THE PENNSYLVANIA STATE UNIVERSITY
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The Effects of ZNF804A Expression on Neuron Growth and Protein Expression as It Relates to Schizophrenia

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ABSTRACT

Schizophrenia is a neurodevelopmental disorder that results in the establishment of psychiatric symptoms around late adolescence or early adulthood. The DSM-5 diagnoses schizophrenia by the presence of 1 of 3 positive symptoms (either hallucinations, delusions, or disorganized speech), and at least one more additional symptom (another of the 3 positive symptoms, catatonic behavior, or negative symptoms) (American Psychiatric Association, 2013). Beyond the positive and negative symptoms, those suffering with schizophrenia also experience cognitive symptoms. Even though 1% of the worldwide population is suffering from this disorder and its symptoms, there are very few treatments available; and those that are available have significant side effects (Van et al., 2014) (Lally & Maccabe, 2015). As such, a better understanding of the etiology and mechanisms by which this disorder is caused is essential. Previous research has cited both environmental factors, such as poor maternal habits or prenatal infections, and genetic factors, such as copy number variations and single nucleotide polymorphisms, as the cause of this disorder (Lewis et al., 2015) (Yolken, 2004) (Tao et al, 2014). The purpose of this research was to determine the effect of ZNF804A on the development of schizophrenia. ZNF804A is a gene that has substantial backing from previous research as being a significant risk factor for schizophrenia; though little is known about the mechanisms in which it affects the development of the said disorder. As such, this study utilized genetic editing to generate ZNF804A KO mice to examine the different effects of this knockout on the production of various proteins and neuron growth to try and determine how this gene results in the development of schizophrenia. This study found a significant difference in NPY expression between heterozygous and wild type mice (p-value of 0.0036), as well as between heterozygous

and homozygous mice (p-value of 0.0239). This is consistent with previous findings and suggests the role of NPY in the development of schizophrenia in relation to ZNF804A KO. On the other hand, this study did not find any significant differences in SST, PV, FUS, SATB2 FEZ1, or CTNNB1 expression, or in cortex layer cell concentration (studied with CTIP2, FOXP2, and BRN2). This is not consistent with previous research and illustrates the need for further research into the mechanism of schizophrenia development as schizophrenia clearly has a complex multi-factorial etiology.

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Chapter 1

Introduction

Schizophrenia: Background and Diagnosis

Schizophrenia is a chronic, severe psychiatric and neurodevelopmental disorder. It affects the feelings, thoughts, moods, attention, and behaviors of those living with this mental illness. It has been shown not only to affect behavioral control in moments of stress, but also the individual's speech, movement, reaction, and aggression following the disorder's onset. Diagnosis of this disorder is performed according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) (American Psychiatric Association, 2013). These standards are based on multi-dimensional evaluations, including the age at onset of the disorder, symptom development and persistence, and symptom description. In order to be diagnosed, a patient must have two of the five classifying symptoms, 1 of which must be either delusions, hallucinations, or disorganized speech. The additional two classifying symptoms for the presence of the disorder are disorganized or catatonic behavior, and negative symptoms, such as avolition. Any of the present symptoms must be observable for a significant amount of time within a one month period (American Psychiatric Association, 2013). Further, diagnostic criteria recognizes the disturbance post onset which the disorder provokes on the individual's life and functioning compared to preceded onset, or in children, compared to their expected level of functioning. As mentioned previously, schizophrenia is a neurodevelopmental disorder. As such, symptoms begin to appear in adolescence and early adulthood and persist throughout life. In 2013, the

DSM-5 modified their classification of schizophrenia to schizophrenia spectrum and other psychotic disorders. Further, the 5th edition of the DSM began recognizing the cognitive symptoms of schizophrenia, in addition to the already recognized positive and negative symptoms (American Psychiatric Association, 2013).

Schizophrenia: Positive Symptoms

The positive symptoms of schizophrenia are defined as the addition of sensation. These can include hallucinations, delusions, disorganized thoughts and agitated body movements. Hallucinations and delusions, however, are the most commonly associated symptoms with schizophrenia. Hallucinations are sensory experiences that occur in the absence of related external stimulation. These sensory experiences can occur in any of the sensory modality: auditory, visual, tactile, and olfactory. One or multiple types of these hallucinations are experienced by about 70% of schizophrenic patients (Chaudhury, 2010). On the other hand, delusions are firm beliefs that conflict with reality or rational argument. Around 90% of patients suffering from schizophrenia experience these delusional symptoms (Elahi, Algorta, Varese, Mcintyre, & Bentall, 2017).

Schizophrenia: Negative Symptoms

As opposed to positive symptoms, negative symptoms are defined by the removal of function. Examples of common negative symptoms experienced by those suffering with schizophrenia are emotional deficits, depression, reduced speech, social withdraw, and diminishing effective responses. Negative symptoms are usually recognizable within the early

stages of schizophrenia and are important in identifying the initiation of psychotic change (Mitra, Mahintamani, Kavoor, & Nizamie, 2016). Specifically, depression is experienced by over 80% of those with schizophrenia within one or more phases of the first psychosis episodes (Upthegrove et al., 2010).

Schizophrenia: Cognitive Symptoms

Finally, cognitive symptoms of schizophrenia affect the cognitive functioning of the individuals. Examples of these symptoms are problems with decision making, attention, learning ability, and memory (McCleery et al., 2014). As mentioned previously, these symptoms only began to be recognized as associated with schizophrenia as of the DSM-5. One of the important differences between cognitive symptoms and positive/negative symptoms, is cognitive symptoms are present before the onset of psychotic symptoms (Kahn & Keefe, 2013).

Schizophrenia: Effect and Treatment

Schizophrenia is one of the most common disorders, with 1% of the worldwide population suffering from it. Men are slightly more at risk of suffering from this mental disorder, as the odds ratio is 1.15 for prevalence in men compared to women (Van et al., 2014). Because of the disruption the symptoms of this disorder cause, those suffering from it have a reduced lifespan as they become at an increased risk of physical illness, self-mutilation and suicide (Tiihonen, Tanskanen, & Taipale, 2018).

Because of the prevalence, and the course of this disorder, that thereby requires lifetime care and treatment, the cost of those suffering with schizophrenia is substantial. In 2013,

the economic burden of schizophrenia in the United States, was estimated at about $155.7 billion (Cloutier, et al., 2016). As such, it is imperative for the health and economic sake of the patients, that a greater understanding of the neurological mechanisms leading to this disorder is reached. As it stands there is little understanding of the cause or neurological mechanisms of schizophrenia. Resultantly, there is a lack of adequate and safe treatments for the disorder. Right now, the majority of approved treatments target reduction of psychopathological symptoms, but do not attend to the cognitive function impairments. The current options for treatment of schizophrenia are classified between first and second generation antipsychotics, though both are known to cause undesirable side effects, such as akathisia, dyskinesia, weight gain, sexual problems resulting from hormonal changes, and sleep disturbance (Lally & Maccabe, 2015). Other treatments, like gene and cell therapy, are under research and development (Shetty & Bates, 2016). To design effective treatments, however, a better understanding of the etiology of schizophrenia must be solidified.

Etiology

An abundance of research into the mechanisms of schizophrenia point to the fact that this mental illness is not one with a simply etiology. Rather, it seems there is a complex mixture of risk factors that contribute to the development of the disorder, and not one single factor. These include a mixture of environmental factors, such as maternal stress, malnutrition, brain injury, and drug abuse; as well as genetic factors, like chromosomal abnormalities, and gene mutations.

Etiology: Environmental Effect

Numerous studies have illustrated that environmental factors do play a role in the development of schizophrenia. Specifically, much research has focused on the environment of the embryo. The human brain is much more vulnerable during the embryonic stage and, as such, the maternal environment in which the fetus is developing plays a role in the fetus's future mental health. Previous studies illustrate that maternal psychological stress during pregnancy leads to a decrease in maternal immune functioning, and an increase risk of the mother smoking, practicing poor dietary habits, and consuming alcohol. Resultantly, these maternal habits cause a problematic environment for the brain development of the fetus, in turn increasing the offspring's risk of psychiatric disorders, like schizophrenia (Lewis et al., 2015). In addition to maternal habits contributing to the environmental effect, prenatal viral infections have been documented as an early indicator of postnatal psychiatric disorders. For instance, pregnant women carrying the herpes simplex virus have a higher than average blood HSV-1 antibody that results in the offspring being more likely to be diagnosed with schizophrenia (Yolken, 2004).

Etiology: Genetics

As mentioned, the environment isn't the only contributor to schizophrenia's development, but rather genetics play a substantial role as well. A variety of family studies show the various relationships between genetics and this mental disorder. In 1996, Rudin discovered that an individual has a higher risk of developing schizophrenia if one of their relatives suffered from psychosis, alcoholism, or both (Zerbin-Rudin & Kendler, 1996). Later, it was further determined that individuals with schizophrenic relatives have a 4.8% risk of having it themselves

(Riley & Kendler, 2006). This is ten times the average risk of developing schizophrenia when no relatives are documented as having been diagnosed with schizophrenia (a 0.5% risk). Similarly, Finnish researchers looked at 144 offspring from schizophrenic mothers and 178 from healthy mothers, and found that 9.1% of the offspring born to schizophrenic mothers developed a form of schizophrenia themselves; while only 1.1% of those born to healthy mothers developed schizophrenia (Tienari, 1991). Twin studies have also been used to identify genetic components of schizophrenia. In 2006, Riley and Kendler identified significantly higher levels of concordance of schizophrenia among monozygotic twins compared to dizygotic (48% verses 17%, respectively) (Riley & Kendler, 2006). In total, the heritability of schizophrenia was estimated to be about 80% (Cardno & Gottesman, 2000).

The relationship between genetics and the environment also seems to play a role in schizophrenic development. Gene-environment interactions were examined through adoption studies, in which the environment was controlled, but the genetics of the adopted children differed. Those who were adopted from a biological family that contained at least one blood relative with schizophrenia, showed a higher risk of schizophrenia than those adopted from a family without a lineage of schizophrenia (Riley & Kendler, 2006).

