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CANDIDATE GENE STUDIES IN PATIENTS WITH AUTISM SPECTRUM DISORDER

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CANDIDATE GENE STUDIES IN PATIENTS WITH AUTISM SPECTRUM DISORDER

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ABSTRACT

Autism Spectrum Disorder is a grouping of disorders that range from the diagnosis of Asperger Syndrome to Autistic Disorder (formally known as autism). Attention Deficit Disorders and Pervasive Developmental Disorder-Not Otherwise Specified are also a part of this spectrum of disorders. Autism Spectrum Disorder affects one out of every 110 children and has a male to female ratio of 4:1. This has led to the need to identify genes that may be causative for this disorder.

Several genome-wide scans have been conducted and have identified locations in the human genome that may contain causative genes for Autism Spectrum Disorder. One such area was found to be located at chromosome 7q and included the *MET* gene. Campbell et al. (2006) identified the rs1858830 C variant in the MET gene and a study found it to be associated with ASD. Screening of the rs1858830 C variant in the *MET* gene was conducted in a unique population comprised of individuals diagnosed with Autistic Disorder and controls. Results indicated that this variant was associated with Autistic Disorder.

The high male to female ratio of individuals affected with Autism Spectrum Disorder raises the possibility that genes on the X chromosome may be involved in these disorders. Alterations in *NLGN4X*, which is involved in neuronal synapse formation and located at Xq22.2, have previously been identified that are associated with Autism Spectrum Disorder. We screened a cohort of individuals diagnosed with Autistic Disorder for any alterations in *NLGN4X*. We identified two alterations in *NLGN4X* within this cohort. Further studies were conducted to observe cell morphology after

transfection with the altered NLGN4X proteins in PC12 cells, a rat pheochromocytoma cell line. Fluorescence microscopy determined that neuronal cells expressing the two altered forms of *NLGN4X* had altered cell morphology. Cellular localization studies also identified a difference between the location of the altered NLGN4X proteins within the cell and the location of wild type NLGN4X.

To further identify causative genes for Autism Spectrum Disorder, the translocation breakpoint region at chromosome 2q21 of a patient with a chromosomal translocation t(2;16) and autistic like characteristics was partially mapped. The breakpoint at chromosome 2q was narrowed to a region of only 80kb in size and one candidate Autism Spectrum Disorder gene within this region was identified.

The understanding and identification of Autism Spectrum Disorder causative genes is very important given the large number of individuals that are affected. The wide range of phenotypes associated with this disorder implies the involvement of many genes that lead to different phenotypes. The studies within this dissertation determined that Autistic Disorder is associated with an alteration in the *MET* gene, identified two alterations, one being *de novo,* in *NLGN4X* that are associated with Autistic Disorder, and provided cellular studies of the *NLGN4X* alterations in a neuronal cell line. Additionally, this research revealed candidate genes located at chromosome 2q21 that may be associated with Autism Spectrum Disorder.

DEDICATION

My parents, Ralph and Vickie Boyter, and family, Brooks and Brissey Jackson, have been an excellent resource of support and guidance, and this work would not have been possible without them. I am very grateful for everything my family has provided and sacrificed during my time completing this work. This work is dedicated to Ralph and Vickie Boyter and Brooks and Brissey Jackson.

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TABLE OF CONTENTS

Page

TITLE PAGE i

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

BACKGROUND 1

CHAPTER

I. FURTHER EVIDENCE THAT THE rs1858830 C VARIANT IN THE PROMOTER REGION OF THE *MET* GENE IS ASSOCIATED WITH AUTISTIC DISORDER 9

Introduction 9
Materials and Methods 11
Results 13
Discussion 17

II. CELLULAR LOCALIZATION AND FUNCTIONAL ANALYSIS OF TWO ALTERATIONS IDENTIFIED IN *NLGN4X* 21

Introduction 21
Materials and Methods 22
Results 35
Discussion 47

III. IDENTIFICATION OF CANDIDATE GENES FOR AUTISM SPECTRUM DISORDER IN A PATIENT WITH A t(2;16)(q21q13) TRANSLOCATION 51

Introduction 51
Materials and Methods 52

Page

Results....58
Discussion....64

CONCLUSIONS....67

APPENDIX A....70

REFERENCES CITED....71

LIST OF TABLES

Table		Page
1.1	*MET* rs1858830 allele genotypes	15
1.2	*MET* rs1858830 allele genotypes by type of family	15
1.3	*MET* rs1858830 allele frequencies	16
1.4	*MET* rs1858830 allele genotypes by racial group in the South Carolina Population	18
2.1	*NLGN4X* primer list	25
2.2	*NLNG4X* alterations identified in the mutation screen	35
2.3	*NLGN4X* protein prediction	38
3.1	Translocation primer list	57

LIST OF FIGURES

Figure		Page
B1.1	Schematic representation of *NLGN4X* functioning at the postsynaptic neuron	6
1.1	*MET* PCR amplification and *Eag I* endonuclease digestion	14
2.1	Sequence identification of *NLGN4X* alterations and normal *NLGN4X*	36
2.2	Family analysis of the c. 865 G>A alteration identified in *NLGN4X*	37
2.3	NLGN4X western analysis	40
2.4	Cellular localization and function of NLGN4X transfected PC-12 cells	41
2.5	Cellular localization and function of NLGN4X transfected PC-12 cells	42
2.6	Average neurite length of NLGN4X transfected cells	43
2.7	GS28 cellular localization in NLGN4X transfected PC-12 cells	44
2.8	NLGN4X cell fractionation	46
2.9	NLGN4X cell fractionation of c. 775 T>C	47
3.1	FISH analysis of chromosome 2 breakpoints involved in a t(2;16)(q21;q13) translocation	59
3.2	Agilent Human Genome CGH microarray analysis	60
3.3	Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 microarray analysis	61
3.4	Quantitative RT-PCR	62
3.5	PCR amplification of FISH probes	63
3.6	Schematic representation of the location of FISH probes and genes	64

Background

Autism was first identified and formally described in 1943 by Dr. L. Kanner from a study of eleven children that he diagnosed with the syndrome (Kanner, 1943). A year later Dr. Kanner published an additional paper and identified the syndrome as *infantile autism*, indicating that the syndrome begins even in early infancy (Kanner, 1944). The terms *infantile autism*, *childhood autism*, and *autistic child* were utilized until fairly recently to identify an individual with the disorder (Ornitz, 1973). Hans Asperger, in 1944, was conducting research in child psychiatric disorders and noted four individuals that displayed focused and unusual behaviors (Asperger, 1944). He predicted that these individuals would later in life be very successful in their area of focus. Asperger diagnosed these individuals as having *autistic psychopathy*, which would later be identified as Asperger syndrome (Asperger, 1944). Asperger syndrome is currently known as high functioning autism (American Psychiatric Association, 1994). The identification and description of these syndromes led to the formation of the current three subgroups for autism: Autistic Disorder, Asperger syndrome, and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) (American Psychiatric Association, 1994). All three of these subgroups fall in the Autism Spectrum Disorder classification.

Autism Spectrum Disorder has a range of phenotypes that includes mental retardation, developmental delays, impairments in communication and social skills, and abnormal behaviors and interests (Folstein and Mankoski, 2000; Lamb et al., 2005;

Muhle, Trentacoste, and Rapin, 2004; Rutter, 2005; Veenstra-VanderWeele and Cook, 2004). The diagnosis of one of the three subgroups in Autism Spectrum Disorder is determined based upon social interaction, language utilized during social interaction, and restricted patterns of interest. The diagnosis of Autistic Disorder is given to an individual that has abnormalities in all three of these categories occurring before the age of three. The diagnosis of Asperger syndrome or PDD-NOS may be given if criteria are not met for a diagnosis of Autistic Disorder (Centers for Disease Control and Prevention, 2009). Autism Spectrum Disorder has a prevalence of one in 110 children, while Autistic Disorder has a prevalence of one in 500 children (Centers for Disease Control and Prevention, 2009; Fombonne, 1999; Herba, et al., 2008; Yeargin-Allsop et al., 2003). The male to female ratio of the individuals diagnosed with Autistic Disorder is 4:1 (Fombonne, 1999; Ylisaukko-oja et al., 2005). The high prevalence of Autism Spectrum Disorder and the early age of onset have driven the desire to identify causative genes for this spectrum of disorders.

Genome-wide linkage scans were used to identify locations in the human genome that may contain causative genes for Autism Spectrum Disorder. Genome-wide association studies have identified chromosomal regions 2q, 4, 7q, 15q, 16p, 17q, 19, and 22q as putative locations for Autism Spectrum Disorder genes (Ashley-Koch et al., 1999; Barrett et al., 1999; Folstein and Rosen-Sheidley, 2001; Gutknecht et al., 2001; Herba et al., 2008; International Molecular Genetic Study of Autism Consortium, 1998; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al., 2006; Trikalinos et al., 2006; Vincent et al., 2000).

Previous studies identified alterations in the *MET* gene, located at chromosome 7q31, that were associated with Autism Spectrum Disorder (Campbell et al., 2006). The *MET* gene is unique because it is involved not only in the metastasis of a variety of cancers, peripheral organ development and repair, and immune function and gastrointestinal repair, but it may contribute to the development of the cerebral cortex and cerebrum (Blume-Jensen and Hunter, 2001; Campbell et al., 2007; Gherdi et al., 2003; Ierci, Forni, and Ponzetto, 2002; Park et al., 1987; Powell, Mars, and Levitt, 2001; Powell et al., 2003; Tahara et al., 2003). Since MET is involved in brain development, the rs1858830 C variant may contribute to the cognitive dysfunction identified in ASD patients. Previous studies in mice demonstrated that *MET* contributes to brain development (Campbell et al., 2007; Powell, Mars, and Levitt, 2001; Powell et al., 2003; Levitt, 2005; Ierci et al., 2002; Gutierrez et al., 2004; Tyndall et al., 2007; Tyndall et al., 2006). This is a result of *MET* involvement in neuronal migration, dendritic arborization, and synapse formation in the developing brain (Campbell et al., 2007). Studies have observed reduced signaling of MET in the brains of mice resulting in a reduction of migration and differentiation of neocortical and hippocampal interneurons. The mice with these abnormalities had seizures, increased anxiety, and abnormal social behavior (Campbell et al., 2007; Powell, Mars, and Levitt, 2001; Levitt, 2005; Powell et al., 2003).

The reduction of MET expression also leads to a decrease in granule cells and a smaller cerebellum (Ierci, Forni, and Ponzetto, 2002). Granule cells are very small, but numerous, neurons that are found in the cerebellum, hippocampus, and olfactory bulb, that are responsible for receiving sensory information (Gilbert, 2003). The granule cells

of the cerebellum account for half the neurons in the entire central nervous system (Gilbert, 2003). A reduction in the size of the cerebellum is a classic feature in the brains of individuals affected with Autism Spectrum Disorder (Bauman and Kemper, 2005). The cerebellum is a part of the brain that is responsible for coordinating movement, long term memory, and olfaction (Gilbert, 2003). Based upon known functions of the hippocampus and cerebellum, and autistic phenotypes resulting from abnormalities of the cerebellum and hippocampus that have been previously described, it is very likely that alterations of the *MET* gene may lead to Autism Spectrum Disorder.

The role the *MET* gene plays in gastrointestinal repair is relevant because of the presence of gastrointestinal problems in individuals with Autism Spectrum Disorder (Goodwin, Cowen, and Goodwin, 1971; Horvath et al., 1999; Jyonouchi et al., 2005; Wakefield et al., 2000; Wakefield et al., 1998). However, the alterations in *MET* would not account for all gastrointestinal problems. Individuals with Autism Spectrum Disorder have a difficult time functioning in society and difficulty communicating their needs or problems, and this could result in gastrointestinal problems being untreated and reducing the individual's ability to interact with society. Further, the identification of alterations in this gene may indicate the need to test an individual with Autism Spectrum Disorder for gastrointestinal problems.

The 4:1 male to female ratio observed in Autism Spectrum Disorder implies the possibility that there are genes residing on the X chromosome that cause Autism Spectrum Disorder (Centers for Disease Control and Prevention, 2009; Ylisaukko-oja et al., 2005). Specifically, alterations of *NLGN4X* at chromosome Xp22.3 have been found

to be associated with Autism Spectrum Disorder (Jamain et al., 2003). *NLGN4X* is involved in the production of cell-adhesion molecules that are important for the formation and organization of functional synapses (Talebizadeh et al., 2004). The synapse is the specialized junction between the neurite (or axon) of a neuron and its target. The synapse is established as neurites extend from presynaptic neurons and grow until they reach their specific postsynaptic partner(s) (Sanes and Yamagata, 1999). The neurite target or postsynaptic partner may either be another neurite or muscle (Gilbert, 2003). At the synapse, the neurotransmitter or chemical message is released and functions to depolarize or hyperpolarize the membrane of the target cell (Gilbert, 2003). The stability of synapses is achieved through trans-synaptic adhesion complexes that link the presynaptic and postsynaptic domains (Fannon and Colman, 1996; Yamagata et al., 2003).

NLGN4X is a member of the neuroligin family of proteins that forms the neuroligin-neurexin complex that links the presynaptic and postsynaptic domains of neuronal synapses (Benson et al., 2000; Scheiffele, 2003; Waites et al., 2005). Neuroligins are the postsynaptic proteins, while neurexins are the presynaptic proteins of the trans-synaptic adhesion complex formed by neuroligin and neurexin. Previous studies have indicated that neurexin functions as a neuroligin receptor during synapse formation (Dean et al., 2003). Neuroligins recruit neurexins, and this action triggers the assembly of presynaptic active zones. This occurs when the extracellular domain of neuroligin on the postsynaptic neuron signals the induction of presynaptic specialization on the presynaptic neuron (Scheiffele et al., 2000). All of these activities illustrate how neuroligins are essential in the formation and stabilization of synapses.

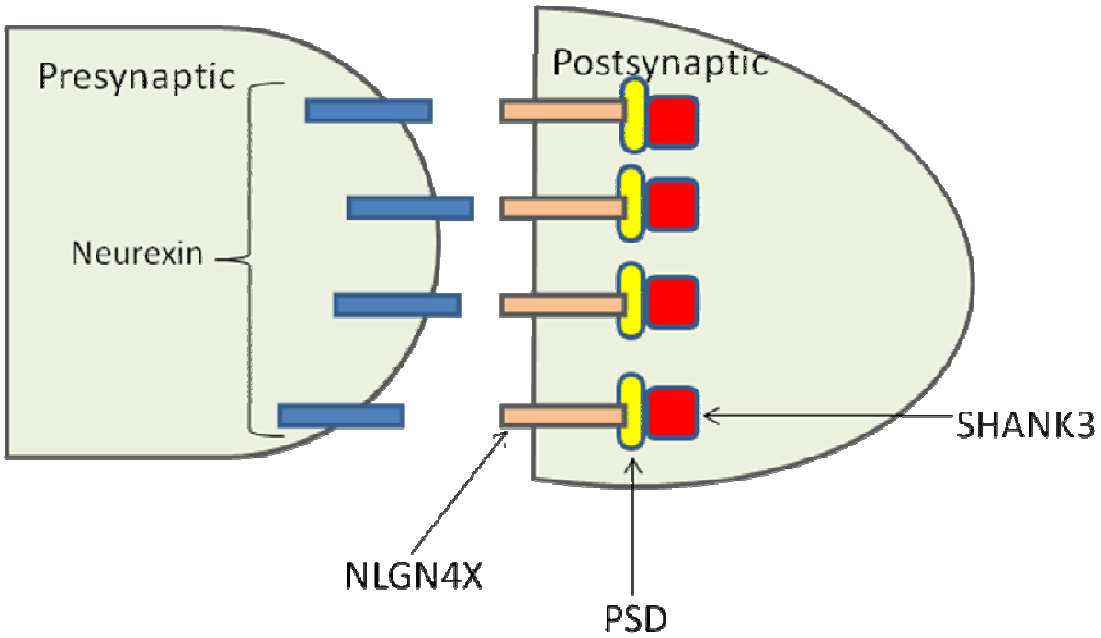

Figure B1.1 Schematic representation of *NLGN4X* functioning at the postsynaptic neuron. NLGN4X along with PSD (postsynaptic density protein) and SHANK3 form a complex that helps to stabilize the synapse.

The studies in this dissertation investigated causative genes for Autism Spectrum Disorder. The rs1858830 C variant in the *MET* gene was previously identified and associated with Autism Spectrum Disorder by Campbell et al. (2006). We attempted to determine the importance of this variant and its association with Autistic Disorder. We also attempted to identify alterations in *NLGN4X* and study the *in vivo* effects of identified *NLGN4X* alterations in a neuronal cell line. Finally, the chromosomal region located at 2q21 has been implicated as a location for causative Autism Spectrum Disorder genes (Ashley-Koch et al., 1999; Barrett et al., 1999; Folstein and Rosen-Sheidley, 2001; Gutknecht et al., 2001; Herba et al., 2008; International Molecular Genetic Study of Autism Consortium, 1998; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al.,

2006; Trikalinos et al., 2006; Vincent et al., 2000). We attempted to locate the translocation breakpoint at chromosome 2q21 in a patient with a t(2;16) translocation and identify causative genes for Autism Spectrum Disorder located in this region.

