Molecular characterization of three mental illness susceptibility genes

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Molecular Characterization of Three Mental Illness Susceptibility Genes

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Bipolar disorder and schizophrenia are devastating mental disorders which share an overlapping constellations of symptoms, most notably the presence of psychosis. Overlapping genetic factors are involved in their pathogenesis and recently, genome-wide linkage scans and association studies have identified many new genes. However, studies focusing on functional follow-up for these genes are very rare, leaving a big gap in comprehending how these genes contribute to the risk of illness.

The goals of this thesis were to focus on three candidate genes: NRG1, ST8SIA2 and NCAM1, each associated with psychotic mental illness, and determine how these genes could be functionally involved in disease susceptibility. Working with case-control cohorts, analysis of the target genes was undertaken at the DNA, RNA and protein levels to identify possible variation examine its potential functional impact and identify any alteration in normal activity which may affect disease status.

The results from this thesis have provided a distinctive contribution to the knowledge about the mechanisms of risk provided by three candidate genes. Using this candidate gene approach, a novel schizophrenia risk mechanism was identified in which the well-known schizophrenia-associated haplotype 'HAPICE' was coupled with increased NRG1 type III expression and high nucleotide diversity in an Australian cohort (Chapter 3). In addition, several novel splice isoforms of ST8SIA2 were identified that could have a significant role in determining the functions of this candidate gene (Chapter 5). Analysis of protein expression identified an increase in the non-glycosylatable form of NCAM1 (120kDa), and a corresponding decrease in the glycosylatable NCAM1 isoforms and glycosylated NCAM (PSA-NCAM) in the dorsolateral prefrontal cortex of female schizophrenia patients (Chapter 4). A decrease in PSA-NCAM, caused by either a deficit in ST8SIA2 function or by reduced substrate availability through modulation of NCAM1, could affect axon migration and synaptic plasticity, therefore resulting in brain defects associated with psychotic mental illness.

This thesis illustrates the importance of functionally characterizing candidate genes informed from gene mapping studies, particularly those that may have weaker evidence in the GWAS data due to heterogeneity (allelic, locus and phenotypic) but may still have a neurobiological impact on disease pathogenesis in specific individuals.

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Abstract

Bipolar disorder and schizophrenia are devastating mental disorders which share an overlapping constellations of symptoms, most notably the presence of psychosis. Overlapping genetic factors are involved in their pathogenesis and recently, genome-wide linkage scans and association studies have identified many new genes. However, studies focusing on functional follow-up for these genes are very rare, leaving a big gap in comprehending how these genes contribute to the risk of illness.

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The results from this thesis have provided a distinctive contribution to the knowledge about the mechanisms of risk provided by three candidate genes. Using this candidate gene approach, a novel schizophrenia risk mechanism was identified in which the well-known schizophrenia-associated haplotype 'HAPICE' was coupled with increased NRG1 type III expression and high nucleotide diversity in an Australian cohort (Chapter 3). In addition, several novel splice isoforms of ST8SIA2 were identified that could have a significant role in determining the functions of this candidate gene (Chapter 5). Analysis of protein expression identified an increase in the non-glycosylatable form of NCAM1 (120kDa), and a corresponding decrease in the glycosylatable NCAM1 isoforms and glycosylated NCAM (PSA-NCAM) in the dorsolateral prefrontal cortex of female schizophrenia patients (Chapter 4). A decrease in PSA-NCAM, caused by either a deficit in ST8SIA2 function or by reduced substrate availability through modulation of NCAM1, could affect axon migration and synaptic plasticity, therefore resulting in brain defects associated with psychotic mental illness.

This thesis illustrates the importance of functionally characterizing candidate genes informed from gene mapping studies, particularly those that may have weaker evidence in the GWAS data due to heterogeneity (allelic, locus and phenotypic) but may still have a neurobiological impact on disease pathogenesis in specific individuals.

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Publications, presentations and awards

Published Manuscripts

Shaw AD, **Tiwari Y**, Kaplan W, Heath A, Mitchell PB, Schofield PR, Fullerton JM. Characterization of genetic variation in ST8SIA2 and its interaction region in NCAM1 in patients with bipolar disorder. ***PLoS One***. 2014 Mar 20;9(3):e92556

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Anderson-Schmidt H, Beltcheva O, Brandon MD, Byrne EM, Diehl EJ, Duncan L, Gonzalez SD, Hannon E, Kantojärvi K, Karagiannidis I, Kos MZ, Kotyuk E, Laufer BI, Mantha K, McGregor NW, Meier S, Nieratschker V, Spiers H, Squassina A, Thakur GA, **Tiwari Y**, Viswanath B, Way MJ, Wong CC, O'Shea A, DeLisi LE. "Selected rapporteur summaries from the XX World Congress of Psychiatric Genetics, Hamburg, Germany, October 14-18, 2012". ***Am J Med Genet B Neuropsychiatr Genet.*** 2013 Mar;162B(2):96-121. doi: 10.1002/ajmg.b.32132. Epub 2013 Jan 22.

Oral Presentations

Yash Tiwari, Janice M. Fullerton, Peter R. Schofield, and Cyndi Shannon Weickert. "NRG1 isoforms and their effect on transcriptional regulation of in schizophrenia". ***Australasian Schizophrenia Conference*** (Sydney, Australia, September 2010).

Poster Presentations

Yash Tiwari, Alex Shaw, Cyndi Shannon Weickert, Peter R. Schofield, Janice M. Fullerton. **"**Characterization of transcriptional and protein variations in major isoforms of NCAM1, a pivotal regulator of neural development, in schizophrenia". ***XXth World Conference on Psychiatric Genetics*** (Hamburg, Germany, October 2012).

Yash Tiwari, Cyndi Shannon Weickert, Peter R. Schofield, Janice M. Fullerton. "Next generation re-sequencing of 48 Bipolar Disorder cases to identify novel DNA variants in disease susceptibility genes *ST8SIA2* and *NCAM1"*. ***Gene Mappers Conference*** 2011 (Hobart, Australia, April 2011)

Yash Tiwari, Cyndi Shannon Weickert, Peter R. Schofield, Janice M. Fullerton. "Characterization of genomic and transcriptional variation of the sialyltransferase *ST8SIA2*

gene, an early developmental generalized susceptibility gene for mental illness". ***XVIII World Congress on Psychiatric Genetics*** (Athens, Greece, October 2010).

Yash Tiwari, Janice M. Fullerton, Peter R. Schofield, and Cyndi Shannon Weickert. "Characterization of DNA variants and their effect on transcriptional regulation of NRG1 isoforms in schizophrenia". ***XVIII World Congress on Psychiatric Genetics*** (Athens, Greece, October 2010).

Awards

Travel Award to attend XXth World Congress of Psychiatric Genetics, Hamburg, Germany (September 2012).

Poster presentation finalist within the Early Career Investigator Program for XXth World Congress of Psychiatric Genetics (Hamburg, Germany September 2012) and XVIIIth World Congress on Psychiatric Genetics (Athens, Greece, October 2010).

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Abbreviations

ABCCC	Australian Bipolar Case Control Cohort
ABS	Australian Bureau of Statistics
ACTB	Beta-Actin
ADHD	Attention Deficit Hyperactivity Disorder
AEBSF	4-(2-Aminoethyl) Benzenesulfonyl Fluoride
AMPA	A-Amino-3-Hydroxyl-5-Methyl-4-Isoxazole-Propionate
ANK3	Ankyrin-3
APA	American Psychiatric Association
ASRB	Australian Schizophrenia Research Bank
BDGP	The Berkeley Drosophila Genome Project Software
BDNF	Brain-Derived Neurotrophic Factor
BLAT	Gene sequence alignment tool
bp	Base pairs
BPD	Bipolar Disorder cases
BP-I	Bipolar I Disorder
BP-II	Bipolar II Disorder
CACNA1C	Calcium channel, voltage-dependent, l type, alpha 1c subunit
CAMs	Cell Adhesion Molecules
CAO	Clinical Assessment Officer
cDNA	Complementary DNA
CFG	Convergent Functional Genomics
cM	CentiMorgan
CNVs	Copy Number Variations
COMT	Catechol-O-Methyl Transferase
CON	Unaffected controls
CR	Cajal-Retzius cells
CRD	Cystein-Rich Domain
CREB	cAMP Response Element-Binding Protein
CSF	Cerebro Spinal Fluid
DAOA	D-Amino Acid Oxidase Activator
DBDs	DNA Binding Domain
DG	Dentate Gyrus
DGKH	Diacyl-Glycerol Kinase
DIGS	Diagnostic Interview for Genetic Studies
DIP	Diagnostic Interview for Psychosis
DISC1	Disrupted In Schizophrenia gene 1
DMSO	Di-Methyl Sulfoxide
DNA	Deoxyribonucleic Acid

DRD2	Dopamine Receptor 2
DSM	Diagnostic And Statistical Manual Of Mental Disorders
E13	Embryonic day 13
E64	Embryonic day 64
EDTA	Ethylene-Diamine-Tetraacetic Acid
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assays
Emx2	Homeobox Protein Emx2
ENCODE	Encyclopedia of DNA Elements
ErbB4	Receptor Tyrosine-Protein Kinase erbB-4
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
FIGS	Family Interview for Genetic Studies;
fMRI	Functional Magnetic Resonance Imaging
Fn-III	Fibronectin III domain
GABA	Gamma Amino-Butyric Acid
GAD	General Anxiety Disorders
GAIN	Genetic Association Information Network
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF	Glial Derived Neurotrophic Factor
GPI	Glycosylphosphphatidylinositol-Linked
GRA	Genetic Repositories Australia
GWAS	Genome Wide Association Studies
HREC	Human Research Ethics Committee at the university of NSW
HRP	Horseradish Peroxidase
ICD	Intracellular Domains
Ig domains	Immunoglobulin domains
IGF	Insulin-like Growth Factor
iGluRs	Ionotrophic Glutamate Receptors
INDEL	Insertions and Deletion polymorphisms
IPSCs	Induced Pluripotent Stem Cells
kb	Kilobase
L1CAM	Neural Cell Adhesion Molecule L1
LD	Linkage Disequilibrium
LOD	Logarithm of an Odds Ratio
LRP	Long Range PCR
LTD	Long Term Depression
LTP	Long-Term Potentiation
MAF	Minor Allele Frequency

mGluRs	Metabolic Glutamate Receptors
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
MZ	Mono-Zygotic
NaAc	Sodium Acetate
NCAN	Neurocan
NCBI	National Centre for Biotechnology Information
Neu5Ac	N-Acetyl Neuraminic Acid
NGF	Nerve Growth Factor
NHMRC	National Health And Medical Research Council
NMDA	N-Methyl-D-Aspartate
NRG1	Neuregulin 1
NRXN1	Neurexin
NT-3	Neurotrophin-3
OB	Olfactory Bulb
OFC	Orbito Frontal Cortex
OPCs	Oligodendrocyte Progenitor Cells
OR	Odds Ratio
Pax6	Paired homeobox transcription factor 6
PCR	Polymerase Chain Reaction
PFC	Pre-Frontal Cortex
PGC	Psychiatric Genomics Consortium
PI-3 Kinase/Atk	Intracellular signalling cascade
PLINK	Whole genome association analysis toolset
polySTs	Polysialyltransferases
PSA/PolySia	Poly-Sialic Acid
PSA-NCAM	Polysialylated NCAM
PSTD	Polysialyl-Transferase Domain
PTSD	Post-Traumatic Stress Disorder
qPCR	Quantitative PCR
RIN	RNA Integrity Number
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid
RNA-SEQ	RNA Sequencing
RT-PCR	Real-Time PCR
SCZ	Schizophrenia Cases
SEC exon	The secreted exon in NCAM1
SM-L	Sialyl-Motif-L
SMRI	Stanley Medical Research Institute based case control cohort

SM-S	Sialyl-Motif-S
SNP	Single Nuclear Polymorphism
SRI	Schizophrenia Research Institute
ST8SIA2	Sialyltransferase 8b
ST8SIA4	Sialyltransferase 8D
STEP-UCL	Systematic Treatment Enhancement Program - University College London
SVZ	Sub-Ventricular Zone
SZD	Schizoaffective Disorder
TBP	TATA box Binding Protein
TCF4	Transcription Factor 4
TFBS	Transcription Factor Binding Site
TMD	Transmembrane Domain
TRANSFAC	Database on eukaryotic regulatory DNA elements
TRC	Tissue Resource Centre, Sydney
UBC	Ubiquitin C gene
UCSC	University of California, Santa Cruz
USUHS	Uniformed Services University of the Health Sciences
UTR	Untranslated Region
VASE	Variable Alternative Spliced Exon
VCFS	Velo-Cardio-Facial Syndrome
VIPR2	Vasoactive Intestinal Peptide Receptor 2
VRK2	Vaccinia-Related Kinase 2
VS-DOMAIN	Very Small Domain in ST8SIA2
VZ	Ventricular Zone
WHO	World Health Organization
WTCCC	Wellcome Trust Case Control Consortium
ZNF804A	Zinc Finger Protein 804A

Chapter 1

Introduction

1.1 Mental health and mental illness

Good mental health is a measure of emotional well being or absence of psychological disorders like depression and anxiety (Westerhof G J, Keyes C L 2010). It plays an important part in a person's capability to communicate and co-exist with people around him/her. According to the World Health Organization (WHO), mental health is "a state of well-being in which the individual realizes his or her own abilities, can cope with the normal stresses of life, can work productively and fruitfully, and is able to make a contribution to his or her community" (Promoting Mental Health, WHO 2005). Individuals with mental illness or unusual mental health can experience a distortion in the way they perceive things, understand them and respond; and as a result, they could feel isolated or discriminated against by society, causing them further disappointment and unhappiness. Mental illness causes a huge financial burden on the nations, with annual costs in Australia anticipated to be $20 billion, which includes individual productivity and labor loss (Australian Bureau of Statistics, 2009).

Mental illness is a serious problem and third fundamental cause of non-fatal burden of disease and injury in Australia (Health and Ageing Factbook, 2006). Within a span of 12-months, one in five Australians experience some sort of mental illness (Australian Bureau of Statistics, 1997) and in 2007, almost half (45%) of all Australians had experienced it at some point in their life (Australian Bureau of Statistics, 2008). Males had a higher prevalence of psychotic disorders (5.4%), compared to females (3.5%). Elevated rates of psychotic illness were observed in 25-34 year-old males (7.4 cases per 1000) (National Survey of Psychotic Illness, 2010) (Morgan V A, *et al.* 2012).

Having a mental illness is no-one's fault, and definitely not "bad blood, or punishment or the evil eye", as believed previously (www.health.qld.gov.au). A combination of different genetic and environmental factors leads to a mental illness. In order to categorize and evaluate the type of mental illness, two widely recognized systems in use are:

a) The "International Classification of Diseases (ICD-10) produced by the World Health Organization (WHO; http://apps.who.int/classifications/icd10/browse/2010/en); and
b) Diagnostics and Statistical Manual of mental disorders (DSM – IV) published by the American Psychiatric Association (APA).

In general, mental illness can be broadly classified into adolescent disorders, cognitive disorders, substance-related disorders, mood disorders, anxiety disorders, psychotic disorders, and disorders of sleep, personality, eating and identity. Of particular relevance to the background of this thesis are psychotic disorders.

1.2 Psychotic Disorders

With lifetime prevalence of 3% in general population (Perala J, *et al.* 2007), psychotic disorders are associated with substantial illness, disability and even death (Heckers S 2009; McGrath J, *et al.* 2008). They are a collection of disorders in which 'psychosis' predominates the complex of symptoms, such that patient loses touch with reality or is unable to differentiate between fantasy and reality. Genetic risk factors, together with environmental stressors play a key role in further development of these disorders (Rutter M, *et al.* 2001). Risk factors could be baseline (e.g. family history of disorder), distal (e.g. substance abuse) or proximal (e.g. psychopathological variations) (Heckers S 2009; Schultze-Lutter F 2009; Woods S W, *et al.* 2009). Clinicians and researchers follow various strategies to assess the risk for psychotic disorders which include, family history analysis, developmental history, assessment of combined effect of several factors, changes in proximal factors such as perceiving thoughts and making decisions (Heckers S 2009).

1.2.1 Psychosis

Psychosis is generally defined as erroneous symptoms that impair a person's ability to diagnose reality. Exclusive psychotic signs include hallucinations and delusions, hearing voices, distorted thinking, and noticeable unusual psychomotor acts (Goghari V M, *et al.* 2012; Heckers S 2009). Assessment of the degree of psychosis could be reliably assessed on a dimensional rating scale known as The Lifeteime Dimensions of Psychosis Scale (LDPS) (Levinson D F, *et al.* 2002), plotting degree of psychosis (0-100 scale, 20 being

diagnostic threshold) on *y*-axis and development of illness on *x*-axis. Based on this scale, there are 4 distinct stages, namely; asymptomatic and symptomatic risk stage, first episode and long-term course. Studies have reported psychotic symptoms in both schizophrenia and bipolar disorder (Craddock N, *et al.* 2005; Edmiston E E, *et al.* 2011; Kerner B, *et al.* 2007; Potash J B, *et al.* 2003; Potash J B, *et al.* 2001). However, numerous unusual brain conditions including brain lesions resulting from head injuries, infections or substance abuse could also lead to psychotic symptoms (Mace C J, Trimble M R 1991; Schwarzbold M, *et al.* 2008).

1.2.2 Schizophrenia

Schizophrenia is a devastating psychiatric disease with world-wide prevalence of 1% (Craddock N, *et al.* 2005; Murray C J, *et al.* 1994; Perala J, *et al.* 2007). Around the globe, this psychiatric illness affects males and females fairly evenly and occurs at comparable rates in all ethnicities (nimh.nih.gov). In a Canadian study conducted in 2004, direct healthcare and non-healthcare cost of schizophrenia in Canada was found to be $2.02 billion (Canadian), which increased to $4.83 billion when lost productivity, labor and lives were added (Goeree R, *et al.* 2005). Important symptoms of this disorder are delusions, distorted thinking, erratic behavior, cognitive disability, and psychosocial health issues (Arai M, *et al.* 2006). The main features that distinguish schizophrenia from other psychotic illnesses and drug ingestion include poor social skills, social withdrawal and unusual thinking. Five subtypes of schizophrenia are recognized by DSM-IV: paranoid, catatonic, disorganized, residual and undifferentiated (McGlashan T H, Fenton W S 1991). Individuals with a paranoid subtype of schizophrenia are very distrustful of others and show recurrent hallucinations and delusions. Whereas, individuals with catatonic subtypes are extremely reserved, negative and secluded. Individuals with disorganized or hebephrenic subtypes of this disease are vocally illogical and show unsuitable feelings according to the condition and individuals with residual subtypes do not actively suffer from hallucinations or delusions, but they are not motivated or interested in everyday life activities. Individuals with undifferentiated subtypes meet the overall characteristics for schizophrenia but do not categorize under any of above-mentioned subtypes.

The etiology of schizophrenia is unknown. However, family, twin, and adoption studies suggest a role of genetic factors in its transmission, with heritability estimates of around 85%. Concordance rates for this disease are higher for monozygotic twins (41-65%), compared to dizygotic twins (0-28%) (Craddock N, *et al.* 2005), showing clear evidence for both genetic and environmental risk factors (Craddock N, *et al.* 2005; Tsuang M T, *et al.* 2001). Different studies link schizophrenia with disturbed neurodevelopment and distorted brain connectivity (Begre S, Koenig T 2008; Francis F, *et al.* 2006; Innocenti G M, *et al.* 2003; Lewis D A, Levitt P 2002).

The age of onset for schizophrenia is generally earlier for males compared to females (Beratis S, *et al.* 1994). The average age of onset is 18 years in men and 25 years in women (Andreasen N C, Black D W 2006). However, females could have a 'first episode' after the age of 30 years (Wynn Owen P A, Castle D J 1999). Late onset cases have been reported, although it is rare for a person to be diagnosed with schizophrenia after 45 years of age (Mueser K T, McGurk S R 2004). Schizophrenia rarely occurs in children (1:40000), compared to adults (1:100). Age of onset was found to vary depending on the subtype diagnosis, and was found to be earliest for disorganized type and latest for paranoid schizophrenia (Beratis S, *et al.* 1994).

There is no cure for schizophrenia. Treatment is performed with different antipsychotic agents or neuroleptics for severe psychotic episodes or to maintain stable condition. Common atypical drugs available for schizophrenia, like risperidone, olanzapine, and zotepine etc., with exception of Ziprasidone, were found to be associated with obesity (Taylor D M, McAskill R 2000). Risperidone and amisulpride were found to raise prolactin levels causing disorders like galactorrhoea and sexual dysfunction (Wieck A, Haddad P 2002). Unlike bipolar disorder, schizophrenia is not classified as a mood disorder; however mood disturbance can be seen in some patients during the course of illness (Kohler C, *et al.* 1998). Schizophrenia patients are at high risk for suicide, with about one-third attempting (Jarbin H, Von Knorring A L 2004) and 10% successfully completing suicide (Radomsky E D, *et al.* 1999).

1.2.2.1 Co-morbid conditions in schizophrenia

Having schizophrenia further increases a patient's risk to develop other co-morbid conditions. Common co-morbid conditions found associated with schizophrenia include substance abuse, extreme drinking of alcohol, smoking, anxiety, depression and diabetes. Forty to sixty two percent of people with schizophrenia are obese or over weight, either due to unhealthy diet or lack of exercise (Allison D B, *et al.* 1999), comorbid diabetes or as a consequence of pharmacological treatment. An Australian survey reported smoking problems in 73% of men and 56% of women with a psychotic disorder and drinking problems in 38% of patients with psychosis (Jablensky A, *et al.* 2000). Co-morbid conditions complicate schizophrenia symptoms and make it difficult to treat patients having this disorder. A study reported that 46% of patients admitted to hospitals or clinics had an undiagnosed physical illness that either contributed to or aggravated their psychiatric condition (Hall R C, *et al.* 1981). Patients with co-morbid conditions are often physically sick, have unproductive life style and face problems following their treatment plan effectively. Interestingly, some studies have recognized marijuana intake as a risk factor for this disorder (Muller-Vahl K R, Emrich H M 2008; Zullino D F, *et al.* 2008), while others have identified it as a self medication or potential cure for schizophrenia (Coulston C M, *et al.* 2011; Deiana S 2013).

1.2.3 Bipolar Disorder

Bipolar Disorder is a severe mood disorder with a life time prevalence of approximately 1% in both males and females (Goodwin and Jamison, 1990). It is characterized by instability in mood, ranging from extreme highs (manic-phase) to severe lows (depressive-phase) with reversion to normal mood in between their extremes. This disorder sometimes illustrates psychotic features and cognitive impairments (Craddock N, *et al.* 2005). Lifetime suicide rates range from 8% to 20% for this disorder (Goodwin F K, *et al.* 2003).

DSM-IV recognizes several subtypes of bipolar disorder such as bipolar-I disorder, bipolar-II disorder, and cyclothymia (mild-form); each differ in the disability-span and the description of the phases with manic or depressive episodes (Fallin M D, *et al.* 2004).

Bipolar-I is the most severe and typically involves lifelong periodic mood dysregulation, with longer episodes (> 5 days) of manic excitement with or without depression (Solomon D A, *et al.* 2006) which requires hospitalization in extreme situations. Bipolar-II does not generally require hospitalization, but involves shorter episodes (a few hours to a few days) of life-long depression and less–severe mania (hypomania) with mild impairment in thinking and decision making but no psychotic episodes (Judd L L, *et al.* 2008); lastly, cyclothymia is a mild form with short episodes of hypomania and mild depression (nimh.nih.gov).

Although, the etiology of this disorder is unknown, family, twin, and adoption studies suggest that genetic factors play a key role in its development. Heritability estimates were found to be 58-74% (Faraone S V, *et al.* 1990) and increased risk for developing bipolar in first-degree relatives was 2-10% (Craddock N, Jones I 1999; Lichtenstein P, *et al.* 2009). Although, bipolar-I is found at similar prevalence rates in both males and females, bipolar-II is slightly more common in females compared to males, with a ratio of approximately 3:2 (Andreasen N C, Black D W 2006). The mean age at diagnosis for bipolar disorder is 28 years (Joyce P R 1984) with males having an earlier age of diagnosis compared to females (Andreasen N C, Black D W 2006). Onset has also been reported in both children and older adults (60-70 year old), but early or late diagnosis is not very common (Fallin M D, *et al.* 2004; Stone K 1989). The age of diagnosis is typically later than onset of first symptoms, which usually present as unipolar depression. A study by Hu *et al.* (2012) highlights that in China people with bipolar disorder were often incorrectly diagnosed with major depressive disorder. Further, a study by Xiang *et al* (2012) found that bipolar disorder-II patients were receiving the same antidepressants as major depressive disorder patients. This common misdiagnosis leads to patients receiving treatments for major depression, which have no effect on manic symptoms and can sometimes exacerbate or change the course of illness (Parker G B, *et al.* 2014).

There is no cure for bipolar disorder. Current pharmacological treatments for manic symptoms include antipsychotics, mood stabilizers like lithium, valproic acid, benzodiazepines, carbamazepine, lamotrigine, gabapentin (Chen C H, Lin S K 2012;

Vacheron-Trystram M N, *et al.* 2004). Electroconvulsive therapy (ECT) is also used in patients needing immediate response, such as those with suicidality or with medication intolerance (Agarkar S, *et al.* 2012). Patients with bipolar disorder-I and bipolar disorder-II patients are often prescribed the same antipsychotics, mood stabilizers and antidepressants, however there is evidence that some drugs may be more effective in a particular subtype [for example, lamotrigine for bipolar-II disorder (Parker G B, *et al.* 2014)]. It was observed by Goodwin et al. (2003) that suicide attempts/deaths were 1.5-3 times higher when the mood-stabilizing drug Divalproex was used as compared to lithium. Therefore there is a need for better understanding of these disorders to perform correct diagnosis and tailor effective treatments.

1.2.3.1 Co-morbid conditions in bipolar disorder

Several mental disorders co-occur with bipolar disorder to further complicate its understanding and management. Some of these common co-morbid mental conditions include but are not limited to substance abuse, attention deficit hyperactivity disorder (ADHD), anxiety and panic disorder (Bizzarri J V, *et al.* 2007; McElroy S L 2004; Strakowski S M, *et al.* 1998). Data from the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD) program showed increased rates for drug and alcohol abuse in cases with bipolar disorder, irrespective of age of onset of the disease (Perlis R H, *et al.* 2004). Another study reported presence of metabolic syndrome (which includes conditions such as hypertriglyceridemia, high blood pressure, obesity, and fasting hyperglycemia) in 171 patients with bipolar disorder (Fagiolini A, *et al.* 2005).

Other studies have reported an overlap between bipolar disorder and eating disorders, leading to obesity in patients suffering from bipolar disorder (Dickerson F B, *et al.* 2006; Simon G E, *et al.* 2006). Other co-morbid conditions reported are migraine, thyroid (Kupfer D J 2005), cardiovascular defects (Weeke A, *et al.* 1987), and type II diabetes (Calkin C V, *et al.* 2013).

1.3 Genetic studies

1.3.1 Linkage studies

Genetic linkage is a process which is said to have occurred if two genetic loci are passed on jointly from parental generation to progeny more often than calculated under laws of independent inheritance. The process is very helpful in mapping proximity of genetic loci by use of observations of recombination events in related individuals. Linkage analysis is used to map genes for simple and complex genetic disorders, and is expressed as a logarithm value of the odds score (LOD score) (Dawn Teare M, Barrett J H 2005). LOD score is basically a ratio between the odds of birth sequence with a given linkage value to the odds of birth sequence with no linkage. Positive LOD score indicates the presence of linkage, while a negative score indicates little or no linkage. Genetic linkage findings are said to have attained genome-wide significance if LOD score is ≥ 3 (Lander E, Kruglyak L 1995).

Linkage studies have been performed on bipolar disorder families and different chromosomal regions have been reported to reach genome-wide significance. These regions include: 4p16 (Blackwood D H, *et al.* 1996), 5q21-q33 (Sklar P, *et al.* 2004), 6q16 and 17q25 (Dick D M, *et al.* 2003), 6q22 (Middleton F A, *et al.* 2004), 8q24 (McInnis M G, *et al.* 2003), 12q (Morissette J, *et al.* 1999), 13q (Detera-Wadleigh S D, *et al.* 1999), 16p (Ekholm J M, *et al.* 2003), 18q (Schulze T G, *et al.* 2003), and 22q12 (Kelsoe J R, *et al.* 2001). In addition, our group identified a significant linkage peak on 15q25-26 (McAuley E Z, *et al.* 2009); supporting the previous linkage peak identified in the Vazza *et al*., (Vazza G, *et al.* 2007) study. Although, these findings were genome-wide significant, they were not replicated in meta-analyses studies. Two large meta-analyses for bipolar disorder were carried out with genome scan information to assess the collective response. The first one, Badner and Gershon *et al* (2002) confirmed 13q and 22q, whereas Segurado *et al* (2003) on the other hand identified regions 9p, 10q, and 14q and areas of chromosome 18.

For schizophrenia, genetic linkage studies have recognized various areas showing genome-wide significance, although these are also plagued with failures to replicate in subsequent

studies. Regions include 1q21-22, 1q42, 5q21-33, 6q16-25, 6p24-22, 8p21-22, 10p15-p11, and 13q32-34 and 22q11-12 (Craddock N, *et al.* 2005), 15q26 (Vazza G, *et al.* 2007). Two meta-analysis of schizophrenia linkage using different methods were performed by Badner and Gershon *et al* (2002) and Lewis *et al* (2003). The study by Badner *et al* provided support for chromosome 8p, 13q, and 22q. While, Lewis *et al* provided strong support for 2q and moderate support for 1q, 3p, 5q, 6p, 8p, 11q, 14p, 20q and 22q. Chromosomal regions 8p and 22q were supported by both studies, but the majority of other regions were only supported by single study (Craddock N, *et al.* 2005).

The linkage findings in both bipolar disorder and schizophrenia were unsatisfactory because even the most robust findings failed to illustrate consistent replications. These inconsistencies in the findings further signify the complex etiology of these illnesses and both locus and phenotypic heterogeneity in the linkage samples. It is now clear that bipolar disorder and schizophrenia are multi-gene disorders, rather than caused by single gene/loci and many different combinations of genes could be implicated in different families, each with individual genes of modest effect and assistance from environmental risk factors, a situation for which linkage studies are not well suited (Barnett J H, Smoller J W 2009).

1.3.2 Association studies

Since linkage findings were not able to identify a replicated locus for bipolar disorder or schizophrenia, scientists started using association studies, which are more powerful to detect loci of small effect. Candidate gene approaches have associated polymorphisms present in genes such as *BDNF* (Muller D J, *et al.* 2006; Neves-Pereira M, *et al.* 2002; Sklar P, *et al.* 2002), *DRD2* (Fan H, *et al.* 2010; Massat I, *et al.* 2002) and *COMT* (Funke B, *et al.* 2005) with psychosis (Pidsley R, Mill J 2011). For bipolar disorder, various genome-wide association studies, including mega-analysis by the psychiatric GWAS Consortium (PGC) (Ferreira M A, *et al.* 2008) and independent studies around the world, have suggested involvement of genes including *ANK3*, *BDNF*, *CACNA1C*, *DAOA*, *DISC1*, *NRG1*, *NCAM1* and *TPH2* (Baum A E, *et al.* 2008; Belmonte Mahon P, *et al.* 2011; Chen D T, *et al.* 2011; Chubb J E, *et al.* 2008; Cichon S, *et al.* 2011; Ferreira M A, *et al.* 2008;

Green E K, *et al.* 2012; Kato T 2007; Ripke S, *et al.* 2011; Seifuddin F, *et al.* 2012; Sklar P 2008; Smith E N, *et al.* 2009; Steinberg S, *et al.* 2012; Swami M 2011). In addition, our group (McAuley E Z, *et al.* 2009) and others (Lee M T M, *et al.* 2011) have reported *ST8SIA2* as a susceptibility gene for psychotic and mood disorders. Hence, *ST8SIA2* was selected as a candidate gene for this thesis, and is discussed later in section 1.11 and Chapter 5.

In schizophrenia, various association studies have reported *DISC1* (Chubb J E, *et al.* 2008), *NRG1* (Stefansson H, *et al.* 2003; Stefansson H, *et al.* 2002), *NCAN* (Muhleisen T W, *et al.* 2012), *NRXN1* (Rujescu D, *et al.* 2009), *ITIH3/4*, *CACNA1C* and *SDCCAG8* (Hamshere M L, *et al.* 2013) and *TCF4* (Steinberg S, *et al.* 2011) as conferring risk to schizophrenia. In addition, structural variants, such as Copy Number Variant (CNV) deletions or duplications at 4q32, 5q14.3, 8q23.3, 11q25, 16p13.1 and 17q12 (Bergen S E, *et al.* 2012; Ingason A, *et al.* 2011; Magri C, *et al.* 2010), micro-deletions at 1q21.1, 22q11.2 (VCFS region), 3p29, 3q29, 15q11.2, 15q13.1 (Grozeva D, *et al.* 2012; Karayiorgou M, *et al.* 1995; Levinson D F, *et al.* 2011) and micro-duplications in *VIPR2* (Vacic V, *et al.* 2011), 16p11.2 (McCarthy S E, *et al.* 2009) have also been identified to confer risk to schizophrenia. Findings in both bipolar disorder and schizophrenia have been inconsistent and not well replicated when studied in independent cohorts (Green E K, *et al.* 2012; Seifuddin F, *et al.* 2012); reviewed in Craddock N, *et al.* (2005).

Some of the difficulties experienced have been in the clinical heterogeneity and genetic intersection with other diseases. Another major challenge historically is that individual studies have typically utilized small or medium sample sizes, which are underpowered to detect associations with many genes of small effect, so it has become apparent that it is essential to get extremely large numbers of patient samples to effectively detect the many contributing loci, each having a small effect in increasing disease risk (Barnett J H, Smoller J W 2009; Belmonte Mahon P, *et al.* 2011; Ripke S, *et al.* 2011; Sullivan P 2012). Clinical misclassification or overlap of cases and controls in contributing datasets present another challenge with such large studies, as reported by Cross-Disorder Group of the Psychiatric Genomics Consortium 2013.

1.4 Similarities between bipolar disorder and schizophrenia

Bipolar disorder and schizophrenia have clinical, demographic, therapeutic, and genetic similarities (Martin L F, *et al.* 2007). Demographically, they share similar lifetime prevalence in males and females and a strong genetic component (Perala J, *et al.* 2007; Potash J B, *et al.* 2003; Takei N, *et al.* 1992; Vazza G, *et al.* 2007). Twin and adoption studies have confirmed that genetic factors are responsible for approximately 80% heritability in both disorders (Cannon T D, *et al.* 1998; Cardno A G, *et al.* 1999; Kieseppa T, *et al.* 2004; McGuffin P, *et al.* 2003; Sullivan P F, *et al.* 2003). Both are multi-gene disorders and involve a major non-genetic environmental factor contribution (Lichtenstein P, *et al.* 2009). Twin studies have also indicated genetic associations amongst psychotic characteristics of bipolar disorder and schizophrenia (Cardno A G, *et al.* 2002). Occurrence of bipolar disorder at high rates in relatives of probands with schizophrenia has also been found (Pope H G, Jr., Yurgelun-Todd D 1990; Tsuang M T, *et al.* 1980). In both the disorders, first-degree relatives were at increased risk, which increased significantly for half-siblings and even more for full-siblings (Lichtenstein P, *et al.* 2009) further highlighting the impact of family history in these disorders.

Bipolar disorder and schizophrenia have medical and genetic similarities (Martin L F, *et al.* 2007). Both disorders share common polygenic variation which contributes to both disorders (Cross-Disorder Group of the Psychiatric Genomics C 2013; Purcell S M, *et al.* 2009). Also, we know that there are linkage regions of 3p21, 6q, 10q24 (Cross-Disorder Group of the Psychiatric Genomics C 2013), 13q, 18q and 22q (Badner J A, Gershon E S 2002; Berrettini W 2003; Craddock N, *et al.* 2005) and overlapping genes such as *CACNA1C* (Ferreira M A, *et al.* 2008; Hamshere M L, *et al.* 2013), *CACNB2*, *DAOA* (G72), *DISC1* (Chubb J E, *et al.* 2008; Thomson P A, *et al.* 2005), *NRG1* (Georgieva L, *et al.* 2008), *ZNF804A* (Williams H J, *et al.* 2011b), *NCAN* (Muhleisen T W, *et al.* 2012), *NCAM1* (Arai M, *et al.* 2004; Atz M E, *et al.* 2007), *ST8SIA2* (Arai M, *et al.* 2006; Vazza G, *et al.* 2007) which contribute to increasing risk to both disorders [reviewed in (Craddock N, *et al.* 2005)].

Clinically, the disorders could cross traditional diagnostic boundaries (Williams H J, *et al.* 2011a) (Craddock N, *et al.* 2005;2006). In addition to psychosis, both bipolar disorder and schizophrenia show pre-frontal cortex (PFC) abnormalities in the anterior-posterior and left-right temporal brain areas (al-Mousawi A H, *et al.* 1996), and unsystematic cholinergic responses within the hippocampus (Martin L F, *et al.* 2007). According to prospective observational studies, both disorders share similar characteristics in neurocognitive functioning and psychopathology (Jabben N, *et al.* 2009). Cognitive impairments are common to both diseases, but tend to be less severe in bipolar disorder (Mann-Wrobel M C, *et al.* 2011). Mood disturbances are reported in schizophrenia, as are psychotic symptoms in bipolar disorder (Baynes D, *et al.* 2000; Kohler C, *et al.* 1998; Kraepelin E 1919;1921), making it important to study these related illnesses together. Alterations in regulation and expression of genes involved in dopamine signaling are also implicated in both bipolar disorder and schizophrenia (Zhan L, *et al.* 2011).

Although, the condition "schizoaffective disorder" is described as an intermediate between the two conditions, it is often difficult to draw a line. Explanation of schizoaffective disorder, according to ICD10 and DSM-IV has a very broad and unclear definition and could be used only when cases cannot be matched to features of schizophrenia or bipolar disorder (Craddock N, *et al.* 2009). The definition of schizoaffective disorder has subsequently been revised to improve the reliability, diagnostic stability, and validity of this disorder in the recently updated DSM-V.

1.5 Differences between bipolar disorder and schizophrenia

While there are some similarities between bipolar disorder and schizophrenia, there are also considerable differences.

Brain abnormalities in people suffering from schizophrenia include the lateral and third ventricle enlargements, reduction in the volume of hippocampus, temporal lobe and temporal cortex on the left side (Honea R, *et al.* 2005; McDonald C, *et al.* 2006), while people suffering from bipolar disorder show slight enlargement of the right ventricles and existence of the white matter hyper-intensities (Kaladjian A, *et al.* 2006; Pillai J J, *et al.*

2002). Therefore, the abnormalities in the structure of the superior temporal gyrus or hippocampus appear to be more related to schizophrenia as compared to bipolar disorder (Altshuler L L, *et al.* 2000; Hirayasu Y, *et al.* 1998). Grey matter abnormalities in the anatomical studies are often observed in schizophrenia compared to bipolar disorder (Kubicki M, *et al.* 2002; Zipursky R B, *et al.* 1997). The theory of clinical distinction between schizophrenia and bipolar disorder is further boosted by the neurophysiological endophenotype results, for example, two common schizophrenia endophenotypes include suppression of P50 auditory induced responses (Adler L E, *et al.* 1982; Olincy A, Martin L 2005) and inhibition of leading saccades during smooth pursuit eye movements (Ross R G, *et al.* 1999). In a study performed by Martin *et al.* (2007) they observed differences in these endophenotypes between schizophrenia and bipolar disorder patients with 95% sensitivity and 83% specificity.

Cognitive deficits are more severe in schizophrenia cases compared to bipolar disorder. Schizophrenia cases have shown decreased IQ (Wobrock *et al.*, 2009), verbal learning, memory performances (Barrett *et al.*, 2009; Simonsen *et al.*, 2011; Rossell *et al.*, 2008) when compared to bipolar cases. Looking at the specific domains of cognitive impairment, Diwadkar *et al.* (2011) showed working memory was affected in schizophrenia cases while attention was affected in bipolar cases.

Although, there is an overlap between the candidate genes for bipolar disorder and schizophrenia, there are also a number of genes which are implicated in one disease but not the other. For the genes which do overlap, differences in the expression levels and their proteins have been noted (Iwamoto K, *et al.* 2004; Karege F, *et al.* 2010). A recent example is region-specific variations in NRG1 cleavage in schizophrenia and bipolar disorder patients described in Marballi *et al.* (2012). These differences further support the clinical distinction between these mental disorders.

Lastly, coming to the lifestyle differences, people suffering from bipolar disorder could live normal lives and perform regular job, while people suffering from schizophrenia often find it hard to live a normal life and interact with people around them (Jones P B, *et al.* 1993).

The results from Gilvarry *et al* (2000) show that people suffering from bipolar disorder have a better neuropsychological performance than their schizophrenia counterparts. Schizophrenia patients suffer from more cognitive impairments (Goldberg T E 1999) and birth complications are more frequent and severe in people with schizophrenia compared to bipolar disorder patients (Murray R M, *et al.* 2004; Verdoux H, Bourgeois M 1993). On the other hand, bipolar disorder patients are more likely to have a history of suicidal behavior compared to schizophrenia patients (King J, *et al.* 2012).

1.6 Brain development

Brain development is a process that involves multiple steps, functioning in a highly controlled and genetically planned manner (Stiles J, Jernigan T L 2010). Understanding the neurobiology behind brain development could provide us with the opportunity to understand the basic processes that are involved in proper development, and defects that could lead to possible developmental brain disorders. With the demarcation of neural progenitor cells, brain development in humans begins in the third gestational week (GW3) and through different processes like interneuron maturation, neurulation, neuronal proliferation, neural migration, synaptogenesis, myelination and apoptosis lasts throughout the lifespan (Catts V S, *et al.* 2013; Faigle R, Song H 2012; Stiles J, Jernigan T L 2010). Gastrulation begins 13 days post conception or embryonic day 13 (E13), where a two-layered embryo is converted into a three layered structure. Neural progenitor cells (neural stem cells) are a multipotent population of cells which can produce many neuronal cell types in the brain and CNS. They are formed from the upper 'epiblast' layer of gastrula as a result of molecular signaling through the 'primitive node'.

