Abstract

The significant impact of microRNAs (miRNAs) on disease pathology is becoming increasingly evident. These small non-coding RNAs have the ability to post-transcriptionally silence the expression of thousands of genes. Therefore, dysregulation of even a single miRNA could confer a large polygenic effect. Schizophrenia is a genetically complex illness thought to involve multiple genes each contributing a small risk. Large genome-wide association studies identified miR-137, a miRNA shown to be involved in neuronal maturation, as one of the top risk genes. To assess the potential mechanism of impact of miR-137 in this disorder and identify its targets, we used a combination of literature searches, Ingenuity Pathway Analysis (IPA), and freely accessible bioinformatics resources. Using TargetScan and the Schizophrenia Gene Resource (SZGR) database, we found that in addition to CSMD1, C10orf26, CACNA1C, TCF4, and ZNF804A, five schizophrenia risk genes whose transcripts are also validated miR-137 targets, there are other schizophrenia-associated genes that may be targets of miR-137, including ERBB4, GABRA1, GRIN2A, GRM5, GSK3B, NRG2 and HTR2C. IPA analyses of all the potential targets identified several nervous system functions as the top canonical pathways including synaptic long-term potentiation, a process implicated in learning and memory mechanisms and recently shown to be altered in patients with schizophrenia. Among the subset of targets involved in nervous system development and function, the top scoring pathways were Ephrin receptor signaling and axonal guidance, processes that are critical for proper circuitry formation and were shown to be disrupted in schizophrenia. These results suggest that miR-137 may indeed play a substantial role in the genetic etiology of schizophrenia by regulating networks involved in neural development and brain function.

3.2 Introduction

First discovered in *C. elegans* (Ruvkun et al., 2004), miRNAs are small noncoding RNA sequences that play a significant role in the regulation of gene expression, particularly at the post-transcriptional level. Regulation by miRNAs is a complex process, in which some miRNAs are capable of targeting and repressing hundreds to even thousands of transcripts (Selbach et al., 2008). Additionally, many mRNAs are targeted by several miRNAs (Hu et al., 2008; Selbach et al., 2008). It is estimated that the expression of at least 30% of human genes are regulated by miRNAs (Lewis et al., 2005; Selbach et al., 2008). With such high levels of potential regulatory influence, some miRNAs may have enormous impact on gene expression and such an impact may play a role in the pathophysiology or etiology of diseases with an elusive genetic basis, such as schizophrenia. This disease is genetically complex and very little is understood about its genetic basis or underlying mechanisms (Hamshere et al., 2012). Several recent lines of research suggest that miRNAs may be involved. First, the gene encoding the DiGeorge syndrome critical region gene 8 protein (DGCR8), one of the components of the nuclear miRNA processing complex, is located in a chromosomal location (22q11.2) associated with high risk for schizophrenia (Stone et al., 2008). Also, a SNP in the gene for a particular miRNA, miR-137 was found to be one of the common alleles associated with schizophrenia (Kwon et al., 2013; Ripke et al., 2011). This review examines the role of this miRNA in brain development and function and explores the potential functional impact of its known and putative targets on schizophrenia. By identifying the putative and validated miR-137 targets, and examining their potential contribution to functional networks, we hope to shed more light on the possible role of this miRNA in the etiology of the disease.

3.2.1 microRNA

microRNAs (miRNAs) are small non-coding RNAs with the ability to silence the expression of multiple target genes by binding to specific sequences of mRNAs. A single miRNA can impact hundreds to thousands of targets and can affect pathways controlling a large variety of processes, from normal development to oncogenesis. Pairing is primarily based on sequence complementarity of a "seed" sequence within the miRNA to a binding site of the mRNA, generally in the 3' UTR of the mRNA being suppressed (Bartel, 2009). The mechanisms by which miRNAs suppress gene expression are still not well elucidated; however mRNA destabilization and translational repression have been demonstrated as dominant methods of repressing subsequent protein expression (Carthew and Sontheimer, 2009).

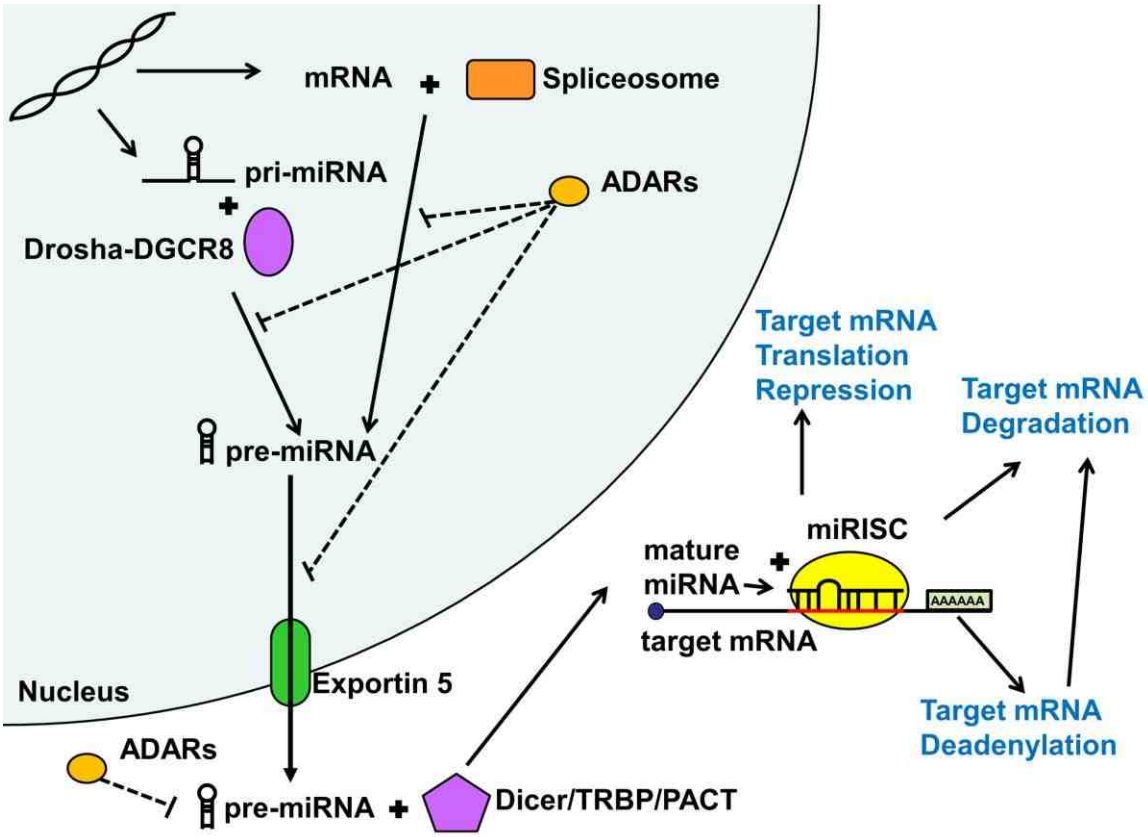

Figure 3.1 MicroRNA biogenesis and function. MicroRNAs (miRNA) are generated via the mirtron or canonical pathway. Primary microRNA (pri-miRNA) from the canonical pathway is further processed by the Drosha-DGCR8 complex into precursor miRNA (pre-miRNA). Pre-miRNAs derived from both pathways are then exported from the nucleus by exportin 5. In the cytoplasm, this pre-miRNA is further processed by the Dicer/TRBP/PACT complex into a duplex miRNA. One of the strands (guide strand or mature miRNA) is then loaded into the miRISC complex along with the target mRNA. Depending on the degree of sequence complementary, miRNAs lead to translational repression, deadenylation or degradation of the target mRNAs, all reducing downstream protein expression. Adenosine deaminases acting on RNA (ADARs) may inhibit various steps of miRNA processing thus reducing or shifting miRNA function. See text for more detail.

As shown in Figure 3.1, miRNAs are either first transcribed in the nucleus as primary miRNAs (pri-miRNAs) or less commonly spliced from introns (Lin et al., 2006). The pri-miRNAs are then processed in the nucleus by the microprocessor complex involving the RNAse III enzyme Drosha complexed with the targeting protein DGCR8 into approximately 89 nucleotide long sequences termed pre-miRNAs (Carthew and

Sontheimer, 2009; Cuperus et al., 2011; Lindow and Gorodkin, 2007). Intron derived miRNAs termed mirtrons are excised by the RNA spliceosomal components and do not require further processing. These pre-miRNAs are then exported out of the nucleus by Exportin5 (Lindow and Gorodkin, 2007) and further processed in the cytoplasm by a protein complex containing Dicer and its associated proteins the *trans*-activation response RNA binding protein (TRBP) and the protein activator of the interferon induced protein kinase (PACT) into an approximately 22 nucleotide long double stranded RNA (Carthew and Sontheimer, 2009). These double stranded sequences then separate and a single strand termed guide strand (mature miRNA) is loaded with a complementary mRNA into an Argonaute containing microRNA-induced silencing complex (miRISC) where the miRNA ultimately binds the target sequence (Carthew and Sontheimer, 2009) to repress ensuing protein expression.

Adenosine deaminases acting on RNA (ADARs) can also modify and regulate miRNA function, both in the nucleus and cytoplasm (Figure 3.1). Adenosine deaminases can modify miRNA processing and function not only by editing pri- and pre-miRNA sequences but also through steric hindrance in the absence of RNA editing (Heale et al., 2009). Alterations in miRNA sequence can lead to shifted (Kawahara et al., 2007), reduced, or eliminated targeting or reduction in mature miRNA production(Luciano, 2004). ADAR miRNA editing seems to play an important role in mammalian brain development (Ekdahl et al., 2012). Surprisingly, very few studies have addressed the potential role of ADAR mRNA editing in psychiatric illness, with conflicting results (Silberberg et al., 2012; Zhu et al., 2012). However, given that the studies only addressed

mRNA editing effects, there may be an alternative large scale regulatory impact of ADARs on miRNA regulation of gene expression, which remains to be explored.

3.2.2 Schizophrenia and miRNAs

Schizophrenia is a severe mental illness that has an average lifetime development risk of 0.7%, with an average annual incidence rate of 15 per 100,000 (Blanchard et al., 2011; Tandon et al., 2008). Heritability of the disease is estimated at 81% based on twin studies (Sullivan PF, 2003). The term schizophrenia meaning, "split mind" was coined by Bleuler in 1911 to describe the dissociation of thought, ideas, identity, and emotion that characterize the illness (Moskowitz and Heim, 2011). The disease is described by negative symptoms of social withdrawal, positive symptoms of psychosis including hallucination and delusion, cognitive impairment, and in some cases mood dysregulation (van Os and Kapur, 2009). These symptoms lead to secondary disparities in health and premature mortality, stressing the need for better understanding of the underlying mechanisms.

This mental illness is largely heterogenetic, with different patients having different associated genetic alterations and varied symptomology (Green et al., 2012; Wahlsten, 2012). Evidence also suggests that it is a polygenic disorder, in which many common genetic variants may each contribute a small increased risk (Purcell et al., 2009). Given this evidence for such polygenicity and the fact that single miRNAs have the potential to regulate hundreds to thousands of transcripts (Selbach et al., 2008), it seems plausible that disruption of a miRNA could lead to abnormal expression levels of many genes, which could in turn contribute to schizophrenia vulnerability. Many miRNAs were found

to be differentially expressed in blood samples and postmortem tissue of patients with schizophrenia, (Beveridge et al., 2009; Gardiner et al., 2011; Kim et al., 2010; Lai et al., 2011; Miller et al., 2012; Moreau, et al., 2011; Perkins et al., 2007; Santarelli et al., 2011) providing further evidence that miRNAs may impact the disorder.

One miRNA of particular interest is hsa-miR-137. The recent study from The Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium (Ripke et al., 2011), with an initial sample size of 21,856 and a replication sample of 29,839 found several new significant loci to be associated with schizophrenia, the strongest association being the single-nucleotide polymorphism (SNP) rs1625579 within an intron containing the primary transcript of miR-137. Several of the other associated loci contained predicted targets of miR-137 (Ripke et al., 2011) supporting evidence that miR-137 might play a role in the disorder. A subsequent smaller GWAS study examining a smaller sample of patients with treatment-resistant schizophrenia from the United Kingdom receiving clozapine treatment, also found significant association for CACNA1C but did not replicate the finding for CSMD1 or miR-137, yet when the data was grouped with the earlier GWAS study both were found to be significant (Hamshere et al., 2012).

Besides GWAS studies, two additional experimental approaches are beginning to shed light onto the possible link between miR-137 and schizophrenia. First, using luciferase reporter assays, Kwon et al., 2011 confirmed that transcripts of other genes associated with schizophrenia in the largest GWAS study (Ripke et al., 2011), namely CSMD1, C10orf26, CACNA1C, and TCF4 can in fact be regulated by miR-137. Likewise, (Kim et al., 2012) recently demonstrated that ZFN804A another gene highly associated with the

illness (O'Donovan et al., 2008; Steinberg et al., 2011) can also be silenced by miR-137 in vitro. While further research is required to confirm the association of the SNP in miR-137 with risk for schizophrenia, the findings that miR-137 can regulate the expression of other schizophrenia associated genes provides new clues on how miR-137 may play a role in the illness. Along these lines, a recent post-mortem tissue study suggests that the T risk allele of rs1625579 may be associated with decreased miR-137 expression in the dorsolateral prefrontal cortex of patients (Guella et al., 2013), further suggesting a potential dysregulation of the miRNA's targets.

3.2.3 Role of miRNA in brain development and function

miR-137 is expressed in embryonic and adult brains (Sun et al., 2011) and was found to be highly enriched in synaptosomes from P15 rats (Siegel et al., 2009). Using *in situ* hybridization high expression of this microRNA was observed in the dentate gyrus, an area highly active in adult neurogenesis (Smrt et al., 2010).The involvement of this miRNA in neuronal development was confirmed by several functional experiments. Overexpression of miR-137 was shown to decrease proliferation of mouse embryonic neural stem cells leading to their premature neuronal differentiation (Sun et al., 2011) and similar effects were observed in adult mouse neural stem cells derived from the subventricular zone or from brain tumors (Silber et al., 2008). However, overexpression of miR-137 in the adult neural stem cells derived from the subgranular layer of the dentate gyrus was found to disrupt the expression of stage specific differentiation markers such as DCX and NeuN as well as dendritic arborization in these newly generated neurons (Smrt et al., 2010). This apparent discrepancy is likely due to distinct roles of miR-137 in different stages of neuronal differentiations as well as the intrinsic properties

of cells in different neurogenic zones. In a recent study, overexpression of miR-137 was shown to decrease maturation and increase proliferation while antagonism of miR-137 in adult neural stem cells increased neuronal differentiation and reduced proliferation (Szulwach et al., 2010). These experiments suggest that a balanced expression of miR-137 is necessary to maintain appropriate neuronal differentiation and proliferation and thus regulate neurogenesis. Given the important role that neurogenesis plays in learning, memory, and mood regulation, disruption of these essential functions may have significant effects that could lead to some of the symptomology seen in schizophrenia (DeCarolis and Eisch, 2010).

3.2.4 Association of miR-137 and cognitive function

Recent genetics imaging studies have also found a correlation of miR-137 with schizophrenia. Firstly, utilizing gene set enrichment analysis to assess the contribution of gene regulatory networks to the illness, (Potkin et al., 2010) found that miR-137 was implicated in two individual GWAS imaging genetics studies of patients performing the Sternberg Item Recognition Paradigm (SIRP) working memory task (Potkin et al., 2009a; Potkin et al., 2009b). Given the findings of the large 2011 GWAS study (Ripke et al, 2011), a subsequent study examining subjects at risk for schizophrenia and bipolar disorder differentiated their cohorts based on this SNP (Whalley et al, 2012). Subjects with two copies of the "T" risk allele were assigned as risk positive and those carrying one or no copies of the allele were classified as risk negative subjects. A reduced response in the right posterior medial frontal gyrus region to increasingly difficult sentence completion tasks was noted only in the risk positive group across all the groups: schizophrenia at risk, bipolar at risk, and controls. This suggests that miR-137 may have

a general effect on executive function. Also, schizophrenia at risk subjects had differential activation of the left amygdala and left pre/postcentral gyrus, suggesting a more schizophrenia-specific effect of the allele as well (Whalley, et al., 2012).

Recent studies also examined the role of this miRNA on other functional endophenotypes. The risk allele in miR-137 was potentially associated with the P300 endophenotype in schizophrenia patients (Decoster et al., 2012). In addition, when combined with greater negative symptoms, the rs1625579 SNP genotype predicted membership of patients in a subgroup with severe cognitive deficits, (Green et al., 2012). In this study, cognitive functioning was evaluated by a battery of tests such as the Letter-Number Sequencing test to assess working memory, and the Controlled Oral Word Association Test to assess executive function. First patients were categorized based on their performance with this battery of tests into either a cognitive deficit group or a cognitive spared group. Patients with more severe negative symptoms and the "G" protective allele were surprisingly more likely to have been previously grouped in the cognitive deficit group (Green et al, 2012). Another study examining carriers of the risk allele among psychosis patients, including those with schizophrenia, schizoaffective disorder and bipolar affective disorder I, found that carriers, particularly those homozygous for the "T" risk allele, had lower scores for psychotic symptoms and a subtle deficit in performance of episodic memory and attention control tasks (Cummings et al., 2012).

While studying the clinical effects of chromosome 1p21.3 microdeletions, the region containing the *MIR137* gene,(Willemsen et al., 2011) found an association with intellectual disability and autism disorder spectrum-like behavior. Furthermore,

lymphoblastoid cell lines from these patients were found to have reduced levels of miR-137 and enhanced levels KLF4 and the previously verified target genes MITF and EZH2. The authors also confirmed that miR-137 is highly expressed in the hippocampus, occipital cortex, and frontal cortex in human postmortem tissue, as well as in the synaptosomal fractions in mouse brain preparations, providing further evidence that miR-137 may play a role in synapse formation during brain development and function.

3.3 Targets of miR-137

To further understand the possible mechanism of miR-137 in schizophrenia, we used available databases and the literature to identify putative and validated targets. Using the list of potential and experimentally-verified targets, we then evaluated their chromosomal location and temporal patterns of expression. Finally, we examined how these targets cluster within biological pathways to identify which functions would be affected if miR-137 levels were dysregulated as shown by initial post-mortem tissue studies (Guella et al, 2013).

3.3.1 Putative targets

Putative targets were identified by querying the publically available TargetScan Human release 6.2 database updated June 2012 for hsa-miR-137 (Friedman et al., 2009a). Selecting for target genes respective to site conservation resulted in 1144 putative target genes. The Ensemble cytoband location for each gene was identified by querying for each gene offered in the freely available GeneCards encyclopedia at www.genecards.org (Stelzer et al., 2011). All subsequent analyses were performed using this list, as TargetScan offers several advantages over other target prediction algorithms given its

unique consideration for sequence context in addition to conservation and seed sequence complementarity (Friedman et al., 2009; Grimson et al., 2007). This list was then evaluated to identify targets studied for experimental validation in the following section.

Examining genes that may play a role in schizophrenia, we compared our putative target lists against a schizophrenia-associated gene list of 278 protein-coding genes from the publicly available schizophrenia gene resource (SZGR) ,a database of a variety of schizophrenia related gene lists (Jia et al., 2010). We chose to use the association studies gene list that is derived from the SchizophreniaGene (SZGene) database and further evaluated for consistency across studies using a combined odds ratio (OR) method (Sun et al., 2008). Of the 1144 TargetScan putative target list, 25 genes intersected with the SZGR schizophrenia associated gene list. These genes are listed in Table 3.1, including cytoband location information identified from GeneCards. Estimating that there are 20,000 genes in the genome, and that 278 are considered risk genes for schizophrenia, the probability that a randomly chosen sample of 1144 genes contains 25 or more of these risk genes is 0.017. Therefore, this result suggests that miR-137 targets are enriched in schizophrenia risk genes.

