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WHITE MATTER NEURONS: mRNA EXPRESSION AND ANALYSIS

A Major Qualifying Project Report

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ABSTRACT

Current neurological research indicates that the improper migration of neurons into subcortical white matter in humans may play a role in the etiology of schizophrenia, major depressive disorder, autism, and other psychological disorders. The purpose of this project was to isolate mRNA from both white and gray matter samples from mentally ill and healthy individuals in an effort to elucidate mRNA gene expression differences between white matter neurons and gray matter neurons, if any. These differences in gene expression may open new avenues of research and treatment in patients suffering from psychiatric disorders with genetic etiologies.

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BACKGROUND

Brain Morphology and Neuronal Function

The central nervous system (CNS) can be divided into three main groups of tissue: neurons, glial cells, and the extracellular matrix. Each group can vary considerably in both organization and morphology, but in general, neurons are the information-processing cells of the CNS, glial cells maintain, support, and propagate the information-processing power of the neurons, and the extracellular matrix provides additional support to the neurons and glial cells. Brain matter can also be divided into white and gray matter (**Figure-1**). The diagram shows the distribution of white and gray matter within the human brain. Neurons are found predominately within gray matter, while white matter is composed of mostly glial cells and myelinated axons (interstitial white matter neurons, IWMNs).

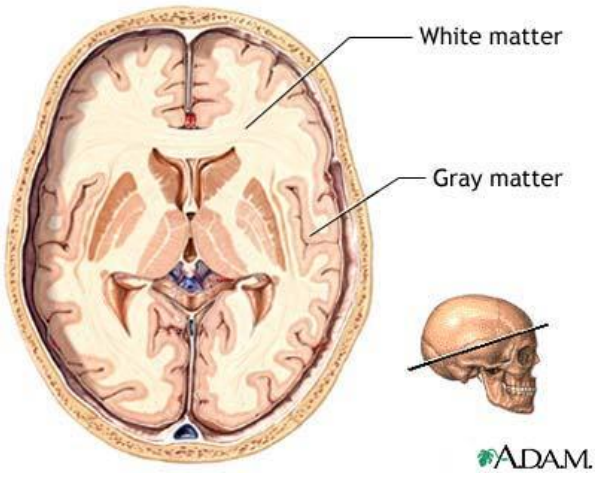


Figure-1: Diagram of the Positions of Brain White and Gray Matter. Gray matter consists mostly of neurons, while white matter contains mostly glial cells and myelinated neurons. (Dugdale and Zieve, 2010)

Figure-2 shows a neuronal cross-section, delineating myelinated (blue) and non-myelinated axons, as a key distinction between white and gray matter.

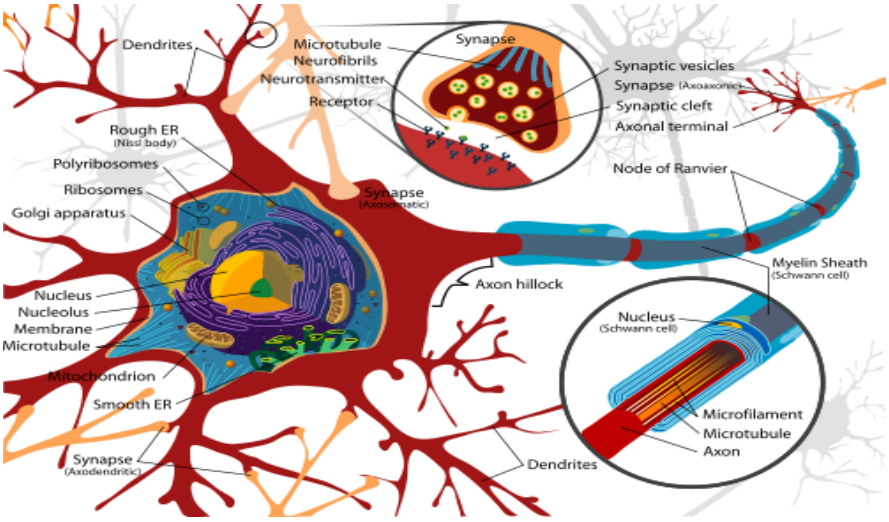


Figure-2: Diagram of a Neuron. Note the difference between myelinated axons (blue) (found predominately in white matter) and non-myelinated axons (found predominately in gray matter). (Villareal, 2007)

Psychiatric Disorders and Neuronal Density in Subcortical White Matter

The complex interactions between white and gray matter has been the subject of much scientific research in the past twenty years. It has become apparent that morphological abnormalities in the white matter regions of the brain may have relevance to psychiatric disorders, especially in the subcortical region of the brain. Post-mortem psychiatric patients diagnosed with schizophrenia appear to show significantly increased densities of IWMNs within superficial or surface subcortical white matter (Yang et al., 2011). Furthermore, these results have been duplicated time and again especially for a deficit subtype schizophrenia, although there are discrepancies between the studies in regards to the definitions of "superficial" and "deep" white matter where the IWMNs

were measured (Akbarian et al., 1993; Akbarian et al., 1996; Anderson et al., 1996; Eastwood and Harrison, 2003; Eastwood and Harrison, 2005; Kirkpatrick et al., 2003). Additionally, neurons within the grey matter of schizophrenics have displayed decreased dopamine, NMDA, and DARPP-32 receptor levels, although expression of these proteins within IWMNs remains unclear (Akbarian et al., 1996; Albert et al., 2002; Conner et al., 2011).

This type of white matter neuronal density analysis has been extended beyond just schizophrenia. Increased densities or altered distributions of white matter neurons both in "superficial" and "deep" white matter have been implicated as a causative factor in other complex neurological disorders, such as autism and bipolar disorder (Dupont et al, 1995; Connor et al, 2011). In the case of bipolar disorder, MRI studies linked an increased volume of abnormal subcortical white matter with increased rates of adult onset cognitive impairments and mood-related disorders, while meta-analysis of similar studies confirms a volumetric difference in subcortical white matter, as well as variety of other volumetric differences in other brain regions in patients with bipolar disorder and major depressive disorder, although the differences vary between disorders (Dupont et al., 1995; Pillai et al., 2002; MacDonald et al., 2004; Arnone et al., 2009). In a perhaps more telling study, teenagers suffering from their first manic episode were shown to display axonal disorganization in white matter with diffusal tensor imaging, an MRI technique. The authors of the study suggest that this disorganization may be the result of a developmental deficiency (Adler et al., 2006). White matter microstructure abnormalities in individuals with bipolar disorder have been extremely well characterized through use of the diffusal tensor imaging technique. In general, the studies confirm that the

microstructural elements of white matter in the subcortical and cortical areas are significantly altered in bipolar sufferers (Cannon et al., 1998; Beyer et al., 2005; McIntosh et al., 2005; Hulshoff et al., 2006; Schneider-Axmann et al., 2006; Yurgelun-Todd et al., 2007; McIntosh et al., 2008; Rüsch et al., 2008; Sussmann et al., 2009).