1.6.1 Neural induction

Development of nervous system begins with neural progenitor cells forming a specific structure called the 'neural plate', which will eventually convert into the brain and spinal cord (see Figure 1.1 for summary of processes). This region is also called the 'ventricular zone' (VZ). The neural plate folds to form 'neural tube' between E20-27 and a specialized structure known as 'notocord' and various signaling pathways like the Wnt/beta-catenin

and sonic hedgehog (Shh) pathways are involved (Hari L, *et al.* 2002; Ruiz i Altaba A, *et al.* 2002; Zechner D, *et al.* 2003). Elementary brain structures and CNS are shaped by the conclusion of embryonic period i.e. GW8. Neurogenesis begins on embryonic day 42 (E42) and typically continues robustly until early postnatal life (Bystron I, *et al.* 2008; Fung S J, *et al.* 2011; Stiles J, Jernigan T L 2010; Weickert C S, *et al.* 2000). Neural progenitors divide in asymmetrical fashion to produce billions of neurons (post mitotic cells) during the process of neurogenesis (Bayer S A, *et al.* 1993). This is a critical period in the development of neocortex, the central information processing system of brain. The neocortex exchanges information through subcortical nuclei and both contain cell bodies of neurons giving them gray colored appearance termed as 'gray matter'. The neocortex later forms cortical areas with help of signaling molecules like Emx2 and Pax6 (Bishop K M, *et al.* 2000).

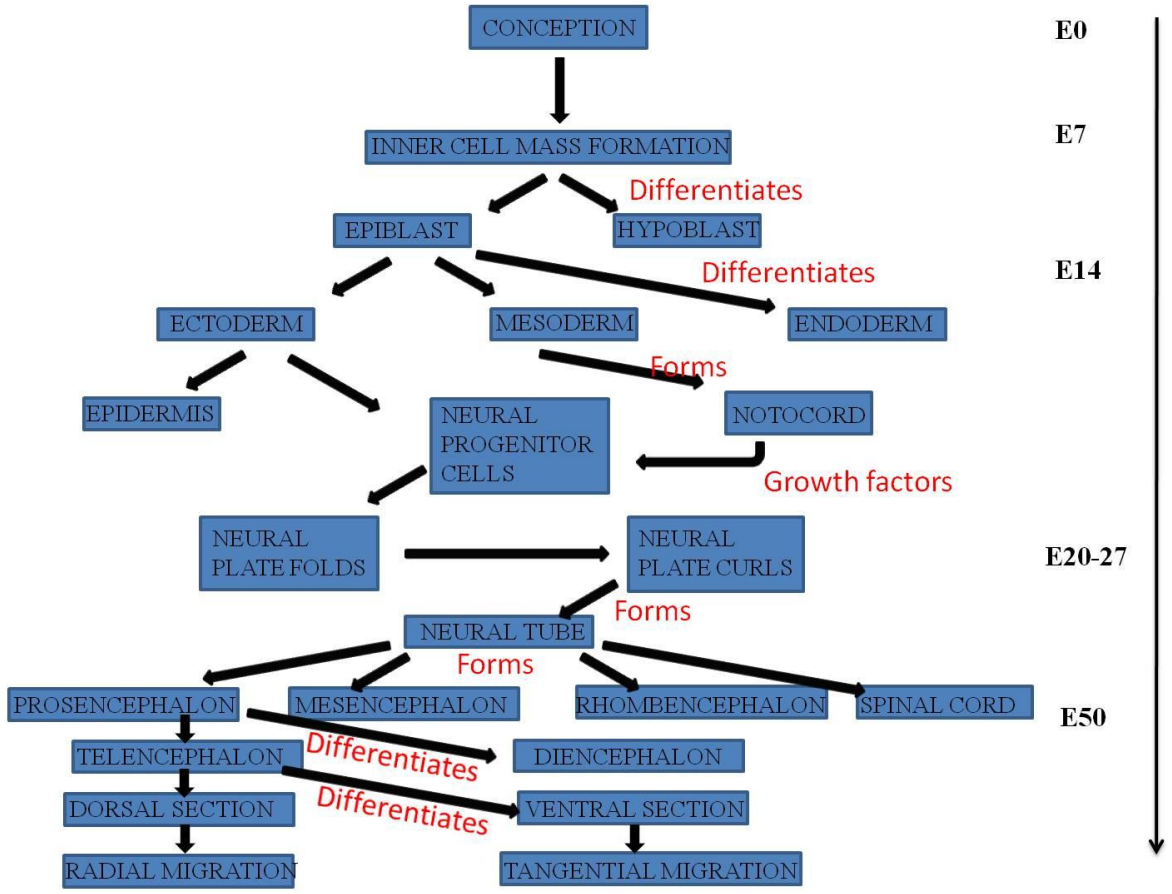


Figure 1.1: Neural induction and migration during early brain development. Inner cell mass differentiates into two layers Epiblast and Hypoblast. Epiblast then gives rise to Ectoderm, Mesoderm and Endoderm. Ectoderm forms Epidermis and Neural progenitor cells. Mesoderm forms Notocord. Notocord is present between Ectoderm and Endoderm and releases special growth factor to activate Neural progenitor cells to form Neural plate. Neural plate then folds and curls to form Neural tube. Neural tube later differentiates into forebrain (Prosencephalon), Midbrain (Mesencephalon), Hindbrain (Rhombencephalon) and Spinal cord. Prosencephalon differentiates into Telencephalon and Diencephalon. Dorsal cells in Telencephalon undergo radial migration to become pyramidal neurons while Ventral cells undergo tangential migration to populate the cortex as interneurons and they also give rise to the basal ganglia. Embryonic days are shown in the right.

1.6.2 Neuron migration

Newly formed neurons start their journey from their birth place in the VZ towards the pial surface (developing neocortex) by somal translocation, which occurs over short distances (Nadarajah B, Parnavelas J G 2002). Later, with increase in brain volume, neurons require special scaffold structures called 'radial glial guides' to radially migrate (Poluch S, Juliano

S L 2007) greater distances towards the developing cortical plate (Noctor S C, *et al.* 2001; Parnavelas J G, *et al.* 2002; Rakic P 1972). Radial glia cells express ErbB4, while NRG1 is expressed by granule cells. A study by Rio *et al.*, (1997) found that activation of ErbB4 by soluble NRG1 plays a significant role in radial cell migration. During this process of migration and development of cortical layers, Cajal-Retzius cells (CR) manage orientation of neurons into accurate layers, through molecular signaling via Reelin (Huang Z 2009).

In another proliferative region around ventral telencephalon, neurons pursue 'tangential migration' to form the basal ganglia and interneurons of the cortex. Polysialylated NCAM (PSA-NCAM) plays an important role in this migration, and is discussed later.

1.6.3 Synapse formation

After correct positioning in the cortex, migrated neurons form simple connections with other neurons with help of neural recognition molecules like NCAM1. Appropriate control of these connections is essential for development of extremely synchronized brain connectivity (Shapiro L, *et al.* 2007) and this is attained through cell adhesion molecules like NCAM1 (Rougon G, Hobert O 2003). Interactions between like molecules are known as homophilic interactions, while non-like molecules show heterophilic interactions. Within neurons, the information flows in the form of electric-chemical impulses. Connections between two neurons are called synapses and is established through neuronal processes, which could be long connecting fibers (called axons) or short connecting fibers (called dendrites), to send or receive electrochemical input signals over long or short distances, respectively. White colored myelin proteins cover axons for efficient transmission of these signals over extended distances; due to their appearance, axonal tracts are termed 'white matter pathways'. To travel long distances, axonal projections use various guidance cues through their tips which are known as the 'growth cone' (Tosney K W, Landmesser L T 1986). NRG1-ErbB signaling is found to be critical for myelination process as NRG1 activates ErbB2/3 receptors and this initiates a complex interaction between adaptor proteins and enzymes to activate different signaling pathways. Integrin/Laminin and ligands of Gpr126 are also believed to be involved in this process to control Schwann cell

myelination (Newbern J, Birchmeier C 2010). How signals are integrated from these distinctive molecules is still a subject of study, but it is known that increased NRG1 expression in mice (driven by Thy-1 promoter) leads to increased myelin sheath thickness (Michailov G V, *et al.* 2004). Recognition molecules on the surface of the neuronal growth cone guide this growth in a highly regulated manner (Cremer H, *et al.* 1997; Doherty P, *et al.* 1990). Axon guidance errors are reported to occur due to premature appearance of polysialic acid-free NCAM; as a result axons stray from their path causing various brain abnormalities like hypoplasia of Anterior Commissure, reduced rostrocaudal extent of Corpus Callosum and Interior Commissure (Muhlenhoff M, *et al.* 2009; Oltmann-Norden I, *et al.* 2008).

1.6.4 Postnatal neurogenesis and migration

While the majority of neuronal cell birth (neurogenesis) occurs prenatally, postnatal neurogenesis has been reported in sub-ventricular zone (SVZ) of the lateral ventricles to olfactory bulb (Sanai N, *et al.* 2004), and sub-granular zone of the dentate gyrus (DG) (Curtis M A, *et al.* 2007; Sanai N, *et al.* 2007; van Praag H, *et al.* 2002). In the SVZ, stem cells or astrocytes (type B cells) form migratory neuroblasts (type A cells) and putative precursors (type C cells) (Doetsch F, *et al.* 1999) (shown in Figure 1.2). Type A cells are PSA-NCAM positive (Rousselot P, *et al.* 1995; Weickert C S, *et al.* 2000) and migrate in chains towards olfactory bulb (OB) following the rostral migratory stream (RMS). Type A cells migrate through glial tubes composed of type B cells, while type C cells are seen as small clusters along chains of type A cells (Doetsch F, *et al.* 1997; Lim D A, Alvarez-Buylla A 1999). After reaching OB, Type A cells differentiate into cortical inhibitory interneurons or GABAergic interneurons (Doetsch F, *et al.* 1997). It is reported that mutations in NCAM or enzymatic removal of polysialic acid results in inhibition of tangential migration and reduction in size of OB (Ono K, *et al.* 1994; Rockle I, *et al.* 2008). In the adult brain, other cell types, oligodendrocyte progenitor cells (OPCs) or glial progenitors continue to reproduce and travel to different locations; for example, cortex, hippocampus, striatum, and SVZ or forebrain. OPCs respond to injury and could

differentiate into oligodendrocytes and astrocytes (Cayre M, *et al.* 2009; Stiles J, Jernigan T L 2010).

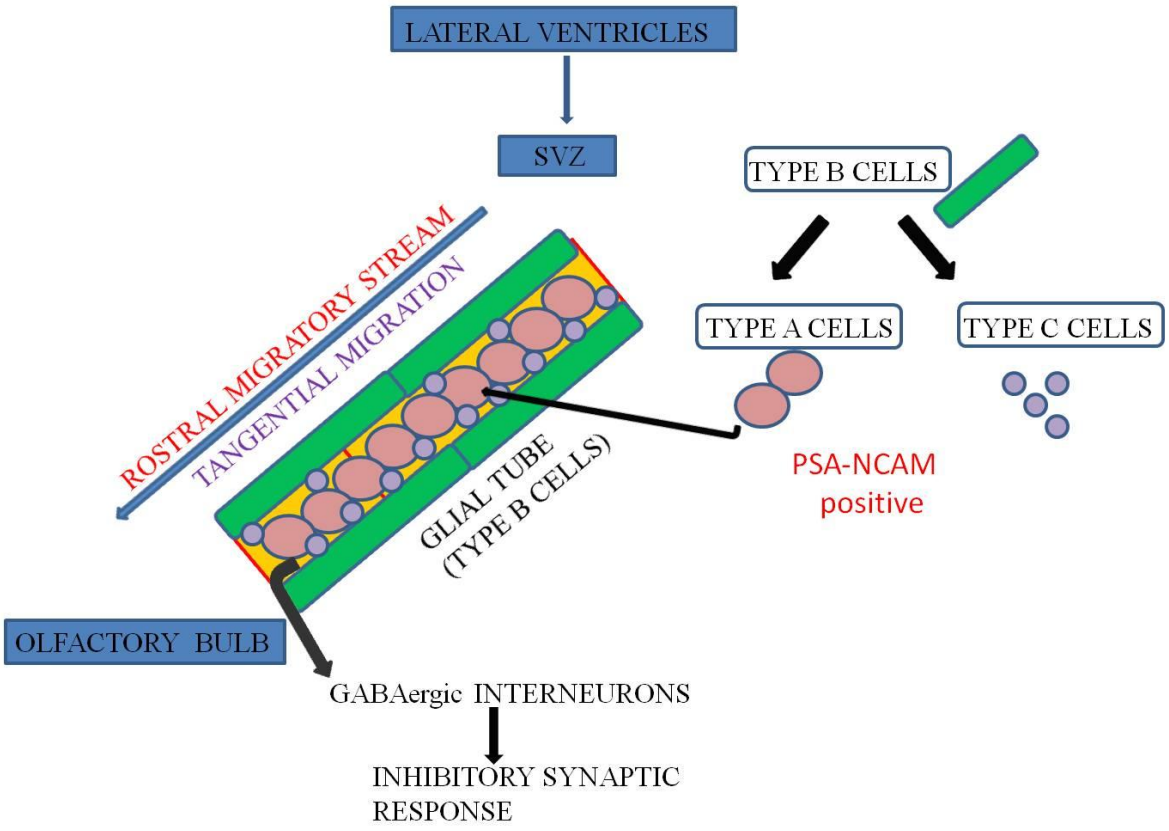


Figure 1.2: Postnatal neurogenesis and migration in sub-ventricular zone (SVZ) of lateral ventricles. Type B cells or astrocytic-like slow proliferating cells form PSA-NCAM positive Type A cells or migratory neuroblasts and Type C cells or rapidly proliferating precursors. Type A cells migrate tangentially through glial tubes (also made up of Type B cells) to reach Olfactory Bulb following Rostral Migratory Stream. After reaching Olfactory Bulb Type A cells become GABAergic interneurons and result in inhibitory postsynaptic potential.

1.6.5 Cell death

Selective neurons and progenitor cells follow a programmed cell death or 'suicide' program called 'apoptosis' (Kerr J F, *et al.* 1972) to correct for errors in production and migration of neurons (Buss R R, Oppenheim R W 2004). Apoptosis is normally controlled by a series of cellular and environmental factors communicated through signaling (Artavanis-Tsakonas S, *et al.* 1999; Park E, *et al.* 2013). It is reported that availability of neurotrophins, produced at synaptic sites, could defend cells against apoptosis (Levi-Montalcini R 1964; Oppenheim R

W 1989). Gascon *et al* (2007a) reported that absence or inactivation of polysialic acid chain on NCAM could cause decreased cell survival because there is deficiency in uptake of neurotrophins like NGF and BDNF. The change also causes an increased p75 neurotrophin receptor expression, as PSA-NCAM which controls expression of this receptor in developing neurons, is inactivated.

1.7 Neuronal communication

1.7.1 Cell adhesion

Cell adhesion molecules (CAMs) are the proteins required to maintain adhesive interactions (Buckley C D, *et al.* 1998) between cell surfaces and their extracellular matrix in a co-ordinated manner during various processes of brain development (Chothia C, Jones E Y 1997). In addition to their adhesive function, CAMs also provide effective signals to regulate several activities, like neuronal migration, synaptogenesis, gene expression and apoptosis (Buckley C D, *et al.* 1998). CAMs could be classified in a number of different ways, such as, on the basis of the tissue in which they were originally documented (Ogou S I, *et al.* 1983), their calcium reliance (Hatta K, *et al.* 1988), or their activity such as cell-cell or cell-extracellular matrix communications (Kerrigan J J, *et al.* 1998). More recently, they have been grouped into five protein super families; namely immunoglobulins, cadherins, proteoglycans, integrins and selectins (Kerrigan J J, *et al.* 1998). The first CAM identified in mammalian system was the Neural Cell Adhesion Molecule (NCAM) (Rutishauser U, *et al.* 1976) which is a target gene for this thesis and is described further in section 1.10 and Chapter 4.

1.7.2 Synaptic plasticity

Excitatory synaptic contacts between two neurons can cause two forms of long-lasting changes, required for information processing and storage of neuronal networks (Muller D, *et al.* 1996). The first one, long-term potentiation (LTP), is induced by short periods of high frequency stimulation resulting in an amplified effectiveness of transmission (Bliss T V, Gardner-Medwin A R 1973); and the second one, long term depression (LTD) is induced

by long periods of low frequency stimulation resulting in a reduced synaptic efficiency (Dudek S M, Bear M F 1992). Cell adhesion molecules like NCAM (Luthl A, *et al.* 1994) and its post translationally modified form PSA-NCAM are essential for regulating synaptic plasticity (Kochlamazashvili G, *et al.* 2010). The removal of PSA from NCAM has been shown to completely block stimulation of LTP and LTD (Muller D, *et al.* 1996). Regulated NRG1-ErbB4 signaling is important in activity dependent maturation and plasticity of the excitatory synapse. ErbB4 activity is essential for the maintenance of existing neural connections. Increase in erbB4 causes an increase in AMPA synaptic currents and as a result size of dendritic spine is increased. Loss of ErbB4/NRG1 signaling weakens AMPA synaptic currents and hence the size of dendritic spine is also reduced (Li B, *et al.* 2007). NRG1 and its signaling are described in more detail in section 1.9.

1.8 Additional factors involved neurodevelopment, plasticity and communication

1.8.1 Neurotransmitters

Communication between neurons is very important in regulating different events such as neural differentiation, integration, proliferation, and activity dependent adult neurogenesis (Faigle R, Song H 2012). It is mainly performed through exchange of neurotransmitters, and the small diffusible molecules involved in this process that activate one or more types of receptors. Neurotransmitters include amino acids (for e.g. glutamate, glycine, GABA), biogenic-amines (e.g. dopamine and serotonin), peptides (somatostanin) and others (acetylcholine, adenosine triphosphate) (Valenzuela C F, *et al.* 2011) (Figure 1.3). Amino acids are the principal neurotransmitters in the brain (constituting 90%), with acetyl-choline in 5% and biogenic-amines in 1% of synapses [reviewed in (Snyder S H, Ferris C D 2000)]. Functionally, neurotransmitters can be excitatory, inhibitory, or both. Excitatory neurotransmitters activate receptors to depolarize cell membrane and increase the likelihood that the target neuron will transmit an action potential. Glutamate activates a number of receptors that include ionotropic glutamate receptors (iGluRs); [e.g. NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)

receptors] or metabotropic glutamate receptors (mGluRs) (Hollmann M, Heinemann S 1994). iGluRs are involved in rapid synaptic transmission through ligand-gated ion channels that allow the flow of Na^{+} and/or Ca^{2+} in response to activation. mGluRs, on the other hand, are involved in slow synaptic transmission via activation of several downstream second messenger molecules utilizing G-proteins (Volpi C, *et al.* 2012; Yang J L, *et al.* 2011). Inhibitory neurotransmitters activate ionotrophic receptors resulting in hyperpolarization of the membrane and generation of decreased action potential. The most common inhibitory transmitter is GABA, which is synthesized from glutamate, a reaction catalysed by glutamate decarboxylase. Both GABA and glutamate have been reported to control cell differentiation, proliferation, and migration (Manent J B, Represa A 2007). Dopamine and acetylcholine utilize both excitatory and inhibitory receptors to maintain neural development and plasticity (Belousov A B, *et al.* 2001; Drever B D, *et al.* 2011; Lester D B, *et al.* 2010). Neurotransmitters are released from synaptic vesicles via exocytosis. Synaptic vesicles, post neurotransmitter releasing process, are recycled through endocytosis and used again during synaptic transmission (Hori T, Takahashi T 2012). Variations in the activities of excitatory and inhibitory neurotransmitters have been linked to both schizophrenia and bipolar disorder (Beasley C L, *et al.* 2002; Guillozet-Bongaarts A L, *et al.* 2013; Zhan L, *et al.* 2011).

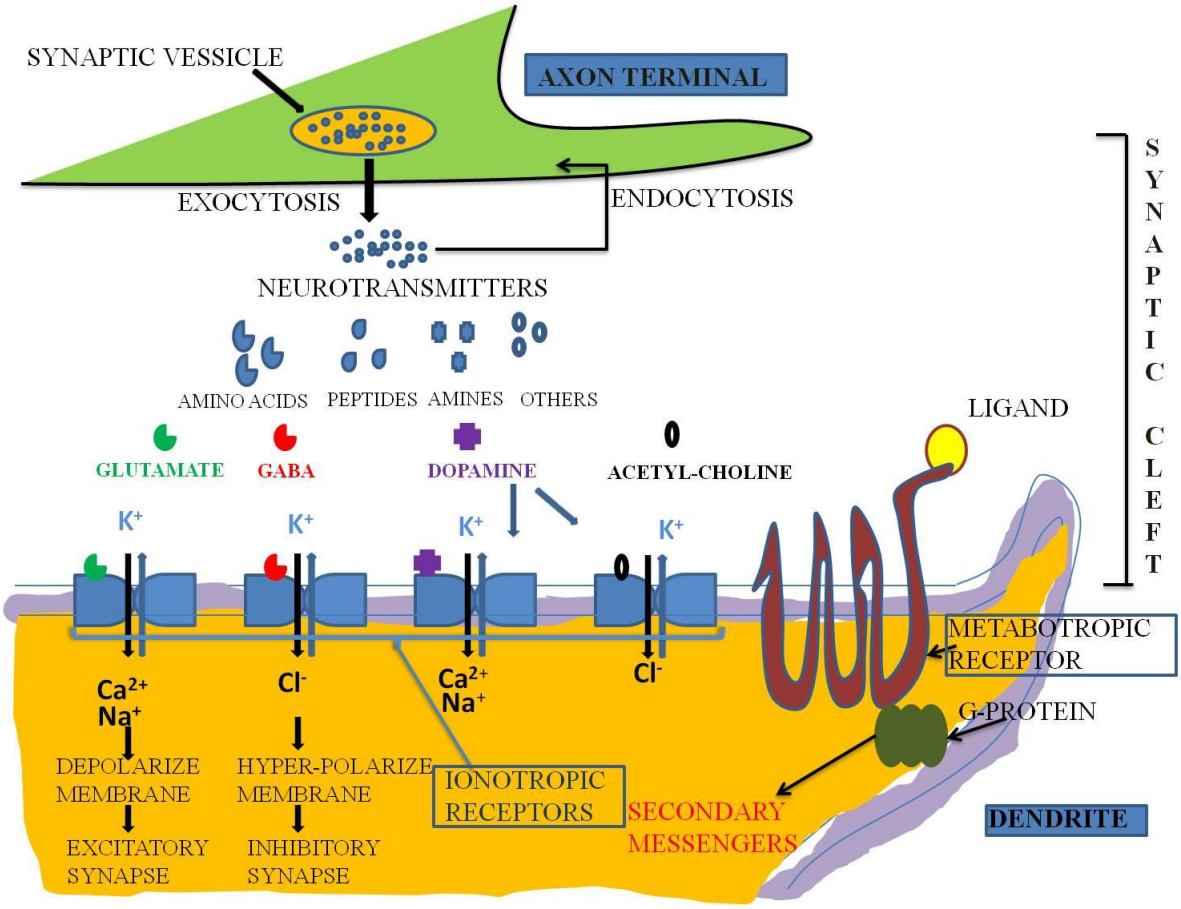


Figure 1.3: Schematic representation of neurotransmitter and receptor interactions at synapse. Neurotransmitters are released from synaptic vesicles through exocytosis and unused neurotransmitters are recycled back through endocytosis. Neurotransmitters could be amino acids (GABA, Glutamate), peptides, amines or others. Receptors could also be broadly classified into two categories ionotropic receptors and metabotropic receptors. Metabotropic receptors are activated by ligands and use G-proteins for their signal transduction. Their synaptic transmission is slow as compared to Ionotropic receptors that use voltage regulated ion gates. Glutamate neurotransmitter mediates inflow of Ca^{2+}/Na^{2+} ions and outflow of K^{+} ions through excitatory ionotropic receptors and therefore with a net + charge inside the cell it depolarize membrane to generate excitatory post synaptic response. GABA on the other hand, mediates inflow of Cl^{-} ions and outflow of K^{+} ions through inhibitorytory ionotropic receptors and therefore with a net - charge inside the cell it hyperpolarize membrane to generate inhibitory post synaptic response. Neurotransmitter like Dopamine and Acetyl choline could use both excitatory or inhibitory ionotropic receptors.

1.8.2 Transcription factors

Transcription factors are proteins or protein complexes that adhere to particular DNA sequences, through their DNA binding domain, and engage in binding of RNA polymerase enzyme to organize the transfer of hereditary information from DNA to mRNA (Lawson C L, *et al.* 2004). Transcription factors are engaged in a variety of roles across development, primarily by triggering and/or suppressing the transcription of their specific genes

(Kawamura A, *et al.* 2008; Showell C, *et al.* 2004). Common transcription factors involved in brain development activities are: Pax6 (paired homeobox transcription factor), CREB (cAMP response element-binding protein), Emx2 and Sox group of factors. Pax6 is expressed in adult immature neurons traveling in rostral migratory stream to the olfactory bulb (Kohwi M, *et al.* 2005). CREB plays a vital role in cell growth and development. It is also a final phosphorylation substrate for various signaling pathways (Delghandi M P, *et al.* 2005; Faigle R, Song H 2012; Hansen T V, *et al.* 1999), while Emx2 is significant for the development of cortex and correct morphogenesis of the CNS (Cecchi C 2002; Heins N, *et al.* 2001; Mallamaci A, *et al.* 1998). The Sox family is crucial for normal development, as its low levels have been associated with reduced adult neurogenesis (Ferri A L, *et al.* 2004). DNA polymorphisms present in the gene sequence, especially near the promoter region, could play a role in altering the efficiency with which transcription factors bind to a particular DNA sequence (Butter F, *et al.* 2012; Kasowski M, *et al.* 2010; Mottagui-Tabar S, *et al.* 2005). This alteration could result in erroneous timing or levels of gene transcription. Transcription factor binding efficiency will be studied for selected SNPs in candidate genes chosen for this thesis.

1.8.3 Growth factors

This large group of extracellular proteins encourage differentiation of neurons, their growth and maintenance, through the process of signal transduction (Faigle R, Song H 2012). In order to carry out their role, growth factors attach to ligand-specific receptors from tyrosine kinase family to autophosphorylate and activate their intracellular domain (Oliveira S L, *et al.* 2013). Activation of specific receptors subsequently activates downstream signaling pathways like PI-3 Kinase/Atk and the Ras/Raf/MEK/Erk pathway. Many families of growth factors are known, common examples being FGF (fibroblast growth factor), NGF (nerve growth factor), IGF (insulin-like growth factor), and EGF (epidermal growth factor) (Faigle R, Song H 2012). FGF is a family of growth factors vital in controlling neuron growth and differentiation in CNS (Garcia-Gonzalez D, *et al.* 2010). NGF is involved in neuron proliferation and differentiation, in addition to apoptosis and migration of oligodendrocytes and Schwann cells (Cattaneo E, McKay R 1990; Chen Y, *et al.* 2009).

IGF, on the other hand can provide defense against apoptosis (Gualco E, *et al.* 2010), while still playing a role in growth, differentiation and proliferation of neural progenitor cells (Rechler M M, Nissley S P 1985). EGF is involved in migration and proliferation of adult and embryonic neural progenitor cells (Burrows R C, *et al.* 1997; Ciccolini F, *et al.* 2005).

Neurotrophic factors are also a type of growth factor that are extracellular signaling proteins and play important roles in controlling cell growth, maintenance, neurotransmitter expression and ion channels (Huang E J, Reichardt L F 2001). In mammals, the main neurotrophic factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) (Hallbook F 1999). Neurotrophin function involves triggering various signaling pathways through two different families of receptors: Trk and p75NTR-TNF family of receptors. Pathways triggered by neurotrophins include signaling through Ras, other members of the G protein family, the MAP and other kinase cascades (Huang E J, Reichardt L F 2001; Iida N, *et al.* 2001; Patapoutian A, Reichardt L F 2001).

1.9 Neuregulin 1 (NRG1)

NRG1 is a growth factor involved in brain development (Anton E S, *et al.* 2004; Chen Y, *et al.* 2010; Fazzari P, *et al.* 2010; Flames N, *et al.* 2004; Mei L, Xiong W C 2008) through its critical roles in neuronal migration (Anton E S, *et al.* 1997) and dendrite formation (Bermingham-McDonogh O, *et al.* 1996). Studies have also highlighted its significant role in synapse formation (Hahn C G, *et al.* 2006; Roy K, *et al.* 2007; Shamir A, *et al.* 2012; Ting A K, *et al.* 2011)

NRG1 has been linked in the etiology of schizophrenia as well as various other diseases including cancer, cardio-vascular disease and multiple sclerosis (Esper R M, *et al.* 2006; Sheng Q, *et al.* 2010). *NRG1* was first linked to schizophrenia in the Icelandic population, where association was observed between a core-risk haplotype (HAPICE) at the 5' end of *NRG1* and schizophrenia (Stefansson H, *et al.* 2002). Since then, HAPICE association has been replicated in samples from Scotland (Stefansson H, *et al.* 2003) and UK (Williams N M, *et al.* 2003a). Significant associations of "HAPICE" have been reported from Irish

(Corvin A P, *et al.* 2004), Chinese (Yang J Z, *et al.* 2003), and South African samples (Hall D, *et al.* 2004), whereas negative findings have been reported in Japanese (Iwata N, *et al.* 2004) and other samples (Hall D, *et al.* 2004; Thiselton D L, *et al.* 2004). *NRG1* has been associated with bipolar disorder as well, in American (Goes F S, *et al.* 2009), British (Prata D P, *et al.* 2009) and Scottish cohorts (Prata D P, *et al.* 2009; Thomson P A, *et al.* 2007) providing evidence that this gene may be a risk gene common to both bipolar disorder and schizophrenia.

1.9.1 Roles of different mRNA and protein isoforms of NRG1

In vertebrates, there are four NRG genes (NRG1-4), and their diversity is additionally amplified by alternate RNA splicing and promoter usage (Fischbach G D, Rosen K M 1997; Lemke G 1996). The *NRG1* gene, located at chromosome 8p13, is approximately1.4Mb in size and uses alternative promoters to form many structurally and functionally different isoforms (Falls D L 2003). In human *NRG1*, there are 20 exons, 6 known alternative promoters and over 30 known mRNA transcripts (Mei L, Xiong W C 2008) of this gene (Falls D L 2003). *NRG1* encodes around 15 protein isoforms involved in various brain functions like neuron signaling, axon guidance, neuronal differentiation and myelination, interactions with ErbB receptor, synaptogenesis, and neurotransmission (Corfas G, *et al.* 2004). These are classified into three main functional isoforms, Type I (contact-independent), Type II (contact-independent), and Type III (contact-dependent or insoluble).

Type I NRG1 comprises a immunoglobulin-like domain adjacent to high glycosidic region (shown in Figure 1.4); Type II NRG1, on the other hand possesses a GC-specific domain and a IgG-like domain; and Type III has a unique cystein-rich domain (CRD) (Buonanno A, Fischbach G D 2001). Type I NRG1 is required for neuron generation in cranial ganglia, while type III NRG1 plays a fundamental role in Schwann cell development (Meyer D, *et al.* 1997). Type II NRG1 has a role in special memory and learning (Taylor A R, *et al.* 2012). Particular NRG1 mRNA isoforms (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009; Parlapani E, *et al.* 2010) are elevated in the hippocampus and

frontal cortex of schizophrenia and this changed NRG1 isoform expression levels have been associated with DNA variations in promoter and intronic regions of schizophrenia (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009).

1.9.2 Signaling through ErbB receptors

Neuregulins represent a family of proteins that are involved in cell signaling with members of EGF receptor family receptor tyrosine kinases (Buonanno A, Fischbach G D 2001; Harrison P J, Law A J 2006). NRG1 is capable of performing both paracrine and juxtacrine signaling. Paracrine signaling activates ErbB receptors on nearby cells via EGF domains, while juxtacrine signaling activates ErbB receptors in direct contact with the NRG1 expressing cell. Contact-independent (soluble) Type I, II and IV NRG1 show unidirectional signaling, where membrane-free N-terminal fragments (NTFs) disperse through extracellular space and take up the role of a ligand to activate ErbB receptors in a paracrine fashion. Whereas contact-independent (insoluble) Type III NRG1, activates ErbB receptors through membrane-attached N-terminal fragments (NTFm) in a juxtacrine fashion (Harrison P J, Law A J 2006). NTFs or NTFm interact with ErbB3/4 and form a hetero-dimer with help of ErbB2, after this, the autophosphorylation of the tyrosine residues takes place in the cytoplasmic domain of the receptor. This autophosphorylation creates binding sites for various adaptor proteins such as PI3-kinase and Shc, which then modify transcriptional functions in the cell by activating various pathways such as PI3K and MAPK (Harrison P J, Law A J 2006).

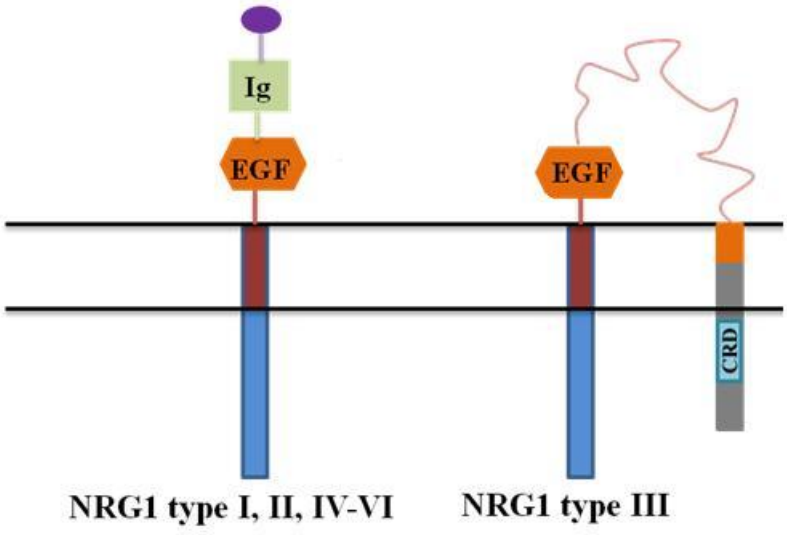


Figure 1.4: Six main functional isoforms of NRG1 could be divided into two main types. NRG1 prototypes I, II, IV-VI or a soluble form and NRG1 prototype III **or** a membrane-bound form. (EGF = Epidermal Growth Factor; Ig = Immunoglobulin domain; CRD = Cystein Rich Domain).

Bi-directional signaling has been reported in Type III NRG1 in its interactions with ErbB family of receptors (Canetta S E, *et al.* 2011). In the process, type III NRG1, in addition to its normal role of as a ligand, also serves as a receptor (Bao J, *et al.* 2004; Bao J, *et al.* 2003). Type III NRG1 has a Intracellular Cystine Rich Domain (NRG1-ICD) that releases from plasma membrane after proteolysis by γ-secretase complex. This NRG1-ICD could either get translocated into the nucleus where it interacts with fusion proteins and enhances promoter activity of certain genes to cause change in gene expression (transcriptional effect) or not get translocated into the nucleus and interact with non-nuclear targets like LIM-Kinase1 (Arber S, *et al.* 1998) to cause neurite growth or actin reorganization (non-transcriptional effect) (Bao J, *et al.* 2004; Bao J, *et al.* 2003; Canetta S E, *et al.* 2011; Hancock M L, *et al.* 2008).

NRG1/ErbB signaling is important in synaptic plasticity and brain development (Anton E S, *et al.* 2004; Fazzari P, *et al.* 2010; Flames N, *et al.* 2004). It is also involved in myelination (Brinkmann B G, *et al.* 2008), cell signaling (Newbern J, Birchmeier C 2010) and even cardio-vascular events (Lopes-Conceicao L, *et al.* 2011). NRG1/ErbB4 signaling

is also associated with schizophrenia at both genetic and transcriptional levels (Law A J, *et al.* 2007; Nicodemus K K, *et al.* 2006; Norton N, *et al.* 2006; Silberberg G, *et al.* 2006; Walsh T, *et al.* 2008). Increased expression levels of NRG1 and ErbB4 have been reported in post-mortem brain tissues (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2007; Law A J, *et al.* 2006; Silberberg G, *et al.* 2006) and in neurons derived from induced pluripotent stem cells (IPSCs) of schizophrenia (Brennand K J, *et al.* 2011), but the precise mechanism by which these genes grants risk to schizophrenia or mental illness in general, remains hard to pin down.

1.10 Neural Cell Adhesion Molecule 1 (NCAM1)

NCAM1, a transmembrane protein is part of the immunoglobin superfamily (Arai M, *et al.* 2004; Kasper C, *et al.* 2000). NCAM1 has multiple roles and is broadly expressed in post-mitotic neurons and Schwann cells (Arai M, *et al.* 2004; Seilheimer B, Schachner M 1988; Wang B 2009). It has been a subject of curiosity to neuroscientists because of its role in neurodevelopment (Ronn L C, *et al.* 1998), neurogenesis (Gascon E, *et al.* 2010) and synaptic plasticity (Chipman P H, *et al.* 2010; Gray L J, *et al.* 2010; Kiss J Z, Muller D 2001). Structurally, NCAM1 typically composes 5 immunoglobulin (Ig) domains, 2 fibronectin III (Fn-III) repeats and a transmembrane domain or a GPI (glycosylphosphphatidylinositol-linked) anchor (Atkins A R, *et al.* 2004).

1.10.1 Cell adhesion

NCAM1 was the first molecule found to be involved in homophilic binding between neural cells (Albach C, *et al.* 2004). NCAM1 supports neurite outgrowth, their migration and synaptogenesis through both homophilic (Ca^{2+}- independent) and heterophilic (cell-cell and cell-extracellular matrix) communications. Crystallographic data suggests that homophilic interactions (NCAM-NCAM) in NCAM1 could be anti-parallel (*trans)* (involving Ig2-Ig3, Ig1-Ig3, Ig2-Ig2 modules between NCAM domains) or parallel (*cis)* (involving formation of dimers between Ig1 and Ig2 modules situated on the same cell surface, which could further interact to form zipper-like adhesion complexes). Heterophilic interactions involve communications with other molecules like heparan, FGF receptor, neural cell adhesion

molecule L1 (L1CAM) and GDNF (Heiland P C, *et al.* 1998; Horstkorte R, *et al.* 1993; Senkov O, *et al.* 2012). Interestingly, L1CAM binds ErbB receptors through Ig-like domains coupling cell adhesion and neuregulin signaling (Donier E, *et al.* 2012).

1.10.2 Roles of different mRNA and protein isoforms

NCAM1 gene is located on chromosome 11q23.1. Alternative splicing of NCAM1 mRNA gives rise to its three major protein isoforms, distinguished by their size in kiloDaltons (kDa) at 180kDa, 140kDa and 120kDa (NCAM180, NCAM140, and NCAM120, respectively) (Cunningham B A, *et al.* 1987). Structurally all these isoforms contain 5 immunoglobulin (Ig) domains and 2 fibronectin III (Fn-III) repeats (Atkins A R, *et al.* 2004). NCAM180 and NCAM140 are long and short transmembrane isoforms, respectively. They differ in cytoplasmic domain with NCAM180 having an extra exon, 18, providing additional 100 amino acids (Senkov O, *et al.* 2012) (Figure 1.5)

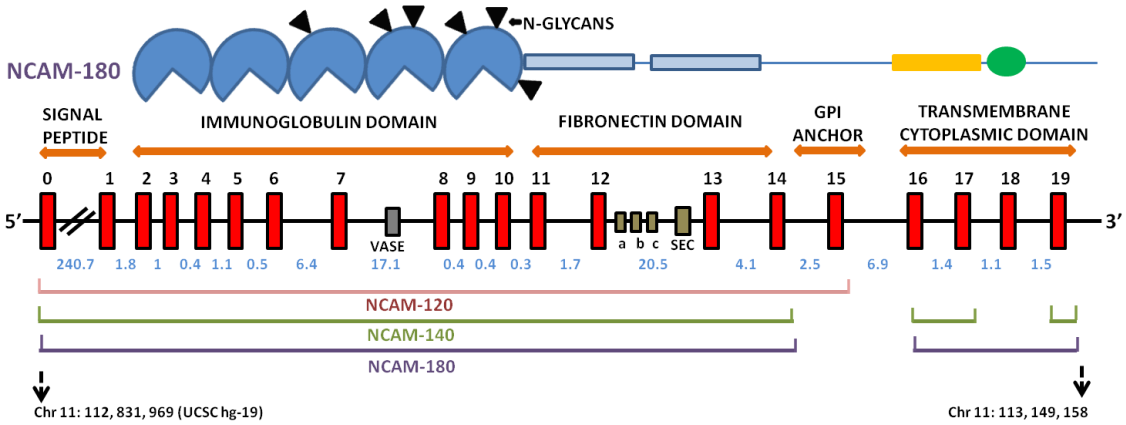


Figure 1.5: Figure showing genomic structure of *NCAM1* and also the positions of different exons, introns and functional domains. PolySTs catalyze the formation of sialic acid chain on the 5th and 6th N-glycans of 5th Ig domain, close to FN domain.Alternative splicing result in three major isoforms of NCAM1 i.e. NCAM180, NCAM140, and NCAM120. Exon 0-14 are common in all these isoforms. Isoform 180 consists of exons 0 to 14 and 16 to 19, missing exon 15, which is essential for GPI anchor. Isoform 140 is similar to isoform 180 but lacks exon, 18. NCAM120 is composed of exons 0 to 15 only (Arai M, *et al.* 2004). Chromosome positions are taken from UCSC human genome version 19 (UCSC hg-19), Variable Alternative Spliced Exon (VASE) represents a 10 amino acid sequence included in the fourth Ig domain of NCAM1 between exon 7 and 8. SEC (secratory) are small exons between exon 12 and 13. In addition, three small-exons denoted as the exon a, b and c, are also located between exon 12 and 13. Numbers in blue represents region in kb between two exons.

NCAM120 does not contain the transmembrane and intracellular domains and is adhered to the membrane with GPI anchor (Brennaman L H, *et al.* 2011). Mature neurons express NCAM180 in their postsynaptic densities (Fux C M, *et al.* 2003), while axon shafts and growth cones of budding neurons express NCAM140 (Stork O, *et al.* 2000). NCAM120 is expressed essentially in oligodendrites and myelin sheaths (Muhlenhoff M, *et al.* 2009).

In humans and rodents, NCAM180 and NCAM140 expression declines over development (Figure 1. 6) (Oltmann-Norden I, *et al.* 2008), while NCAM120 expression which is non-existent in fetal brains, increases and is expressed at constant levels in adult brain (Brennaman L H, Maness P F 2008; Cox E T, *et al.* 2009). A cleavage product of NCAM180 is approximately 105-115 kDa and is also reported in dorso-lateral prefrontal cortex (DLPFC) and hippocampus (Ronn L C, *et al.* 1998; Vawter M P, *et al.* 2001). Vawter *et al.* 2001 study also associated this cleavage product with ventricular enlargement, a condition common in schizophrenia.