Gene Symbol	Full Name	Cytoband
ACSL6	acyl-CoA synthetase long-chain family member 6	5q31.1
ATXN1	ataxin 1	6p22.3
BRD1	bromodomain containing 1	22q13.33
C18orf1	chromosome 18 open reading frame 1	18p11.21
CHGA	chromogranin A (parathyroid secretory protein 1)	14q32.12
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2q34
FOXP2	forkhead box P2	7q31.1
FXYD6	FXYD domain containing ion transport regulator 6	11q23.3
FZD3	frizzled homolog 3 (Drosophila)	8p21.1
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	5q34
GRIA1	glutamate receptor, ionotropic, AMPA 1	5q33.2
GRIA4	glutamate receptor, ionotropic, AMPA 4	11q22.3
GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A	16p13.2
GRM5	glutamate receptor, metabotropic 5	11q14.3
GSK3B	glycogen synthase kinase 3 beta	3q13.33
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	Xq23
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	18p11.21
MLC1	megalencephalic leukoencephalopathy with subcortical cysts 1	22q13.33
NRG2	neuregulin 2	5q31.2
NRG3	neuregulin 3	10q23.1
PLXNA2	plexin A2	1q32.2
SYN2	synapsin II	3p25.2
SYN3	synapsin III	22q12.3
TNXB	tenascin XB; tenascin XA pseudogene	6p21.33
TSNAX	translin-associated factor X	1q42.2

Table 3.1. SZGR associated miR-137 target genes*

*This list is derived from the intersection of the 1144 TargetScan putative target list with the SZGR schizophrenia associated gene list. Cytoband location information was identified using GeneCards.

3.3.2 Experimentally verified targets

Twenty-six experimentally verified targets were identified using the publicly available database TarBase (Vergoulis et al., 2012) and manual literature searches (Table 3.2). The functional targeting of these targets by miR-137 was confirmed by luciferase expression reporter and Western blot assays in a variety of cell lines. A PubMed search of miR-137 resulted in 44 articles, 16 of which had abstracts mentioning target gene validation experiments, which were further evaluated for their relevance. Only targets reported in articles using functional validation assays were included except for KLF4, which was confirmed by qPCR in an animal model over-expressing miR-137 (Willemsen et al, 2011). A subsequent search of hsa-miR-137 yielded no more unique articles from the previous search. Of note, ZNF804A, a gene implicated in schizophrenia in several studies (O'Donovan et al., 2008; Steinberg et al., 2011; Walton et al., 2013) (O'Donovan et al., 2008 Steinberg et al., 2011, and Walton et al., 2012) was experimentally verified (Kim et al., 2012) although it was not included in the TargetScan putative target list, presumably because of the poor conservation of its binding site.

Verified Target	Assay	Cell Lines	Reference	Source
CTBP1	Ago2 binding assay, luciferase assay	HEK293, A375	Deng et al., 2011	TarBase
CDC42	Western blot, luciferase assay	SW116, Lovo, Hela, AGS, MKN45	Liu et al., 2011; Chen et al., 2011a	TarBase
CDK6	luciferase assay, Western blot	U251,OSCC, HEK293	Silber et al., 2008; Kozaki et al., 2008; Chen et al., 2011b	TarBase
KDM1A (LSD1)	Luciferase assay, Western blot	HCT116, HEK293, neural stem cells	Balaguer et al.2010; Sun et al., 2011	TarBase, Literature Search
E2F6	Western blot	OSCC	Kozaki et al. 2008	TarBase
NCOA2	Western blot	OSCC	Kozaki et al. 2008	TarBase
MITF	luciferase assay, GFP reporter	HEK293, A375, WM852	Haflidadóttir et al., 2010; Chen et al., 2011b; Bemis et al., 2008	Literature Search
KDM5B (Jarid1b)	Western blot, luciferase assay	mouse ESC, HEK293	Tarantino et al., 2010	Literature Search
SPTLC1	luciferase assay	rat primary astrocytes	Geekiyanage and Chan, 2011	Literature Search
PTBP1	luciferase assay	Neuro2a cells	Smith et al., 2011	Literature Search
CSMD1#	luciferase assay	HEK-293T	Kwon et al., 2011	Literature Search
C10orf26#	luciferase assay	HEK-293T	Kwon et al., 2011	Literature Search
CACNA1C#	luciferase assay	HEK-293T	Kwon et al., 2011	Literature Search
TCF4#	luciferase assay	HEK293T	Kwon et al., 2011	Literature Search
CDK2	Western blot	M23 and SP6.5	Chen et al., 2011b	Literature Search
RB1 (p-Rb)	Western blot	M23 and SP6.5	Chen et al., 2011b	Literature Search
MAPK1 (p-ERK1/2)	Western blot	M23 and SP6.5	Chen et al., 2011b	Literature Search
MAPK3 (p-ERK1/2)	Western blot	M23 and SP6.5	Chen et al., 2011b	Literature Search
MET (c-Met)	Western blot	M23 and SP6.5	Chen et al., 2011b	Literature Search
ESRRA	luciferase assay	HepG2	Zhao et al., 2012	Literature Search
PTGS2 (Cox-2)*	Western blot, luciferase assay	U87 and LN229	Chen et al., 2012	Literature Search
MIB1	luciferase assay	DIV6 primary neurons	Smrt et al., 2010	Literature Search
MSI1	Western blot, luciferase assay	U251,Daoy, HeLa	Vo et al., 2011	Literature Search
EZH2	luciferase assay	HEK293T	Szulwach et al., 2010	Literature Search
KLF4	quantitative reverse transcription PCR in overexpressing miR-137 animal model	LCL	Willemsen et al., 2011	Literature Search
ZNF804A	luciferase assay	HEK293T, Be2C	Kim et al., 2012	Literature Search

Table 3.2. Experimentally verified targets of hsa-miR-137

*indicates genes in SZGR associated list

# indicates genes associated with schizophrenia but not in SZGR list

3.3.3 Chromosomal location of target genes

Cytoband data was gathered from the UCSC database table browser, assembly dated February 2009 http://genome.ucsc.edu/ (Karolchik, 2004). miR-137 target gene location data was identified using the GeneCards database. This data was graphed using the Matlab Bioinformatics Toolbox (Figure 3.2). Target genes are located throughout the genome with a few localized "hot spots" (shown by red vertical lines in the figure). There are several "hotspots" located in Chromosomes 1, 11, 12, 16, and one each in chromosome 3,14,17,19, and X. Comparison of the cytoband locations with miR-137 targets and those known to be affected by copy number variations (CNVs) in schizophrenia and autism spectrum disorders (ASD) (Liu et al., 2012; Sullivan et al., 2012) revealed several regions of overlap. While the proportion of overlapping cytobands did not reach global statistical significance with a hypergeometric probability test, it is important to note that one of these regions mapped to NRXN1, a 2p16.3 gene with associated deletions in both schizophrenia and ASD and whose transcript is a putative miR-137 target.

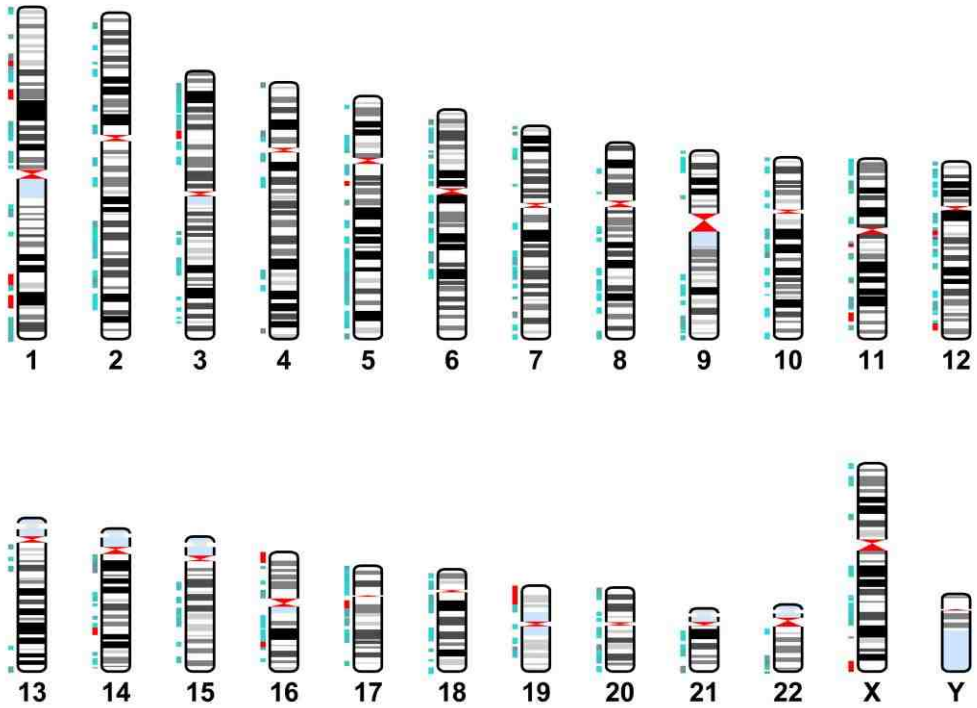

Figure 3.2 miR-137 Target Gene Locations. Karyotype representing cytoband locations of the miR-137 TargetScan putative target genes. Blue depicts cytobands with 2-5 target genes. Red indicates regions containing 6 or more target genes.

3.4 Functional relevance of target genes

3.4.1 Expression of target genes

Given that the onset of schizophrenia generally occurs in adolescence and early adulthood, we reviewed the temporal expression patterns of miR-137 target genes. Using the freely available BrainCloud expression database developed by the Lieber Institute for Brain Development (LIBD) and the National Institute of Mental Health (NIMH) (Colantuoni, et al., 2011) that uses the expression profiles in the dorsolateral prefrontal cortex of human postmortem tissue across the lifespan, we examined the expression patterns of all available experimentally verified target genes (Table 3.2) and putative targets common to the SZGR association list (Table 3.1). Except for ATXN1, CDC42, GRIN2A, KDM1A (LSD1), and SYN3 we found expression patterns for all these genes.

Of the 46 examined genes, about 41 percent have peak expressions during prenatal life, 13 percent during prenatal and postnatal combined, 20 percent postnatal, 4 percent both post-natal and adult and 22 percent during adulthood (Figure 3.3). Comparison of the patterns of expression of miR-137 targets vs. whole brain transcriptome (Kang et al, 2011) revealed that the targets have an atypical temporal distribution with peak expressions occurring more often during the prenatal period, or during adult life. The findings that 74 percent of target genes have peak expressions prior to adulthood, particularly prenatally, suggest that these target genes may be particularly relevant to the development of the schizophrenia. The unexpectedly large proportion of genes with peak expression in adulthood raises the possibility that miR-137 targets are involved in the ongoing decline in cognitive function and gray matter density observed in schizophrenia over the lifespan.

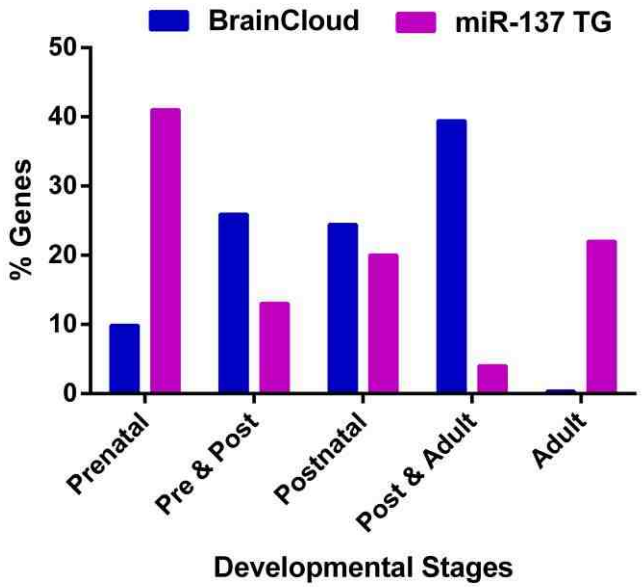

Figure 3.3 Peak Expression Life Stage for Genes of Interest. Temporal expression data for all available miR-137 putative targets and verified targets is based on the BrainCloud Database (Kang et al., 2011). Comparison of the temporal expression profiles of miR-137 targets and whole brain transcriptome (BrainCloud) using Chi square analysis revealed that the temporal expression profile frequency distributions were significantly different ($p < 0.01$). The prenatal stage is defined as week 14 through birth, postnatal stage is defined as birth through age 20 years, and adult stage is defined as age 20 years and older.

3.4.2 Pathway analysis

Pathway analysis was performed using the putative target gene list derived from TargetScan, containing 1144 target genes and the 8 experimentally verified target genes (Table 3.2) not contained within the putative list. This analysis was performed in three levels using the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, CA, USA, www.ingenuity.com) generating a canonical pathway analysis and related network analyses for each level. The networks are given a score based on the probability of inclusion of the number of molecules in the generated networks over the probability of a network being generated by chance with random molecules. This score is generated as a negative log p value based on a right tailed Fisher's exact test (Calvano et al., 2005). Canonical pathway analysis is performed by comparing the dataset of interest against

known canonical (signaling and metabolic) pathways within the database. A negative log p value is also assigned to the pathways based on a Fisher's exact test of the probability of the number of molecules from the user-created dataset included in the given pathways, versus being included based on chance alone.

A core analysis was first performed with this data set analyzing molecules in all tissue types in mammals. Of the 1144 putative target genes, 1142 were mapped in the IPA software and usable for the analysis. In addition the 8 experimentally-verified transcripts not included in this putative target list were all mapped in IPA and included in the analysis, so that a total of 1150 target genes were used in the analysis. The top scoring canonical pathways corresponding to targets expressed a) in all tissues, b) only in the nervous system (NS) or c) those associated with NS development and function are shown in Figure 3.4.

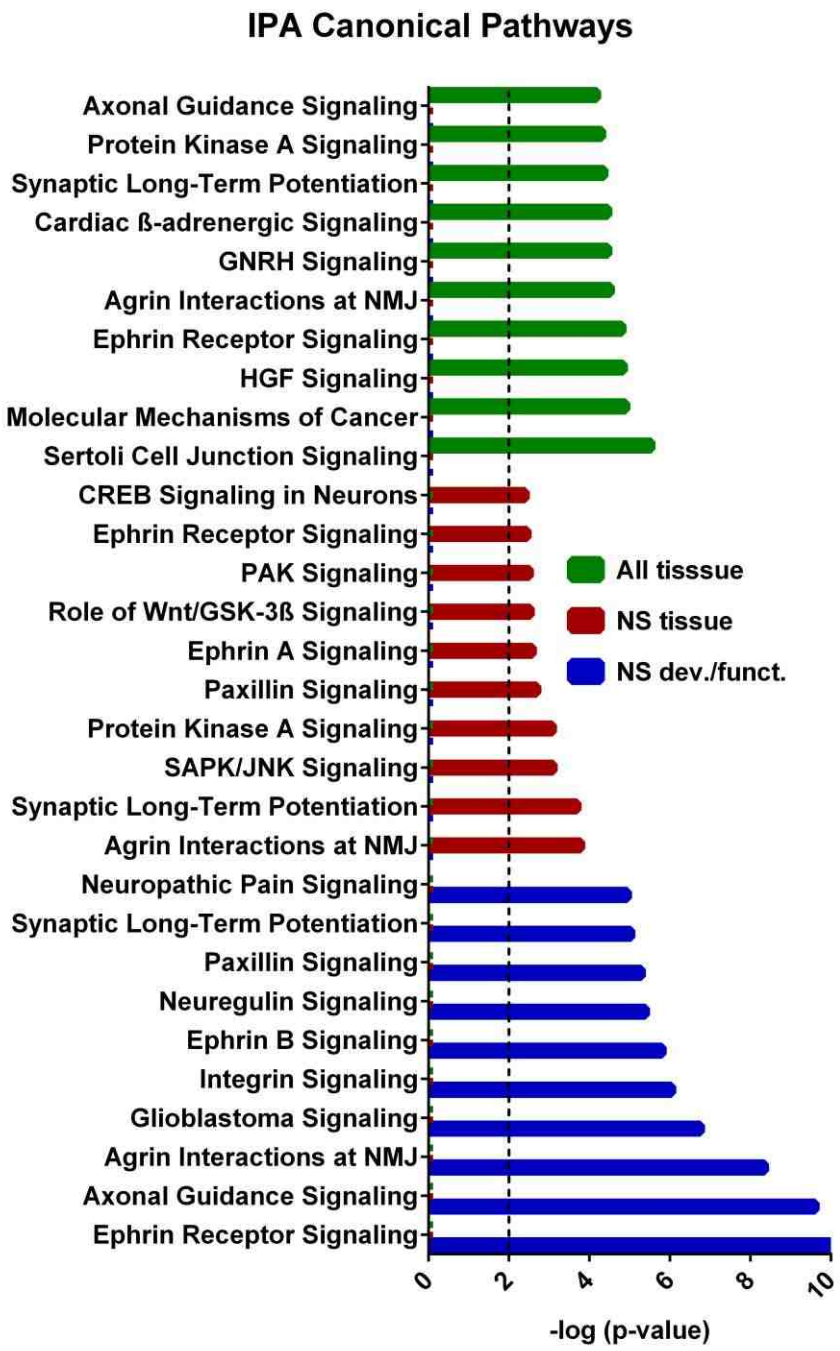

Figure 3.4 IPA canonical pathway analyses from each tier of core analysis. The X axis represents negative log p values based on the probability that molecules in the uploaded dataset were included the predefined IPA canonical pathways by true association as opposed to inclusion of molecules based on chance alone. Pathways not involved in nervous system were removed from the nervous system tissue analysis and nervous system development and function graphical displays. Only the top ten pathways with the largest negative log p values are shown. The dashed line indicates the threshold of significance for a p value of 0.01. GNRH, Gonadotropin-releasing hormone, HGF, hepatocyte growth-factor, NMJ, neuromuscular junction.

Interestingly, while *Sertoli cell-signaling* was the top scoring pathway for targets expressed in all tissues, this set also included several nervous system-specific pathways. Among these, the top scoring pathways were *agrin interaction at the neuromuscular junction,* and *synaptic long-term potentiation.* The top physiological system associated with miR-37 targets, containing 202 genes, was *nervous system development and function.* Using this set, we found that the top pathways were *Ephrin receptor signaling,* and *axonal guidance,* processes known to be involved in neuronal development and cognition.

The biological significance of the molecules included in these canonical pathways is best illustrated by their associated interactive networks (Figures 3.5-3.7). As shown in Figure 3.5, the top networks of targets expressed in all tissues contained a large number of nervous system associated molecules, depicted in yellow, confirming past studies that miR-137 is involved in nervous system development (Siegel et al., 2009; Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010).