Numerous mechanisms in which schizophrenia is transmitted genetically have been studied. Two of these methods are development of schizophrenia through copy number variation (CNV) and by single nucleotide polymorphisms (SNP). CNV refers to a change in the number of copies of a large chromosomal region present after transcription of the original chromosome. These chromosomal abnormalities can be in the form of deletions, insertions or translocation within the chromosome. In a study conducted by Steinberg and colleagues, they identified two ZNF804A deletions associated with a diagnosis of schizophrenic and no ZNF804A CNV

identified in any of the healthy control subjects (Steinberg et al., 2011). Previous studies note CNVs to be the cause of approximately 2% of schizophrenic cases (Bassett, Scherer, & Brzustowicz, 2010). The copy number variation of the gene is believed to cause the varied abundance of the gene within cells that then leads to the development of psychosis.

Single nucleotide polymorphisms, however, are the alterations of a single nucleotide within a specific gene. In 2008, genome wide association studies (GWAS) identified ZNF804A as the first gene with significant association to schizophrenia (O'Donovan et al., 2008). Since then, more genes have been determined to be associated with schizophrenia, but ZNF804A remains one of the most significantly associated. The SNP of ZNF804A that shows the strongest evidence of determining schizophrenia's development is rs1344706 (G/T), where T is both the risk allele and major allele (Tao et al, 2014). Specifically, the T/T risk genotype showed a 1.2 fold increase risk of developing schizophrenia. Studies have also identified the T allele resulting in lower visual memory, increased personal bias, and diminished attention skills (Tao et al, 2014).

ZNF804A

ZNF804A is a gene, previously known as C2orf10, that is located on chromosome 2q32.1 and encodes a 4.7kb mRNA transcript with a 3.6kb coding sequence between 595bp to 4224bp. The mRNA encodes for a protein of 1209 amino acids with one zinc finger domain on exon 2. The protein is known as zinc finger protein 804a. The encoding mRNA contains three small exons (exon 1,2 and 3), and one large exon (exon 4) (Figure 1). Though little is known about the mechanisms by which ZNF804A results in schizophrenia, there are a number of studies backing

up the relationship between the gene and the disorder, each with a robust number of participants/cases. For instance, O'Donovan and colleagues utilized 6,666 case studies and 9,897 controls in a study that identified a relationship between ZNF804A and schizophrenia (O'Donovan et al., 2008). Williams and colleagues also ran a meta- analysis including 18,945 patients with schizophrenia, 21,274 patients with bipolar disorder, and 38,675 controls, and also found similar findings (Williams et al., 2011). Finally, in 2014, the Schizophrenia Working Group of the Psychiatric Genomics Consortium published the largest GWAS study on schizophrenia at the time. Their study compared 36,989 cases of schizophrenia with 113,075 control cases that supported the previous findings that ZNF804A was among the top genes associated with schizophrenia. They also determined that rs1344706 was not the only significant SNP associated with schizophrenia, but rather rs11693094 showed significance as well (Figure 1) (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Rs1344706 may regulate the mRNA level of ZNF804A, as Riley and colleagues determined that it binds to the transcription factors MYT1I and POU3F1/OCT-6. In addition, upstream and downstream targets of ZNF804A's transcription and translation are also associated with schizophrenia risk (Riley et al., 2010). Immunostaining of this gene, ZNF804A, has shown expression within dendrites and neuronal spines, indicating the function of ZNF804A on neurogenesis regulation and dendritic spine plasticity.

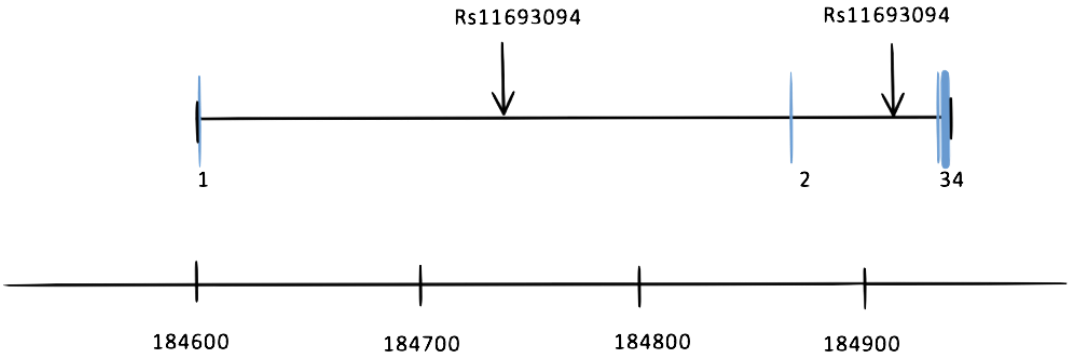

Figure 1. Location of Mutations

Animal Models

Research utilizing animal models has been a significant contributor to better understanding the mechanisms by which these genetic variations lead to schizophrenia. There are four categories of animal models for schizophrenia: neurodevelopmental models, pharmacological models, lesion models, and genetic models. In the following research, genetic models were utilized. By altering the genetic makeup of a mouse, expression of specific genes can be altered and thereby give a means to study the effects of the gene on functioning and disorder development. To generate genetic animal models, researchers can use models such as deletion/knockout, insertion/knockin/transgenesis, and mutation/mutagenesis. Previous research has used CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/ Cas9 (CRISPR-associated protein 9) for simplified gene manipulation and knockout (KO). CRISPR/Cas9 contains a Cas9 protein, a guide RNA, and a protospacer adjacent motif that together create a

double-strand break on the target DNA (Cong et al., 2013). Homology-directed repair (HDR) and non-homologous end joining (NHEJ) then rejoin the DNA strands. HDR also has the ability to insert large fragments into the DNA at the break site, while NHEJ contributes insertions, deletions or mutations at said break site (Wang & Qi, 2016).

Aims

Leading up to the following research, genome editing was utilized to generate ZNF804A knockout (KO) mice. We then genotyped the mice to determine which were wild type, heterozygous for the mutation, and homozygous. We utilized behavioral tests, such as open field, plus maze, and social interaction tests to determine the presence of schizophrenic symptoms in the knockout mice. This research aimed to study the molecular mechanisms by which ZNF804A KO and wild type mice differ and how this downregulation of ZNF804A affects various neurite growth.

Chapter 2

Methods

Sample Preparation

Well plates containing adherent cultured cells, which were previously prepared, were obtained. Exactly 80µl of 1% Radioimmunoprecipitation assay buffer (RIPA buffer) was added to each well and the wells were gently disrupted then left on ice for 15 minutes. A 200µl pipette was used to scratch into the well to pick up the cultured cells and transfer them to a new 1.5µl tube. The samples were then placed on ice for 10 minutes. Afterwards, the samples were centrifuged for 15 minutes at maximum speed. The supernatant was transferred into a new 1.5µl tube , avoiding the pellet. Exactly 1µl of the supernatant was transferred to a separate tube to calculate the concentration of genomic material. This dictated the volume of each sample utilized in the western blot protocol.

Western Blot

A variety of samples were run, with a variety of primary and secondary antibodies used (see results). For each set of primary and secondary antibodies, a 15% lower gel was made using the following:

30% Acrylamide	7.5 mL
dH2O	2.4 mL
1.5 M Tris-HCl, pH 8.8	3.75mL
Glycerol	1.35mL
10% APS	74µl
TEMED	5.6µl

Once the lower gel was solidified, the 5% upper gel was made using the following:

30% Acrylamide	660µl
dH2O	2.7 mL
0.5 M Tris-HCl, pH 6.8	1.25mL
Glycerol	400µl
10% APS	20µl
TEMED	6µl

Once the upper gel was solidified, the gels were secured in a plastic tub, fresh buffer was added to the tub until the gels were completed submerged, and fresh buffer was pipetted into the wells to remove any left over gel. Samples were denatured for 5 minutes at 100 degrees Celsius, then placed on ice until loaded. The samples were loaded according to their protein concentration, with an average loading volume of 20µl (including an equal amount of loading buffer in each sample). Ladder was loaded into the gel with 2µl on one side of the samples and 1µl on the other. Electrophoresis was run in two steps. The first running 20 minutes at 80V and the second for an hour and 20 mins at 115V.

A fresh membrane was obtained and aligned with the gel in fresh transfer buffer to prepare for protein transfer. Cassettes were assembled, fresh buffer added to center of tub and reused buffer to surrounding areas. The transfer was run overnight at 40V in 4 degrees Celsius. The cassettes were dissembled and the membrane was blocked for 1 hour on the shaker at room temperature. The blocking buffer used was 5% milk (5g nonfat dry milk mixed in 100mL of 1x TBS buffer). The membrane was then incubated overnight on a shaker at 4 degrees Celsius in the primary antibody dilution (dilutions of primary antibody to blocking buffer varied depending on the antibody). The membrane was then washed three times in 1xTBST for 10 minutes on the room temperature shaker. Next, the membrane was incubated in the secondary antibody dilution (dilutions of secondary antibody to blocking buffer varied depending on the antibody) at room

temperature for one hour on the shaker, covered. The membrane was washed three more times in 1xTBST for 10 minutes on the room temperature shaker, this time covered. Finally, the membrane was imaged using an image scanner.

Perfusion

Perfusion was performed on each mouse by first administering approximately 450μl of Avertin into the designated mouse using a BD syringe. The mouse was then returned to its cage until it reached anesthetic state. Complete anesthetic state was confirmed by checking loss of corneal reflex upon air blown into eyes, and loss of pain reflex by lack of movement when paws were squeezed tightly. Once in this state, the mouse was pinned down to a Styrofoam perfusion board above a collection bag (for blood and hair). Tweezers were used to pull up the skin and hair over the sternum. Scissors were used to cut the skin, parallel to the spine to expose the outer abdominal wall and diaphragm and then cut through them, while being sure to avoid cutting any organs. Once the heart was exposed, the cut skin was pinned back. A 25-gauge needle at the end of a tube connected to a pump was obtained. The alternative end of the tubing was placed into a beaker of 1xPBS. The needle was then inserted into the left ventricle of the heart, being sure not to pierce through the other side. The pump was turned on and rapid perfusion began at high speed. The right atrium was then severed to allow for drainage. At least 50 mL of 1xPBS was pumped through each mouse. Once it appeared the majority of the blood had been removed from the circulatory system (organs begin to look more white), the tubing in the 1xPBS was switched to a container of PFA (1:8 ratio of formaldehyde and 1xPBS respectively). A minimum of 50mL of PFA was pumped through each mouse. Perfusion was complete upon the organs and tail

becoming stiff; at which point the pump was turned off and the needle removed. The head of the mouse was then removed using scissors and the skin was peeled back to reveal the skull. The skull was then removed in pieces using tweezers to chip it off. Once fully exposed, the nerves around the brain were cut using the tweezers and the brain was placed in a 15mL tube containing fresh PFA solution. The brains were then stored in 4 degrees Celsius until slicing.