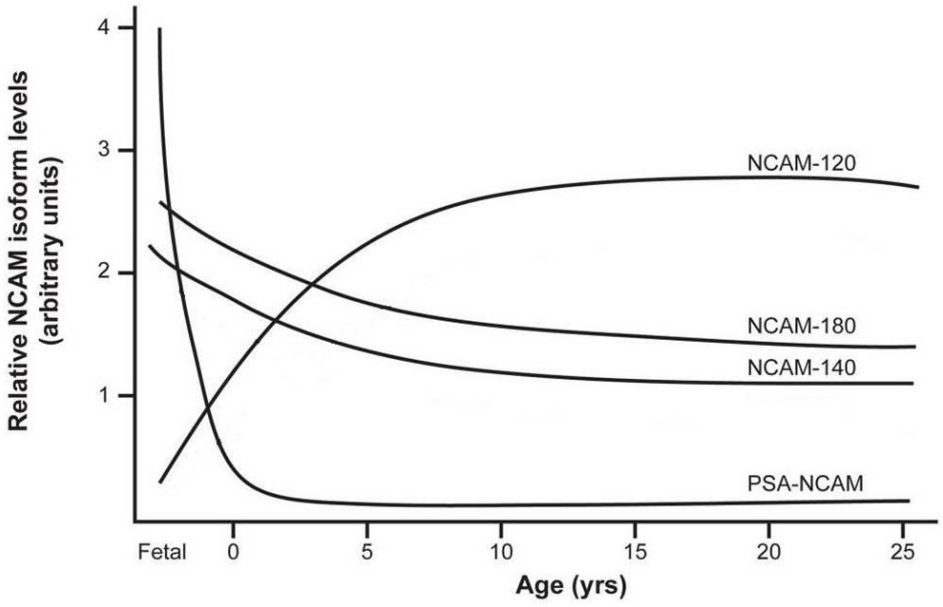


Figure 1. 6: Developmental profile of three major isoforms of NCAM1 and its polysialylated form (PSA-NCAM) in humans. Figure modified from *(Cox E T, et al. 2009).*

Previously, different studies have provided evidence that both increase and decrease in different NCAM1 isoform expression has been linked to schizophrenia, at both mRNA and protein level (Gibbons A S, *et al.* 2009; Gray L J, *et al.* 2010). Inequity of NCAM1 and polysialyltransferases (PolySTs) has been linked to defective brain connectivity (Hildebrandt H, *et al.* 2009). Elevated levels of VASE are reported in the hippocampus and PFC of bipolar disorder (Vawter M P, *et al.* 1998b) and in the CSF of schizophrenia, but not bipolar disorder (Vawter M P, *et al.* 2000).

Many illustrated roles of NCAM have been linked to its posttranslational modification, PSA-NCAM. In the brain, NCAM140 and NCAM180 act as the predominant carriers of PSA, whereas the majority of NCAM120 remains PSA free (Muhlenhoff M, *et al.* 2009). Studies by Nelson *et al.*, (Nelson R W, *et al.* 1995) demonstrate that of NCAM's six N-glycosylation sites, sites 5 and 6 on the fifth Ig domain (Ig5) bear majority of NCAM's PSA (Figure 1. 7). FN1 α-helix is not required for polyST recognition, but does play an important function in orientation of Ig5 N-glycans for polysialylation process (Colley K 2010). The FN1-acidic patch consists of four amino acid residues (Asp^{497}, Asp^{511}, Glu^{512}, and Glu^{514}) (Mendiratta S S, *et al.* 2005); mutations in three of these residues (Asp^{511}, Glu^{512}, and Glu^{514}) have been reported to diminish NCAM polysialylation (Foley D A, *et al.* 2009).

NCAM1 has been identified as a susceptibility gene for both bipolar disorder and schizophrenia (Arai M, *et al.* 2004; Atz M E, *et al.* 2007; Gibbons A S, *et al.* 2009; Sullivan P F, *et al.* 2007; Vawter M P 2000). It was found to be notably associated with bipolar disorder in Japanese individuals (Arai M, *et al.* 2004). Mice lacking *NCAM1* illustrated errors in spatial learning, cell migration and synaptic plasticity (Angata K, *et al.* 2004) to further highlight importance of this gene in proper brain development.

1.10.3 Post-translational glycosylation (PSA-NCAM)

Sialic acid (Sia), is a common name for the family of N- and O-substituted derivatives of a 9-carbon sugar molecule, neuraminic acid. It is a fundamental constituent of brain gangliosides and polysialic acid (Wang B 2009). In neuronal cells, monomers of Sia (e.g.

N-acetylneuraminic acid or Neu5Ac) can be joined together to form linear, anionic chains of α2,8-linked sialyl residues (Wang B 2009) called PSA. PSA is an important constituent for development of brain and its cognitive abilities (Wang B 2009).

PSA is synthesized in the Golgi apparatus, where it is found as homopolymer of α2,8-linked sialic acid attached to the N-linked and O-linked glycans of a small group of proteins. These proteins include NCAM1 (Finne J, *et al.* 1983; Rothbard J B, *et al.* 1982), voltage-dependent sodium channel α-subunit (Finne J, *et al.* 1983; Rothbard J B, *et al.* 1982; Zuber C, *et al.* 1992), two polySTs (Close B E, Colley K J 1998), CD36 (Yabe U, *et al.* 2003), fetuin (Colley K 2010), synCAM1 and neuropilin-2 (present on dendritic cells) (Curreli S, *et al.* 2007; Muhlenhoff M, *et al.* 2013). Interestingly, PSA on CD36 and neuropilin-2 is found exclusively on O-glycans. The process of polysialylation is protein specific and requires polySTs to distinguish specific features of their glycoprotein substrates. NCAM is the most extensively polysialylated glycoprotein in the brain (McCoy R D, *et al.* 1985). Polysialylation of NCAM by polySTs is very efficient and this allows the polymerization of PSA chains on the glycans of NCAM to form PSA-NCAM (Angata K, *et al.* 2000). Attachment of PSA reduces the homo- and heterophilic adhesive functions of NCAM (Cunningham B A, *et al.* 1987; Hoffman K B 1998; Kasper C, *et al.* 2000; Rutishauser U 2008). It also enlarges its extracellular hydrodynamic radius (Figure 1. 7) increasing intermembrane distances (Fujimoto I, *et al.* 2001; Oltmann-Norden I, *et al.* 2008), and thereby assisting in migration of neuronal cells, their outgrowth and pathfinding (Acheson A, *et al.* 1991; Durbec P, Cremer H 2001; Tang J, *et al.* 1994) and synaptogenesis (Angata K, *et al.* 2002; Muller D, *et al.* 1996; Rutishauser U, Landmesser L 1996). PSA masks NCAM to ensure configuration of NCAM contacts in a time- and site-specific manner (Figure 1. 8) (Weinhold B, *et al.* 2005). Expression of PSA-NCAM is high during brain development, but it gradually diminishes postnatally (Angata K, Fukuda M 2003; Oltmann-Norden I, *et al.* 2008; Rutishauser U 2008), such that in the adult brain it is confined to areas of high plasticity and regeneration, including the hippocampus, PFC, thalamus, SVZ and amygdala (Bonfanti L 2006; Gascon E, *et al.* 2007b; Rutishauser U 2008).

Studies have reported that *in vitro* removal of PSA, using endoneuraminidase N (endo-N), results in complete failure of long-term potentiation (LTP) and long-term depression (LTD) (Muller D, *et al.* 1996). While, *in vivo* removal resulted in distorted learning and formation of memories (Becker C G, *et al.* 1996; Lopez-Fernandez M A, *et al.* 2007). As mentioned, PSA modified NCAM1 plays a critical role in neural development (Angata K, *et al.* 2000), while disruption of this process has been suggested as an etiological factor in psychiatric disorders (Arai M, *et al.* 2006; Barbeau D, *et al.* 1995). Loss of PSA in adults, coincides with completion of major morphogenetic events, and conversion of PSA-NCAM back to NCAM (Muhlenhoff M, *et al.* 2009). Thus, it is thought that PSA and NCAM interaction play an important role in brain development and related disorders (Arai M, *et al.* 2006; Barbeau D, *et al.* 1995; Senkov O, *et al.* 2012).

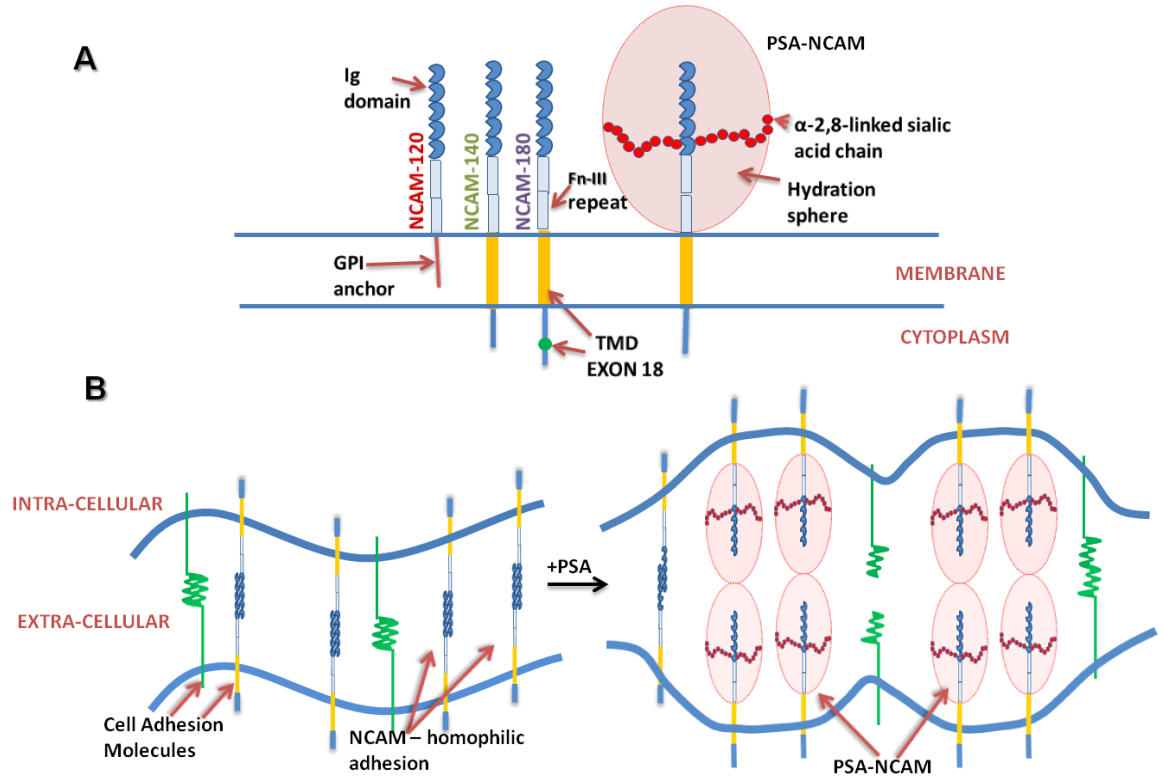


Figure 1. 7: Schematic representation showing A) Major isoforms of NCAM i.e. NCAM120, NCAM140 and NCAM180 and their mode of attachment on the membrane, figure also shows formation of 'hydration sphere' around PSA-NCAM due to polyanionic nature of PSA; B) NCAM which is normally

present on the membrane as an adhesive molecule becomes non-adhesive post translation process, PSA restricts adhesive properties of NCAM and increases intercellular spaces.

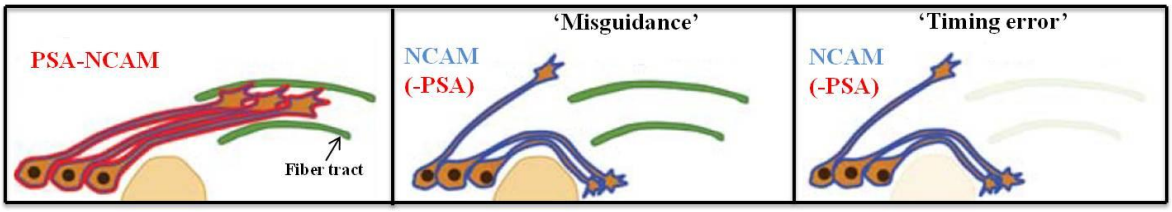


Figure 1. 8: Schematic representation showing importance of PSA-NCAM in regulating axon guidance and pathfinding. Figure shows PSA allows proper axon guidance and pathfinding. Whereas, consequences of no PSA on NCAM could cause axons to migrate outside fiber tracts (misguidance) or axons could start to migrate before fiber tracts are formed properly (timing error). [Figure modified from reference (Muhlenhoff M, *et al.* 2009)]

1.11 Polysialyltransferases (PolySTs)

There are six alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase genes in the human genome, of which protein products of two (*ST8SIA2* and *ST8SIA4*) are responsible for synthesizing long (>50 residues) polysialic acid chains. ST8SIA2 and ST8SIA4 are together referred as polySTs. The protein products of other four *ST8SIA* genes synthesize shorter (<10 residues) chains and are referred to as oligo- or mono-STs (Harduin-Lepers A, *et al.* 2008). They catalyze the transfer of sialic acid chains from its activated sugar nucleotide precursor, CMP-Sia, to its major acceptor substrate (Wang B 2009). Each PolyST, by itself is capable of controlling PSA synthesis (Angata K, *et al.* 2004) and their mRNA levels parallel PSA-NCAM expression (Brocco M A, Frasch A C 2006). Although expressed in diverse spatial and temporal patterns among neural tissues, these polySTs synthesize PSA chains of the identical average length (Angata K, *et al.* 2004; Colley K 2010). Both PolySTs can be auto-polysialylated *in vivo* (Close B E, Colley K J 1998) and have 59% amino acid resemblance (Angata K, *et al.* 2004).

1.11.1 Polysialyltransferase-2 (ST8SIA2)

Alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 or ST8SIA2 (also known as SIAT8B and STX) is a member of an enzyme family sialyltransferase. ST8SIA2 protein is

encoded by a single gene comprising 6 exons, which spans a region of 70.3 kb on human chromosome 15q25-26. The resulting protein is a type II membrane protein with their N-terminus towards the cytoplasm and C-terminus exposed towards the extracellular matrix. ST8SIA2 protein has a molecular mass of 42.5 kDa and has six N-linked glycosylation sites (Close B E, Colley K J 1998). Detailed structure of ST8SIA2 has four main protein domains. First, the transmembrane domain (TM) is present from amino acid (aa) 8-25, presenting a common feature of all sialyltransferases (Nakata D, *et al.* 2006). Second, Sialyl-motif-L (SML) domain is present from aa 139-186 and is involved in binding both substrate CMP-Neu5Ac and acceptor glycoproteins (Datta A K, Paulson J C 1995; Datta A K, *et al.* 1998). Third, polysialyltransferase domain (PSTD) is present from aa 246-277 and is a structurally unique poly-basic motif, also called 'polymerization domain'. It is present only in polySTs (absent in mono-oligoSTs) and is located immediately upstream and contiguous with Sialyl-motif-S (Nakata D, *et al.* 2006; Sevigny M B, *et al.* 1998). Fourth and last, Sialyl-motif-S (SMS) domain is present from aa 278-300 and is involved in the binding of CMP-Neu5Ac only (Datta A K, Paulson J C 1995; Datta A K, *et al.* 1998). ST8SIA2 is the foremost enzyme for polysialylation in hippocampus (Brocco M A, Frasch A C 2006) and plays a dominant role in PSA synthesis on glycolsylatable forms of NCAM1 (Galuska S P, *et al.* 2006; Hildebrandt H, *et al.* 2009), which is a 1500-fold better substrate for ST8SIA2 than the next best substrate, Fetuin (Colley K 2010). Therefore, precise control in ST8SIA2's expression during early brain development is crucial to allow processes to reach their target and neurons to set up connections (Dityatev A, *et al.* 2004). High level ST8SIA2 expression is found in early embryonic stages and in the neonate, whereas in adults, expression is low (Nakayama J, *et al.* 1998), but detectable.

Our group and others have implicated the region on 15q26 as harboring a risk locus for mental illness (McAuley E Z, *et al.* 2009; Vazza G, *et al.* 2007) and have implicated the *ST8SIA2* gene as a susceptibility gene for bipolar disorder, schizophrenia and autism (Anney R, *et al.* 2010; Arai M, *et al.* 2006; Lee M T M, *et al.* 2011; McAuley E Z, *et al.* 2012; Sato C, Kitajima K 2013). Cognitive disturbances seen in the mice that lack ST8SIA2 were decreased social motivation and increased aggressive behavior (Calandreau

L, *et al.* 2010), common traits of both bipolar disorder and schizophrenia patients. Hence *ST8SIA2* poses a good functional candidate gene for investigation of its role in bipolar disorder and schizophrenia susceptibility, and will be explored as a subject in this thesis (Chapter 5).

1.11.2 Polysialyltransferase-4 (ST8SIA4)

Alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 or ST8SIA4 (also known as SIAT8D and PST) is also a type II membrane protein, golgi-resident and a member of family sialyltransferase that plays a dominant role in PSA synthesis on NCAM1 (Nakayama J, *et al.* 1998). The *ST8SIA4* gene is located on 5q21 and has 5 exons (Arai M, *et al.* 2006). Mice lacking *ST8SIA4* show disturbed cognitive performance (Eckhardt M, *et al.* 2000), a feature of schizophrenia (Mohamed S, *et al.* 1999). Structurally, ST8SIA4 protein is very similar to ST8SIA2 in having all the major catalytic and transmembrane domains including the PSTD (Angata K, Fukuda M 2003). Unlike ST8SIA2, which is expressed principally in embryonic tissues, ST8SIA4 is constantly expressed in a range of tissues (Angata K, *et al.* 1997; Brocco M, *et al.* 2003; Hildebrandt H, *et al.* 1998). ST8SIA4 could be auto-polysialylated *in vitro* as opposed to ST8SIA2 (Muhlenhoff M, *et al.* 1996).

1.11.3 Differences between the two PolySTs

Other differences between the two polySTs include; ST8SIA2 has a greater affinity for NCAM1 and is also identified to be more efficient than ST8SIA4, in processing complete NCAM1 protein *in-vivo* (Colley, 2010; Galuska *et al.*, 2006; Oltmann-Norden *et al.*, 2008). However, *in-vitro* ST8SIA4 is known to form more highly polysialylated N-glycans on NCAM1 (Angata K, *et al.* 2002). *ST8SIA4* deficiency causes impairment in synaptic plasticity, while ST8SIA2 deficit results in faulty infrapyramidal mossy fibers and errors in configuration of hippocampal ectopic synapses (Angata K, *et al.* 2004) and deficiency of PSA from neuronal surfaces (Brocco M A, Frasch A C 2006). The antiepileptic drug, valproic acid, which is one of the most frequently used medications used to treat bipolar disorder, up-regulates ST8SIA4 mRNA levels but down regulates the level of ST8SIA2 mRNA (Beecken W D, *et al.* 2005). During the first postnatal days, unavailability of

ST8SIA2, but not ST8SIA4, considerably reduces PSA levels (Oltmann-Norden I, *et al.* 2008), illustrating the opposite temporal expression pattern of the two genes – where ST8SIA2 is expressed highly early on in development with decline into adulthood, whereas ST8SIA4 is expressed at low levels early in development with increasing expression into adulthood.

Summary:

In summary, both bipolar disorder and schizophrenia cause a huge financial burden on the nations, with their direct (e.g. hospital and clinic fees) and indirect costs (e.g. reduced work commitment and increased government support) [reviewed by Insel *et al* (2008)]. Living with such disorders is extremely difficult not only for the patients, but also for the people around them. Therefore, it is important to understand underlying mechanisms that cause such disorders and translate them into better diagnoses and treatments in future. Both the disorders share overlying collection of symptoms, most remarkably the presence of psychosis. Recently, genome-wide linkage scans have identified many new genes for both bipolar disorder and schizophrenia. However, studies focusing on functional follow-up for these genes are very rare, which is largely due to nature of such studies being very labour intensive and expensive. Also, there is no availability of high throughput methods for functional characterization of genes and their isoforms. Therefore, at present, a big lag exists in the discovery of putative risk genes from GWAS, and the crucial downstream functional investigation to figure out how the genetic polymorphisms contribute to risk of illness. When considering functional studies, only a small number of studies have used post-mortem human brain samples to explore these mental disorders in the context of schizophrenia or bipolar disorder risk genes. Harrison *et al* (2011) precisely made a point, "Considering psychiatric disorders are brain disorders, direct examination of the brain should be as integral to their biological study as is the study of bone marrow for a hematologist".

1.12 Aims of this thesis

The goals of this thesis are to focus on three candidate genes (i.e. *NRG1, NCAM1* and *ST8SIA2*), associated with both schizophrenia and bipolar disorder, and determine how these genes could be functionally involved in the disease susceptibility. Of these three genes, *NRG1* is the most replicated susceptibility gene for mental illness (Agim Z S, *et al.* 2013; Fukui N, *et al.* 2006; Stefansson H, *et al.* 2003; Stefansson H, *et al.* 2002; Williams N M, *et al.* 2003a), with support from both genetic association as well as functional studies (Harrison P J, Law A J 2006; Hashimoto R, *et al.* 2004; Law A J, *et al.* 2004; Parlapani E, *et al.* 2010). On the other hand, *ST8SIA2* and *NCAM1* have not been as strongly replicated, but have shown associations with both bipolar disorder (Arai M, *et al.* 2004; Lee M T M, *et al.* 2011) and schizophrenia (Atz M E, *et al.* 2007; Vazza G, *et al.* 2007) in smaller cohorts. I have selected *ST8SIA2* as a candidate gene, mainly because it has shown strong association with bipolar disorder in our groups' findings (McAuley E Z, *et al.* 2009; McAuley E Z, *et al.* 2012). During early embryonic stages of brain development it plays a very important role in the polysialylation of its prime substrate NCAM1 (Hildebrandt H, *et al.* 1998; Hildebrandt H, *et al.* 2010), a step very critical for normal brain development (Hildebrandt H, *et al.* 2009; Senkov O, *et al.* 2012; Vogt J, *et al.* 2012). While none of these selected candidate genes are represented in the top GWAS hits from large GWAS and Psychiatric GWAS Consortium studies (Ferreira M A, *et al.* 2008; Green E K, *et al.* 2012; Hamshere M L, *et al.* 2013; Levinson D F, *et al.* 2012; Ripke S, *et al.* 2011) each has shown independent association with mental illness in multiple genetic mapping studies and play a crucial role in neural development (Hildebrandt H, *et al.* 2009; Liu X, *et al.* 2011; Senkov O, *et al.* 2012; Vogt J, *et al.* 2012). In this thesis all three candidate genes (*NRG1, NCAM1* and *ST8SIA2*) will be examined for genetic, expression and protein changes using human post-mortem brain and case-control cohorts comprising of schizophrenia and bipolar disorder patients, in addition to normal unaffected controls. Specifically, the thesis aims to assess variation in gene structure through targeted sequencing (Chapters 3, 4 and 5) and examine the potential functional impact of DNA polymorphisms on transcription factor binding (Chapters 3, 4 and 5) and mRNA splicing (Chapters 4 and 5). Alterations in mRNA

expression and examination of splicing (Chapter 3, 4 and 5) and protein expression levels or activity (Chapters 4 and 5) will be undertaken. The overall aim is to provide evidence that could link variations in the candidate genes with deficits in brain development which could result in psychotic mental illness.

Chapter 2

Materials and Methods

2.1 Cohort descriptions

2.1.1 Post mortem brain cohorts

Considering psychiatric disorders like bipolar disorder and schizophrenia are brain disorders, studies using human brain tissue become very important. However brain tissue resources are scarce, and complicated by artifacts caused by the process of death, and limitations in the quality and quantity of samples obtained. Despite these issues, post-mortem human brain serves as a vital resource to study underlying pathogenic molecular mechanisms of these devastating mental disorders. In my studies, tissues from post-mortem brains were used to extract DNA, RNA and proteins.

Before starting to work with post-mortem brains, it is important to know about their quality and correct diagnosis. It is also important to set inclusion and exclusion criteria of different samples in the cohort. Post-mortem studies are subjected to many potential confounds which differ between diagnostic groups [for example, subject medication history (antipsychotics) and smoking history etc.], and this can make it difficult to distinguish between primary effects specific to the illness and other secondary effects. Therefore, accurate recording and assessment of all potential confounders is very important, otherwise it could bias the main experimental findings and influence the interpretation. In my experimental analysis, data was corrected for significantly associated confounders before performing analyses for main effects. Additionally, factors associated with only one group were tested for impact on measures of interest through correlations or by dichotomous grouping.

In human brain, the frontal cortex is thought to play an important role in programming, updating and sustaining internal representations of tasks for working memory (D'Ardenne K, *et al.* 2012). Different regions of frontal cortex such as, orbitofrontal (OFC), dorsolateral prefrontal (DLPFC), ventro-lateral prefrontal (VLPFC), and medial prefrontal (MPFC) cortices have been found to be associated with bipolar disorder (Foland L C, *et al.* 2008; Kronhaus D M, *et al.* 2006; Passarotti A M, *et al.* 2012; Sinclair D, *et al.* 2012b) and schizophrenia (Becker T M, *et al.* 2008; Gonzalez-Blanch C, *et al.* 2008; Pinkham A E, *et*

al. 2008; Sinclair D, *et al.* 2012b; Taylor S F, *et al.* 2007). In my studies, I have focused on the DLPFC region because it is believed to regulate executive functions and organizes behavioral responses and strategies in learning new tasks (Frey B N, *et al.* 2007; Mega M S, Cummings J L 1994). The DLPFC also represents the primary site of higher cognitive function impairment (Frith C, Dolan R 1996). Patients suffering from schizophrenia and bipolar disorder were reported to have structural, neurochemical, and cytoarchitectural abnormalities in this region (Chan M K, *et al.* 2011; Rajkowska G, *et al.* 2001), including reduced neuron size and glial density (Cotter D, *et al.* 2002; Rajkowska G, *et al.* 2001). Evidence also suggests irregular neurotransmission (Raust A, *et al.* 2007), signal transduction (Funk A J, *et al.* 2012), neural connectivity (Kyriakopoulos M, *et al.* 2012), GABAergic (Rajkowska G, *et al.* 2007) and glutamatergic activities (Karolewicz B, *et al.* 2010) in the DLPFC of patients suffering from psychiatric illness, making it an important region to focus on.

2.1.1.1 Tissue Resource Centre (TRC) brain tissue specimens

This cohort has been assembled by the NSW Tissue Resource Centre Brain Bank with support from the Schizophrenia Research Institute (SRI) to create a common resource for post-mortem brain research into the molecular and pathological causes of schizophrenia. Dissection of brains and different regions were performed by trained pathologists under full ethical clearance from the Sydney Local Health Network Ethics Review Committee (X11-0107 and HREC/11/RPAH/147) and informed written consent from the next of kin. The use of these samples for this study was approved by Human Research Ethics Committee at the University of NSW (#HREC: HC12435). Grey matter tissue from DLPFC was available through Schizophrenia Research Laboratory (SRL) at NeuRA, that included individuals with schizophrenia (n = 30) or schizoaffective disorder/schizoaffective disorder (n = 7) and normal unaffected controls/unaffected controls (n = 37).

Affected cases with schizophrenia/schizoaffective disorder met the criteria for Diagnostic and Statistical Manual for Mental Disorders, 4th edition. Cases with a prolonged agonal life support, or cases with a history of cerebral infarction, head injury, or neurodegenerative disease (e.g., Alzheimer's disease), were excluded. Other factors such as brain-hemisphere,

gender etc. were matched on a group-wise basis. Unaffected controls were screened for medical history and psychiatric problems, including drug/alcohol abuse by telephonic interviews of family members. All samples were from regions around Sydney, which included mostly Caucasian (72) with 2 Asians (1 schizoaffective and 1 control). Samples included 20 females (13 schizophrenia, 7 unaffected controls) and 54 males (24 schizophrenia and 30 unaffected controls).

Further details regarding the clinical information, brain collection and processing of its tissue for this cohort has been described previously (Weickert C S, *et al.* 2010). Paired case-control samples based on their demographic characteristics are described in Table 2.1. Other demographic information such as tobacco use status and clinical correlations such as antipsychotic history, chlorpromazine equivalent neuroleptic (mean daily dose, last dose, and total lifetime use), age of onset, agonal state immediately preceding death and manner of death (natural/suicide), were also collected and included in statistical analysis.

SCHIZOPHRENIA CASES									CONTROLS								
Matched pair	indID	GENDER	AGE	PMI	PFC_pH	RIN	RNAyield	HEMI	Matched pair	indID	GENDER	AGE	PMI	PFC_pH	RIN	RNAyield	HEMI
1	2	M	27	10	6.56	7	0.556945	L	1	18	M	18	33	6.79	7	0.656233	L
2	12	M	27	38.5	6.82	8	0.697238	L	2	69	F	21	39.5	6.83	8.1	0.687883	R
3	5	M	27	33	6.84	8.2	0.716637	R	3	49	F	33	24	6.91	8	0.636301	L
4	54	M	30	26	7.01	7.2	0.676807	L	4	68	M	24	43	6.95	7.1	0.693051	R
5	25	M	32	26	6.99	7.9	0.679626	L	5	47	M	34	20.5	6.51	7.1	0.617933	R
6	22	M	30	24	6.77	7.3	0.607995	L	6	88	M	37	11	6.23	6.9	0.660384	R
7	61	M	33	48	6.75	8.3	0.636554	L	7	17	M	37	21	6.8	7.6	0.691639	L
8	65	F	33	50	6.93	8.1	0.697813	R	8	30	M	37	24	6.94	7.5	0.674944	R
9	62	M	34	26	6.95	7.3	0.644389	R	9	7	M	38	13.5	6.73	7.7	0.644233	L
10	34	M	40	21.5	6.5	7.6	0.692583	L	10	29	M	43	13	6.71	7.4	0.663746	R
11	15	M	44	29.5	6.52	7.3	0.676681	L	11	56	M	44	50	6.72	7.2	0.674861	L
12	3	F	51	12	5.7	6.2	0.60522	R	12	59	M	46	29	5.84	6.7	1.09856	L
13	4	M	51	21	6.53	6.9	0.700697	L	13	37	M	46	25	6.65	7.3	0.676728	R
14	32	M	51	18	6.68	7.9	0.589426	L	14	74	F	49	15	6.64	8.3	0.405359	R
15	63	M	52	46	6.35	7.1	1.049513	R	15	40	M	50	19	6.61	6.9	0.621748	L
16	13	M	52	8.35	6.7	7.7	0.678291	R	16	20	M	50	29	6.68	8.1	0.616426	R
17	14	F	56	39	7.09	7.6	0.686118	R	17	90	F	51	37.5	7.15	7.7	0.560884	R
18	55	F	54	29	6.41	7.2	0.656695	R	18	60	M	53	27	6.7	7.1	0.716039	R
19	42	M	54	27.5	6.19	6.3	0.665646	R	19	82	M	54	29	6.8	6	0.571161	R
20	43	M	57	48	6.72	6.5	0.663509	R	20	85	M	55	20	7.19	7.3	0.581922	L
21	64	F	55	33.5	6.3	7.6	0.747951	L	21	48	F	56	23	6.47	7.1	0.554326	R
22	24	M	56	15	6.16	6.7	0.441408	L	22	26	M	56	24	6.64	7.1	0.583805	R
23	35	M	55	72	6.37	6.6	0.645103	R	23	84	M	56	37	6.98	6.2	0.68544	R
24	51	M	57	28	6.41	7.4	0.680211	R	24	38	M	58	12	6.56	7.6	0.651876	L
25	52	F	56	34	6.81	7.7	0.576311	L	25	80	M	57	18	6.86	8.4	0.706787	L
26	11	M	57	38	6.98	7.5	0.551161	L	26	28	M	59	20	6.96	7.4	0.5352	R
27	31	F	58	19	6.34	6.8	0.635906	R	27	75	M	60	25	6.02	7.1	0.543471	R
28	83	M	59	26.5	6.87	7.6	0.628456	R	28	9	M	60	21	6.52	7.8	0.676817	L
29	71	F	68	32	6.24	7.5	0.688964	L	29	39	F	60	13	6.55	7.4	0.798047	L
30	23	F	61	17	6.43	6.8	0.625162	R	30	81	M	60	21.5	6.95	6.9	0.625872	R
31	53	F	61	42	6.91	6.8	0.65858	R	31	86	M	61	27.5	6.31	6.6	0.54543	R
32	33	F	66	12.5	6.33	6.6	0.636842	R	32	72	M	62	37.5	6.57	7.5	0.654113	R
33	1	M	67	5	6.82	8.4	0.641716	L	33	46	M	74	10	6.31	7.4	0.555328	L
34	21	F	67	27	6.44	6.3	0.58256	L	34	70	M	64	39.5	7.01	7	0.658127	R
35	44	M	73	14	6.79	7	0.680115	L	35	79	M	78	6.5	6.29	7.5	0.655102	L
36	45	F	73	20	6.9	7.2	0.702713	L	36	78	F	78	11	6.37	6.1	0.665736	R
37	41	M	75	36	6.56	6.9	0.547692	L	37	58	M	73	48	6.57	8.1	1.055466	R

Table 2.1: TRC post-mortem brain cohort demographics. Pairs were matched for age, sex, brain pH, RIN and RNA yield. Table also shows other demographic variables (indID: individual ID, PMI: post-mortem interval, hours; RIN: RNA integrity number; HEMI: Brain hemisphere R-right, L-left; Gender: M-male, F-female).

2.1.1.2 Stanley Medical Research Institute (SMRI) brain tissue specimens

This cohort has been compiled by Dr Maree Webster, Dr Michael Knable and colleagues, at the Stanley Medical Research Institute (Bethesda, MD, USA). Brain dissections were performed by qualified medical examiners under full ethical clearance from the Department of Psychiatry of the Uniformed Services University of the Health Sciences (USUHS) in Bethesda, MD and written consent was obtained from the next of kin. The use of these samples for this study was approved by Human Research Ethics Committee at the University of NSW (#HREC07261). RNA extracted from DLPFC of individuals with schizophrenia (n = 35), bipolar disorder (n = 31), and unaffected controls (n = 34), was obtained from the SMRI. RNA was reverse transcribed to synthesize cDNA, diluted and plated out in the SRL laboratory at NeuRA. Shan Y Tsai (Research assistant) and Duncan Sinclair (PhD candidate) performed the cDNA synthesis and made respective dilution plates.

Affected cases met the criteria for Diagnostic and Statistical Manual for Mental Disorders, 4th edition. Cases with Alzheimer's disease and other cerebral pathology were excluded. A qualified pathologist contacted the family of deceased patient to make preliminary diagnosis regarding deceased's birth and development, education, job history, family history of severe psychiatric disorders, any hospitalizations, medications and drug or alcohol use. Family members were contacted by psychiatrist, in case of missing medical records. Unaffected controls were included after structured telephone interviews with first-degree family member. Interviewer requested information as described above. The cohort had all Caucasian samples, except one sample each from African-American and Hispanic ethnicity. Samples included 66 males (26 schizophrenia cases, 15 bipolar disorder cases and 25 unaffected controls) and 34 females (9 schizophrenia cases, 16 bipolar disorder cases and 9 unaffected controls).

Further details regarding the brain collection, clinical information, tissue processing and quality screening for this cohort has been described previously (Torrey E F, *et al.* 2000). Demographic characteristics for this cohort have been summarized in Table 2.2. Other demographic information included family history, age of onset, illness duration, psychiatric

diagnosis, cause of death, medical diagnosis, medications at the time of death, brain weight, post-mortem interval, antipsychotic medications (mean daily dose, last dose, and total lifetime use) and smoking history.

No.	Diagnosis	PMI	Gender	Age	Brain pH	Race		No.	Diagnosis	PMI	Gender	Age	Brain pH	Race
1	BPD	62	F	29	6.74	C		51	CON	28	F	38	6.7	C
2	BPD	49	F	42	6.65	C		52	CON	52	M	35	6.7	C
3	BPD	28	F	41	6.44	C		53	CON	21	M	47	6.81	C
4	BPD	53	F	59	6.2	C		54	CON	29	M	45	6.94	C
5	BPD	48	M	29	6.39	C		55	CON	24	F	34	6.87	C
6	BPD	19	F	49	5.87	C		56	CON	37	M	42	6.91	C
7	BPD	32	M	42	6.65	C		57	CON	10	F	44	6.2	C
8	BPD	35	M	35	6.3	C		58	CON	29	F	33	6.52	C
9	BPD	16	M	64	6.1	C		59	CON	31	M	46	6.67	C
10	BPD	32	F	63	6.97	C		60	CON	38	M	40	6.67	C
11	BPD	38	F	49	6.39	C		61	CON	11	M	31	6.13	C
12	BPD	24	F	33	6.51	C		62	CON	58	F	39	6.46	C
13	BPD	23	M	56	6.07	C		63	CON	36	M	47	6.57	C
14	BPD	60	M	29	6.7	C		64	CON	50	F	41	6.17	C
15	BPD	28	M	45	6.35	C		65	CON	13	M	37	6.5	C
16	BPD	70	M	41	6.71	H		66	SCZ	13	F	53	6.49	C
17	BPD	19	M	44	6.74	C		67	SCZ	26	F	44	6.58	C
18	BPD	18	F	48	6.5	C		68	SCZ	38	M	53	6.17	C
19	BPD	44	M	54	6.5	C		69	SCZ	9	M	50	6.2	C
20	BPD	17	F	35	6.1	C		70	SCZ	30	F	47	6.47	C
21	BPD	35	F	58	6.5	C		71	SCZ	19	M	42	6.48	C
22	BPD	84	M	59	6.65	C		72	SCZ	43	M	51	6.63	C
23	BPD	23	M	51	6.67	C		73	SCZ	36	F	32	6.8	C
24	BPD	37	F	44	6.37	C		74	SCZ	32	M	44	6.67	C
25	BPD	26	F	56	6.58	C		75	SCZ	38	F	59	6.93	C
26	BPD	39	F	43	6.74	C		76	SCZ	35	M	45	6.66	C
27	BPD	22	M	35	6.58	C		77	SCZ	42	F	54	6.65	C
28	BPD	62	F	50	6.51	C		78	SCZ	26	M	39	6.8	C
29	BPD	57	F	43	5.92	C		79	SCZ	54	M	41	6.18	C
30	BPD	23	M	48	6.9	C		80	SCZ	65	M	43	6.67	C
31	BPD	12	M	19	5.97	C		81	SCZ	26	M	42	6.19	C
32	CON	46	M	49	6.5	C		82	SCZ	30	M	46	6.72	C
33	CON	31	M	48	6.86	C		83	SCZ	33	M	31	6.2	C
34	CON	18	M	45	6.81	C		84	SCZ	52	F	45	6.51	C
35	CON	45	F	49	6.72	C		85	SCZ	34	M	40	6.18	C
36	CON	49	M	50	6.75	C		86	SCZ	28	M	19	6.73	C
37	CON	28	F	44	6.59	C		87	SCZ	30	M	37	6.8	C
38	CON	9	M	53	6.4	C		88	SCZ	10	M	52	6.1	C
39	CON	31	M	51	6.7	C		89	SCZ	15	M	24	6.2	C
40	CON	33	F	38	6	C		90	SCZ	9	M	44	5.9	C
41	CON	47	M	60	6.8	C		91	SCZ	80	M	39	6.6	C
42	CON	22	M	34	6.48	C		92	SCZ	29	M	33	6.5	C
43	CON	23	M	49	6.93	C		93	SCZ	18	M	43	6.3	C
44	CON	24	M	35	7.03	C		94	SCZ	47	M	35	6.4	C
45	CON	31	M	55	6.7	C		95	SCZ	13	M	47	6.3	C
46	CON	13	M	32	6.57	C		96	SCZ	27	F	36	6.49	C
47	CON	11	M	47	6.6	C		97	SCZ	35	M	38	6.68	AA
48	CON	22	M	51	6.71	C		98	SCZ	35	F	47	6.5	C
49	CON	24	M	48	6.91	C		99	SCZ	16	M	52	6.52	C
50	CON	28	M	53	6	C		100	SCZ	26	M	43	6.42	C

Table 2.2: Table showing demographic variables for the Stanley cohort, which is divided in three main groups i.e. BPD (bipolar disorder), CON (unaffected controls), and SCZ (schizophrenia). Table also shows no. of individuals in each group; their unique IDs; PMI: post-mortem interval, hours Gender: M-male, F-female; Age in years; Brain pH and Race (C=Caucasian, AA=African American, H=Hispanic).

2.1.1.3 Maryland Brain (MB) and Tissue Brain Bank Developmental Cohort

This cohort has been compiled by Dr Joel Kleinman, Dr Barbara Lipska and colleagues (Lipska B K, *et al.* 2006), at the University of Maryland Brain and Tissue Bank to help researchers in understanding brain characteristics, across human development. Brains from deceased individuals were collected with informed consent from the next of kin under NIMH protocol 90-M-0142. Frozen tissue from the middle frontal gyrus of 68 individuals from 6 weeks to 49 years of age was obtained from the University Of Maryland Brain and Tissue Bank. The study was approved by Human Research Ethics Committee at the University of NSW (#HREC07261). RNA was extracted from this frozen tissue and then reverse-transcribed to synthesize cDNA by Shan Y Tsai (Research assistant) and Duncan Sinclair (PhD candidate). Samples were classified into groups of neonates (day 1 – day 90; n = 11), infants (day 91 – day 365; n = 14), toddler (year 1 – year 5; n = 9), school age (year 5 day 1 – year 12 day 364; n = 9), teenage (year 13 – year 19; n = 8), young adults (year 20 – year 25 day 364; n = 9) and adults (year 26 and above; n = 8). Synthesized cDNA was used in ST8SIA2 splice isoform detection studies (Chapter 5).

Demographic information about the individuals was gathered through telephonic interviews of next-of-kin. Demographic information included family history, PMI, gender, age, pH, race, average RIN and cause of death. The cohort included Caucasian (n = 33), African-American (n = 33) and Hispanic (n = 2) samples. Demographic characteristics for this cohort have been summarized in Table 2.3.