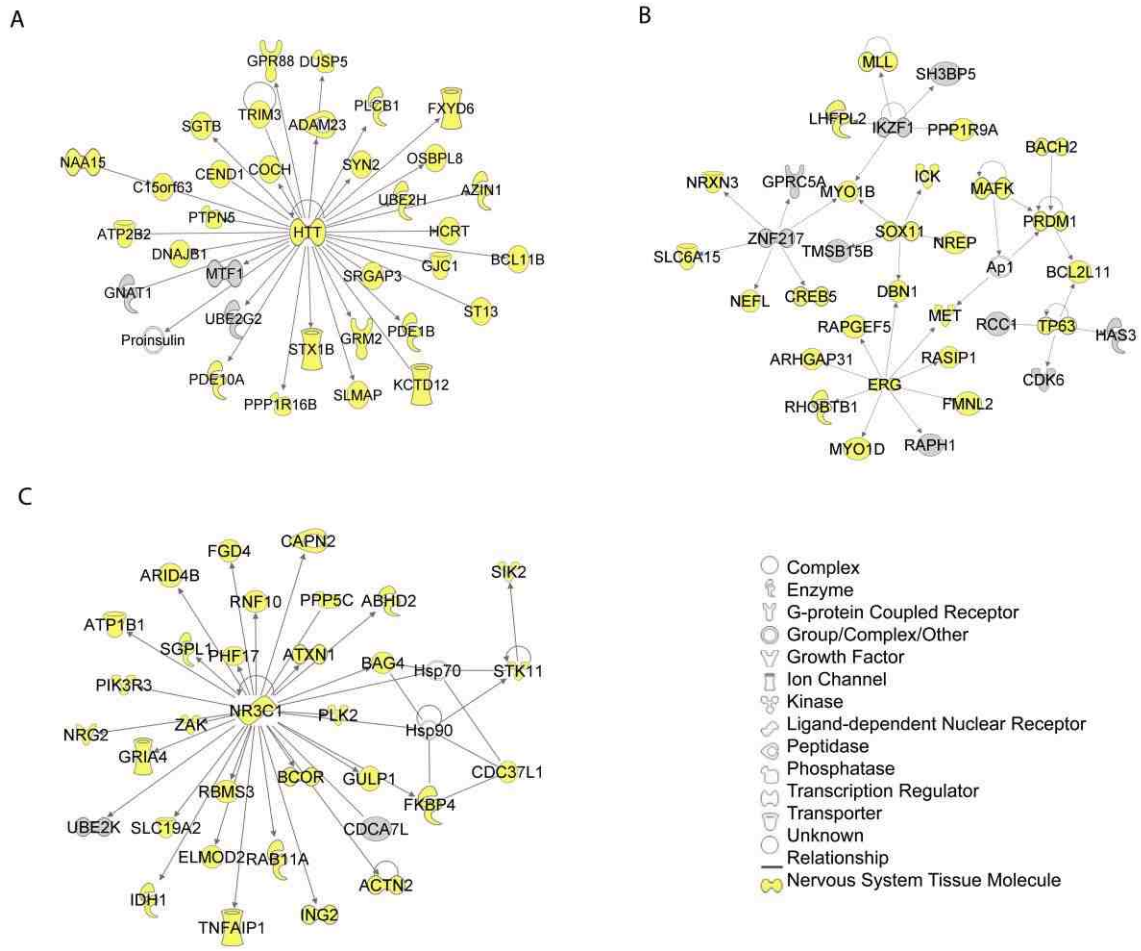

Figure 3.5. Top 3 scoring IPA network analysis generated networks for all mapped 1150 TargetScan putative targets and verified targets. The top 3 scoring networks identified were: (A)"Hereditary Disorder, Neurological Disease, Skeletal and Muscular Disorders," with a score of 39, (B)"Cellular Development, Connective Tissue Development and Function, Cancer," with a score of 39, and (C) "Protein Synthesis, Endocrine System Development and Function, and Lipid Metabolism," with a score of 36. Yellow indicate molecules expressed in the nervous system. White molecules are those not included in the uploaded dataset but added by IPA. Gray molecules indicate those that were included in the uploaded dataset. Relationships depicted by lines with arrows represent "act on," while lines without arrows represent binding. See figure keys for identification of the types of molecules included.

A subsequent core analysis was then performed with the 929 nervous system tissue associated subset of molecules of the original 1150 putative and verified target genes used in the previous analysis. This resulted in networks (Figure 3.6) including a substantial number of target genes found in the SZGR database and the experimentally

verified list, as well as many other schizophrenia-associated genes. Finally, the top association networks of targets involved in nervous system development and function also contained many of the schizophrenia associated and verified target genes (Figure 3.7).

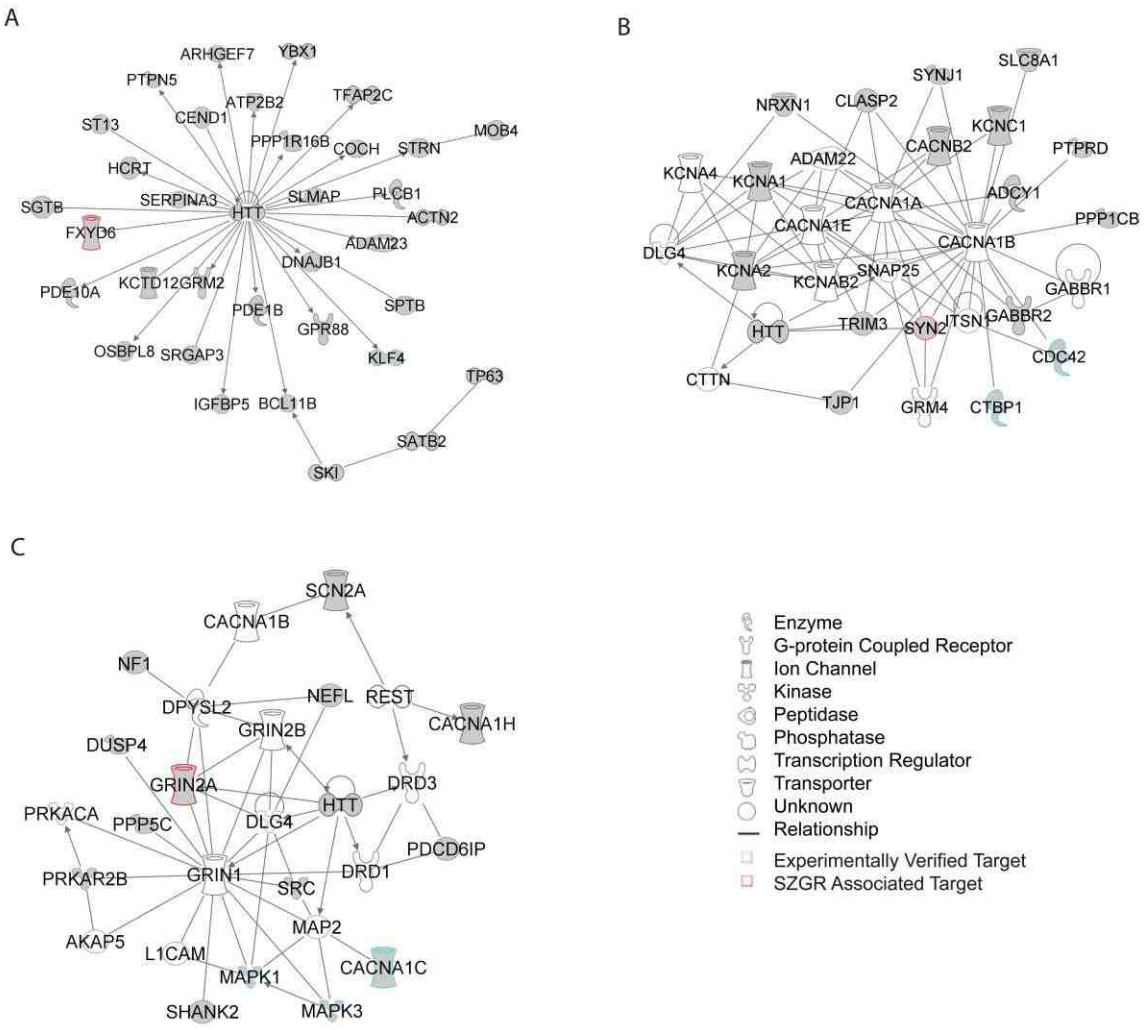

Figure 3.6 Top 3 scoring IPA network analysis generated networks for the 929 nervous tissue specific putative and verified targets. The top 3 scoring networks identified by this analysis were: (A) "Hereditary Disorder, Neurological Disease, Skeletal and Muscular Disorders," with a score of 35, (B) "Neurological Disease, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function," with a score of 10, and (C) "Behavior, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function," with a score of 8. White molecules are those not included in the uploaded dataset but added by IPA. Gray molecules indicate those that were included in the uploaded dataset. Relationships depicted by lines with arrows represent "act on," while lines without arrows represent binding. See figure legend keys for identification of the types of molecules included. Blue outlines depict experimentally validated targets and those in red indicate SZGR associated targets.

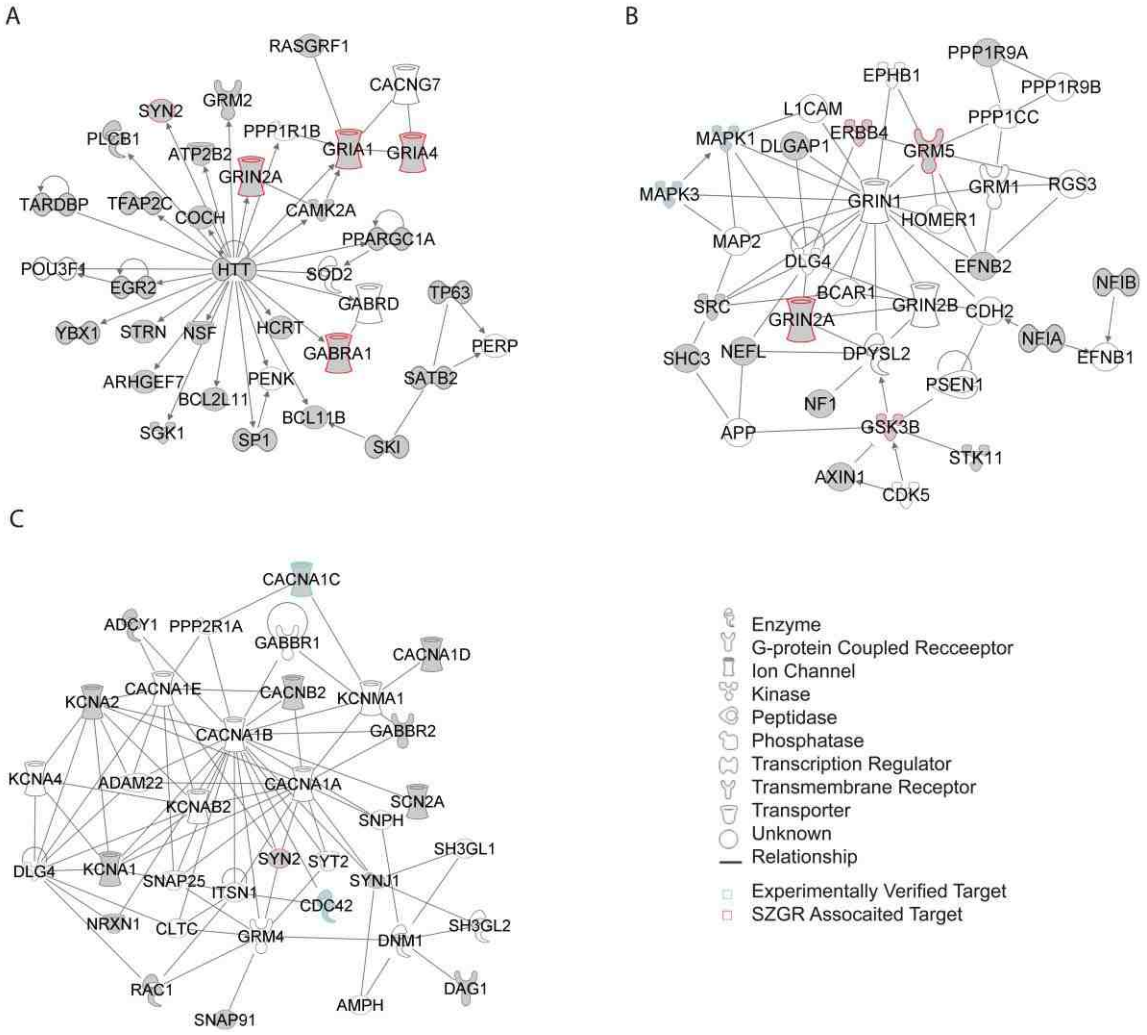

Figure 3.7.Top 3 scoring IPA network analysis generated networks for 202 nervous system development and function subset of putative and verified targets. The three top scoring networks identified were: (A) "Behavior, Hereditary Disorder, Neurological Disease," with a score of 29, (B) "Cell Morphology, Cellular Assembly and Organization, Cellular Development," with a score of 12, and (C) "Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Neurological Disease," with a score of 10. White molecules are those not included in the uploaded dataset but added by IPA. Gray molecules indicate those that were included in the uploaded dataset. Relationships depicted by lines with arrows represent "act on," while lines without arrows represent binding. See figure keys for identification of the types of molecules included. Blue outlines depict experimentally validated targets and those in red indicate SZGR associated targets

3.5 Conclusions and perspectives

There is evidence that schizophrenia is a highly complex polygenic disorder with multiple genes contributing only a small risk. Given that individual miRNAs can affect the expression of up to thousands of genes post-transcriptionally, and that differential expression of miRNAs between patients with schizophrenia and controls has been shown in many different studies; it seems likely that miRNAs may play a role in the etiology of the disease. miR-137 in particular was shown to have a SNP (rs1625579) with the highest association with schizophrenia in the largest schizophrenia GWAS study performed to date (Ripke et al, 2011). Although the mechanisms by which this SNP may cause a dysregulation in miR-137 processing is not completely understood, recent studies suggest that carriers of the risk allele have abnormal levels of mature miR-137 in the cerebral cortex (Guella et al, 2013). Considering the biological pathways associated with miR-137 targets, there are several possible mechanisms, discussed below, by which alterations in miR-137 expression may contribute to the development and pathophysiology of schizophrenia.

Imaging genetics studies have shown an association of the miR-137 risk allele with reduced fMRI responses in both patients and at risk subjects while performing cognitive tasks (Potkin et al., 2010, Potkin et al., 2009a; Potkin et al., 2009b; Whalley, et al., 2012). In addition, this SNP was also shown to have a potential association with the P300 endophenotype (Decoster et al., 2012) and a significant association with cognitive deficits in patients with schizophrenia using a variety of tasks (Green et al., 2012, Cummings et al., 2012). Furthermore, a recent study (Lett et al, 2013) found that the risk allele was associated with earlier age-at-onset of psychosis, decreased hippocampal

volume, and reduced white matter integrity throughout the entire brain. Given that miR-137 plays a role in neurogenesis, neurodevelopment, dendritic arborization and is located in the synapse (Siegel et al., 2009; Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010) it is enticing to propose that abnormal expression of this miRNA may lead to abnormal synapse formation which could in turn play a role in the cognitive deficits, psychotic symptoms and brain structural abnormalities found in these patients.

By utilizing the freely accessible TargetScan, GeneCards, and UCSC databases, we found that the miR-137 potentially targets over a thousand genes, with a variety of functions and potential impact. As depicted in Figure 3.2, these putative target genes can be found throughout nearly every chromosome, with some particular regions of high concentration. This list of putative target genes also includes some known to be associated with schizophrenia (e.g., ERBB4, GABRA1, GRIN2A, GRM5, GSK3B, NRG2 and HTR2C) as well as many experimentally verified as true targets. Subsequent analysis of these putative and verified genes using IPA demonstrates that this list of genes includes many nervous system specific genes. Expression Data from BrainCloud also confirms that many of the genes such as FXYD6, BRD1, GSK3B, CDK6, CDC42, and CACNA1C have higher expression levels in onset risk time periods, suggesting their relevance in contributing risk for development of schizophrenia. These target genes also form networks involved in neuronal function and development; with the top canonical pathways associated being *agrin interaction at the neuromuscular junction, synaptic long term potentiation (LTP*), *Ephrin receptor signaling*, and *axonal guidance signaling*. Sertoli cell signaling was the top scoring pathway identified by the first tier of analysis of all 1150 putative and verified targets of miR-137, which may be related to the increased

risk of schizophrenia with increasing paternal age (Frans et al., 2011). The identification of agrin interaction at neuromuscular junctions as a top canonical pathway may also be of interest given the association with neurological soft signs and schizophrenia (Sewell et al., 2010). Also, given that several of the same target transcripts play a role in cell-cell interactions in the periphery and the CNS, it is likely that this miRNA has similar roles in other tissues. However, given the enrichment of this mRNA in developing neurons, we focus our discussion in this cell type.

Many of the top scoring pathways identified: LTP signaling, Ephrin receptor signaling, and axonal guidance signaling, are closely linked to learning and memory and have shown to be associated with schizophrenia. A recent study suggests evidence of impaired LTP in schizophrenia patients with a simultaneous deficit in motor learning(Frantseva et al., 2008). In addition, many schizophrenia animal model studies have also demonstrated an impairment of LTP (Pollard et al., 2011). Of particular interest, a study examining ERBB4 (one of the miR-137 putative target genes), and synaptic potentiation, demonstrated that mice with a full ErbB4 knock-out and mice with a conditional ErbB4 knock-out in only parvalbumin expressing cells exhibit increased hippocampal LTP and lack theta-pulse evoked LTP reversal (Shamir et al., 2012). These mice have increased locomotor activity in the open field test; such hyperactivity in response to novelty is used often as a model of positive symptomatology in schizophrenia. The mice also exhibit deficits in prepulse inhibition of the acoustic startle response, a test believed to model sensorimotor gating, which has been shown to be reduced in patients with schizophrenia. These findings provide further evidence that miR-137 may indeed impact LTP in

schizophrenia leading to the cognitive dysfunction, a clinical feature still poorly understood and inadequately treated (Blanchard et al., 2011).

Axonal guidance has also been implicated in schizophrenia. Ephrin signaling plays a role in axonal guidance by controlling axon motility (Xu and Henkemeyer, 2012). A recent imaging study found an association between polymorphisms in RELN and PCDH12, genes involved in neuronal guidance and synaptic formation, and alterations in the patients' brain structure as seen by MRI (Gregorio et al., 2009). Another imaging study found an association with SNPs in RELN, PLXNA2 and other genes involved in axonal guidance and neuronal development and prediction of DLPFC inefficiency during a working memory task (Walton et al., 2013). There is also evidence linking Ephrin receptor signaling and LTP. Particularly two studies found that postsynaptic EphB receptors and presynaptic B-ephrins are necessary for the induction of LTP of the mossy fibers in the hippocampus, a N-methyl-D-aspartate (NMDA) receptor independent form of LTP (Armstrong et al., 2006). Of note, many genes involved in glutamate signaling such as GRIA1, GRIA4, GRIN2A, GRM2, and GRM5 were identified in the generated networks and were included in the LTP signaling cascade of the IPA canonical pathway. The potential role of miR-137 in the expression of these glutamate receptors could explain not only the altered glutamate signaling observed in schizophrenia (Coyle, 2006; Sendt et al., 2012), but also the LTP disruption in the illness, which may in turn contribute to the associated cognitive deficits.

Interestingly, we also found that the Huntington's associated protein huntingtin (HTT) was identified as a hub in all three tiers of IPA network analyses. While the normal function of this protein is still largely unknown, it is possible that HTT has been included

in the IPA database as having many biological relationships largely because it is well studied. However, recent evidence suggests that HTT is involved highly in neuronal development, playing a role in early neuronal survival, regulation of axonal transport, regulation of BDNF production particularly in the cortex, and controlling synaptic activity (Zuccato et al., 2010). Given the role of miR-137 in neuronal development, these two molecules may work in concert to regulate this process. Further research will be necessary to determine if HTT plays a role in schizophrenia.