Brain Slicing

One by one the brains were obtained for brain slicing. The designated brain was removed from its tube of PFA. The brain was then trimmed by removing the cerebellum and a small part of the prefrontal cortex, as the desired section of interest is the hippocampal and cortex regions. The brain was then mounted using superglue, on the cerebellum side, to the vibrotome's specimen disc. The disc was screwed into the vibrotome. Next, the blade was screwed into the vibrotome and the settings were programmed. The speed was set to 9.5, the frequency to 6 or more, and the feed to 50. The limits were then set for the start and stop position for slicing. The slice holding chamber was then filled with 1xPBS and the blade lowered until it reached just before the surface of the brain. The machine was run on continuous and brain slices were picked up one at a time using a paint brush and placed in a 6 well plate with 3mL of 1xPBS in each well. The samples were placed in the wells clockwise so each well contained a similar representation of the brain. Once all of the sample was collected, the plate was covered and sealed with paraffin and placed in 4 degrees Celsius.

Immunostaining

A 48-well plate was obtained and one brain slice of the cortex region of each mouse was placed into a corresponding well. Approximately 200µl of blocking buffer (1X PBS/5% donkey serum/0.3% Triton™ X-100) was added to each well. The samples were placed on the shaker at room temperature for an hour. Primary antibody was prepared in the meantime by diluting primary antibodies with blocking buffer using the designated dilutions specified by each antibody. The blocking buffer was aspirated from the wells and 200µl of the diluted antibody was added. The plate was then incubated overnight at room temperature.

On day two the primary antibody was removed from each well and refrigerated for future uses in a falcon tube. About 300µl of a wash buffer (.05%: PBS +Triton) was added to each well to wash the samples. The samples were washed for 5 minutes on the shaker. The wash buffer was then aspirated and fresh wash buffer was added. The samples were then shook for an additional 5 minutes. A third wash was then performed the same as the first two. The secondary antibody dilutions were prepared in the meantime by adding corresponding dilution levels of each secondary antibody (according to the provided antibody information) to fresh blocking buffer (1X PBS/5% donkey serum/0.3% Triton™ X-100). The wash buffer was removed and 200µl of the secondary antibody dilution was added to each well. The plate was covered with aluminum foil and placed on the shaker at room temperature for two hours. After two hours, the secondary antibody dilution was aspirated and three more washes performed (following the same procedures as previously described).

The samples were then mounted on coverslip slides using Prolong® Gold Antifade Reagent with DAPI. They were left to dry overnight at room temperature in the dark. Then, long-term they were placed in 4 degree Celsius in a box to protect them from light exposure.

The samples were imaged using immunofluorescence microscopic imaging.

Analysis

The immunofluorescence images were used to count and compare cell types across genotypes. The raw counts were then all divided by the image area ($0.25mm^2$) to produce cell counts across a $1mm^2$ area. The average, standard deviation (SD), and error bar for each genotype (hetz, homo, wt) across each cell type were then calculated. The averages and error bars were graphed for comparison (see results section). The cell counts across $1mm^2$ were also run through ANOVA to determine significance.

The western blot images were run through Image J to calculate the signal intensity of each protein expression specified for comparison across genotypes. Once the intensity was calculated, it was normalized by dividing the signal by the corresponding signal of ACTB. The average of all wild type signals obtained for the specified protein detection was calculated. Each normalized signal was then divided by this average. The average for each genotype was then obtained so that there was a single value for each genotype per protein detected. The standard deviations and error bars for each genotype per protein were calculated. A bar graph was constructed for each protein detection using the averages of each genotype for said protein and their corresponding error bars. Finally, the normalized and un-normalized data were run through ANOVA to compare every condition to all other conditions.

Chapter 3

Results

Cell Counting Data

Images were generated of the cortex region of each sample and categorized by treatment and genotype for comparison (Figures 2 and 4). The concentration of cells per 1mm^2 area of cortex were calculated for each treatment of each sample (organized by genotype) (Table 1 and 5). The averages were generated for each treatment per genotype (Table 2 and 6), as well as the standard deviation (Table 3 and 7) and error bars (Table 4 and 8). Finally, a graph was generated to illustrate the variation within genotypes for each cell type (based on treatment), as well as the variation between cell type expression for each genotype (Figures 3 and 5).

Figure 4 illustrated similar expression of CTP2 across heterozygous (hetz) and homozygous (homo) mice; in addition to similar expression of FOXP2 across heterozygous and homozygous mice. Wild type mice (wt) showed greater expression of both these cells types compared to heterozygous and homozygous mice. CTP2 was the most highly expressed cell type across all genotypes, followed by FOXP2, then BRN2. An ANOVA test was run to determine significance, but not one of the variations across genotypes for any of the cell types was significant.

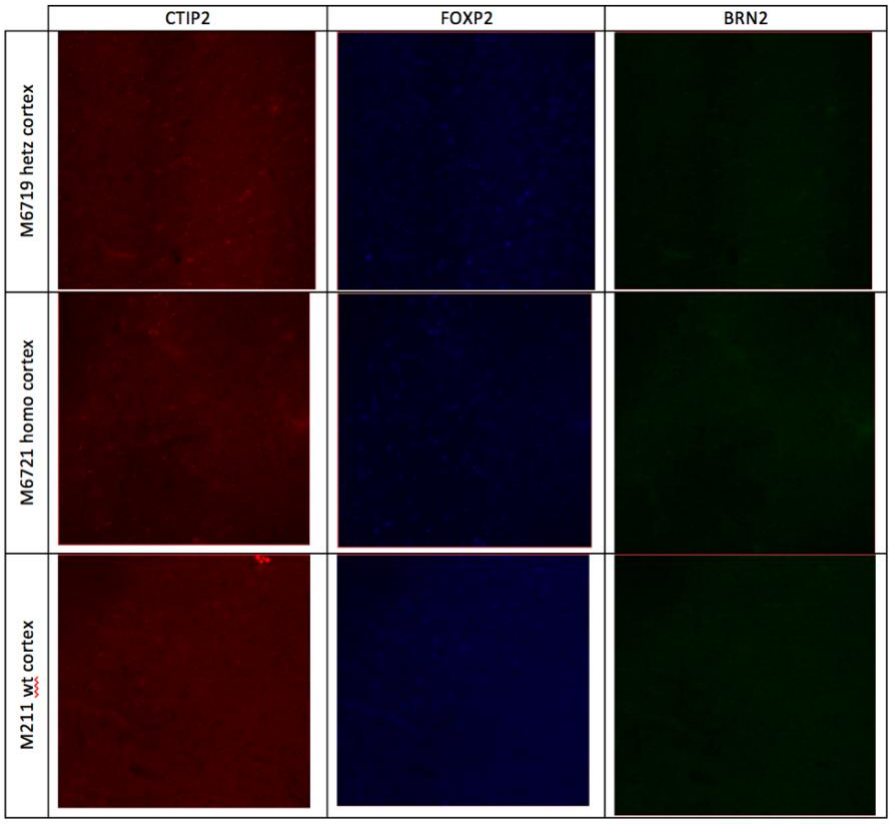

Figure 2 Cell Distribution Across Cortex Layers of Male Mice (Hetz, Homo, Wt)

Table 1 Cell Concentration Across Cortex Layers in Varied Genotypes of Male Mice

Samples	CTIP2	FOXP2	BRN2
m0024 hetz	240	152	88
m0025 hetz	204	160	52
m0029 hetz	272	140	72
m4712 hetz	568	372	152
m6719 hetz	760	392	196
m4709 homo	344	72	8
m6721 homo	472	412	120
m211 wt	348	156	196
m4710 wt	676	628	84
m6717 wt	368	128	80

Table 2 Average Cell Concentration Across Cortex Layers in Male Mice (Hetz, Homo, Wt)

Samples	CTIP2	FOXP2	BRN2
hetz	408.8	243.2	112
homo	408	242	64
wt	464	304	120

Table 3 Standard Deviation Within Genotypes For Specified Cortex Layer Cell Concentrations

Samples	CTIP2	FOXP2	BRN2
hetz	243.8425722	127.1031078	60.06662967
homo	90.50966799	240.4163056	79.19595949
wt	183.869519	280.941275	65.84831053

Table 4 Error Bar For Specified Cortex Layer Cell Concentrations For Each Genotype

Samples	CTIP2	FOXP2	BRN2
hetz	109.0497134	56.84223782	26.86261342
homo	64	170	56
wt	106.1571163	162.2015207	38.01753981