For autism, similar studies have linked increased subcortical white matter volume with autism and autism spectrum disorders. In 2003, Herbert et al. performed MRI morphometric analysis on the brains of 17 male autistic children, and 15 male control brains, and found that there were significant increases in brain volume in male autistic children (Herbert et al., 2003). These general findings of young autistic children with increased or abnormal brain volumes, have been replicated in several other studies (McAlonan et al., 2004; Hazlett et al., 2005; McAlonan et al., 2009; Chawarska et al., 2011; Courchesne et al., 2011). In terms of cellular structure and white matter abnormalities, several studies have noted that ASD patients tend to have altered microstructure in the subcortical region, although conclusions vary from study to study (Lainhart, 2006; Cheng et al., 2010; Ameis et al., 2011; Vissers et al., 2011; Jeong et al., 2011; Muller et al., 2011; Anderson et al., 2011). It should be noted that these volume increases were not limited to subcortical or cortical regions of the brain; generally the autistic brains showed individual differences while still tending to be oversized. Interestingly, a similar study was conducted on adults living with ASD, but it was reported that white matter volume was significantly decreased rather than increased, adding an interesting side to the tissue regulation story of autism (Ecker et al., 2012).

While these studies are informative in terms of the physical location and structure of IWMNs within the brain, they do not address any genetic differences these cells may

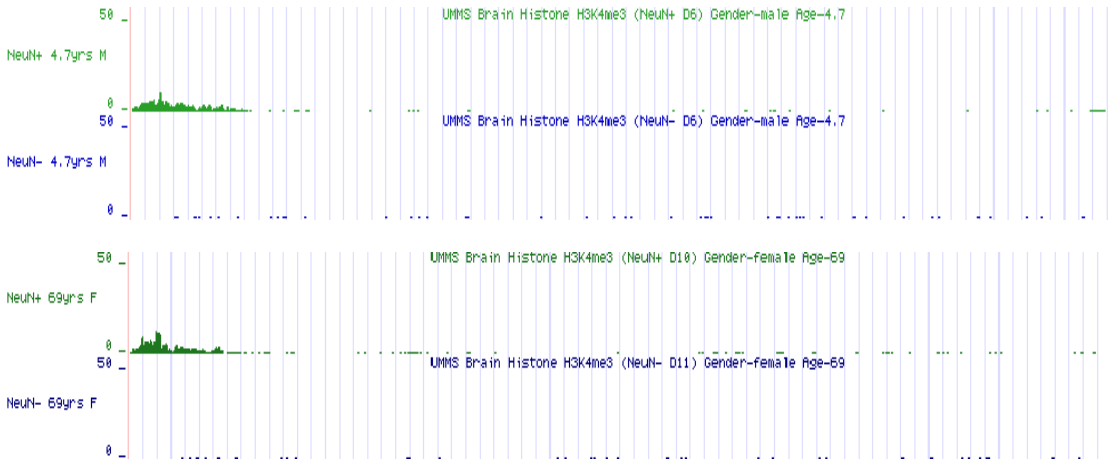
possess, especially between normal and psychiatric patients. Currently, there are two main theories regarding patient IWMNs. The "tombstone" theory states that the increased IWMN density in psychiatric individuals represents the remnants of an earlier developmental problem that occurred during early infancy or childhood, but this density increase has little effect on brain transcription. Alternatively, the second theory states that IWMNs are transcriptionally active and alter the transcriptome of white matter within the brain, and this transcriptional alteration may have psychiatric or developmental consequences. This project will investigate whether these white matter neurons are in fact transcriptionally active, and if so, whether they are expressing various neuronal gene products related to psychiatric disorders. White matter and gray matter neuronal tissue will be isolated from patient autopsy brains, and the up-regulation of specific target mRNAs will be monitored by RT-PCR and RNA-Seq whole transcriptome analysis. The project will assay a variety of genes, carefully selected on the basis of Chip-Seq genome data provided by Cheung et al. (2010).

Gene Selection

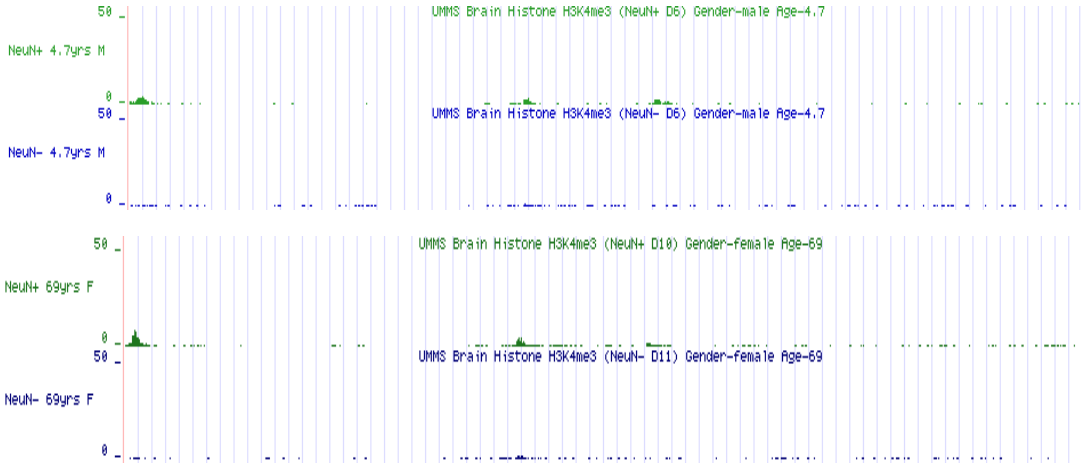
Chip-Seq data was used to help identify potential genes for analysis in this project. **Figure-3** shows our lab's previous Chip-Seq data (Cheung et al., 2010) for nine genes. Age and gender-matched samples were tested in neuronal cells (green) and non-neuronal cells (blue), for male (upper row) and female (lower row) samples, for the amount of methylation of H3Kme3, a general indicator of gene expression. Overall, most of the 9 genes analyzed (*SHANK3*, *GRIN1*, *GRIN2D*, *GLRA1*, *GLRA3*, *GABRG3*, *GABRG5*, *GABRA5*, and *GABRD*) showed a noticeable increase in gene expression in

neuronal cells, indicating their potential relevance to this particular study, so were chosen for this project for analysis by RNA-Seq and qRT-PCR.