GROUP	PMI (HRS)	GENDER	AGE	pH	Race		GROUP	PMI (HRS)	GENDER	AGE	pH	Race
Neonate	27	M	39D	6.47	AA		Schoolage	17	M	11Y	7	C
Neonate	17	M	54D	6.63	AA		Schoolage	12	F	11Y 198D	6.44	C
Neonate	11	M	56D	6.86	C		Schoolage	16	M	12Y 154D	6.82	C
Neonate	24	M	56D	6.36	C		Schoolage	18	F	12Y 353D	6.85	C
Neonate	26	F	60D	6.64	C		Schoolage	17	M	5Y 144D	6.74	C
Neonate	27	F	62D	6.52	AA		Schoolage	18	M	6Y	6.05	AA
Neonate	19	F	66D	6.48	C		Schoolage	20	F	8Y	6.78	C
Neonate	25	M	68D	6.5	AA		Schoolage	12	F	8Y 336D	6.41	C
Neonate	21	F	71D	6.36	H		Schoolage	17	F	9Y 356D	6.89	H
Neonate	24	F	73D	6.12	C		Teenager	13	M	15Y	6.76	AA
Neonate	24	F	89D	6.65	AA		Teenager	20	M	15Y 54D	6.9	C
Infant	19	M	118D	6.36	C		Teenager	7	F	16Y 125D	6.6	C
Infant	22	M	119D	6.54	C		Teenager	16	F	16Y 250D	6.81	C
Infant	27	M	129D	6.66	C		Teenager	19	M	17Y 138D	6.84	C
Infant	9	M	139D	6.54	AA		Teenager	16	M	17Y 180D	6.67	C
Infant	5	M	141D	6.81	AA		Teenager	16	M	17Y 251D	6.83	AA
Infant	18	F	175D	6.47	AA		Teenager	12	M	17Y 300D	6.8	C
Infant	22	F	188D	6.82	AA		Young Adult	18	M	20Y 50D	6.5	AA
Infant	10	M	196D	6.17	AA		Young Adult	13	M	21Y 341D	6.96	C
Infant	24	M	198D	6.71	AA		Young Adult	7	M	21Y 355D	6.25	AA
Infant	21	F	245D	6.58	AA		Young Adult	12	M	22Y 185D	6.75	C
Infant	18	M	301D	6.65	AA		Young Adult	4	M	22Y 334D	6.84	AA
Infant	10	F	332D	6.38	AA		Young Adult	14	F	23Y 228D	6.57	AA
Infant	18	M	332D	6.87	AA		Young Adult	7	M	24Y 338D	6.92	C
Infant	14	F	92D	6.54	AA		Young Adult	16	F	25Y 137D	6.73	C
Toddler	24	F	1Y 211D	6.9	C		Young Adult	32	F	25Y 37D	6.54	C
Toddler	11	F	2Y 75D	6.89	AA		Adult	13	M	35Y 363D	6.73	C
Toddler	22	F	2Y 163D	6.74	AA		Adult	19	F	38Y 154D	6.98	AA
Toddler	44	F	2Y 260D	6.47	C		Adult	8	M	38Y 231D	6.37	AA
Toddler	14	M	2Y 273D	6.16	AA		Adult	18	M	42Y 342D	6.49	C
Toddler	27	M	2Y 71D	6.64	AA		Adult	18	M	46Y 65D	6.75	AA
Toddler	18	M	4Y 232D	6.92	C		Adult	12	M	47Y 162D	6.56	C
Toddler	19	M	4Y 313D	6.74	AA		Adult	12	F	48Y 251D	6.12	C
Toddler	13	M	2Y	6.89	AA		Adult	7	F	49Y 79D	6.78	AA

Table 2.3: Table showing demographic variables for the MB cohort which is divided in seven groups (Age group; PMI: post-mortem interval, hours; Gender: M-male, F-female; Age; pH: brain pH; Race: African American (AA) Caucasian (C) or Hispanic (H).

2.1.2 Case-control cohorts

2.1.2.1 Australian Bipolar Case Control (ABCCC) cohort

This cohort has been recruited by Prof Phil Mitchell and colleagues, over many years and has been used in previous studies by our group (McAuley E Z, *et al.* 2012). The families were recruited through the Black Dog Institute and Mood Disorders Unit, University of

New South Wales, Sydney. Families were established through a proband of bipolar I disorder using an interview for genetic studies (Family Interview for Genetic Studies; FIGS). Families in which relatives suffered from schizophrenia without mood disturbance were excluded. Selection for inclusion in the case-control cohort comprised of one affected individual and spouse or completely unrelated family member, for cases and controls respectively. Additional singleton cases were ascertained through specialized bipolar clinic (Mitchell P B, *et al.* 2009), run through the Black Dog Institute. Samples were matched for gender, although for age, the control group was older to lessen the probability of a later diagnosis. Selected individuals had psychotic features (50.5% of total), family history of bipolar disorder (70% of total), family history of depression (17% of total) and family history of bipolar disorder or depression (87% of total). The study was approved by the Human Research Ethics Committee of UNSW (#HREC04144, HREC09394 and HREC05237). Written informed permission was obtained from all the individuals. Assessment was performed using Diagnostic Interview for Genetic Studies (DIGS) by qualified medical practitioners, psychologists and psychiatric nurses trained in this instrument. Cohort included Bipolar I disorder (BPI; n = 158), Bipolar II disorder (BPII; n = 41), Schizoaffective Disorder-Manic type (SZ/MA; n = 10), Major Depressive Disorder (MDD; n = 10) or unaffected controls (n = 166). All individuals were Caucasian and of Anglo-Celtic descent and half of them were females (n = 192). DNA from leukocytes was extracted by Genetic Repositories Australia, an NHMRC funded enabling facility, and extracted DNA was used in *NCAM1* genotyping study (Chapter 4); and targeted-resequencing studies of *NCAM1* (Chapter 4) and *ST8SIA2* (Chapter 5).

2.1.2.2 Australian Schizophrenia Research Bank (ASRB) cohort

This cohort has been compiled by Prof Bryan Mowry and colleagues, in collaboration with Schizophrenia Research Institute (SRI), to study a large sample of people with schizophrenia and healthy controls in Australia. Schizophrenia cases were recruited through hospital inpatient units, community mental health services, outpatient clinics and rehabilitation services, non-government mental illness support organizations, large-scale national, multi-media advertising campaign and through direct phone calls. Healthy

controls were recruited through multi-media campaign and direct telephone calls. All the research participants were screened by trained clinical assessment officer (CAO) using detailed telephone checklist based on Diagnostic Interview for Psychosis (DIP). DIP is used to establish a lifetime diagnosis of a psychotic disorder, as well as present and lifetime substance use disorder diagnoses, according to DSM-IV and ICD-10 criteria. Inclusion criteria for schizophrenia cases were: (i) screen positive on the Diagnostic Interview of Psychosis (Castle D J, *et al.* 2006); (ii) Caucasian ethnicity; (iii) adequate English proficiency in order to complete the interview; and (iv) DNA availability at the time of testing. Schizophrenia cases were excluded if they had other organic brain disorder, brain injury, mental retardation (IQ <70), movement disorders, and current diagnosis of substance dependence. Controls were excluded if they had a personal or family history of psychosis or bipolar 1 disorder. Eligible participants provided written informed consent prior to participation in this study, which was approved by Wolston Park Hospital Institutional Ethics Committee. They were allocated the unique ASRB ID code and their details were entered into the ASRB system. DNA was extracted from the blood sample collected at the time of clinical interview, using ASRB protocols (Jablensky A, *et al.* 2000; Loughland C, *et al.* 2010). The cohort comprised 128 cases (85 men and 43 women) and 128 age-, gender- and ethnically-matched controls. Extracted DNA was used in *NRG1* targeted-resequencing study to verify the frequency of novel variants in the Australian population (Chapter 3).

Detailed demographic characteristics, clinical information, tissue processing and quality screening related information were available through ASRB system and accessed through (www.schizophreniaresearch.org.au).

2.1.3 Summary of cohorts studied in this thesis

To clarify the use of the different post-mortem brain and case-control cohorts in different parts of this thesis, a summary table is provided (Table 2.4). The TRC cohort was used in western blotting (Chapter 4 and 5), qRT-PCR (Chapter 3, 4, 5), and splice isoform detection (Chapter 5) experiments. All the individuals from the TRC cohort were included in *NRG1* Sanger sequencing (Chapter 3); Western blotting studies with NCAM1 (Chapter

4) and ST8SIA2 (Chapter 5); RT-PCR studies with NRG1 (Chapter 3), NCAM1 (Chapter 4), and ST8SIA2 (Chapter 5); ST8SIA2 splice isoform detection and screening studies (Chapter 5).

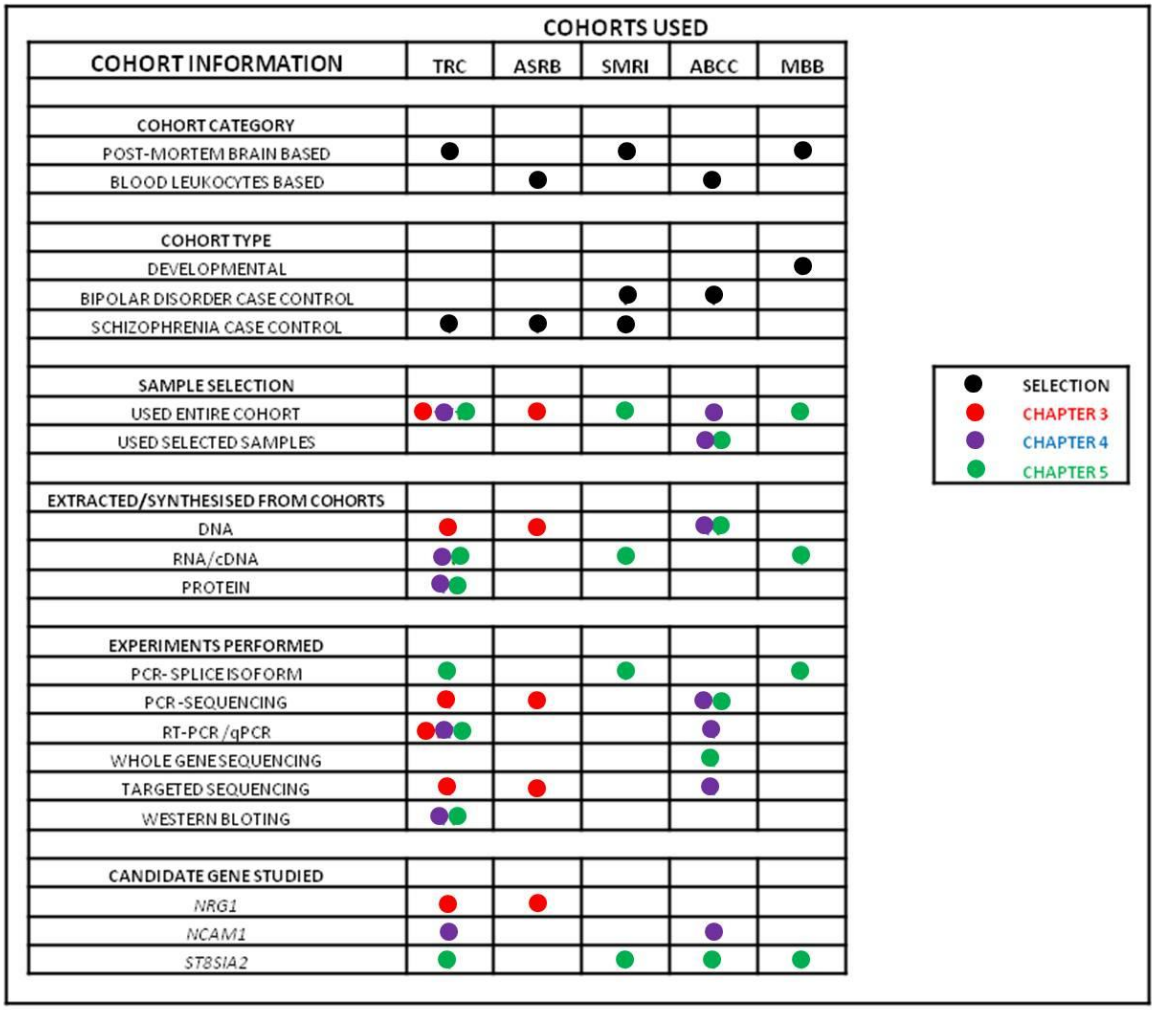


Table 2.4: Table summarizing cohorts used in different parts of this thesis. Vital information about them such as, cohort category, nature of cohort, sample selection within cohort, form in which the material was extracted, various experiments performed in the cohort, different genes studied and their relevant chapters.

cDNA from SMRI cohort was used in ST8SIA2 splice isoform detection and screening experiments (Chapter 5). All the individuals from the SMRI cohort were included in ST8SIA2 splice isoform detection and screening studies (Chapter 5). All the individuals from the MB cohort were included in ST8SIA2 splice isoform detection and screening studies (Chapter 5).

All the samples in ABCC cohort were used for *NCAM1* genetic association study (Chapter 4), while selected 48 DNA samples, from bipolar disorder cases, were selected for *NCAM1* (Chapter 4) and *ST8SIA2* (Chapter 5) resequencing study (Table 2.5).

Sample No.	Gender	Diag/hap	Family linkage to 15q25-26	Haplotype information		Sample No.	Gender	Diag/hap	Family linkage to 15q25-26	Haplotype information
1	Male	BPI	-	-		25	Female	BPI	-	1 risk
2	Male	BPI	-	-		26	Male	BPI	Yes	1 risk
3	Female	BPI	Yes	-		27	Female	BPI	Yes	2 risk
4	Male	BPI	Yes	-		28	Female	BPI	Yes	1 risk
5	Female	BPI	Yes	1 risk		29	Male	BPI	Yes	-
6	Male	BPI	Yes	2 risk		30	Male	BPI	Yes	1 protective
7	Male	BPI	Yes	-		31	Female	BPII	Yes	2 risk
8	Female	BPI	-	-		32	Female	BPI	-	1 risk + 1 protective
9	Male	BPI	-	-		33	Male	BPI	Yes	1 risk
10	Male	BPI	Yes	-		34	Female	BPI	-	1 risk
11	Male	BPI	Yes	-		35	Female	BPI	Yes	-
12	Female	BPI	Yes	1 risk		36	Male	BPI	-	-
13	Male	BPI	Yes	1 risk		37	Female	BPI	-	-
14	Female	BPII	Yes	2 risk		38	Female	BPI	-	-
15	Male	BPI	Yes	-		39	Male	BPI	-	-
16	Male	BPI	Yes	-		40	Male	BPI	-	2 risk
17	Female	BPI	Yes	-		41	Female	BPI	-	-
18	Male	BPI	Yes	-		42	Female	BPI	-	-
19	Male	BPI	Yes	-		43	Female	BPI	-	1 risk + 1 protective
20	Female	BPI	Yes	1 risk		44	Male	BPI	-	2 protective
21	Male	BPI	Yes	-		45	Female	BPI	-	1 protective
22	Female	BPI	Yes	-		46	Female	BPII	-	-
23	Female	BPI	Yes	1 risk		47	Male	BPI	-	1 risk + 1 protective
24	Female	BPI	Yes	1 risk		48	Female	BPI	-	2 protective

Table 2.5: Description of 48 individuals from ABCC cohort selected for Roche 454 sequencing. Diagnosis i.e. Bipolar I (BPI) or Bipolar II (BPII); Haplotype information i.e. copies of risk or protective haplotype; Gender information i.e. M-male, F-female, is provided with their sample ID. Samples that showed previous family linkage to 15q25-26 chromosomal region are also shown.

For resequencing study, samples included individuals those who contributed to bipolar linkage peak at 15q25-26 (n = 28), identified by our group (McAuley E Z, *et al.* 2009). Forty-five individuals with bipolar 1 disorder having 0, 1 or 2 copies of risk haplotype and three bipolar 2 individuals, with 2 copies of risk haplotype (McAuley E Z, *et al.* 2012) formed the selected samples. Table 2.5 shows details of the 48 samples used in the 454 resequencing study.

All the samples from ASRB cohort were included in *NRG1* Sanger sequencing study to estimate the prevalence of a novel *NRG1* 5-SNP haplotype in the Australian population (Chapter 3).

2.2 Sample preparation

2.2.1 DNA extraction from brain tissue

This procedure was performed by myself and Duncan Sinclair (PhD candidate). Frozen brain (DLPFC) of 74 post-mortem brains was obtained through the Tissue Resource Center, Sydney, Australia. Brain tissue was weighed (while frozen), and then pulverized on dry ice, using extraction buffer. Genomic DNA was extracted according to the protocol provided by the manufacturer of the extraction buffers, TRIZOL Reagent method (Life Technologies Inc., Grand Island, NY, USA). Extracted genomic DNA was used in sequencing studies of *NRG1* (Chapter 3).

2.2.2 Protein extraction from brain tissue

This procedure was performed by myself and Shan Tsai (research assistant). Frozen brain tissues (DLPFC) from TRC cohort were used in the process. Crude protein was extracted by homogenization of brain tissue in extraction buffer (0.05 M Tris pH 7.5, 50% glycerol, 2 mM AEBSF, 0.015 mM aprotinin, 0.038 mM leupeptin, 0.030 mM pepstatin A, 0.028 mM E-64, 0.08 mM bestatin; Sigma, St Louis, MO, USA). Protein concentrations were determined using the Bradford protein assay according to the manufacturer's instructions (96 Well Plate Assay Protocol, BioRad, Hercules, USA), with bovine serum albumin as standard. Protein extracts were used in Western blot studies of NCAM1 and ST8SIA2 (Chapter 4 and Chapter 5, respectively).

2.2.3 cDNA synthesis from RNA

cDNA synthesis from commercially bought RNA was performed by myself. In the process, 24 ng/µl fetal whole brain RNA (Clontech Laboratories**,** Inc., San Jose, CA) was reverse transcribed into cDNA. Superscript III First-Strand Synthesis System for RT-PCR was used

with Random Hexamers, according to the manufacturer's protocol (Invitrogen, CA, USA). This cDNA was used in splice isoform detection studies of ST8SIA2 (Chapter 5).

cDNA synthesis from TRC, SMRI and MBC RNA was performed by Shan Y Tsai (research assistant) in SRL, methods are described in (Sinclair D, *et al.* 2012a) and (Weickert C S, *et al.* 2010). cDNA plates were prepared, dried-down and stored at -80 ºC. Dried-down plates were hydrated, centrifuged and used for all qPCR experiments. Each plate had seven serially diluted standards, synthesized by pooling all the samples together in triplicates, on each plate ranging from 0 to 100 ng/µl. Negative-control (no cDNA) was also present in three wells. Medium-dilution plates (3.8 ng/µl cDNA per well) from TRC cohort were used in NRG1 isoform expression studies (Chapter 3). High-dilution plates (0.38 ng/µl cDNA per well) from TRC cohort were used in NCAM1 isoform expression studies (Chapter 4), while low dilution (19 ng/µl cDNA per well) and (38 ng/µl cDNA per well) were used for ST8SIA2 expression and splice isoform detection studies, respectively (Chapter 5). Low dilution (38 ng/µl cDNA per well) plate from SMRI was used in ST8SIA2 splice isoform detection and screening experiments (Chapter 5) and parallel diluted cDNA plate from MBC was used in ST8SIA2 splice isoform detection across human development (Chapter 5).

2.2.4 DNA extraction from peripheral lymphocytes

DNA from peripheral lymphocytes from the ABCC cohort was extracted by Genetic Repositories Australia (GRA), an enabling facility, which is supported by National Health and Medical Research Council of Australia (NHMRC). Extracted DNA was used for *NCAM1* SNP genotyping studies (Chapter 4), *NCAM1* targeted sequencing (Chapter 4), and *ST8SIA2* whole gene sequencing (Chapter 5).

DNA from the ASRB cohort was received already extracted, courtesy of Prof Brian Mowry (Jablensky A, *et al.* 2000; Loughland C, *et al.* 2010). Extracted DNA was used in estimating the prevalence of novel *NRG1* risk-haplotype in Australian population (Chapter 3).

2.3 PCR and Gel electrophoresis

2.3.1 PCR

PCR was performed to amplify the copies of specific DNA sequence, before conducting targeted-sequencing studies in *NRG1* (Chapter 3), *NCAM1* (Chapter 4), and *ST8SIA2* (Chapter 5). PCR was also performed with cDNA template, to detect splice isoforms of ST8SIA2 in TRC, MB and SMRI (Chapter 5).

The reaction mixture for the primary PCRs consisted of DNA/cDNA (30-50 ng total) template in a total volume of 15 µl with final concentrations of 1 X 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 1.5 mM $MgCl_2$, 0.2mM dNTP mixture, 0.2 µM concentration of each primer from the external sets (Sigma); 1.0 U (unit) of Platinum® Taq DNA polymerase (Invitrogen). The PCR reactions were mixed by pipetting, briefly centrifuged, and then run on a PTC-240 DNA Engine Tetrad 2 Cycler (MJ Research, Bio-Rad Laboratories, Copenhagen, Denmark). In general, reactions were thermally cycled once at 94 °C for 2 min (initializing step); 34 times at 94 °C for 30 s (denaturing step), 60 °C for 45 s (annealing step), and 72 °C for 1 min (extension/elongation step); and then once at 72 °C for 10 min (final elongation step), followed by final hold at 10 ºC. Annealing temperature for primer pairs was optimized before performing the final run. Dimethyl-Sulfoxide (5% v/v final concentration) was also added in some reactions to get rid of non-specific bands.

2.3.1.1 Primer pairs for short-range PCR

Short-Range PCR primer pairs were designed to cover the area of interest, that included upstream intron sequences in *NRG1* (Chapter 3) and specific exon sequences in *ST8SIA2* (Chapter 5). Several resources were used for primer design: In-Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) was used to confirm primer positions and to ensure the specificity of each primer pair, REPEAT MASKER (http://www.repeatmasker.org/) was used for screening DNA sequences in FASTA format against a library of repetitive elements, and Primer3 (v 0.4.0, http://frodo.wi.mit.edu/primer3/) was used for primer sequence design.

Primer pairs used for different short range PCR reactions are shown in the Table 2.6.

Primer Name	Design Strand	Primer Sequence (5'-3')	DMSO (5%)	Annealing Temp.
NRG1_promIII.1	Forward	AACCTAACCCAAAAGGCATG	–	60 ºC
NRG1_promIII.1	Reverse	GAGCATCACTGCAAACAGGA	–	60 ºC
NRG1_promIII.2	Forward	CAGCTAGAGCAATGCCCAGT	Yes	57 ºC
NRG1_promIII.2	Reverse	CCTTTCATGGTGACTCCACA	Yes	57 ºC
NRG1_promIII.3	Forward	AAATGGCGGTTGTTGTAAGC	Yes	57 ºC
NRG1_promIII.3	Reverse	CCAAGCATGATAACCCATAGTTT	Yes	57 ºC
NRG1_promI.1	Forward	ACTTCCCGACCACAACACA	Yes	57 ºC
NRG1_promI.1	Reverse	CCGGAGTTCCAACAAGTTTC	Yes	57 ºC
NRG1_promI.2	Forward	CCATCACATCAAACCAAGGA	Yes	57 ºC
NRG1_promI.2	Reverse	CCCTGCAAAGGACAAAAGTT	Yes	57 ºC
NRG1_promI.3	Forward	GGCTCAAGCAATCCACTCAT	Yes	57 ºC
NRG1_promI.3	Reverse	CAGGAATTTTAGGGCTGAGG	Yes	57 ºC
NRG1_promIV.1	Forward	AGTTGGGGGAGCTTGGTATT	Yes	57 ºC
NRG1_promIV.1	Reverse	CCTTGCCCTCCTCCTTTTAT	Yes	57 ºC
NRG1_promIV.2	Forward	TGCACTTTGTGGAGTGGTCA	Yes	57 ºC
NRG1_promIV.2	Reverse	GCTTCTATGGCTATTGGAACA	Yes	57 ºC
NRG1_promIV.3	Forward	GGCAATCCACATTCCCTTAC	–	60 ºC
NRG1_promIV.3	Reverse	ATCCTTTGAGATCCGTCTGC	–	60 ºC
NRG1_promI.1b	Forward	TTGTTCCAACTCACCACTTCC	Yes	57 ºC
NRG1_promI.1b	Reverse	TATCACCGTCCTGCTCACG	Yes	57 ºC
NRG1_promI.3b	Forward	TGCTGAGTGAGATTTTCTTTGC	Yes	60 ºC
NRG1_promI.3b	Reverse	AGGGGAAGAGCCTACGATTT	Yes	60 ºC
ST8SIA2_External	Forward	CTGCTCGTGGTCTTCCTCAT	–	60 ºC
ST8SIA2_External	Reverse	CCCCTGCTCATGTAGGCTCT	–	60 ºC
ST8SIA2_Internal	Forward	TCAGAGATCGAAGAAGAAATCG	–	Touchdown_PCR*
ST8SIA2_Internal	Reverse	TTAAACTCCAAGGGCATGGT	–	Touchdown_PCR*
ST8SIA2_Delta_2	Forward	CGAAGAAGAAATCGGAGCTG	–	60 ºC
ST8SIA2_Delta_2	Reverse	GAGCGTCTGGTTATGTCTCCA	–	60 ºC
ST8SIA2_Delta_5	Forward	CAGCTTCGTCATCAGATACTGG	–	58 ºC
ST8SIA2_Delta_5	Reverse	CCTGGGAGGTGTAGCCATACT	–	58 ºC
ST8SIA2_Delta_2345	Forward	CGAAGAAGAAATCGGATACTGG	–	60 ºC
ST8SIA2_Delta_2345	Reverse	CCTGGGAGGTGTAGCCATACT	–	60 ºC

Table 2.6: Primers used for short-range PCRs for *NRG1* and *ST8SIA2* genes. DMSO (5% v/v final conc.), was used in some reactions as shown. Annealing temperature for different primer sets is also shown.

2.3.1.2 PCR purification

PCR products in each well were mixed with 125 mM EDTA (2 µl), 3M NaAc (2 µl) and 100% ethanol (50 µl). This mixture was purified using MultiScreen$_{96}$-PCR filter plate (Millipore, Bedford, MA) on vacuum suction system, through which the vacuum pressure was applied for 30 min. The plate was detached from the vacuum suction and any remaining liquid under the plate was removed with a Kim-wipe. Ethanol (75%, 70 µl) was

then added to each sample and the vacuum was reapplied. In the next step, Milli-Q-grade water (30 µl) was added to rehydrate the samples in plate, which were then resuspended on a shaker at 480 rpm for 15 min. After properly mixing the samples through pipetting, the purified product was transferred from MultiScreen$_{96}$-PCR filter plate to clean PCR plate.

2.3.1.3 Nested PCR

Because ST8SIA2 is expressed at very low levels in the adult brain and RNA quantity was very limited, a nested PCR was performed to amplify the cDNA template prior to detection of specific ST8SIA2 splice isoforms in TRC, SMRI and MB cohorts. For nested PCR, external primer pairs (Table 2.6) were designed to cover the ST8SIA2 cDNA sequence between exon 1 and exon 6 (Figure 2.1). The external primer set was aimed to amplify a 1045-bp sequence. Reaction-samples with the external primer sets were cycled one time at 94 °C for 2 min; 34 times at 94 °C for 30 sec, 60 °C for 45 sec, and 72 °C for 1 min; and then one time at 72 °C for 10 min. PCR product from endpoint-PCR was purified (using protocol described in 2.3.1.2), and used as a template for an internal or nested PCR.

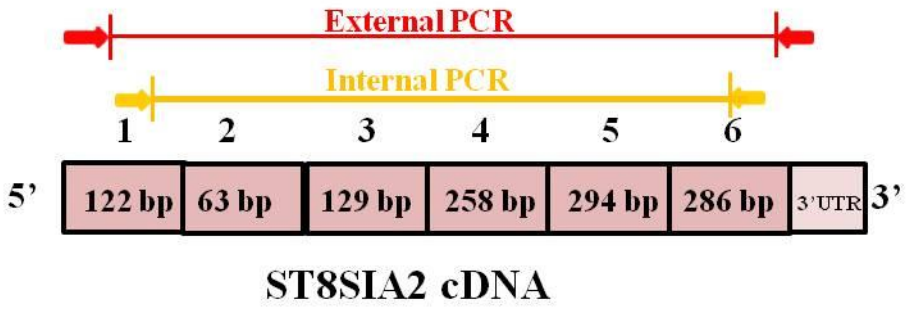


Figure 2.1: Schematic diagram showing positions of internal and external PCR primers and region covered by them. External PCR product was 1044 bp, while the internal PCR product was 983 bp.

The inner primer set (Table 2.6) complementary to positions 43 to 1,086 and 76 to 1,058 of the ST8SIA2 mRNA, respectively, was aimed to amplify a specific 983-bp sequence. The reaction mixture for the nested PCRs was identical, except that 1 µl of the 'first reaction purified product' was used in a total 10 µl reaction. After the reaction mixtures were thermally cycled once at 94 °C for 2 min, "touch-down" PCR protocol was performed which included 14 "touch-down" cycles for the increased specificity (94 °C for 30 sec, 66

°C for 30 sec, 72 °C for 1 min 10 s, with annealing temperature decreasing 0.5 °C at each consequent cycle) followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 sec, and 72 °C for 1 min 10 s and a final extension at 72 °C for 5 min. The number of amplification cycles and annealing temperatures were optimized and negative control reaction was included for each primer set by not adding cDNA/primary amplification product in the PCR reaction.

2.3.1.4 Long-range genomic PCR

Long Range PCR (LRP) was performed for amplicons that were used for targeted next generation sequencing of *NCAM1* (Chapter 4) and *ST8SIA2* (Chapter 5). PCR reactions were carried out using the Expand Long Range dNTPack kit (Roche, Mannheim, Germany). Reactions were executed following the manufacturer's instructions in 50 µl reaction mixtures. Briefly, 50 µl reaction volumes consisted of 10 µl of 5x Expand Long Range Buffer with 12.5 mM $MgCl_2$, 2.5 µl of 10 mM PCR Nucleotide Mix, 0.3 µM of each primer, 0.7 µl of 5U/µl Expand Long Range Enzyme Mix, with 500 ng genomic DNA, DMSO (5%) was added to PCR mix of all amplicons except amplicons ST8-2 and ST8-3. Thermal cycling conditions were followed using the protocol provided by the manufacturer. A negative control was performed for each primer set by excluding the DNA from the PCR reaction. All PCRs were optimized to determine the most appropriate running conditions that did not compromise sensitivity.

LRP primer pairs were designed to cover the area of interest, which included whole *ST8SIA2* gene and possible regulatory regions around it (Chapter 5) and glycosylatable region of *NCAM1* (Chapter 4). Primer design process was similar to that described for short range primers (section 2.3.1.1). Primers pairs were designed to keep amplicon average size of approx. 13 kb and an overlap of approximately 400 bp between adjacent amplicons. Primer pairs used for different LRP reactions are shown in the Table 2.7.

Amplified products from LRP were quantified by comparing the intensity of amplified product with a molecular weight marker after gel electrophoresis. Abundance was estimated by comparing the intensity of the known loaded amount of product with the intensity of the band closest in size to the amplified product. This approach was used rather

than optical density measurements due to the interfering nature of the amplification buffer on the absorbance ($A_{260/280}$) readings, and the need to account for the length of the product in estimation of molar quantity. After determining concentration all nine amplicons (approximately 5 ng/µl each) for each individual, products were pooled at an apparent equimolar ratio to generate 1 µg of final LRP product. This was a requirement for the 454-sequencing procedure to ensure that each amplicon is adequately represented in the sequencing run. Final LRP products for forty eight samples were then sent to Ramacciotti Centre, UNSW for Roche/454 GS-FLX sequencing.

2.3.2 Gel Electrophoresis

Successful PCR amplification (short and long range) was confirmed by gel electrophoresis, which typically employed 2% agarose (Invitrogen, Carlsbad, CA) in 1x TAE buffer (40mMTris acetate, pH 8.3, and 1 mM EDTA) run at 80 volts for 2 h. All agarose gels were stained in ethidium bromide (final concentration 0.5 µg/ml), and were imaged using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA) under UV illumination and were quantitated by the manufacturer's software Bio-Rad Quality One. Molecular weight marker (100 bp ladder; New England Biolabs, MA, USA) was used for SRP products, while 1 kb ladder (New England Biolabs, MA, USA) and DNA molecular weight marker XV (Roche, Mannheim, Germany) were used for estimating the size and abundance of the LRP products.

2.4 Sequencing

2.4.1 Roche 454 Next Generation sequencing

Next-gen Sequencing is a comparatively new approach to overcome limitations faced with Sanger sequencing such as, sequencing cost, generating speed, its throughput, scalability and resolution. Next-gen sequencing approach is capable of sequencing large DNA sequences (> 1 Gb) in one sequence run. Therefore, I used Roche 454 next-gen sequencing to study whole *ST8SIA2* gene (approx. 95 kb, Chapter 5) and glycosylation site of its primary substrate, NCAM1 (approx. 6.6 kb, Chapter 4). Amplicon sizes varied from 5 to 14.5 kb, with an average size of 13 kb (Chapter 5; Figure 5.1). Adjacent amplicons

overlapped with approximately 400 bases to allow for SNP detection below the primer binding sites.

2.4.1.1 Long range PCR Primers

Primers were ordered from Sigma-Aldrich (St. Louis, MO), and are shown in Table 2.7. The annealing temperature was 60 °C for all amplicons, except amplicons ST8SIA2_3 and ST8SIA2_5, which were 59 °C and 58 °C, respectively. Amplicons were checked by gel-electrophoresis, purified and then pooled as described above.

S No.	Gene	Primer Name	Design Strand	Primer Sequence (5'-3')
1	*ST8SIA2*	ST8SIA2_1	Forward	AGCGTCTTTTCAGGAGGTGA
2	*ST8SIA2*	ST8SIA2_1	Reverse	CTACCCTGACCCAGCAACAT
3	*ST8SIA2*	ST8SIA2_2	Forward	CGCTTCCTGCTCTCATTTTC
4	*ST8SIA2*	ST8SIA2_2	Reverse	GTTTCCTCCTTGCCATCGT
5	*ST8SIA2*	ST8SIA2_3	Forward	TCCCAGTGAAGAGCACAGTC
6	*ST8SIA2*	ST8SIA2_3	Reverse	TTCCCATTGCCCTGAGTATC
7	*ST8SIA2*	ST8SIA2_4	Forward	TAAGGTGGAGCTAGGGACCA
8	*ST8SIA2*	ST8SIA2_4	Reverse	GGAGACCAAATGCCTTGAAA
9	*ST8SIA2*	ST8SIA2_5	Forward	TCCCATCTGTGATTCCATGA
10	*ST8SIA2*	ST8SIA2_5	Reverse	GGGCAGGATCTTCTTTCTCC
11	*ST8SIA2*	ST8SIA2_6	Forward	AATGACAAGTGCCCCATAGC
12	*ST8SIA2*	ST8SIA2_6	Reverse	ACTGCAAACTCATGCTGACAA
13	*ST8SIA2*	ST8SIA2_7	Forward	ACCCACTTTTTCTCCCCAGT
14	*ST8SIA2*	ST8SIA2_7	Reverse	TATGAGCACGGACAGCAATC
15	*ST8SIA2*	ST8SIA2_8	Forward	ATCAGCCTTTCCCAACAGC
16	*ST8SIA2*	ST8SIA2_8	Reverse	CCACTCCCTCTACCCAATTTC
17	*NCAM1*	NCAM1	Forward	CATCTTTCAACCAACCAGCA
18	*NCAM1*	NCAM1	Reverse	CCTATTCCCCTCCAGCTACC

Table 2.7: Primers used for targeted sequencing of *ST8SIA2* and *NCAM1* genes. Primers were used in Long-Range PCR described in [2.3.1.4].

2.4.1.2 Quality control

DNA quality and concentration for all samples were checked before PCR. Primers were optimized and PCRs were checked several times for quality check before performing the main-cohort PCR run. Negative control was included in all PCR runs. All the samples were checked to have single clean PCR product by gel electrophoresis. All PCR products were purified to get rid of primer traces that could obstruct sequencing process. Roche/454 GS-FLX sequencing was performed by Ramacciotti Centre, UNSW.

2.4.2 Sanger sequencing

Sanger sequencing was performed to study DNA sequences from purified PCR products, which were all under 1200 bp in length. This sequencing approach was used in analyzing various target regions in *NRG1* (Chapter 3) and for splice isoform detection studies (Chapter 4 and 5).

DNA quality of samples was checked before conducting template PCR. Primers were optimized and PCRs were for quality check before performing the main-cohort PCR run. A negative control was included in all PCR runs. All the samples were checked to have single clean PCR product by gel electrophoresis. All PCR products were purified to get rid of unused traces of primers, as they could hinder the sequencing process.

Sequencing reaction was performed on purified PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), which included BigDye reagent, and 5x Sequencing buffer. Briefly, for a 20 µl reaction, 2 µl BigDye, 4 µl 5x buffer, 0.25 µl forward/reverse primer (20 µM final concentration), 11.75 µl Milli-Q-grade water and 2 µl clean PCR product (20-40 ng/µl). Reactions were mixed by pipetting the samples, which were then centrifuged before running on a PTC-240 DNA Engine Tetrad 2 Cycler (Bio-Rad). Reactions were thermally cycled once at 94 °C for 1 min; 29 times at 96 °C for 10 s, 50 °C for 10 s, and 60 °C for 4 min; followed by final reaction-hold at 10 ºC. After PCR-run was finished, samples were purified using EDTA, NaAc and ethanol (as described in 2.3.1.2), dried and wrapped in aluminum-foil. Samples were then submitted to the Ramacciotti Centre, UNSW for sequencing according to standard protocols using an ABI 3730 capillary sequencer (Applied Biosystems).

2.5 Genotyping

Manual genotyping of 6 SNPs comprising the *ST8SIA2* risk haplotype was performed to study the effect of risk or protective haplotypes on the gene expression in brains of individuals affected with schizophrenia/schizoaffective disorder or bipolar disorder, compared to unaffected controls. Manual genotyping in ABCC cohort was performed by Erica McAuley (PhD student) (McAuley E Z, *et al.* 2012), while Alex Shaw (Post-doc)

performed genotyping in TRC cohort (work not yet published). Restriction digest was performed for SNPs (rs4586379, rs2035645, rs11074070, rs3784735) with *Xba*I, *BstE*II, *Hpy*CH4IV and *Alw*NI, respectively). SNPs (rs4777974, rs11637898) were genotyped with direct Sanger sequencing. Haplotypes were inferred using PLINK software (Purcell S, *et al.* 2007) by Jan Fullerton. I used this genotype information for my statistical analysis performed in Chapter 4 and Chapter 5.

2.5.1 Taqman Allelic discrimination

This experiment was performed to gather genotype information for *NCAM1* SNPs (Chapter 4) in ABCC cohort. The primers and probes for all the selected SNPs (Table 2.8) were purchased from Applied Biosystems (Foster City, CA, USA).

No.	NCBI SNP Reference	Assay ID	Design Strand	Context Sequence
1	rs11607273	C__31503329_10	Forward	CTGTGCAGGGGCAGCATTGCATCAT**[A/G]**GAAAGATCACCTACCCGGAGCCAGG
2	rs17115109	C__33641424_10	Reverse	CCCGTCCTTTAAATTGGTGATCAGA**[A/G]**CATCTTGCTTGGGTGTGTCTACATT
3	rs686050	C____613361_20	Forward	AGCAGCGAAGAAAAGGTATCATGCT**[C/G]**CCCAGGAGTTTCAGGGCCTTGGAAT
4	rs646558	C___2998872_20	Forward	AGTCCGTAAGTAAAGCCAGCTGCCC**[A/C]**CCTTTTCCCAGCCCACCTTCTTCTC
5	rs12802504	C___2866878_10	Reverse	CAGTTCATTCTACCTATAACCTGTG**[C/T]**TTCACTGGCATCATCCTGCATGACA
6	rs1836796	C___2866877_10	Reverse	GGCCTCTTCCTTCCTTTTGCAAAAA**[G/T]**CTGCCTCAACATAGCAAATCTGTCA
7	rs2574823	C___2866872_20	Reverse	AAGTGTGAACTGTCCACAAAAGAGG**[C/G]**CTTGGGGGATGAGTGCTTCTTGGAG
8	rs7928046	C__29205308_10	Forward	ATTAGACAATCAATTATGATGTCAT**[A/G]**TTAGACAGAATGCTGAAGAATTCTA
9	rs12279261	C__11339066_10	Forward	CTCCTCTCCCTCCCATAGCAAGAGC**[A/G]**TTCCTCACGGCCTCTTTCAAGAGAA
10	rs12785741	C__31503346_10	Forward	CAACTGAGTCTCCAGGTTTCCTCTG**[C/G]**TCTGGATTTGATCAGTAAATGAATG
11	rs12271908	C__26702777_10	Forward	CACTTAATGGACTCTTGTTTTCAAG**[C/T]**TGCCAGTTAGTAAGCATGGCTTACC
12	rs2303377	C___2998870_1_	Forward	TTTTTTCCCCACCTTCATTTTTCTT**[C/T]**CTTCAGCGGCATCTGCTAGCTCGTC
13	rs2303378	C__15971552_10	Forward	CAGGTGATAATTCAACTCAGTTGGT**[A/G]**GAAAGCAGCGTCGGGTTTTAGAGGA
14	rs12574998	AHWRLKX	Forward	GCAGTGGGGGCCCAGGTCACTGCTC**[T/C]**GCCACAATTCCCCTTCCTCTGCACAT

Table 2.8: Primers and probes used for genotyping *NCAM1* gene polymorphisms. Probes corresponding to different alleles were labeled with VIC and FAM fluorescent dyes (Applied Biosystems). Polymorphic bases are bold and underlined. All SNPs were intronic.

Each reaction contained 2.5 µl TaqMan Universal PCR Master Mix, 0.125 µl TaqMan SNP Genotyping Assay, 1.375 µl distilled water and 1 µl DNA (10 ng/µl), with a final reaction volume of 5 µl. For each SNP, a positive control was included. The plate also contained at least two no template controls (i.e. without any DNA). Before analyzing the whole cohort, a pilot test was conducted to confirm the accuracy of the assay and proper differential clustering of homozygote and heterozygote genotypes. After a successful pilot test, sample analysis was carried out in 384-well microplates (Applied Biosystem). Fluorescence-

readings were measured with 7900HT Fast Real-Time PCR System (Applied Biosystem) and analysed with ABI's recommended SDS software version 2.3. A representative image of genotyping post-read result is shown in Figure 2.2. Systematic genotype missingness was determined as part of the quality control measures prior to association analysis. The average missingness rate per SNP was around 2%. There were 13 subjects with a high missingness (>40%) and these were excluded from analysis (4 controls, 9 cases), but most subjects had less than 10% missingness.