By IPA analysis of the putative and experimentally validated targets of miR-137, we identified that a large majority are expressed in the nervous system, forming networks involving genes associated with schizophrenia. The top canonical pathways identified by these analyses are widely known to be associated with learning and memory and synaptic formation, suggesting that the genetic impact of this miRNA may play a role in the processes of cognition and neuronal development. While our own analyses and the results of the literature support a role of miR-137 in the etiology of schizophrenia, further analysis is necessary to understand the full impact of this miRNA. In particular, it will be important to evaluate the involvement of LTP, Ephrin receptor signaling, axonal guidance, and glutamate signaling. Further elucidation of the role of miR-137 in schizophrenia is merited as the negative symptoms and cognitive deficits associated are still inadequately treated and can have such a grave impact on patients. This miRNA may provide a new avenue for exploring the underlying mechanisms involved in the etiology of the disease as well as discovering new biomarkers and therapeutic targets.

3.6 Conflict of interest

This research was performed with no financial or commercial interest.

3.7 Acknowledgements

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4. Meta Gene Set Enrichment Analyses Link miR-137-regulated Pathways with Schizophrenia Risk

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4.1 Abstract

Background: A single nucleotide polymorphism (SNP) within *MIR137*, the host gene for miR-137, has been identified repeatedly as a risk factor for schizophrenia. Previous pathway analyses suggest that potential targets of this microRNA (miRNA) are also highly enriched in schizophrenia-relevant biological pathways, including those involved in nervous system development and function.

Methods: In this study, we evaluated the schizophrenia risk of miR-137 target genes within these pathways. Gene set enrichment analysis of pathway-specific miR-137 targets was performed using the stage 1 (21,856 subjects) schizophrenia genome wide association study data from the Psychiatric Genomics Consortium and a small independent replication cohort (244 subjects) from the Mind Clinical Imaging Consortium and Northwestern University .

Results: Gene sets of potential miR-137 targets were enriched with variants associated with schizophrenia risk, including target sets involved in axonal guidance signaling, Ephrin receptor signaling, long-term potentiation, PKA signaling, and Sertoli cell junction signaling. The schizophrenia-risk association of SNPs in PKA signaling targets was replicated in the second independent cohort.

Conclusions: These results suggest that these biological pathways may be involved in the mechanisms by which this *MIR137* variant enhances schizophrenia risk. SNPs in targets and the miRNA host gene may collectively lead to dysregulation of target expression and aberrant functioning of such implicated pathways. Pathway-guided gene set enrichment

analyses should be useful in evaluating the impact of other miRNAs and target genes in different diseases.

4.2 Background

MicroRNAs (miRNAs) are a class of noncoding RNAs involved in post-transcriptional gene expression regulation by binding to complementary sequences within target mRNAs (Bartel, 2009). miRNAs play a role in a variety of cellular processes and diseases (Henrion-Caude et al., 2012), including psychiatric disorders (Mellios and Sur, 2012). Several lines of evidence support a role of miR-137 in schizophrenia, a severe mental illness characterized by symptoms of delusions, hallucinations, and diminished sociability Not only was the strongest associated SNP identified in the first large schizophrenia genome wide association study (GWAS) located in the host gene of miR-137 (Ripke et al., 2011) but also the other top four polymorphisms mapped to validated target genes of this miRNA (Kwon et al., 2011). This miRNA is involved in several steps in neuronal development, from regulation of neuronal proliferation and differentiation (Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010a), to dendritic arborization (Smrt et al., 2010) suggesting that the risk allele may impact these processes. Additionally, imaging genetics studies have found distinct alterations associated with the *MIR137* risk variant in subjects with schizophrenia. One recent functional magnetic resonance imaging (fMRI) study identified alterations in brain activity patterns during a sentence completion task within the amygdala and pre and postcentral gyrus only in risk allele carrier subjects that were also at risk for schizophrenia development (Whalley et al., 2012). Structural imaging analyses also identified patient-specific alterations in risk allele carriers such as reduced whole brain

functional anisotropy, reduced left hippocampal volume and enlarged right and left lateral ventricle volume (Lett et al., 2013). In contrast, no volumetric alterations were found in protective-allele carrying patients or healthy risk allele carriers (Li and Su, 2013). The disease-specific risk allele associations found in these studies suggest that, in agreement with the evidence of polygenic risk in schizophrenia (Purcell et al., 2009), other genetic factors besides the *MIR137* SNP may underlie disease-specific abnormalities in brain structure and function. Polymorphisms within multiple miR-137 targets may in part increase genetic risk by potentially enhancing dysregulation by this miRNA. Variants within or adjacent to miRNA recognition sites in 3' UTRs can alter binding and binding availability of miRNAs to target mRNAs, leading to altered gene expression and phenotypic or disease states (Abelson, 2005; Wang et al., 2008) Therefore, collective polymorphisms within miR-137 target genes in schizophrenia-relevant pathways may disrupt regulation by this miRNA, and/or lead to a general disruption of the pathways in the patients.

Initial bioinformatics analyses of the function of putative and validated targets suggest that miR-137 target genes are involved in many schizophrenia relevant pathways, including axonal guidance signaling, Ephrin receptor signaling, synaptic long term potentiation (LTP), and protein kinase A (PKA) signaling, among others (Wright et al., 2013). Besides LTP, little is known about the role of these miR-137 regulated pathways in schizophrenia. Preliminary SNP by SNP association analyses performed by the PGC found significant enrichment of risk associated SNPs within a subset of predicted miR-137 target genes (Ripke et al., 2011) and this was replicated with a larger set of predicted targets, using a joint gene set enrichment analysis (Ripke et al., 2013). However, no

studies to date have examined the collective risk of miR-137 target SNPs across biological pathways.

4.2.1 Overall Goals and Contribution

The goals of this study were to assess the schizophrenia-risk of both experimentally validated and high confidence predicted miR-137 targets, and to evaluate for the first time the risk association of these targets in a pathway-specific manner. These analyses were performed using meta gene set enrichment analyses of specific target gene sets (Segrè et al., 2010). Gene set enrichment analysis (GSEA) is particularly useful in the case of polygenic diseases such as schizophrenia (Purcell et al., 2009) as it allows for examination of the collective effect of multiple polymorphisms. Furthermore, analysis of gene sets in a pathway specific framework can also increase the power to detect collective moderate risk associations and can allows for evaluation of more biologically relevant and interpretable genetic effects particularly with genetically complex disorders (Juraeva et al., 2014). In this study we identified pathway-specific miR-137 target gene sets, and evaluated their risk association both within the PGC Stage 1 GWAS data (Ripke et al., 2011) and within a smaller independent dataset including subjects from the Mind Clinical Imaging Consortium (MCIC) (Gollub et al., 2013) and Northwestern University (NU) (Wang et al., 2013). The evaluation of pathway-specific gene sets in this manner allows for an estimation of schizophrenia-risk due to miR-137 dysregulation.

4.3 Methods

4.3.1 miR-137 Target Curation and Prediction

Experimentally validated targets and 2 indirectly regulated genes (*MAPK1* and *MAPK3*) were curated (36 in total) from the literature as described previously for the identification of 26 regulated genes in Wright et al., 2013. Additionally *HTT* (Kozlowska et al., 2013), *TBX3* (Jiang et al., 2013), *GLIPR1* (*RTVP-1*) (Ariel Bier et al., 2013), *CLDN11*, *GABRA1*, *NRXN1*, *NEFL*, *ZNF365*, *NECAP1*, and *RAPGEF5* (Boudreau et al., 2014) were included as validation experiments were published since Wright et al., 2013.. Targets were predicted using TargetScan version 6.2, released June 2012 (Lewis et al., 2005). Target prediction databases are known to include false positive miRNA-mRNA interactions and to exclude true interactions (Zheng et al., 2013). TargetScan offers two scoring systems to improve confidence in target-miRNA prediction: the probability of conserved targeting (Pct) score and the context score. The Pct score (with a range from 0 to 1, with 1 indicating highest) is derived by evaluating the conservation of the interaction site sequence across species (Friedman et al., 2009). Highly conserved binding sites are more likely to be functionally relevant and effective in inducing subsequent mRNA repression (Friedman et al., 2009; Nielsen et al., 2007) However, target interactions that may have evolved later in primates and humans are less likely to be conserved (Friedman et al., 2009; Glazov et al., 2008) and may be more relevant to higher order cognition and complex behavior phenotypes, such as those affected in schizophrenia. Thus some human-specific or primate-specific targets may be lost based on conservation score (Farh et al., 2005; Grimson et al., 2007). The context score provides confidence for the less conserved targets and improved confidence for

conserved targets. This score (with a range from 0 to -1, with -1 indicating more probable binding) is based on evaluation of site efficacy including seed site interaction type, nearby nucleotides , site location, and seed site interaction stability among other criteria (Garcia et al., 2011; Grimson et al., 2007). Therefore, site conservation and site efficacy were both used, either separately or combined, to better capture the potential impact of miR-137 on biological pathways.

The four predicted miR-137 target gene lists, each including validated targets, that were curated for further analysis (Appendix A, Table A.1) included: a) the full target list as predicted by TargetScan (full target list), b) targets with Pct scores greater than or equal to 0.9 (conserved target list), c) targets with the best 50% of context scores (context target list), and d)the high Pct and low context scoring targets (intersection target list). The Pct score cutoff of 0.9 was based on previous work of Ripke et al., 2011. The context score, with an equal percentage of predicted targets as that chosen for the Pct score, was -0.12. Finally, the intersection of targets with Pct scores greater than or equal to 0.9 and with context scores below -0.12 was used as a higher confidence predicted list that represents more plausible conserved targets.

4.3.2 Pathway Selection Criterion

Selection of gene sets was based on prior pathway analysis of the full list of TargetScan predicted targets and validated targets using Ingenuity Pathway Analysis (IPA) as described previously in Wright et al., 2013. From this analysis, it was determined that several possibly schizophrenia-relevant pathways were significantly enriched with potential miR-137 target genes. The top ten enriched pathways for potential targets, listed

in Table 4.1 were selected for pathway-specific gene set enrichment analyses. Gene sets of miR-137 target genes of varied prediction confidence were created for each pathway using the target gene lists described above (Appendix A, Table A.1).

Evaluated Gene Set in MAGENTA	**Full List**	**Conserved List**	**Context List**	**Intersection List**	**Validated List**
Gene Set size	1154	560	597	311	36
Pathway Specific Gene Sets:					
Sertoli cell junction signaling	27	16	15	11	4
Mechanisms of cancer	40	25	22	17	8
Hepatocyte growth factor (HGF) signaling	18	13	12	9	6
Ephrin receptor signaling	25	16	12	10	3[a]
Agrin interactions at neuromuscular junctions	14	9	8	6	3[a]
Gonadotropin Releasing Hormone (GNRH) signaling	20	12	6	6	3[a]
Cardiac –B adrenergic signaling	20	10	4	2	1
Synaptic long term potentiation (LTP)	19	11	7	4	3
Protein kinase A (PKA) signaling	42	26	18	10	4
Axonal guidance signaling	42	20	18	13	3[a]

Table 4.1. Gene Sets of Potential hsa-miR-137 Targets Evaluated in MAGENTA

This table shows the number of potential miR-137 target genes evaluated in each gene list and the number of genes in each pathway-specific gene set derived from each target gene list. MAGENTA= Meta Gene Set Enrichment of Variant Analysis

[a]identical gene sets

4.3.3 Meta Gene Set Enrichment of Variant Analysis (MAGENTA)

The MAGENTA software program (Segrè et al., 2010) evaluates enrichment of modest associations with a disease or trait within gene sets using GWAS disease association p values and odds ratios. MAGENTA includes SNPs within a region from 110 kb upstream to 40 kb downstream of each gene's transcript boundaries. The SNP with the smallest disease association p-value within this region is determined for later analysis as the "gene's best" association p-value. Such a procedure helps overcome the "watering-down" effects that occur when analyzing the average SNP p-value across a gene, where unassociated SNPs can depreciate gene association. The following confounds are addressed by correcting the smallest gene SNP p-values with step-wise linear regression: gene size, number of SNPs per gene kb, number of independent SNPs per gene kb, number of recombination spots per gene kb, linkage disequilibrium units per gene kb, and genetic distance per gene kb (Segrè et al., 2010).

MAGENTA uses corrected best gene disease association p-values to evaluate the enrichment of each gene set with, in this case, genes containing a schizophrenia-associated variant. Gene sets are compared to 10,000 random gene sets of identical size. The gene set p-value is calculated as the fraction of random gene sets with a sum rank p-value equal or smaller than that of the tested gene set. Gene sets with a one-tailed Mann-Whitney like rank-sum based false discovery rate (FDR) (Sabatti et al., 2003) q-value of <0.05 were considered significantly enriched with associated SNPs based on the FDR gene score enrichment cutoff of 75%. This cutoff is based on the fraction of p-values lower than 75% of all gene p-values and is suggested for polygenic diseases such as is

proposed for schizophrenia (Purcell et al., 2009) where association values may be more modest (Segrè et al., 2010)

4.3.4 Database and GWAS Information

To determine whether the gene sets in Table 4.1 are enriched in schizophrenia risk variants, p-values from two independent GWAS were evaluated. The first p-values were derived from the stage 1 GWAS study reported in Ripke et al., 2011, the GWAS in which the schizophrenia risk association was discovered for the miR-137 host gene SNP, rs1625579. This analysis included 21, 856 subjects (9,394 cases and 12,462 controls) of European ancestry from the Psychiatric GWAS Consortium (PGC) (TableS3). Genotyping was performed using Affymetrix and Illumina Chips across samples. Quality control was conducted as described in Ripke et al., 2011. The unadjusted p-values and odds ratios for the 1.2 million SNPs evaluated in this GWAS are available on the PGC website, https://pgc.unc.edu/Sharing.php. GWAS p-values and odds ratios were loaded into MAGENTA (Segrè et al., 2010) with one gene set file including all gene sets in Table 4.1 to allow cross-comparison of gene sets, as permutation differences can cause slightly different results.

To evaluate if the PGC MAGENTA results were replicable, subjects were analyzed from the Mind Clinical Imaging Consortium (MCIC) (Gollub et al., 2013) and Northwestern University (NU) (Wang et al., 2013) (Appendix A, Table A.2). All participants provided written informed consent, and the Institutional Review Board at each site approved this project. Genotyping was conducted at the Mind Research Network Neurogenetics Core lab using Illumina HumanOmni-Quad 1M and 5M BeadChips respectively. Only

Caucasian subjects were used to avoid population-specific effects. Caucasians were identified using the Enhancing Neuroimaging Genetics through Meta-Analysis (ENIGMA) multi-dimensional scaling (MDS) protocol within the imputation protocol (http://enigma.ini.usc.edu/protocols/genetics-protocols/). Genotype data from each dataset was merged using PLINK after updating the MCIC SNP locations to match that of the more recent NU data. Quality control was performed before and after merging similarly to that of Ripke et al. 2011, using PLINK with the following thresholds: Hardy Weinberg equilibrium P $<10^{-6}$, minor allele frequency < 0.05, missing rate per SNP < 0.02, missing rate per individual < 0.02. Relatedness and population stratification testing was performed using PLINK. Outliers were identified and removed as well as one individual per pair that appeared to be related, according to pi-hat values of 0.05 or greater per pair of individuals. After all quality control and pruning, a total of 244 individuals remained (103 cases and 141 controls) and 539,288 SNPs.

A GWAS study using logistic regression covarying for chip type was performed on the merged MCIC and NU genotypic data with a genomic inflation factor of 1.00737. The p-values obtained were evaluated using MAGENTA for the previously significantly enriched gene sets found in the PGC data.

4.4 Results

4.4.1 MAGENTA Analyses of miR-137 Predicted and Validated Target Lists

Gene sets for each of the target lists of different prediction confidences were first evaluated for enrichment of schizophrenia risk SNPs (Table 4.2). The higher confidence predicted lists, i.e., the conserved, context, and intersection, were all significantly

enriched with schizophrenia-associated variants. As shown in Figure 4.1, the IPA network derived from the 77 intersection targets that were associated with neurological disease contains many nervous system expressed genes including some schizophrenia-associated genes that interact with one another. The validated target list was not significantly enriched with variants, likely due to the small size of this set. The full list of putative targets, although trending, was not enriched either, possibly due to a higher inclusion of false positive miRNA-target interactions. This suggests that the *high confidence predicted miR-137 target genes* overall contain SNPs that are associated with schizophrenia.

Gene Set	**MAGENTA Gene Set Size**	**Nominal GSEA p-value with 75% cutoff**	**FDR q-value with 75% cutoff**
Full List	1061	1.32E-02	5.11E-02
Conserved List	548	1.50E-03	**1.96E-02**
Context List	585	3.90E-03	**1.89E-02**
Intersection List	329	2.50E-03	**1.90E-02**
Validated List	36	9.08E-02	9.75-02

Table 4.2. Curated hsa-miR-137 Target Gene Lists Show Enrichment for Association with Schizophrenia

This table shows the results of the target gene lists including the number of genes after conversion to ENTREZ IDs , the gene set enrichment analysis (GSEA) p-value before false discovery rate (FDR) correction, and the FDR q-value used to determine significance at a threshold of q <0.05. Significant gene lists are shown in bold. MAGENTA= Meta Gene Set Enrichment of Variant Analysis

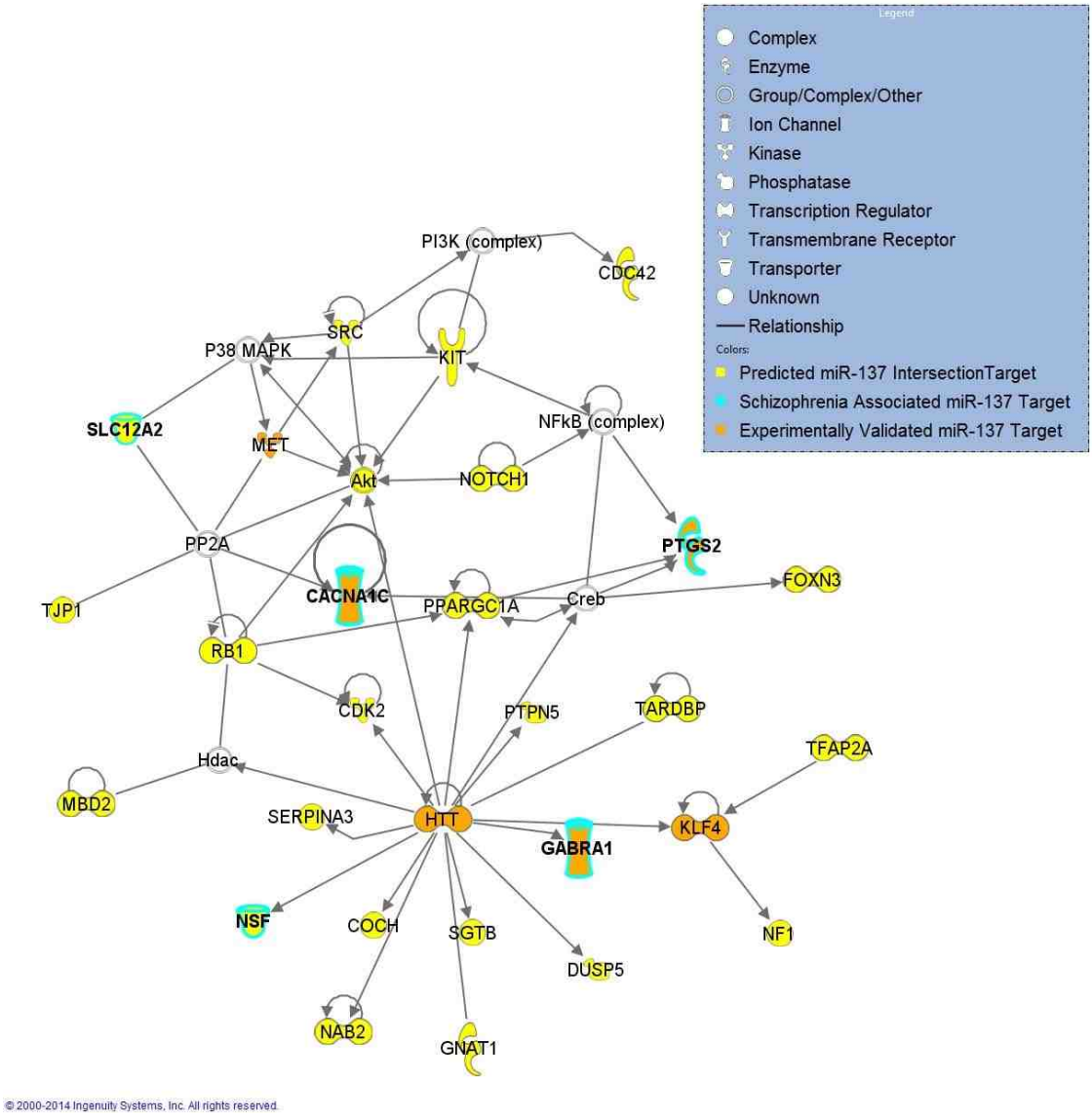

Figure 4.1. miR-137 Intersection Target Network of Neurological Disease

Top network derived from a core analysis using Ingenuity Pathway Analysis with the 77 genes of the intersection target list that are associated with Neurological Disease. Predicted targets are indicated in yellow, targets associated with schizophrenia are outlined in light blue and validated targets are indicated in orange.