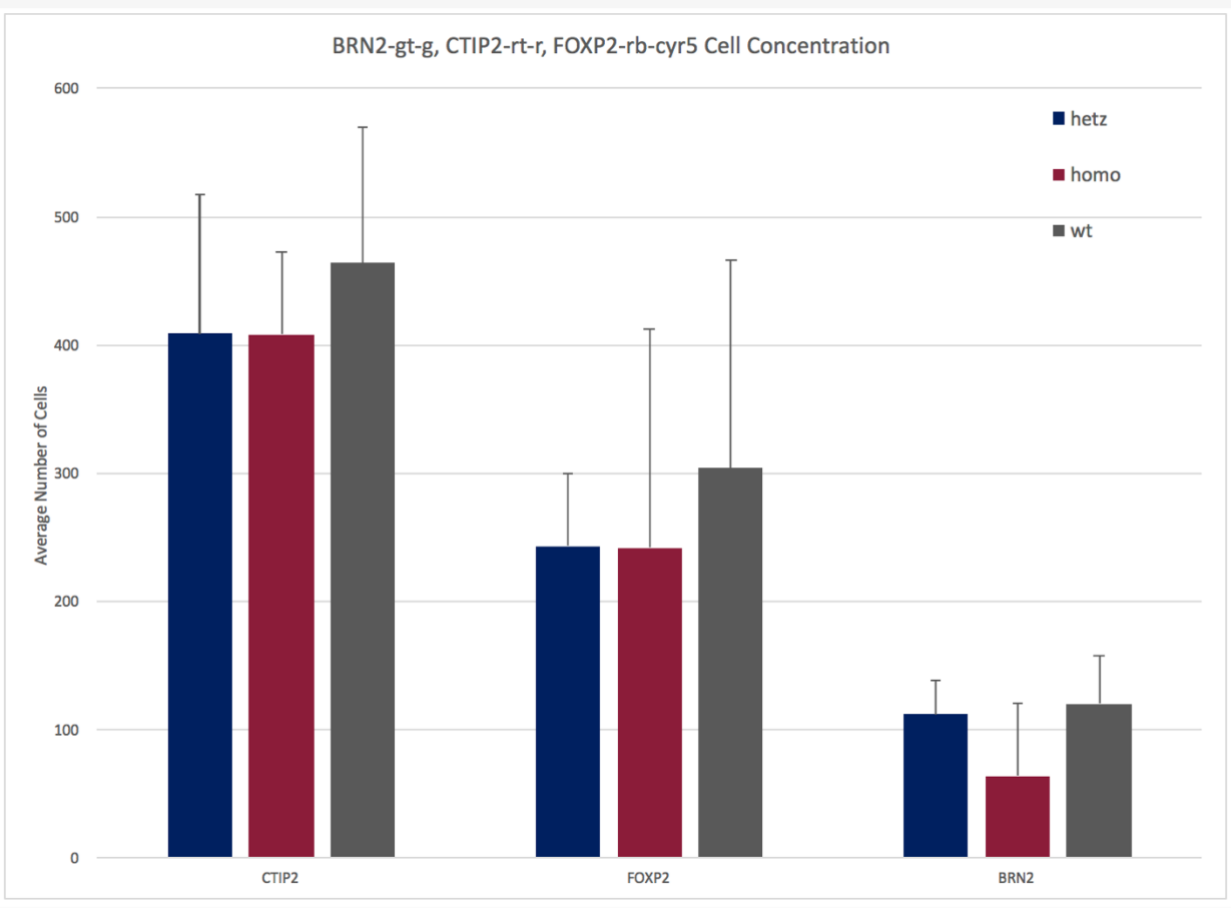

Figure 3 Graph of Different Cortex Layer Cell Concentrations Across Varied Genotypes

Figure 5 showed similar expression distribution of SST and PV cell types across genotypes, though NPY cells showed lower expression across all genotypes. For all cell types, heterozygous mice showed the lowest expression, followed by homozygous and finally wild type with the highest expression. An ANOVA test was again run to determine significance across genotypes for all the cell types. Significance was only found within the NPY cell type. It was determined that wild type expression of NPY was significantly varied from heterozygous expression of NPY, with a p-value of 0.0036 (Table 9). It was also determined that heterozygous

expression of NPY was significantly different from homozygous expression, with a p-value of 0.0239 (Table 9). The wild type expression of NPY was not significantly different than the homozygous expression, however.

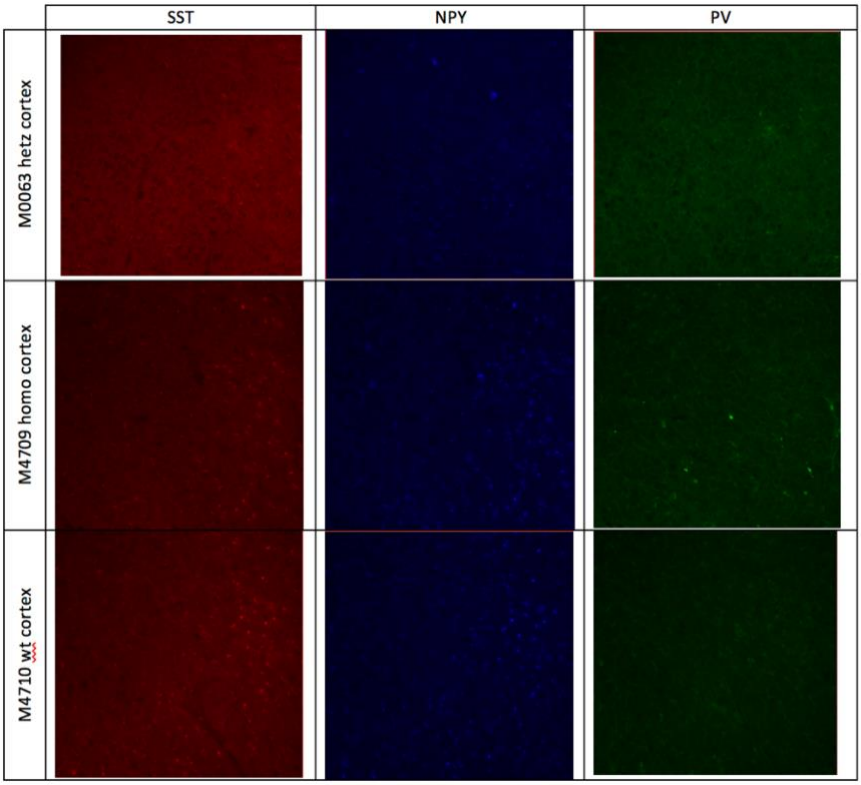

Figure 4 Cell Distribution of Inhibitory Neurons in Male Mice (Hetz, Homo, Wt)

Table 5 Inhibitory Neuronal Cell Concentrations Across Genotypes of Male Mice

Samples	SST	NPY	PV
m0062 hetz cortex	196	100	244
m0063 hetz cortex	186	134	270
m4712 hetz	392	214	312
m6719 hetz	296	240	384
m0025 hetz	192	146	572
m0029 hetz	372	176	476
m4709 homo	504	328	458
m6721 homo	282	282	476
m211 wt	356	340	468
m4710 wt	580	392	588

Table 6 Average Inhibitory Neuron Concentrations For Each Genotype

Samples	SST	NPY	PV
hetz	272.3333333	168.3333333	376.3333333
homo	393	305	467
wt	468	366	528

Table 7 Standard Deviation of Inhibitory Neuron Concentrations For Each Genotype

Samples	SST	NPY	PV
hetz	94.38997122	52.20600221	127.4639818
homo	156.9777054	32.52691193	12.72792206
wt	158.391919	36.76955262	84.85281374

Table 8 Error Bar For Inhibitory Neuron Concentrations For Each Genotype

Samples	SST	NPY	PV
hetz	38.53454439	21.31301115	52.03695268
homo	111	23	9
wt	112	26	60

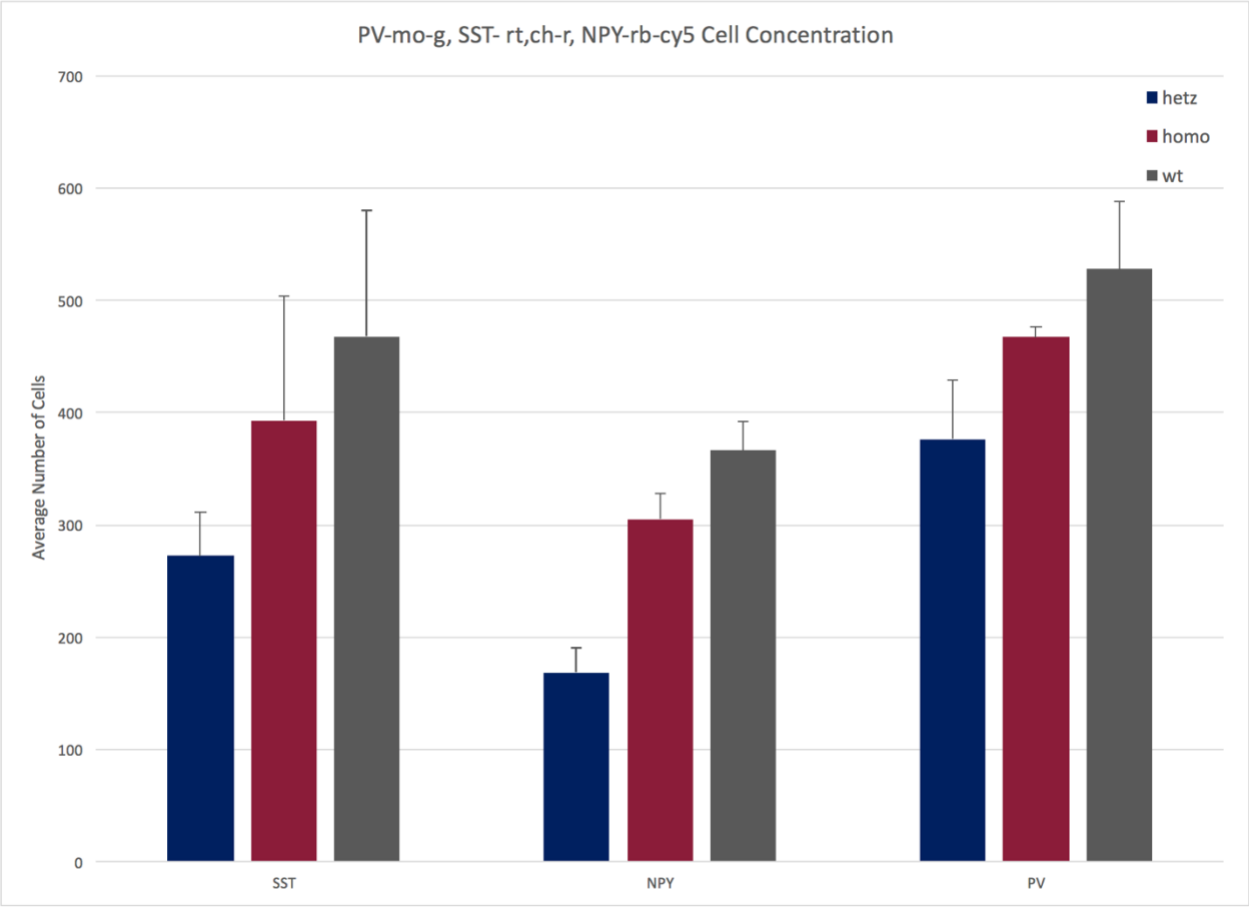

Figure 5 Graph of Different Inhibitory Neuronal Cell Concentrations Across Varied Genotypes

Table 9 ANOVA Test For Significance Between Genotypes For NPY Inhibitory Neurons

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
wt vs. hetz	**197.7**	**82.57 to 312.8**	**Yes**	**	**0.0036**	**A-B**
wt vs. homo	61	-79.96 to 202.0	No	ns	0.4516	A-C
hetz vs. homo	**-136.7**	**-251.8 to -21.57**	**Yes**	*	**0.0239**	**B-C**

Western Blot Data

Western Blots were run and their membranes scanned into Image J to measure the various bands signal intensities for identifying various proteins across genotypes. The intensities were then normalized according to ACTB corresponding signals (Table 10). The average of the wild type signals was taken and all the normalized signals for each genotype were divided by said average. The average of these signals were then taken for each genotype and graphed. The standard deviation and error bar were also calculated and the error bar graphed as well. An ANOVA test was run to determine significance, but not one of the variations across genotypes for any of the cell types was significant, nor was the comparison between cell types. ANOVA was run again using the un-normalized data and still not significance was found between any comparisons, though the p-values did improve. The comparison between wild type and homozygous mice for the signals generated by STAB2 detection were the closest to being significant with a p-value of 0.10.