The first study involved screening a cohort containing individuals diagnosed with Autistic Disorder and a cohort of controls to identify the significance of the rs1858830 C variant of the *MET* gene and Autistic Disorder. This cohort of individuals diagnosed with Autistic Disorder is quite unique because it represents a very small percentage of individuals that fall within Autism Spectrum Disorder. Results obtained from screening the cohorts for the rs1858830 C variant indicated an association with the *MET* rs1858830 C variant and Autistic Disorder.

The second study involved screening a cohort containing individuals diagnosed with Autistic Disorder and a cohort of controls to identify alterations in *NLGN4X*. Two alterations were identified in *NLGN4X*, one of which is a *de novo* alteration, within the Autistic Disorder cohort. Further studies were conducted to observe the cellular localization and cell morphology of these alterations in a neuronal cell line. Fluorescence microscopy determined that neuronal cells expressing the two alterations identified in *NLGN4X* did have altered cell morphology. The cells transfected with the altered NLGN4X protein had reduced neurite lengths and a reduction in the number of neurites per transfected cell as compared to cells transfected with wild type NLGN4X protein. Cellular localization studies also identified a difference between the location of the altered NLGN4X protein within the cell and the location of wild type NLGN4X.

The final study attempted to identify causative genes for Autism Spectrum Disorder located in the 2q21 chromosomal region. Previous genome-wide linkage scans identified 2q as possibly containing genes associated with Autism Spectrum Disorder (Ashley-Koch et al., 1999; Barrett et al., 1999; Folstein and Rosen-Sheidley, 2001; Gutknecht et al., 2001; Herba et al., 2008; International Molecular Genetic Study of Autism Consortium, 1998; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al., 2006; Trikalinos et al., 2006; Vincent et al., 2000). A patient with karyotype 46XY, t(2;16)(q21;q13) and autistic characteristics was studied to identify the translocation breakpoint in the chromosomal 2q21 region. The breakpoint at chromosome 2q21 was narrowed to a region of only 80kb and a potential candidate gene within this region was identified.

In summary, this work found an association between Autistic Disorder and the *MET* gene rs1858830 C variant. Two alterations were identified in *NLGN4X* that are associated with Autistic Disorder and further studies provided information about the cellular localization and morphology associated with these alterations. Finally, this work identified a candidate gene, *C2orf27A*, located in the 2q21 chromosomal region, that may be involved in Autism Spectrum Disorder.

Chapter 1

FURTHER EVIDENCE THAT THE rs1858830 C VARIANT IN THE PROMOTER REGION OF THE *MET* GENE IS ASSOCIATED WITH AUTISTIC DISORDER.

Introduction

Autistic Disorder is a neurodevelopmental disorder characterized by social impairments, communication impairments, repetitive behaviors, and a restricted pattern of interests and behaviors (Folstein and Mankoski, 2000; Lamb et al., 2005; Muhle, Trentacoste, and Rapin, 2004; Rutter, 2005; Veenstra-VanderWeele and Cook, 2004). The prevalence of Autistic Disorder is one in 500 children, and onset can occur as early as three years of age (Fombonne, 1999; Yeargin-Allsop et al., 2003). Pervasive Developmental Disorder (PDD), which encompasses autistic signs and symptoms that vary in severity, has a much higher prevalence of one in 166 children (Fombonne, 1999; Herba et al., 2008; Yeargin-Allsop et al., 2003). The term Autism Spectrum Disorder (ASD) includes these two diagnoses and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS). This high prevalence of both Autistic Disorder and PDD has increased the desire to identify the causes that may lead to these disorders.

Genome-wide linkage scans have attempted to identify potential regions within the human genome where genes that cause autism may be located. These studies have

identified various chromosomal regions (2q, 4, 15q, 16p, 17q, 19, and 22q) that are putative locations of autism genes (Barrett et al., 1999; Herba et al., 2008; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al., 2006), including a region at chromosome 7q that is associated with autism (Ashley-Koch et al., 1999; Folstein and Rosen-Sheidley, 2001; Gutknecht et al., 2001; International Molecular Genetic Study of Autism Consortium, 1998; Trikalinos et al., 2006; Vincent et al., 2000). The *MET* gene is located within this region, at chromosome 7q31, and a report by Campbell et al. (2006) found a variant within the promoter region, rs1858830 C, to be associated with autism in two separate cohorts (individuals in the cohorts had diagnoses that ranged from autism to ASD).

Recently, there have been other studies identifying a correlation between the *MET* gene and autism. A positive correlation between the *MET* rs1858830 C allele variant and autism was found in a third cohort with diagnosis falling in the range of ASD (Campbell et al., 2008). A study by Sousa et al. (2009) found a positive correlation between rs38845 in the *MET* gene and autism in two cohorts with diagnosis falling in the range of ASD, but was unable to find a correlation between the rs1858830 C allele variant and autism. These combined results include five unrelated cohorts that show a positive association between the *MET* gene and autism.

The *MET* gene is known to play a role in the metastasis of a variety of cancers and in mediating hepatocyte growth factor/scatter factor signaling in peripheral organ development and repair (Blume-Jensen and Hunter, 2001; Gheradi et al., 2003; Park et al., 1987). Recent studies also indicate that *MET* contributes to the development of the

cerebral cortex and cerebellum (Campbell et al., 2007; Ierci, Forni, and Ponzetto, 2002; Powell, Mars, and Levitt, 2001; Powell et al., 2003). The cerebral cortex and cerebellum both have been shown to be disrupted during development in individuals with autism (Bauman and Kemper, 2005). *MET* is also involved in immune function and gastrointestinal repair (Campbell et al., 2007; Tahara et al., 2003). The involvement of *MET* in gastrointestinal repair is of some interest due to the fact that some individuals with autism also exhibit gastrointestinal problems (Campbell et al., 2006).

In the present study, we tested for an association of the *MET* rs1858830 C variant with Autistic Disorder and PDD in two unrelated cohorts. The study utilized cohorts of patients with Autistic Disorder and PDD from South Carolina and Italy, respectively. The cohort of individuals diagnosed with Autistic Disorder is very unique because it represents a narrowly defined population within Autism Spectrum Disorder.

Materials and Methods

This study included a South Carolina cohort composed of 174 patients (110 Caucasians, 56 African Americans, and eight unknowns) between the ages of five and 21 years with Autistic Disorder from the South Carolina Autism Project (SCAP) (Schroer et al., 1998). The South Carolina cohort diagnoses were established utilizing the Autism Diagnostic Interview-Revised (ADI-R) or the Childhood Autism Rating Scale (CARS) tests. An Italian cohort composed of 65 patients ranging in diagnosis from Autistic Disorder (n = 14) to PDD (n = 51) was also studied. The Italian cohort was composed of Caucasian patients that met the ADI-R and/or the Autism Diagnostic Observation

Schedule (ADOS) criteria for Autistic Disorder or PDD. In both cohorts, all patients had negative results for high-resolution chromosome analyses and FMR1 molecular testing. Four SCAP patients carried balanced chromosomal rearrangements that did not involve any known autism locus.

The South Carolina control cohort consisted of 180 Caucasian males, 89 African American males, 11 Caucasian females, and 89 African American females. The Italian control cohort consisted of 55 Caucasian males and 71 Caucasian females. In both cohorts all of the control individuals were 18 years of age or older, and none of these individuals ever demonstrated any features of developmental delay or autistic traits.

Screening for the *MET* promoter rs1858830 variant was conducted using genomic DNA amplified using primer metF (gatttccctctgggtggtg) and primer metR (caagccccattctagtttcg) in a polymerase chain reaction (PCR). Amplification was carried out in 20 µL reactions containing 105 ng genomic DNA, 2µL of 1 µM of each appropriate primer, 0.08 µL of dNTPs, 2 µL of 5X PCR buffer, 1µL DMSO, 0.2µL Hot Start Antibody, and 0.2 µL Go Taq DNA polymerase. Amplification conditions consisted of an initial denaturation of 95^{o}C for five minutes, then 30 cycles consisting of a denaturation step at 95^{o}C for 30 seconds, annealing temperature of 55^{o}C for 30 seconds, and an extension at 72^{o}C for 60 seconds. A final extension at 72^{o}C lasted for seven minutes. PCR products were examined on a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet light. After digesting overnight at 37^{o}C in a 20 µL reaction containing 1X Buffer, 1 µL Eag I enzyme, and 10 µL PCR product, the DNA was analyzed on a 3.5% TBE agarose gel containing ethidium bromide and visualized

under ultraviolet light. Individuals with the genotype GG had fragments of 378 base pairs, 240 base pairs, and 34 base pairs. An individual with the genotype CC had fragments of 412 base pairs and 240 base pairs, and an individual with the genotype GC had all of these fragments.

The Hardy-Weinberg equilibrium and odds ratio tests were performed for the different groups using Microsoft Excel (Redmond, OR). Chi-square tests comparing the allelic and genotypic proportions between cases and controls were performed using Epi Info™ 3.4.3 (Atlanta, GA). Family-based association testing was performed using an additive model with the FBAT program (Horvath, Xu, and Laird, 2001).

Results

To characterize the rs1858830 C variant in the promoter region of the *MET* gene (Campbell et al., 2006), we used restriction endonuclease digestion. The chromosome 7q region containing the variant was amplified by polymerase chain reaction (PCR) and the amplicon was digested with the restriction endonuclease *Eag I* (Figure 1.1). Genotype frequencies for the case and control cohorts from the South Carolina and Italian populations are shown in Tables 1.1 and 1.2. All case and control cohorts were found to be in Hardy-Weinberg equilibrium.

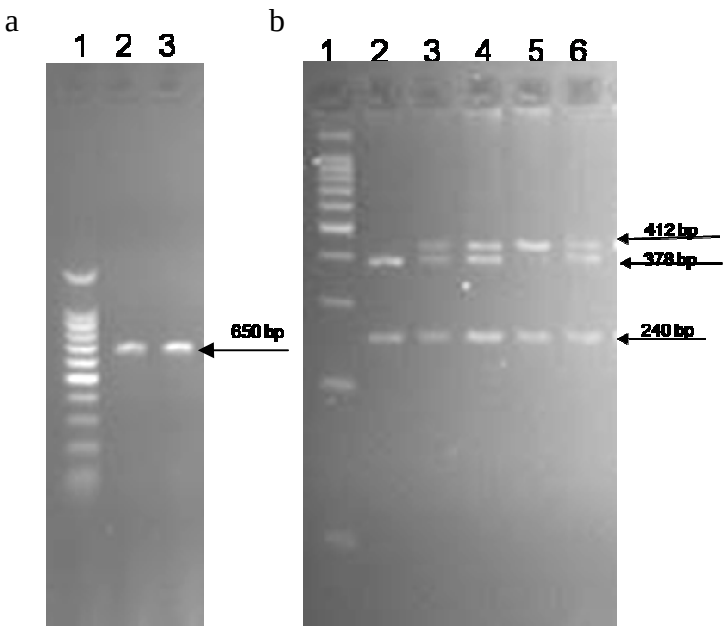

Figure 1.1 *MET* PCR amplification and *Eag I* endonuclease digestion. (a) *MET* PCR product 650 bp. Lane 1 is a 100 base pair marker. Lane 2 and 3 are PCR products using primers metF and metR. (b) Digestion of *MET* PCR product with endonuclease *Eag I*. Lane 1 is a 100 base pair marker. Lane 2 contains the product of an individual with genotype GG for the rs1858830 C variant in the promoter region of *MET*. Lanes 3, 4, and 6 contain the digested products of individuals with the genotype GC for the rs1858830 C variant in the promoter region of *MET*. Lane 5 contains the digested DNA amplicon of an individual with the genotype CC for the rs1858830 C variant in the promoter region of *MET*.

Table 1.1 *MET* rs1858830 allele genotypes

Cohort	GG	GC	CC	Total
South Carolina Autistic Disorder[1]	55	91	28	174
South Carolina Controls	159	164	46	369
Italian Autistic Disorder	5	8	1	14
Italian PDD[2]	12	21	18	51
Italian Controls	34	65	27	126

Results from comparison of these groups with the appropriate controls are as follows:
[1]χ^2 = 6.7, df = 2, *P* = 0.04 and [2]χ^2 = 3.7, df = 2, *P* = 0.16.

Table 1.2 *MET* rs1858830 allele genotypes by type of family

Cohort	GG	GC	CC	Total
South Carolina cases from simplex families[1]	50	78	26	154
South Carolina cases from multiplex families	5	13	2	20
South Carolina Controls	159	164	46	369

[1]Results from the comparison of cases from simplex families with the SC controls are as follows:
χ^2 = 5.5, df = 2, *P* = 0.06.

The genotypic proportions within the South Carolina cohort, consisting of patients diagnosed with Autistic Disorder, were found to be significantly different from the South Carolina controls (χ^2 = 6.7, df = 2, *P* = 0.04; Table 1.1). There was no significant difference in genotypic proportions between the Italian controls and the PDD patients within the Italian cohort (Table 1.1). This association approached significance for South Carolina cases from the simplex families (χ^2 = 5.5, df = 2, *P* = 0.06) as compared to controls (Table 1.2).

In the South Carolina cohort, a significant association with Autistic Disorder was found when comparing the CC or the CG genotype to the GG genotype (OR= 1.64; 95%

CI = 1.12-2.40; χ^2 = 6.5, df = 1, *P* = 0.01). This association was also significant for cases from simplex families (OR = 1.58; 95% CI = 1.06-2.34; χ^2 = 5.1, df = 1, *P* = 0.02). In the Italian cohort, no significant association with PDD was found when comparing the CC or the CG genotype to the GG genotype (OR = 1.20; 95% CI = 0.56-2.56; χ^2 = 0.2, df = 1, *P* = 0.64).

Allelic frequencies for the South Carolina and Italian patient and control groups are found in Table 1.3. The frequency of the C allele variant was found to be significantly increased in South Carolina Autistic Disorder patients as compared to South Carolina controls (χ^2 =5.8, df = 1, *P* = 0.02). There were no significant differences found in the allele frequencies between the Italian patient and control groups.

Table 1.3 *MET* rs1858830 allele frequencies

Cohort	G	C
South Carolina Autistic Disorder[1]	0.58	0.42
South Carolina Controls	0.65	0.35
Italian Autistic Disorder[2]	0.64	0.36
Italian PDD[3]	0.44	0.56
Italian Controls	0.53	0.47

Results from comparison of these groups with the appropriate controls are as follows: [1]χ^2 = 5.8, df = 1, *P* = 0.02; [2]χ^2 = 1.3, df = 1, *P* = 0.25; and [3]χ^2 = 2.2, df = 1, *P* = 0.14.

Family-based association testing (Horvath, Xu, and Laird, 2001) indicated no overtransmission of the C allele variant to affected individuals (Z = 0.10 and *P* = 0.92) in the South Carolina cohort families (Horvath, Xu, and Laird, 2001). The allele frequency

of the *MET* rs1858830 G allele was 0.59 and the C allele was 0.41. Seventy-five of 174 families (43%) were informative for this analysis.

Discussion

We found an association between the *MET* rs1858830 promoter C variant and Autistic Disorder in our South Carolina cohort of patients. This study is the fourth independent cohort to find correlation between the *MET* rs1858830 C allele variant and autism. Campbell et al. (2006, 2008) found a positive correlation between the *MET* rs1858830 C allele variant and autism in a cohort of 204 Italian families with autism, a US cohort of 539 families, and an unrelated cohort of 101 families from the US. Sousa et al. (2009) found a correlation between the *MET* gene rs38845 variant in two autistic cohorts (a 335 IMGSAC family cohort and an 82 family autistic Italian cohort), but no correlation with the rs1858830 C allele variant and autism. These cohorts included patients with either Autistic Disorder or PDD-NOS.

There is a possibility that the association observed between the *MET* rs1858830 C allele variant and Autistic Disorder in the South Carolina cohort is due to population stratification. As shown in Table 1.4, Caucasians within this cohort have a higher frequency of the C allele variant and also comprise a larger proportion of cases. To further examine this, a Cochran-Mantel-Haenszel test was run using SAS Version 9.2 (The SAS Institute, Cary, NC) to analyze the association of the CC and CG genotypes versus the GG genotype and Autistic Disorder while controlling for racial group (OR=

1.48, 95% CI = 0.99-2.21; χ^2 = 3.7, df = 1, *P* = 0.05). While stratification may have some influence on the new borderline significant results, it appears that the CC and CG genotypes remain associated with Autistic Disorder in the South Carolina cohort, especially in Caucasians.