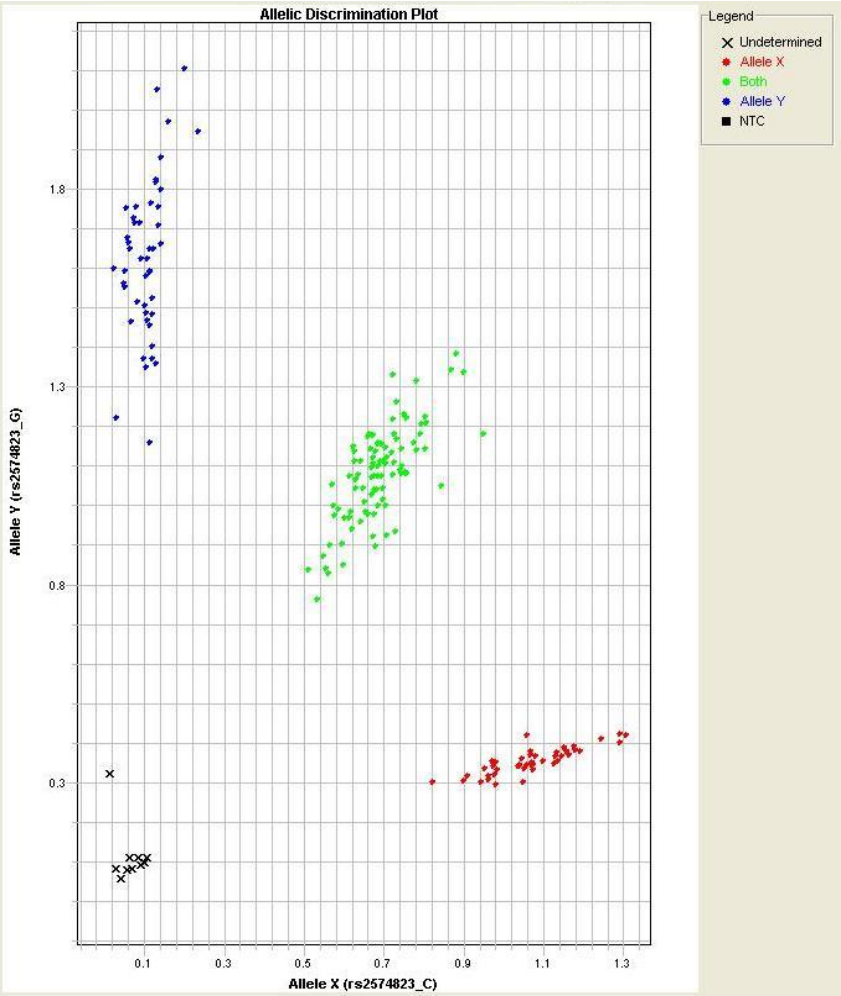


Figure 2.2: Representative genotyping assay result (post-read image) of the NCAM1 Taqman probe for rs2574823. Red dots are CC homozygotes, blue dots are GG homozygotes, green dots have both alleles (heterozygous), crosses indicate samples with undetermined genotype.

Data was independently cross-checked by Dr. Jan Fullerton (PhD supervisor). Positive controls for restriction digest experiments were the samples with known genotype. DNA obtained from GRA was further examined by agarose gel electrophoresis, to check for degradation and quality. The absorbance at 260 and 280 nm, of the low intensity purified product was checked by NanodropTM 1000 Spectrophotometer (Biolab, Musgrave, VIC, Australia), and the ratios were checked to confirm that they are in right concentration range (i.e. ratio > or = 1.8). Duplicates were included in genotyping reactions to confirm genotype accuracy. A negative control was performed for each primer set. All PCR reactions were optimized to settle on the most suitable running conditions that did not compromise sensitivity.

2.6 Quantitative PCR – mRNA transcript quantification

Quantitative PCR was performed in TRC cohort to investigate if there are differences in mRNA expression of different NRG1 isoforms (Chapter 3), NCAM1 isoforms (Chapter 4) and ST8SIA2 probe (Chapter 5), comparing schizophrenia and unaffected controls. Quantitative PCR technique was chosen because of its accuracy and sensitivity in quantitative analysis of mRNA gene expression. qPCR was also used to confirm my ST8SIA2-Delta_5 finding (Chapter 5).

Gene sequences were derived from NCBI database. For *NCAM1* (Chapter 4) and *ST8SIA2* (Chapter 5), pre-designed TaqMan gene expression assays (Applied Biosystems, Foster City, CA) targeting specific exon junctions were used to determine relative gene transcript levels. Whereas, for ST8SIA2-Delta_5 and some NRG1 transcripts, custom designed probes were used, as shown in Table 2.9.

Quantitative Real-Time PCR was performed in 384-well format on Prism 7900HT Fast Real Time PCR system (Applied Biosystems). Reactions were performed in a 10 µl volume, which contained 5 µl of 1x Taqman universal master mix, 0.5 µl of 20 x probe, 3 µl cDNA. Appropriate cDNA was used from low (3.8 ng/µl), medium (0.38 ng/µl), or high dilution (0.038 ng/µl) cDNA plates, on the basis of relative expression of each transcript

after comparison with a standard curve. Amplification conditions were: 50 °C for 2 min and 95 °C for 10 min; then 40 cycles of 95 °C for 15 sec, 60 °C for 1 min.

Experimental machine runs were checked to have completed without errors. No signal was observed for the negative template control in any experimental reaction. All gene expression amplifications from each subject were performed in triplicate, and relative quantities were determined from the standard curve, which was created by serial dilutions of cDNA, pooled from all cohort samples. Standards of known concentration were included on every qPCR plate to determine sample expression by relative standard curve method.

GENE	PROBE NAME	ISOFORMS DETECTED	TAQMAN ASSAY	CUSTOM-MADE PROBE (5'-3')
NRG1	Type II	GGF-2, HRGβ-1d, HRG-β3b, HRG-γ3	-	'CACTGGGACAAGCC'
NRG1	Type IV	HRG-β1b, HRG-β1c and HRG-β1d	-	'ACCACAGCCTTGCCT'
NRG1	Type III	SMDF	Hs01103792_m1	-
NRG1	pan-NRG1	all isoforms (excluding SMDF, ndf43c)	Hs00247620_m1	-
NRG1	Type I	HRG-β3b and HRG-γ3 (excluding HRG-β1b, HRG-β1c)	Hs01108479_m1	-
NCAM1	NCAM180	180 kD	Hs00941826_m1	-
NCAM1	NCAM140	140 kD	Hs00969851_m1	-
NCAM1	NCAM120	120 kD	Hs00941844_m1	-
NCAM1	NCAM (+VASE)	(+VASE)	Hs00941837_m1	-
NCAM1	NCAM (-VASE)	(-VASE)	Hs00354746_m1	-
ST8SIA2	ST8SIA2 (Exon 3-4)	ST8SIA2 (Exon 3-4)	Hs00195648_m1	-
ST8SIA2	ST8SIA2 (Delta_5)	ST8SIA2 (Delta_5)	-	'TCGTCATCAGATACTGG CTGACCAA'
ACTB	-	-	Hs99999903_m1	-
GAPDH	-	-	Hs99999905_m1	-
UBC	-	-	Hs00824723_m1	-
TBP	-	-	Hs00427620_m1	-

Table 2.9: Taqman probes for quantification and normalization of different mRNA isoform expression. Custom designed primer and probe combinations were used to specifically target particular NRG1 isoforms and *ST8SIA2* delta_5 isoform, while available Taqman assays (Applied Biosystems) were used for all other isoforms. The isoforms detected by each probe is also shown. The geometric mean of four endogenous control genes (*ACTB, GAPDH, UBC, and TBP*) was used for transcript normalization.

If the percent variance of the triplicates was greater than 30% of the mean quantity value, and the amplification curve of one replicate looked erroneous, that replicate was considered an outlier and removed from further analysis. The mean was then recalculated based on the reliable values. Normalization to the geometric mean of four housekeeper genes (*UBC, ACTB, GAPDH, TBP*) was then performed, by dividing quantity mean of each tested transcript by the geometric mean of the four housekeepers. Suitability of the 4 transcripts selected as reference genes is published by our group in manuscript titled "Selection of reference gene expression in a schizophrenia brain cohort" (Weickert C S, *et al.* 2010). Geometric means from four housekeepers were chosen since there is no universal reference gene whose mRNA levels are constant in all tissue and cells. These housekeepers are stably expressed in DLPFC, and their geometric-mean did not significantly differ in patients compared to controls (Weickert C S, *et al.* 2010). Experiments relating to expression of the housekeepers in the TRC cohort was performed by Ms Shan Tsai (Research assistant), using the protocol described in (Weickert C S, *et al.* 2010), and I used geometric means from that round of cDNA synthesis. cDNA used for the qPCR studies came from the same batch used for the reference genes. Individuals, whose normalized expression values were greater than 2 standard deviations from the group mean, were removed.

2.7 Western Blotting – Protein quantification

Western blotting was performed in TRC cohort to investigate if there are differences in protein expression of different NCAM1 isoforms, PSA-NCAM (Chapter 4) and ST8SIA2 enzyme (Chapter 5), comparing schizophrenia cases and unaffected controls.

The linear range for quantification for each protein isoforms was determined from a six point standard curve (range: 0-40 μg protein for PSA-NCAM and ST8SIA2; and 0-4 μg for NCAM1 isoforms). For loading, crude protein was prepared in 1x loading buffer, and was heated to 95 °C (NCAM1) or 65 °C (PSA-NCAM and ST8SIA2) for 10 min prior to loading. Samples were kept on ice for 10 min. before being separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Mini-PROTEAN-Tetra Cell, Bio-Rad), using a 7.5% (w/v) polyacrylamide resolving gel and run at 100V for 90 minutes at 25 °C. Protein sizes were determined using Precision Plus Protein™ Dual Xtra Standards (Bio-

Rad). After electrophoresis, proteins were electrophoretically transferred on a 0.2 μm nitrocellulose membrane (Bio-Rad) using Criterion Blotter apparatus (Bio-Rad), surrounded with ice, at 100 V for 2 h, and transfer verified by membrane staining with Ponceau S solution (sc-P3504; Sigma-Aldrich, St. Louis, Missouri, USA).

2.7.1 Matched pair design

Case-control samples from TRC are matched on age, pH, RNA yield, and RNA integrity (RIN) (Weickert C S, *et al.* 2010). To directly compare paired-samples, and to reduce gel to gel variation, all gels were loaded according to a matched pair design. Two replicate experiments were performed for each study, where each experiment had 6 gels containing 4 matched pairs per gel. Matched pairs were randomized within each gel, and the experimenter was blind to diagnosis during experimentation and quantification.

2.7.2 Immuno-staining

Nonspecific protein binding was blocked by washes with 20mM Tris base and 137mM sodium chloride (TBS) containing 5% milk powder and 0.05% Tween 20 (TBS-T, Sigma-Aldrich), for an hour at room temperature and incubated overnight at 4 °C with the primary antibody. Different primary antibodies used, are shown in Table 2.10. After 3x 15 min. TBS-T rinse, blots were incubated for 2 hours at room temperature, with horseradish peroxidase (HRP)–conjugated secondary antibody, diluted in 5% milk with TBS-T. Secondary antibodies were specific to primary antibodies used in the experiment, and are shown in Table 2.10. Loading controls were obtained by subsequent immunostaining using a primary monoclonal mouse antibody against Anti-Actin IgG (β-actin) (1:1000, clone C4, Chemicon-Millipore), and as secondary antiserum, a peroxidase-conjugated 1:10000 goat anti-mouse serum (Chemicon-Millipore). In addition, another loading control, a primary monoclonal antibody against Anti-Alpha Tubulin IgG (α-tubulin) (1:5000, Sigma-Aldrich), and as a secondary anti-serum, a peroxidase-conjugated 1:5000 donkey anti-mouse serum (Chemicon-Millipore) was used to compare the two housekeepers.

TARGET PROTEIN	PRIMARY ANTIBODY	DILUTION USED	SECONDARY ANTIBODY	DILUTION USED
PSA-NCAM (20 µg: loaded on gels)	Mouse monoclonal antibody target: PSA-NCAM Anti-Polysialic Acid-NCAM clone 2-2B Cat. # MAB5324 Chemicon-Millipore	1:200	Goat Anti-Mouse IgG Antibody Cat. # 12-349 Chemicon-Millipore,	1:1000
NCAM (1.5 µg: loaded on gels)	Rabbit polyclonal IgG antibody target: N-terminus (aa 1-300) NCAM (H-300) Cat. # sc-10735 Santa-Cruz	1:2000	Goat Anti-Rabbit IgG Antibody Cat. # A 8275 Sigma-Aldrich	1:5000
ST8SIA2 (20 µg: loaded on gels)	Rabbit polyclonal IgG antibody target: ST8SIA2 Cat # ab91411 Abcam	1:100	Goat Anti-Rabbit IgG Antibody Cat. # A 8275 Sigma-Aldrich	1:8000

Table 2.10: Primary and secondary antibodies used against three target proteins, their catalogue numbers, and dilutions. Optimized concentration of each protein, which was loaded on the gels, is also mentioned.

2.7.3 Quantification

Protein bands/smear were detected by chemilluminescence using the HRP substrate (Immobilon Western, Millipore Corporation, Billerica, MA, U.S.A) and visualized on hyperfilm (Amersham Hyperfilm ECL, UK). Immunoreactive bands were quantified using Chemidoc-Quantity One 1D Software (Bio-Rad, CA, USA). Background intensity was subtracted from the band intensity measurements, and all images were below saturation. A distinct band-metric was applied uniformly to all lanes for each protein band, with values expressed as trace intensity x mm^2. For PSA-NCAM, there was a smear rather than discrete bands due to attachment of differential PSA chains. Therefore, the contour function was used to determine the intensity of the smear, with values expressed as intensity per mm^2 (INT/mm^2). Figure 2.3 shows how NCAM, PSA-NCAM and β-actin gels were quantified. Final values were the means ± 2 S.D. of two replicate experiments.

Necessary steps were taken throughout the experiment to maintain quality. Protein concentrations were checked by Bradford assay. Antibody concentrations were optimized in pilot tests (Figure 2.4-2.6). All steps in the experiment including tube labeling, making

buffer, sample transfer, and loading was cross checked by Dr Alex Shaw (a research officer). Protein samples were kept on ice, and buffers were made fresh. During electrophoresis, special care was taken to avoid sample over-run. Transfer-tank was kept cold while transferring proteins onto nitrocellulose membrane. Protein quantification steps, images and final results were cross checked by Jan Fullerton (PhD supervisor).

A number of proteins were considered for normalization, including β-actin (43kDa), and α-tubulin (41 kDa). The band intensity values were first normalized to β-actin, but the normalized values exhibited greater variance (both amongst samples within a single experiment and between identical samples in replicate experiments) than non-normalized values (more than 100%).

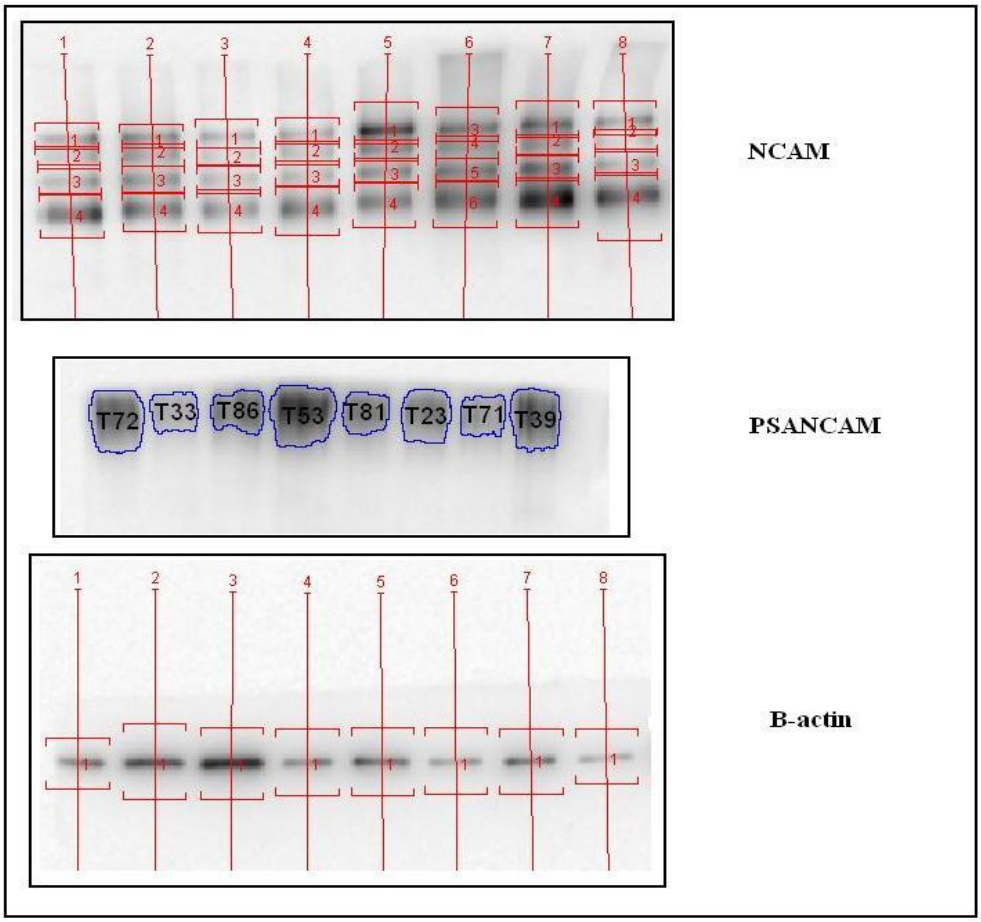


Figure 2.3: Representative western blot quantification image showing quantification approach used for NCAM (4 bands), PSA-NCAM (smear) and B-actin (single band). A discrete band metric was applied uniformly to all lanes for NCAM and B-actin with values expressed as Trace intensity x mm^2. Same approach

was used for ST8SIA2 gels. For PSA-NCAM, there was a smear rather than discrete bands. Therefore, contour function was used, with values expressed as intensity per mm2 (INT/mm^2).

The variance for individual NCAM1 isoforms were considerably reduced by calculating expression relative to total NCAM1 expression (ie sum of NCAM 120, 140, 180 + D isoforms), which was selected as the more accurate estimation of relative isoform expression. There were differences in expression of β-actin and both PSA-NCAM and ST8SIA2 isoforms, the former of which was in the linear quantification range at a much lower protein concentration than PSA-NCAM or ST8SIA2. Hence the quantification of β-actin was deemed to be unreliable for accurate normalization. Antibodies to α-tubulin were tested as an alternative housekeeping control, but α-tubulin expression was even lower than β-actin (Chapter 4; Figure 4.7). I was limited by the choice of housekeepers available to normalize my data. Therefore, to allow for comparison of subjects run on different gels, the raw protein intensity from each subject was expressed as a percentage of the average values of the four controls on that gel (% average control). To obtain this value, each of the four case values were divided by the average of the four measurements of the normal controls on that gel, and multiplied by 100. The direction of effect was the same for β-actin normalized values and % average control normalized values, except that the later has less variance (around 40%). Correlation values (r^2) for the normalized values across the two replicate experiments were more than 0.40 for all studies (Table 2.11).

Protein studied	Correlation Value between two rounds (r^2)
PSA-NCAM	0.678
NCAM-120	0.59
NCAM-140	0.41
NCAM-180	0.49
NCAM-D	0.63
Total NCAM	0.57
ST8SIA2	0.615

Table 2.11: Correlation values between two rounds of western blot experiments performed for each protein isoform studied.

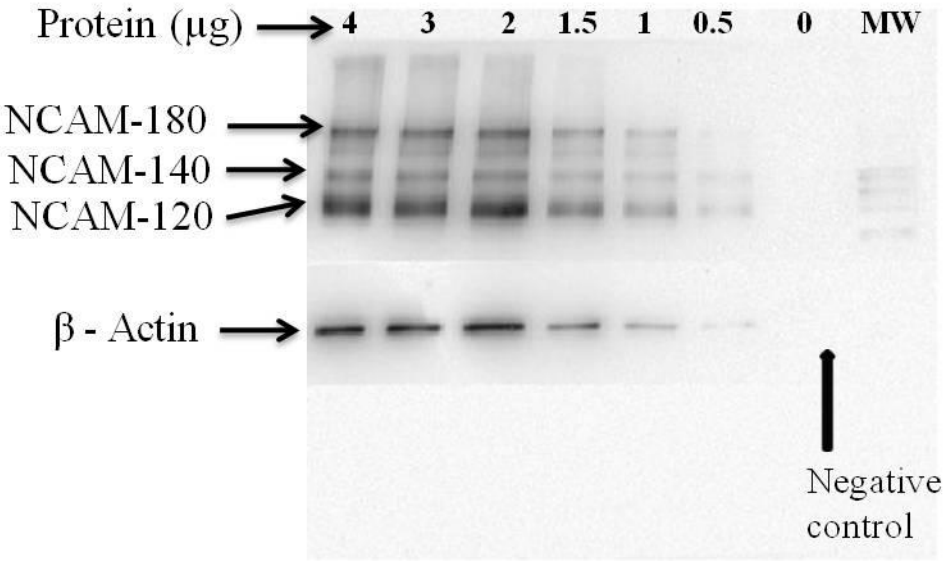


Figure 2.4: NCAM1 standard curve with negative control. Three NCAM1 isoforms are shown as NCAM-180, NCAM-140 and NCAM120. Protein concentrations are shown in µg at the top. MW is the molecular weight marker.

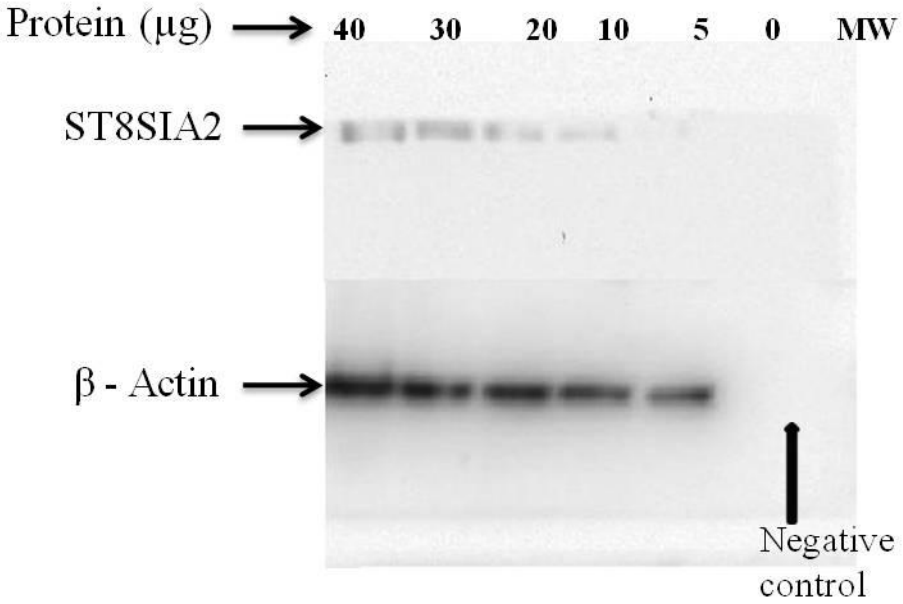


Figure 2. 5: ST8SIA2 standard curve with negative control. ST8SIA2 band was observed at 43 kD. Protein concentrations are shown in µg at the top. MW is the molecular weight marker.

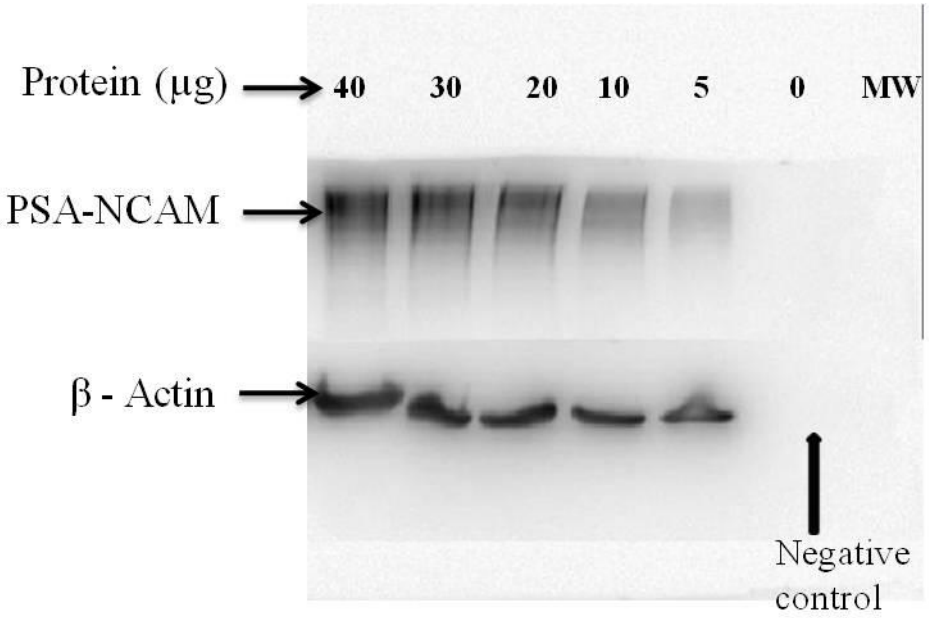


Figure 2. 6:PSA-NCAM standard curve with negative control. PSA-NCAM was observed as a smear between 250 kD and 160 kD. Protein concentrations are shown in µg at the top. MW is the molecular weight marker.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays (EMSA) were conducted in Chapter 3 to determine whether NRG1 alternative alleles affect transcription factor binding *in-vitro*. Complementary 23-mer primer probes targeting both minor and major alleles of novel SNPs were designed and ordered from (Operon Technologies, Inc., CA). A probe containing a known transcription factor binding site (NFκB) was included as a positive control. Primer-probe sequences used for both minor and major alleles of SNPs tested and NFκB are shown in Table 2.12. Each probe had a non-complementary 5' overhang (agct) to enable radioactive probe labelling.

Primers were heat denatured and annealed (heteroduplex formation) by slow cooling overnight. Annealed duplex overhangs were then blocked by using 1U DNA polymerase (Klenow) enzyme and 3 mmol/mL dNTPs using either cold dCTP or γ^{32}P-dCTP for the cold competitor or hot probe, respectively. Hot probes were diluted to 20000 CPM/µL before use. Nuclear extracts were prepared for HEK293 and SKNMC cells (supplied by Ms Giti Agahi, a research assistant) by washing with 1× phosphate buffered saline (PBS)

before harvesting cells for counting by haemocytometer. Cells were aliquoted to 5×10^6 cells per tube and lysed in the presence of 0.007% NP-40 before centrifugation. For binding reactions, the protein concentration was diluted to 2 mg/mL.

PROBE NAME	FORWARD/REVERSE COMPLIMENT	SEQUENCE
NFκB	Forward	agctGGGTCTGTGAATTCCCGGGGGT
NFκB	Reverse	agctACCCCCGGGAATTCACAGACCC
YT_1395_A (rs117469567*)	Forward	agctTAAAAGTTAAGTTTCATTA
YT_1395_A (rs117469567*)	Reverse	agctATAATGAAACTTAACTTTT
YT_1395_T (rs117469567*)	Forward	agctTAAAAGTTATGTTTCATTA
YT_1395_T (rs117469567*)	Reverse	agctATAATGAAACATAACTTTT
YT_848_A (rs117129618*)	Forward	agctTTTAAACAGAACATAACTC
YT_848_A (rs117129618*)	Reverse	agctCTGAGTTATGTTCTGTTTA
YT_848_G (rs117129618*)	Forward	agctTTTAAACAGAGCATAACTC
YT_848_G (rs117129618*)	Reverse	agctCTGAGTTATGCTCTGTTTA
rs36213231_T	Forward	agctAGGCTCCTCCTGGTGGCG
rs36213231_T	Reverse	agctCACGCCACCAGGAGGAGC
rs36213231_C	Forward	agctAGGCTCCTCCCGGTGGCG
rs36213231_C	Reverse	agctCACGCCACCGGGAGGAGC
YT4.2_2_C (rs201058425*)	Forward	agctTAAAAGAACACGAGAGAAA
YT4.2_2_C (rs201058425*)	Reverse	agctTTTTCTCTCGTGTTCTTTT
YT4.2_2_T (rs201058425*)	Forward	agctTAAAAGAACATGAGAGAAA
YT4.2_2_T (rs201058425*)	Reverse	agctTTTTCTCTCATGTTCTTTT
JF3.2_2_DEL (rs200516494*)	Forward	agctTCTTTTAAAAAACAGTCAT
JF3.2_2_DEL (rs200516494*)	Reverse	agctATGACTGTTTTTTAAAAGA
JF3.2_2_CA (rs200516494*)	Forward	agctTCTTTTAAAACAAACAGTC
JF3.2_2_CA (rs200516494*)	Reverse	agctATGACTGTTTGTTTTAAAA

Table 2.12: Primer-probes for EMSA assay. Custom designed probes were used to specifically target particular *NRG1* SNPs. Each probe comprised a forward and reverse primer, which were annealed to form a heteroduplex with a non-complementary 5' overhang (agct) to enable radioactive probe labelling. Identified novel SNPs, that now have an rs number (after submission to dbSNP 137 build) are denoted by asterisks '*'.

Binding reactions were performed by combining 4 µl binding buffer (20 mmol/mL HEPES, 2 mmol/mL EDTA, 2 mmol/mL EGTA, 25% glycerol, 187.5ug/mL dI-dC (Polydeoxyinosinic-Deoxycytidylic Acid; Sigma-Aldrich, St. Louis, Missouri), 2 µl nuclear extract, and 4 µl cold competitor probe and incubating for 5 min at room temperature before the addition of the hot labeled probe and further incubation for 10–15 min. Protein-bound duplexes were then loaded on a 5% acrylamide, 0.5× TBE gel pre-cooled to 4 °C, and run at 200 V for 2 hours.

Tested protocols were followed closely throughout the experiment to maintain quality. I undertook a radiation safety course at UNSW, to work with radioactive substances. I was also supervised by Dr Jan Fullerton (PhD supervisor) during various experimental steps. Special care was taken to work behind perspex shielding and to wipe out radioactive chemicals. For experiments, a positive control, which was a sequence variant in the promoter of the NFκB gene that is known to alter protein binding was used for every reaction. If there was no protein binding to NFκB probe, the experiment was discarded. Experimental results were cross checked and verified by Dr Jan Fullerton.

2.9 Analysis and Statistics

2.9.1 Statistical analysis

Statistical analysis was performed using Statistica Software version 7.1 for Windows (Statsoft, Inc., Tulsa, OK, USA). The Kolmogorov–Smirnov (K-S) test was used to determine whether the mRNA and protein expression data was normally distributed. For positively skewed data, a natural logarithm transformation was used to create data conforming to normal distribution, to enable the use of parametric statistics for data analysis.

2.9.2 Effects of demographic and clinical variables

The effects of quantitative demographic variables such as post-mortem interval (PMI), age, brain pH, RNA integrity (RIN, for mRNA studies) and RNA yield (for mRNA studies); and clinical covariates such as age of onset, illness duration, chlorpromazine equivalents (daily, last and life time levels)] on mRNA or protein expression were assessed by correlation analysis. Significant correlations were defined when the p value from the Pearson correlation was less than 0.05 ($p \leq 0.05$) and where medium to large effect size effects ($r \geq 0.25$) were observed (Kraemer H C, *et al.* 2003).

2.9.3 Group differences

Levene's test was used to assess homogeneity of variance. For variables with no significant demographic correlated variables, one-way ANOVAs and factorial ANOVAs were

performed to assess the effects of diagnosis, and to detect interaction effects of gender and brain hemisphere. For ANCOVA studies, only demographic covariates with medium to large effect sizes ($r \geq 0.25$) and Pearson correlation was less than 0.05 ($p \leq 0.05$) were included (Kraemer H C, *et al.* 2003). In addition, the effects of clinical variables such as antipsychotic history, chlorpromazine equivalent neuroleptic (mean daily dose, last dose, and total lifetime use), age of onset, agonal state immediately preceding death and manner of death (natural/suicide) were also analysed on protein and mRNA expression in the schizophrenia sample, although these were not used in ANCOVA to examine group differences between cases and controls. Values exceeding a 5% significance threshold ($p<0.05$) were considered significant. Post-hoc Fisher least squares difference (LSD) test was used to identify specific significant group differences.

2.9.4 Ratio analysis

To investigate how the differential expression of substrate (NCAM1), enzyme (ST8SIA2) and product (PSA-NCAM) relate to each other, different ratio analyses were performed in Chapters 4 and 5. Protein expression levels were studied as a ratio of total NCAM protein level to PSA-NCAM protein level; total "glycosylatable" NCAM (sum of NCAM-180, NCAM-D, and NCAM-140) level to PSA-NCAM protein level (Chapter 4), and ST8SIA2 enzyme level to PSA-NCAM protein level (Chapter 5).

Chapter 3

How does gene and mRNA variation in neuregulin-1 (NRG1) increase risk for mental illness?

3.1. Introduction

NRG1 plays an important role in neurodevelopment through its involvement as a growth factor in range of different processes such as neuronal migration, cell signaling and synaptic plasticity (Anton E S, *et al.* 2004; Chen Y, *et al.* 2010; Fazzari P, *et al.* 2010; Flames N, *et al.* 2004; Mei L, Xiong W C 2008). Interestingly, *NRG1* has also been identified as a candidate gene for mental illness. *NRG1* was initially associated with schizophrenia in the Icelandic population (Stefansson H, *et al.* 2002) through linkage studies. Further fine mapping and haplotype analysis identified a core risk haplotype (HAPICE) within 5' region of NRG1. This haplotype comprised of 5 SNPs (SNP8NRG221132, SNP8NRG221533, SNP8NRG241930, SNP8NRG243177 and SNP8NRG433E1006) and two microsatellite markers (478B14-848 and 420M9-1395), covering a region of approximately 290 kb on Chromosome 8 (Stefansson H, *et al.* 2002). Since that finding, several other studies have associated this HAPICE region (Georgieva L, *et al.* 2008; Petryshen T L, *et al.* 2005; Stefansson H, *et al.* 2003; Thomson P A, *et al.* 2007; Williams N M, *et al.* 2003b) and other haplotypes in 5' region of *NRG1* (Corvin A P, *et al.* 2004; Fukui N, *et al.* 2006; Tang J X, *et al.* 2004; Yang J Z, *et al.* 2003; Zhao X, *et al.* 2004) with schizophrenia. In addition, other parts of this gene were also found to be associated with schizophrenia and mental illness in general, making it an important gene to more closely examine, particularly in regards to the mechanism by which NRG1 may increase disease risk (Petryshen T L, *et al.* 2005; Thomson P A, *et al.* 2007; Walker R M, *et al.* 2010).

NRG1 is one of the most replicated susceptibility genes for schizophrenia (Harrison P J, Law A J 2006) and although it has not been a 'top hit' in any GWAS for schizophrenia or bipolar disorder, supportive evidence has been reported in three GWAS datasets (Agim Z S, *et al.* 2013). Meta-analysis involving 22 studies has established replication at the haplotype level, but not at the particular SNP level (Munafo M R, *et al.* 2008; Munafo M R, *et al.* 2006), suggesting that specific risk alleles are likely to differ across populations and individuals. Therefore, the precise way in which genetic variation in *NRG1* impacts on

disease susceptibility remains uncertain, as is the mechanism through which HAPICE risk haplotype confers risk to mental illness, making it an interesting gene to investigate. In this chapter, I will look to find answers to these questions.

NRG1 genotypes have been also associated with schizophrenia-related phenotypes. A study by Mata *et al.* (2009) reported an association between *NRG1* SNP8NRG243177 and increased lateral ventricle volume of people experiencing their first episode of schizophrenia. A study by Krug *et al.* (2010) found that *NRG1* genotype significantly increases brain activation during episodic memory processing in BA9, BA24 and fusiform gyrus. It is well documented that *NRG1* SNPs associated with mental illness are non-coding (intronic, upstream of transcription start site, exonic substitution) (Law A J, *et al.* 2006) and only 1 missense mutation in transmembrane domain (TMD) of *NRG1* (exon 11), has been identified so far (Walss-Bass C, *et al.* 2006). It would be interesting to see if any known/novel SNPs present in intronic or exonic regions are associated with mental illness in our Australian cohort.

The *NRG1* is a large gene, approximately 1.4 Mb in size (Falls D L 2003). To carry out different functions, it uses alternative promoters and generates different mRNA and protein isoforms. The main functional isoforms of NRG1 can be classified as, type I and II (contact-independent or soluble) and type III (contact-dependent or insoluble) (Figure 3.1). NRG1 types IV-VI were reported by Steinthorsdottir *et al* (2004) and are similar to type I and II in their proteolysis, but are less abundant.

Interestingly, different studies, largely in non-caucasian (African American or Asian) cohorts have reported specific alteration of NRG1 mRNA transcripts (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009; Parlapani E, *et al.* 2010) in the frontal cortex and hippocampus of patients with schizophrenia. Looking at expression levels of different isoforms of this gene, Hashimoto *et al.* (2004) found that NRG1 type I expression is increased in DLPFC of schizophrenia cases. Also, Law et al. (2006) found this increase in NRG1 type I expression in schizophrenia cases. Collectively, this variation in transcript expression has been further related to promoter region and polymorphisms in

the 5' intronic region, associated with schizophrenia (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009).

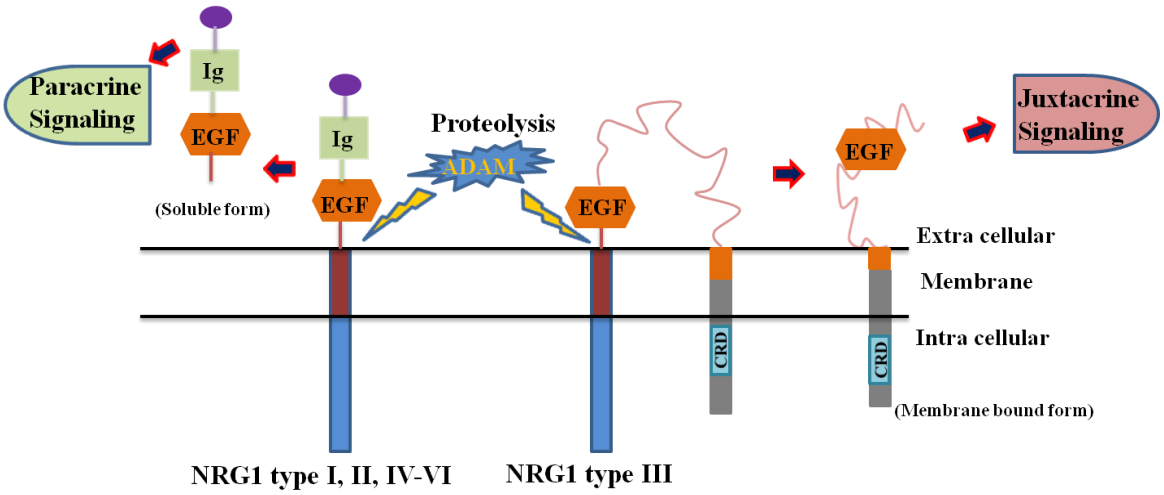


Figure 3.1: Six main functional isoforms of NRG1 and the effect of proteolysis by A-Disintegrin And Metalloprotease (ADAM) family of proteases. NRG1 prototypes I, II, IV-VI, post-proteolysis, give rise to a soluble form, which is then involved in paracrine signaling. While NRG1 prototype III forms a membrane-bound form after proteolysis to facilitate juxtacrine signaling. (EGF = Epidermal Growth Factor; Ig = Immunoglobulin domain; CRD = Cystein Rich Domain).

Functionally, NRG1 signals through high affinity tyrosine kinase receptor, ErbB4, to control neural development and synaptic plasticity (Anton E S, *et al.* 1997; Bermingham-McDonogh O, *et al.* 1996; Li B, *et al.* 2007; Lopez-Bendito G, *et al.* 2006) other common ligands of ErbB4 include EGF (Iwakura Y, Nawa H 2013), YAP65 (Omerovic J, *et al.* 2004), STAT5A (Williams C C, *et al.* 2004), PDZ domain proteins (Garcia R A, *et al.* 2000). Impaired NRG1-ErbB signaling is connected to abnormal behavior linked to schizophrenia (Barros C S, *et al.* 2009; Rimer M, *et al.* 2005; Savonenko A V, *et al.* 2008; Stefansson H, *et al.* 2002).

Post-mortem human brain serves as a vital resource to study underlying pathogenic molecular mechanisms of bipolar disorder and schizophrenia. Therefore, in this study, a post-mortem brain cohort where cases and controls are matched for age, RNA integrity, post-mortem interval, brain pH and hemisphere, is used. Given that most *NRG1* SNPs associated with mental illness are non-coding, this chapter aims to characterize the genetic

variation in upstream regulatory sequences of alternative promoters of the candidate gene *NRG1*; to identify putative functional variations and alterations in NRG1 transcript expression levels which may increase risk to mental illness.

3.2. Results

3.2.1. Genomic variation and diversity

To investigate the genetic variation between subjects with either schizophrenia or controls in the upstream regulatory regions, three main promoters of NRG1, namely type I, II and III were studied. Type IV was included because of its close proximity to type II. Two HAPICE microsatellite repeats were also included in this resequencing study (Figure 3.2).

Considering the regions of interest were both discrete and small (< 3500 bp), TRC genomic DNA from 37 schizophrenia cases and 37 controls (Chapter 2; Table 2.1) was sequenced by the Sanger dideoxy-sequencing (Chapter 2; Section 2.4.2). Post sequencing, 919 kb (12.5 kb per person) of good quality sequence was available for variant detection. Sequence traces for each sample were analyzed through Lasergene SeqMan software 7.0 (DNASTAR, Madison, WI). Identification of SNPs was performed by manually looking at the chromatograms and their novelty was determined by comparing them against NCBI dbSNP (build 130). Results were cross-checked on PhredPhrep software (Ewing B, Green P 1998; Ewing B, *et al.* 1998), by Dr Jan Fullerton (PhD supervisor). The human genome reference assembly (NCBI36/hg18) was used to report base pair positions of SNPs identified in this study.

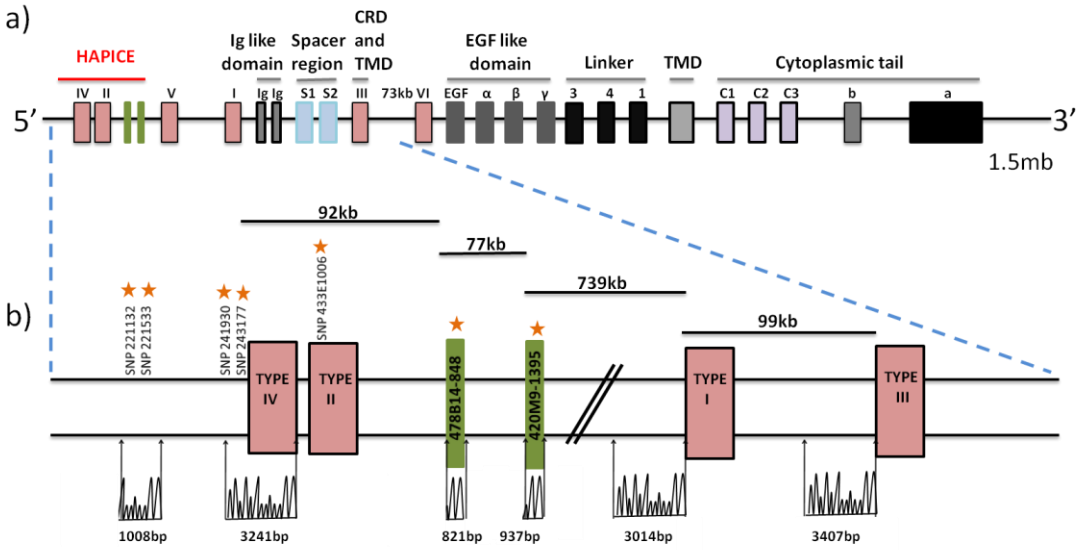


Figure 3.2: Schematic representation of *NRG1* genomic structure and regions that were included for Sanger sequencing. a) Different protein domains of NRG1 and its six alternative promoters. Promoters I-IV are main, while V-VI are minor promoters. The position of the HAPICE haplotype is shown in the upstream promoter region. **b)** Respective positions and size (in bp) of the target regions selected for sequencing are indicated. Stars represent 5-SNPs and two microsatellite markers in the HAPICE region. Alternative promoters are in pink, microsatellites in green, different structural domains are presented in shades of grey and black and spacer region is blue. Wavy lines below represent area sequenced by Sanger sequencing.