Table 10 ACTB Signals Utilized For Normalization Of Data

ZNF KO ACTB	
Sample	**Signal**
wt-1	10867.539
wt-2	9004.539
wt-3	8716.882
wt-4	10850.347
hetz-1	9244.347
hetz-2	8280.69
hetz-3	9047.518
hetz-4	9823.518
homo-1	8279.225
homo-2	8373.811
homo-3	8795.933
homo-4	8111.075

The first protein signals calculated were for FUS (Table 11) and the average expressions were graphed for each genotype (Figure 7). No significant differences in FUS protein expression were found across genotypes.

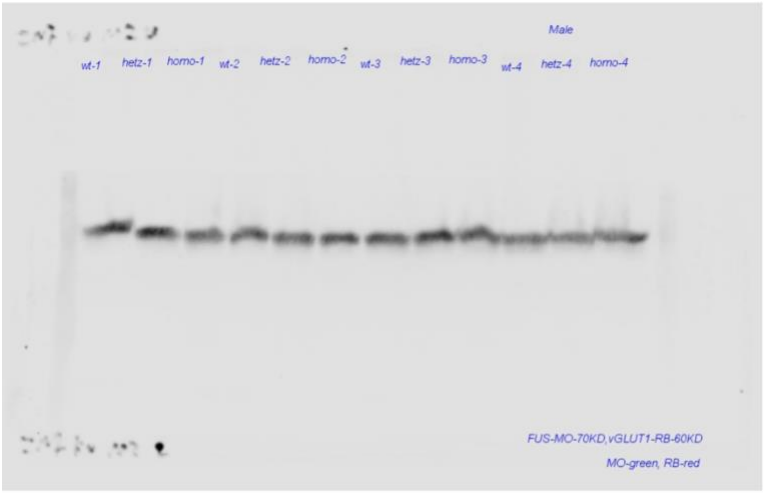

Figure 6 Western Blot ZNF804A KO FUS Signal

Table 11 ZNF KO FUS Expression Across Genotypes

ZNF KO FUS-MO-70KD						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	1.55534045	1.582745349	0.982685213	1	0.132392816	0.066196408
wt-2	1.299721567		0.821181732			
wt-3	1.774632115		1.121236664			
wt-4	1.701287263		1.074896391			
hetz-1	1.945150884		1.228972738	1.08972291	0.135426338	0.067713169
hetz-2	1.559135221		0.985082801			
hetz-3	1.871041311		1.182149303			
hetz-4	1.52368805		0.962686797			
homo-1	1.592280703		1.006024566	1.191685202	0.17705117	0.088525585
homo-2	1.738460442		1.09838291			
homo-3	2.229271822		1.408484204			
homo-4	1.984523876		1.253849129			

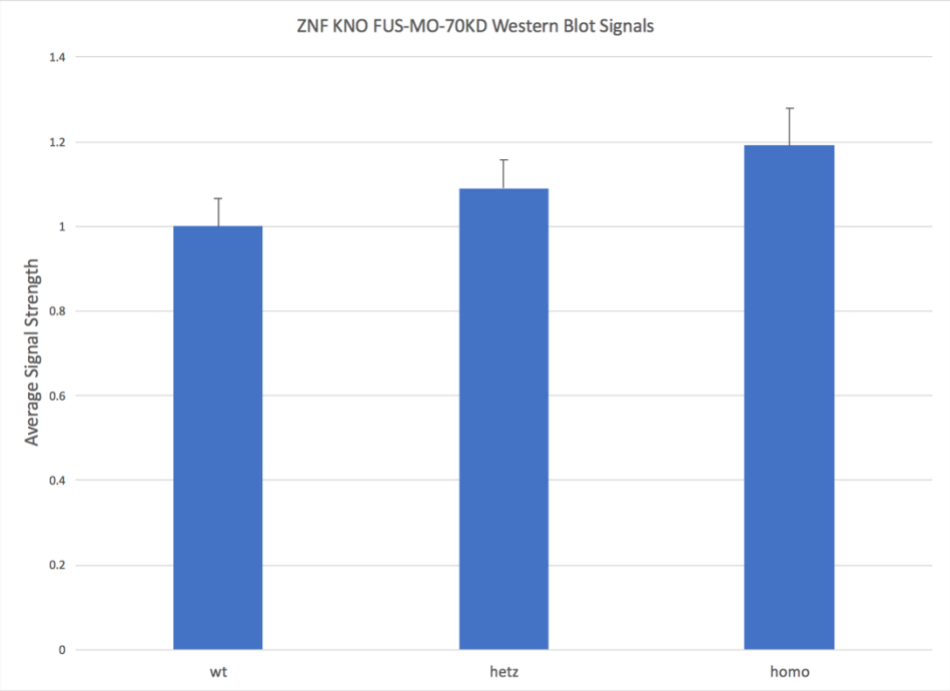

Figure 7 Graph of FUS Average Expression Across Genotypes

The second protein signals calculated were for SATB2 (Table 12) and the average expressions were graphed for each genotype (Figure 9). While there were no significant differences in SATB2 protein expressions across genotypes, the comparison between wild type and homozygous mice for this protein did generate a p-value of 0.10.

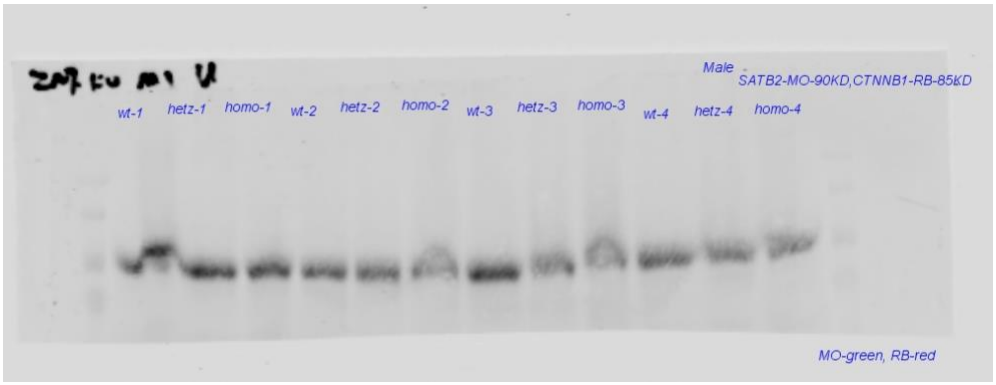

Figure 8 Western Blot ZNF804A KO SATB2 Signal

Table 12 ZNF KO SATB2 Expression Across Genotypes

ZNF KO SATB2-MO-90KD						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	1.711679526	1.56643249	1.092724734	1	0.232540738	0.116270369
wt-2	1.058677294		0.675852487			
wt-3	1.911143808		1.220061394			
wt-4	1.584229331		1.011361384			
hetz-1	1.864670807		1.190393342	0.833716717	0.251890635	0.125945318
hetz-2	1.302300206		0.831379721			
hetz-3	1.007871518		0.64341842			
hetz-4	1.049001283		0.669675386			
homo-1	1.532964769		0.978634431	0.781789571	0.137661747	0.068830873
homo-2	1.167113006		0.745077119			
homo-3	1.168687045		0.746081975			
homo-4	1.029717516		0.657364759			

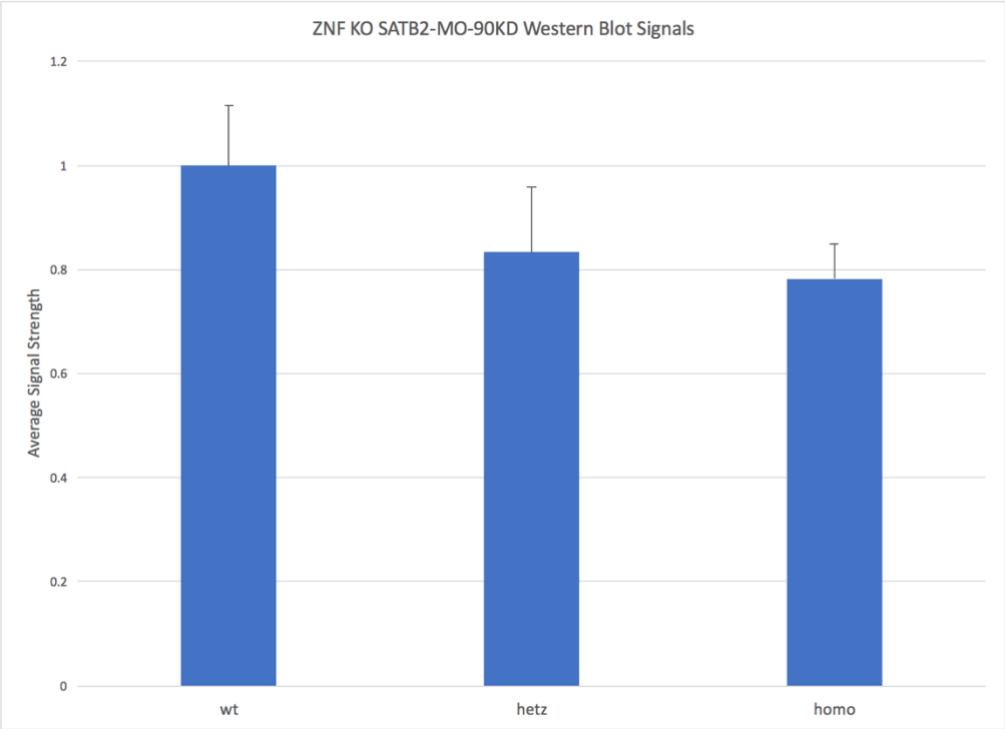

Figure 9 Graph of SATB2 Average Expression Across Genotypes

The third protein signals calculated were for FEZ1 (Table 13) and the average expressions were graphed for each genotype (Figure 11). There were no significant differences in FEZ1 protein expressions across genotypes.