Table 1.4 *MET* rs1858830 allele genotypes by racial group in the South Carolina population

Population		**Allele frequency**		**Allele genotypes**			
Racial Group	**Cohort**	**G**	**C**	**GG**	**GC**	**CC**	**Total**
African American	Autistic Disorder	0.69	0.31	26	25	5	56
Caucasian	Autistic Disorder	0.51	0.49	26	61	23	110
Other	Autistic Disorder	0.69	0.31	3	5	0	8
African American	Control	0.73	0.27	96	67	15	178
Caucasian	Control	0.58	0.42	63	97	31	191

Our study did not find any association of the *MET* rs1858830 C allele with PDD within the Italian cohort. This may be evidence that there is no correlation between PDD and the C allele variant. This result may also be due to the small sample size in this cohort. Although both the South Carolina and Italian cohorts contained approximately twice as many controls as cases, we would have needed at least 300 cases and 600 controls to have 80% power to detect an allele frequency difference of ten percentage points between the two groups (Power and Precision, Biostat, Englewood, NJ). However, smaller sample sizes can give you an indication of potential significant findings with larger sample sizes.

The involvement of MET signaling in interneuron development along with its involvement in gastrointestinal repair is a possible key to its association with Autistic Disorder. MET is a receptor for hepatocyte growth factor (HGF) in the forebrain. HGF provides trophic support, stimulates axonal outgrowth, and enhances neuronal differentiation (Tahara et al., 2003). Alterations in MET signaling may lead to a disruption in the involvement of MET with HGF leading to alterations in GABAergic neuron development in the forebrain (Levitt, Eagleson, and Powell, 2004; Powell et al., 2001, 2003). Reduced MET signaling could possibly explain the epilepsy and atypical sleep patterns found in some individuals with Autistic Disorder. Epilepsy and atypical sleep patterns may indicate an imbalance in the glutamatergic (excitatory) projection neurons and GABAergic inhibitory interneurons in the neocortex (Levitt, Eagleson, and Powell, 2004; Powell et al., 2003; Tuchman and Rapin, 2002). This is probably significant due to this balance of excitation-inhibition being relatively conserved across mammalian species (Levitt, Eagleson, and Powell, 2004).

MET is involved in the normal development of the cerebellum and cerebral cortex (Campbell et al., 2007; Ierci, Forni, and Ponzetto, 2002; Powell et al., 2003; Powell, Mars, and Levitt, 2001). Mice with alterations in MET expression have altered organization of the cerebellum and cerebral cortex. Altered organization of the cerebellum and cerebral cortex has also been found in individuals with autism (Bauman and Kemper, 2005). This indicates that a reduction in MET signaling may lead to abnormalities in the organization of the cerebellum and cerebral cortex.

HGF also serves as a critical regulator of intestinal wound healing (Tahara et al., 2003). A previous study indicates that HGF and MET together may function in the repair of gastrointestinal abnormalities (Tahara et al., 2003). The rs1858830 C variant found in the promoter region of *MET* may lead to an altered interaction of HGF and MET resulting possibly in their inability to correct intestinal abnormalities. This may also account for the gastrointestinal problems found in individuals with Autistic Disorder.

In conclusion, this study reinforces an association with Autistic Disorder and the *MET* rs1858830 C allele variant as noted by Campbell et al. (2006). The results from our study indicate that *MET* may play a stronger role in individuals with Autistic Disorder as compared to other diagnoses contained within the spectrum of autism. Our results, in conjunction with other studies (Campbell et al., 2006, 2008; Sousa et al., 2009), suggest the rs1858830 C variant in the promoter region of the *MET* gene is likely a significant risk factor for Autistic Disorder.

Chapter 2

CELLULAR LOCALIZATION AND FUNCTIONAL ANALYSIS OF TWO ALTERATIONS IDENTIFIED IN *NLGN4X*

Introduction

Autism is a disorder associated with developmental delay, impairments in communication and social skills, and abnormal patterns of behaviors and interests. Autistic Disorder is a type of Autism Spectrum Disorder (ASD). Autism Spectrum Disorder classification includes Asperger syndrome and Pervasive Development Disorder-Not Otherwise Specified (Rutter, 2005). Autistic Disorder occurs before the age of three years and varies in the degree of severity of social skills, behavior, and cognition. The male to female ratio is 4:1 (Ylisaukko-oja et al., 2005). The exact nature of the male predisposition for autism has not been identified, but several previously identified alterations of the X chromosome are associated with autism (Jamain et al., 2003; Laumonnier et al., 2004; Ylisaukko-oja et al., 2005). Specifically, Jamain et al. (2003) and Laumonnier et al. (2004) identified mutations in *NLNG4X* in patients diagnosed with ASD.

NLGN4X, which is located at chromosome Xp22.3, is a member of the neuroligin family. The neuroligin family in humans is comprised of five neuroligin genes: *NLGN1* (3q26), *NLGN2* (17p13), *NLGN3* (Xq13), *NLGN4X* (Xp22.3), and *NLGN4Y* (Yq11.2) (Chih et al., 2004 and Talebizadeh et al., 2004). This family of genes is involved in the

production of cell-adhesion molecules essential for the formation and stability of functional synapses (Talebizadeh et al., 2004). Alterations in the *NLNG4X* gene have been associated with ASD (Jamain et al., 2003; Ylisaukko-oja et al., 2005).

Alterations in *NLGN4X* may lead to the abnormal development of synapses, resulting in altered communication processes in the brain and cognitive development. Phenotypes such as impairments in communication and social skills, and deficits in cognition resulting from altered communication processes and cognitive development of the brain, are observed in individuals with ASD (Jamain et al., 2003; Ylisaukko-oja et al., 2005). The higher incidence of Autistic Disorder in the male population does support the involvement of genes located on the X chromosome (Ylisaukko-oja et al., 2005). Further research is needed to understand how alterations in *NLGN4X* may lead to Autistic Disorder. This study is designed to identify any alterations in *NLGN4X* in patients with Autistic Disorder and how these alterations may lead to the Autistic Disorder phenotype.

Materials and Methods

Subjects

This study included 204 subjects with Autistic Disorder from 178 different families. DNA samples from these Autistic Disorder patients and their families were provided by the South Carolina Autism Project (SCAP). The SCAP is composed of individuals diagnosed specifically with Autistic Disorder using the Autism Diagnostic Interview-Revised test, Bayley Scales of Infant Development, and the Stanford-Binet Intelligence Scale: Fourth Edition (Schroer et al., 1998).

Case Reports

Autism 17702

This individual is a male that was diagnosed with autism at 5.5 years of age. His height was measured at the 40th percentile, weight was greater than the 98th percentile, and his head circumference was at the 70th percentile. The individual's mother smoked during pregnancy and had several episodes of vaginal blood spotting. He was delivered by cesarean birth at 40 weeks gestation. He only spoke a few words at the age of two then stopped speaking until age four. His gross motor skills were noted to be clumsy and he had outbursts of anger that involved tearing things, yelling, and episodes of cursing. He has a history of ear infections with the placement of tubes in his ears but no other significant childhood illness. He was hospitalized once due to his aggressive behavior.

Autism 15627

This individual is a female that was diagnosed with autism at the age of four years and eight months. Her height and weight were both above the 95th percentile and her head circumference was measured at the 60th percentile. She is the older of two children and her sibling is reported to be normal. Her mother had cryosurgery on her cervix during the third week of pregnancy and developed hypertension during the last trimester of pregnancy. She was delivered vaginally at 38 weeks gestation after induction. She has a history of ear infections and had tubes placed in her ears and an adenoidectomy. She had normal vocabulary development, but there were concerns about her cognitive and social development around 18 months of age. She was very hyperactive, provided

inappropriate responses to questions, played inappropriately with toys, and did not play well with other children.

Mutation Screening

Alterations in patients Autism 17702 and Autism 15627 were identified in *NLGN4X* using by single-strand conformation polymorphism (SSCP) analysis in the SCAP cohort (unpublished data by Gao and Schwartz). The alteration c. 775 C>T was confirmed in patient Autism 17702 and the alteration c. 864 G>A was confirmed in patient Autism 15627 by sequence analysis using primers 1 and 2 and primers 3 and 4 (Table 2.1), respectively. Amplification was carried out in 50µL reactions containing 105 ng genomic DNA, 5µL of 10µM of each appropriate primer, 0.08µL of dNTPs, 10µL of 5X PCR buffer, and 0.5µL Go Taq DNA polymerase. Amplification conditions included an initial denaturation of 95^{o}C for 5 minutes, then 30 cycles consisting of a denaturation step at 95^{o}C for 30 seconds, optimal annealing temperature for 30 seconds, extension at 72^{o}C for 30 seconds, followed by a final extension at 72^{o}C for 7 minutes. PCR products were purified using a Qiagen kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). Samples were sequenced using the recommended standard procedure for the MegaBASE 1000 (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). Sequencing results were analyzed using a SeqMan program which is part of the DNASTAR Lasergene 7 Program (DNASTAR, Inc., Madison, WI).

Table 2.1 *NLGN4X* primer list

Primer Name	Sequence (5'-3')
Primer 1	ACCAAAAATCTCTTGTGTTCT
Primer 2	TTCTTGGTTCAGGGTATTTGC
Primer 3	TTCTGTCCTGTGTTTCATTTG
Primer 4	AGGATCTGGGGGTCGTCTGG
Primer 5	TTTTTAAGTACCGGTGACCAG
Primer 6	CCTCTGAGTAGTGGGACAGG
Primer 7	CACCGCCCTGTCCAGCTAGA
Primer 8	AGGATCTGGGGGTCGTCTGG
Primer 9	AGAAGGCCATCATTCAGAGC
Primer 10	CATGTTGCAGCCGACCTT

Polymorphism Study

One thousand normal X chromosomes were screened to determine if either of the c. 775 C>T or c. 865 G>A point mutations were a rare single nucleotide polymorphism (SNP). The alteration c. 775 C>T was tested using polymerase chain reaction (PCR) and enzymatic digestion. Genomic DNA from control individuals was amplified using primers 5 and 6 (Table 2.1). The amplification was carried out in a 50µL reaction containing 105ng genomic DNA, 5µL of 10µM of each appropriate primer, 0.8µL of dNTPs, 10µL of 5X PCR buffer, and 0.5µL of Go Taq DNA polymerase. Amplification conditions consisted of an initial denaturation of 95ºC for 5 minutes, then 30 cycles consisting of a denaturation step at 95ºC for 30 seconds, annealing temperature of 60ºC for 30 seconds, and an extension at 72ºC for 60 seconds. A final extension at 72ºC lasted for 7 minutes. PCR products were examined on an agarose gel containing ethidium bromide and visualized under ultraviolet light. The PCR product was digested overnight

at 37ºC with the restriction endonuclease *Mnl I.* The digestion reaction of 20µL contained 2µL of 10X buffer, 1µL of *Mnl I* enzyme, and 10µL of PCR product. The alteration created an enzymatic site not present in normal sequences (Figure 2.2). This band difference was observed on a 1.5% TBE agarose gel with ethidium bromide that was allowed to run for 2 hours.

The screening of the controls for the c. 865 G>A alteration was conducted using allelic specific primers. If the altered allele is present it would be the only allele to amplify because primer 7 (Table 2.1) is specific for the alteration. A primer set for a control gene was used with every PCR to ensure that the PCR conditions were optimal. The amplification was carried out in a 50µL reaction containing 105ng genomic DNA, 5µL of 10µM of each appropriate primer, 0.8µL of dNTPs, 10µL of 5X PCR buffer, and 0.5µL of Go Taq DNA polymerase. Products obtained after amplification were electrophoresed on a 1.5% agarose gel with ethidium bromide and analyzed under ultraviolet light.

Family Analysis

Family members of the patient with the c. 865 G>A alteration were available for testing. An analysis was conducted with genomic DNA of family members of the patient utilizing PCR and enzymatic digestion. The amplified region had an extra *HpyCH4III* enzymatic site if the alteration was present. Amplification was carried out with primers 9 and 10 (Table 2.1). The amplification conditions were denaturation at 95^{o}C for 5 minutes followed by 30 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 30

seconds with an extension of 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. The amplification was carried out in a 50µL reaction containing 105ng genomic DNA, 5µL of 10µM of each appropriate primer, 0.8µL of dNTPs, 10µL of 5X PCR buffer, and 0.5µL of Go Taq DNA polymerase. The PCR product was digested with *HpyCH4III* overnight at 37°C in a 20µL reaction containing 2µL of 10X Buffer, 2µL of 10X BSA, 2µL of 10X Spermidine, and 10uL of PCR product, the digested product was analyzed on a 1.5% TBE agarose gel containing ethidium bromide and visualized under UV light. To confirm the presence of both alleles in the father, the DNA amplicon from PCR, utilizing primers 9 and 10, was subcloned into the pCR 2.1 vector (Invitrogen Corporation, Carlsbad, CA). Positive colonies were isolated and sequenced to obtain a single strand sequence. Colonies were sequenced using the recommended standard procedure for the MegaBASE 1000 (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). Sequencing results were analyzed using a SeqMan program which is part of the DNASTAR Lasergene 7 Program (DNASTAR Inc., Madison, WI).

NLGN4X Construct

Wild type *NLGN4X* was obtained from the clone MHS1011-7509691, that was purchased from OPEN BIOSYSTEMS. This clone contained the full-length *NLGN4X* cDNA. The full-length cDNA was amplified from the plasmid by PCR with cDNAF (caccatgtcacggccccaggga) and cDNAR (tactctagtggtggaatgtcc)primers. Amplification was carried out in 25µL reactions containing 1µL of the clone, 2.5µL of 10µM of each

primer, 4µL of 2.5mM dNTPs, 2.5µL of 10X PCR buffer, and 0.5µL PFU DNA polymerase. Amplification conditions included an initial denaturation step at 95°C for 2 minutes, then 30 cycles consisting of a denaturation step at 95°C for 30 seconds, 55°C optimal annealing temperature for 30 seconds, extension at 72°C for 4 minutes and 30 seconds, followed by a final extension at 72°C for 10 minutes. PCR samples were placed on ice and one unit of GoTaq was added to each sample. Samples were incubated at 72°C for 10 minutes. The PCR product was isolated by gel extraction. The product was purified using the Qiagen Gel Extraction kit. The purified PCR product was cloned into the pcDNA3.1D/Vs-His-TOPO expression vector following the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). The construct was sequenced to confirm the proper reading frame using the standard procedure for the 3730 DNA Analyzer (Life Technologies Corporation, Carlsbad, CA) . Sequencing results were analyzed using the SeqMan program which is part of the DNASTAR Lasergene 7 Program (DNASTAR, Inc., Madison, WI).

c. 775 C>T and c. 865 G>A Constructs

The two alterations, c. 775 C>T and c. 865 G>A, were created using Stratagene's QuikChange II Site-Directed Mutagenesis kit. The pcDNA3.1D/Vs-His-+ NLGN4X construct was used as a template for these reactions. The c. 775 C>T alteration was created with primer c. 775 C>T (gctggggcctcccgtgtcagctgt) and primer c. 775 C>T antisense (acaggctgacacgggaggccccagc). The c. 865 G>A alteration was created with primer c. 865 G>A (cctgtccagctgggcaatgaactaccagcc) and primer c. 865 G>A antisense

(ggctggtagttcattgcccagctggacagg). A reaction for each alteration was conducted in a total volume of 50µL containing 5µL of 10X reaction buffer, 10ng of dsDNA template, 1.25µL of c. 775 C>T primer or c. 865 G>A primer at 100ng/µL, 1.25µL of c. 775 C>T antisense primer, 1.25µL c. 865 G>A antisense primer at 100ng/µL, 1µL 10µM dNTP mix, and 40.5µL of double distilled water, and 1µL of *Pfu Ultra* HF DNA polymerase (2.5U/µL). The reaction proceeded in a thermal cycler at 95°C for 30 seconds, then 12 cycles of 95°C for 30 seconds, 55°C for one minute, and 68°C for five minutes. After cycling was complete the products were placed on ice for two minutes. One microliter of the restriction enzyme *Dpn* I (10U/µL) was added to the PCR products and this reaction was allowed to incubate for one hour at 37°C. The *Dpn* I treated DNA was transformed in XL1-Blue Supercompetent cells following standard transformation procedure. The transformations were spread on plates containing 100mg of ampicillin and LB agar. Colonies were picked and grown in liquid cultures and plasmids were purified using Quiagen Midi prep kit. The constructs were sequenced to confirm the mutation using the standard protocol for the 3730 DNA Analyzer (Life Technologies Corporation, Carlsbad, CA) . Sequencing results were analyzed using the SeqMan program which is part of the DNASTAR Lasergene 7 Program (DNASTAR, Inc., Madison, WI).

Cell Line Maintenance

The PC-12 cell line was obtained from American Type Culture Collection (ATCC) and is a rat pheochromacytoma cell line derived from a cancerous adrenal gland (American Type Culture Collection, Manassas, VA). The cells were grown at 37°C with

5% CO_2. Standard procedures for passage of the cell line were followed. These protocols included removal of cells twice per week to obtain a 1:2 ratio of cells to cell culture media (500mL F12-K, 75mL Horse serum, 15mL fetal bovine serum (FBS), 5mL of penicillin, 5mL of 10,000 units per mL streptomycin, and 5mL of 200mM Glutamine (L-Glut)). This included removing the growth media from the cells, washing with 10mL of Phosphate Buffered Saline (PBS) (Sigma), and adding 3mL of Trypsin+EDTA (Sigma). After three minutes, the 3mL of Trypsin+EDTA (Sigma) was removed from the cells and 7mL of growth media is added back to the cells. Nine mililiters of this mixture was removed that could be used for further studies or discarded. Thirteen mililiters of growth media was added back to the remaining 1mL of cell mixture and the cells were allowed to continue growing at 37°C at 5% CO_2.