Panel-A: GRIN1 Expression



Panel-B: Shank3 Expression

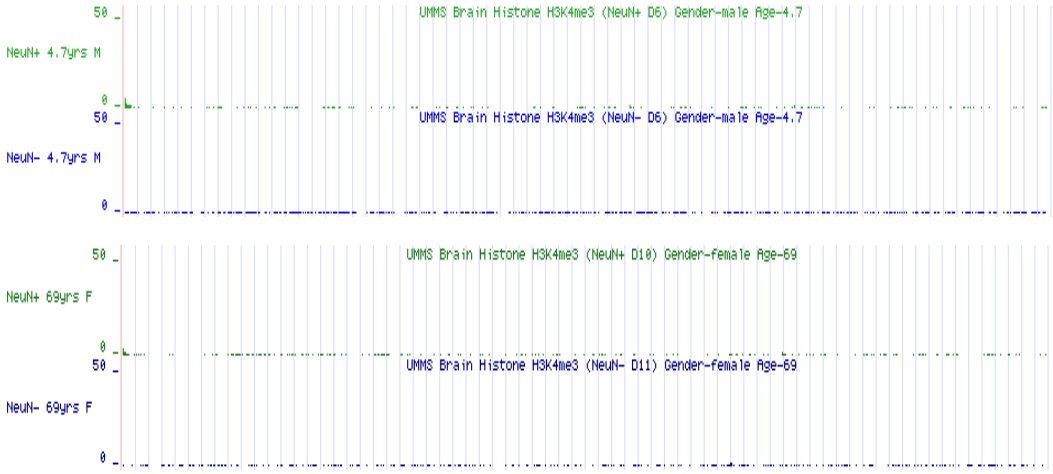


Panel-C: Grin2D Expression

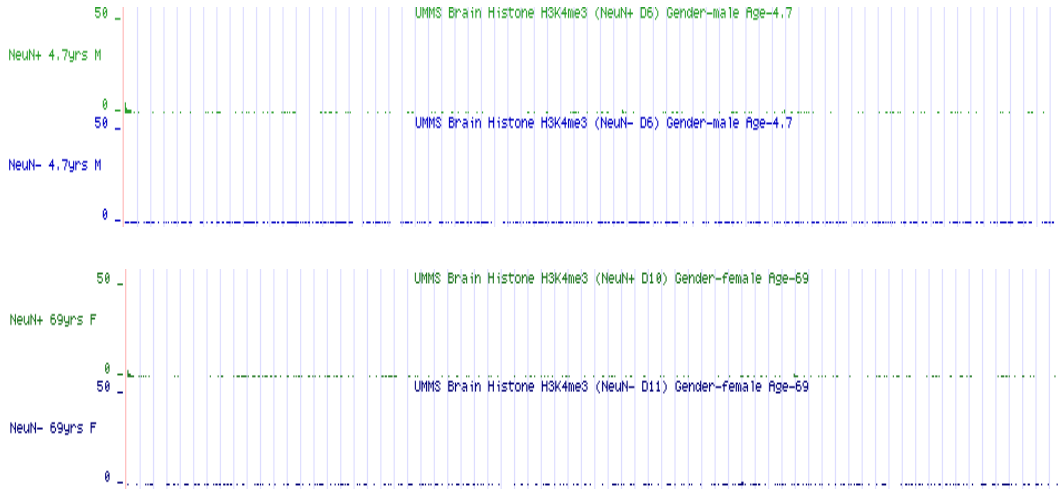
Panel-D: GLRA1 Expression

Panel-E: GLRA3 Expression

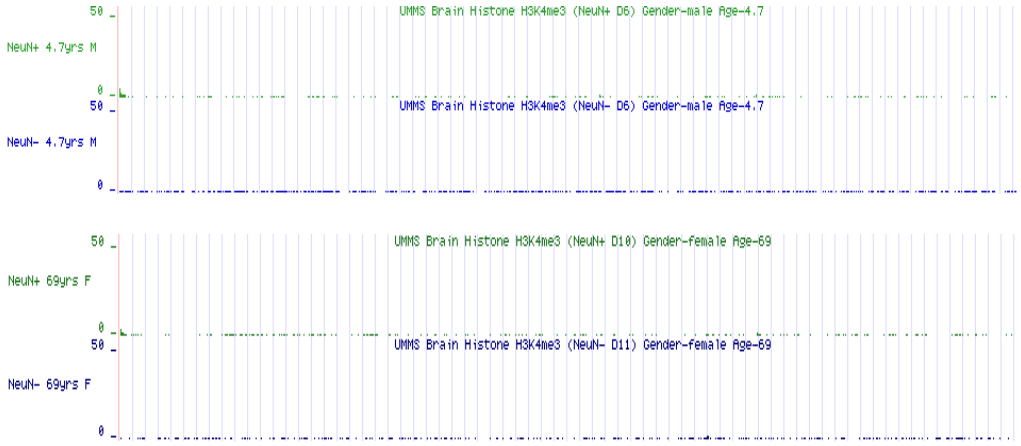
Panel-F: GABRG3 Expression



Panel-G: GABRG5 Expression



Panel-H: GABRA5 Expression



Panel-I: GABRD Expression

Figure-3 (A-H): CHIP-Seq Data for Potential Genes of Interest. The above graphs show the relative levels of gene expression for the listed target gene in both neuronal (green) and non-neuronal tissue (blue), for male (upper row) and female (lower row) samples, as assayed by H3Kme3 methylation activity. Note that most assayed genes display neuronal expression, but little or no non-neuronal expression. Bars indicate levels of H3Kme3 modification, a marker of gene transcriptional activation.

Target Gene Functions

Several genes were selected for assay by RNA-Seq and RT-PCR for their expression in neurons (discussed above), possible relevance to IWMNs in general, and to schizophrenia, autism, and major depressive disorders. These genes have functions relating either to synapse formation or inhibitory neurotransmitters.

SHANK3

SHANK3, also known as ProSAP2, encodes a synaptic scaffolding protein that may serve as an organizer of other scaffolding proteins in the postsynaptic region (Baron et al., 2006). The shank3 protein has also been shown to form the postsynaptic density

complex with neurexins and neuroligins, which are cell-adhesion proteins located in glutamatergic synapses (Meyer et al., 2004). **Figure-4** shows a localization experiment for *SHANK3*. The protein can be seen to localize to synaptic spines and postsynaptic densities in rat brains (Wendholt et al., 2006).

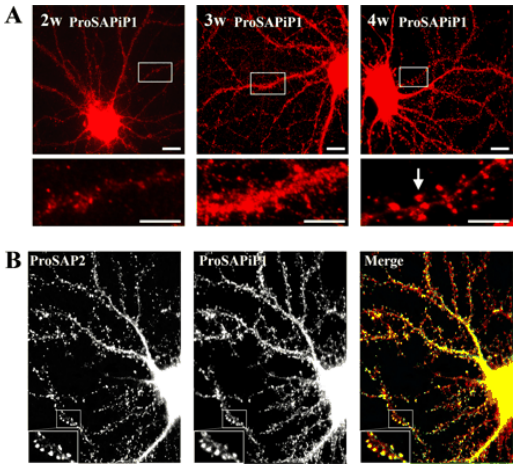


Figure-4: Neuronal Localization of Protein SHANK3. Note the localization of the protein in synaptic spines and in post-synaptic densities. This protein has been linked to autism spectrum disorders. (Wendholt et al., 2006)

Later studies implicated *SHANK3* with autism spectrum disorders (ASD) and other pervasive development disorders. Moessner and associates (2007) determined through DNA sequencing and microarray analysis that *SHANK3* variants were found in 1% of 400 individuals with ASD, or their immediate families, but not in the control population. Combined with another study of large sample size (Durand et al., 2007), Moessner and colleagues found that *SHANK3* deletions, duplications, and variants may be associated with 1% of all autism spectrum disorder patients (Moessner et al., 2007). **Figure-5** shows a diagram of the *SHANK3* gene and the positions mutated in the disorders.