Sixty-eight DNA variants were identified through this sequencing study, with twenty-six SNPs identified as being novel (not reported before; as at dbSNP130 release). The position of these SNPs and their distribution across schizophrenia and control groups is shown in Table 3.1.

NRG1 region	Base-pair position (NCBI Build 36)	Sequencing Region size (bp)	All observed SNPs		SCZ observed SNPs		CON observed SNPs	
			dbSNPs (130)	Novel	dbSNPs (130)	Novel	dbSNPs (130)	Novel
upstream HAPICE	31592860-31593868	1008	2	3	3	3 (1)	3	2 (0)
promoter IV/II	31613334-31616575	3241	9	8	9	8 (2)	7	3 (0)
Microsatellite 478B14-848	31708115-31708936	821	3	4	3	3 (3)	3	1 (1)
Microsatellite 420M9-1395	31784622-31785559	937	7	3	3	3 (2)	3	1 (0)
promoter I	32522326-32525340	3014	15	1	13	1 (1)	13	0 (0)
promoter III	32620730-32624137	3407	6	10	6	6 (2)	6	7 (4)
Total		**12428**	**42**	**29**	**37**	**24 (11)**	**35**	**14 (5)**

Table 3.1: Summary of the SNPs reported during targeted resequencing. The average minor allele frequency (MAF) of novel SNP variations was 0.044±0.038. The total numbers of novel SNPs observed in each *NRG1* region studied are shown, with the number of SNPs exclusive to that group shown in parentheses. Numbers of novel SNPs are higher in schizophrenia (SCZ) group, especially in HAPICE region (no. 1-4). Numbers in brackets for the novel SNP indicate SNPs that were novel in our study, but were included in dbSNP (130) at the time of manuscript submission.

Novel DNA variations were present in both schizophrenia and control groups. However, the total number of novel variants was higher in schizophrenia group (n = 24 vs 14; Fishers exact test: X^2 = 10.8; p = 0.001). Interestingly, this difference was enhanced when the number of novel SNPs carried by each individual were considered. Overall, more than 3 novel variants were rarely identified in single control individual, while 7 schizophrenia cases had more than 3 novel variants in the resequenced regions (Fishers exact test: X^2 = 7.815; p = 0.05), with up to 6 novel variants observed in one schizophrenia individual (Figure 3.3). This study was an investigatory study. Replication study in a larger cohort is required to validate the results.

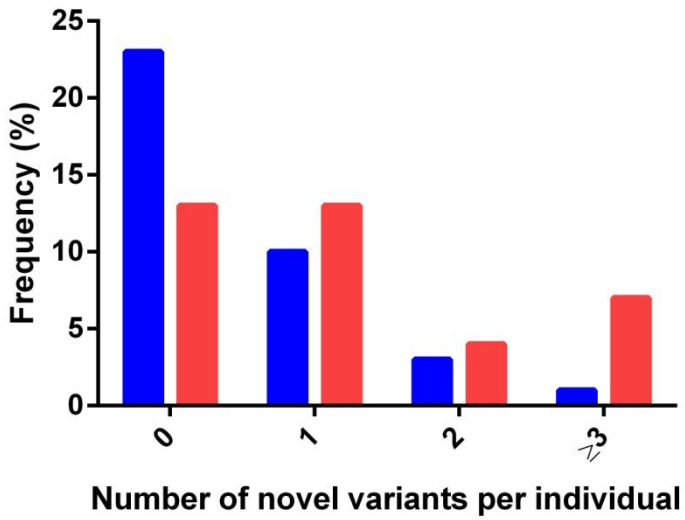


Figure 3.3: Schizophrenia patients (SCZ, in red) showed an increased novel variant load when compared to unaffected controls (CON, in blue) (Fishers exact test: χ^2 = 7.815; p = 0.05; df = 3). CON group has more individuals with no novel variants. Only one individual from CON group with 3 novel variants is represented in the ≥3 category, compared to 7 individuals from SCZ group with 3, 5 and 6 novel variants (n = 3, 3 and 1 respectively).

An interesting result from this study was the identification of a novel 5-SNP haplotype (CAGAA; Figure 3.4) in intron 1, which was detected in 4/37 (approximately10%) of schizophrenia cases studied. This haplotype was missing in all 37 control samples. When the prevalence of this haplotype was examined in a wider population sample (the ASRB cohort), in 256 chromosomes studied, this novel haplotype was not observed in any of the schizophrenia cases and in only one unaffected control individual at a frequency of 0.0039, thus, demonstrating that this haplotype is rare and may not necessarily be associated with only schizophrenia. However, this haplotype should be screened in a larger cohort, possibly also involving bipolar patients if possible, to investigate its distribution and specificity for association with mental illness.

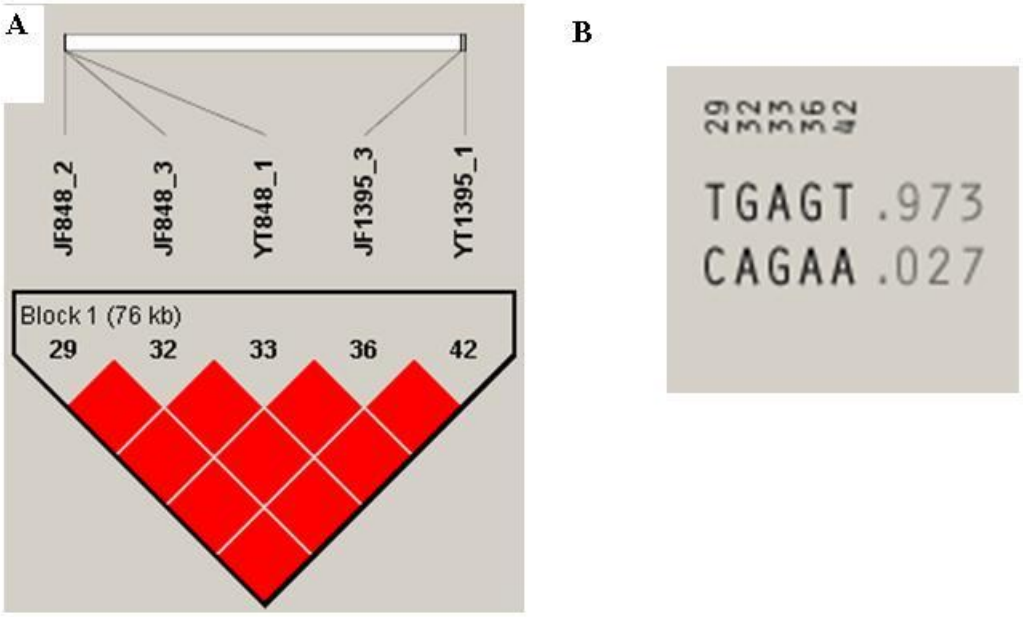


Figure 3.4: Linkage disequilibrium relationship between five novel SNPs forming a haplotype in intron 1 region of NRG1. A) SNPs JF848_2, JF848_3, YT848_1, JF1395_3 and YT1395_1 represent novel SNPs identified in this study, and each are in high LD.. These novel SNPs now have rs numbers (via NCBI) and are shown in Table 3.2. The block represented was determined via the solid spine of LD method in HAPLOVIEW (Barrett *et al.* 2005). B) Two haplotypes were identified using these SNPs as per the haplotype block shown in panel A. The haplotype CAGAA represents the rare novel allele at each SNP, and frequencies are shown as determined in the TRC post-mortem brain cohort.

Nucleotide diversity (Θ) calculations were then performed to study genetic diversity/variation in different *NRG1* regions that were sequenced. Calculations took into consideration the number of SNPs (K) identified in genomic length (L) in a sample of (n) alleles. Predictions were performed based on formula that is described in the previous published literature (Halushka M K, *et al.* 1999; Licinio J, *et al.* 2009).

$$\theta = K/aL \;,\; S(\hat{\theta}) = \sqrt{a\theta L + b(\theta L)^2}/aL \quad a = \sum_{i=2}^{n} \frac{1}{(i-1)} \;,\; b = \sum_{i=2}^{n} \frac{1}{(i-1)^2} \;,$$

Results indicated high nucleotide diversity in all the regions that were sequenced. In particular the 5' upstream ($\Theta = 18.0 \times 10^{-4}$) and the two micro-satellite region showed very high nucleotide diversity ($\Theta = 15\text{-}30.1 \times 10^{-4}$) and diversity was elevated in schizophrenia cases compared to unaffected controls (Figure 3.5).

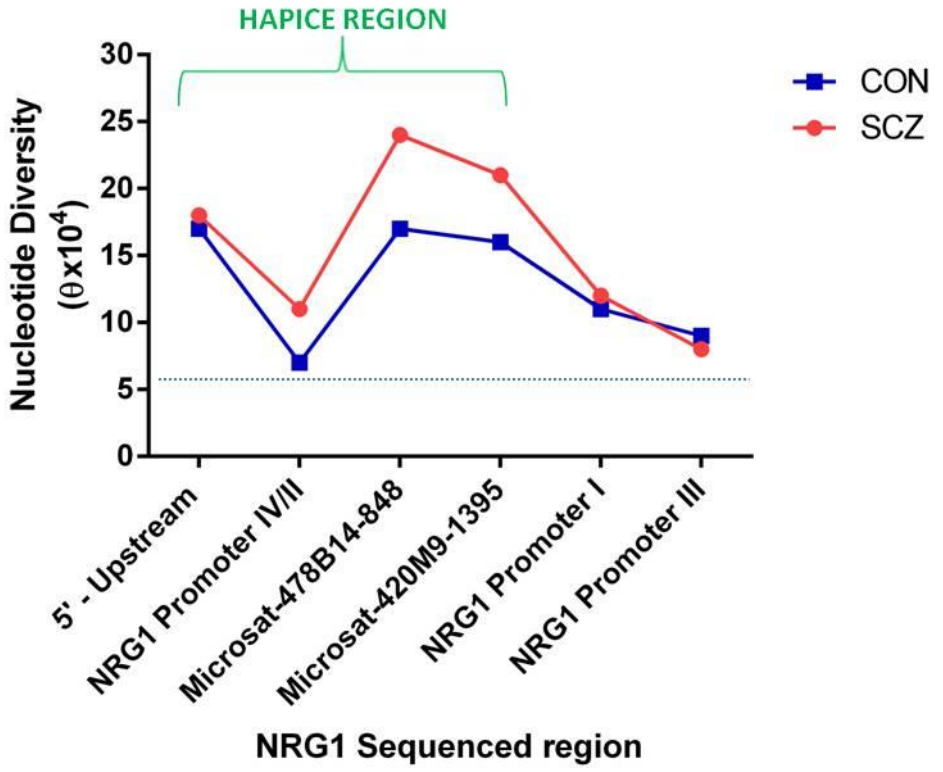


Figure 3.5: Greater nucleotide diversity was observed in schizophrenia group (red) than control group (blue), predominantly in the HAPICE risk haplotype region. Blue dashed line illustrates average value of nucleotide diversity ($\theta = 5.4 \times 10^{-4}$) from non-coding regions of 75 other human genes (Halushka M K, *et al.* 1999). Green bracket shows the regions that are included within the HAPICE LD block.

In an analysis to investigate how nucleotide diversity in intronic *NRG1* region compares to intronic nucleotide diversity of other genes, it was found that *NRG1* HAPICE region is genetically very diverse ($\Theta = 10.0 \times 10^{-4}$), surpassing average nucleotide diversity found in promoter and intronic regions of BDNF ($\Theta = 6.0 \pm 2 \times 10^{-4}$) (Licinio J, *et al.* 2009) and of 75 other human genes ($5.4 \pm 1.5 \times 10^{-4}$) (Halushka M K, *et al.* 1999).

3.2.1.1. Genetic similarity of TRC cohort to other cohorts

To explore whether the individuals contributing to TRC post-mortem brain cohort were genetically similar to the Icelandic population, where the HAPICE was first discovered (Stefansson H, *et al.* 2002), I compared the frequencies of the risk haplotype across the Australian TRC cohort and the Icelandic population. To find out the frequency of the HAPICE 5-SNP haplotype in the TRC cohort, genotypes were phased using PLINK

software (Purcell S, *et al.* 2007) and haplotypes were selected with best posterior probability (mean p value = 0.91 ± 0.15). Risk haplotype frequencies for HAPICE (5-SNP) were similar between schizophrenia groups in the TRC cohort (35.2%) and the Icelandic cohort (32.1%). The risk haplotype frequency for the Scottish cohort was 31.4% (Stefansson H, *et al.* 2003), again very comparable to both groups. In the control group, the frequencies for HAPICE were 29.8% in the TRC cohort and 25.1% in the Icelandic population.

While the frequencies were comparable in the TRC cohort compared to the cohorts in which disease association in *NRG1* was observed, the frequency difference of either haplotype between schizophrenia and unaffected control group was not significant in the TRC cohort ($p>0.844$). Given that risk haplotype frequencies for the TRC cohort and the Icelandic population are very similar, it might be possible to replicate the main association finding in our Australian cohort, if both sample sizes were equivalent. However, this is not possible given small sample size of TRC cohort. The population studied to show the association of HAPICE with schizophrenia comprised of 478 schizophrenia cases and 394 controls (Stefansson H, *et al.* 2002), whereas the TRC cohort is a post-mortem brain cohort of only 37 schizophrenia cases and 37 controls. A post-mortem brain cohort is not likely to achieve sample numbers comparable to the power of lymphocyte-based case-control cohorts for genetic association. However, given that the Australian haplotype frequencies are similar to those discovery cohorts, it is possible that the mechanism of risk of the HAPICE haplotype might be equivalent in Australian and Icelandic populations. Future studies could involve Australian cohorts with large sample size.

3.2.1.2. Functionality of DNA variation

Given that there was increased novel variant load in schizophrenia cases compared to unaffected controls, I next investigated if these novel variants had any functional consequences, particularly on gene transcription. Considering that the regions selected for sequencing were promoter regions, it is likely that a functional variation in these regions might affect transcription, by obstructing or changing the binding of transcription factors.

Bioinformatic predictions were therefore performed to find out whether any of the known or novel DNA variants are capable of affecting transcription factor binding. TRANSFAC software was used to predict the effects of alternative alleles for each novel SNP on transcription factor binding, by comparing the sequence containing each allele to a consensus containing each allele to a consensus binding sequence for human transcription factors. TRANSFAC is accessible via http://www.transfac.gbf.de/TRANSFAC

On comparing TRANSFAC results for known and novel variants, it was found that novel DNA variants were more likely to be functional and 85% (22/26) of them were predicted to alter putative transcription factor-binding sites (Table 3.2). Bioinformatic predictions indicated possible functional effects of DNA variations; however these are only predicted binding sites on the basis of flanking sequence conservation and similarity to known binding sites. Hence, I then tested some of these DNA variations functionally by direct experimentation. The Electrophoretic Mobility Shift Assay (EMSA) technique was chosen to functionally test the interaction between nucleic acid and nuclear proteins. This method was specifically chosen because it was fast and sensitive, plus the technique was well understood and optimized in our laboratory. Also, as the minor allele of the novel SNPs tended to be quite low in frequency (Table 3.2), correlations between genotype and mRNA expression were not possible due to low sample numbers in the rare allele genotype groups.

NRG1 gene region	BP position (build 36)	SNP name	dbSNP (137) name	Minor allele	Major allele	MAF (SCZ)	MAF (CON)	TRANSFAC binding change
Upstream prom IV/II	31593076	JF221XXX_4	rs200938633	C	T	0.014	0.000	Removes TATA
Upstream prom IV/II	31593103	JF221XXX_1	rs71523425	C	T	0.068	0.054	Removes Hb, creates cap
Upstream prom IV/II	31593216	JF221XXX_2	rs74506441	C	T	0.095	0.041	Creates CDP-CR
Upstream prom IV/II	31613752	JF4.3_1	rs200059112	C	T	0.014	0.000	Removes cdxa, reduces Oct-1
Upstream prom IV/II	31614472	JF4.2_4	rs77626248	C	A	0.108	0.041	Creates c-ETS, GATA
Upstream prom IV/II	31614502	YT4.2_1	rs73584584	C	T	0.054	0.014	Reduces Sox-5, removes SRY
Upstream prom IV/II	31615552	YT4.2_2	rs201058425	C	T	0.014	0.000	Increases TATA
Upstream prom IV/II	31615555	YT4.2_3	rs76063839	A	G	0.027	0.041	Creates SRY, improves HNF-3
Intron 1	31708375	JF848_2	rs144930522	C	T	0.042	0.000	Removes cdxa
Intron 1	31708615	JF848_3	rs143001928	A	G	0.056	0.000	Removes SRY, creates cdxa
Intron 1	31708702	YT848_1	rs117129618	G	A	0.054	0.000	No change
Intron 1	31708937	NOR_848_4	rs199803466	C	T	0.000	0.027	Increases AP-1
Intron 1	31784714	JF1395_2	rs201472757	A	G	0.014	0.000	Increases Sox-5
Intron 1	31784774	JF1395_3	rs148797477	A	G	0.054	0.000	Reduces SRY, removes c-Myb
Intron 1	31785235	YT1395_1	rs117469567	A	T	0.054	0.000	Reduces SRY, removes cdxa
Upstream prom I	32524132	JF1.2_1	rs200575150	T	G	0.027	0.000	Creates SRY
Upstream prom III	32620979	NOR3.3_1	rs192753677	A	G	0.000	0.014	Introduces GATA-1, removes AML-1a
Upstream prom III	32621144	JF3.3_5	rs201900567	DEL	AA	0.014	0.014	Removes HFH-2
Upstream prom III	32621357	JF3.3_6	rs111526496	A	G	0.014	0.027	Removes AML-1a
Upstream prom III	32621907	JF3.3_7	rs113060920	T	C	0.029	0.000	Creates GATA, reduces deltae
Upstream prom III	32622150	NOR3.2_4	rs117532293	T	C	0.000	0.014	Creates S8
Upstream prom III	32622768	NOR3.2_2	rs117347889	G	A	0.000	0.014	No change
Upstream prom III	32622867	NOR3.2_3	rs191779973	A	G	0.000	0.014	No change
Upstream prom III	32622943	JF3.2_2	rs200516494	DEL	CA	0.027	0.014	Removes SRY, HFH-2
Upstream prom III	32623136	JF3.1_3	rs199939899	DEL	TGA	0.014	0.000	Introduces C-/EBP
Upstream prom III	32624045	JF3.1_2	rs199493414	C	T	0.014	0.000	No change

Table 3.2: Detailed information about 26 novel variants according to (dbSNP130) identified in *NRG1* re-sequenced regions. The gene region and base pair location (NCBI build 36) of each SNP identified is given, along with the minor and major alleles of the variant. The name assigned to each SNP upon discovery is given and the corresponding NCBI dbSNP137 name for each novel variant after submission for novel variants to NCBI is also shown. The minor allele frequency (MAF) in all 74 individuals (ALL), the 37 cases with schizophrenia (SCZ) and the 37 controls (CON) are given. The predicted transcription factor binding changes for each SNP (TRANSFAC) are listed.

The EMSA technique involved mixing solutions of nuclear protein extracts with probes, which had specific nucleic acid sequences of interest. The principle behind the method is that functional DNA variants in nucleic acids will form a complex with DNA binding proteins and this protein-nucleic acid complex would travel more gradually in a polyacrylamide than the equivalent free nucleic acid (non-functional DNA variants). The protein-DNA complexes were analysed by polyacrylamide gel electrophoresis, where hot-samples were run as ^{32}P labeled and cold lanes comprised of equivalent amount of hot probe and 10 fold more unlabeled probe as cold competitor. Protein extracts used in this experiment were obtained from either nuclear extracts from cultured HEK293 cells (provided by Ms Giti Agahi) or from brain tissue (SMRI cohort). Experimental procedure and probe sequences are detailed in (Chapter 2; Table 2.12). Testing all the SNPs was beyond the timeframe of the PhD, therefore 5 SNPs were chosen for EMSA experiments. Primarily, the SNPs were chosen if they were novel and were predicted to have an effect on transcription factor binding. For example, the two SNP were chosen because rs117469567 was predicted to reduce activity of SRY, a sox-like male specific transcription factor; and rs113060920 was predicted to create binding site for transcription factors recognizing GATA sequence. Other SNPs examined were rs117129618, rs201058425 and rs200516414. SNP rs117129618 was a novel SNP that was identified in our study but which affected no predicted transcription factor binding site. I included both SNPs with and without predicted functional effects to determine if the bioinformatic predictions were correct. Finally, results showed that two of the five probes tested were functional and showed differential binding of the novel and wild-type alleles in proteins from both transformed neuronal cell lines (HEK 293) and directly from brain tissue (SMRI). Dark spots in Figure 3.6 indicate the presence of protein-nucleotide complex, while paleness/absence of spot in the same area indicates a ‘shift’ in the electrophoretic profile and therefore confirming difference in migration.

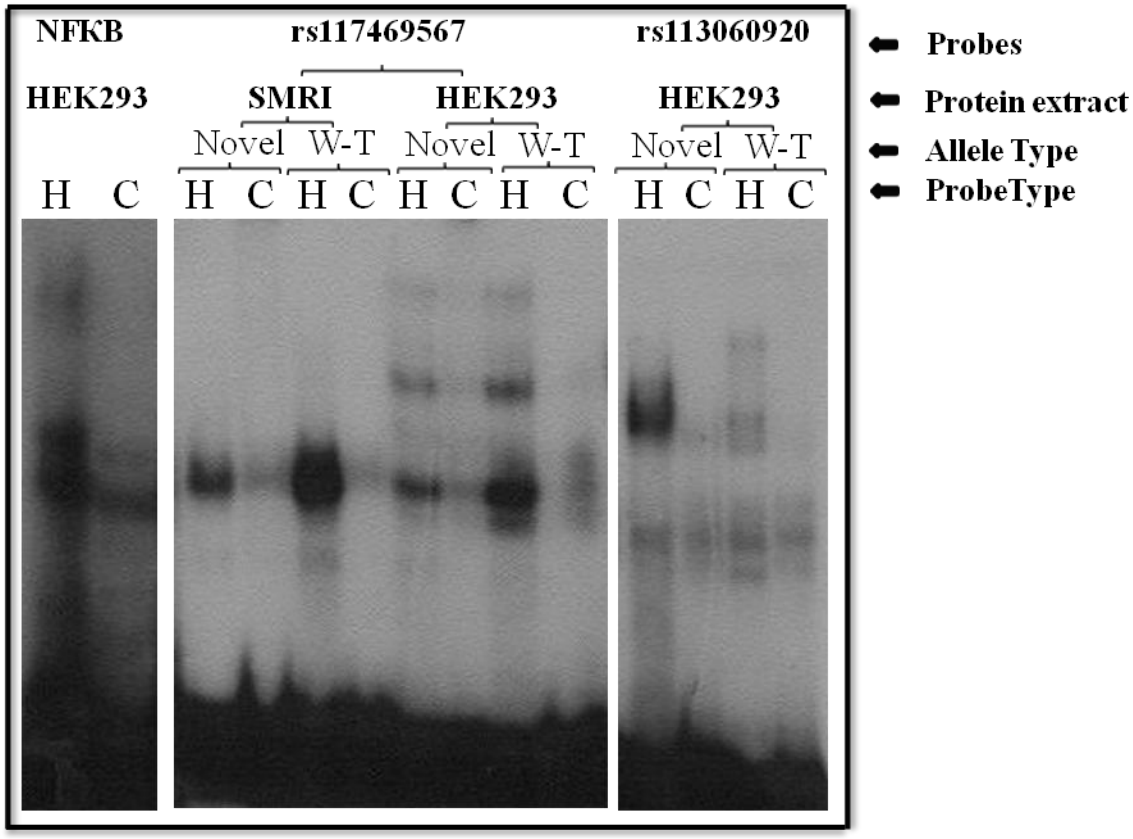


Figure 3.6: Electrophoretic Mobility Shift Assay results to show protein binding to two novel SNPs (rs117469567 and rs113060920). Each Hot lane (H) represents the probes labeled with ^{32}P, and (C) represents the cold competitor lane. Alleles are marked as 'Novel' or Wild-Type (W-T). Protein extracts used were either nuclear extracts from cultured HEK293 cells or proteins extracted directly from SMRI DLPFC tissue. Figure illustrates differential protein binding between 'H' and 'C' probe reactions for minor and major alleles in rs117469567 and rs113060920. NFκB was used as a positive control. SNP rs117469567 was observed at MAF of 0.054 and 113060920 was observed at MAF of 0.0285 in TRC cohort (i.e. in 2 and 1 schizophrenia patients, respectively).

Analysis of DNA variation in the *NRG1* gene therefore identified a high novel variant load in schizophrenia cases, and some of the novel variants identified were found to be functional through bioinformatic predictions and *in vitro* test.

3.2.2. mRNA variation

This study was performed to investigate relative abundance of different NRG1 transcripts in DLPFC of human post-mortem brain. Five transcripts were studied including those driven from four main NRG1 promoters and a 'pan' probe which assessed all NRG1 transcripts (Table 3.3). Data was normalized to geometric mean of four housekeepers (Chapter 2; Section 2.6). Outlying data points with values ± 2.5 times the standard

deviation were excluded from the main data analyses to remove the possible effects of outliers on the statistics. NRG1 type IV transcript level in DLPFC tissue was found to be extremely low i.e. at the boundary of detection and hence its quantification was considered as unreliable.

PROBE NAME	ISOFORMS DETECTED	TAQMAN ASSAY	CUSTOM-MADE PROBE (5'-3')
NRG1_Type I	HRG-β3b and HRG-γ3 (excluding HRG-β1b, HRG-β1c)	Hs01108479_m1	-
NRG1_Type II	GGF-2, HRGβ-1d, HRG-β3b, HRG-γ3	-	'CACTGGGACAAGCC'
NRG1_Type III	SMDF	Hs01103792_m1	-
NRG1_Type IV	HRG-β1b, HRG-β1c and HRG-β1d	-	'ACCACAGCCTTGCCT'
NRG1_PAN	all isoforms (excluding SMDF, ndf43c)	Hs00247620_m1	-

Table 3.3: Five NRG1 transcripts studied for mRNA variation. NRG1 type I, III and NRG1_PAN had pre-existing Taqman assay available, whereas custom made probes were used for NRG1 type II and IV. HRG = heregulins; SMDF = sensory and motor neuron derived factor; GGF = glial growth factor.

3.2.2.1. mRNA Expression differences by diagnosis

Tests were performed to study the effect of disease diagnosis on the expression of different NRG1 transcripts. No significant effect of diagnosis (all $p>0.45$), diagnosis x gender (all $p>0.29$) or diagnosis x hemisphere (all $p>0.30$), was seen for any of the NRG1 transcripts. Results were contrary to previous reports (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Parlapani E, *et al.* 2010).

3.2.2.2. Expression differences by genotype

In the absence of any significant effects of diagnosis on mRNA expression, tests were performed to study the effect of the specific HAPICE risk haplotype on the expression of different NRG1 transcripts. Both 5-SNP and 7-marker versions of the risk haplotype were included in the analysis. A significant effect of the 5-SNP haplotype (ANCOVA $F(2,71) = 3.883$, $p = 0.025$) and 7-marker haplotype (ANCOVA $F(2,71) = 3.15$, $p = 0.048$) on type III isoform expression was seen (Table 3.4). However, while the number of individuals in each group for the 7-marker haplotype conformed to expectations under Hardy-Weinberg equilibrium, the number of individuals with 2 copies of risk was very small (n = 57, 16 and 1 for 0, 1 and 2 copies, respectively) so the genotypes had to be assessed under a dominant

model with one degree of freedom. Effects of 5- and 7-marker HAPICE risk haplotype on expression of other NRG1 isoforms are shown in Table 3.4.

Isoform studied	HAPICE (5-SNP) haplotype	HAPICE (7-marker) haplotype
NRG1 pan probe	F(2,69) = 1.47, p = 0.235	F(1,72) = 0.594, p = 0.443
NRG1 type I	F(2,69) = 0.158, p = 0.854	F(1,70) = 0.00026, p = 0.987
NRG1 type II	F(2,69) = 0.569, p = 0.568	F(1,68) = 0.137, p = 0.711
NRG1 type III	**F(2,71) = 3.883, p = 0.025**	**F(1,72) = 5.747, p = 0.019**
NRG1 type IV	F(2,52) = 0.213, p = 0.808	F(1,53) = 1.8106, p = 0.184

Table 3.4: ANCOVA results to show the effect of HAPICE 5-SNP and 7-marker haplotypes on the expression of the NRG1 isoform. Results indicate that only NRG1 type III isoform expression was related to HAPICE (5-SNP) haplotype and also showed significant association with HAPICE (7-marker) haplotype.

An allele-dose related effect of NRG1 type III expression with genetic risk was established for 5-SNP haplotype (Figure 3.7). This effect was found in both schizophrenia cases and unaffected controls. Results were contrary to Nicodemus *et al* (2009) finding, which reported an increase in NRG1 type I transcript.

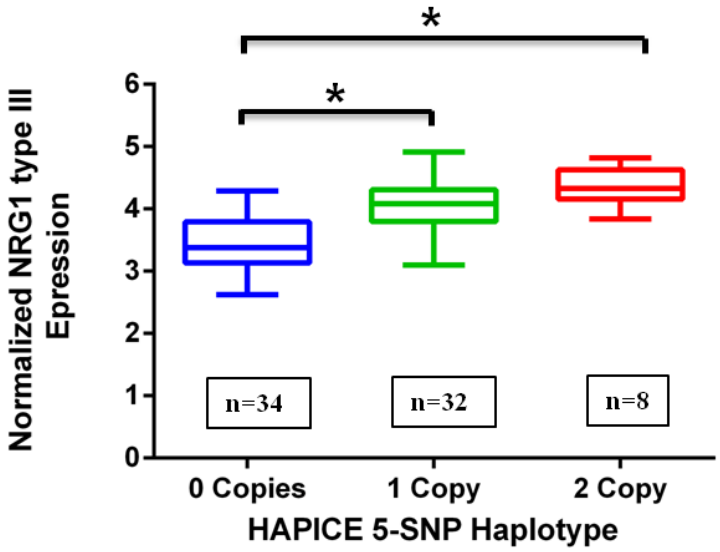


Figure 3.7: The effect of the HAPICE 5-SNP haplotype on NRG1 type III isoform expression. The HAPICE 5 SNP risk haplotype GCGTG has a significant effect on type III isoform expression (ANCOVA F(2,69) = 4.434, p = 0.015; with RIN and PMI as co-variates; Post-hoc Fisher -LSD: p = 0.0148 or 0.0207 (for 1 or 2 copies compared with 0 copies)) with mean expression values (±s.e.) for individuals (n) with 0 copies (n = 34), 1 copy (n = 32) or 2 copies (n = 8) were 3.442±0.167, 4.070±0.172, 4.278±0.347, respectively. Error bars represent standard error.

Furthermore, four out of the five HAPICE SNPs individually showed significant or indicative effects on type III isoform expression: SNP8NRG221533 ($F(2,70) = 3.51$, $p = 0.035$); SNP8NRG241930 ($F(2,68) = 4.14$, $p = 0.02$); SNP8NRG243177 ($F(2,71) = 4.418$, $p = 0.016$) and SNP8NRG221132 ($F(2,72) = 3.48$, $p = 0.066$). There were no interactions involving genotype × diagnosis (p-values all >0.58). These results further support my haplotype findings, although they do not indicate if any of the directly genotyped SNPs are causing the effect or whether SNPs in LD with those genotyped are driving increased expression of type III NRG1.

3.2.2.3. Demographic correlations

Demographic factors that were included to test their impact on expression of different NRG1 transcripts were as follows: Brain pH, RNA integrity (RIN), post mortem interval (PMI) and age of individuals. Significant correlations with brain pH and RIN values were observed for pan NRG1, NRG1 type II and NRG1 type III, and significant correlations with PMI were observed for pan-NRG1 and NRG1 type I (Table 3.5). In addition, a significant correlation with age was detected for pan-NRG1 mRNA. All significant demographic variables were included as covariates, in ANCOVA tests when statistical significance was determined.

NRG1 isoform studied	Demographic factors	r(X,Y)	r^2	t	*p* (uncorrected)	N
Pan NRG1	Age	-0.23	0.05	-2.02	**0.047**	74
Pan NRG1	pH	0.32	0.10	2.85	**0.006**	74
NRG1 type II	pH	0.43	0.19	4.08	**0.000**	74
NRG1 type III	pH	-0.33	0.11	-2.93	**0.005**	70
Pan NRG1	PMI	0.41	0.16	3.77	**0.000**	74
NRG1 type I	PMI	-0.27	0.07	-2.30	**0.024**	72
Pan NRG1	RIN	0.39	0.16	3.64	**0.001**	74
NRG1 type II	RIN	0.29	0.09	2.61	**0.011**	74
NRG1 type III	RIN	-0.25	0.06	-2.16	**0.034**	70

Table 3.5: Demographic factors that were significantly associated with NRG1 transcript expression levels. N = no. of individuals; Age = age at death; pH = brain pH; PMI = post-mortem interval; RIN = RNA integrity

3.2.2.4. Clinical correlations

Clinical factors that were studied for their effect on expression of different NRG1 transcripts were as follows: age of disease onset, habit of smoking, duration of illness, history of anti-depressant, any use of daily chlorpromazine (antipsychotic), last chlorpromazine and life-time chlorpromazine dose levels. Results showed a significant correlation between age of disease onset and NRG1 type III isoform mRNA levels (r = −0.334, p = 0.043) signifying elevated expression of NRG1 type III transcripts is linked to an earlier age of onset of schizophrenia in the TRC cohort (Figure 3.8). This is consistent with the direction of effect of the HAPICE haplotype, which is associated with increased NRG1 type III expression. No significant correlation was observed between any of the clinical factors with other NRG1 transcript levels (all p>0.14).

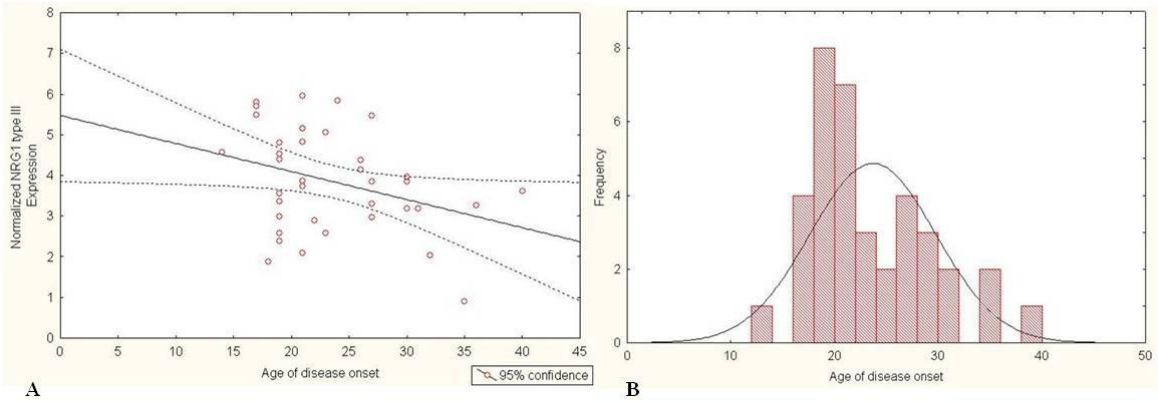


Figure 3.8: Age of onset at first diagnosis in post-mortem brain cohort. (A) Correlation of age of onset with normalized NRG1 type III expression. NRG1 type III expression is presented on the *y* axis as the standardized residual from the regression equation after accounting for the significant demographic effects of brain pH and post-mortem interval (PMI) on expression. The linear regression line showing a significant correlation (r = −0.334, p = 0.043) is shown (black line) along with the mean 95% confidence intervals (black curved lines). (B) Frequency plot of age distribution in the sample, with age of onset on the *x* axis and frequency on the *y* axis.

3.3. Discussion

The overall goal of this chapter was to investigate how variations in *NRG1* at the genetic level and the expression of its different isoforms at the mRNA level, could confer risk to mental illness. At the genetic level, I observed an increased novel variant load in people suffering from schizophrenia, mainly in the HAPICE LD region. I also found high

nucleotide diversity in 5' upstream regulatory regions of NRG1, which was higher than the average values found in intronic regions of other genes. At the mRNA level, I found a significant haplotype effect of the HAPICE 5-SNP and 7-marker risk haplotypes on the level of membrane bound NRG1 type III mRNA expression. A significant correlation between NRG1 type III mRNA expression and age of disease onset suggest HAPICE risk haplotype contributes to increased NRG1 type III expression levels, which to some extent may lead towards an earlier age of disease onset.

NRG1 was identified as a risk gene for schizophrenia after its core risk haplotype (HAPICE), was found to be associated with the disease in the originally studied Icelandic population (Stefansson H, *et al.* 2002). Since the original finding, many studies have established HAPICE, or more generally the 5' region of *NRG1* as conferring risk for mental illness (Corvin A P, *et al.* 2004; Fukui N, *et al.* 2006; Georgieva L, *et al.* 2008; Green E K, *et al.* 2005; Stefansson H, *et al.* 2003; Tang J X, *et al.* 2004; Thomson P A, *et al.* 2007; Williams N M, *et al.* 2003b; Yang J Z, *et al.* 2003; Zhao X, *et al.* 2004). However, the actual mechanism by which this risk haplotype and region conveys vulnerability to mental illness remained unidentified. Results from this study have indicated high nucleotide diversity in 5' upstream regulatory regions of *NRG1*. This nucleotide diversity was higher than the average values found in intronic regions of BDNF (another susceptibility gene for schizophrenia) (Licinio J, *et al.* 2009) and other human genes (Halushka M K, *et al.* 1999). My study, did not however study the whole *NRG1* gene or its complete intronic sequences, and the calculations are based on the analysis of selected regulatory and upstream regions.

Since nucleotide diversity is a measure of genetic variation, the presence of high nucleotide diversity in regions of *NRG1*, suggests that this gene is relatively unstable and more prone to genetic variation. An elevated novel variant load was observed in schizophrenia cases, when compared to unaffected controls, principally in HAPICE LD region. Although, this elevated variant load was not linked to the precise risk haplotype, this region appears to be a site for genetic alterations, consistent with it being a region which confers disease risk. A majority of novel variations identified in this study were bioinformatically predicted to have a functional role (by disrupting transcription factor binding sites), and EMSA

experiments showed that 50% of bioinformatically predicted SNPs (2/5) could actually have a functional role. Genetic *de novo* mutations have been linked to mental illness before (Karayiorgou M, *et al.* 1995; Malhotra D, *et al.* 2011; Xu B, *et al.* 2008) and the applications of advanced technical approaches, like exome sequencing, support *de novo* mutational model for schizophrenia (Xu B, *et al.* 2011).

My study involved working with post-mortem brain samples (n = 74; 37 schizophrenia cases, 37 unaffected controls) and as a result the sample size was a lot smaller, when compared to HAPICE discovery study (478 schizophrenia cases, 394 unaffected controls) (Stefansson H, *et al.* 2002). However, a similar allele frequency difference was observed in the 5-SNP HAPICE risk haplotype, between cases and controls, in the TRC cohort and the Icelandic population. Hence, assuming the TRC cohort is representative of the Australian population at large; it seems that the HAPICE haplotype in Australian subjects may carry similar vulnerability factors as are carried in the Icelandic population, which increase risk to schizophrenia.

Previous post-mortem brain studies of NRG1 have been conducted in cohorts of mixed ethnicity, largely involving African American samples, with no ethnic matching to explain variations in genetic diversity across the populations (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009). The post-mortem brain cohort study in this chapter provides data from one of the larger ethnically matched Caucasian post-mortem brain studies world-wide, going beyond the Caucasian sample numbers from other previous post-mortem brain studies. For example, sample numbers in previous studies which were the benchmarks for NRG1 post-mortem brain research prior to my study were: n = 8 (5 schizophrenia cases and 3 unaffected controls) (Hashimoto R, *et al.* 2004) and n = 45 (20 schizophrenia cases and 25 unaffected controls) (Law A J, *et al.* 2006; Nicodemus K K, *et al.* 2009).

Previous studies have shown that expression levels of NRG1 type I, II and III are altered in PFC and hippocampus of human brain (Hashimoto R, *et al.* 2004; Law A J, *et al.* 2006; Parlapani E, *et al.* 2010). An increase in NRG1 type I expression was reported in DLPFC

(Hashimoto R, *et al.* 2004) and this finding was replicated in hippocampus (Law A J, *et al.* 2006). While a study conducted in Caucasian based cohort with anterior PFC found a significant decrease in NRG1 type I and significant increase in NRG1 type II (Parlapani E, *et al.* 2010).

A later finding reported association of NRG1 type IV isoform with the HAPICE risk haplotype (Law A J, *et al.* 2006). NRG1 type IV is specific to human brain and encodes a full length transcript of NRG1 and the promoter that drives its expression lies within the HAPICE (Tan W, *et al.* 2007), hence was considered to be the mechanism through which *NRG1* exerted its effect in increasing risk to schizophrenia. However, the results for this transcript were not able to be accurately quantified in my study due to extremely low abundance of type IV transcript in the TRC DLPFC. Low abundance of NRG1 type IV has also been reported by Liu et al (2011) in both humans and rats. In addition, Moon et al (2011) study reported a low expression of NRG1 type IV in DLPFC of patients suffering from major depressive disorder (MDD). A highlight of my study is the significant association observed between the HAPICE risk haplotype and increased level of membrane bound NRG1 type III mRNA expression. Type III NRG1 has a Intracellular Cystein Rich Domain (NRG1-ICD) that releases from plasma membrane after proteolysis by γ-secretase complex and translocated into the nucleus where it could enhance promoter activity of certain genes to cause change in gene expression.