4.4.2 MAGENTA Analyses of miR-137 Target Pathway Gene Sets

The schizophrenia-risk of miR-137 validated and predicted targets was assessed after targets were classified within canonical pathways according to IPA. Meta gene set enrichment of variant association analysis using MAGENTA software (Segrè et al., 2010) revealed several pathway relevant gene sets of miR-137 targets significantly enriched with schizophrenia-associated variants (Table 4.3). Ephrin receptor signaling, axonal guidance signaling, and Sertoli cell junction signaling gene sets were significantly enriched with schizophrenia-associated variants from four out of five gene lists (Table 4.3). The enrichment found for nearly all gene sets specific to these pathways using higher confidence target lists, strongly suggests that these pathways are indeed enriched with miR-137 target genes associated with schizophrenia risk. Additionally, synaptic LTP gene sets from the intersection and validated gene lists, and PKA signaling gene sets from the full and validated target lists, were enriched in risk genes. The mechanism of cancer gene set was also significantly enriched, but only from the validated target list. Overall, analysis of pathway specific gene sets derived from the multiple potential target lists provided higher confidence for the potential impact of this miRNA within these pathways.

Gene Set	Gene List	MAGENTA Gene Set Size	Nominal GSEA p-value with 75% cutoff	FDR q-value with 75% cutoff
Axonal guidance signaling	Conserved	19	8.00E-04	4.85E-03
	Context	18	5.60E-03	1.99E-02
	Intersection	13	5.80E-03	2.13E-02
	Full	40	1.14E-02	2.82E-02
Ephrin receptor signaling	Conserved	16	4.10E-05	7.00E-04
	Full	25	1.00E-03	8.43E-03
	Context	12	2.20E-03	1.18E-02
	Intersection	10	3.80E-03	1.36E-02
Synaptic LTP	Validated	3	1.69E-02	1.89E-02
	Conserved	13	2.19E-02	3.23E-02
Mechanisms of Cancer	Validated	8	2.59E-02	2.75E-02
PKA signaling	Full	46	1.03E-02	2.97E-02
	Validated	4	4.73E-02	4.18E-02
Sertoli cell junction signaling	Intersection	11	7.40E-03	1.84E-02
	Conserved	16	6.40E-03	1.84E-02
	Context	15	1.48E-02	2.75E-02
	Validated	4	5.24E-02	4.88E-02

Table 4.3. Significantly Enriched hsa-miR-137 Pathway-Specific Gene Sets

This table shows the results for the pathway-specific gene sets of Table 4.1 including the number of genes after conversion to ENTREZ IDs, the gene set enrichment analysis (GSEA) p-value before false discovery rate (FDR) correction, and the FDR q-value used to determine significance at a threshold of $q < 0.05$. All gene sets pass the significance threshold. MAGENTA= Meta Gene Set Enrichment of Variant Analysis

4.4.3 MCIC and NU Replication Cohort Results

MAGENTA analysis of a replication cohort using the MCIC (Gollub et al., 2013) and NU (Wang et al., 2013) dataset GWAS association p-values revealed one significantly enriched gene set. The PKA signaling gene set from the validated target list, including *TCF4* and *PTGS2* , as well as *MAPK1*, *MAPK3* (experimentally validated indirectly regulated genes), was significantly enriched with a nominal GSEA p-value of 0.003 and an FDR q-value of 0.014. Appendix A, Table A.3 shows the top SNPs from this analysis. As shown in Figure 4.2, the canonical PKA signaling pathway from IPA is enriched with predicted and validated miR-137 targets, suggesting that this pathway may be involved in the mechanism of the miRNA in schizophrenia.

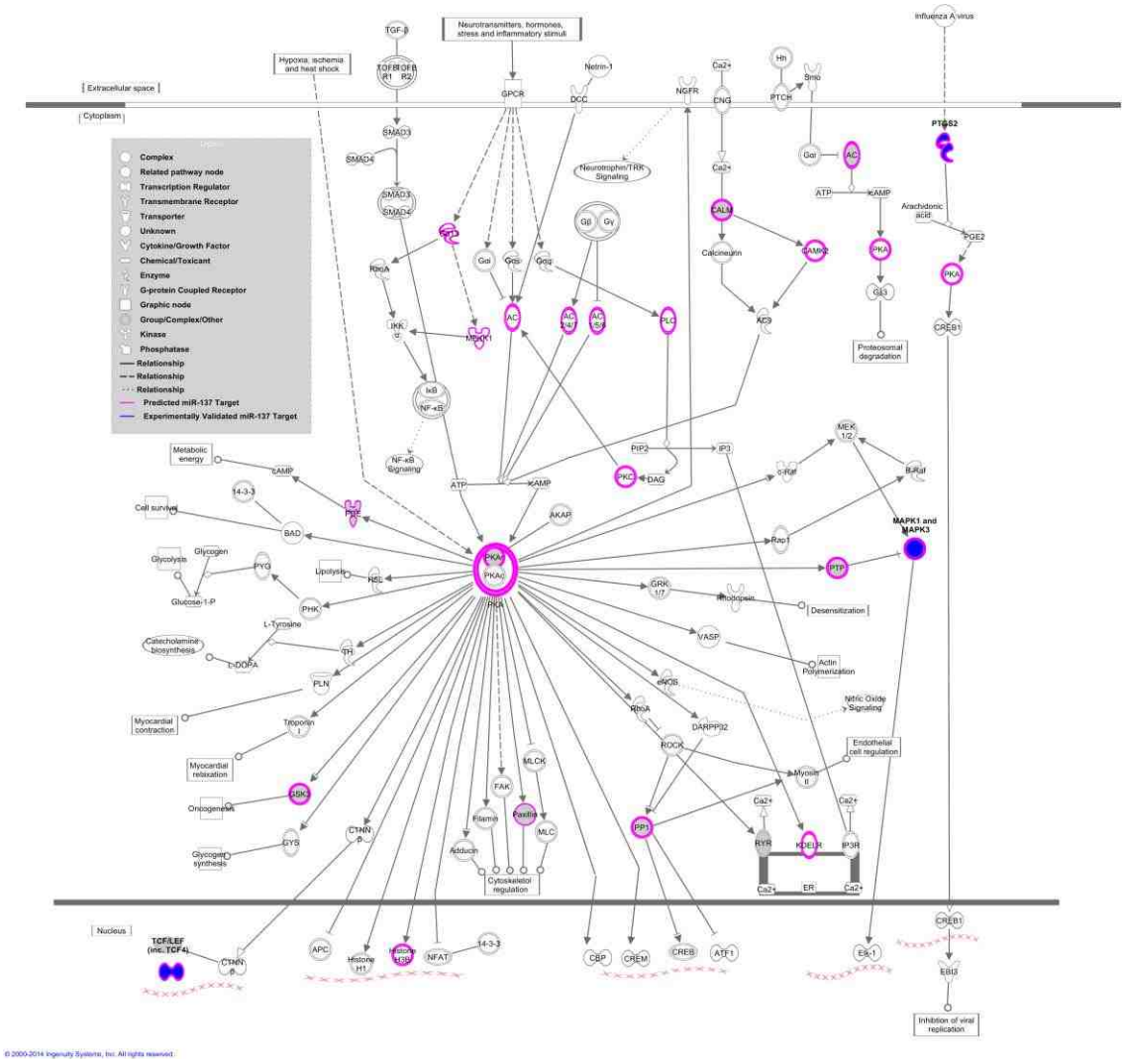

Figure 4.2. miR-137 targets within Protein Kinase A (PKA) Signaling Pathway

This figure depicts the canonical PKA signaling pathway according to Ingenuity Pathway Analysis. Predicted miR-137 targets based on TargetScan are indicated in pink and validated targets are indicated in dark blue.

4.5 Discussion

Current research about the recently discovered *MIR137* host gene SNP in schizophrenia suggests that this variant impacts some endophenotypic measures in patients and controls in a different manner (Lett et al., 2013; Whalley et al., 2012). Therefore, it is likely that

the interaction of this SNP with additional variants present in patients may increase both dysregulation by this miRNA and schizophrenia risk. These additional variants may disrupt targeting efficiency or lead to general disruption of pathway-specific genes, collectively altering biological processes required for proper brain functioning.

We have previously shown that miR-137 target genes fall within certain biological pathways more frequently than expected by chance (Wright et al., 2013). Data presented in this study clearly indicates that the genes in these miR-137 regulated pathways are also enriched with schizophrenia risk variants, suggesting a potential mechanism for the association of the *MIR137* risk variant. These pathways include Ephrin receptor signaling targets, axonal guidance signaling targets, synaptic LTP targets, Sertoli cell junction signaling targets, and PKA signaling targets. As described below, there is supporting evidence for how each of these may indeed enhance the risk of schizophrenia or impact the etiology of the disorder.

Ephrin receptor signaling is closely linked to axonal guidance and synaptic LTP, particularly NMDA dependent plasticity (Filosa et al., 2009). Interactions of the Ephrin receptors across adjacent neurons or glial cells and neurons help to guide axonal repulsion (Xu and Henkemeyer, 2012), dendritic spine stability, synaptogenesis (Lin and Koleske, 2010), and control synapse glutamate concentrations (Filosa et al., 2009), all of which impact LTP. Schizophrenia imaging genetics studies have found associations between axonal guidance signaling genes and prediction of fMRI measures of dorsolateral prefrontal cortex (DLPFC) inefficiency during a working memory task (Walton et al., 2013). A link between altered LTP and schizophrenia was shown more directly in a transcranial direct current stimulation (tDCS) study, which found altered

LTP-like plasticity in patients (Hasan et al., 2013). Additionally, schizophrenia animal models using NMDAR antagonists, have shown effects on both LTP and behavior measures demonstrating similar alterations to the cognitive, negative, and positive symptoms found in humans (Wiescholleck and Manahan-Vaughan, 2013). This suggests that Ephrin receptor signaling and axonal guidance alterations leading to changes in NMDA driven synaptic LTP alterations could lead to all three spectra of symptoms associated with the disorder.

The enrichment of Sertoli cell junction signaling gene sets is compelling as increased risk of schizophrenia is associated with increased paternal and grandpaternal age (Frans et al., 2011). Sertoli cells create the supportive niche for the spermatogonial stem cells and create the blood-testis barrier (Kaur et al., 2014). It is suggested that reduced Sertoli cell population with age may reduce both germ cell production and quality (Paul and Robaire, 2013). Perhaps alterations in Sertoli cell function via dysregulation by this miRNA could also reduce germ cell quality of patient fathers and grandfathers.

Finally, the miR-137 validated target PKA signaling gene set (*MAPK1*, *MAPK3*, *TCF4*, and *PTGS2*) is of particular interest given that enrichment of schizophrenia-risk associated variants within these targets was replicated in an independent cohort. PKA signaling modulates glutamate signaling and responds to dopamine signaling, both highly implicated in schizophrenia (Sarantis et al., 2009). PKA signaling also appears to play a critical role in the synergistic interactions between these two neurotransmitter signaling cascades within the hippocampus and prefrontal cortex through activity of MAPK1 and MAPK3 (ERK1/2) (Sarantis et al., 2009).

PKA signaling is also critical for maturation of prefrontal cortex D1 excitability in adolescence, a region well known for alterations in schizophrenia and a time period of particular vulnerability (Heng et al., 2011). Inhibition of phosphodiesterase 4 (PDE4), an enzyme implicated in schizophrenia and involved in the auto-inhibition of PKA signaling, increases D1 signaling in pyramidal neurons of the prefrontal cortex and enhanced sensory gating behavior in mice as measured by prepulse inhibition (PPI) (Juraeva et al., 2014). Interestingly, both schizophrenia and control subjects carrying a schizophrenia-risk associated variant within the *TCF4* gene and mice moderately overexpressing TCF4, a transcription factor downstream of PKA signaling, (Figure 4.2) also have disrupted PPI activity (Brzozka et al., 2010 and Quednow et al., 2014). Evidence for a role of this molecule in schizophrenia is extensive (Quednow et al., 2014). *TCF4* mRNA expression is increased in human induced pluripotent stem cells (hiPSC) from schizophrenia patients (Brennand et al., 2011) and increased in postmortem DLPFC samples of miR-137 risk SNP carriers (Guella et al., 2013).

The remaining PKA gene set molecule, *PTGS2*, encoding the COX-2 protein, is gaining attention as a schizophrenia drug target because inhibitors appear to be beneficial in symptom treatment (Baheti et al., 2013; Müller et al., 2010). *PTGS2* mRNA expression is altered in the prefrontal cortex of patients (Tang et al., 2012). This risk gene is relevant to the inflammatory basis theories for schizophrenia (Feigenson et al., 2014). As depicted in Figure 4.2, *PTGS2* is responsive in inflammatory processes such as infection, a potential risk factor for the disorder.

Given the current bioinformatics tools one limitation of this analysis is the lack of evaluation for the possible creation of new binding sites from polymorphisms in

unpredicted target genes. Also, the TargetScan algorithm may have missed other true targets, especially in gene lists where the scope is more constrained. On the other hand, the algorithm likely predicted false positive interactions, so analysis of the higher confidence target lists derived from a variety of target prediction scoring lists helps verify the pathway vulnerabilities. The experimentally validated target list was limited by current lack of annotation. Our current tools do not allow evaluation of how target SNPs might impact regulation. Given the heterogeneity and polygenicity of schizophrenia, our replication sample size was perhaps too small to allow full replication of many of our results (Purcell et al., 2009). However, the replication of associated variants within the validated target PKA signaling gene set suggests that other signaling pathways gene set risk association may be replicated in a larger sample. Moreover, the use of the PGC stage 1 dataset (Ripke et al., 2011) provided a unique opportunity for use of a very large dataset giving confidence to our findings. Further replication with larger sample sizes will help validate our results.

Despite these limitations, our analysis of the enrichment of schizophrenia-associated variants within pathway specific gene sets of potential miR-137 targets suggests that these pathways are particularly vulnerable to dysregulation by this miRNA.

4.6 Conclusions

Genetic association studies indicate that variants within miRNAs and targets can have great impact on specific diseases (Abelson, 2005; Wang et al., 2008). These studies often evaluate variants within one risk gene of interest at a time, and discover alterations in miRNA binding to that specific target risk gene. However, each miRNA has the capacity

to target hundreds of targets and impact many different pathways, so determining possible variants associated with disease that impact miRNA regulation, can be challenging. Thus studies like this, evaluating many putative and validated targets, are necessary first steps to guide further research on the impact of specific miRNAs in diseases.

Many schizophrenia relevant pathways were previously identified to have an overrepresentation of miR-137 target genes. Our findings of schizophrenia-associated variants within PKA signaling and other pathways provide a map to guide further investigation of the role of this miRNA in this illness.

4.7 Competing Interests

The authors declare that they have no competing interests.

4.8 Author's Contributions

CW helped design the study, carried out the analyses, and drafted the manuscript. NPB and JT designed the study and assisted in interpreting the data and writing the manuscript. VC, LW, and SE revised the manuscript and assisted in data interpretation. All authors have read and approved the final manuscript.

4.9 Acknowledgements

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was shared through support from the National Institutes of Health grants (P50 MH071616, R01 MH056584, 1R01 MH084803 to LW) and 1U01 MH097435 (LW and JAT). The database for annotation, visualization, and integrated discovery (DAVID) gene ID conversion tool was used to convert target gene official gene symbols into Entrez IDs. GeneCards was used for genes that could not be converted with this tool. Preliminary data was presented in a poster entitled, 'Meta gene set variant enrichment analysis of miR-137 predicted and validated targets reveals schizophrenia vulnerable pathways' at the 2014 International Imaging Genetics Conference (IIGC).

5. Polymorphisms in *MIR137* and microRNA-137 regulated genes influence gray matter structure in schizophrenia

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To Submit To Schizophrenia Research

5.1 Abstract

Background: Evidence suggests that microRNA-137 (miR-137) is involved in the genetic basis of schizophrenia. Risk variants within the host gene of this miRNA influence structural and functional brain imaging measures, and miR-137 itself is predicted to regulate hundreds of genes. We evaluated the influence of a *MIR137* risk variant (rs1625579), in combination with variants in miR-137 regulated genes, *TCF4*, *PTGS2*, *MAPK1* and *MAPK*, on brain imaging measures. These genes were selected based on previous work showing their relationship with increased risk of schizophrenia.

Methods: We identified the *MIR137* risk variant genotype and a genetic risk score based on genotypes in *TCF4, PTGS2, MAPK1* and *MAPK3* in 221 Caucasian subjects (89 schizophrenia patients and 132 controls). We evaluated the effects of rs1626679 genotype with the risk score in a three-way interaction with diagnosis, on the expression of multivariate gray matter concentration (GMC) patterns.

Results: We found that only schizophrenia subjects homozygous for the *MIR137* risk SNP show a decrease in occipital, parietal, and temporal lobe GMC (including Brodmann area19, 39, and 40) with increasing miR-137 regulated gene risk score.

Conclusions: Variants within or upstream of *MIR137* and regulated genes may in part influence gray matter measures in the implicated regions in patients with schizophrenia. These genes are all involved in the PKA signaling pathway suggesting that dysregulation of the pathway through MIR137 may underlie the gray matter loss seen in the disease.

5.2 Introduction

Evidence for the role of miR-137 in the genetic basis of schizophrenia is increasingly accumulating, although the mechanism of association is not known. Interest in the miRNA began after a single nucleotide polymorphism (SNP), rs1625579, within the host gene (*MIR137*) was identified as the top associated SNP in the first large schizophrenia genome wide association study (GWAS) (Ripke et al., 2011). Four other new variants were identified in this GWAS within genes now experimentally verified as miR-137 targets (Kwon et al., 2013). A subsequent and larger GWAS identified another SNP near the miR-137 host gene (Ripke et al., 2013). The largest schizophrenia GWAS to date, including over 150 000 subjects also identified a SNP, rs1702294, located closely to the previously identified rs1625579 SNP (Ripke et al., 2014). The consequences of these SNPs on miR-137 biogenesis has not yet been determined. However, post mortem tissue analysis suggests that the rs1625579 risk genotype predicts lower miR-137 expression in the dorsolateral prefrontal cortex (DLPFC) (Guella et al., 2013).