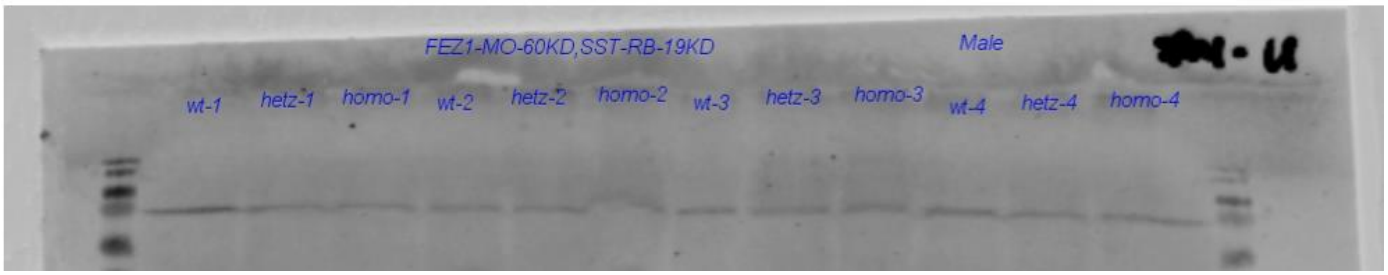

Figure 10 Western Blot ZNF804A KO FEZ1 Signal

Table 13 ZNF KO FEZ1 Expression Across Genotypes

ZNF KO FEZ1-MO-60KD						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	0.502499508	0.354517717	1.417417195	1	0.400505094	0.200252547
wt-2	0.201558807		0.568543679			
wt-3	0.269906399		0.76133402			
wt-4	0.444106154		1.252705106			
hetz-1	0.340362899		0.960073031	0.790007957	0.155505582	0.077752791
hetz-2	0.21146015		0.596472728			
hetz-3	0.26498908		0.747463575			
hetz-4	0.30347514		0.856022494			
homo-1	0.255613647		0.721017977	0.90143276	0.133591461	0.066795731
homo-2	0.312189685		0.880603901			
homo-3	0.359639821		1.014448091			
homo-4	0.350852384		0.989661072			

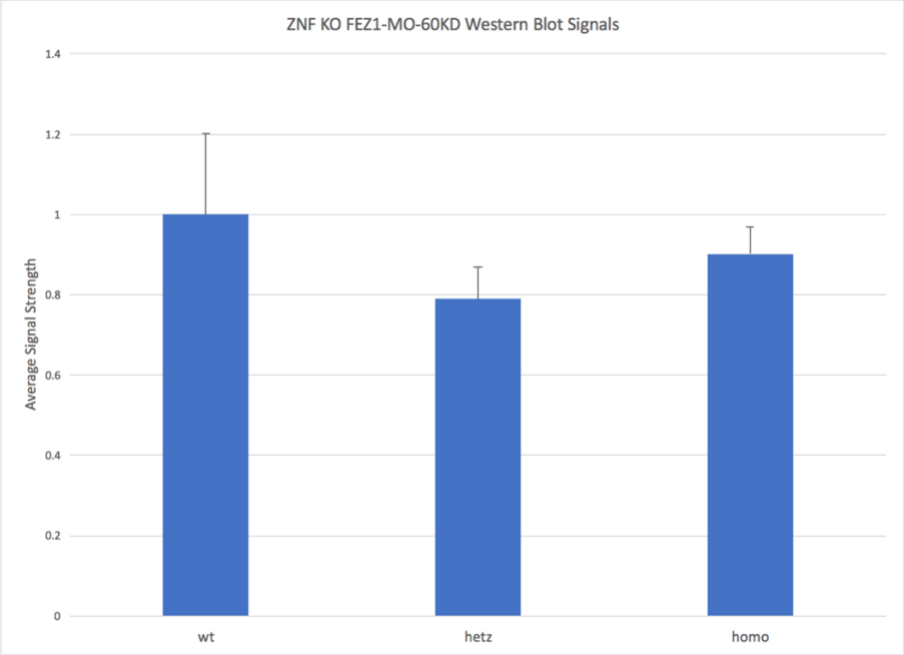

Figure 11 Graph of FEZ1 Average Expression Across Genotypes

The next signals calculated were for the protein CTNNB1 (Table 14) and the average expressions were graphed for each genotype (Figure 13). There were no significant differences in CTNNB1 protein expressions across genotypes.

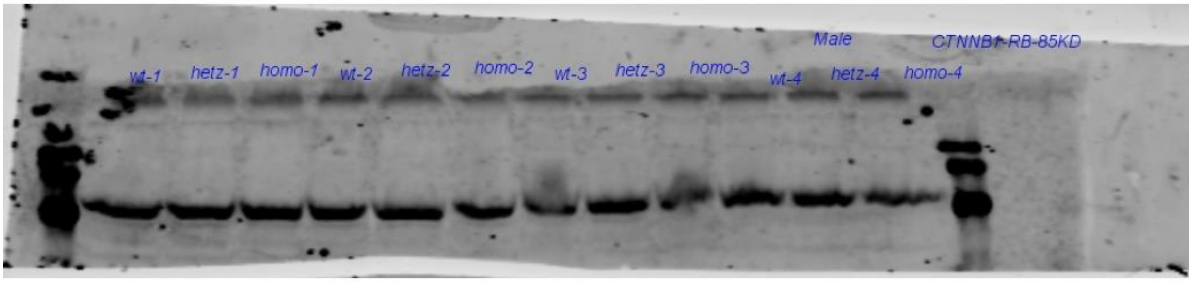

Figure 12 Western Blot ZNF804A KO CTNNB1 Signal

Table 14 ZNF KO CTNNB1 Expression Across Genotypes

ZNF KO CTNNB1-RB-85KD						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	1.269416194	1.258646358	1.008556682	1	0.172950886	0.086475443
wt-2	0.94747744		0.752774943			
wt-3	1.401878615		1.11379865			
wt-4	1.415813182		1.124869725			
hetz-1	1.522881294		1.209935804	1.044996707	0.119697505	0.059848752
hetz-2	1.278267897		1.015589398			
hetz-3	1.162627788		0.923712829			
hetz-4	1.297348218		1.030748796			
homo-1	1.439216454		1.143463726	1.044199454	0.083257087	0.041628544
homo-2	1.237750719		0.983398324			
homo-3	1.361855488		1.082000102			
homo-4	1.218288698		0.967935664			

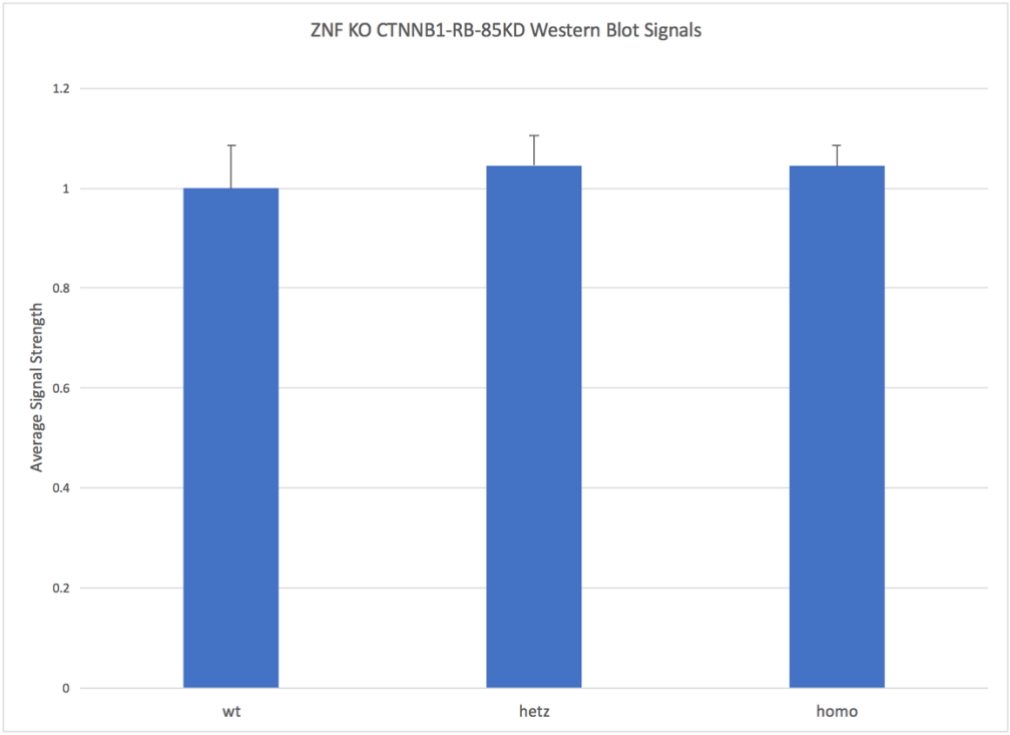

Figure 13 Graph of CTNNB1 Average Expression Across Genotypes

The next signals calculated were for the protein SST (Table 15) and the average expressions were graphed for each genotype (Figure 15). There were no significant differences in SST protein expressions across genotypes.