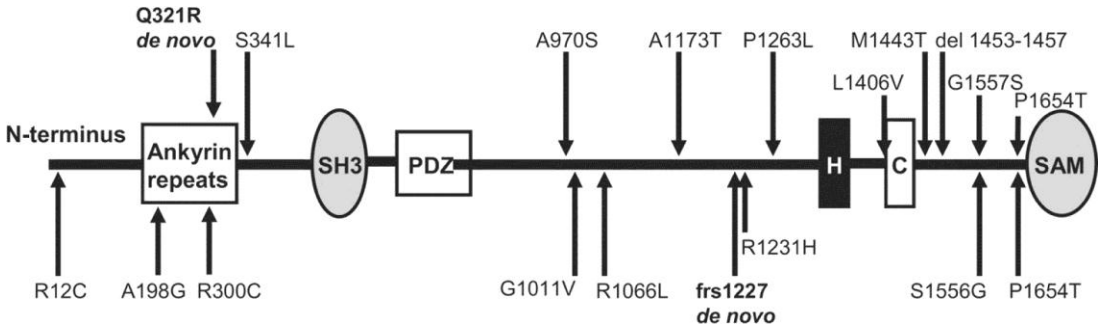


Figure-5: Diagram of the SHANK3 Gene and Sites Mutated in Autism Spectrum Disorders. The mutation sites listed above the line represent those discovered by Moessner and colleagues (2007), while those shown below the line represent those found by Durand and colleagues (2007). The SH3 box (gray) represents a Src homology-3 domain. The PDZ box (white) represents a PDZ domain. The H box (black) represents a homer–binding site. The C box represents a cortactin-binding site. The SAM box represents a sterile α motif. (Moessner et al., 2007)

It should be noted that while *SHANK3* mutations are "relatively" common (1%) in autism spectrum disorders, mutations in the genes encoding other proteins in the postsynaptic density complex (*NGLN3, NGLN4,* and *NRXN1*) have been found to be comparatively very rare. In the case of *NGLN3,* only one individual expressing a variant has ever been identified (Jamain et al., 2003). Thus, currently it seems likely that *SHANK3* is a better target for study than the other proteins forming the post-synaptic density complex.

NMDA Receptor Subunits GRIN1 and GRIN2D

GRIN1 and *GRIN2D* are genes encoding N-Methyl *D*-Aspartate (NMDA) receptor subunits NR1 and NR2d. The NMDA receptor is composed of a four-part assembly of two NR1 subunits and two subunits from the NR2A-2D subfamily or the

NR3 subfamily. NMDA-Rs are interesting in that they have two prerequisites before channeling ions across the cell membrane; 1) the neuron containing the receptor must depolarized, and 2) ligands glycine and glutamate must be bound to the receptor (Kleckner and Dingledine, 1988; Purves et al., 2008). NMDA-Rs are thought to help balance and control interplay between neuronal excitation and inhibition, as well as play a role in long term potentiation and long term depression. Both of these neuronal actions are thought to play a role in memory formation and synaptic plasticity (Villman and Becker, 2007).

With respect to schizophrenia (SCZ), NMDAR antagonists like PCP and ketamine often produce SCZ-type cognitive deficits, and individuals suffering from anti-NR1 encephalitis suffer from SCZ-type deficits and memory loss (Krystal et al., 2002; Vincent and Bien, 2008; Dalmau et al., 2008; Amitai and Markou, 2010). A separate team of researchers based in Shanghai Jiao Tong University found a positive correlation between *GRIN2B* gene variants and schizophrenia in a Chinese population (Li and He, 2006). More conclusively, mice that have been genetically engineered to only develop about 5% of the normal NR1 levels are phenotypically normal until adolescence, upon which time they display behavioral and social deficits that can be improved with treatment of anti-psychotic drugs haloperidol or clozapine (Ikeda et al., 1995; Sakamura et al., 1995; Ebralidze et al., 1996).

Furthermore, NMDAR dysfunction and the failure to balance excitation/inhibition of neurons has become a leading theory regarding the neurobiological etiology of autism spectrum disorders (Polleux and Lauder, 2004). NMDAR function has also been implicated with bipolar disorder; two drugs, lithium and valproate, used to treat bipolar

disorder have been found to change NMDAR receptor function, implying a specific role in NMDAR function and bipolar disorder pathogenesis (Mundo et al., 2003; Hayden and Nurnberger, 2006). However, it should be noted that no *GRIN1* or *GRIN2D* variants or truncated transcripts have ever been discovered; so it seems safe to conclude that any non-functional *GRIN1* and *GRIN2D* is likely to be lethal (Forrest et al., 1994; Tarabeux et al., 2011). Importantly, few studies have detailed whether the expression level of *GRIN1* or *GRIN2D* play a role in the etiology of schizophrenia or autism, or how expression levels of these genes change between IWMNs and gray matter neurons. One German study in particular links *GRIN1* function to schizophrenia and lifelong depressive symptoms, showing that German schizophrenics who also suffered from lifelong depression displayed greatly increased rates (p-value < 0.01) of *GRIN1* variants (Alexander et al., 2007).

GLRA1 and GLRA3

GLRA1 and *GLRA3* encode glycine receptor chloride channels that comprise a major part of the inhibitory system within the CNS (although the receptor also has a limited excitatory role during development) (Lynch, 2004). *GLRA1-3* and *GLRB1* exist in humans as homo-pentamers. **Figure-6** shows a top view of the complete *GLRA1* pentamer and its ligand binding sites (shown as red in the figure). The glycine receptor channels are especially important for synaptogenesis, as glycine receptors have been shown to directly trigger rises in Ca^{2+}, which are essential for the proper function of the synapse (Kirsch and Betz, 1998).

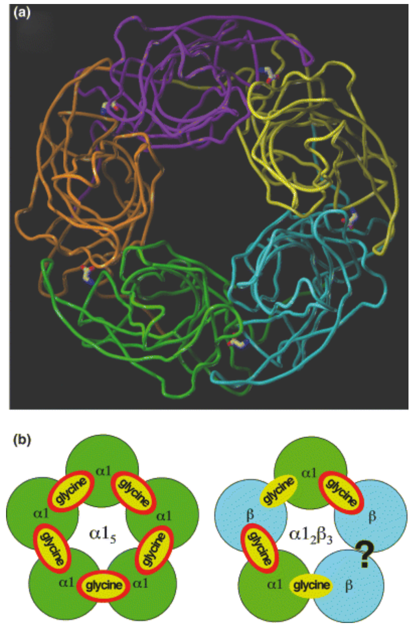


Figure-6: Diagram of the Structure of the GLAR1 Protein. The protein exists in human brain as a pentamer. The glycine binding sites are shown as red circles in the lower diagram. (Betz and Laube, 2006)

In terms of pathology, *GLRA3* has recently been theorized to be a causative factor in the development of autism spectrum disorders (Ramanathan et al., 2004). Ramanathan and colleagues reported the karyotyping and analysis of an autistic individual, age 11, who displayed the genetic deletion of one set of alleles for 33 genes, including the following genes, *AMPA 2*, *GLRA3*, *GLRB*, *NPY1R* and *NPY5R*, that are responsible for neurological growth and development. It should be noted that the ratio between active *GLRA1-3* and *GLRB* protein changes as an individual develops; in infancy the *GLRA2* receptor protein and mRNA predominate, but by early adolescence the *GLRA1* and *GLRB* protein have replaced the vast majority of the *GLRA2* (Takahashi, 2005; Betz and Laube, 2006). Thus, it seems likely that proper management of glycine receptor expression is integral to the proper development and function of the adult brain.

GABA Receptor Subunits GABRG3, GABRG5, GABRA5, and GABRD

Finally, *GABRG3*, *GABRG5*, *GABRA5*, and *GABRD* are related genes that encode subunit proteins for $GABA_A$, a well-studied hetero-pentameric receptor for gamma-aminobutyric acid (GABA), the main inhibitory immune-transmitter in the CNS. Like glycine receptors as described above, $GABA_A$ receptors bind their GABA ligand to transport Cl^- ions through the cell membrane to inhibit the firing of the neuron. $GABA_A$ receptors are widespread throughout the human body, although the distribution and subunit composition of the $GABA_A$ receptors varies depending on the tissue and stage of development. $GABA_A$ receptor density in the brain is greatest during infancy, and then diminishes as the individual develops (Chugani et al., 2001).