Functional studies indicate that the miRNA is involved in controlling neuronal proliferation, differentiation, and dendritic arborization (Silber et al., 2008; Smrt et al., 2010; Sun et al., 2011; Szulwach et al., 2010) all of which are important for proper neurogenesis, a process implicated in schizophrenia (O'Reilly et al., 2014; Toro and Deakin, 2007). RNA expression studies suggest that the miRNA regulates expression of predicted target genes involved in several schizophrenia relevant pathways (Collins et al., 2014) and confirms regulation of genes involved in neuronal differentiation (Hill et al., 2014). Bioinformatics studies indicate that a significant number of target genes are

associated with schizophrenia risk and further predict that the miRNA regulates many schizophrenia relevant pathways (Wright et al., 2013).

Gray matter loss is well described in schizophrenia (Vita et al., 2012). Imaging genetics studies seek to determine how genetic factors contribute to specific regions of loss and to provide insight about how plausible genetic factors may influence the pathophysiology of the disorder (Hariri et al., 2006). Studies using this approach indicate that the rs1625579 SNP influences a variety of structural and functional measures in patients and controls. Some effects appear to be common among risk allele carriers regardless of diagnosis, while other effects appear to be unique to schizophrenia carriers. Findings of common genotypic effects within homozygous risk allele carriers across diagnostic groups include: higher activation of the DLPFC during a working memory task (van Erp et al., 2014) and decreased activation of the posterior right medial frontal gyrus during a sentence completion task (Whalley et al., 2012). Effects of the genotype specific to schizophrenia or schizophrenia-at-risk status include: reduced hippocampal volume, increased ventricle volume, (Lett et al., 2013) increased mid-posterior corpus callosum volume (Patel et al., under review), and decreased white matter integrity measures (Kelly et al., 2014; Lett et al., 2013), as well as, altered activation of the amygdala and pre- and post-central gyrus during a sentence completion task (Whalley et al., 2012).

Given that miR-137 is known to regulate many schizophrenia risk genes, (Wright et al., 2013) its effects on schizophrenia-related phenotypes may be modulated by genotypic variation in its target genes. To our knowledge, no studies have yet evaluated the impact of the *MIR137* risk variant in combination with subsets of target gene variants, on brain imaging phenotypes. Beginning with a set of miR-137 regulated genes that are also

associated with schizophrenia risk, we examined the interaction of risk gene genetic variation, *MIR137* risk variants, and schizophrenia disease status on brain measures.

The genes of interest include *TCF4*, *PTGS2*, *MAPK1* and *MAPK3*. *TCF4* and *PTGS2* (which encodes the COX-2 protein) are experimentally validated target genes directly regulated by this microRNA (Kwon et al., 2013 and Chen et al., 2012). There is substantial evidence for TCF4 (Quednow et al., 2014) in schizophrenia and more recently for PTGS2 (Müller et al., 2010; Tang et al., 2012). *MAPK1* and *MAPK3* are associated with schizophrenia (Yuan et al., 2010) and are indirectly regulated by miR-137. Regulation of the activation of the *MAPK1* and *MAPK3* protein products erk1/2 by miR-137 has been consistently demonstrated experimentally (Chen et al., 2011; Liang et al., 2013; Zhu et al., 2013). These genes group within the PKA signaling pathway according to Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, CA, USA, www.ingenuity.com). Experimental evidence suggests the genes are indeed involved in this pathway (Taurin et al., 2008; Hino et al., 2005; Tamura et al., 2002; He et al., 2010). The PKA signaling pathway was shown previously to be overrepresented with predicted miR-137 target genes (Wright et al., 2013).

We use multivariate analyses (Chen et al., 2014; Turner et al., 2012; Xu et al., 2009) to avoid region by region or voxel by voxel analyses, to reduce the number of phenotypes under consideration, while capturing gray matter concentration (GMC) variation throughout the brain. Here we evaluated the impact of the rs1625579 genotype along with a genetic risk score combining SNPs within miR-137 regulated genes associated with schizophrenia-risk and grouping within the PKA pathway (Wright et al., 2014, unpublished observations) on structural patterns of GMC loss in schizophrenia.

5.3 Materials and Methods

5.3.1 Subject demographics

Subject imaging and genetic data was derived from the Mind Clinical Imaging Consortium (MCIC) shared repository (Gollub et al., 2013) and Northwestern University (NU) datasets (Wang et al., 2013). A total of 221 Caucasian subjects were analyzed from both datasets. The schizophrenia subject cohort was identified according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for diagnosis of schizophrenia, schizoaffective disorder or schizophreniform disorder. For MCIC, controls were excluded based on past or present psychiatric illness including drug abuse or drug dependence, or use of antidepressants, anti-anxiety medication or sleep aids for greater than 2 months or within 6 months prior to the MRI scan. All MCIC subjects were excluded for IQ<70, a history of head injury with prolonged loss of consciousness, history of skull fracture, or severe/disabling medical conditions. NU subjects were excluded for drug abuse or dependence, severe medical disorders, head injury history with loss of consciousness, or diagnosis of mental retardation according to DSM-IV. See Table 5.1 for subject demographic information and Table 5.2 for patient clinical information. All subjects provided informed consent and all subject data was collected according to IRB standards.

Dataset	MCIC	NU	MCIC/ NU Merged	Mean Age	Age Range	MCIC Handedness	MCIC Mean Parent SES	MCIC Mean Years of Education
Controls	90 (61% male)	42 (55% male)	132 (59% male)	32	14-60	82 Right 5 Mixed 3 Left	2.7 range 1-5	15.3 range 12-21
Cases	60 (75% male)	29 (69% male)	89 (73% male)	34	17-61	54 Right 4 Mixed 1 Left 1 Unknown	2.6 range 1-5	13.6 range 7-22
All Subjects	150 (67% male)	71 (61% male)	221 (65% male)	33	21-61	136 Right 9 Mixed 4 Left 1 Unknown	2.65 range 1-5	14.45 range 7-22

Table 5.1. Subject Demographics

Handedness, parent socioeconomic status (SES) and subject years of education was only available for MCIC subjects. Parent SES was reported by 100 % of controls and 95% of cases. Years of education was reported by 99% of controls and 93% of cases. Parent SES score was based on the following: 1= Situation of wealth, education, top-rank social prestige; 2 = College or advanced degree; professional or high-rank managerial position; 3 = Small businessman, white-collar and skilled worker; high school graduate; 4= Semi-skilled worker, laborer; education below secondary level; 5 =Unskilled and semi-skilled worker; elementary education.

Category	**Mean Duration of Illness** (in years)	**Mean Positive Symptoms** (SAPS)	**Mean Negative Symptoms** (SANS)	**Mean Disorganized Symptoms score** (SAPS)	**Mean Chlorpromazine Equivalence** (4 MCIC subjects were not medicated with antipsychotics)
MCIC	11.7 range 0.25- 42	4.7 range 0-10	8.0 range 0-15	1.7 range 0-6	594 range 0-2750
NU	NA	2.1 range 0-10	4.5 range 0-18	NA	NA

Table 5.2. MCIC Schizophrenia Subject Clinical Information

Table shows available clinical information for the subjects used in this study. For the MCIC subjects analyzed, 98% of subjects reported duration of illness, 100% reported symptom scores, and 96% reported medication dosages. For the NU subjects analyzed, 100% of subjects reported positive symptoms scores and 82 % reported negative symptom scores. SAPS= Scale for the Assessment of Positive Symptoms; SANS= Scale for the Assessment of Negative Symptoms. Chlorpromazine equivalencies were calculated as in **(**Andreasen et al., 2010**)**.

5.3.2 Genetic data

Genotyping was performed at the Mind Research Network Neurogenetics Core lab using the Illumina Human Omni-Quad 1M BeadChip for MCIC and the Illumina Human Omni-Quad 5M chip for NU. Using PLINK, the genotype data from MCIC and NU was merged after updating SNP locations of MCIC to match that of the more recent NU data. Quality control was performed before and after merging with the following thresholds: Hardy Weinberg equilibrium P $<10^{-6}$, minor allele frequency < 0.05, missing rate per SNP < 0.02, missing rate per individual < 0.02. Caucasian subjects were identified using the Enhancing Neuroimaging Genetics through Meta-Analysis (ENIGMA) multi-dimensional scaling (MDS) protocol within the imputation protocol (http://enigma.ini.usc.edu/protocols/genetics-protocols/). Genetic outliers and related subjects were identified with identity-by-state (IBS) and identity-by-decent (IBD) testing in PLINK. One individual subject was removed from each pair or group of subjects with pi-hat values of 0.05 or greater. MCIC subjects were genotyped separately for the rs1625579 SNP at the Mind Research Network Neurogenetics Core lab using a custom TaqMan® assay.

5.3.2.1 miR-137 regulated gene genetic risk score

Genetic risk scores were based on variants previously identified to be the top SNPs associated with schizophrenia risk within miR-137 regulated genes or their respective regulatory regions, in a gene set enrichment analysis (Wright et al., 2014, unpublished observation). These SNPs were rs2276195 (*TCF4*), rs10489401 (*PTGS2*), rs9610608 (*MAPK1*) and rs7202714 (*MAPK3*), see Table 5.3 for SNP information.

The risk score was calculated across these SNPs for each subject based on the number of risk alleles (0, 1, or 2) per SNP weighted by its respective odds ratio and summed for all four SNPs. The risk allele and odds ratio used to create risk scores were derived from the psychiatric genomics consortium (PGC) recent 2014 GWAS (Ripke et al., 2014), http://www.med.unc.edu/pgc/downloads. This risk score was rounded to two decimal places.

Gene	SNP	Location	Minor/ Major Allele	Minor Allele frequency in cases	Minor Allele frequency in controls	Chi-Square	P value	OR for Minor Allele	PGC risk allele	PGC 2014 odds ratio
MIR137	rs1625579	Intronic	G/T	0.163	0.178	0.1705	0.679	0.899	T	1.120
miR-137 Regulated Gene Risk Score SNPs										
TCF4	rs2276195	Intronic	T/C	0.163	0.296	10.18	0.001	0.464	T	0.990
PTGS2	rs10489401	Upstream	G/A	0.287	0.398	5.758	0.016	0.608	A	1.005
MAPK1	rs9610608	Upstream	G/A	0.191	0.102	7.037	0.008	2.073	A	0.995
MAPK3	rs7202714	Upstream	T/C	0.393	0.265	8.062	0.004	1.796	T	1.039

Table 5.3. SNP Information

Table shows information for all single nucleotide polymorphisms (SNPs) used in this study. All risk score SNPs were found to have significantly different minor allele frequencies between cases and controls. OR = odds ratio; PGC = psychiatric genomics consortium. All SNPs are within110 kb upstream to 40 kb downstream of each gene's transcript boundaries.

5.3.3 Imaging data

Structural T1 MRI scans were conducted across 4 sites (University of Minnesota (UMinn), Massachusetts General Hospital (MGH), the University of Iowa (UIowa), and the University of New Mexico (UNM)) for the MCIC dataset and one site (Northwestern University) for the NU dataset. MCIC scans were collected in the coronal orientation using Siemens (1.5T), GE Signa (1.5T), and Siemans Trio (3) scanners with voxel sizes of 0.625 X 0.625 X 1.5, 0.664 X 0.664 X 1.6, and 0.625 X 0.625 X 1.5 respectively. NU

scans were collected in sagittal orientation with a Siemans (1.5T) scanner with 1 X 1 X 1.25 voxel size.

5.3.3.1 Image pre-processing

All T1 Images were co-registered to the same stereotactic space using an affine transformation, resliced to 2 x 2 x 2 mm, and segmented into cerebrospinal fluid (CSF) and gray and white matter using the Statistical Parametric Mapping 5 software (SPM5) unified segmentation method (Ashburner and Friston, 2005). Images were visual inspected and evaluated for correlation to an averaged image across all subjects. Images with a correlation value <0.9 to the mean image from all subjects were dropped from further analysis. Site of scan, gender, and subject age were then regressed out of the gray matter images to allow further analysis to be more sensitive to group and genotypic imaging differences. These regressed gray matter images were then smoothed with a full width half maximum (FWHM) Guassian kernel of 10mm. See (Chen et al., 2014) for more details on the methods used here.

5.3.3.2 Multivariate SBM image analysis

Independent Component Analysis (ICA) was performed on the pre-processed gray matter images. This analysis was performed using the SBM module of the GIFT toolbox (http://mialab.mrn.org/software/gift/index.html). Estimation of the appropriate number of independent components to capture the variance within the subject image files was performed using a minimum description length (MDL) method (Li et al., 2007) and was determined to be 18. We performed a group ICA using the Infomax algorithm and the ICASSO algorithm 20 times with a bootstrap and random initialization each time. The

imaging data was decomposed into these 18 GMC components and loading coefficients or weights respective to the contribution of each structural component to each subject's image. These loading coefficients were used to determine group contribution differences across these GMC components. The spatial maps for the components were all visually inspected and none were removed as being artifactual.

5.3.4 Imaging genetics statistical analysis

A multiple regression analysis was performed using the loading coefficients for the 18 independent components as dependent variables and diagnosis as the grouping factor to determine which components captured imaging variance due to diagnostic group. Tests were deemed significant based on Bonferroni multiple testing correction ($p < 0.002$).

A multivariate analysis with the rs1625579 genotype, diagnosis, and genotyping chip type included as factors, and the miR-137 regulated gene risk score included as a covariate was then performed on the loading coefficients of the structural GMC components showing a diagnosis effect. The main effect of rs1625579, chip type, and risk score, two-way interactions between genotype and risk score, genotype and diagnosis, risk score and diagnosis and a three-way interaction between genotype, diagnosis, and risk score were all considered. Given that only 5 subjects were homozygous for the G allele, the rs1625579 genotype was analyzed in two groups, those homozygous for the T allele and those not.

5.4 Results

5.4.1 Risk score results

The miR-137 regulated gene risk score distribution had a skewness of .610 and kurtosis of -0.182, suggestive of normality. Mean risk score for patients was 0.025 with a 0.023 standard deviation; for controls, it was 0.012 and 0.022. A two sample t test determined that the patients' risk score was significantly greater than the controls' ($t = 4.07$, $df = 162$ $p < 0.001$, equality of variances not assumed). This remained significant ($p < 0.001$) after removing two subjects with extreme single frequency scores. All further analyses were performed following the removal of these subjects ($N = 219$). The range of risk scores for both diagnostic groups was -.02 to 0.08 following this removal.

5.4.2 Imaging results

Three components had loading coefficients significantly associated with diagnostic group following Bonferroni correction for multiple comparisons ($p < 0.002$). See Appendix B, Table B.1 and Appendix B, Figure B.1 for the brain regions captured by these components.

5.4.3 Imaging genetics results

Of the three components significant for diagnosis, one component had a significant a three-way interaction between the rs1625579 genotype, the miR-137 regulated gene risk score, and diagnosis ($F = 6.3$, $df = 1$, $p = 0.013$). The main effect of the genotype, risk score, and the two-way interactions evaluated were not found to be significant. Post hoc

univariate analysis of the loadings for this component for the two diagnostic groups separately, accounting for genotyping chip type as a random factor, and evaluating for two way interactions between risk score and rs1625579 genotype revealed that the identified three-way interaction in the multivariate analysis was driven by a two-way interaction in the schizophrenia subjects alone. No significant interaction or main effect was found in the healthy control group and only the interaction between risk score and rs1625579 genotype was significant for the schizophrenia subjects ($F = 5.98$, $df = 1$, $p = .017$). Figure 5.1 and table 5.4 show the areas of GMC variance in this particular component, with the greatest weightings in the spatial map being in the occipital lobe and regions of the parietal and temporal lobe.

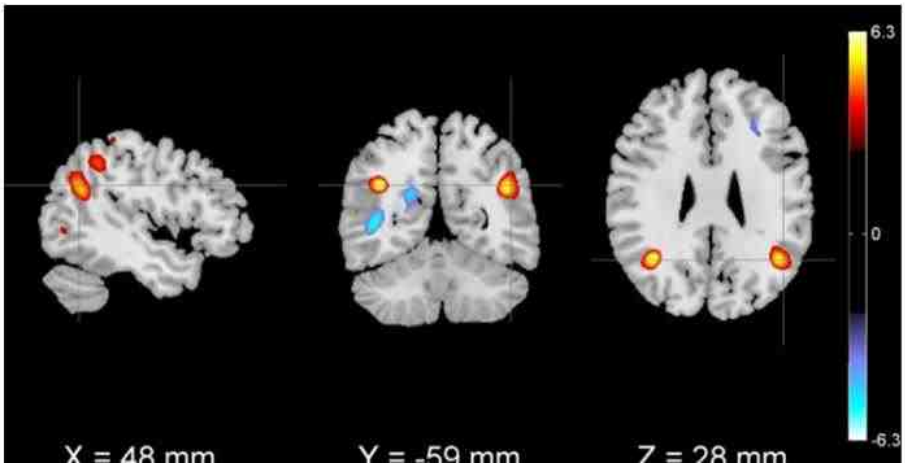

Figure 5.1. The spatial component showing a genetic and diagnosis interactive effect

This figure shows the regions within the occipital lobe (Brodmann area 19), angular gyrus (Brodmann area 39), supramarginal Gyrus, and the inferior parietal lobule (Brodmann area 40) with greater gray matter concentration (GMC) in controls than patients. The spatial map is overlaid on a template brain, thresholded with z scores >|3.5|. The heat map coloring indicates z score intensity, with red indicating findings of GMC greater in controls and blue indicating areas of GMC greater in patients. White indicates areas with greatest z scores. The bar on the right indicates z score.

Finding Directionality	Area	L/R Brodmann Area	L/R volume (cm^3)	L/R Max Z score region and coordinates (x, y, z)
Positive CT>SZ	Inferior Occipital Gyrus/ Inferior Parietal Lobule	19/40	0.8/0.6	6.4 (-38, -68, 2)/5.2 (36, -41, 37)
	Angular Gyrus	39/39	0.4/0.8	6.3 (-40, -55, 29)/5.9 (44, -55, 27)
	Middle Occipital Gyrus	19/NA	1.0/0.0	5.8 (-40, -70, 5)/NA
	Occipital Gyrus/ Inferior Temporal Gyrus	19/47	0.4/0.3	5.8 (-42, -70, 2)/4.7 (42, -66, 2)
	Angular Gyrus/ Inferior Parietal Lobule	39/40	0.4/1.5	5.3 (-40, -51, 27)/5.7 (40, -41, 37)
	Angular Gyrus	39/39	0.3/0.5	4.7 (-40, -55, 32)/5.4 (44, -57, 30)
	Inferior Parietal Lobule, Supramarginal Gyrus	NA/40	0.0/1.3	NA/5.3 (44, -43, 39)
	Middle Temporal Gyrus	39/39	0.3/0.3	5.1 (-40, -59, 29)/4.6 (46, -57, 23)
	Fusiform Gyrus	19/NA	0.1/0.0	4.3 (-40, -70, -5)/NA
	Superior Parietal Lobule	7/NA	0.1/0.4	3.8 (-34, -65, 51)/4.3 (32, -67, 49)
	Calcarine/Lingual Gyrus	18/18	0.2/0.1	3.8 (-6, -95, -5)/3.6 (20, -82, -1)
Negative SZ>CT	Middle Occipital Gyrus	18/NA	0.7/0.0	5.8 (-30, -77, 13)/NA
	Middle Temporal Gyrus	37/NA	0.8/0.0	5.7 (-44, -56, 5)/NA
	Cuneus	23/48	1.0/0.6	4.9 (-18, -55, 25)/4.4 (34, 15, 29)
	Middle Frontal Gyrus	NA/48	0.0/0.4	NA/4.1 (30, 27, 26)
	Temporal Gyrus	20/NA	0.3/0.0	4.1 (-46, -23, -24)/NA
	Precuneus	23/NA	0.1/0.0	3.9 (-14, -52, 17)/NA
	Lingual Gyrus	18/18	0.1/0.1	3.8 (-18, -72, 2)/3.8 (16, -70, 3)

Table 5.4. Brain Regions in the Spatial Pattern Showing Genetic and Diagnostic Effects

Table shows the brain regions of gray matter concentration (GMC) variance between patients and controls for component 11. Positive findings indicate areas where GMC is greater in controls. Negative findings indicate areas where GMC is greater in patients. Findings are shown for the left (L) and right (R). CT = Control; SZ= Schizophrenia.