PC-12 Cell Transfection

PC-12 cells were transfected with constructs using Neuromag in a 24-well format. The PC-12 cells were seeded at a density of 150,000 cells per well in 1% plating media (50mL DME and 0.5mL of FBS). Poly L-Lysine coating (100µL of poly L-Lysine and 10mL of PBS) of 500µL was added to each of the wells, and the plate was incubated at 37°C overnight. Cells were then transfected with 1µg of the appropriate vector with the NeuroMag (Boca Scientific, Boca Raton, FL) transfection reagent. After adding the vector/transfection mixture at a ratio of 1µg:3.5µL (DNA:NeuroMag) to the cells, the cells were placed on a magnet for 20 minutes on a level surface. The plates were placed back in the incubator at 37°C for five hours. After five hours of incubation, 400µL of

warm growth media was added to each well. The cells were incubated at 37°C overnight and then seeded onto 18 mm diameter, 1.0 mm thick microscope cover glass slips (Fisher) that had a poly L-Lysine and laminin coating (10mL of PBS, 100μL of 10mg/μL poly L-Lysine, and 100μL of 1mg/μL laminin). Nerve growth factor (Sigma) treatment was added to the appropriate cells on slips to allow the cells to grow extensions that mimic neurites. The slips were then placed back a 37°C incubator for 36-48 hours. Forty-eight hours later the cells were used in immunofluorescence studies or protein was harvested from the cells.

Fluorescence Microscopy

PC-12 cells transfected with V5-tagged *NLGN4X*, V5-tagged c. 775 C>T *NLGN4X*, or V5-tagged c. 865 G>A *NLGN4X* were stained with anti-V5 mouse monoclonal antibody (Invitrogen Corporation, Carlsbad, CA) to visualize the V5-tagged protein. Dapi (4;6-diamidino-Z-phenylindole) (Molecular Probes) was used to stain the nucleus of each cell. Rhodamine Phalloidin (Molecular Probes) was used to stain the cytoskeleton of each cell. The anti-V5 mouse monoclonal antibody was visualized by the use of an Alexa Fluor 594 goat anti-mouse Immunoglobulin G secondary antibody (Invitrogen Corporation, Carlsbad, CA).

The growth media was aspirated off the transfected cells and the cells were washed twice in PBS. The cells were fixed by adding 1mL of 4% para-formaldehyde to each well for 30 minutes. Triton X-100, at a concentration of 0.1%, was added to each well for five minutes to make the cells permeable. The Triton X-100 was aspirated off

and the cells were washed twice with PBS. Quenching solution (1.02g sodium acetate in 50mL PBS) was added to each well to remove background fluorescence. Five hundred microliters of Quench was added for seven and half minutes, and then aspirated off and 500µL more of Quench was added for an additional seven and half minutes. The Quench was removed and cells were washed with block (2% horse sera and 0.4% BSA in PBS). This first 500µL of block was removed and then 500µL of block was added back and allowed to incubate with cells for 30 minutes at room temperature. At the end of 30 minutes the primary antibody diluted 1:400 in PBS was added and allowed to incubate at room temperature for one hour with the cells. The primary antibody was then aspirated off and the cells were washed three times with block. Each rinse was allowed to incubate at room temperature for five minutes. The secondary antibody was diluted 1:2000 in blocking solution and 488 Phalloidin was added to the cells and allowed to incubate for one hour at room temperature. The secondary antibody and 488 Phalloidin were removed and the cells were washed three times with block with incubation at room temperature for five minutes after each rinse. The Dapi stain, at a concentration of 1:1000, was added to each well and incubated for five minutes. It was then aspirated off and the cells were rinsed with PBS. Each slip was then mounted with eight microliters of anti-fade ProLong Gold (Invitrogen Corporation, Carlsbad, CA) mounting media onto microscope slides. The slides were viewed with the Zeiss Ax10 inverted Fluorescence Microscope. Images were captured with the AxioCam MRCS and analyzed with the AxioVision Release 4.6.3 software. Data were stored and analyzed in Microsoft Excel (Redmond, WA). Unpaired t-tests were used to compare the length and number of neurite extensions on the cells. An

F-test was run before the t-test to check if the variances were equal, and this information was then used to run the t-test.

Western Analysis

Protein was isolated from PC-12 cells with 1X Lamelli's sample buffer and then boiled for 5 minutes at 95°C. The protein was loaded on a precast 4 to 20% gradient SDS-PAGE gel (Pierce, Rockford, IL). The gel was allowed to run for one hour and thirty minutes at 100 volts. The protein was transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). After transfer the membrane was rinsed in Tris-Buffered Saline and Tween 20 (TBST) twice and placed in block (5% nonfat dry milk dissolved in TBST) for one hour at room temperature. At the end of the one hour block the membrane was rinsed in TBST once and incubated with a primary antibody overnight at 4°C with continual agitation. The primary antibody was removed and the membrane was rinsed with TBST for one hour, while changing the TBST three times during the hour. The membrane was incubated with the appropriate secondary antibody for one hour at room temperature with constant agitation. The SuperSignal®West Dura Extended Duration Substrate (Pierce, Rockford, IL) was used for chemiluminescent detection.

Cell Fractionation

Cell fractionation was performed to confirm cellular localization of the V5-tagged protein. This was conducted utilizing the FractionPREP Cell Fractionation system following the manufacturer's protocol (BioVision, Mountain View, CA). In brief,

transfected PC-12 cells were fractionated by exposure to a corresponding cell fraction buffer and then the fraction was separated by centrifugation. Cells were washed with PBS, 1.1 mL of Cytosol Extraction buffer with Protease Inhibitor Cocktail (PIC) and DTT were added to the cells, and then the 60-mm plates that cells were grown on were scraped. This mixture was transferred to an eppendorf tube and allowed to incubate on ice for 30 minutes. After the incubation, the samples were centrifuged for 10 minutes and the resulting supernatant was the cytosolic faction. The remaining pellet was resuspended in 1.1 mL of Membrane Extraction Buffer-A with PIC and DTT. After the pellet was resuspended, 5.5μL of Membrane Extraction buffer-B was added and samples incubated on ice for one minute. Samples were centrifuged for five minutes and the resulting supernant was the membrane/particulate fraction. The remaining pellet was resuspended in 500μL of Nuclear Extraction Buffer with PIC and DTT. The samples were kept on ice for 40 minutes and vortexed every 10 minutes. After the incubation, samples were centrifuged for 10 minutes and the resulting supernatant was the nuclear fraction. The remaining pellet was the cytoskeletal fraction that then was dissolved in 1X Lamelli sample buffer. Ten percent of each fraction was loaded onto a 4 to 20% precast gradient SDS-PAGE gel (Pierce, Rockford, IL). The cell fractions for the c. 775 C>T alteration were loaded onto an additional 4 to 20% precast gradient SDS-PAGE gel (Pierce, Rockford, IL) at 50% of each fraction since amounts of protein were not detectable using only 10% of the fraction.

Results

The SCAP cohort was screened to identify any alterations in *NLGN4X*, a gene previously associated with ASD. The screen identified two missense mutations from unpublished data by Gao and Schwartz that had not been reported previously (Table 2.2). These alterations were confirmed by sequence analysis (Figure 2.1). The two alterations were not observed in 1000 random X control chromosomes, thus indicating that the two alterations were rare single nucleotide polymorphisms.

Table 2.2 *NLGN4X* alterations identified in the mutation screen

Patient	Type of Mutation	Alteration Base Pair	Alteration Amino Acid	Domain
17702	Missense	c. 775 C>T	p. S258P	Extracellular noncatalytic acetylcholinesterase homolog
15627	Missense	c. 865 G>A	p. A288T	Extracellular noncatalytic acetylcholinesterase homolog

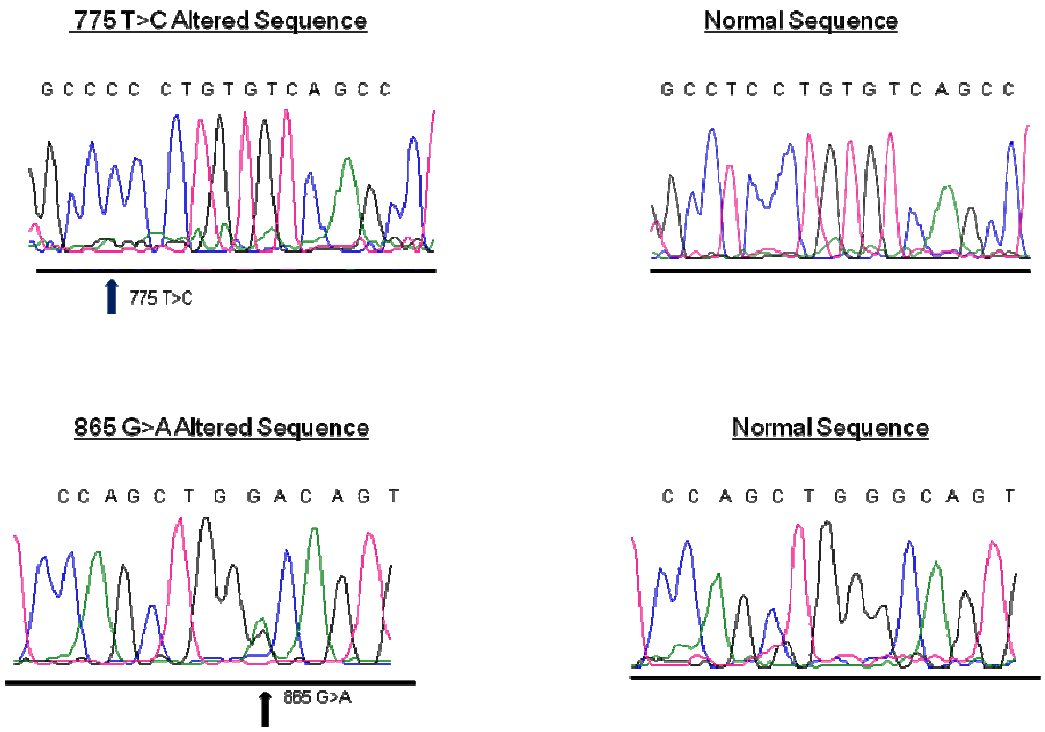

Figure 2.1 Sequence identification of *NLGN4X* alterations and normal *NLGN4X*. (a) Sequence of c. 775 C>T *NLGN4X* alteration and normal *NLGN4X* sequence. (b) Sequence of c. 865 G>A *NLGN4X* alteration and normal *NLGN4X* sequence.

As genomic DNA from the family of the patient with the c. 865 G>A alteration was available, an analysis was conducted to determine if this was a *de novo* mutation. The screening of the family determined that the father carries the alteration. The father also appears to have both the altered and a normal allele (Figure 2.2). Primers for the Y chromosome confirmed that the sample also contains material from this chromosome indicating that the sample is from a male (Figure 2.2). Sequence analysis confirmed that the father does have one normal allele and one altered allele. The father may have a duplication on his X chromosome that contains the *NLGN4X* gene or may have two X

chromosomes. This may also be a result of sample contamination. New samples from this family are needed for further studies and to rule out sample contamination.

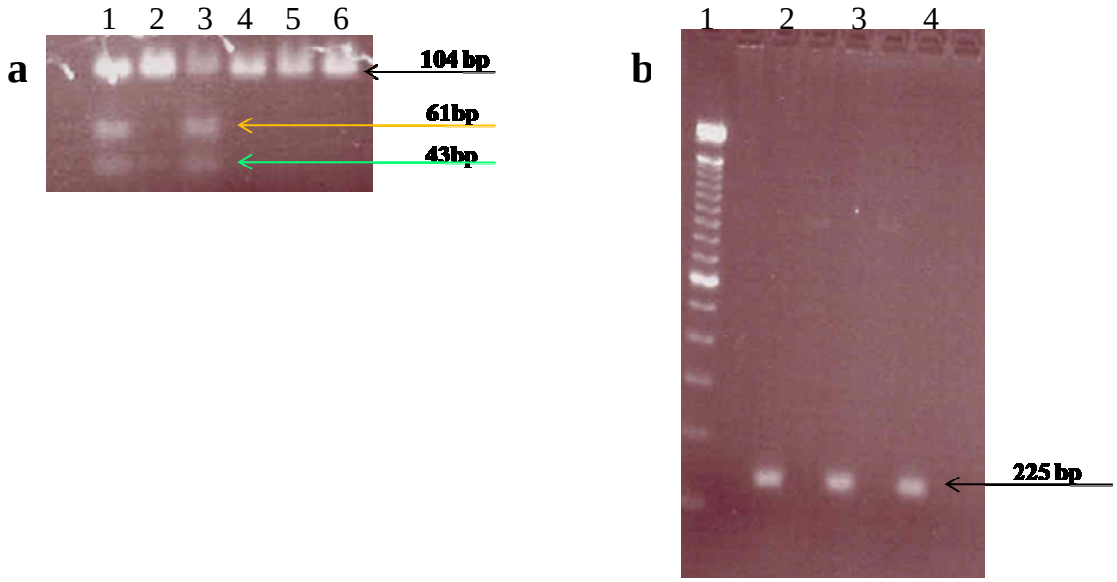

Figure 2.2 Family analysis of the c. 865 G>A alteration identified in *NLGN4X*. (a) Agarose Electrophoresis picture of individuals digested with *HpyCH4III*. The c. 865 G>A change creates an enzymatic site resulting in products that are 61 and 43 base pairs in size. If the change is not present the product is undigested resulting in a product that is 104 base pairs. The lanes contained the following samples: 1) Patient with c. 865 G>A NLGN4X alteration, 2) Mother of patient, 3)Father of patient, 4) Brother of patient, 5) Random male control, 6) Random female control. (b) The arrow indicates the 225 base pairs amplicon from PCR analysis to identify Y chromosome material. The lanes contained the following samples: 1) Marker, 2) Father of patient, 3) Brother of patient, 4) Random male control. PCR confirms that the DNA from the father contains Y chromosome material.

Protein structure analysis indicates the alterations p. S258P and p. A288T lead to an alteration in protein structure (Table 2.3). These alterations are located within the extracellular noncatalytic acetylcholinesterase homolog domain. The extracellular noncatalytic acetylcholinesterase domain is involved in the presynaptic binding of

neurexin (Laumonnier et al., 2004). Splice site analysis indicates that these alterations do not increase splicing or alter a splice site.

Table 2.3 NLGN4X protein prediction

Protein Prediction Program	Protein Prediction Website	p. S258P	p. A288T
Pmut	http://mmb2.pcb.ub.es:8080/PMut/	Pathological	Neutral
iPTREE	http://210.60.98.19/IPTREEr/iptree.htm	Negative	Negative
PANTHER	http://www.pantherdb.org/	Deleterious	Deleterious
Polyphen	http://genetics.bwh.harvard.edu/pph/	Possibly Damaging	Benign
SIFT	http://sift.jcvi.org/	Not Tolerated	Not Tolerated

The bioinformatic results obtained indicated that the two identified alterations in *NLGN4X* had potentially damaging effects which led us to study these alterations *in vivo* (Table 2.4). The alterations in *NLGN4X* and wild type *NLGN4X* were transfected into the PC-12 cell line to determine cellular localization and function. PC-12 cells were transfected with wild type *NLGN4X*, c. 775 T>C *NLGN4X*, c. 865 G>A *NLGN4X*, or lac Z (control). Western analysis also indicated that the NLGN4X protein was of appropriate size and transfected PC-12 cells were expressing the protein (Figure 2.3). There does appear to be a difference in the size of the NLNG4X protein expressed *in vitro* as compared to the protein expressed *in vivo*. This may be a result of posttranslational modifications that may have taken place *in vivo*.

PC-12 cell line transfections were visualized using a Zeiss Ax10 inverted Fluorescence Microscope (Figures 2.4 and 2.5). The cells transfected with wild type *NLGN4X* appear similar to untransfected cells and cells transfected with lacZ. The cells transfected with wild type NLGN4X have a normal cell body, appropriately mimic neurite formation after treatment with nerve growth factor, and form an appropriate number of neurite extensions (after treatment with nerve growth factor). The wild type protein was observed at the cell membrane and was found in the neurite extensions and observed in the structures that mimic synapses. These structures would be the correct location based on *NLGN4X* involvement in synapse formation. PC-12 cells transfected with mutant c. 775 C>T *NLGN4X* were very different in appearance when compared to cells transfected with wild type *NLGN4X*, lac Z, or untransfected cells. Cells expressing the mutant c. 775 C>T NLGN4X protein have an abnormal cell body that appeared larger in size. The cell body appears more like a flattened body than the normal cell body that has the stretched appearance from the growth of the neurite extensions. The neurite extensions of these cells were significantly shorter (t-test *P*-value=1.58E-12 and Figure 2.5) and had significantly less neurite extensions (after treatment with nerve growth factor; t-test *P*-value=5.8E-05) then the wild-type cells. The altered protein did not localize to the cell membrane nor into the extensions the same way as the cells transfected with wildtype *NLGN4X* (Figures 2.4 and 2.5). The altered protein appears to stay localized close to the nucleus. PC-12 cells transfected with the c. 865 G>A *NLGN4X* were different from cells transfected with wildtype NLGN4X, lac Z, or untransfected cells. The cell body was larger and abnormal in shape. The cell body had

more of a blob shape instead of the normal stretching shape observed in neuronal cells. These cells did not form proper neurite extensions. The extensions were shorter in length and fewer in number (Figure 2.6). The altered protein was transported to the cell membrane and into the neurite extensions, but the neurite extensions do not appear to form correctly.