$GABA_A$ receptor dysfunction is another highly promising area of autism research. SNP variants of *GABRG3* and *GABRA5* in particular have been determined to be genetic risk factors for autism, and are present at a much higher rate in autistic populations than in control populations (Menold et al., 2001). Even more telling, PET scans of autistic children using a radioactive GABA-R antagonist, flumazenil, revealed that autistic children possess significantly less GABA-binding sites in the limbic and hippocampal areas of the brain, while other important neurotransmitter receptors remain unaffected (Blatt et al., 2001). Furthermore, sophisticated statistical techniques performed by Ashley-Koch and associates revealed that while not statistically significant, *GABRG3* was linked to ASD; they go on to state that perhaps variants of *GABRG3* are only genetic risk factors in some ASD subpopulations (Ashley-Koch et al., 2006).

$GABA_A$ receptor dysfunction has also been implicated with suicide victims and individuals suffering from major depressive disorder. A study was conducted by Merali and colleagues concerning mRNA expression levels of GABA receptors subunits, and determined that mRNA expression of all GABA receptor subunits were significantly diminished in the fronto-lateral cortex region of the brain (Merali et al., 2004). Thus, it seems reasonable to assume that gene variants or changes in gene expression levels for GABA receptor subunits may have some role in complex psychiatric disorders.

qRT-PCR and RNA-Seq

The main techniques used in this project to assay potential changes in specific mRNAs between white and gray matter neurons are quantitative RT-PCR and RNA sequencing whole transcriptome analysis (RNA-Seq). For the RNA-Seq, RNA was purified from human brain tissue and sent for analysis at Eurofins, an international bioanalytics firm. Whole Transcriptome Shotgun Sequencing (WTSS) or a variant of the protocol was implemented at the company. This technique allows for the analysis of all mRNA present within the sample cells. Generally, the process begins with the removal of ribosomal RNA, which normally accounts for nearly 90% of a cell's transcriptome (Mortazavi et al., 2008). Further preparation of the RNA occurs, although the exact steps change given the needs of the particular sequencing project and the machines utilized. Next, the RNA is converted to DNA via the action of the enzyme reverse transcriptase, and polymerase chain reaction is performed, a four step process that duplicates DNA with each successive cycle. Further processing and statistical steps are usually required, but

these tend to vary given the differences between PCR machines and the desired analysis. Finally, the RNA is mapped and duplicative sequences removed, using a reference genome and the transcript data is assembled into a coherent picture.

Quantitative RT-PCR (qRT-PCR) is a similar process, although it lacks the high-throughput sequencing power of the WTSS described above. Generally when using qRT-PCR it is only possible to screen an RNA sample population for the presence of a few target mRNAs at time, although the general principle of using labeled primers and RT-PCR cycles is similar to WTSS. In the case of qRT-PCR, random hexamer primers, or oligo-dT that hybridizes to mRNA poly(A) segment, are used with reverse transcriptase to produce cDNA, then the cDNA is amplified with gene-specific primers. In the case of real time RT-PCR, the primers are fluorescently labeled, which allows the amplification process to be assayed throughout each cycle. While PCR proceeds, only the genes that have been chosen to be amplified are duplicated. In this particular project, a series of qPCR runs were made using primers specific for *1SHANK3*, *GRIN2D*, and *GABRG3*.

PROJECT PURPOSE

As stated in the Background section, there are currently two main theories about the role of interstitial white matter neurons (IWMN) in psychiatric disorders. The two theories differ in whether IWMNs are transcriptionally active. The "tombstone" theory states that the increased IWMN density observed in some psychiatric individuals represents the remnants of an earlier developmental problem, but the increase does not alter the adult IWMN transciptome, as IWMNs are transcriptionally inactive. The second theory states that IWMN neurons are transcriptionally active, so the increase in IWMNs in psychiatric disorders will alter the transcriptome of white matter and its function. This project will investigate whether IWMNs are transcriptionally active, and if so, whether they are expressing gene mRNAs previously shown to correlate with psychiatric disorders. White matter and gray matter neuronal tissue will be isolated from patient autopsy brains, and the up-regulation of specific target mRNAs will be monitored by qRT-PCR and by RNA sequencing.

METHODS

Brain Tissues

Brain tissues were obtained by our lab through a variety of NIH grants. Gray and white matter tissues were dissected by Dr. Yin Lin prior the beginning of this project, and stored at -80ºC until RNA purification.

RNA Purification

RNA was isolated from white and gray matter human brain samples through use of an RNeasy Lipid Tissue Mini Kit (Qiagen). Aseptic technique was practiced, as well as precautionary safety regulations for the handling of human brain tissue. RNA purification began with the thawing of approximately 100 mg of human brain samples on ice for approximately two minutes, before being homogenized in a 7 ml glass dounce homogenizer with 1.0 ml of QIAzol Lysis Reagent. After the tissue was homogenized, it was transferred to an O-ring tube and allowed to incubate for five minutes at room temperature. Then 200 µl of chloroform was added to the homogenized solution, and the solution was vortexed for 15 seconds. The solution was allowed to incubate for another two minutes at room temperature, before being placed on ice. This process was repeated until six samples had been processed.

Next, the samples were centrifuged for 15 minutes at 4ºC at 12,000g. After centrifugation, the samples had separated into an upper aqueous phase, and a thicker pink tissue phase at the bottom of the tube. The upper aqueous phase containing RNA was carefully transferred into a fresh Eppendorf tube, and one volume of 70% ethanol was

added to the solution. The tube was vortexed, and then added to the provided RNeasy spin-through column. The RNeasy column was immediately centrifuged at 11,000g for 15 seconds, and the flow-through from the column was discarded. Depending on the volume of the solution mixed above, another round of centrifugation with the remaining solution and the same column, was sometimes necessary to process the entire Eppendorf tube's worth of solution.

At this point, another solution comprised of 10 µl of DNase1 stock solution and 70 µl of Buffer RDD (provided by the RNeasy Lipid Tissue Mini Kit) was prepared. The 80 µl prepared solution was then pipetted directly into the center of the flow-through column membrane. The flow-through column was then allowed to incubate for another 15 minutes at room temperature. After this period of incubation, 350 µl of RW1 buffer was added to the center of the column membrane, and the column was centrifuged at 11,000g for 15 seconds. Next, 500 µl of Buffer RPE was added to column, and was centrifuged for another 15 seconds. Finally, another 500 µl of Buffer RPE was added to the column, and centrifuged for 2 minutes. The column was then placed in a fresh, RNAse-free Eppendorf tube, while the flow-through and the original tube were discarded. Then 30 µl of RNAse free water was added to the membrane of the column, and the column was centrifuged at 11,000g for 1 minute to elute the RNA from the column membrane into solution.

Each sample was tested for RNA concentration using a NanoDrop-1000 Spectrophotometer, and was then stored at -80ºC until needed.

RNA-Seq and qRT-PCR

After the full range of brain tissue samples had been processed, around 30 µl of each purified RNA solution was sent to a third-party RNA sequencing corporation to fully process using a proprietary WTSS method. Additionally, a smaller set of samples had been sent for processing slightly earlier in the year, in order to get an idea of what to expect from the larger body of samples.