The findings include previously well-known schizophrenia relevant regions including the angular gyrus (Brodmann area 39), the supramarginal gyrus and the inferior parietal

lobule (Brodmann area 40). Appendix B, Figure B.1 depicts the areas of GMC variance captured by the other two components. Figure 5.2 shows the relationship between the rs1625579 genotype and the genetic risk for the associated component for both diagnostic groups. The increased genetic risk by MIR137 genotype and regulated gene risk genotypes is associated with GMC loss in subjects with schizophrenia but not in controls.

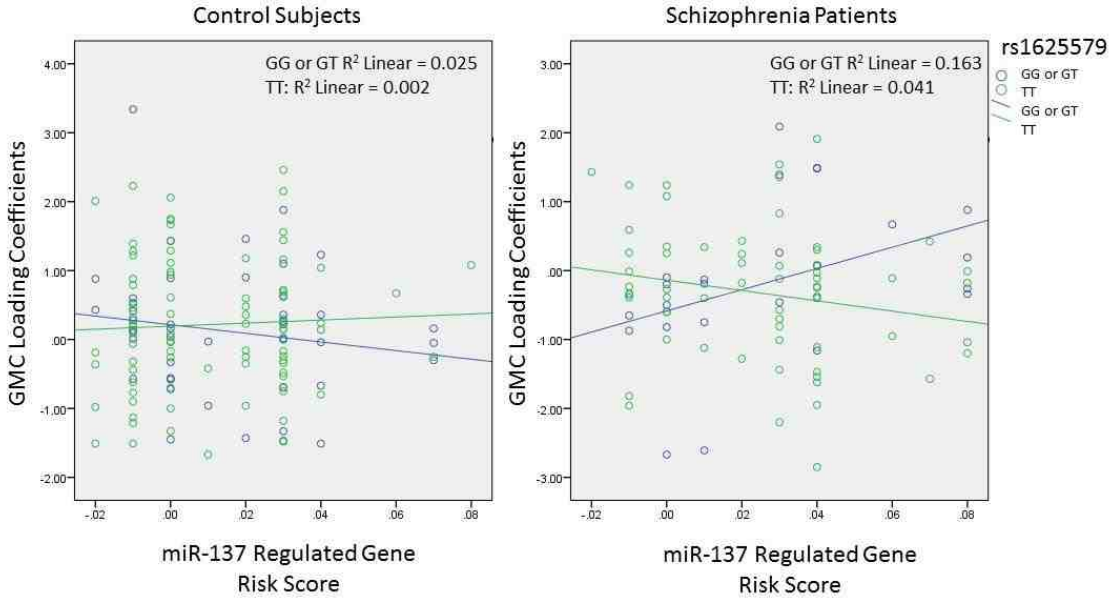

Figure 5.2. Relationship between GMC, diagnosis, rs1625579 genotype, and miR-137 regulated gene risk score

This figure shows the interaction between the rs1625579 genotype and the miR-137 regulated gene risk score on structural loading coefficients, and the directionality of the relationship across diagnostic groups. The solid blue lines indicate the best-fit trend line for subjects with the GG or GT rs1625579 genotype and the solid green lines indicate that of the TT subjects.

5.5 Discussion

Our results indicate a schizophrenia-specific interaction of the rs1625579 genotype with cumulative risk summed across a subset of regulated risk gene SNPs. The interactive effect of this genotype with the miR-137 regulated risk score is reduced GMC within the occipital, parietal, and temporal lobe. Within patients with schizophrenia, those with two risk alleles (TT) showed decreasing GMC in these regions with increasing genetic risk score, while those with only a single or no risk allele did not. This pattern was not present in healthy controls, and if anything was reversed.

This suggests that within schizophrenia, the rs1625579 *MIR137* SNP in conjunction with specific genotypes in the PKA pathway may cause altered regulation of the genes within our risk score and lead to decreased GMC within Brodmann area 19 of the occipital lobe, Brodmann area 39 of the angular gyrus, and Brodmann area 40 of the supramarginal gyrus and inferior parietal lobule. These regions have all previously been implicated in schizophrenia and are associated with exploratory eye movement dysfunction (Qiu et al., 2011), theory of mind or self-sensing deficits (Guo et al., 2014), hallucinations (Curcic-Blake et al., 2013; Chen et al., 2013; Diederen et al., 2012; Sommer et al., 2012), adaptive control deficits (Becerril and Barch, 2013), and attention deficits (Thimm et al., 2011; Huang et al., 2010). This raises promising possibilities that these genetic variations within people with schizophrenia could be related to GMC and functional loss within these networks, leading to previously unexplained variation in their clinical symptoms and capabilities.

Interestingly, a risk genotype for a variant within another miR-137 experimentally validated target gene, *CACNA1C*, was associated with reduced activity in Brodmann area 40 during orientation of attention in an fMRI study (Thimm et al., 2011). Gray-matter volume decrease of the supramarginal gyrus was also associated with poor cognitive test scores in unaffected relatives of schizophrenia patients, further suggesting genetic liability of alterations in this region (Bhojraj et al., 2011). The 22q.11.2 deletion, one of the highest known risk factors for schizophrenia is associated with altered miRNA biogenesis (Sellier et al., 2014) and cortical morphological reduction of this region, (Kates et al., 2011). The effect of the *MIR137* risk SNPs on the biogenesis of the miRNA has not been determined, but postmortem tissue analysis (Guella et al., 2013) suggests that the biogenesis may be disrupted with the rs1625579 risk genotype. Therefore, other findings implicating this region with reduced miRNA biogenesis may be in part due to a reduction of miR-137.

Very few studies have evaluted the role of PKA signaling on GMC in the brain regions implicated here. However, evidence suggests that this signaling pathway (Funk et al., 2012) and each of the investigated genes within it are associated with schizophrenia overall (Müller et al., 2010; Quednow et al., 2014; Yuan et al., 2010). Further research is required to determine the exact mechanism of the association presented in this study, to determine the replicability of these findings, and to determine if these genetic factors also influence exploratory eye movement, self-sensing, hallucinations, adaptive control, or attention characteristics. Our analysis is a first step into determining the combined effect of *MIR137* variants with miR-137 regulated gene variants and our results suggest a key structural and functional region is affected by rs1625579 vulnerability.

5.6 Role of funding source

This work was supported by The National Institutes of Health (5R01MH094524-03 to VDC and JAT; R21DA034452-01 to NPB). The MCIC dataset was collected through support from the Department of Energy (DE-FG02-99ER62764 to VDC) and the National Institutes of Health (5P20RR021938/P20GM103472 to VDC). The NU dataset was shared through support from the National Institutes of Health grants (P50 MH071616, R01 MH056584, 1R01 MH084803 to LW) and 1U01 MH097435 (LW and JAT).

5.7 Contributors

C. Wright, V. Calhoun, N. Perrone-Bizzozero, and J. Turner designed the analyses. C. Wright performed the analyses and wrote the paper. C. N. Gupta and J. Chen assisted with the imaging analyses. V. Patel assisted with the genetic data. J. Turner revised the manuscript. All authors approve the manuscript. S. Ehrlich and L. Wang assisted in subject data collection and manuscript revisions.

5.8 Conflict of interest

All authors have no conflicts of interest to report.

5.9 Acknowledgements

The authors would like to thank the participants that enrolled in the studies used in this analysis, as well as Marilee Morgan for assistance with genotyping.

6. General Discussion

6.1 Findings and significance

The goals of this project were: 1) to identify and characterize all predicted and experimentally validated target genes; 2) to determine enrichment of schizophrenia associated variants within pathway relevant miR-137 target gene sets; and 3) to evaluate the effect of the *MIR137* schizophrenia risk SNP, rs1625579, in addition to target gene polymorphisms on structural gray matter.

The results of this project increase our understanding of the influence of miR-137 in schizophrenia in three ways:

1) We identify that the putative and confirmed targets of this miRNA fall into pathways relevant for schizophrenia development.

2) We identify that many of these same putative and confirmed targets are associated with schizophrenia risk.

3) We determine that a particular gene set of target genes affects gray matter in brain regions previously identified to mediate schizophrenia phenotypes.

At the onset of this project, the first genetic association study linking rs1625579 with schizophrenia included an evaluation of the enrichment of associated SNPs within a limited number of predicted target genes and found a significant enrichment (Ripke et al., 2011). This same research group later replicated this finding using a joint set enrichment method (Ripke et al., 2013). At that time, no other studies had investigated the targets of this miRNA or their possible role in schizophrenia. Given that miRNAs can regulate

hundreds of target genes (Bartel, 2009) and thus a great number of biological pathways and functions, it is highly probable that the mechanism for the involvement of miR-137 in schizophrenia would involve target genes that play some critical role in schizophrenia relevant pathways.

With our first goal, we identified many target genes to be associated with schizophrenia and we determined that these target genes have unique temporal expression profiles, with peak expression levels during developmentally critical time-periods. miR-137 target genes were found to be overrepresented in several schizophrenia relevant pathways. This was the first evaluation of miR-137 target genes in a pathway approach and demonstrated that pathways involved in neuronal development and plasticity, such as axonal guidance signaling, Ephrin receptor signaling, and long-term potentiation, were indeed enriched with targets of the miRNA. Additionally we identified other miR-137 target enriched pathways that maybe more distantly impactful, such as Sertoli cell junction signaling. Since then, two recently published studies evaluated enrichment of dysregulated genes within pathways, following over-expression and inhibition of miR-137. Their results identified an overrepresentation of dysregulated genes within pathways of MHC genes, synapse formation, calcium channel signaling (Collins et al., 2014), and neuronal differentiation (Hill et al., 2014). All of these pathways have been previously associated with schizophrenia (Mukherjee et al., 2014; Yang et al., 2014; Berridge, 2013; O'Reilly et al., 2014 and Toro and Deakin, 2007) and brain development (Durand et al., 1996; Sun et al., 2009; Wu et al., 2011; and Rosenberg and Spitzer et al., 2011). These studies further suggest that the miRNA may contribute to developmental regulation of the nervous system. Therefore, consistent with the neurodevelopmental basis theory for

schizophrenia, (Gupta and Kulhara, 2010), the miRNA may contribute to schizophrenia risk by altering regulation of the nervous system processes during development.

Our next goal was to evaluate the schizophrenia risk contribution of target genes within our previously identified target enriched pathways. We identified several target gene sets specific to neuronal development and plasticity pathways among others that had significantly more schizophrenia-associated variants than 10,000 random gene sets of the same size. This included target gene sets from the following pathways: Ephrin receptor signaling, axonal guidance signaling, synaptic LTP, and Sertoli cell junction signaling, and PKA signaling. We successfully replicated significant enrichment of associated variants within a regulated gene set of genes involved in PKA signaling in a separate cohort. This analysis was the first evaluation of schizophrenia risk contribution of miR-137 targets in a pathway specific manner. Additionally our study was the first to analyze risk contribution of experimentally validated targets rather than putative targets alone. Our findings suggest that the miRNA may contribute to schizophrenia risk by disrupting regulation of targets within these pathways.

The final goal of our studies was to determine if variants of target genes may in concert with the miR-137 host gene (*MIR-137*) variant alter gray matter measures. We chose to focus our investigation on the polymorphisms within the target genes and downstream regulated genes of the PKA signaling gene set associated with schizophrenia risk in the second aim, as this schizophrenia association for this gene set was replicated in a separate cohort. This was the first analysis of the influence of the *MIR-137* risk SNP rs1625579 genotype in combination with miR-137 regulated gene SNP genotypes on brain imaging measures. A risk score was used to investigate the contribution of regulated gene variants

on gray matter concentration. Gray matter concentration variance among all subjects was determined using Source Based Morphometry, a multivariate approach using independent component analysis (Ashburner and Friston, 2005). This produces loadings for each subject that represent weights for each subject's contribution to each component (in this case, brain regions of gray matter concentration) found to vary within the subjects analyzed. Three components were found to capture gray matter concentration variance between patients and controls. miR-137 regulated gene risk score was evaluated with the rs1625579 genotype in a multivariate analysis with the subject loadings for these imaging components to determine if these genotypes would affect gray matter concentration in the areas found to be different in patients and controls. A significant three-way interaction was identified for the rs1625579 genotype, the risk score of miR-137 regulated genes, and diagnosis for one component. This component includes schizophrenia relevant areas such as Brodmann area 39 and 40 of the angular gyrus, supramarginal gyrus, and inferior parietal lobule and Brodmann area 19 of the occipital lobe which are known to be involved in several schizophrenia phenotypes including: exploratory eye movement alterations (Qiu et al., 2011), hallucinations (Curcic-Blake et al., 2013), and deficits of attention (Thimm et al., 2011; Huang et al., 2010), self-sensing (Guo et al., 2014) , and adaptive control (Becerril and Barch, 2013). Therefore, mir-137 and these regulated genes involved in the PKA signaling pathway may genetically underlie these associated phenotypes. Suggestive that this miRNA may indeed impact these functions, attention orientation deficits and reduced activity within Brodmann area 40 was found in subjects carrying a risk genotype within another experimentally validated miR-137 target, *CACNA1C* (Thimm et al., 2011). Furthermore, Cummings et al., 2012 found that patients

homozygous for the rs1625579 risk T allele performed worse in attention related tasks. Our imaging genetics findings in this analysis can help guide further studies to determine the influence of this miRNA on the phenotypes associated with schizophrenia. This may one day lead to more specific drug targets for particular symptom subtypes or lead to discovery of functional and structural biomarkers to assist in diagnosis.

Overall, our analyses suggest that miR-137 regulates schizophrenia associated target genes involved in neuronal development and plasticity pathways relevant to schizophrenia. Many of these target genes have variants associated with schizophrenia that may disrupt regulation by the miRNA. A conglomerate of target gene polymorphisms disrupting miR-137 regulation within a pathway may lead to altered functioning of the pathway. For pathways involved in neuronal development, this may alter development of the brain, such as gray matter structure, and lead to a predisposition for the development of schizophrenia. Alterations in pathways involved in plasticity may also lead to altered development, as well as continued alterations of brain functioning which may contribute the ongoing symptom expression associated with the disorder.

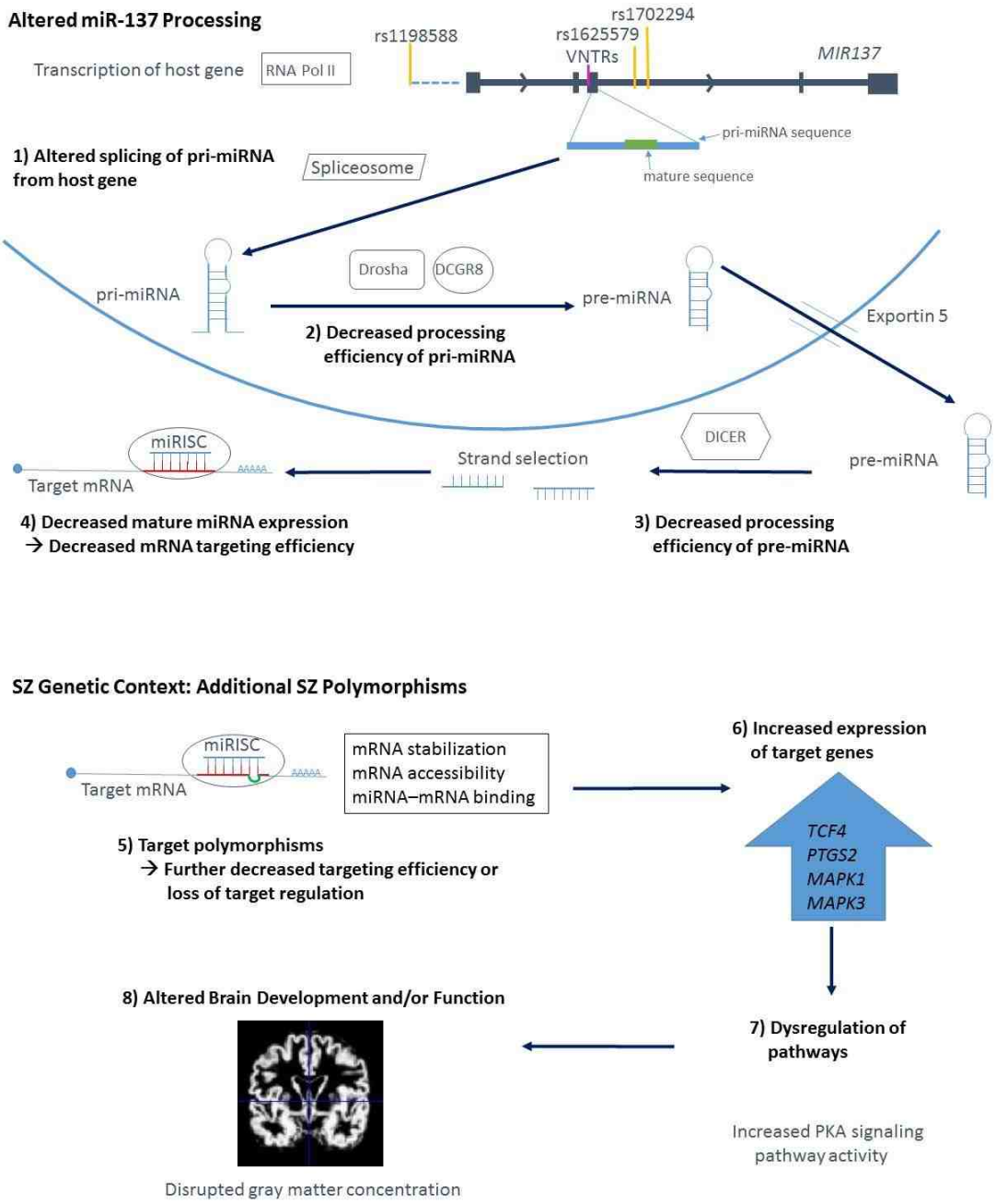

Figure 6.1 Model of the mechanism for the influence of *MIR137*, miR-137, and miR-137 regulated genes in schizophrenia

This figure shows the potential effects of the *MIR137* schizophrenia-risk variants in conjunction with target gene variants on the functional activity of miR-137 in schizophrenia. *MIR137* risk variants may disrupt miR-137 biogenesis by altering the splicing of the pri-miRNA from the host gene. This may lead to decreased production of premature and ultimately mature miR-137. A reduction in the mature miR-137 in combination with target gene alterations that further reduce the regulatory activity of the miRNA, may lead to disrupted activity of pathways highly enriched with miR-137 target genes. This may ultimately lead to altered developmental and plasticity related processes, which may either increase schizophrenia risk or potentiate alterations associated with the disorder itself.