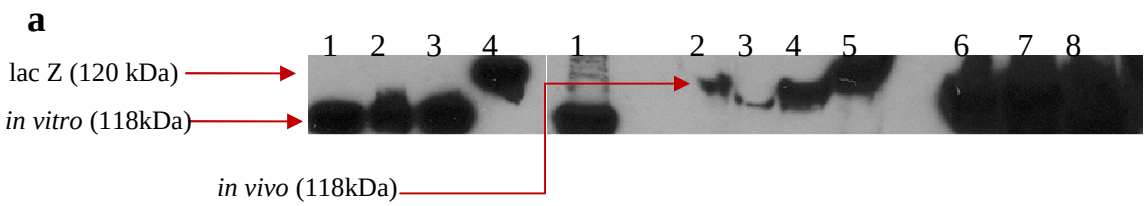

Figure 2.3 NLGN4X western analysis. *In vit*ro expression from the T7 translation of the expression construct used in the transfections and *in vivo* expression from cell lysate of transfected PC-12 cells. a. The lanes contained the following proteins: *in vitro* NLGN4X protein (1), *in vitro* c. 775 C>T NLNG4X protein (2), *in vitro* c. 865 G>A NLGN4X protein (3), *in vitro* lac Z protein (4) and *in vivo* protein of NLGN4X (2 and 6), *in vivo* c. 775 C>T NLGN4X (3 and 7), *in vivo* c. 865 G>A NLGN4X (4 and 8), and *in vivo* lac Z (5).

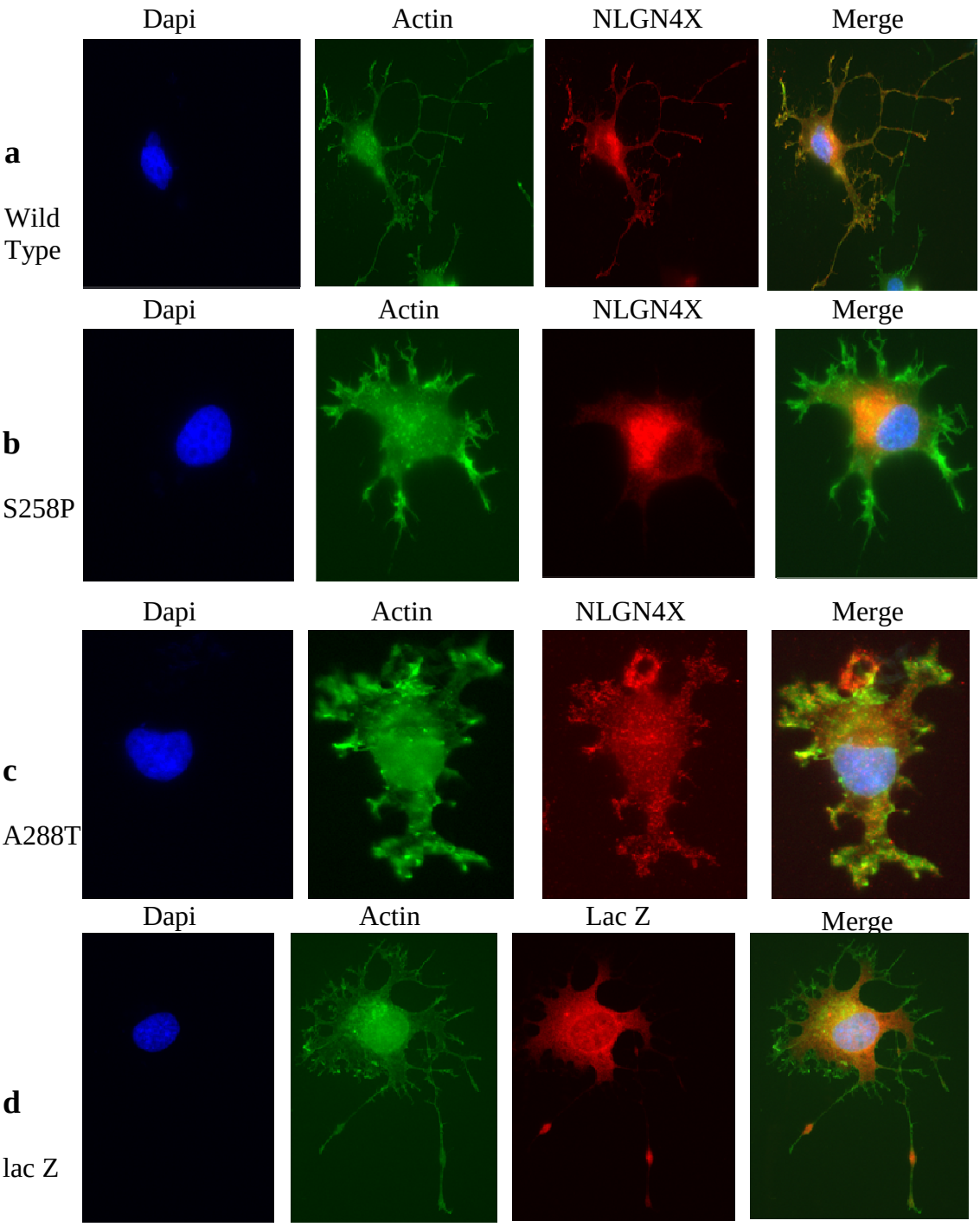

Figure 2.4 Cellular localization and function of NLGN4X transfected PC-12 cells. (a) *NLGN4X* (red) is found throughout the cytoplasm, cell membrane, and neurite extensions. The cellular outline is depicted by phalloidin staining of actin (green) and the nucleus is depicted by the dapi stain (blue). (b) *NLGN4X* with c. 775 C>T alteration (red) is found in the cytoplasm but does not appear to be at the cell membrane or in the neurite extensions. (c) *NLGN4X* with c. 865 G>A alteration (red) is found in the cyptoplasm, cell membrane, and in neurite extensions. (d) lac Z (red) is found in the cytoplasm, cell membrane, and in neurite extensions.

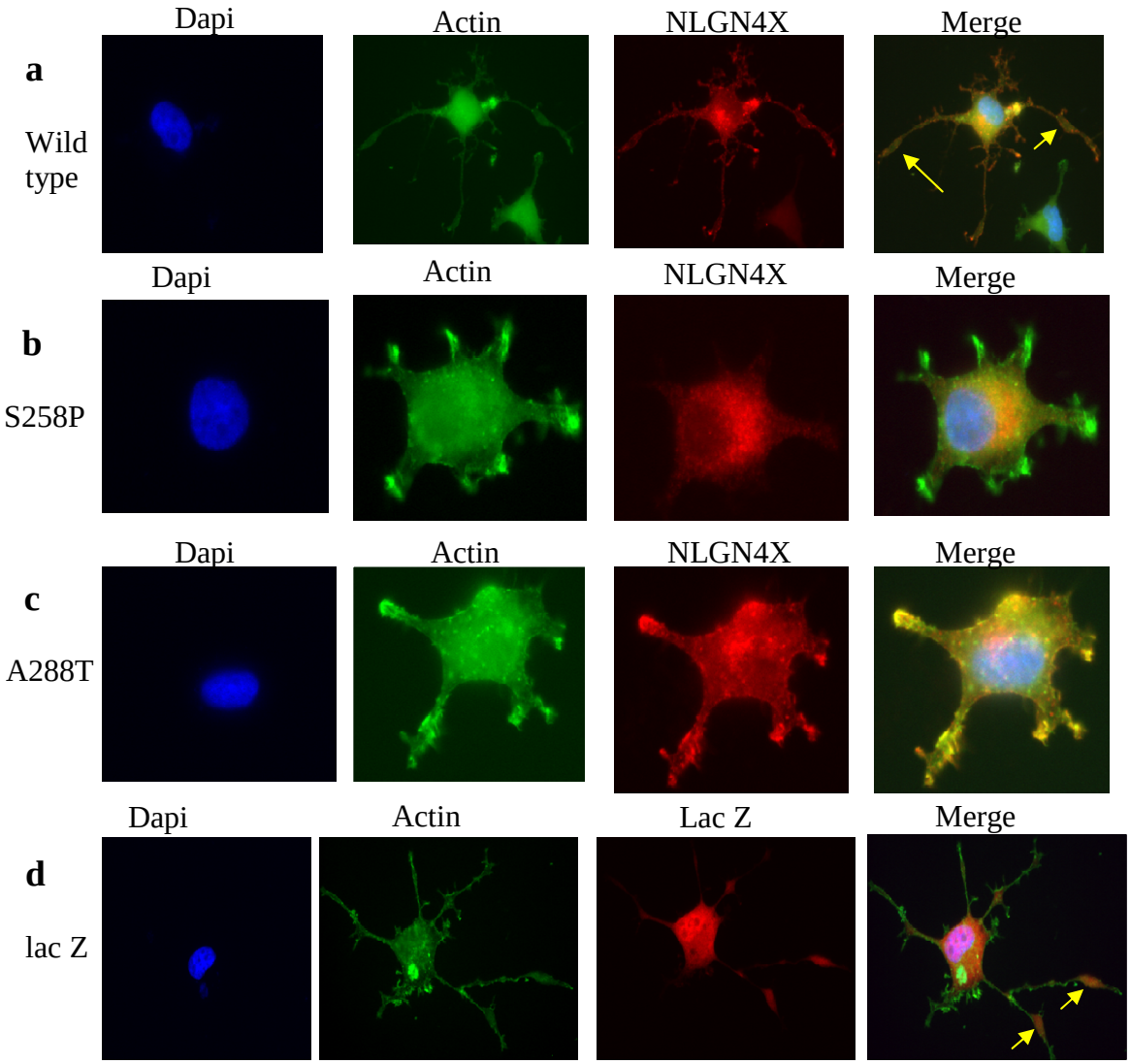

Figure 2.5 Cellular localization and function of NLGN4X transfected PC-12 cells. Yellow arrow indicates synapse formation. (a) *NLGN4X* (red) is found throughout the cytoplasm, cell membrane, and neurite extensions. The cellular outline is depicted by phalloidin staining of actin (green) and the nucleus is depicted by the dapi stain (blue). (b) *NLGN4X* with c. 775 C>T (red) alteration is found in the cytoplasm but does not appear to be at the cell membrane or in the neurite extensions. (c) *NLGN4X* with c. 865 G>A (red) alteration is found in the cyptoplasm, cell membrane, and in neurite extensions. (d) lac Z (red) is found in the cytoplasm, cell membrane, and in neurite extensions.

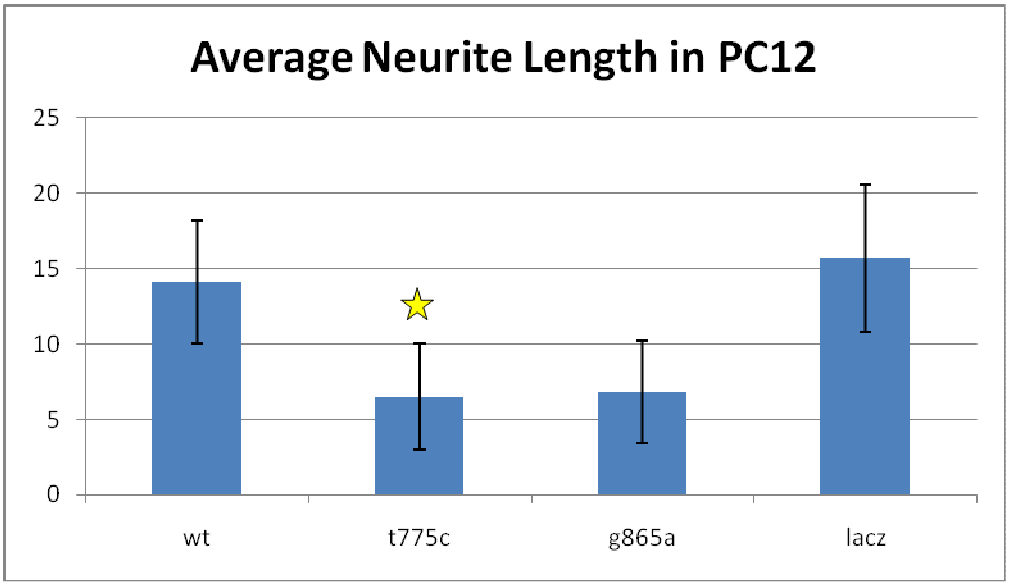

Figure 2.6 Average neurite length of NLGN4X transfected cells. PC-12 cells transfected with wild type NLGN4X are labeled as wt, cells transfected with c. 775 C>T are labeled as t775c, cells transfected with c. 865 G>A are labeled as g865a, and cells transfected with lac Z are labeled as lacz. The star above t775c indicates that the neurite lengths for these cells were significantly shorter than cells transfected with wild type NLGN4X (t-test *P*-value=1.58E-12).

An endogenous Golgi apparatus protein in PC-12 cells was also used to study cellular localization. The Golgi plays a role in modifying and sorting some proteins. If there was a problem modifying or sorting these altered NLNG4X proteins, the protein may be localizing in the Golgi apparatus and not properly transported in the cell.

The GS28 Golgi protein was stained with chicken anti-mouse Alexa fluor 488 (Molecular Probes) after cells were transfected with either wild type *NLGN4X*, mutant c. 775 C>T *NLGN4X*, mutant c. 865 G>A *NLGN4X*, or with lac Z. Colocalization in the Golgi would exist if the protein of interest was located in the Golgi. There does not appear to be any colocalization of the endogenous Golgi protein and wild type *NLGN4X*, the two mutants, or lac Z (Figure 2.7).

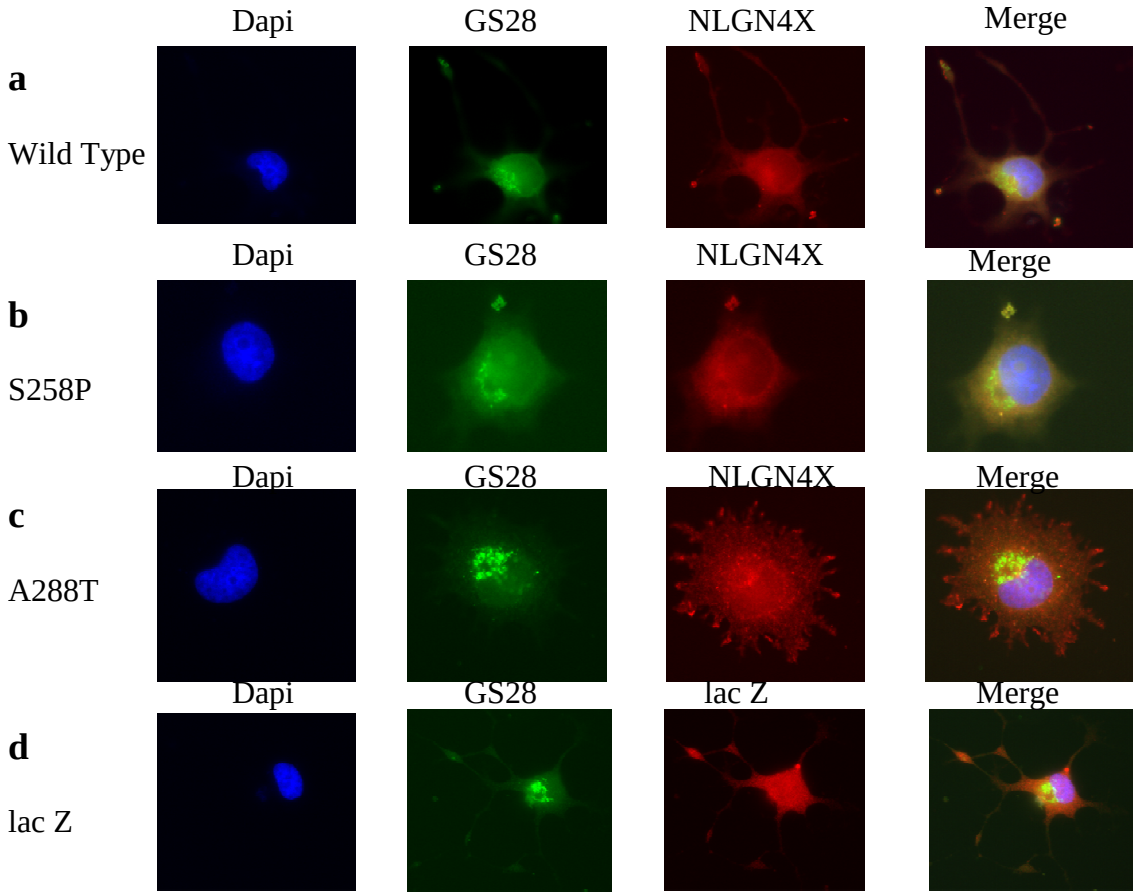

Figure 2.7 GS28 cellular localization in NLGN4X transfected PC-12 cells. (a) NLGN4X (red) is found throughout the cytoplasm, cell membrane, and neurite extensions. The Golgi specific protein, GS28, is depicted by green and the nucleus is depicted by the dapi stain (blue). (b) NLGN4X with c. 775 C>T alteration (red) was found in the cytoplasm but does not appear to be in the Golgi apparatus. (c) NLGN4X with c. 865 G>A alteration (red) was found in the cyptoplasm, cell membrane, and in neurite extensions but does not appear to be located in the Golgi apparatus. (d) lac Z (red) was found in the cytoplasm, cell membrane, and in neurite extensions and not in