While the results of the RNAseq are still being processed at the time this report was written, quantitative RT-PCR was used to provide a secondary set of results that could be used as an additional resource. Primers for *SHANK3*, *GRIN1*, *GRIN2D*, *GLRA1*, *GLRA3*, *GABRG3*, *GABRG5*, *GABRA5*, and *GABRD* were designed and tested for efficacy via a series of standard curve experiments in an Agilent 7500 RT-PCR machine. In order to test the primers for efficacy, a protocol was developed where each primer was tested against various dilutions of template cDNA, or against a random sequence of nucleotides. Template cDNAs were synthesized by Invitrogen based on the RNA we sent them. Several types of random hexamers were mixed to provide a random template mixture for the reverse transcriptase reaction. This mixture was then used in conjunction with the primers to determine the primer's efficiency and specificity.

Each 96-well plate used to test these primers was prepared in the following manner. First, a plate map of the primers to be tested was developed, so that each primer would be tested in quadruplicate with ½ dilution cDNA template, ¼ dilution cDNA template, and $^{1}/_{8}$ dilution cDNA template. Secondly, an empty 96-well plate was placed on ice, as well as the set of forward and reverse primers to be tested. At this point, eight

master mixes containing 31.2 µl of RNAse-free water, 260 µl of KAPA SYBR Fast qPCR buffer, 8 µl of combined forward and reverse primer for each gene, 10.4 µl of ROX low buffer, and finally 10.4 µl of appropriately diluted template cDNA were added to each tube of master mix. Each master mix was pipetted into the appropriate wells, before the qPCR process was initiated on the Agilent 7500 RT-PCR machine.

Of the nine primer sets designed, only the primers for *SHANK3*, *GRIN2D*, and *GRIN1* displayed acceptable levels of specificity. From this point, brain samples were age and gender matched as to minimize confounding factors so that two pairs of gray and white matter samples from each disease individual, and two pairs of gray and white matter samples from control individuals were placed on each plate to analyze. qRT-PCR analysis was performed on each purified RNA brain tissue sample for each of the three targets, as well as the housekeeping ribosomal gene 18S as a reference.

After qRT-PCR analysis had been completed, statistical analysis was performed on the raw data produced by each qRT-PCR run, in order to determine the relative level of expression for each gene for each tissue sample, as well as illuminate any significant differences in gene expression between the sub-groups tested (Control, SCZ, Autism, BPD, and MDD brain samples).

RESULTS

Epigenetic analysis of white matter neurons may reveal gene expression differences in individuals suffering from schizophrenia, autism, and other psychiatric disorders, especially in those genes previously shown to be associated with psychiatric disorders. In this project, several genes were selected for qRT-PCR analysis on the basis of their strong neuronal gene expression based on our lab's previous Chip-seq data (Cheung et al., 2010), and based on their previous association with psychiatric disorders. Brain samples were obtained for autopsy patients for schizophrenia, autism, spectrum disorders, etc. RNA was isolated from white matter and gray matter neurons, and was sent to Eurofins for RNA-Seq third-party analysis. The samples were tested for RIN values using the Agilent 2100 Bioanalyzer, a microfluidics-based analysis device that uses very small volumes of RNA solution to determine RNA integrity.

A subset of the original 9 gene candidates was selected for analysis by qRT-PCR in a 96-well format. **Figure-7** shows example qRT-PCR data for 18S rRNA (green and purple curves, left panel, left side), and for mRNAs for SHANK3, GABRD, and GRIN1 (left panel, right side). As the cycle numbers increase (x-axis), the amplicon levels rise. Note that the 18S amplicons appear early (cycles 8-16), as is typical for a high abundance RNA template. In the melt curve (right panel), note the tight peaks indicating a narrow range of temperatures (X-axis) that successfully amplify this specific gene with these primers; no amplicons appear outside the annealing range of this set of primers. Not all of the samples in the wells exhibited melt curves of this high caliber, so any underperforming primers were excluded from further data analysis.

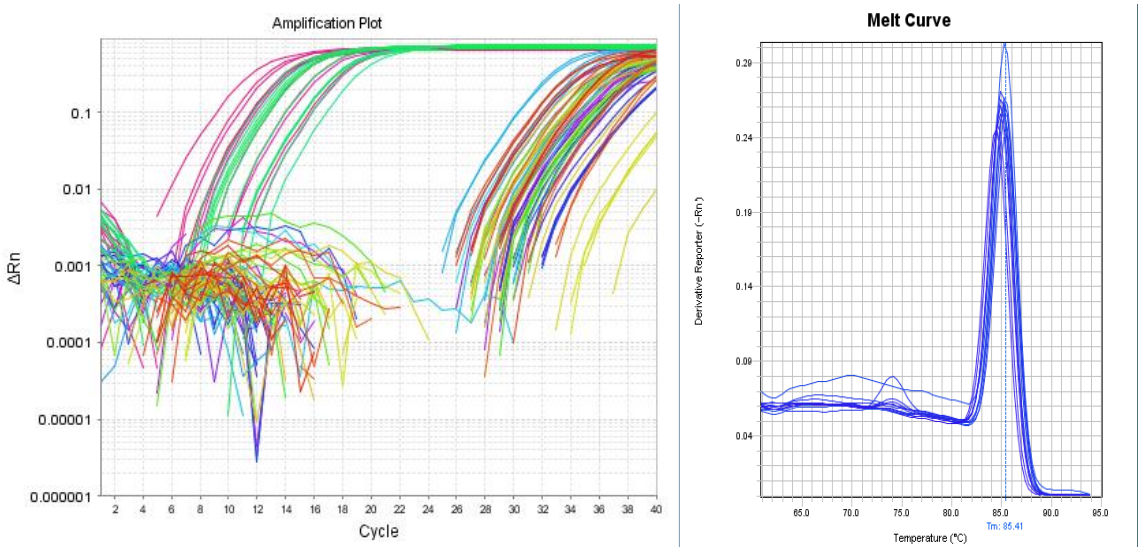
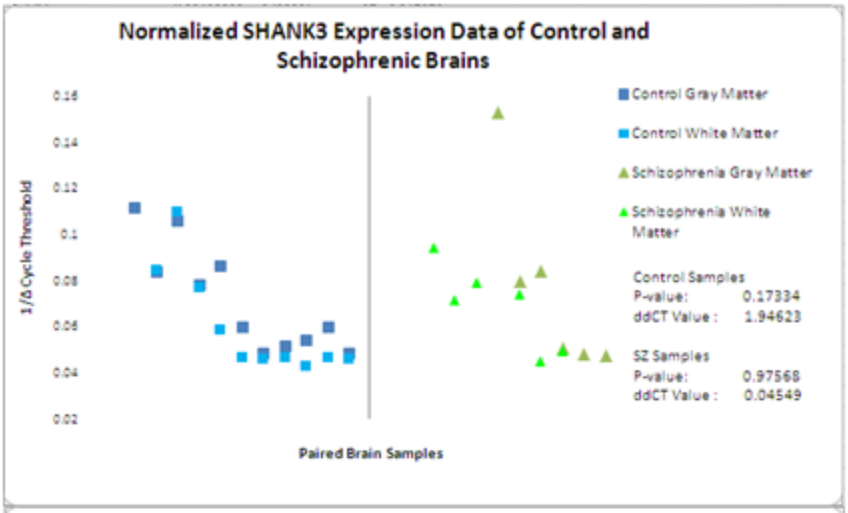
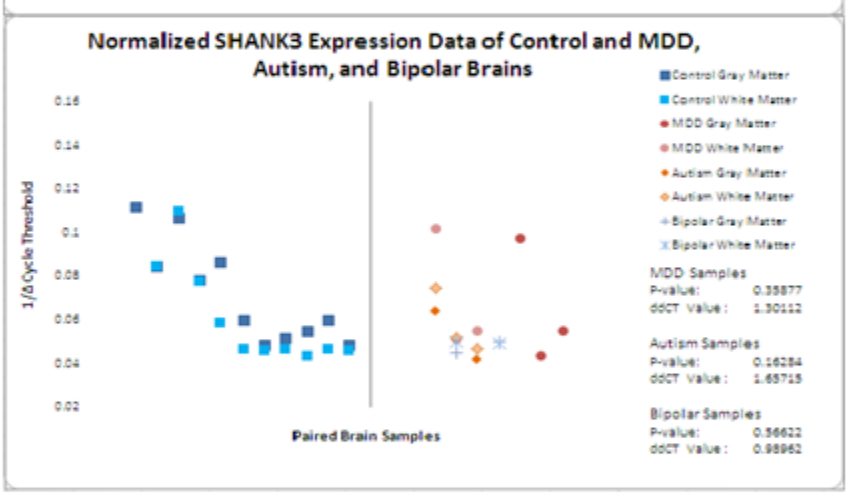
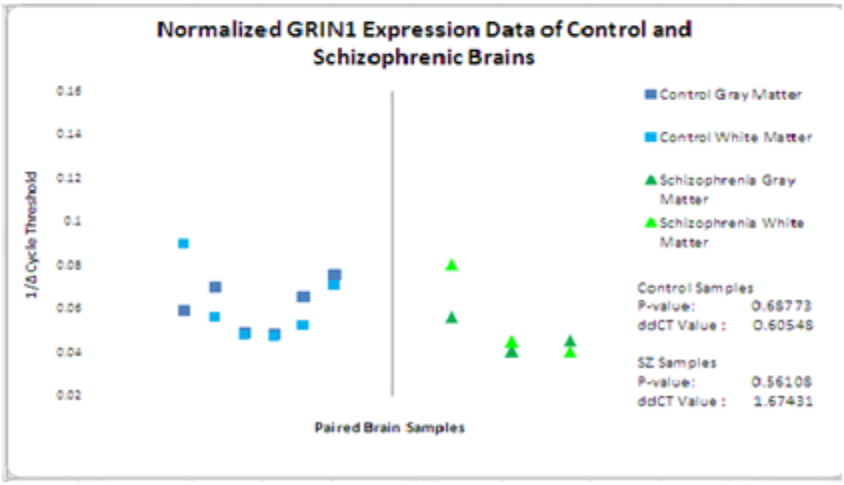