Another major finding of Law *et al* (2006) was association of single SNP SNP8NRG22113 (from the HAPICE region), with NRG1 type I expression. My study did not support the conclusion of Law *et al* (2006) but rather showed suggestive association of the same SNP and significant association of three other HAPICE SNPs (SNP8NRG241930, SNP8NRG221533 and SNP8NRG243177) with NRG1 type III expression. Interestingly, SNP8NRG243177 maps to the upstream region of the NRG1 type IV promoter and has been previously associated with NRG1 type IV transcript upregulation (Law A J, *et al.* 2006). This SNP lies approximately 1.2 Kb upstream of the type IV promoter, so the mechanism by which this SNP might mediate expression of type III expression may be through a long range enhancer. Long range enhancers in other genes have previously been

shown to effect gene transcription over 1 Mb away (Maston G A, *et al.* 2006). My results deviate from the finding of Hashimoto *et al* (2004) and Parlapani *et al* (2010) who reported no effect of SNP8NRG221533 on NRG1 isoforms in PFC, although these studies were in smaller cohorts of human brains.

The exact mechanism defining how dysregulation of the NRG1 isoforms would lead to increased disease risk is not yet known, although the results of my studies implicate that increased NRG1 type-III may play a critical role. NRG1 types I, II, IV-VI are directly subjected to proteolyses and participate in short-distance or paracrine signaling (Mei L, Xiong W-C 2008; Shamir A, Buonanno A 2010). Whereas, Type III NRG1, as a result of its exclusive N-terminal transmembrane domain, remains attached to the membrane (Figure 3.1) and is predominantly involved in contact dependent or juxtacrine signaling (Bao J, *et al.* 2004; Bao J, *et al.* 2003; Hancock M L, *et al.* 2008; Wang J Y, *et al.* 2001). Transgenic mice studies have reported that dysregulation in expression of NRG1 isoforms could cause defects in working memory and hippocampal oscillations (Deakin I H, *et al.* 2012), both of which are directly linked to mental illness (Chepenik L G, *et al.* 2012; Glahn D C, *et al.* 2006; Herold C J, *et al.* 2013; Zilles D, *et al.* 2010). The increased NRG1 type III expression seen in this study was also found to be associated with an earlier age of onset; a clinically relevant finding. This suggests that individuals with HAPICE risk haplotype have elevated NRG1 type III expression levels, which contribute to increased disease risk and an earlier age of onset.

In conclusion, the results from this chapter have contributed in understanding the manner in which the HAPICE haplotype could be contributing towards increased disease risk in mental illness.

Chapter 4

How is the *NCAM1* gene, mRNA or protein altered in psychotic mental illness?

4.1. Introduction

NCAM1 plays a key role in neurodevelopment because of its effect on brain growth (Ronn L C, *et al.* 1998), neuronal plasticity (Chipman P H, *et al.* 2010; Gray L J, *et al.* 2010; Kiss J Z, Muller D 2001) and neurogenesis (Vawter M P 2000). Because of this, NCAM1 has been proposed as a candidate gene for mental illness. Genetically, the polymorphisms in *NCAM1* have been associated with both bipolar disorder and schizophrenia (Arai M, *et al.* 2004; Atz M E, *et al.* 2007) and a recent comparative functional genomics study has classified *NCAM1* as one of the main candidate genes for schizophrenia (Ayalew M, *et al.* 2012).

Three of NCAM's major isoforms are NCAM-180, NCAM-140 and NCAM-120 identified by Cunningham *et al* (1987). Structurally these three isoforms contain 5 immunoglobulin (Ig) domains and 2 fibronectin III (Fn-III) repeats (Atkins A R, *et al.* 2004; Soroka V, *et al.* 2008). NCAM-180 and NCAM-140 are the long and short transmembrane isoforms, respectively. NCAM-120 lacks the transmembrane domain (TMD) and the intracellular domain (ICD) and is linked to the membrane with glycosylphosphphatidylinositol-linked (GPI) anchor (Brennaman L H, *et al.* 2011) (Figure 1.7). The 5^{th} Ig domain of NCAM1 is involved in interactions with polySTs (ST8SIA2 and ST8SIA4) (Thompson M G, *et al.* 2013) and the first Fn-III repeat includes a unique acidic patch (comprising of Asp^{497}, Asp^{511}, Glu^{512}, Glu^{514}) and α-helix that are involved in structural modification and polyST recognition (Mendiratta S S, *et al.* 2006; Mendiratta S S, *et al.* 2005), thus playing a crucial role in PSA-NCAM formation, post-translationally (Close B E, *et al.* 2003). NCAM-140 and NCAM-180 are the main glycosylatable forms, whereas NCAM-120 remains PSA free (Hildebrandt H, *et al.* 2010; Oltmann-Norden I, *et al.* 2008). Different NCAM isoforms exhibit age-dependent expression (Oltmann-Norden I, *et al.* 2008) and their irregular expression has been linked to the mental illness (Gibbons A S, *et al.* 2009; Gray L J, *et al.* 2010; Tanaka Y, *et al.* 2007; Vawter M P, *et al.* 1999). This study is aimed to analyze if there is any dysregulation of different NCAM isoforms and if this effect is specific to disease status.

Studies have reported a proteolytic cleavage product of NCAM-180 (c-NCAM), in the PFC and hippocampus (Cox E T, *et al.* 2009; Vawter M P, *et al.* 2001). NCAM1 also utilizes additional exons in its functional execution; these exons include the Variable Alternative Spliced Exon (VASE) and the Secreted Exon (SEC). While, the VASE represents a 10 amino acid sequence included in the fourth Ig domain of NCAM1 between exon 7 and 8 (Reyes A A, *et al.* 1991; Small S J, Akeson R 1990; Vawter M P, *et al.* 2000), SEC is located between the exons 12 and 13 (Atz M E, *et al.* 2007). In addition, three small-exons denoted as the exon a, b and c, are also located between exon 12 and 13 (Arai M, *et al.* 2004; Barthels D, *et al.* 1992; Hamshere M, *et al.* 1991). Both VASE and SEC isoforms are reported to be increased in the people suffering from mental illness (Vawter M P, *et al.* 2000; Vawter M P, *et al.* 1998b; Vawter M P, *et al.* 1999). Findings also suggest a relationship between the VASE expression and decreased brain plasticity (Vawter M P 2000). Hence, in my thesis studies, I aimed to analyse cleavage products of NCAM.

NCAM1 serves as the principal substrate for ST8SIA2, which is also a potential vulnerability gene for mental illness (McAuley E Z, *et al.* 2012) and is the subject of studies in Chapter 5 of this thesis. Due to the inter-relationship between these two potential risk genes, I wanted to determine whether *NCAM1* gene is also associated with disease risk in the same population, considering NCAM1 protein acts as principle substrate for ST8SIA2 enzyme. Therefore, in this study, I have used ABCC cohort to study genetic variations in the *NCAM1* gene structure.

Irregularities in the expression of NCAM1 isoforms at mRNA and protein level have been connected to mental illness (Gibbons A S, *et al.* 2009; Gray L J, *et al.* 2010; Vawter M P, *et al.* 2001), but in previous studies, mRNA expression and protein expression studies have not been conducted in human post-mortem brain tissue from the same cohort, resulting in missed opportunities to understand the complete picture. It is possible that irregularities which occur at mRNA level are not translated, perhaps due to mRNA decay (Yang E, *et al.* 2003), or that irregularities occur after transcription and pass on further in the form of abnormal expression in their protein levels or generation of cleavage products. For example, the disease associated changes like decreased PSA-NCAM level previously

reported (Barbeau D, *et al.* 1995; Gilabert-Juan J, *et al.* 2012) could be because of decreased expression of the glycosylatable forms of NCAM1, or decreased expression or functionality of ST8SIA2.

Therefore, in this study, I aimed to genotype variations present in the genetic structure of *NCAM1*, as well as to identify if there are any novel variations present in this gene through resequencing studies. Working with the TRC post-mortem brain cohort, I am aiming to study the expression pattern of different NCAM1 isoforms at both mRNA and protein levels, together with PSA-NCAM protein levels, to investigate if there is any variation in their expression in psychotic mental illness.

4.2. Results

4.2.1. Genetic variation

Evidence for NCAM1 genetic association:

Taqman SNP genotyping was performed to study known *NCAM1* gene polymorphisms in ABCC cohort. For this study, genotyping was performed on a limited number of SNPs that were prioritized across the entire *NCAM1* gene (Chapter 2; Table 2.8). Genotype data for the *NCAM1* gene was obtained from the HapMap project for Caucasian and Japanese populations (www.hapmap.org), and linkage disequilibrium structure was studied to select SNPs for genotyping (Figure 4.1).

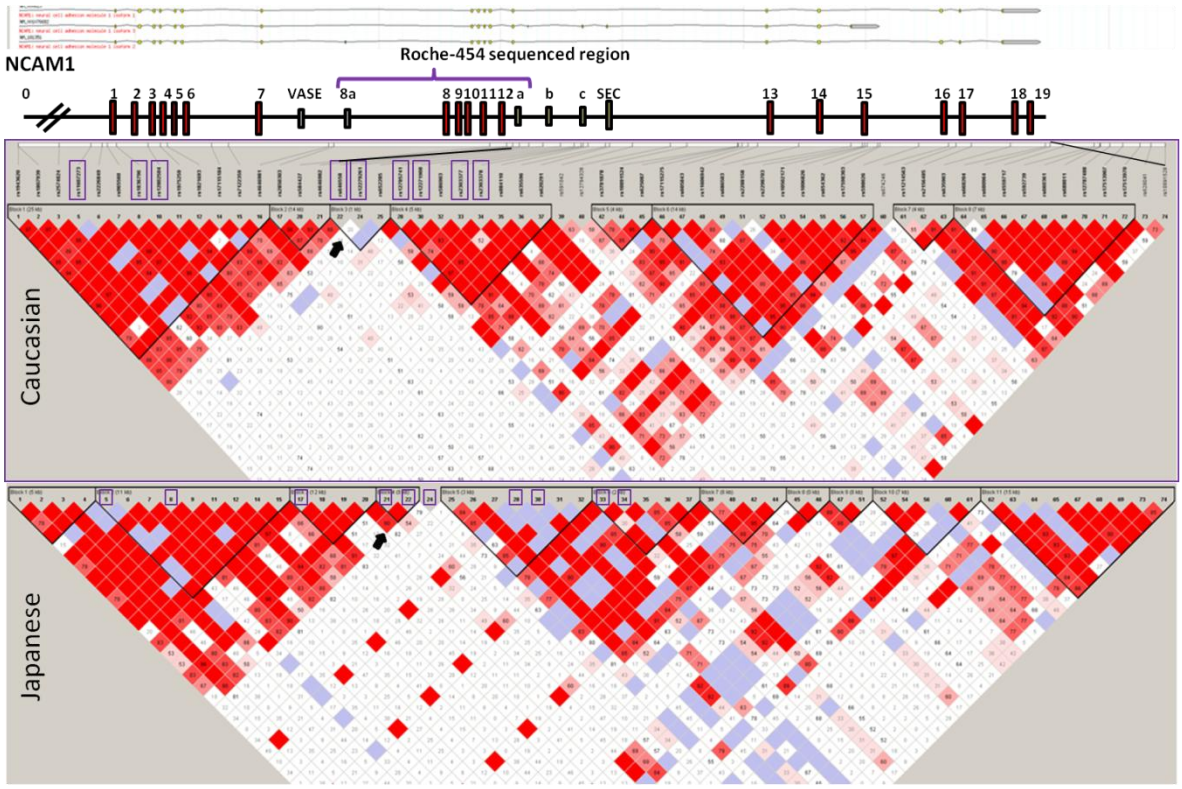


Figure 4.1: Differential linkage distribution of SNPs across *NCAM1* gene in Caucasian and Japanese population. Figure was plotted on Haploview based on HAPMAP-2 data information. Linkage disequilibrium blocks were defined by the solid spine of LD method (D'>0.8). Purple boxes represent SNPs that were chosen for Taqman genotyping. Bold arrow represent most significant SNP rs646558 in Arai *et al* study showing different LD pattern in Caucasian population. Region targeted for Roche 454 resequencing in the next step, is also shown.

Thirteen SNPs were prioritized for genotyping because they were tagged SNPs (r^2 threshold = 0.8), whereas SNP rs12574998 was selected because it was significantly associated in the Arai *et al* (2004) study. In total, the 14 SNPs were selected, which incorporated 4 SNPs that were previously reported to be associated with mental illness (Figure 4.2).

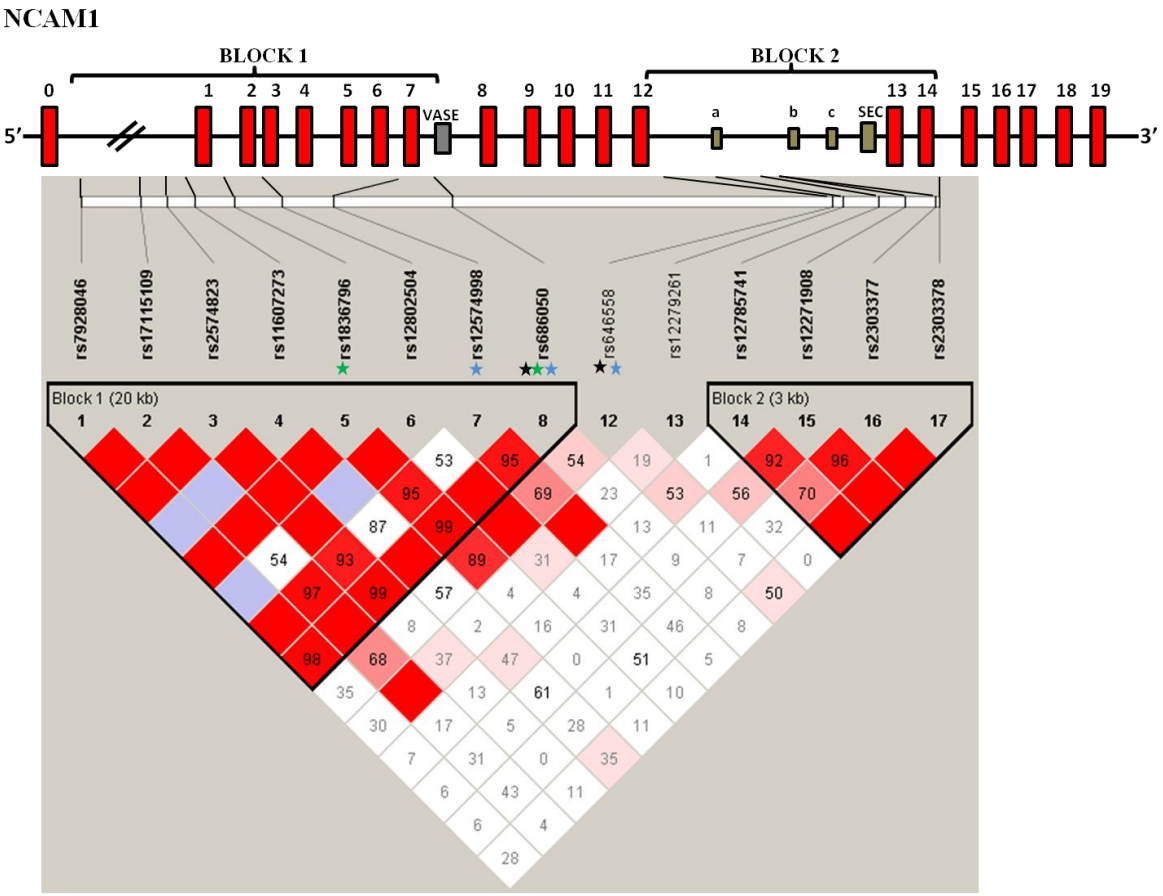


Figure 4.2: Linkage disequilibrium structure and relative positions of genotyped *NCAM1* SNPs in Australian bipolar disorder cohort. Two linkage disequilibrium blocks as defined by the solid spine of LD method (D'>0.8) were identified, with the frequencies of the main haplotypes (>5%) shown. SNPs which were previously reported to be associated with mental illness are marked with black asterisks for Atz *et al* (2007); blue asterisks for Arai *et al.*, (2004) and green asterisks for Sullivan *et al* (2007) studies.

Taqman genotype cluster plots for all SNPs showed good separation between the homozygote and heterozygote genotype groups (for example cluster plot, see Chapter 2; Figure 2.2). The genotype distributions of all SNPs conformed to expectations under Hardy-Weinberg equilibrium. Tests for allelic, genotypic and haplotypic association were conducted using PLINK with the *--assoc, --model* and *--hap-assoc* commands. For SNPs with minor allele frequency (MAF) < 0.2, genotype groups were collapsed into carriers of the minor allele versus major allele homozygotes before testing for association via chi-square test. The allelic and genotypic association analyses were conducted by chi-square, and a summary of the results (353 bipolar disorder cases, 187 controls) is presented in Table 4.1.

SNPs	BP position	Allele	n	MAF (BP)	MAF (CON)	χ2 (allelic)	*p* value (allelic)	OR (95%CI)	χ2 (geno)	df	*p* (geno)
rs7928046	113065024	G/A	529	0.136	0.133	0.013	0.909	1.02 (0.70-1.48)	0.372	1	0.847
rs17115109	113068301	A/G	534	0.115	0.093	1.167	0.28	1.26 (0.83-1.93)	1.52	1	0.218
rs2574823	113069713	C/G	534	0.484	0.489	0.025	0.874	0.98 (0.76-1.26)	1.964	2	0.375
rs11607273	113071249	A/G	529	0.084	0.088	0.057	0.811	0.95 (0.60-1.49)	0.125	1	0.724
rs1836796	113073360	T/G	530	0.403	0.385	0.322	0.571	1.08 (0.83-1.40)	7.39	2	**0.025**
rs12802504	113075938	T/C	534	0.064	0.041	2.354	0.125	1.59 (0.87-2.91)	2.58	1	0.108
rs12574998	113078756	G/A	530	0.115	0.097	0.755	0.385	1.21 (0.79-1.83)	1.01	1	0.314
rs686050	113085244	G/C	531	0.371	0.392	0.472	0.492	0.91 (0.70-1.19)	5.146	2	**0.076**
rs646558	113105907	A/C	532	0.203	0.249	2.958	**0.085**	0.77 (0.57-1.04)	3.454	2	0.178
rs12279261	113106455	G/A	530	0.155	0.159	0.034	0.853	0.97 (0.68-1.37)	0.595	1	0.807
rs12785741	113108387	C/G	532	0.363	0.319	2.096	0.148	1.22 (0.93-1.60)	4.744	2	**0.093**
rs12271908	113109836	T/C	534	0.225	0.232	0.086	0.769	0.96 (0.71-1.29)	0.105	2	0.949
rs2303377	113111501	C/T	534	0.391	0.361	0.953	0.329	1.14 (0.88-1.48)	0.939	2	0.626
rs2303378	113111665	A/G	533	0.283	0.275	0.073	0.786	1.04 (0.78-1.38)	0.092	2	0.955

Table 4.1: Association results for genotyped 14 *NCAM1* SNPs. For each SNP marker, the base pair position (NCBI build 36, hg19) and alternative alleles are given, with the minor allele listed first. The minor allele frequency (MAF) is listed for bipolar disorder (BP) and control (CON) groups (n = 353 and 187 respectively). Allelic and genotypic (geno) association tests were performed by chi-square (χ^2), and the p value and odds ratios (OR) with 95% confidence intervals are given. Two degrees of freedom were used for all genotypic tests, with the exception of SNPs with MAF < 0.2 which were conducted under a dominant model with one degree of freedom. Nominally significant and suggestive p values prior to multiple testing corrections are indicated in bold text.

While no SNPs showed significant allelic or genotypic association after multiple testing correction, nominal association was observed at the genotypic level for rs1836796 (χ^2 = 7.39; p = 0.025), and a trend effect was observed for rs68650 (χ^2 = 5.15; p = 0.076) and rs12785741 (χ^2 = 4.74; p = 0.093). A trend effect was also observed for rs646558 at the allelic level (χ^2 = 2.96; p = 0.085). Interestingly, these three SNPs have shown association with mental illness and cognition in other published studies (Arai M, *et al.* 2004; Atz M E, *et al.* 2007; Sullivan P F, *et al.* 2007).

Linkage disequilibrium (LD) block structures between genotyped SNPs were determined using the solid spine of LD method in HAPLOVIEW (Figure 4.2). On comparing the entire LD structure of *NCAM1* gene in the Australian Caucasian population (Figure 4.2) to the

HAPMAP Caucasian and Japanese populations (Figure 4.1), very similar patterns were observed between the two Caucasian samples and also across to the Japanese samples, with the exception of differential strengths of LD around rs646558. This was the most significant SNP in a Japanese bipolar disorder cohort in Arai *et al* (2004) study, and is immediately adjacent to the LD block in which two of my genotyped SNPs (rs1836796 and rs686050) show suggestive association, indicating that the LD block spanning exons 1-7 may harbor variations which marginally increase risk to mental illness.

To look for gender specific effects, NCAM genetic association was also performed including gender as a covariate (Table 4.2). Interestingly, heterogeneity in the odds ratios of associated alleles with gender was observed at rs646558, where the major C allele is over-represented in male cases (χ^2=6.63, p=0.01, OR=1.78(95%CI=1.14-2.77)) with no effect of either allele in females (χ^2=0.036, p=0.85, OR=0.96(95%CI=0.62-1.48)). Additional gender specific effects were also observed at rs17115109 and rs12574998, both of which seem to be driven by an increase in frequency of the minor allele in female cases (χ^2=4.82, p=0.028, OR=2.17(95%CI=1.07-4.38); and χ^2=4.10, p=0.043, OR=1.99(95%CI=1.01-3.98), respectively) with no effect of either allele in males. This heterogeneity in genetic effects could represent a bonafide difference in risk allele effects across the genders or, as the numbers of males and females in the study were not same, may be due to type II error caused by uneven numbers of subjects with each gender in the analysis. It was particularly interesting that a gender effect was observed with rs646558, given the previously reported evidence of association with this SNP in earlier studies, however those studies did not perform gender specific analyses.

CHR	SNP	BP	A1	MAF	A2	CHISQ	P	OR	SE	CHISQ_BD	P_BD
11	rs7928046	112570234	G	0.13	A	0.03	0.85	1.04	0.19	0.004	0.95
11	rs17115109	112573511	A	0.11	G	1.89	0.17	1.36	0.22	3.31	**0.07**
11	rs2574823	112574923	C	0.45	G	0.02	0.88	0.98	0.13	0.049	0.82
11	rs11607273	112576459	A	0.08	G	0.15	0.691	0.91	0.23	4.4e-05	0.99
11	rs1836796	112578570	T	0.40	G	0.42	0.51	1.09	0.13	1.03	0.31
11	rs12802504	112581148	T	0.05	C	2.36	0.12	1.60	0.31	0.037	0.85
11	rs12574998	112583966	G	0.11	A	1.09	0.29	1.26	0.22	3.57	**0.06**
11	rs686050	112590454	G	0.38	C	0.80	0.37	0.89	0.13	0.36	0.55
11	rs646558	112611117	A	0.22	C	2.75	**0.09**	0.77	0.16	3.85	**0.05**
11	rs12279261	112611665	G	0.16	A	0.06	0.80	0.96	0.18	0.009	0.92
11	rs12785741	112613597	C	0.35	G	1.71	0.19	1.20	0.14	0.078	0.78
11	rs12271908	112615046	T	0.23	C	0.001	0.978	1.00	0.16	2.41	0.12
11	rs2303377	112616711	C	0.38	T	1.67	0.19	1.19	0.14	0.81	0.37
11	rs2303378	112616875	A	0.28	G	5.34e-05	0.99	1.00	0.15	0.20	0.66

Table 4.2: NCAM genetic association data including gender as a covariate. Males (133 cases, 99 controls) and females (217 cases and 89 controls) were included in the analysis. P indicates the p value for overall disease/gene association while controlling for gender, and the P_BD value indicates the p value for heterogeneity of the disease/gene association between the genders.

Resequencing study:

Considering NCAM1's 5th and 6th glycosylation site and acidic patch are involved in enzyme ST8SIA2 recognition and positioning (Foley D A, *et al.* 2009; Thompson M G, *et al.* 2013), this 6.5 kb region (hg19/GRCh37: chr11:113100972-113107571bp) - including exons and introns between exon 8a and exon 12 mini exon-a (Figure 4.1) within *NCAM1* – was considered a region of interest for DNA variation studies. This region was resequenced

to characterize SNPs and to investigate if there are any novel variations that could have a potential functional role. Roche 454 next generation sequencing was performed. Forty-eight samples were selected from the families who contributed to the linkage and association signal with bipolar disorder, in a study performed by our group (McAuley E Z, *et al.* 2009).

After analyzing the sequencing data, I had information about 21 SNPs, including five novel variations. Of the five novel SNPs, one was located in intron 8 (C/G substitution; hg19/GRCh37: at 113102679), while the other four had base-pair insertion or deletions (at 113101620bp, 11310236 bp, 113104997bp and 113105039bp; hg19) (Figure 4.3). Amongst the known SNPs, two (rs117219783 and rs584427) were located in exonic regions. SNP rs117219783 was found to introduce a non-conservative amino acid change (non-polar Glycine to polar Glutamic acid), while the other coding SNP rs584427 was silent (Glycine to Glycine). The position of different SNPs and glycosylation sites are shown in Figure 4.3.

Since only bipolar cases were resequenced in this study, I used Caucasian control data from 1000 genomes (1000genomes.org) to run association analysis. Analysis was performed using PLINK software. Minor allele frequencies for bipolar cases and controls were similar (±5%) with the exception of rs584427, where frequencies were 0.452 and 0.342 for controls and cases, respectively (Table 4.3). Two novel SNPs showed significant allelic association before multiple testing correction, INDEL_113101620 (χ^2 = 5.37; p = 0.02) and INDEL_113104997 (χ^2 = 3.57; p = 0.05). A trend effect was observed for exonic SNP rs584427 (χ^2 = 3.16; p = 0.08). Genotypic associations were not performed for novel SNP variants due to the low minor allele frequency and small cell sizes for genotypic association analysis.

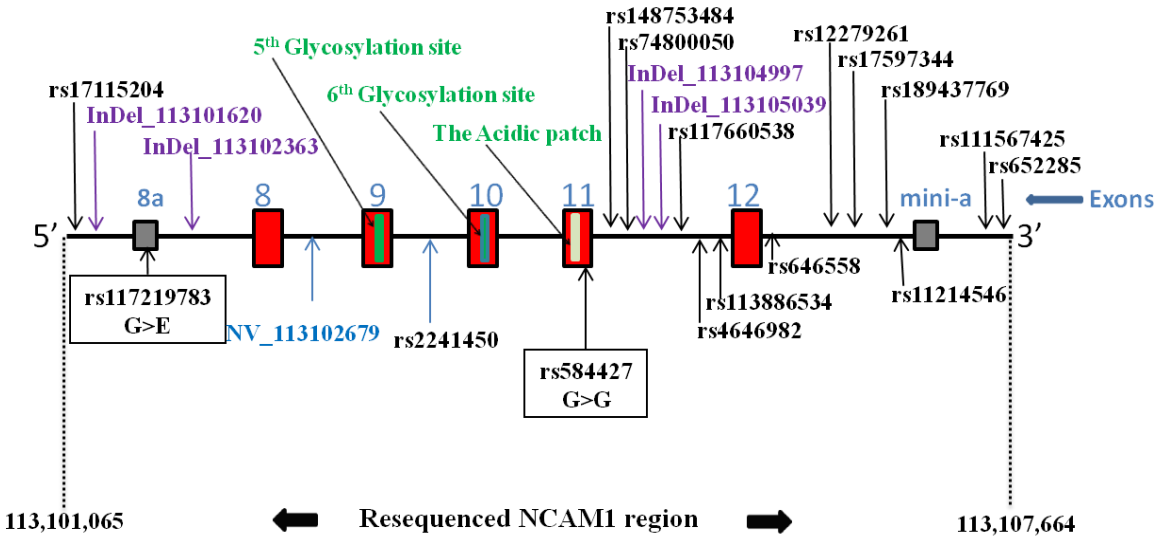


Figure 4.3: Location of *NCAM1* SNPs identified via Roche 454 sequencing. Selected sequencing region included exon 8a-exon 12 mini exon-a, covering the 5th and 6th glycosylation sites as well as the acidic patch in the FN-domain. Figure shows known SNPs as well as novel SNPs identified in this study. Known SNPs are marked with their 'rs numbers' while novel SNPs are listed as NV (novel base substitutions) or InDel (novel insertion-deletion change). Two SNPs that were located in exon 8a and exon 11 are marked with closed boxes and their amino acid change is shown. G represents Glycine (non-polar) and E represents Glutamic acid (acidic polar).

This association analysis should be viewed with caution, as differences in allele frequencies could be due to the difference in genotyping platform and/or sequencing coverage from 1000 Genomes data, which may have affected estimates of genotype frequency. A replication study in a larger cohort using identical genotyping platforms in cases and control subjects is required to confirm the results.

SNPs	BP position	poly	Genotype (BP)	Genotype (CON)	MAF (BP)	MAF (CON)	$\chi 2$ (allelic)	*p* value (allelic)	OR
rs17115204	113101143	C/T	0/1/46	0/1/84	0.01	0.01	0.18	0.67	1.82
INDEL_113101620	113101620	T/-	0/3/45	0/0/85	0.03	0	5.37	**0.02**	NA
rs117219783	113101958	G/A	0/2/45	0/2/83	0.02	0.01	0.37	0.54	1.83
INDEL_113102363	113102363	C/-	0/1/47	0/0/85	0.01	0	1.78	0.18	NA
NV_113102679	113102679	C/G	0/1/46	0/0/85	0.01	0	1.82	0.18	NA
rs2241450	113103259	C/T	0/1/46	0/1/84	0.01	0.01	0.18	0.67	1.82
rs584427	113103996	G/T	6/20/21	13/51/21	0.34	0.45	3.16	**0.08**	0.62
rs148753484	113104198	CACCCCAA/-	0/11/37	0/0/85	0.11	0.088	0.483	0.487	1.337
rs74800050	113104456	C/T	0/3/44	0/3/82	0.03	0.02	0.55	0.46	1.84
INDEL_113104997	113104997	T/-	0/2/46	0/0/85	0.02	0	3.57	**0.058**	NA
INDEL_113105039	113105039	T/-	0/1/47	0/0/85	0.01	0	1.78	0.18	NA
rs117660538	113105158	C/G	0/1/46	0/2/83	0.01	0.01	0.01	0.93	0.9
rs4646982	113105405	T/G	8/14/25	5/41/39	0.32	0.3	0.1	0.75	1.09
rs113886534	113105410	A/G	0/2/45	0/2/83	0.02	0.01	0.37	0.54	1.83
rs646558	113105907	C/A	3/17/27	1/31/53	0.24	0.19	0.93	0.34	1.35
rs12279261	113106455	A/G	0/11/36	3/21/61	0.12	0.16	0.86	0.35	0.7
rs17597344	113106464	G/A	0/4/43	1/09/75	0.04	0.06	0.55	0.46	0.64
rs189437769	113106527	C/T	0/1/46	0/2/83	0.01	0.01	0.01	0.93	0.9
rs11214546	113106528	G/T	0/4/43	1/07/77	0.04	0.05	0.14	0.71	0.8
rs111567425	113107077	G/A	0/1/46	0/6/79	0.01	0.04	1.43	0.23	0.29
rs652285	113107204	G/A	1/07/39	0/9/76	0.1	0.05	1.75	0.19	1.89

Table 4.3: Association results for 21 resequenced *NCAM1* SNPs. For each SNP marker, the base pair position (NCBI build 36, hg19) and alternative alleles are given (poly), with the reference allele listed first. The genotype counts (Genotype) and minor allele frequency (MAF) is listed for bipolar disorder (BP) and control (CON) groups (n = 48 and 85 respectively). Allelic association tests were performed by chi-square (χ^2), and the p value and odds ratios (OR) with 95% confidence intervals are given. Nominally significant and suggestive p values prior to multiple testing corrections are indicated in bold text.

In a bioinformatic study to investigate if any of the associated SNP has a functional role in mRNA splicing or transcription factor binding; only INDEL_113104997 was predicted to

introduce a donor splice site with a score of 0.41, while rs584427 along with INDEL_113104997 was predicted to alter transcription factor binding sites (Table 4.4).

SNP	Allele	Model name	Change type	Allele score
rs584427	Reference	Myf	New Factor	9.229
rs584427	Reference	TAL1::TCF3	New Factor	8.206
rs584427	Alternate	ZNF354C	New Factor	6.916
INDEL113104997	Reference	INSM1	New Factor	8.098
INDEL113101620	Reference	SPIB	New Factor	6.493
INDEL113101620	Alternate	STAT1	New Factor	9.676

Table 4.4: Transcription factor binding results from JASPAR for the 3 *NCAM1* SNPs. All SNPs were tested against all available JASPAR matrix models for Homo Sapiens and significant (with cut-off of allele score 6) SNPs and their models (or transcription factor binding matrix) are presented. Table displays SNP names, whether they are present on reference or alternative allele, their model name, type of change i.e. preference for a new transcription factor and their respective scores. STAT1 is Signal transducer and activator of transcription 1. SPIB is Spi-B transcription factor. INSM1 is Insulinome-associated 1. ZNF354C is Zinc finger protein 354C. TAL1::TCF3 is helix-loop-helix protein TAL1 and its binding partner TCF3. Myf is myogenic factor.

4.2.2. Transcriptional variation

This study was performed to investigate the relative abundance of different NCAM1 mRNA isoforms in the DLPFC of human post-mortem brains. The TRC brain cohort was used in this study. Pre-designed TaqMan gene expression assays (Applied Biosystems) were used to target the NCAM-120, NCAM-140, NCAM-180, NCAM1 (+VASE), and NCAM1 (-VASE) isoforms. Normalization using four housekeeper genes i.e. ubiquitin C, β-actin, glyceraldehyde-3-phosphate dehydrogenase and TATA-binding protein was then performed, by dividing mean of three replicate readings for each NCAM1 mRNA by the geometric mean of the four housekeepers, for each subject. Statistical analysis was performed using the Statistica Software.

First, I determined whether the NCAM1 isoform expression was influenced by the brain cohort demographic variables which may influence the quality of my results. These quantitative variables included the age at death, the time between death and the brain harvesting and its preservation i.e. post-mortem interval (PMI), the quality of RNA after extraction from the brain tissue i.e. RNA integrity (RIN), the brain pH, the age of disease

onset, the duration of illness, the history of anti-depressent, any use of daily chlorpromazine (antipsychotic), last chlorpromazine and life-time chlorpromazine dose levels.

The NCAM1 isoforms that showed significant correlations with the demographic factors are shown in Table 4.5. The brain PMI, illness onset age, and the last chlorpromazine levels did not correlate with any major NCAM1-isoform mRNA measures. Illness duration, daily chlorpromazine and lifetime chlorpromazine levels were found to be significantly and positively correlated with the total NCAM levels. All significant demographic variables were included as the co-variates in the respective ANCOVA analyses.

NCAM1 isoform	**Demographic variables**	**r(X,Y)**	**r^2**	**t**	***p***	**N**
NCAM-120	Age	0.2906	0.0844	2.5044	0.0147	70
NCAM (+VASE)	Age	-0.3475	0.1208	-3.0786	0.0030	71
NCAM-120	pH	-0.3316	0.1099	-2.8982	0.0050	70
NCAM (+VASE)	pH	0.3131	0.0981	2.7389	0.0078	71
NCAM-180	pH	-0.2785	0.0775	-2.3553	0.0215	68
NCAM-140	pH	-0.4567	0.2086	-4.2018	0.0001	69
NCAM-180	RIN	-0.3125	0.0977	-2.6730	0.0095	68
NCAM-140	RIN	-0.4456	0.1986	-4.0747	0.0001	69
NCAM1 isoform	**Clinical variables**	**r(X,Y)**	**r^2**	**t**	***p***	**N**
Total-NCAM	Illness duration	0.3505	0.1228	2.2139	0.0334	37
Total-NCAM	Daily-Chlor	0.3937	0.1550	2.5341	0.0159	37
Total-NCAM	Lifetime-Chlor	0.4778	0.2283	3.2177	0.0028	37

Table 4.5: Table showing the different NCAM1 mRNA isoforms that were significantly correlated with the different demographic and clinical variables. Age = Age at death, pH = brain pH, RIN = RNA integrity; Daily-Chlor and Lifetime-Chlor represents daily and lifetime doses of antipsychotic chlorpromazine.

While assessing the impact of the categorical variables on mRNA transcript expression, I did not observe any significant differences in the expression of any mRNA isoform when testing against diagnosis, brain hemisphere or gender alone (all Student's t-test $p > 0.05$). However, a suggestive increase in NCAM-180 mRNA expression was observed in the right hemisphere ($t = ^{-}1.789$, $p = 0.078$, $df = 66$).

While studying the effect of diagnosis with categorical variables, a diagnosis × hemisphere effect on the NCAM-180 mRNA expression was observed [ANCOVA $F(1, 62) = 4.795$, p

= 0.0323; co-varying for pH and RIN]. The effect was a significant 12.75% increase in the NCAM-180 mRNA expression (Figure 4.4) in the right hemisphere of schizophrenia cases, compared to the right hemisphere of controls (Fishers LSD p = 0.0047), and a 13.7% increase compared to the left hemisphere of schizophrenia cases (Fishers LSD p = 0.0019).

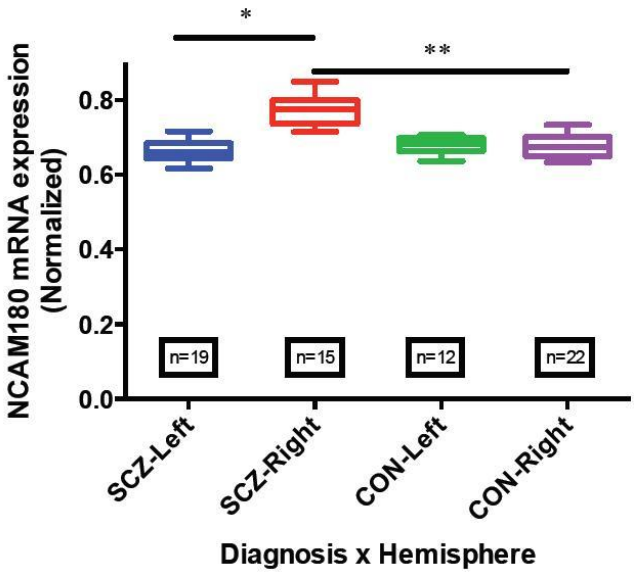


Figure 4.4: Increased expression of NCAM1-180 mRNA in the right hemisphere of DLPFC in schizophrenia. A significant diagnosis x hemisphere effect on NCAM180 mRNA expression [ANCOVA F (1, 62) = 4.795, p = 0.0323; co-varying for pH and RIN], was observed. * (p≤0.001); SCZ = schizophrenia cases, CON = unaffected control individuals; Right and Left = brain right and left hemisphere, respectively. Error bars represent standard error.

A suggestive diagnosis × gender effect on the NCAM-120 mRNA was also observed [ANCOVA F (1, 63) = 2.937, p = 0.0915; co-varying for pH and age], however the post-hoc Fisher LSD tests were not significant.

4.2.3. Protein variation

This study was performed to explore the relative abundance of three major NCAM1 protein isoforms and its post-translational modification in the DLPFC of human post-mortem brains. The TRC brain cohort was used for this study. The linear range for quantification for the PSA-NCAM and NCAM1 protein isoforms was determined from a six point standard curve (range: 0-40 µg for PSA-NCAM; 0-4 µg for NCAM1 isoforms). The

amount of protein considered optimal for quantification was 1.5 µg for all the NCAM1 isoforms (Figure 4.5) and 20 µg for the PSA-NCAM (Figure 4.6).

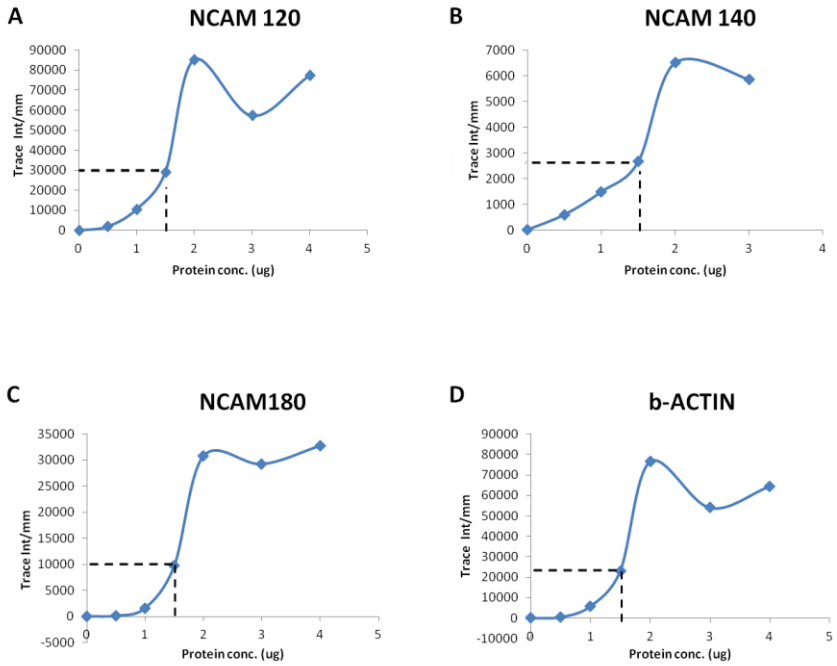


Figure 4.5: Quantification curves for three NCAM1 isoforms and β-actin western blots. The linear range of quantification was found to be around 1.5 µg from a 6 point standard curve (range 0-4ug protein loaded) for: A) NCAM-120; B) NCAM-140; C) NCAM-180; D) b-actin.

Considering the NCAM1 protein expression was very high and the PSA-NCAM protein expression was very low in the DLPFC; problems were encountered while accurately normalizing the raw data, particularly for assessing the ratio of PSA-NCAM to NCAM isoforms. The linear range for NCAM1 protein expression (1.5 µg) was just at the detection range of the β-actin protein (Figure 4.5D) but the linear range for PSA-NCAM (20 µg) was beyond the saturation limit of the β-actin protein (Figure 4.6B).