Cell fractionation studies were conducted to further determine the location of NLGN4X in the PC-12 cells (Figures 2.8 and 2.9). This study supported previously observed cellular localization of *NLGN4X* wild type, c. 775 C>T *NLGN4X* altered protein, c. 865 G>A *NLGN4X* altered protein, and lac Z. The amount of wild type

NLGN4X protein in each cellular fraction compared to the amount of c. 775T>C altered NLGN4X protein in each cellular fraction was different. This indicated a difference between the wild type NLGN4X protein localization and the c. 775T>C NLGN4X alteration protein localization. There was a greater percentage (32.5% vs. 24.6%) of c. 775 T>C NLGN4X in the cytosol fraction, less in the membrane/particulate fraction (45% vs. 59.1%), and more in the cytoskeletal fraction (10% vs. 2.7%) as compared to wild type *NLNG4X*. The amount of wild type NLGN4X in each fraction when compared to the amount of c. 865 G>A NLGN4X in each fraction was different for some fractions but the same for others. The percentages for the cytosol fraction from the c. 865 G>A NLGN4X (18.2% vs. 24.6%) mutant were less than the wild type, the membrane fractions for the mutant and wild type were similar. There was much less c. 865 G>A NLNG4X (1.5% vs. 13.6%) protein in the nuclear fraction than the wild type, and there was no detectable protein for the cytoskeletal fraction from the c. 865 G>A mutant that was treated with nerve growth factor. Western analysis confirmed that cell fractions for different parts of the cell contained known proteins for the different cell fractions (Figure 2.8).

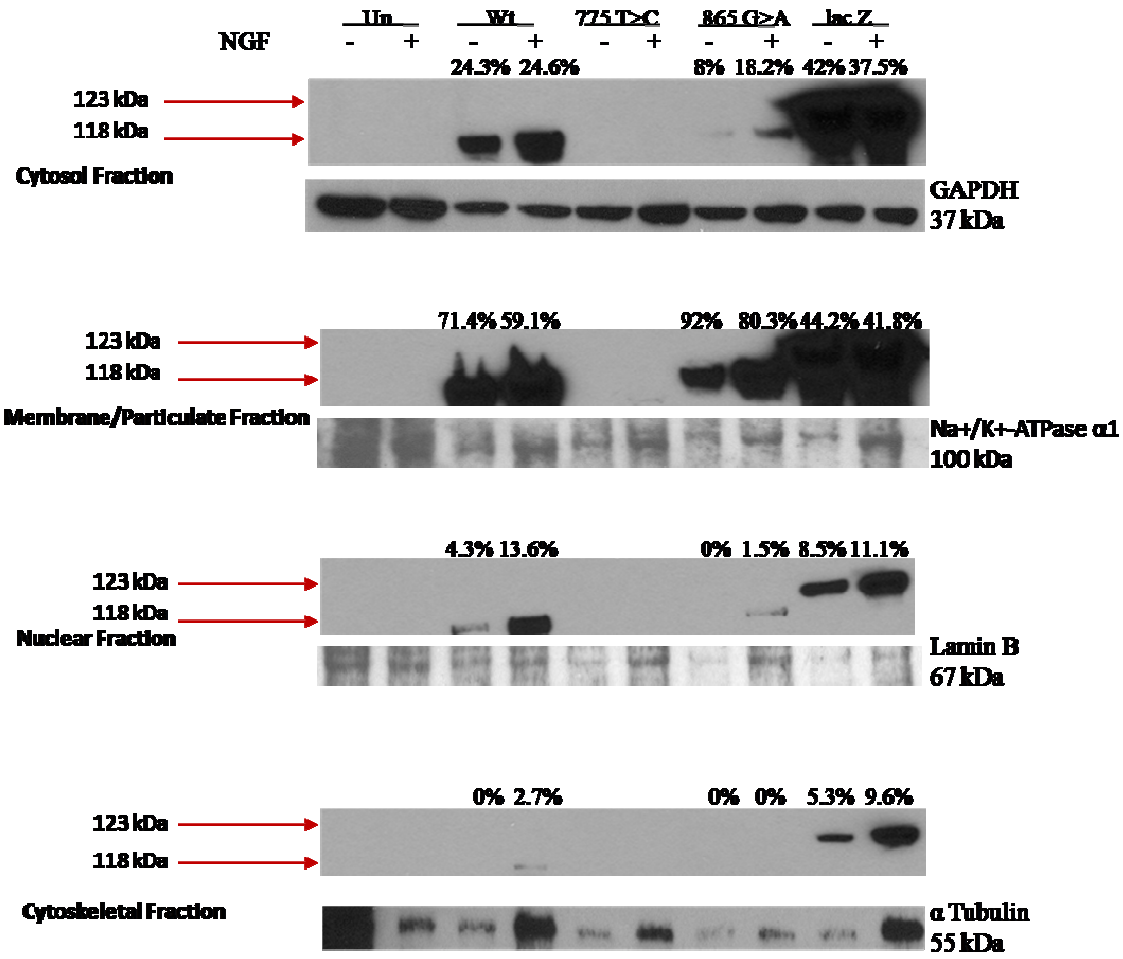

Figure 2.8 NLGN4X cell fractionation. Ten percent of each fraction for each transfection was loaded onto a protein gel and probed with the V5 antibody. Cytosol fraction for untransfected PC-12 cells, PC-12 cells transfected with *NLGN4X*, c. 775 C>T *NLGN4X*, c. 865 G>A *NLGN4X*, and lac Z. Membrane/particulate fraction for untransfected PC-12 cells, PC-12 cells transfected with *NLGN4X*, c. 775 C>T *NLGN4X*, c. 865 G>A *NLGN4X*, and lac Z. Nuclear fraction for untransfected PC-12 cells, PC-12 cells transfected with *NLGN4X*, c. 775 C>T *NLGN4X*, c. 865 G>A *NLGN4X*, and lac Z. Cytoskeletal fraction for untransfected PC-12 cells, PC-12 cells transfected with *NLGN4X*, c. 775 C>T *NLGN4X*, c. 865 G>A *NLGN4X*, and lac Z. Below each cellular fraction is the protein gel probed with a specific antibody for each cellular fraction to confirm that each fraction is specific for its location. The cytosol fraction was probed with the GAPDH antibody, the membrane/particulate fraction was probed with the Na+/K+-ATPaseα1 antibody, the nuclear fraction was probed with the lamin B antibody, and the cytoskeletal fraction was probed with the α tubulin antibody.

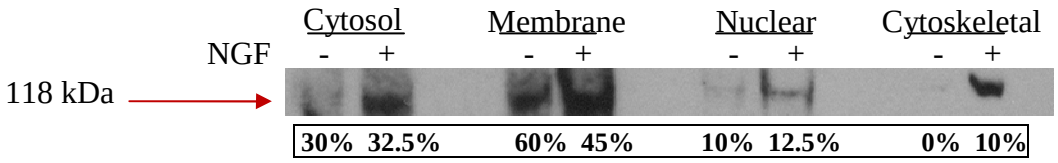

Figure 2.9 NLGN4X cell fractionation of c. 775 T>C. Previously loaded 10% of the c. 775 T>C fractions were not visible after Western analysis. The experiment was repeated loading 50% of each fraction from the c. 775 T>C transfection and probed with the V5 Antibody. Each lane is indicated as having nerve growth factor (NGF) or not having NGF treatment. Each lane is indicated with the representative cellular fraction.

Discussion

The high ratio of males to females (4:1) diagnosed with autism has lead to the assumption that causative genes for ASD are located on the X chromosome. Studies have resulted in the identification of genes on the X chromosome involved with Autistic Disorder (Centers for Disease Control and Prevention, 2009; Ylisaukko-oja et al., 2005). This study screened an autistic population looking for possible alterations in one of these genes on the X chromosome, *NLGN4X*. This study presents the identification of two novel *NLGN4X* alterations (c. 775 C>T and c. 865 G>A), *in vivo* protein expression for these two alterations, and cellular localization of these two alterations.

The c. 775 C>T *NLGN4X* alteration is a *de novo* alteration found in a male proband. The c. 865 G>A *NLGN4X* alteration was identified in a female proband and is possibly of paternal inheritance. This is perplexing since the digestion and sequence analyses of the father indicate that he contains the altered allele and the normal allele. It is expected that the father would only contain one allele for this gene since it is located

on the X chromosome. The father may have a XXY karyotype, be mosaic, or it is possible that the sample is contaminated with the proband's DNA. Testing of the father's sample did confirm that it contained material from the Y chromosome.

The autistic population screened for this study was from the SCAP (Schroer et al., 1998). This population represents a group of individuals diagnosed with Autistic Disorder only. The participants were identified using the Autism Diagnostic Interview-Revised to test adaptive function. Cognitive skills were tested using Bayley Scales of Infant Development and Stanford-Binet Intelligence Scale: Fourth Edition (Schroer et al, 1998).

The *NLGN4X* gene belongs to the neuroligin family (Chih et al., 2004). This family of genes is composed of neuronal cell-surface proteins located in the synaptic structures (Chih et al., 2004 and Talebizadeh et al., 2004). Every member contains a conserved domain that is critical for presynaptic neurexin binding, a transmembrane domain, and a short cytoplasmic tail (Talebizadeh et al., 2004). Their location in the glutamatergic synapses suggests a role in targeting excitatory synapses. Previous studies also indicate their involvement in the ability to trigger the formation of functional presynaptic elements (Benson et al., 2000; Scheiffele, 2003; Waites et al., 2005). This leads to axon specialization, and their involvement in synaptogenesis (Scheiffle et al, 2000). *NLGN4X* plays an important role in the normal function of all these processes. Abnormal function in autistic individuals may lead to an alteration in synapses or abolish the formation of synapses required for communication processes.

PC-12 cells treated with nerve growth factor can mimic neuronal cells and their formation of neurites. Functional cellular analysis from PC-12 cells treated with nerve growth factor demonstrates that cells transfected with c. 775 C>T or c. 865 G>A (altered NLGN4X protein) do not form proper neurite extensions as compared to cell transfected with wild type NLGN4X protein and controls. The cells form extensions that are shorter in length and fewer in number. The protein that results from the c. 775 C>T alteration does not appear to traffic properly through the cell to reach the cell membrane or the location for neurite extension formation. The NLGN4X protein containing the c. 865 G>A alteration expressed in PC-12 appears to localize to the same locations as wild type NLGN4X but does not form neurite extensions of equal length or number. In cells transfected with wild type NLGN4X and lac Z, a control protein, a structure that mimics a synapse, can be observed. These structures are not observed in cells with either the c. 775 C>T or c. 865 G>A altered NLGN4X protein. These results indicate that these novel alterations identified in *NLGN4X* may lead to abnormal formation of synapses or possibly the absence of synapse formation. Both of these novel alterations are located in the extracellular noncatalytic acetylcholinesterase homolog domain of *NLGN4X*. This is the major extracellular domain of NLGN4X and all neuroligins that mediates binding to neurexin. This is the domain in neuroligins that also forms homomultipliers which are structural associations that are important for function (Comoletti et al., 2003; Dean et al., 2003).

Alterations in the extracellular noncatalytic acetylcholinesterase homolog domain could lead to altered association with neurexin which could result in synapses not being

formed and/or poor stability. The abnormal formation of synapses in the individuals with these alterations may lead to their diagnosis of autism. Further study of brain development and the presence of abnormal synapse formation are needed to more clearly understand how these alterations affect cognitive problems observed in these individuals.

This study screened a cohort of individuals diagnosed with Autistic Disorder and a cohort of controls and identified two alterations in *NLGN4X*. The c. 775 T>C *NLGN4X* alteration is a *de novo* alteration, while both alterations were determined to not be rare single nucleotide polymorphisms. Studies were conducted that observed aberrant cellular localization and cell morphology due to the presence of the two *NLGN4X* alterations expressed in PC-12 neuronal cell line. Fluorescence microscopy determined that neuronal cells expressing the two alterations identified in *NLGN4X* did have altered cell morphology. Cellular localization studies also identified a difference between the location of the altered NLGN4X protein within the cell and the location of wild type NLGN4X in the cytosol, membrane, nucleus, and cytoskeleton of the cell.

Future studies are needed to determine if the father of the patient with the c. 865 G>A NLGN4X alteration has a karyotype of XXY or if his sample used for these studies was contaminated with the patient's sample. Future studies are also needed to further understand *NLGN4X*'s role in synapse formation and cognitive function.

Chapter 3

IDENTIFICATION OF CANDIDATE GENES FOR AUTISM SPECTRUM DISORDER IN A PATIENT WITH A t(2;16)(q21q13) TRANSLOCATION

Introduction

Autism Spectrum Disorder (ASD) is a collection of neurodevelopmental disorders that affect development, communication, social skills, behaviors, and interests of affected individuals. Autistic Disorder is the most severe and attention deficit disorders are the least severe of the disorders in the spectrum. Autism Spectrum Disorder affects one in 110 children (Centers for Disease Control and Prevention, 2009). This high prevalence has increased the need to identify causative genes for ASD.

Genome-wide linkage scans have previously identified chromosomal locations that may contain causative genes for ASD. These studies identified that the 2q chromosomal region potentially contains causative gene(s) for ASD (Barrett et al., 1999; Herba et al., 2008; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al., 2006). These genes could be identified by mapping the breakpoints of cytogenetically visible chromosomal aberrations in individuals with ASD.

An individual with autistic features and a 46XY, t(2;16)(q21;q13) karyotype was studied. Currently there are no known causative genes for ASD located in the 2q21 chromosomal region that contains the translocation breakpoint. This study narrowed the

translocation breakpoint on chromosome 2q21 to an area of 80kb and identified a putative causative gene in this region.

Materials and Methods

Case Report

CMS5326 is a male whose height was measured at the 2nd percentile and head circumference was measured at the 90th percentile at 31 years of age. At his birth he had a "pin hole" anal opening that was covered with a membrane. He has autistic features that include repetitive behaviors and poor social skills. His karyotype was determined to be 46, XY, t(2;16)(q21;q13).

Molecular Cytogenetics

BAC clones were identified and selected by searching for the breakpoint region on chromosome 2q21 using the NCBI (http://www.ncbi.nlm.nih.gov/genome/guide/human) database. BAC clones were obtained from BACPAC (Chori BACPAC Resources). DNA was purified using Qiagen Mini-Prep colums. DNA was labeled by incorporation of digoxigenin-11-dUTP or biotin-16-dUTP (Roche) by nick translation using DNA polymerase (Life Technologies). Labeled probes were combined with 50X human Cot-1 DNA (Gibco-BRL) and a chromosome 2 specific labeled centromeric alphoid probe to a final concentration of 20ng/μL. This product was then denatured at 76°C for 10 minutes and allowed to preassociate at 37°C for 20 minutes before hybridization.

Metaphase chromosome spreads were obtained from lymphoblastoid cells and FISH was performed using previously described methods (Ross et al., 1997; Vervoort et al., 2002). Replication banding was also performed in conjunction with FISH to provide secondary confirmation of chromosome identity. Labeled probes were visualized with rhodamine-labeled anti-digoxigenin and chromosomes were counterstained with DAPI. Images were examined under a Zeiss Axiphot fluorescence microscope equipped with FITC, DAPI, and triple band pass filter sets.