Figure 6.1 shows a suggested model for how the miR-137 host gene (*MIR137*) variants (specifically, rs1625579 or rs1702294), miR-137 and its targets may be involved with the pathophysiology of schizophrenia. Depicted in the top half of the model, variants within the host gene may alter the biogenesis of miR-137. miR-137 is coded within an exon of long noncoding gene *MIR-137*. The mechanisms involved in the excision of these unusual pre-miRNAs derived from noncoding sequences is not well known. SNPs can alter Drosha and Dicer processing, by decreasing efficiency due to altered immature miRNA sequence stability or may alter the cleavage sites leading to shifted or altered seed sequences. Processing by these enzymes appears to be more structure-based than sequence-based, as variants outside of the pre-miRNA and mature miRNA sequences can alter processing (Sun et al., 2009). Therefore, for unusual miRNAs like miR-137, variants downstream or upstream of the pri-miRNA sequence may disrupt the excision of the pri-miRNA from their noncoding host genes. Like other processing influential SNPs, the rs1625579 variant, or other nearby schizophrenia associated variants such as rs1702294, could shift the excision or reduce excision efficiency. Given that postmortem tissue analysis suggests that the rs1622579 genotype is associated with reduced mature miRNA expression (Guella et al., 2013), it seems probable that these SNPs may reduce processing efficiency. These SNPs may reduce the stability of the host gene transcript, leading to reduced production of the pri-miRNA, and subsequently reduced production of the mature miRNA. Alternatively, these SNPs may shift excision to produce a less stable pri-miRNA sequence, leading to reduced pre-miRNA and mature miRNA production.

Many of the imaging findings suggest that the rs1622579 risk genotype influences structural and functional MRI measures in schizophrenia patients in a distinct manner

than healthy controls. In agreement with the polygenic basis of the illness (Purcell et al., 2009), this suggests that there are additional variants within these patients responsible for these disease specific MRI measure alterations. These additional variants may be SNPs within target genes that disrupt targeting by the miRNA. The effect of these SNPs are illustrated in the bottom half of the model. These SNPs may disrupt target gene binding affinity and availability for the miRNA. Thus, an overall reduction of miR-137 production coupled with reduced targeting may lead to substantially reduced regulation of target genes. This may then alter functioning of pathways highly concentrated with targets and lead to alterations in brain development or functioning that may ultimately lead to the development of schizophrenia or progression of the disorder.

6.2 Limitations

There are several limitations associated with the analyses discussed in this dissertation. Our pathway analysis with IPA is limited, in that pathway definition is dependent on the software annotation used for analysis. It is not obvious how broadly or narrowly inclusive a pathway should be defined. Regardless it is clear that these molecules interact with one another to provide a biological function and thus variants within several molecules can be disruptive, but the function disrupted may be less clear. However, this still provides a closer estimate biologically of how miR-137 target gene regulation may underlie schizophrenia. Many of the studies included putative target genes predicted with TargetScan. This prediction algorithm predicts targets based on conservation of seed site sequence, miRNA seed and mRNA sequence complementarity, nearby sequence complementarity, and binding stability. However, it is suggested that as many as 30% of predicted targets by such algorithms are false positives (Zheng et al., 2013). To account

for these false positives, experimentally validated targets and predicted targets restricted by prediction scores were also included in evaluations. Unfortunately, annotation of validated miR-137 targets remains very limited at this time and largely includes genes involved in cancer. Additionally the current validation experiments do not determine co-expression of miRNAs and their respective targets in the same place and time. However, these targets are still more likely to be true target genes than other predicted targets.

In our evaluation of schizophrenia association of polymorphisms within target gene sets, we could not assess the possibility that polymorphisms may create new miR-137 targets. However, some falsely predicted targets might make up a population of low binding predicted targets that may become a true target with the right polymorphism. Additionally, polymorphisms can be falsely associated in a GWAS. However, the data set used was so large (over 21,000 subjects) that this is less likely. (Shen and Carlborg, 2013). Our replication analysis in a separate cohort also provide more confidence for our results; however, only one target gene set demonstrated to be enriched with associated variants in the replication cohort. Our replication cohort was rather small, and we may not have had statistical power enough to allow for replication of other target gene set associations.

The effect of these identified polymorphisms within *MIR137* and miR-137 target genes on regulation by the miRNA was not evaluated in these studies. Annotation of the effect of these SNPs is currently very limited. However, such annotations should be more available in the future. Further studies could also bioinformatically assess the effect of each of these target gene polymorphisms as well as the influence of the MIR137 variants on miR-137 biogenesis and function.

Our imaging genetics cohort proved to be large enough to evaluate the influence of variants of miR-137 regulated genes on gray matter concentration measures; however, we did not have imaging and genetic data for additional subjects to perform a replication analysis for the third aim findings. Although we found a significant interactive effect of diagnosis, the rs1625579 genotype, and the miR-137 regulated gene based risk score on gray matter concentration within schizophrenia relevant brain regions, our methods could not evaluate how these genetic factors may specifically interact. More research is necessary to determine if these polymorphisms affect these genes and regulation by the miRNA in schizophrenia. Further studies will also be necessary to determine this significance of these variants on the functional activity of these and other regions. We can also not rule out that some of the GMC findings may be due to medication effects rather than disease status. However, regardless if medication is more responsible for the GMC losses found instead of schizophrenia itself, these genetic variants may still influence GMC medication effects. Thus, these genetic variants may be important in determining patient drug response. Additionally, our studies did not evaluate the influence of the interaction between age and diagnosis on gray matter. However, additional studies in our lab suggest this interaction does not cause significant gray matter alterations. Finally, many studies will be necessary to evaluate the influence of other miR-137 regulated genes not examined in this analysis on imaging measures.

6.3 Future directions

The studies presented here provide an estimate for how this miRNA and its target genes may be involved in schizophrenia. As miR-137 can regulate many targets and pathways, our studies reduce the dimensions of further research trajectories. Therefore, our findings

provide a guide for further research in this area, so that it may be more effective and efficient.

The effect of the *MIR137* SNP rs1625579 on miR-137 expression and function is not well studied and no studies have analyzed the effect of the very recently identified *MIR137* SNP rs1702294. Guella et al., 2013 found reduced miR-137 expression in the DLPFC of rs1625579 risk allele homozygotes. This was a limited study however, that used only 10 control subject samples and 7 schizophrenia subject samples. Further research using postmortem tissue and cell culture assays can help determine the effect of this genotype and the rs1702294 genotype on miR-137 expression. RNA sequencing of postmortem tissue from patients and controls can determine if the risk genotypes are associated with a reduction or alteration in the premature and mature forms of the miRNA. Comparison of RNA sequencing or Northern Blot analysis following HEK293 cells, which are known not to express miR-137(Kozlowska et al., 2013), transduced with lentivirus vectors containing the host gene *MIR137* with the alleles of the associated variants (rs1625579 and rs1702294) can assist in determining if the *MIR137* variants alter biogenesis of the pri-, pre-, or mature miRNA.

As stated in the limitations, the current studies did not evaluate the effect of target gene variants on miR-137 regulation. Bioinformatics studies could first determine which variants are more likely to disrupt pathway functioning and miR-137 regulation. This could be done by determining targets that contain non-synonymous SNPs, SNPs within promoter regions, SNPs within the 3'UTR, or the miR-137 seed site sequence. Non-synonymous SNPs, SNPs that alter subsequent protein amino acid sequence, may lead to alterations in protein function, which could disrupt pathway functioning. SNPs in

promoter regions may alter transcription of target genes; this coupled with disrupted miR-137 regulation may lead to further altered expression of gene products and effect pathways containing such genes. SNPs within 3' UTRs or seed site sequences may alter miR-137 regulation by altering binding or binding availability of the miRNA due to secondary structure alterations (Abelson, 2005; Wang et al., 2008).

In vitro studies can determine if these non-synonymous SNPs alter the function of implicated targets and the function of other downstream pathway molecules. Northern blot analysis can help determine if SNPs within promoter regions leads to altered gene expression of targets. Luciferase studies using plasmids expressing target 3'UTRs and overexpression of the miR-137 primary sequence could confirm if s 3' UTR and seed site SNPs alter regulation by the miRNA. Structural and functional MRI analyses can help further determine the consequences of polymorphisms believed to effect target and pathway regulation by the miRNA.

The studies described in this dissertation suggest that the PKA signaling pathway may be involved in the mechanistic role of miR-137 in schizophrenia. Evaluation of the regulation and function of the target genes within this pathway, with the mentioned methods above are an obvious potential first next step. Our imaging genetics findings suggest that variants of miR-137 regulated genes within this pathway affect structure within brain regions known to be involved in attention, hallucination, self-sensing, and adaptive control. Functional MRI studies could help determine if carriers of the identified risk SNPs also have altered activity in these areas and measures of these phenotypes. One simple first step would be to evaluate if positive symptom score is higher in subjects with the rs1625579 risk allele and higher miR-137 regulated gene risk score.

No studies have evaluated the influence of variants within miR-137 regulated genes and *MIR137* on symptom scores and schizophrenia relevant phenotype measures. Limited studies have evaluated the effect of the rs1625579 genotype alone. Green et al., 2012 found subjects carrying the protective G allele, and who had more severe negative symptoms scores performed worse at cognitive tasks. Perhaps in these patients additional variants responsible for the worsened negative symptoms may also worsen cognitive deficits with a slightly different genetic basis. In support of our findings, Cummings et al., 2012 found that the T allele was associated with attention deficits, however, these subjects also scored lower for positive symptoms. This suggests that these particular variants may affect the other implicated schizophrenia associated phenotypes rather than hallucination. However, it is possible that this is again due to a genetic variant and medication interaction. Perhaps risk TT carriers are more likely to be responsive to treatment of positive symptoms regardless of baseline status. Longitudinal studies evaluating prodromal subjects, such as that of Addington et al., 2007, may help to determine the influence of these genotypes on treatment response. Such studies can also assist in determining if our GMC findings are due to disease status rather than medication effects.

While we are still far from determining the impact of this miRNA in schizophrenia, the use of a pathway approach with the methods described above may streamline our investigation and our results so far suggest avenues of further research. Determination of pathways dysregulated by miR-137 may ultimately provide alterative drug target candidates for future treatments.

6.4 Conclusions

Evidence of miRNA impact in psychiatric illness is ever growing. Substantial evidence for miR-137 involvement in schizophrenia is apparent. However, it is unclear how the miRNA influences the disorder. The studies presented here provide the first thorough investigation of miR-137 targets and the consequences of their possible dysregulation in the disorder. The results of these studies suggest that the miRNA may affect pathways involved in neuronal development and plasticity through the regulation of target genes. These pathways may be dysregulated in schizophrenia and may lead to altered gray matter concentration, which may influence important phenotypes associated with the disorder, such as hallucinations, attention and adaptive control deficits and exploratory eye movement deficits. These alterations may be part of the progression of schizophrenia development itself or may predispose individuals to development of the disorder. Further research is necessary to elucidate if targets are indeed dysregulated by the miRNA in schizophrenia and the consequences of such dysregulation. Overall, these studies provide a guide for future research to clarify the role of this miRNA and its targets in this devastating disorder. Determination of the mechanism of this miRNA involvement may ultimately inspire novel treatments that may improve patient wellbeing.

Appendix A: Supplemental Data for Meta Gene Set Enrichment Analyses Link miR-137-regulated Pathways with Schizophrenia Risk Article

Target List Name	Criteria	TargetScan Predicted Target Genes	Validated and Predicted Overlap	Total Target Genes Including Validated targets	Relevance
Full List	None	1144	26	1154	Most Inclusive
Conserved List	Pct ≥ 0.9	537	13	560	Conserved
Context List	Context ≤ -0.12	577	16	597	Less Conserved, Strong Binding
Intersection list	Pct ≥ 0.9 and Context ≤ -0.12	283	8	311	High Confidence Conserved
Validated List	Experimental Validation Study	None	None	36	Highest Confidence, Least Inclusive

Table A.1. Curated target gene lists of hsa-miR-137

This table depicts the scoring criteria for each curated list, the number of predicted targets for each gene list, the number of validated targets that were also predicted, and the total number of targets in the final list listed by gene symbols. The relevance column indicates how each gene list contributed to the analysis. Pct = probability of conserved targeting. For further details see Methods section.

Dataset	PGC	MCIC	NU	MCIC and NU Merged
Controls	12,462 (49% male)	97 (59% male)	44 (52% male)	141 (57% male)
Cases	9,394 (66% male)	73 (75% male)	30 (67% male)	103 (73% male)
Total Subjects	21,856 (56% male)	170 (66% male)	74 (58% male)	244 (64% male)

Table A.2. Dataset demographics

This table shows the demographic information of each dataset used in this analysis. PGC = Psychiatric Genomics Consortium; MCIC = Mind Clinical Imaging Consortium; and NU = Northwestern University. All subjects were Caucasian. Subjects from MCIC and NU and nearly all PGC subjects were diagnosed based on the DSM-IV criteria. Only one cohort of subjects within the PGC was diagnosed according to ICD-10. Case subjects included those diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder. Controls were excluded with a past history of psychiatric illness or substance abuse. DSM = Diagnostic and Statistical Manual of Mental Disorders; ICD = International Classification of Diseases.

Gene	Top SNP	Minor /Major Allele	Minor Allele frequency in cases	Minor Allele frequency in controls	Chi-Square	P value	OR for Minor Allele
PTGS2	rs10489401	G/A	0.2864	0.3901	5.651	0.017	0.6276
MAPK3	rs7202714	T/C	0.3627	0.273	4.447	0.034	1.515
TCF4	rs2276195	T/C	0.165	0.2908	10.39	0.001	0.4821
MAPK1	rs9610608	G/A	0.1796	0.09574	7.348	0.0067	2.068

Table A.3. Significant association of SNPs in validated PKA signaling target genes with schizophrenia in the replication cohort

This table shows the top SNPs from the validated PKA signaling target gene set for the merged MCIC and NU data. All SNPs have significantly different frequencies between cases and controls. Analyses were performed in PLINK. OR = Odds Ratio

Appendix B: Supplemental Data for Polymorphisms in MIR137 and microRNA-137 regulated genes influence gray matter structure in schizophrenia Article

Component Number	**F**	**df**	**P value**	**Effect Size (Cohen's d)**	**Loadings Directionality**	**Brain Region Label** (Positive/***Negative***)	**L/R Volume (cm^3)**	**Brodmann Area**
1	61	1	<.001	1.04	CT>SZ	Superior Temporal Pole	2.2/2.4	38
						Superior Temporal Pole	3.2/2.8	38
						Medial Frontal Gyrus	1.6/2.4	32,10
						Superior Temporal Gyrus	1.0/1.0	NA
11	11	1	0.001	0.62	CT>SZ	Middle Occipital Gyrus	1.0/0	19
						Angular Gyrus/Inferior Parietal Lobule	0.4/1.5	39,40
						Inferior Parietal Lobule/Supramarginal Gyrus	0.0/1.3	40
6	12	1	0.001	0.48	CT>SZ	Calcarine	2.9/4.0	17
						Superior Occiptal Gyrus	3.7/3.5	17
						Middle Temporal Gyrus	0.2/2.2	***21,39***
						Sub-Gyral	0.6/1.2	***NA***

Table B.1 Imaging Components Showing Differences in GMC between Patients and Controls

This table shows the major findings, significance value and effect size for the three components that captured GMC differences between patients and controls. CT= Control; SZ=Schizophrenia.

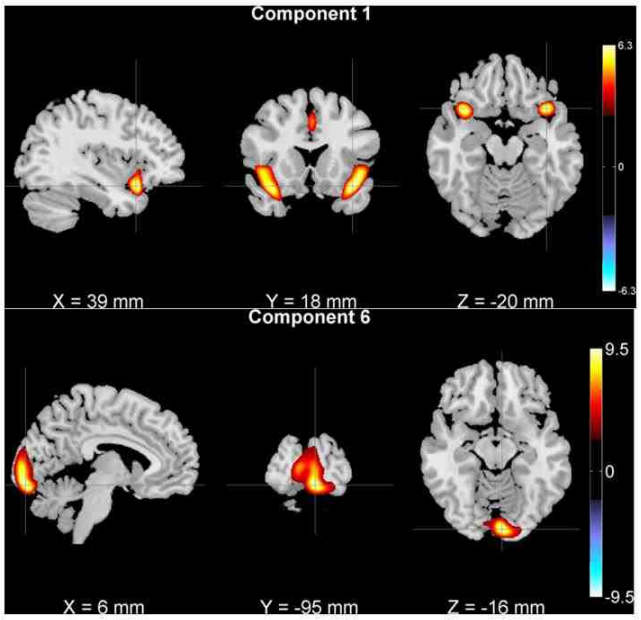

Figure B.1 Regions of GMC Variance between Patients and Controls for Component 1 and 6

This figure shows the regions of GMC variance between cases and controls for the components found to significantly capture diagnostic variance but not genotypic variance. The spatial map is overlaid on a template brain, thresholded with z scores >|3.5|. The heat map coloring indicates z score intensity, with red indicating findings of GMC greater in controls and blue indicating areas of GMC greater in patients. White indicates areas with greatest z scores. The bar on the right indicates z score.

Abbreviations Used

ADAR	Adenosine Deaminase Acting on RNA
Ago 2	Argonaute 2
ASD	Autism Spectrum Disorders
CNV(s)	Copy Number Variation(s)
CSF	Cerebrospinal Fluid
CT	Control Subjects
DCGR8	DiGeorge Syndrome Critical Region Gene 8
DLPFC	Dorsolateral Prefrontal Cortex
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-IV
ENIGMA	Enhancing Neuroimaging Genetics through Meta-Analysis
FDR	False Discovery Rate
fMRI	Functional Magnetic Resonance Imaging
FWHM	Full Width Half Maximum
GMC	Gray Matter Concentration
GNRH	Gonadotropin-releasing Hormone
GSEA	Gene Set Enrichment Analysis
GWAS	Genome Wide Association Study
HGF	Hepatocyte Growth Factor
HITS-CLIP	High-Throughput Sequencing of RNA Isolated by Crosslinking Immunopercipitation
HTT	Huntington's Associated Protein Huntingtin
IBD	Identity-By-Decent
IBS	Identity-By-State
ICA	Independent Component Analysis

IPA	Ingenuity Pathway Analysis
L	Left Side
LIBD	Lieber Institute for Brain Development
LTP	Long-term Potentiation
MAGENTA	Meta Gene Set Enrichment of Variant Analysis Software Program
MCIC	Mind Clinical Imaging Consortium
MDL	Minimum Description Length
MDS	Multi-dimensional Scaling
MGH	Massachusetts General Hospital
MHC	Major Histocompatibility Complex
miR-137	microRNA 137
MIR137	microRNA 137 host gene
miRISC	microRNA Induced Silencing Complex
miRNA	microRNA
NIMH	National Institute of Mental Health
NMJ	Neuromuscular Junction
NS	Nervous System
NU	Northwestern University
OR	Odds Ratio
PACT	Protein Activator of the Interferon Induced Protein Kinase
Pct	Probability of Conserved Targeting
PGC	Psychiatric Genomics Consortium or Psychiatric Genome-Wide Association Study Consortium
PKA	Protein Kinase A
PPI	Prepulse Inhibition
R	Right Side

SANS	Scale for the Assessment of Negative Symptoms
SAPS	Scale for the Assessment of Positive Symptoms
SES	Socioeconomic Status
SIRP	Sternberg Item Recognition Paradigm Working Memory Task
SNP	Single Nucleotide Polymorphism
SPM5	Statistical Parametric Mapping 5 Software
SZ	Schizophrenia Subjects
SZGR	Schizophrenia Gene Resource
tDCS	Transcranial Direct Current Stimulation
TRBP	Trans-Activation Response RNA Binding Protein
UIowa	University of Iowa
UMinn	University of Minnesota
UNM	University of New Mexico

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