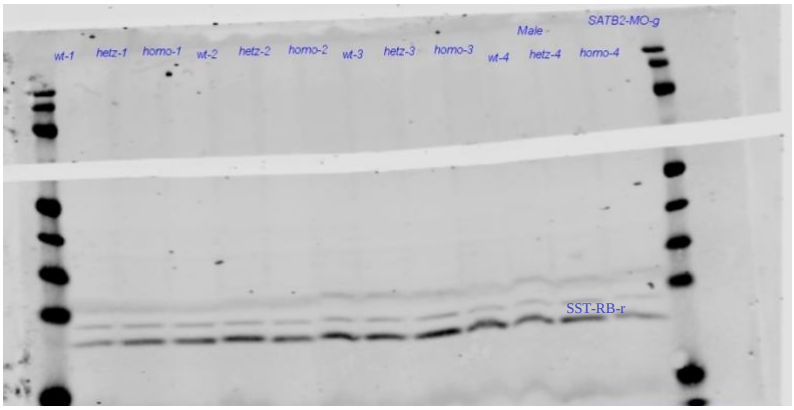

Figure 14 Western Blot ZNF804A KO SST Signal

Table 15 ZNF KO SST Expression Across Genotypes

ZNF KO SST-RB-r						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	0.232091829	0.676643789	0.343004447	1	0.452835145	0.226417572
wt-2	0.811229079		1.198901242			
wt-3	0.92617456		1.368777154			
wt-4	0.737079688		1.089317157			
hetz-1	0.615659613		0.909872556	1.201076388	0.323982956	0.161991478
hetz-2	0.709646338		1.04877389			
hetz-3	1.120386607		1.655799736			
hetz-4	0.805110953		1.189859371			
homo-1	0.762669037		1.127135207	1.241610198	0.396997887	0.198498944
homo-2	1.05661847		1.561557924			
homo-3	1.048052928		1.548899059			
homo-4	0.493170881		0.728848604			

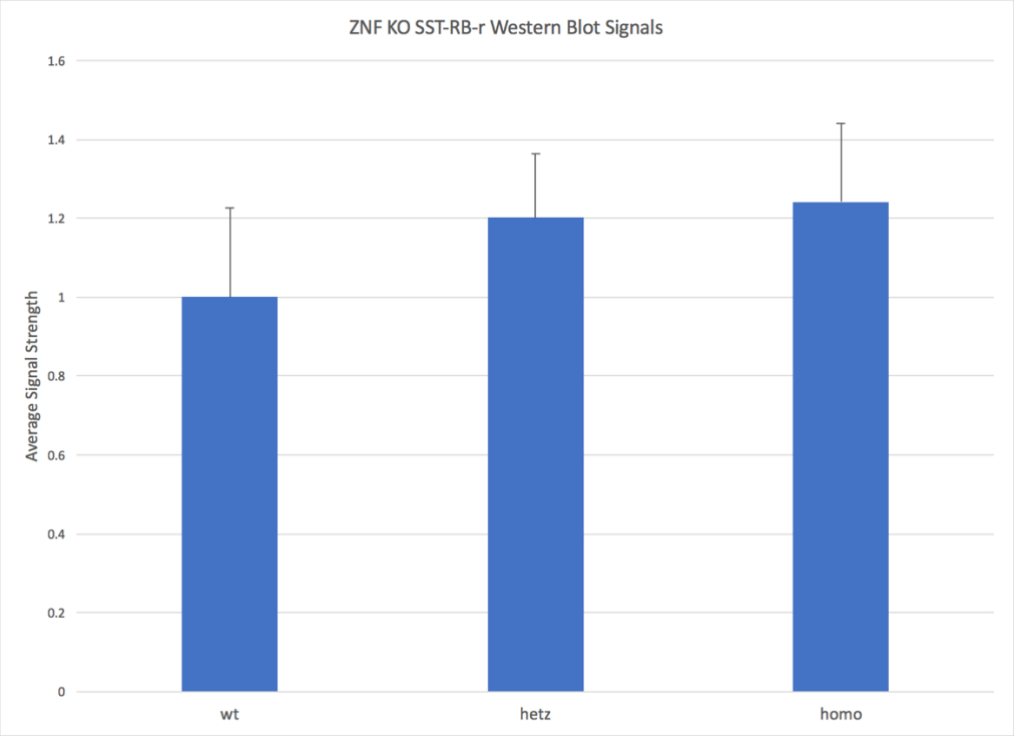

Figure 15 Graph of SST Average Expression Across Genotypes

Finally, the signal intensities were calculated for PV (Table 16) and the average expressions were graphed for each genotype (Figure 17). There were no significant differences in PV expressions across genotypes.

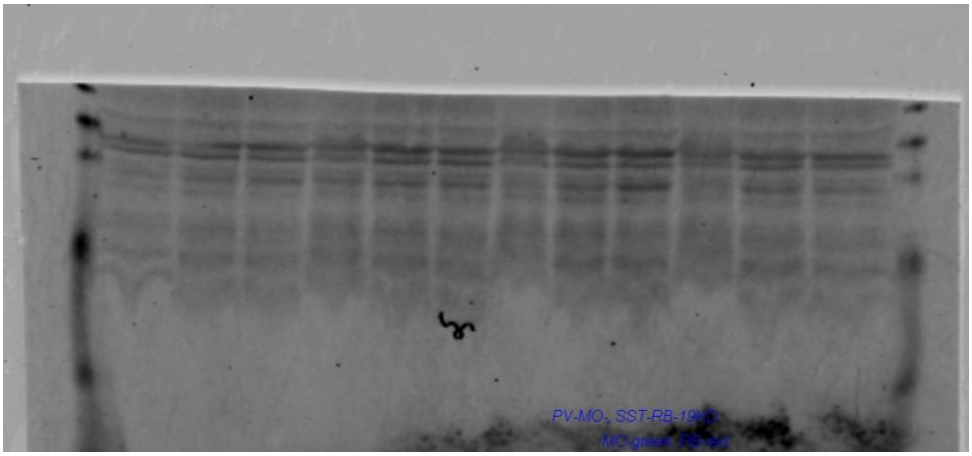

Figure 16 Western Blot ZNF804A KO PV Signal

ZNF KO PV-MO						
Sample	Signal/ACTB	Average Signal of Four WT	(Signal/ACTB)/ Average WT Signal	Average of Previous Col. Per Genotype	Standard Deviation Per Genotype	Error Bar Per Genotype
wt-1	0.644322141	0.878220407	0.733667922	1	0.218463084	0.109231542
wt-2	0.804121288		0.915625828			
wt-3	0.994808853		1.132755336			
wt-4	1.069629348		1.217950914			
hetz-1	0.883367488		1.005860808	1.192015962	0.166116898	0.083058449
hetz-2	1.237713491		1.409342667			
hetz-3	1.045800395		1.190817687			
hetz-4	1.020529602		1.162042687			
homo-1	1.155596233		1.31583851	1.296647227	0.121446234	0.060723117
homo-2	1.070678047		1.219145033			
homo-3	1.282454698		1.460287973			
homo-4	1.046239247		1.191317394			

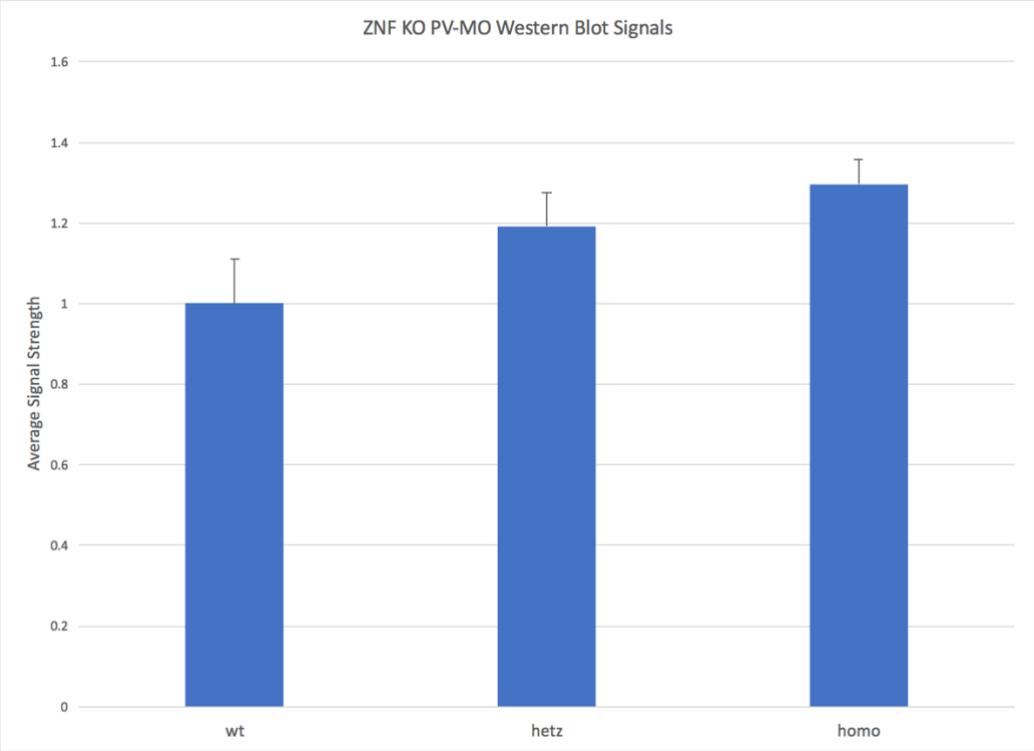

Figure 17 Graph of PV Average Expression Across Genotypes

Chapter 4

Discussion

Schizophrenia is a neurodevelopmental disorder that shows chronic, severe psychiatric symptoms. There is currently very little effective treatment available without harmful side effects. As such, research is needed to identify mechanism and changes within the brain that result in the development of this disorder. A vast number of studies have backed the link between schizophrenia and the gene ZNF804A. This research looked to determine the difference in protein and cell concentrations between the wild type expression of ZNF804A and knockout of ZNF804A expression (heterozygous and homozygous).

The immunostaining protocol was utilized to analyze the difference in cell expression across cortex layers (identified by CTIP2, FOXP2, and BRN2) and the difference in expression of inhibitory neurons (identified by SST, NPY, PV). CTIP2 and FOXP2 are both deep cortical layer markers, while BRN2 is an upper cortical layer marker.

The data illustrated above shows a greater concentration of deep cortical layer marker expression, both through CTIP2 (COUP-TF-interacting protein2) and FOXP2 (Forkhead box protein P2), as compared to the upper cortical layer expression, through BRN2 (POU Class 3 Homeobox 2). There was no significant difference in cortical layer marker expression across the genotypes. However, large error bars were also present and could explain the lack of differentiation between genotypes. Previous research has identified FOXP2 as a critical protein in the development of speech and language. When decreasing the production of this protein, impairments in language occur (French et al., 2007). One could argue, therefore, that ZNF804A KO mice, whom are prone to schizophrenia, should exhibit a decrease in FOXP2 compared to

wild type because of the speech impairments, such as speaking in tongues, associated with the hallucinations of schizophrenia. However, it is not yet known whether these vocal hallucinations are related to FOXP2. CTIP2, on the other hand, regulates the proliferation of epidermal tissue and development and commitment of T cells (Zhang, Bhattacharya, Leid, Ganguli-Indra, & Indra, 2012). As such, it is important to consider these cells when studying schizophrenia as the immune function of many with schizophrenia is decreased (Tiihonen, Tanskanen, & Taipale, 2018). However, the data presented here does not support that CTIP2 plays a critical role in that immune system malfunction. BRN2, the upper cortex layer marker of the cortex, plays a role in neurogenesis, and molecular differentiation and migration (Dominguez, Ayoub, & Rakic, 2013). Resultantly, since this protein is essential for neural growth and migration, the presence of a neurodevelopmental, psychiatric disorder such as schizophrenia, should show an alteration in this proteins expression. The fact that it does not in this study is interesting and requires further testing.