Microarray Analysis

Agilent Human Genome CGH 244 microarray analysis (Agilent Technologies) was used to determine if a deletion existed within the breakpoint region. This technique uses a two-color process to measure DNA copy number changes in an experimental sample relative to a reference sample. The process of dye-swapping was utilized for each experimental sample to provide confirmation of results. The reference sample was normal commercially available genomic DNA. The manufacturer's protocol was followed, which included digestion of one microgram of genomic DNA by adding 5.9µL Digestion Master Mix (2µL nuclease-free water, 2.6µL 10X buffer, 0.2µL of 10µg/µL acetylated BSA, 0.5µL of 10U/µL *Alu I*, and 0.5µL of 10U/µL *Bsa I*). Digestion reactions were incubated at 37ºC for two hours and then transferred to 65ºC for 20 minutes to inactivate the restriction endonucleases. Samples were then labeled individually with cyanine 3-dUTP and cyanine 5-dUTP. Labeling was conducted by adding 21µL of Labeling Master Mix (2µL nuclease-free water, 10.0µL of 5X buffer,

3µL of 1.0mM cyanine 3-dUTP or 3µL of 1.0mM cyanine 5-dUTP, and 1µL Exo-Klenow fragment) to each sample. Samples were allowed to incubate at 37ºC for two hours and then transferred to 65ºC for ten minutes to inactivate the enzyme. Labeled genomic DNA was diluted by adding 430µL of 1X TE to each reaction and purified as follows. Each sample was loaded onto a Microcon YM-30 filter (Agilent Technologies), centrifuged for ten minutes, and the flow-through was discarded. Appropriate purified cyanine 5-labeled sample and purified cyanine 3-labeled samples were combined and 5µL of 1mg/mL of Cot-1 DNA, 11µL of Agilent 10X Blocking Agent, and 55µL of Agilent 2X Hybridization buffer were added to each sample. Samples were incubated at 95ºC for three minutes and then they were incubated at 37ºC for 30 minutes. A gasket slide in the Agilent SureHyb chamber (Agilent Technologies) was loaded with 490µL of sample. The assembled slide chamber was placed in a rotator rack in a hybridization oven at 65ºC with rotation for 24 hours. After this incubation, samples were washed in Oligo aCGH Wash Buffer 2 (Agilent Technologies) at 37ºC overnight. Next, the slides were washed and removed from the assembly in Oligo aCGH Wash Buffer 1 (Agilent Technologies). Slides were scanned using the GenePix 4000B scanner (Agilent Technologies) and the Feature Extraction software v9.5 (Agilent Technologies) was used to extract microarray TIFF images.

The Agilent Human Genome CGH 244 Microarray was found to be probe poor in the 2q21 chromosomal area, so we utilized a different microarray that had better coverage in this area (Agilent Technologies). The Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 array was utilized to identify if any deletions existed in the translocation

breakpoint. This array contains 906,600 thousand unique SNPs probes and 945,826 copy variant probes. A genomic DNA control (Reference Genomic DNA 103) provided with the the Affymetrix array was utilized as the experimental positive control. The DNA control was also used to assess the quality of the sample genomic DNA. Manufacturer's protocols were followed that included digesting 250ng of sample genomic DNA with *Sty I* restriction endonuclease in a *Sty I* digestion master mix and a separate digestion of sample genomic DNA with *Nsp I* restriction endonuclease in a *Nsp I* digestion master mix (11.55µL AccuGENE® water, 2µL 10X NE buffer, 0.2µL 10mg/mL BSA, and 1µL 10U/µL *Sty I* or *Nsp I*). Samples were digested for 120 minutes at 37°C and then the enzyme was denatured at 65°C for 20 minutes. Digested samples (19.75µL of each sample) were ligated using the Sty Adaptor or Nsp Adaptor (Affymetrix) in a ligation reaction containing 2.5µL 10X T4 DNA Ligase Buffer, 0.75µL of 50µM Adaptor Sty I or Adaptor Nsp I, and 2µL of 400U/µL T4 DNA Ligase. Samples were ligated at 16°C for 180 minutes. After ligation 10µL of each sample was amplified using TITANIUM *Taq* DNA polymerase using the primer provided by the manufacturer (Affymetrix). Amplification products were visualized on a 2% TBE gel under ultra violet light to verify that the product had a size between 200 base pairs and 1100 base pairs. The amplified sample for each enzyme was pooled together and purified using the manufacturer's supplied magnetic beads and filter plate and then each sample was quantitated. Amplification products were fragmented using 5µL of 0.1U/µL of Fragmentation Reagent (Affymetrix) at 37°C for 35 minutes. Fragmentation was visualized on a 4% TBE agarose gel under ultra violet light. Fragmented samples were then labeled using

2µL of 30mM DNA labeling Reagent and 3.5µL of 30U/µL TdT enzyme (Affymetrix) at 37°C for four hours. Each reaction was then loaded onto a Genome-Wide Human SNP Array 6.0 and allowed to hybridize for 18 hours. The array was then washed and stained in the Fluidics Station 450 (Affymetrix). The array was then scanned with the GeneChip Scanner 3000 7G (Affymetrix). Results were analyzed using the Genotyping Console 2.1 program (Affymetrix).

Quantitative Real-Time PCR

RNA was extracted from transformed human lymphoblastoid cell lines. RNA was purified by treatment with two units of TURBO™ DNase (Ambion) for every 50µg of RNA for 30 minutes at 37°C. RNA was further purified by precipitation with 7.5M lithium chloride solution (Ambion). Quantitative Real-Time PCR was conducted using the iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad, Hercules, CA) on the iCycler iQ™ Real-Time PCR system (Bio-Rad, Hercules, CA). Experimental primer sequences used for amplification were: FKSG30-RT-F-5'- cacaaagcttaaaggaagtgaaa-3', FKSG30-RT-R-5'- ttcttcttcaatagccataaaatcttc-3', MGC50273-F-5'-aagaggacgcagcctgtcta-3', and MGC50273-R-5'-cgctgcttctggggtaca-3'. The NEPH2F (5'-gggccatcagagctaaagacc-3') and NEPH2R (5'- cttgggggaagtggaggttta-3') primers amplified exon 16 of the *NEPH2* reference gene. Cycling conditions were as follows: denaturation at 95ºC for five minutes followed by the two-step amplification cycles at 95ºC for 10 seconds and 58ºC for 30 seconds repeated 40 times. All samples were analyzed in quadruplicate and the data obtained was analyzed with the iCycler iQ™

software to generate a standard curve for each gene and calculate fluorescence generated from the experimental primer pairs relative to the *NEPH2* gene using the comparative Ct method (Bio-Rad, Hercules, CA).

PCR Analysis

DNA from BAC clones RP11-25F5, RP11-611E16, and RP11-890E24 were amplified using primers listed in Table 3.1. Amplification was carried out in 50µL reactions containing 105 ng DNA, 5µL of 10µM of each appropriate primer, 0.8µL of dNTPs, 10µL of 5X PCR buffer, and 0.5µL Go Taq DNA polymerase. Amplification conditions included an initial denaturation of 95^{o}C for 5 minutes, then 30 cycles consisting of a denaturation step at 95^{o}C for 30 seconds, optimal annealing temperature (Table 3.1) for 30 seconds, extension at 72^{o}C for 30 seconds, followed by a final extension at 72^{o}C for 7 minutes. PCR products were visualized on an agarose gel containing ethidium bromide under ultraviolet light.

Table 3.1 Translocation primer list

Primer Name	Primer Sequence	Annealing Temperature
A Forward	gataccgctgtgctcgtcattg	55ºC
A Reverse	cagttcccgcccagccaggtcta	55ºC
B Forward	gcacagaggggcaggagtagttca	55ºC
B Reverse	agttttcccctgtggcggtagag	55ºC
C Forward	aggccaacagttcccagagtgacc	58ºC
C Reverse	ccttccgggattagaccttgag	58ºC
D Forward	aggccaacagttcccagagtgacc	58ºC
D Reverse	ccttcccgggattagaccttgag	58ºC
E Forward	gggccctttctccaacactat	55ºC
E Reverse	aaaaaccctcccccaaaacag	55ºC
F Forward	tcccacattcagtttctcagtttg	55ºC
F Reverse	ccagcagctacaggtaaggaata	55ºC

Results

CMS 5326 had a karyotype identified to be t(2;16)(q21;q13). BAC clones RP11-611E16, RP11-890E24, and RP11-25F5 were identified to be located in the translocation region on chromosome 2q21 and were used in FISH analysis to map the translocation breakpoint critical region (Figure 3.1). The boundaries of the chromosome breakpoint region were established with BAC probe RP11-611E16, which was proximal to the breakpoint (signal was on the normal and derivative chromosome 2), and BAC probe RP11-25F5, which was distal to the breakpoint (signal on the normal chromosome 2 and derivative chromosome 16) (Figure 3.1). BAC clone RP11-890E24 spans both BAC clones RP11-611E16 and RP11-25F5 according to the NCBI database. However, this clone did not span the breakpoint by FISH analysis. Rather it was found to be distal to the breakpoint (signal was found on the normal chromosome 2 and derivative chromosome 16) (Figure 3.1). At this point a deletion was suspected in the patient that carried this translocation and FISH analysis was followed by microarray analysis to determine if a deletion was present.

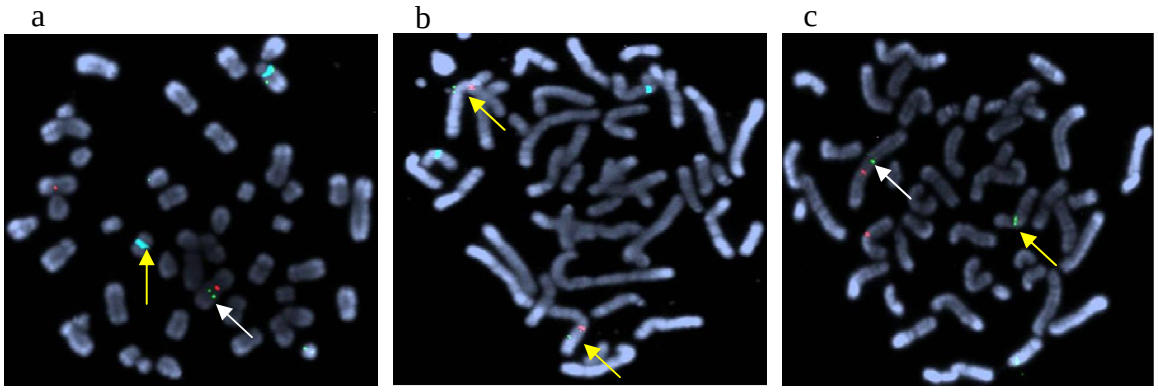

Figure 3.1 FISH analysis of chromosome 2 breakpoints involved in a t(2;16)(q21;q13). (a) FISH analysis of distal probe RP11-25F5 (green). The white arrow indicates a signal on normal chromosome 2 and the yellow arrow indicates a signal on derivative chromosome 16. (b) FISH analysis of proximal probe RP11-611E16 (green). Yellow arrows indicate a signal on normal and derivative chromosome 2. (c) FISH analysis of distal probe RP11-890E24 (green). The white arrow indicates a signal on normal chromosome 2 and yellow arrow indicates a signal on derivative chromosome 16. A centromeric control probe for chromosome 2 is shown in red.

Comparative Genomic Hybridization (CGH) was utilized to determine if a deletion in the translocation breakpoint region on chromosome 2q21 existed. Comparative Genomic Hybridization is a molecular-cytogenetic technique that can detect copy number changes in an individual's DNA. Balanced reciprocal translocations and inversions are not detected since these alterations do alter the copy number of DNA. The Agilent Human Genome CGH 244 Microarray was utilized first. A change in dosage was not observed at the translocation breakpoint 2q21 (Figure 3.2). However, there were only a few probes from the microarray that covered the translocation breakpoint region for chromosome 2. The Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0

microarray was then utilized. This array has over one million unique probes that cover the human genome (906,600 unique SNPs probes and 945,826 copy variant probes). Probe coverage for the translocation breakpoint region was good but no deletion or loss of heterozygosity was identified in this region (Figure 3.3).

a

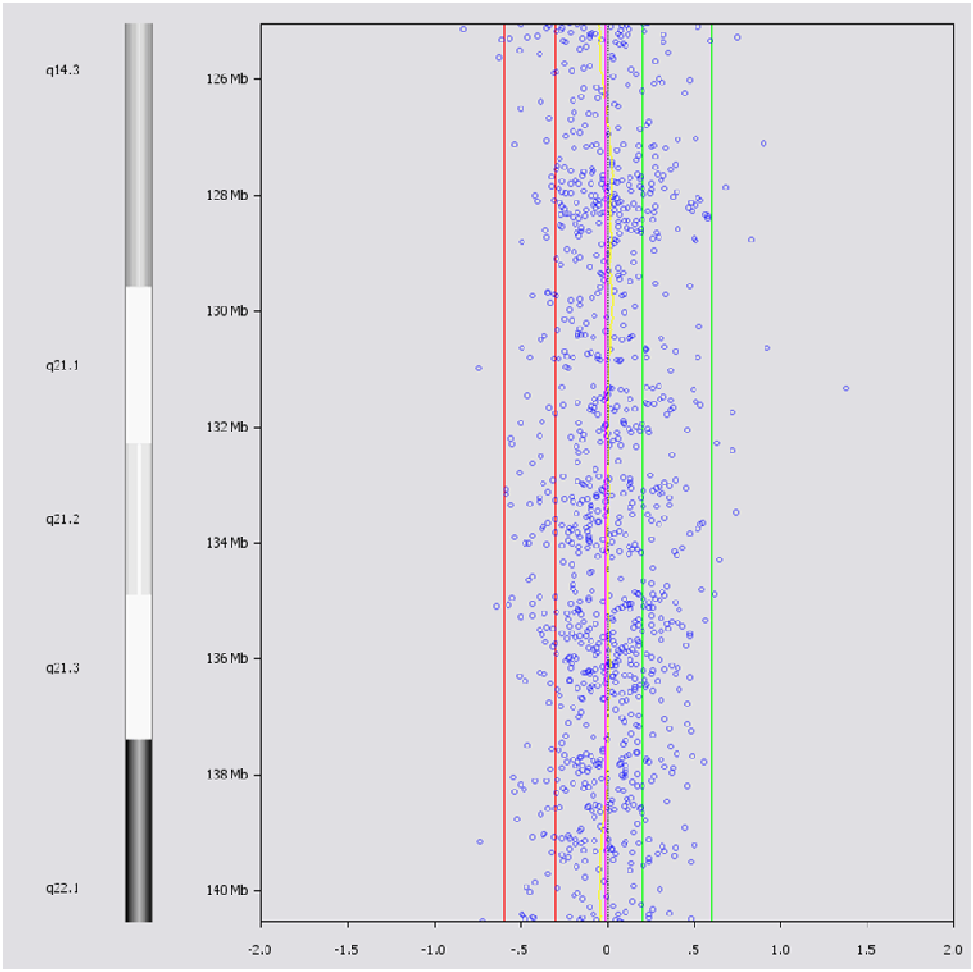

Figure 3.2 Agilent Human Genome CGH 244 Microarray Analysis. (a) Translocation breakpoint region for chromosome 2q21. Blue circles represent probes from the microarray. Probes past the outer green line represent a duplication or gain of gene dosage. Probes past the outer red line represent a deletion or loss of gene dosage. There were not any duplication or deletions identified.

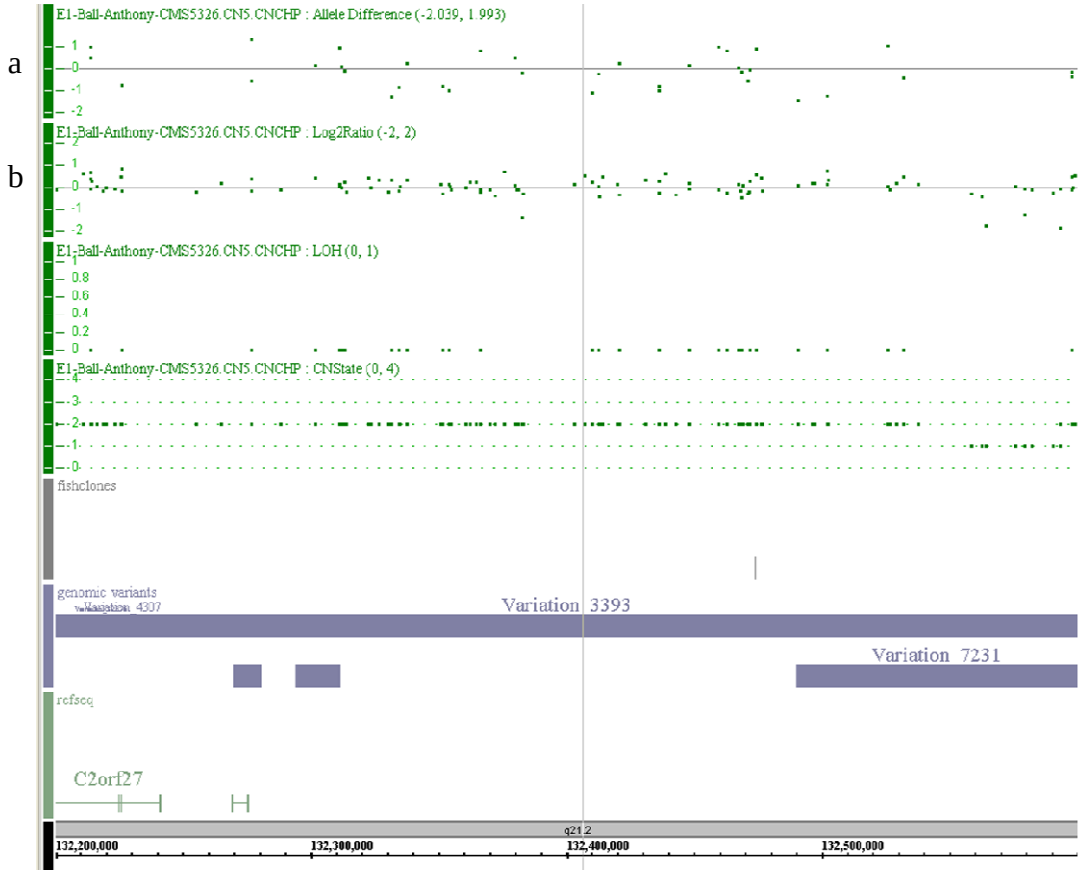

Figure 3.3 Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 Microarray Analysis. (a) Copy number variant probes in the 2q21 breakpoint critical region. There is no loss of heterozygosity identified. (b) SNPs probes in the 2q21 critical region. A duplication or deletion is not identified.