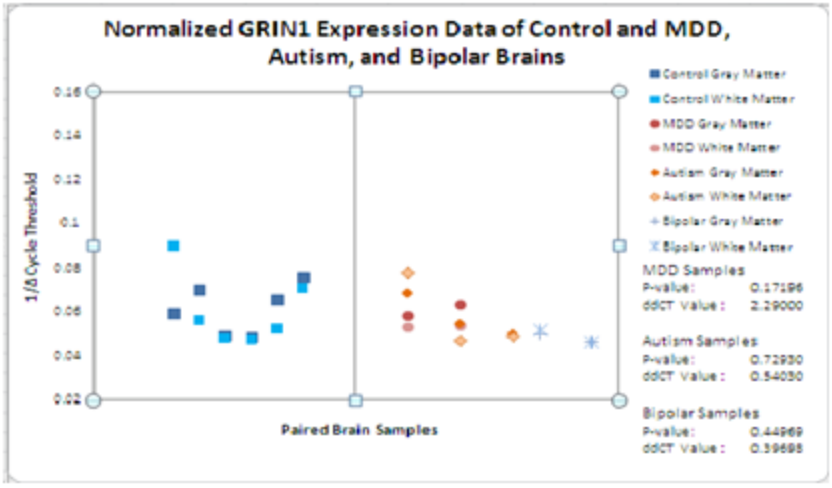
Figure-7: Example of Real Time qRT-PCR Data and Accompanying Melt Curves. The **left panel** denotes an amplification plot for cycles 1-40 (X-axis) relative to amplicon abundance (Y-axis), obtained from a 96-well plate with quadruplicate samples. Shown are curves for the relative expression of *18S* rRNA (green and pink lines rising from the interference on the left side of the panel). Note that the 18S amplicons come up early as expected for a high abundance RNA. The right half of the panel shows the amplicons for mRNAs *SHANK3*, *GABRD*, and *GRIN1*. The **right panel** shows several melting curves for the SHANK3 primers. The X-axis shows the melting temperature tested, and the Y-axis represents amplicon formation. Note the tight specific amplification obtained from this set of primers, as no amplicons formed outside the main annealing temperature for this set of primers.

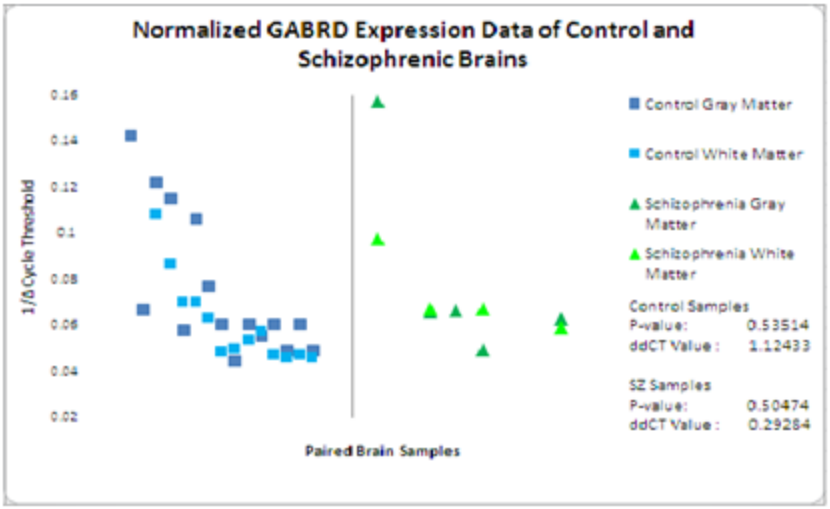
Based on the melting temperature curves for nine genes, the RT-PCR reactions for mRNAs for SHANK3, GRIN1, and GABRD were selected for further analysis. After each 96-well plate of qRT-PCR had been performed, all of the data from acceptable primers were entered into an Excel spreadsheet to determine statistical significance (**Figure-8**). Each plot shows the data for a particular mRNA, for gray matter or white matter neurons, for control brains, schizophrenic brains, major depressive disorder brains, and autistic brains. The Y-axis denotes 1/ΔCycle Threshold, a measure of the mRNA abundance, i.e. at which cycle does the amplicon reach 50% maximal amplification.