The second set of immunostaining data illustrates the distribution of inhibitory neuron expression, SST (neuropeptide somatostatin), PV (parvalbumin), and NPY (neuropeptide Y), across genotypes. SST and PV showed similar expression of inhibitory neuron concentration for each genotype; NPY however, showed a reduced concentration across all genotypes. All three neuron types had the greatest expression under wild type conditions and the least expression under heterozygous conditions. This difference in expression across genotypes was significant among NPY neurons. This research illustrated that wild type expression of NPY was significantly greater than heterozygous expression of NPY, with a p-value of 0.0036. It was also determined that heterozygous expression of NPY was significantly lower than homozygous expression, with a p-value of 0.0239. This corresponds with previous research that NPY

expression is reduced in schizophrenic patients. NPY has been reported to be essential in coping with stress and anxiety to maintain emotional homeostasis. When NPY levels are reduced, psychiatric states are frequently met (Eaton, Sallee, & Sah, 2007). As such, the findings in this study that NPY is reduced in the presence of a knockout of ZNF804A, makes sense as both correlate to schizophrenic symptoms. The findings that are not consistent with previous research are those of PV and SST expression levels in the presence of ZNF804A KO. PV inhibitory neurons are strong regulators of cortical circuitry and cortical gamma oscillation (Inan, Petros, & Anderson, 2013). Resultantly, previous research has noted PV dysfunction as an essential factor in schizophrenia and the cognitive symptoms that come along with it as a decrease in PV results in abnormal oscillations. Similarly, previous studies have reported a decrease in SST expression in patients with schizophrenia, though the precise functional consequences of the decrease in SST remains unclear (Inan, Petros, & Anderson, 2013). Given the previous research, it would have been expected that PV and SST would have seen a more significant reduction in the presence of ZNF804A KO. Though the lack of evidence of this could be the result of numerous factors influencing schizophrenia and therefore PV and SST happen to not be impacted by ZNF804A, though more tests need to be done to determine this and exclude contamination errors as the influencing factor in these results.

Next, the western blot protocol was used to analyze the difference in protein expression across genotypes. The proteins analyzed were FUS (RNA-binding protein fused in sarcoma), SATB2 (special AT-rich sequence-binding protein), FEZ1 (fasciculation and elongation protein zeta 1), CTNNB1 (catenin beta-1), and both SST (neuropeptide somatostatin), and PV (parvalbumin) again. There was no significant difference found for any of the protein across genotypes. As mentioned above, this does not correspond with previous research for PV and SST

(for the same reasons mentioned previously). In addition, research by Zucchi and Ticozzi has linked mutations in the FUS protein to the presence of psychosis, schizophrenia and ALS (Zucchi, Ticozzi, & Mandrioli, 2019). This is not exhibited here as ZNF804A KO FUS levels do not vary significantly from the wild type levels. Similarly, previous research supports the theory that a reduction in SATB2, a transcription factor that assists in chromatin modification within neurons, results in the cognitive and behavioral problems seen in schizophrenia; though that is not shown in the data generated here either (Gigek et. al, 2015). Likewise, schizophrenia symptoms, as well as other psychiatric diseases, have been linked to FEZ1 deficiencies that attenuate the development of neurons and oligodendroglia cells alike (Chen et al., 2017). This is not exhibited by the findings in this study, as the knockout of the schizophrenic risk gene ZNF804A does not show a significant reduction in FEZ1 production. However, this could again be due to the fact that there are many contributing components to schizophrenia's etiology and some may not act in the presence of others. Lastly, CTNNB1 was examined in this study and again showed no significant changes across genotypes. Previous studies, however, have linked an increase in CTNNB1 mRNA expression to schizophrenia (Guo et al., 2019).

The differences between this study and previous ones, as mentioned, could be due to the complex multi-factor etiology of schizophrenia. There is little research into all the risk factors and mechanisms correlated to this disorder, and even less research into their interactions with one another. As such, it is hard to say whether these findings are completely due to multi-factoral etiological circumstances or whether there was contamination and over saturation of antibodies used. More studies and tests must be done to determine this. However, the overall conclusion that can be drawn from this study is that the development of schizophrenia is quite complex.

This study had some limitations that open the door for future research. For instance, this research only examined male mice within their mature stage. Other studies should be done to compare sex and age variations. In addition, many symptoms in mice for autism and schizophrenia overlap; as do many of the molecular mechanisms by which the two disorders develop. As such, future studies should consider autism as well and compare the variations in protein and cell expression levels to help determine where the divergence is in development between the two disorders.

In conclusion, this study found no significant changes in protein or neuron expression between genotypes, with the exception of NYP. Both this research and previous studies support the findings that NPY reduced expression is correlated with schizophrenia. More specifically, KO of ZNF804a (a schizophrenia risk gene) results in this reduction of NPY expression. Further studies must be done to determine the interactions of the other proteins and cell expressions, examined in this study, in relation to schizophrenia and ZNF804A KO.

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ACADEMIC VITA

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EDUCATION

Bachelor of Science: Biological Neuroscience (Major)
Penn State University 2019
University Park, Pennsylvania

Bachelor of Science: Psychological Neuroscience (Major)
Penn State University 2019

Neuroscience (Minor)
Penn State University 2019

Transfer Credits
Lehigh University 2015 (8 Credits Obtained)
Bethlehem, Pennsylvania

High School Diploma
Saucon Valley High School 2015
Hellertown, Pennsylvania

RELEVANT COURSE WORK

Honors Biology: Basic Concepts and Biodiversity (Lab)
Honors Biology: Populations and Communities (Lab)
Honors Biology: Molecules and Cells (Lab)
Honors Biology: Function and Development Of Organisms (Lab)
Sociobiology
Chemistry I and II (Lab)
Organic Chemistry I and II
Statistic
Neurological Bases of Human Behavior
Calculus with Analytic Geometry I and II
Physics (Lab)
Intro Psychology
Biobehavioral Health
Abnormal Psychology 270 and 470
Independent Biological Studies Under Dr. Mao (3.5 years)
General Biochemistry 401 and 402
Clinical Neuropsychology
Physiological Psychology
Neurobiology
Functional Neuroscience
Neurodiseases

LABORATORY SKILLS

Microscopy/Fluorescence Microscopy
PCR
UV Spectrophotometry
DNA Extraction/Isolation
Pipetting
Gel Electrophoresis
Heat Shock/Mating/Brooding
Mega and Homed Edit 9
Genetic Screening
Thorough Understanding of Karst Gene
Buffer Preparation
Brain Dissection
Brain Slicing
Western Blot
Immunofluorescence
Staining
Cell Counting
Genotyping
Cell Lysis
Mini and Maxi Prep
uDISCO Clearing

Larval Dissection
Cell Amplification
Plate Preparation and Purification
Cell Passaging
Viral Cell Collection
Bone Marrow Extraction
Plaque Picking
ELISA
Perfusion

SUMMARY OF FIELD QUALIFICATIONS

- Familiar with fundamental lab procedures including those listed above
- Have worked with Komodo, MEGA, and Blast Search
- Ability to write detailed and concise lab reports
- Very analytical and highly motivated; ability to multitask, attentive to details
- Capable of working alone and in groups
- Excellent communication skills; dedicated to high-quality work
- Mac skills: Word, Excel, PowerPoint
- Fluent in English and some ability to understand and read in Spanish

SCHOLARSHIPS/HONORS/AWARDS

Schreyer Honors College (August 2015- December 2019)
PSU Dean's List (Spring 2016)
Saucon Valley High School Salutatorian (June 2015)
Bethlehem Apparatus Company Scholarship Award for Research (June 2015)
Lions All-Star Soccer Classic Scholarship for Academic and Athletic Achievement (June 2015)
United States Marine Corps Scholastic Excellence Award for Academic Achievement and Leadership As A Scholar (June 2015)
The United States Army Reserve National Scholar/Athlete Award for Academic and Athletic Excellence (May 2015)
Saucon Valley High School Nominee for Colonial League Academic All-Star (January 2015)
National Honors Society (2012-2015)
Sauce Valley Girls Soccer Scholar Athlete (November 2014)

PREVIOUS JOBS/EXPERIENCE

Internship as Research Assistant

Memorial Sloan Kettering Cancer Center/ June 2017 - July 2017

New York, New York

I carried out two long-term projects surrounding treatment for melanoma in mice models. I performed all the related experiments, analyzed all the data, and presented my findings to the head of the lab at the completion of my internship. Specifics on my research are classified. Through this internship, I developed a variety of lab skills, as well as enhanced my ability to effectively communicate my findings.

Hostess

Melt/ June 2016- August 2016
Center Valley, Pennsylvania

Nanny

Private Employer/ May 2015-August 2015
Bethlehem, Pennsylvania

Mentorship

St. Luke's Neurology/ January 2015- June 2015
Bethlehem, Pennsylvania
Shadowed a variety of doctors and physician assistants throughout a range of neurological fields in the hospital. I witnessed and learned about the treatment and examinations surrounding brain tumors, epilepsy, headaches, memory disorders (specifically Alzheimer's disease), multiple sclerosis, peripheral neuropathy, Parkinson's disease, strokes and concussions. I had the opportunity to work both behind the scenes with doctors examining MRIS, CTS, and EEGS, as well as working face-to-face with patients as they were examined, communicated with about their options and situation, and even receiving treatment sometimes. This experience also taught me more about the complexity of the human anatomy and how to interact with patients.

CLUBS AND ORGANIZATIONS SINCE 2015

NAMI Lehigh Valley (May 2019-present)
Suicide Prevention Task Force Northampton County (May 2019-present)
Active Minds (Aug. 2016-present)
Polish Club (Aug. 2016-present)
Science Lion Pride/ Family Relations Chair (Aug. 2015-present)
Blue and White Society (Aug. 2015-present)
THON Rules and Regulations Committee (Aug. 2018-April 2019)
IM Soccer (Aug. 2015-December 2018)
Springfield (Aug. 2016-May 2018)
Global Brigades (Aug. 2015-May 2018)
Apollo (Aug. 2015- May 2016)