Quantitative Real-Time PCR (QRT-PCR) was conducted to identify a deletion within the region on chromosome 2q21 that contained the breakpoint. The boundaries for the breakpoint on chromosome 2q21 that were identified from FISH analysis were used to choose the genes for QRT-PCR analysis. Genes at the proximal and distal end of the breakpoint were chosen for this analysis. Primers for FKSG-30 and MGC50273 were used for expression analysis. Results from this expression analysis indicated a possible

duplication for this region but statistical error is high for this experiment and microarray analysis does not support these results (Figure 3.4).

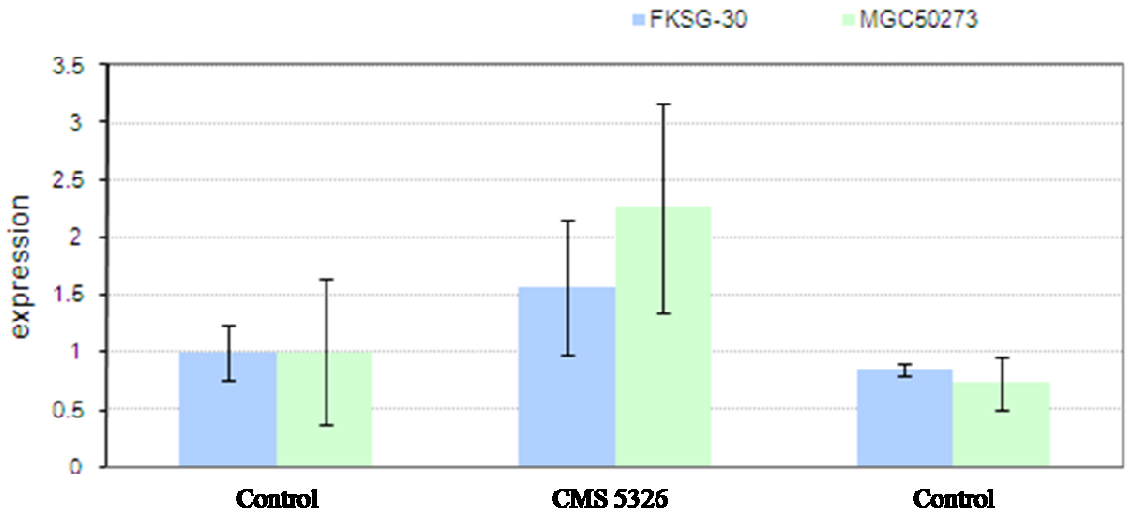

Figure 3.4 Quantitative RT-PCR. Quantitative RT-PCR analysis indicates possible duplication of FKSG-30 and MGC50273 between the translocation patient (CMS5326) and two control patients. Quantitative RT-PCR was performed in quadruplicate.

The results obtained from microarray analysis did not support results obtained from Quantitative RT-PCR analysis. In order to confirm that the FISH probes contained the correct sequence, each FISH probe (RP11-611E16, RP11-890E24, and RP11-25F5) was amplified by PCR to confirm that deletions were not present in any probe used and to confirm that each probe contained the indicated sequence. These results indicated that the areas of the probes that were amplified do not contain any deletions and indicate that the probes likely contain the appropriate sequence information (Figure 3.5). Further analysis of the FISH probe location indicates that *C2orf27A* is the only identified possible

gene in the 80 kb overlap region of the proximal (RP11-611E16) and distal (RP11-890E24) FISH probes (Figure 3.6).

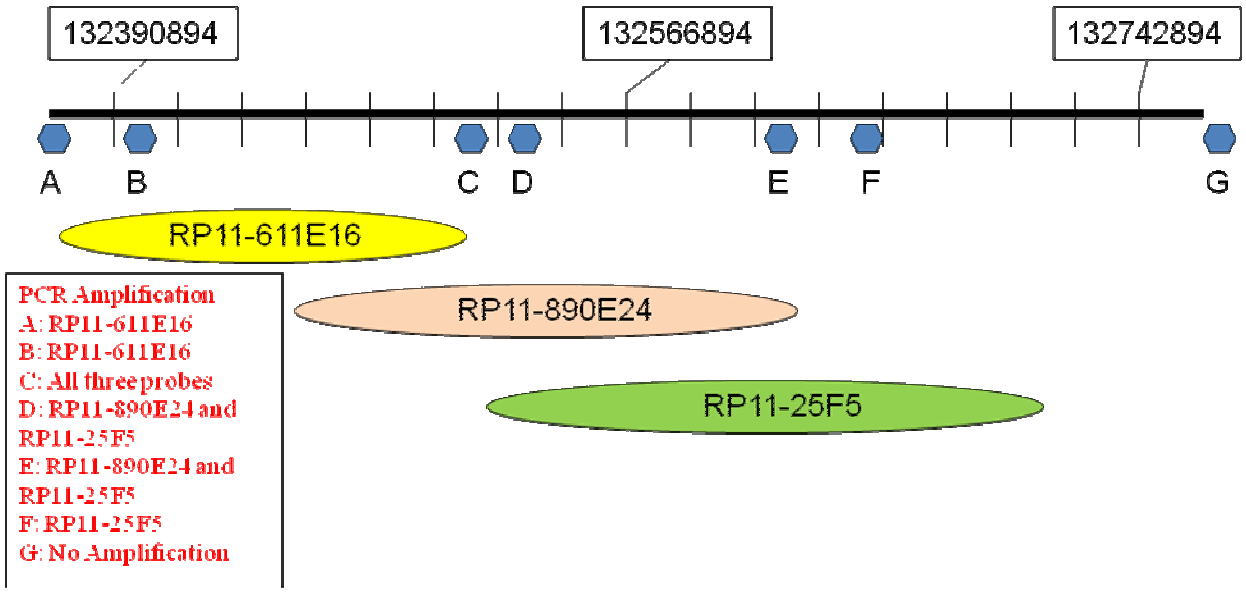

Figure 3.5 PCR Amplification of FISH probes. Blue hexagons indicate primer pairs that were used in the amplification of each FISH probe and their location in the 2q21 translocation region. The yellow oval depicts the area that RP11-611E16 covers in the 2q21 translocation breakpoint region. The blue oval depicts the area that the RP11-890E24 covers in the 2q21 translocation breakpoint region. The green oval depicts the area covered by RP11-25F5 in the 2q21 translocation breakpoint region. The boxes indicate the location of these probes in base pair on chromosome 2 in human genome build 36.1.

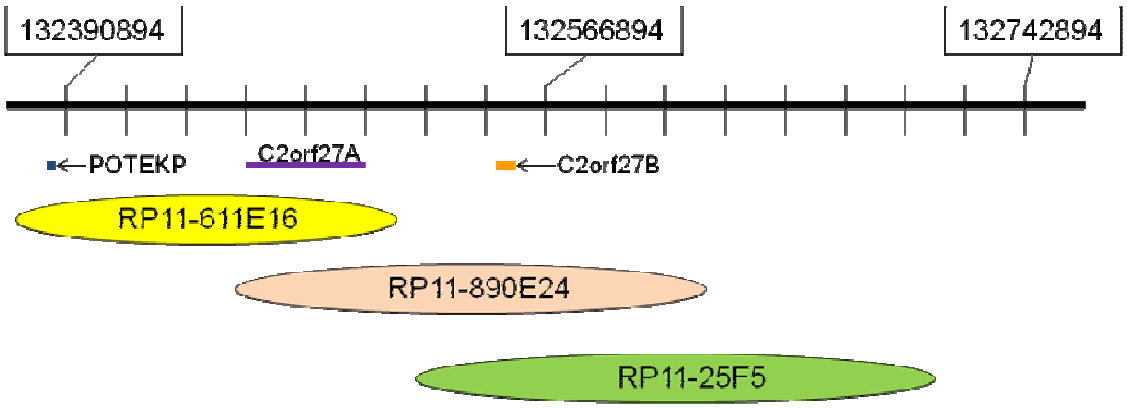

Figure 3.6 Schematic representation of the location of FISH probes and genes. The location of FISH probes and genes found within this region are represented to scale.

Discussion

This study was unable to isolate the translocation breakpoint in the patient with ASD and karyotype 46,XY,t(2;16)(q21;q13). We were able to narrow the translocation breakpoint region at 2q21 to an area of 80kb by FISH. The boundaries of the translocation breakpoint identified by FISH analysis were utilized to analyze gene expression. Genes were chosen from the proximal end and distal end (*FKSG-30* and *MGC50273*) of the breakpoint region (Figure 3.4). Quantitative RT-PCR indicated a possible duplication of these two predicted genes. These results were not supported by data obtained from microarray analysis.

There is only one gene, *C2orf27A*, contained within this 80kb region that may contain the breakpoint. *C2orf27A* (chromosome 2 open reading frame 27A) is a predicted protein coding gene but very little is known about this gene. *POTEKP*

(*FKSG30*) and *C2orf27B* (chromosome 2 open reading frame 27B, *MGC50273*) were the genes utilized for RT-PCR analysis. These two genes lie just outside the 80 kb region that may contain the breakpoint. Very little is known about the predicted protein coding gene *C2orf 27B* (*MGC50273*). Bera et al. (2002) identified the *POTE* gene on chromosome 21q11.2 and a paralog of this gene is located at 2q13-q22. *POTEKP*, located at 2q21.1, is the prostate, colon, lung, breast, ovary, and pancreas ankyrin domain family, member K, pseudogene (Bera et al., 2002). The *POTE* gene is involved in carcinogenesis, localized on the inner side of the plasma membrane, and is mainly involved in facilitating membrane interactions (Bera et al.,2002; Das et al., 2007; Resh, 2006). More studies are needed to confirm that *POTEKP* is actually a pseudogene. It is possible that this gene is still producing a protein and abnormalities in the protein may account for the phenotype observed in the patient with this translocation. Abnormalities of *POTEKP* could lead to improper cell to cell communication in neurons. This may account for the social impairments and repetitive behaviors observed in Autism Spectrum Disorder.

The *TUBA3D* (tubulin, alpha 3d) gene is located at chromosome 2q21.1 and is a member of the *TUBA3* family. This gene is located outside the RP11-611E16 FISH probe. The exact function of this gene is not known, but it is speculated to be involved in formation of the hippocampus and the cortex because abnormalities in this gene lead to lissencephaly in humans (Keays et al., 2007; Nanda et al., 2008). Lissencephaly is an abnormality in the formation of the brain and results in intellectual disability (ID) and possibly epilepsy (Francis et al., 2006). Mutations in the mouse homolog *Tuba1* are

associated with impaired neuronal migration (Keays et al., 2007). The identification of chromosome 2q by genome-wide scans as a putative location for Autism Spectrum Disorder genes and the location of this gene within the translocation area in an individual with autistic features indicate a need for future studies involving alterations in *TUBA3D* and ASD.

C2orf27A is the most likely candidate gene causing the autistic features and macrocephaly observed in this patient due to its location within the 80kb region that contains the chromosomal breakpoint. Array data does not indicate a deletion or duplication within this region. Future studies are needed to identify the cause of the features observed in this patient. Further analyses of *C2orf27A*, putative causative gene, may identify alterations in this gene in this patient.

CONCLUSIONS

Autism Spectrum Disorder includes a wide range of disorders and phenotypes. This implies the involvement of many genes that lead to these disorders and phenotypes. Many studies are currently being conducted to identify causative genes for Autism Spectrum Disorder and several genes have been identified. The studies presented here associated Autistic Disorder with the rs1858830 C variant of *MET*, which is a putative causative gene for Autistic Disorder, identified two alterations in *NLGN4X* (putative causative gene for ASD), provided cellular localization and function for these alterations, and identified a putative causative genes, *C2orf27A*, for ASD.

The first study screened a cohort of individuals diagnosed with Autistic Disorder and controls to confirm an association of the rs1858830 C variant of the MET gene with Autistic Disorder. The results obtained showed an association between the rs1858830 C variant and Autistic Disorder.

NLGN4X is involved in synapse formation and stabilization (Talebizadeh et al., 2004; Ylisaukko-ofa et al., 2005) and has previously been shown to be associated with Autism Spectrum Disorder (Jamain et al., 2003; Laumonnier et al., 2004). The second study identified two alterations in *NLGN4X*, one alteration is a *de novo* alteration, c. 775 C>T. The inheritance of the c. 865 G>A alteration is unclear due to the proband's father. A resample is needed to clarify significance of this alteration in the father. The two alterations were determined not to be rare single nucleotide polymorphisms. Functional cellular studies and cellular localization studies were conducted to determine the effects

of these alterations *in vivo*. The analysis conducted with PC-12 cells indicates that both alterations affect the normal function of *NLGN4X*. Cells transfected with altered *NLGN4X* did not form as many neurties per cell as compared to cells transfected with wild type *NLGN4X*. Cells transfected with altered *NLGN4X* produced cells with neurites that were significantly shorter than cells transfected with wild type *NLGN4X*. These results indicate that *NLGN4X* may have a significant role in cognitive development.

The third study narrowed the translocation breakpoint region to 80kb located on chromosome 2q21 by FISH analysis in a patient with a 46XY, t(2;16)(q21;q13) karyotype. We attempted to identify the breakpoint at chromosome 2q since this region had previously been identified as possibly containing genes that cause Autism Spectrum Disorder. The individual who carries this t(2;16)(q21;q13) translocation has autistic features. Microarray analysis was conducted to further map the breakpoint, but the results did not identify the translocation breakpoint. A candidate gene for Autism Spectrum Disorder, *C2orf27A*, was identified in this 80kb region on chromosome 2q. Future studies are needed to identify the translocation breakpoint and to further study expression levels of this candidate gene.

The studies presented in this work provide a greater understanding of genes that cause Autism Spectrum Disorder by confirming the association with the rs1858830 C variant of the *MET* gene and Autistic Disorder and by studying the *in vivo* functions of two alterations in *NLGN4X*, a gene previously associated with Autism Spectrum Disorder. These studies also identified a candidate gene, *C2orf27A*, for Autism Spectrum Disorder located at chromosome 2q21.

APPENDIX A

Name	Ingredients
Eag I buffer (NEB buffer 3)	100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 1 mM Dithiothreitol pH 7.9 @ 25°C
Mnl I and HpyCH4III buffer (NEB buffer 4)	50 mM potassium acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol pH 7.9 @ 25°C
10X Bovine Serum Albumin (BSA)	In a 15 ml conical tube, dissolve 100 mg BSA (Sigma, A-8022) in 10 ml sdH_2O. Store at -20°C. Used as an enzyme stabilizer in some restriction enzyme digestions.
0.5 M EDTA, pH 8.0	In a 1 L beaker, add 186.12 g EDTA (Mallinkrodt through VWR, MK493104) to ~800 ml sdH_2O. Slowly add 15-20g of NaOH pellets and use the magnetic stirrer to dissolve. The disodium salt of EDTA will not go into solution until the pH approaches 8.0; adjust pH to 8.0 with concentrated HCl (Mallinkrodt through VWR, MK206204) or 4N NaOH. Add sdH_2O to 1 L. Autoclave and store at room temperature (should be refrigerated if not in common use).
10X Spermidine (40 mM)	Weigh out 152 mg Spermidine (Sigma S-2501) and pour it into a 15 ml screw-cap conical tube. Add sd H_2O to the 15 ml mark on the tube. Dissolve by vortexing well. Store at -20°C. Spermidine interacts with DNA to cause conformational changes thought to make recognition sites more available for cutting.
5X Lamelli Buffer	0.5M Tris-HCL pH6.8 1.75ml Glcerol(Glycrin) 4.5ml SDS (0.25g dissolved in 1ml Thris-HCL) 2ml 0.5g 0.25% Bromophenol blue (25mg in 10ml H20) 0.5ml B-mercaptoethanol 1.25ml Total of 10mls Store in fridge and protect from light
10X TBST	12.11 g Tris 87.66 g NaCl 5 mL Tween-20 1 g Na-azide Bring up the mix with 1 liter ddH20 distilled water

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