Normalized SHANK3 Expression Data of Control and Schizophrenic Brains
1/Δ Cycle Threshold
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
Paired Brain Samples
Control Gray Matter
Control White Matter
Schizophrenia Gray Matter
Schizophrenia White Matter
Control Samples
P-value: 0.17334
ddCT Value : 1.94623
SZ Samples
P-value: 0.97568
ddCT Value : 0.04549


Normalized SHANK3 Expression Data of Control and MDD, Autism, and Bipolar Brains
1/Δ Cycle Threshold
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
Paired Brain Samples
Control Gray Matter
Control White Matter
MDD Gray Matter
MDD White Matter
Autism Gray Matter
Autism White Matter
Bipolar Gray Matter
Bipolar White Matter
MDD Samples
P-value: 0.35877
ddCT Value : 1.30112
Autism Samples
P-value: 0.16284
ddCT Value : 1.65715
Bipolar Samples
P-value: 0.56622
ddCT Value : 0.98962


Normalized GRIN1 Expression Data of Control and Schizophrenic Brains
1/Δ Cycle Threshold
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
Paired Brain Samples
Control Gray Matter
Control White Matter
Schizophrenia Gray Matter
Schizophrenia White Matter
Control Samples
P-value: 0.68773
ddCT Value : 0.60548
SZ Samples
P-value: 0.56108
ddCT Value : 1.67431


Normalized GRIN1 Expression Data of Control and MDD, Autism, and Bipolar Brains
1/Δ Cycle Threshold
Paired Brain Samples
Control Gray Matter
Control White Matter
MDD Gray Matter
MDD White Matter
Autism Gray Matter
Autism White Matter
Bipolar Gray Matter
Bipolar White Matter
MDD Samples
P-value: 0.17196
ddCT Value: 2.29000
Autism Samples
P-value: 0.72930
ddCT Value: 0.54050
Bipolar Samples
P-value: 0.44969
ddCT Value: 0.39698


Normalized GABRD Expression Data of Control and Schizophrenic Brains
1/Δ Cycle Threshold
Paired Brain Samples
Control Gray Matter
Control White Matter
Schizophrenia Gray Matter
Schizophrenia White Matter
Control Samples
P-value: 0.53514
ddCT Value: 1.12433
SZ Samples
P-value: 0.50474
ddCT Value: 0.29284

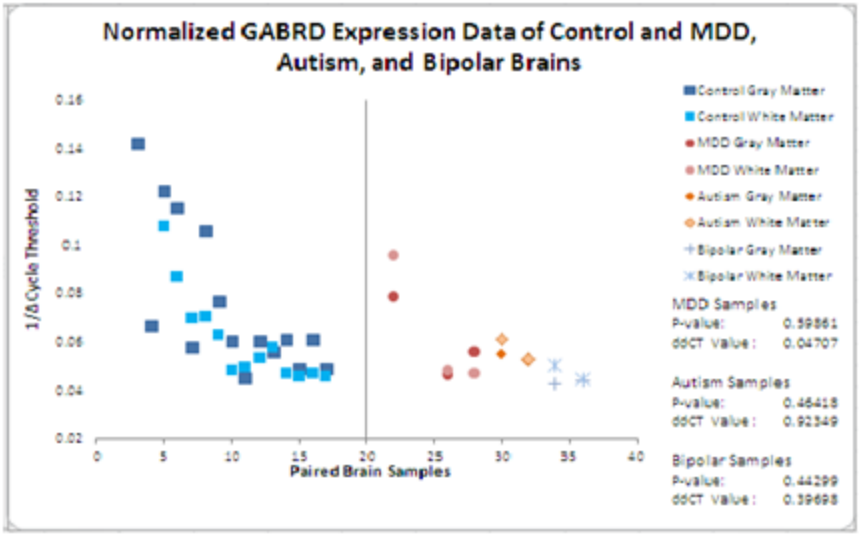

Normalized GABRD Expression Data of Control and MDD, Autism, and Bipolar Brains
1/Δ Cycle Threshold
Paired Brain Samples
Control Gray Matter
Control White Matter
MDD Gray Matter
MDD White Matter
Autism Gray Matter
Autism White Matter
Bipolar Gray Matter
Bipolar White Matter
MDD Samples
P-value: 0.59861
ddCT Value: 0.04707
Autism Samples
P-value: 0.46418
ddCT Value: 0.92349
Bipolar Samples
P-value: 0.44299
ddCT Value: 0.39698

Figure-8: Normalized Gene Expression Data for SHANK3, GRIN1, and GABRD. Each graph displays a gene of interest, comparing its expression between control and different disease sub-groups, for gray and white matter neurons. The Y-axis denotes 1/ΔCycle Threshold, a measure of the mRNA abundance. No difference was seen between the groups in this particular analysis.

Note that for this particular analysis, no strong difference was observed between the control samples and the disease samples for all genes, the calculated p-values for the complete set of data (not shown) indicated no statistically relevant differences.

The results of this first analysis were confusing since previous data had indicated that GRIN1 expression is much lower in white matter tissue, so a second analysis was performed. In the repeat analysis, brain samples with RIN values lower than 6.0 were removed (**Figure-9**). RIN values represent the integrity of the RNA; RIN values less than 6.0 indicate that a sample has been significantly degraded, so that sample is perhaps not the best to use for analysis. This second more restrictive analysis, found that GRIN1 expression for all brain samples was significantly lower ($p=0.0545$) in white matter neurons. The only other potentially significant finding ($p=0.086$) was that GRIN1 may be expressed at significantly higher levels for white versus gray matter neurons in Major Depressive Disorder brains.

Ratios	SHANK3 (dCT)	GRIN1 (dCT)	GABRD (dCT)
Ratio (Gray:White)	1.030	1.132	1.003
Ratio Control (Gray:White)	1.056	1.125	0.924
Ratio Schizophrenia (Gray:W	1.618	N/A	1.222
Ratio MDD (Gray:White)	0.915	1.139	1.061
Ratio Autism (Gray:White)	0.961	N/A	0.822

T Tests	SHANK3	GRIN1	GABRD
All Samples (Gray:White)	0.7353	0.0545	0.9793
Control T-Test (Gray:White)	0.7267	0.4123	0.7424
Schizo. T-Test (Gray:White)	N/A	N/A	0.3324
MDD T-Test (Gray:White)	0.5462	0.0863	0.5919
Autism T-Test (Gray:White)	N/A	N/A	N/A

Figure-9: Analysis of RIN > 6.0 Intact RNA Samples. The CT averages expressed in ratio format are shown in the above table, while the T-test's for each group are displayed in the lower panel. Note the two yellow highlighted boxes for GRIN1 expression are near significance.

DISCUSSION

The results of this project provided RT-PCR data for SHANK3, GRIN1, and GABRD, and indicate that *GRIN1* mRNA expression may be significantly higher than normal in the white matter neurons in individuals with Major Depressive Disorder (MDD) versus normal brains. This gene may play a role in the etiology of the disorder. Previous research has not linked *GRIN1* expression and MDD in patients, although one study (Alexander et al., 2007) did link *GRIN1* variants to German schizophrenics who also suffered from lifelong depression. It should be noted that the vast majority of studies involving *GRIN1* and mood disorders focus on Bipolar Disorder (Mundo et al., 2003; Hayden and Nurnberger, 2006), although it is possible that some of the same mood dysregulation problems affect both BPD and MDD sufferers.

Over the course of the project, several difficulties were overcome, mostly in the form of primer specificity. Only one third of the RT-PCR primers were found to be of acceptable quality after an arduous primer testing process. After this initial primer testing phase was completed, extensive real time RT-PCR analysis was performed. Unfortunately many of the same primer specificity issues were still present, and some samples were not able to be included in the final data analysis.

In the future, valid primer sets could be designed and tested for the remaining six genes of interest: GRIN2D, GLRA1, GLRA3, GABRG3, GABRG5, and GABRA5, as described in the Background section. And the analysis could be extended to other candidate genes. Given that a near-to-significant result was obtained with the limited analysis performed, it seems likely that other significant gene expression differences between gray and white matter neurons could be discovered in patients suffering from

psychiatric disorders, and these differences could be used to correct the defects in patient brains using gene therapy.

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