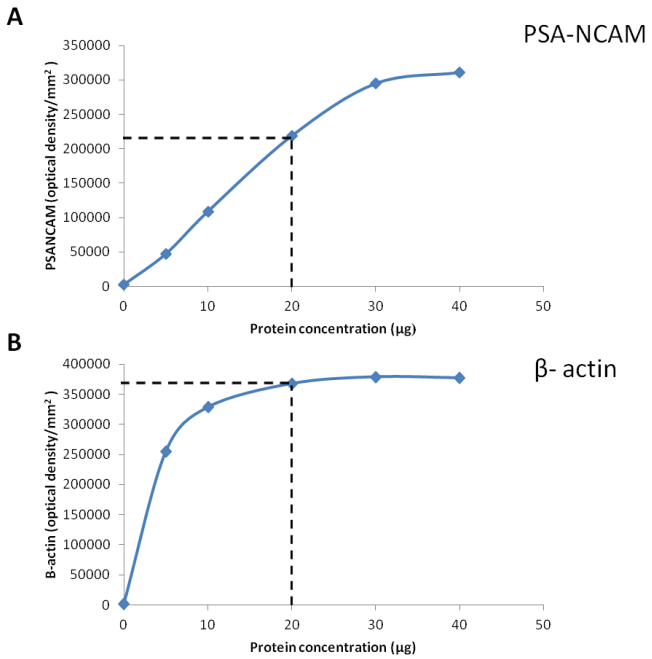


Figure 4.6: Quantification curves for PSA-NCAM and β-actin western blots. The linear range of quantification for PSA-NCAM was found to be around 20 µg from a 6 point standard curve (range 0-40 µg protein loaded) for A) PSA-NCAM and B) β-actin. The quantification of β-actin was not in the linear range at 20 µg (attaining saturation).

Therefore, the β-actin was deemed an unreliable housekeeper for normalization of PSA-NCAM protein. I tried using another housekeeper, α-tubulin, but its expression was weaker than the β-actin at optimum NCAM1 concentration levels (1.5 µg; Figure 4.7) and expression was again attaining saturation when higher levels of protein (20 µg) were loaded per well. Thus, α-tubulin was not an option to replace β-actin as a housekeeper, to solve the problem of very different housekeeper expression relative to target proteins. So rather than normalizing to β-actin, I conducted my experiments in a matched-pair design, such that matched case and control brains (matched for age, brain pH, RIN and RNA yield) were represented on the same gel (described in Chapter 2). I then analysed the expression relative to the average values of the four controls on each gel, which was expressed as a percentage (%control). For NCAM1, expression of each individual protein isoform was also calculated relative to the total NCAM1 detected.

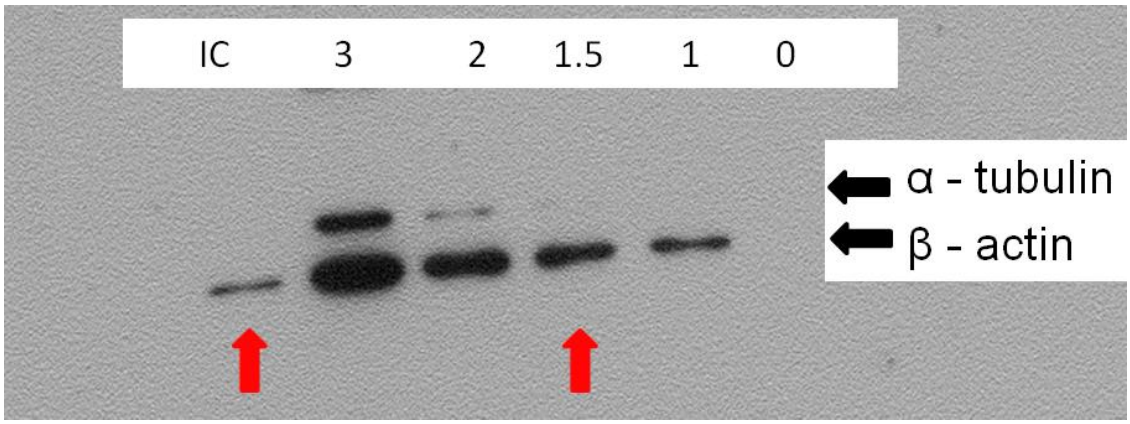


Figure 4.7: A comparison between two commonly used housekeepers i.e. β-actin and α-tubulin. The expression of two housekeepers is shown along the concentration range of 0, 1, 1.5, 2, 3 µg protein. IC represents internal control which is 1.5 µg. Figure shows that α-tubulin expression is weaker than that of β-actin at the desired concentration range of 1.5 µg (shown by two red arrows). Thus, proving that α-tubulin is not an option to replace β-actin in my study, to solve the problem of low housekeeper expression.

Using western blotting (Chapter 2; Section 2.7) and an N-terminus antibody (Chapter 2; Table 2.10), I identified the three major NCAM1 protein isoforms of 120kDa, 140kDa and 180kDa; plus additional minor isoforms at approximately 160kDa and 170kDa (Figure 4.8). As the three major isoforms are also known as NCAM-A, NCAM-B, and NCAM-C, respectively (Kasper C, *et al.* 2000), I designated the ~160-170 kDa isoforms as NCAM-D. Although, the 160-170 kDa bands were visible as a doublet, the resolution of individual bands not sufficient to enable accurate quantification of both bands independently, and hence the 160-170kDa bands were quantified as a single entity.

In the western blots examining PSA-NCAM (Figure 4.8) using mouse monoclonal antibody against PSA-NCAM (Chapter 2; Table 2.10), the polysialylated protein was identified as a smear from approximately 250kDa to 150kDa, rather than a discrete band. This was expected because of uneven polysialic acid chain lengths, which are added to the 140kDa and 180kDa isoforms of NCAM1. Therefore, while quantifying PSA-NCAM protein levels, I used a contour tool, which took the average intensity across the whole smear as a measure of PSA-NCAM quantity.

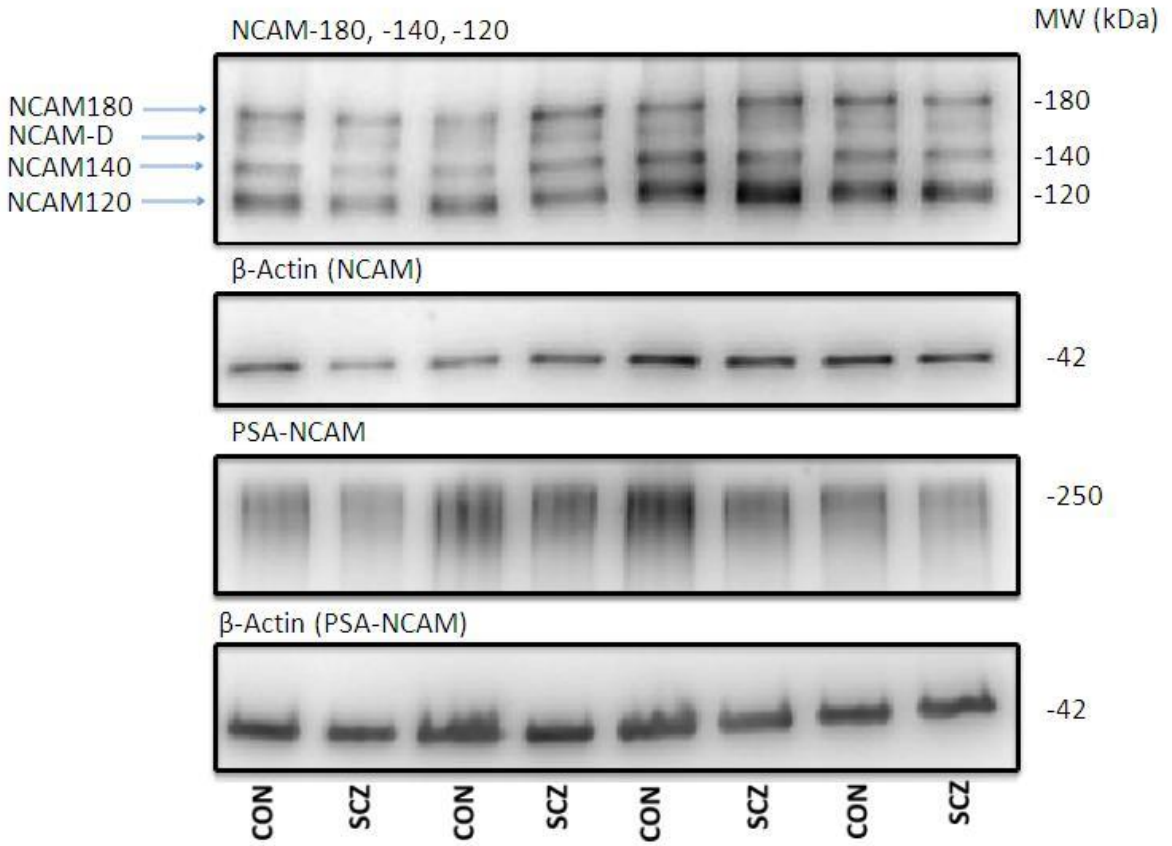


Figure 4.8: Representative western blot showing different NCAM1 isoforms detected in human DLPFC. NCAM isoforms at ~180kDa, ~170kDa, ~160kDa, ~140kDa and ~120kDa were observed with N-terminal NCAM1 antibody. Gels were loaded in a matched pair design, with schizophrenia cases (SCZ) loaded on the same gel as their demographically matched control (CON). Molecular weight (in kDa) for protein marker bands is shown on right hand side. Equal amounts of total protein (1.5 μg for NCAM isoforms, and 20 μg for PSA-NCAM) were loaded, with β-actin used as a loading control.

To ensure the quality of my protein quantification results, I took the averaged values across two duplicate western blot runs, as the final value for each protein isoform. The distribution of each protein measure was assessed, and were found to be normally distributed (Kolmogorov-Smirnov and Lilliefors tests $p>0.1$), with the exception of NCAM-120/Total-NCAM and NCAM-140/Total-NCAM ratios which were log transformed so that parametric statistics could be applied.

The PSA-NCAM and NCAM1 isoform expression levels were examined separately, and expressed as a ratio of each individual isoform to the total NCAM protein levels (sum of NCAM-180, NCAM-D, NCAM-140 and NCAM-120) or as a ratio to "glycosylatable"

NCAM (sum of NCAM-180, NCAM-D, and NCAM-140). The level of NCAM-180 plus NCAM-D was also examined, as some previous studies had not resolved 160-170kDa doublet as a separate entity to the 180kDa band (Gray L J, *et al.* 2010; Jurgenson M, *et al.* 2012), so NCAM-D would likely have contributed to NCAM-180 intensity, in their studies.

First, I determined whether NCAM1 isoform and PSA-NCAM protein expression was influenced by the brain cohort demographic variables which are independent of diagnostic status. The quantitative variables assessed were the same as those listed for the mRNA studies, with the exception of RNA integrity that would not influence post-mortem protein preservation. Demographic and clinical variables that were significantly correlated with NCAM1 protein isoforms are shown in Table 4.6. In this analysis, Age at death, duration of illness, daily and life time chlorpromazine levels did not correlate with any major NCAM1 isoform protein measures.

Isoforms	**Demographic variables**	**r(X,Y)**	**r^2**	**t**	***p***	**N**
NCAM-140	pH	-0.23475	0.055109	-2.03493	0.046	73
NCAM-D	pH	-0.29308	0.085895	-2.58294	0.012	73
PSA-NCAM	PMI	0.265701	0.070597	2.3386	0.022	74
Isoforms	**Clinical variables**	**r(X,Y)**	**r^2**	**t**	***p***	**N**
NCAM-180	Onset_Age	0.316942	0.100452	1.94853	0.060	36
NCAM-180+NCAM-D	Onset_Age	0.318371	0.10136	1.9583	0.058	36
NCAM-180/Total-NCAM	Onset_Age	0.371597	0.138084	2.33388	0.026	36
NCAM-180+D/Total-NCAM	Onset_Age	0.31741	0.100749	1.95173	0.059	36
PSA-NCAM	Onset_Age	0.338018	0.114256	2.12481	0.041	37
NCAM-D/Total-NCAM	Last-Chlor	0.389036	0.151349	2.31305	0.028	32
NCAM-180+D/Total-NCAM	Last-Chlor	0.336879	0.113487	1.95971	0.059	32
NCAM-140+180+D/Total-NCAM	Last-Chlor	0.342841	0.11754	1.99896	0.055	32

Table 4.6: Demographic and clinical correlations with different NCAM1 protein isoforms. pH = brain pH, Onset age = Age of disease onset; PMI = post-mortem interval, Last-Chlor represents last dose of antipsychotic chlorpromazine.

There was no significant difference observed in the Total-NCAM expression with diagnosis alone ($t = 0.987$, $p = 0.326$, $df = 71$) or diagnosis x gender analysis [ANOVA $F(1, 69) = 0.957$, $p = 0.331$]. However, a significant effect of the diagnosis was observed with the NCAM-120, where its expression was increased by 26% in the schizophrenia cases (Figure

4.9) compared to the normal controls [t = 2.047, p = 0.044, df = 72]. No other significant effect of the diagnosis was observed with the other NCAM1 isoforms (all p values ≥ 0.30). Surprisingly, no significant effect of gender x hemisphere was observed for any NCAM1 protein isoform, including NCAM-180 [ANOVA F (1, 69) = 0.025, p = 0.874], NCAM-D [ANOVA F (1, 69) = 0.132, p = 0.717] or NCAM-180+NCAM-D [ANOVA F (1, 69) = 0.073, p = 0.787], indicating inconsistencies with my earlier mRNA expression studies.

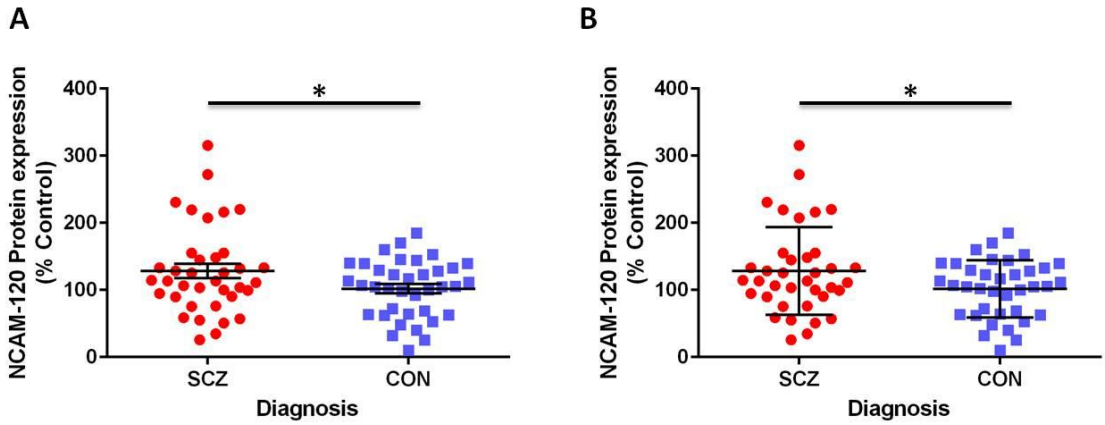


Figure 4.9: Increased expression of NCAM1-120 protein in the DLPFC in schizophrenia. A significant 25.79% increase in NCAM120 protein expression in SCZ vs. CON [t = 2.047, p = 0.044, df = 72]; * represents p = 0.044. Error bars represent standard error in panel A and standard deviation in panel B.

However, looking at the diagnosis x gender effects, I observed few interesting effects, especially with the female schizophrenia patients. Effects included an increase in the NCAM120/Total-NCAM ratio in the female schizophrenic cases (Figure 4.10A) compared to the female controls (Post-hoc Fisher LSD; p = 0.018). I also observed a corresponding 31% decrease in the potentially glycosylatable NCAM1 isoforms (NCAM140+180+D) in females with schizophrenia [ANOVA F(1, 69) = 3.56, p = 0.063] (Figure 4.10B). Consistent with the reduction in availability of the glycosylatable NCAM isoforms in the females with schizophrenia, I also observed a 26% decrease in the level of PSA-NCAM protein in the female schizophrenic cases compared to their unaffected control counterparts [ANCOVA F (1, 69) = 3.5224, p = 0.06; co-varying for PMI]; (post-hoc Fisher LSD; p = 0.193) (Figure 4.10C).

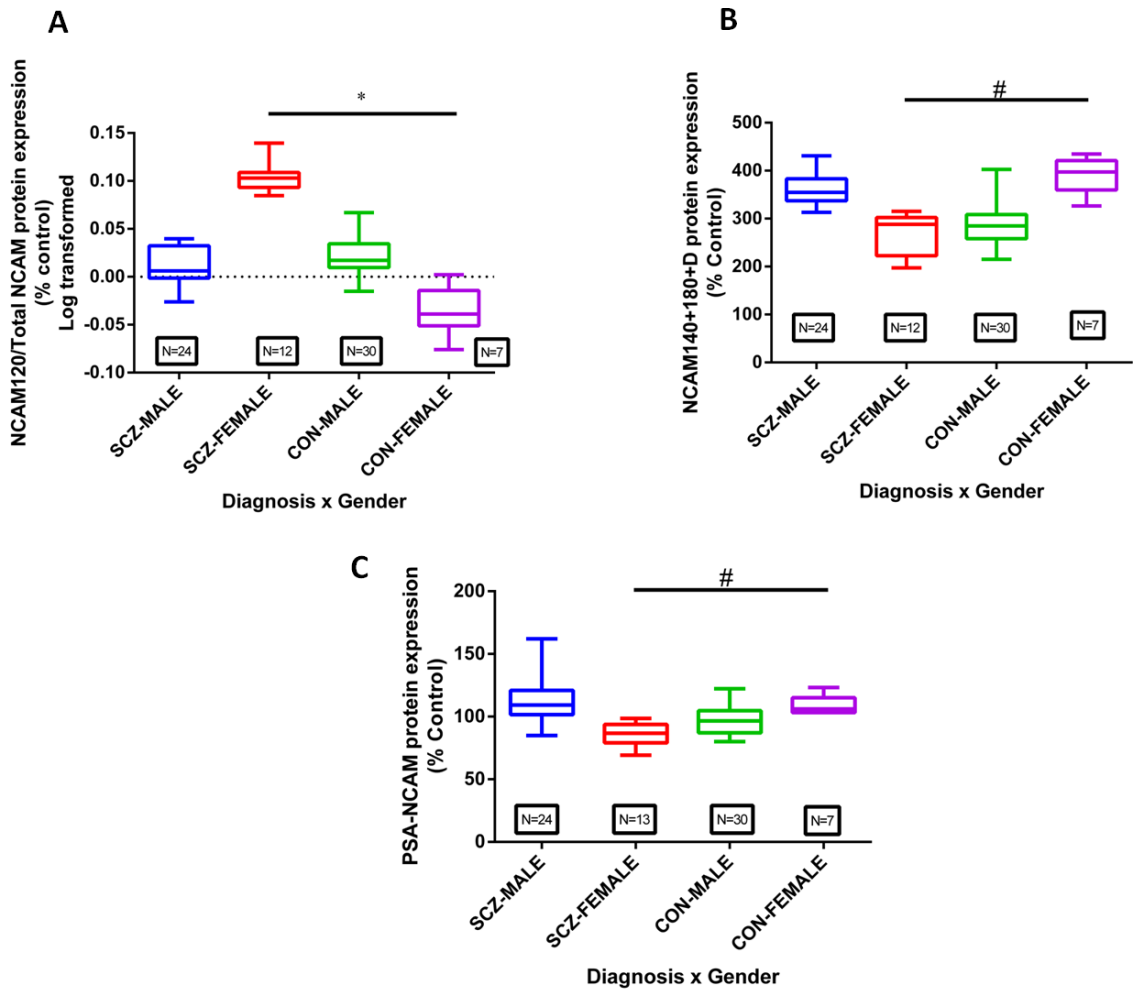


Figure 4.10: Gender specific dysregulation of NCAM1 protein isoforms in schizophrenia. A) A significant increase in the ratio of NCAM-120 isoform was detected in the females with schizophrenia [ANOVA F (1, 69) = 5.042, p = 0.0279]; planned post-hoc comparisons Fisher LSD p = 0.018). **B)** A suggestive decrease in the ratio of the glycosylatable NCAM1 isoforms in females with schizophrenia [ANOVA F(1, 69) = 3.56, p = 0.063]; planned post-hoc comparisons comparing SCZ-females to CON-females (Fisher LSD p = 0.172). **C)** A suggestive decrease in the PSA-NCAM in females with schizophrenia [ANCOVA F (1, 69) = 3.522, p = 0.065, co-varying for PMI], was observed; planned post-hoc comparisons (Fishers LSD), comparing SCZ-females to CON-females ($^{\#}p$ = 0.193). Error bars denote the standard errors. Asterisks indicate the significant group differences (p< 0.05). Numbers inside the boxes (N) represent numbers of samples in each group.

These results broadly suggest dysregulation of NCAM1 protein isoforms in a subgroup of schizophrenia patients, and indicate a reduction in available target protein for PSA-NCAM formation. However as the sample numbers are small in the stratified analysis, this work should be replicated to confirm the results.

4.3. Discussion

The overall goal of this study was to investigate how variations in NCAM1 at the genetic level and the expression of its different isoforms at mRNA and protein level, could confer risk to mental illness. At the genetic level, I found weak evidence for association between novel and known *NCAM1* SNPs and bipolar disorder. At the mRNA level, I found a significant up-regulation of the NCAM-180 isoform in the right hemisphere of schizophrenia cases. This effect was however not repeated at the protein level, where I found an increase in the expression of NCAM-120 isoform (non-glycosylatable isoform) in schizophrenia cases. I also observed some gender-specific effects at the protein level, where the ratio of NCAM-120 isoform expression to the total NCAM1 expression was significantly increased in female schizophrenia patients, and a subsequent decrease the expression of main glycosylatable NCAM1 isoforms (140+180+D) and PSA-NCAM was also observed, although this change was not statistically significant.

NCAM1 is located on chromosomal region 11q, close to the *DRD2* gene, a susceptibility gene for both bipolar disorder (Bocchetta A, *et al.* 1999; Zou Y F, *et al.* 2012) and schizophrenia (Kukreti R, *et al.* 2006). *NCAM1* polymorphisms have previously been related to co-morbid conditions associated with the mental illness such as, alcohol dependence and drug abuse (Nelson E C, *et al.* 2013; Yang B Z, *et al.* 2008). The significant association between polymorphisms in *NCAM1* and mental illness has been shown by Arai *et al* (2004) and Atz *et al* (2007) previously. However Xu *et al* (2008) and Vicente *et al* (1997) findings have reported no significant association signal in the Chinese and Canadian populations, respectively. In my genetic association study using a largely Caucasian Australian bipolar disorder case-control cohort, I genotyped a small selection of SNPs including the major SNPs previously associated with mental illness (rs1836796, rs686050, rs646558, rs2303377). SNP rs646558 which is located in Fn-III repeat (around NCAM1 polysialylation site) has shown significant association with bipolar disorder in the Japanese (Arai M, *et al.* 2004) and schizophrenia in American (Atz M E, *et al.* 2007) based cohorts. This SNP also showed suggestive allelic association in my study, but this association could be due to the difference in genotyping platform and/or sequencing

coverage from 1000 Genomes data, which may have affected estimates of genotype frequency. Heterogeneity was also observed at this SNP when I performed association studies including gender as a covariate, with the finding being driven by males with schizophrenia. A replication study in a larger cohort using identical genotyping platforms in cases and control subjects, with examination of possible gender differences, is required to confirm this association.

NCAM's Ig domain-5 and regions around it (e.g. acidic patch and α-helix) interact with ST8SIA2 in the polysialylation process (Atkins A R, *et al.* 2004; Soroka V, *et al.* 2008). Interestingly, SNPs in this region have shown association with mental illness in the past in Japanese (Arai M, *et al.* 2006) and American populations (Atz M E, *et al.* 2007). However, the same SNPs did not show any significant association in my study that included Caucasian Australian population. Possible reasons for this variation could be differences in allele frequencies and linkage disequilibrium blocks across different populations arising due to evolution, geographical separation, mutation, natural selection, migration or genetic drift between the populations (Olson-Manning C F, *et al.* 2012). Many studies have observed difference in allele frequencies in different populations using microsatellites (Bowcock A M, *et al.* 1991; Jorde L B, *et al.* 1997), Restriction Fragment Length Polymorphisms (RFLP) (Dean M, *et al.* 1994), and blood group markers (Cavalli-Sforza L L, *et al.* 1993). Difference in allele frequencies of SNPs is also well known for a long time in different genes such as human lipoprotein lipase by Nickerson *et al.* (1998) and ApoE by Lai *et al.* (1998).

My sequencing results identified novel SNPs in NCAM1's functional domain region, which further showed suggestive association with mental illness. Novel SNPs were also predicted to play a role in altering transcription factor binding and mRNA splicing to cause functional effects at mRNA and protein level. However, these were only bioinformatic predictions which rely on flanking sequence and only functional studies could confirm their actual effect. I could not test them functionally in this study. However, the presence of these novel SNPs in genetic structure highlights the importance of resequencing studies in characterizing variation which might be functionally important.

At the mRNA level, NCAM1 produces many isoforms and the expression patterns of these isoforms differs during development. In mouse models, NCAM-180 and NCAM-140 expression decreases with age (Oltmann-Norden I, *et al.* 2008), while NCAM-120 expression, which is at low levels in the embryonic brain, elevates with age and is expressed at stable levels in the adult brain (Brennaman L H, Maness P F 2008; Cox E T, *et al.* 2009). In this study, looking at the NCAM1 isoform mRNA expression levels in human DLPFC, I found several NCAM1 isoforms were influenced by the brain cohort demographic variables. Notably, age was found to be positively correlated with the NCAM-120 isoform, highlighting that the expression of this isoform increases with age in humans. This pattern is in general agreement with the previous findings (Brennaman L H, Maness P F 2008; Cox E T, *et al.* 2009).

Irregular expression of the NCAM1 mRNA isoforms has been linked to the mental illness (Gibbons A S, *et al.* 2009; Gray L J, *et al.* 2010; Tanaka Y, *et al.* 2007; Vawter M P, *et al.* 1999). In this study, looking at the expression of different NCAM1 mRNA isoforms, I observed an increased expression in the NCAM-180 mRNA isoform, but only in the right hemisphere of schizophrenic cases. This effect was statistically significant. The erroneous right hemisphere could affect inter-hemispheric communication and this has been reported in schizophrenia previously (Corradi-Dell'Acqua C, *et al.* 2012). Increased NCAM-180 mRNA expression in Brodmann Area 46 (BA46, which lies adjacent to DLPFC) has been reported by Gibbons *et al.*, (2009) in schizophrenia, but their study involved samples from the left hemisphere only. So while both our studies show increased NCAM-180 mRNA in schizophrenia, we find our effects specific to different hemispheres. The consequences of increased NCAM-180 expression could reflect increased synaptic plasticity, as NCAM-180 is involved in the signaling via spectrin, effecting the cognitive functions and brain plasticity in the form of LTP (Doyle E, *et al.* 1992; Fux C M, *et al.* 2003; Leshchyns'ka I, *et al.* 2003; Schuster T, *et al.* 1998). NCAM-180 is very adhesive in nature and bonds with the cytoskeleton through an elongated cytoplasmic region; its increased expression could therefore influence the adhesive properties of neurons and also their shape (Ricard C S, *et al.* 2000; Ricard C S, *et al.* 1999).

This increase in NCAM-180 mRNA however did not reflect in my protein studies, where I observed an unexpected decrease in the NCAM-180 protein isoform. A possible explanation could be that NCAM-180 mRNA was targeted by the cellular machinery for degradation (Tuschl T, *et al.* 1999), their expression was selectively suppressed by miRNAs (Orom U A, Lund A H 2010) or by involvement of different complex post-transcriptional mechanisms. Difference could also be due to difference in *in-vivo* half-lives of different protein isoforms (Greenbaum D, *et al.* 2003).It is also possible that potential errors and noise values in both mRNA and protein quantification are causing this difference or result is an artifact of small sample numbers after stratifying an already small sample. Although, opposite results of a gene isoform at mRNA and protein level have been reported before: a systematic study of 165 cancer-related protein spots and their corresponding mRNA expression by Chen *et al* (2002) reported that only ~20% of proteins correlate well ($r>0.24$) with their mRNAs, with correlation values ranging from -0.4 to 0.44, indicating complexity in the control of protein expression from mRNA. This effect has also been reported in PFC of individuals with psychiatric illness (Sinclair D, *et al.* 2011), so while the inconsistency between mRNA and protein results may indicate false positive effects, it might also indicate changes in downstream processes relating to conversion of mRNA to protein.

NCAM1-VASE isoform is reported to be increased in the PFC and hippocampus of patients suffering from bipolar disorder (Vawter M P, *et al.* 1998b) and in the CSF of patients with schizophrenia (Vawter M P, *et al.* 2000). Previous studies also suggested a relationship between the VASE expression and decreased neural plasticity (Vawter M P 2000) and neurite extension (Doherty P, *et al.* 1992). However, in this study, no significant association was observed between the quantity of VASE isoform transcripts and schizophrenia cases.

Looking at the protein expression, I found no significant change in the overall expression of NCAM1 (NCAM-120+140+180+D) in schizophrenia cases versus controls. No alteration in the total NCAM1 expression in schizophrenia cases compared to unaffected controls has been reported before by Barbeau *et al* (1995) and Breese *et al* (1995). However, I noticed a

significant 26% increase in NCAM-120 in schizophrenic cases compared to the normal controls. This result is consistent with Poltorak *et al* (1996) finding, who observed an increase in NCAM-120, but not in NCAM-180 protein expression in the CSF of schizophrenia cases. Increased NCAM-120 protein expression has previously been observed in the hippocampus, PFC and cortical regions in schizophrenia cases (Tanaka Y, *et al.* 2007; Vawter M P, *et al.* 1998a). If overall expression of NCAM1 remains same, an increase in NCAM-120 in schizophrenia cases compared to controls would mean an increase in non-glycosylatable or 'naked NCAM', and potentially a decrease in glycosylatable forms of NCAM. In fact, an interesting pattern was observed in the female schizophrenia cases, where a significant increase in the NCAM-120/Total NCAM ratio and a significant decrease in main the glycosylatable isoforms i.e. NCAM-180, NCAM-180+D, and NCAM-180+140+D were observed. This effect needs to be verified in a larger cohort.

In rodents, the presence of 'naked NCAM' in early brain development is associated with hypoplasia of anterior commissure, ventricular enlargements and reduced rostrocaudal extent of the interior commissure and corpus callosum by Hildebrandt *et al* (2009). Structural defects in these brain structures have been associated with both bipolar disorder and schizophrenia previously (Rane S, *et al.* 2013; Saxena K, *et al.* 2012; Shiraishi H, *et al.* 1990; Swayze V W, 2nd, *et al.* 1990; Van Horn J D, McManus I C 1992). This suggests that there could be a link between non-glycosylatable NCAM availability, abnormal brain formation through faulty neural migration and possibly development of psychotic mental illness.

Possibly as a consequence of reduced availability of glycosylatable NCAM isoforms, I noticed a subsequent decrease in PSA-NCAM expression, in the same group of females with schizophrenia, this effect was however not significant. The study by Barbeau *et al* (1995) has reported decreased PSA-NCAM protein levels in the post-mortem hippocampal neurons in schizophrenia cases. PSA-NCAM expression was also found to be decreased in cortical layers IV and V in schizophrenia cases (Gilabert-Juan J, *et al.* 2012). Another interesting finding from this study was a significant positive correlation between the age at disease onset and PSA-NCAM and glycosylatable NCAM1 isoforms (NCAM-180+D and

NCAM-140+180+D). Results suggest that the presence of higher levels of PSA-NCAM and glycosylatable NCAM1 play a protective role, and their presence could delay the age of onset of the disease.

The exact pathway that would connect decreased PSA-NCAM expression to mental illness has not been identified, but in addition to acting as a neuronal lubricant to aid cell migration and synaptogenesis, PSA-NCAM is known to prevent excitotoxicity by blocking the Glu-N2B possessing NMDA receptors (Hammond M S, *et al.* 2006). The decreased PSA-NCAM levels could mean no competitive inhibition of GluN2B-containing glutamate receptors. As a result of this unblocking, the Ras-GRF1-p38 MAPK signaling cascade could become functional and effect the neural plasticity and cognition, potentially increasing the likelihood of developing mental illness (Kochlamazashvili G, *et al.* 2012; Kochlamazashvili G, *et al.* 2010; Nacher J, *et al.* 2013; Senkov O, *et al.* 2012).

The main findings of this chapter illustrate the effect of brain hemisphere and gender on the disease pathogenesis. The reason why this NCAM-dysregulation might only be seen in the female schizophrenia patients remains unanswered, but gender effects have been previously reported in schizophrenia (Javed M A 2000; Seeman M V 2000; Usall J, *et al.* 2000). I could not directly compare my gender and hemisphere specific findings with other studies, because Gibbons *et al* (2009) worked with brain tissue only from the left hemisphere and Barbeau *et al* (1995) did not examine the effect of gender. This chapter also presents an alternative approach to normalize western blot data, when traditional housekeepers are well outside the expression range of the target protein and hence fail to standardize the raw values.

This study has some limitations such as, the genetic experiments were performed in bipolar based cohort and the mRNA and protein experiments were performed in schizophrenia based cohort. As a common limitation of all post-mortem brain studies, there were small and uneven sample numbers in each group of the TRC cohort when stratifying the sample for mRNA and protein expression studies. Future studies with a larger sample size and including the same number of males-females and tissues from the right-left brain

hemisphere would be ideal. Also, it will be important to investigate the expression of polyST enzymes in the same cohort, as the abundance of PSA-NCAM is dependent on the availability of both enzyme and substrate (Figure 4.11).

In conclusion, the findings from this chapter identified known and novel SNPs that showed weak evidence for association with mental illness. My results support the findings from published literature, regarding the irregular expression of different NCAM1 isoforms at mRNA and protein level and its possible association with psychotic mental illness. I observed inconsistencies in the alterations of NCAM1 isoform expression at mRNA and protein levels, which may indicate complexity in the control of protein synthesis from mRNA. Finally, I would like to hypothesize that decreased glycosylatable NCAM1 protein expression may lead to decreased PSA-NCAM formation, and altered cell adhesion and cell-cell communication which may influence both disease risk and onset. Further work will be required to prove or disprove this hypothesis.

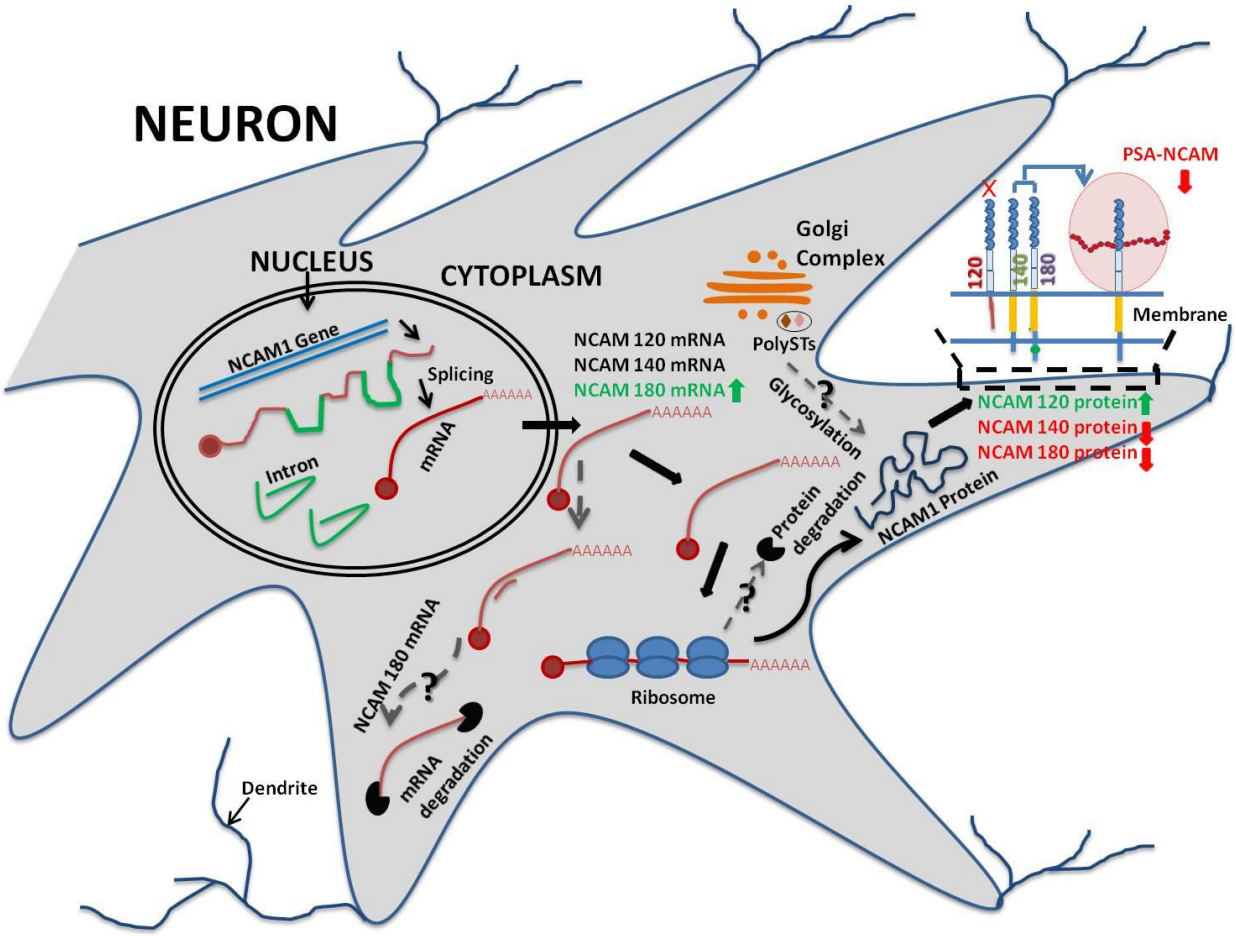


Figure 4.11: Summary of NCAM effects observed in this study. After transcription, an increase in NCAM-180 mRNA was observed this was not reflected in the protein findings. Possible reasons could be NCAM-180-mRNA degradation or NCAM-180-protein degradation. A decrease in the levels of main glycosylatable NCAM1 isoforms at protein level was reflected by a subsequent decrease in PSA-NCAM as NCAM-120 do not contribute towards PSA-NCAM formation. A decrease in PSA-NCAM protein concentration could also involve a role of golgi-based polysialyltransferases (polySTs) enzymes ST8SIA2 and ST8SIA4.

Chapter 5

Do variations in sialyltransferase-2 (ST8SIA2) at genetic, mRNA or protein levels associate with psychotic mental illness?

5.1. Introduction

After proteins are synthesized in the ribosomes, they undergo modification to achieve proper folding, trafficking and stability (Wujek P, *et al.* 2004). One method of modification is by the addition of sugar groups, in a process called glycosylation (Golabek A A, *et al.* 2003; Sola R J, Griebenow K 2009). A common glycosylation process in neuronal cells involves monomers of Sia (e.g. N-acetylneuraminic acid or Neu5Ac) to join together and form linear, anionic chains of α2,8-linked sialyl residues (Wang B 2009). In the human genome, there are six genes (ST8-alpha-N-acetyl-neuraminide-α-2,8 genes) encoding enzymes capable of transfering sialic acid to α2,8-linked oligosaccharides. Protein products of only two (*ST8SIA2* and *ST8SIA4*) out of these six genes are responsible for the synthesis of the long polysialic acid chains. These PSA chains range between 50-200 residues and completely check the adhesive properties of NCAM1 (Galuska S P, *et al.* 2006; Harduin-Lepers A, *et al.* 2008; Hildebrandt H, *et al.* 2009). While, ST8SIA2 is expressed at high levels in the developing brain (McAuley E Z, *et al.* 2012; Oltmann-Norden I, *et al.* 2008), *ST8SIA4* is more abundant in the adult brains (Hildebrandt H, *et al.* 2008;2010). Together, ST8SIA2 and ST8SIA4 are known as the polysialyltransferases (polySTs) and are type II membrane proteins that utilize typical N-linked glycans to glycosylate target proteins, principally NCAM1 via a posttranslational process (Angata K, *et al.* 2000; Galuska S P, *et al.* 2006; Hildebrandt H, *et al.* 2009; Nelson R W, *et al.* 1995).

The ST8SIA2 enzyme is encoded by a gene positioned on human chromosome 15q26.1. This particular genomic region has been identified by linkage studies as a risk locus for a range of disorders, including schizophrenia, bipolar disorder and psychosis, by our group and the others (Maziade M, *et al.* 2003; McAuley E Z, *et al.* 2009; Vazza G, *et al.* 2007) and genetic association studies have established *ST8SIA2* as a general vulnerability gene for bipolar disorder, schizophrenia and autism (Anney R, *et al.* 2010; Arai M, *et al.* 2006; Lee M T M, *et al.* 2011; McAuley E Z, *et al.* 2012). The genomic structure of *ST8SIA2* comprises six exons stretching across 74818 base pairs of DNA. In addition to the main (full-length) transcript of ST8SIA2, one splicing isoform has been described in the literature, which excludes exon 2 (Δ2) (ncbi.nlm.nih.gov). Isoform Δ2 was identified in

brain tissue and lacks a portion of the transmembrane domain (www.genecards.org). This study was planned to determine if there are any additional splicing isoforms of ST8SIA2.

ST8SIA2 enzyme has a molecular weight of 42.5 kDa, and comprises of six potential N-linked glycosylation sites (Close B E, Colley K J 1998), suggesting this enzyme is itself sensitive to regulation by glycosylation (auto-glycosylation). Studies using animal models have revealed major faults in brain development and morphology with disturbance in the equilibrium of polySTs and NCAM1 (Hildebrandt H, *et al.* 2009). Double knockout transgenic studies of both the polySTs (*ST8SIA2*$^{-/-}$ and *ST8SIA4*$^{-/-}$) with normal *NCAM1*, (*NCAM1*$^{+/+}$) showed structural deformations of the anterior commissure, corpus callosum, interior commissure and enlargements of lateral ventricles (Hildebrandt H, *et al.* 2009). Interestingly, deficits in these brain structures have been linked to the mental illness in other studies. Anterior Commissure disruptions were observed in youth suffering from bipolar disorder by Saxena *et al* (2012). A study by Choi *et al* (2011) found reduction in interhemisphere activity with in this region in schizophrenia patients. Ventricular enlargement in lateral hemisphere was observed in schizophrenia cases by Davis *et al* (1998) and Mitelman *et al* (2010). Saxena *et al* (2012) reported disruption of Corpus Callosum in youth suffering from bipolar disorder and Downhill *et al* (2000) study reported a reduction in size of Corpus Callosum in individuals suffering from schizophrenia. These results suggest importance of polySTs in proper brain formation and in developing risk to mental illness. To date, no study has directly investigated the protein levels of ST8SIA2 in the human brain, or explored the correlations between mRNA and protein expression of ST8SIA2 and PSA-NCAM in the same cohort. Therefore, in this study ST8SIA2 protein expression will be studied in TRC cohort and results will be compared to NCAM1 protein expression results from the previous chapter.

In light of the genetic and developmental connections of *ST8SIA2* with mental illness, detailed information about the variations in *ST8SIA2* genomic structure and its expression at both the mRNA and protein levels may help elucidate the mechanism through which this gene is connected to disease. Therefore, the aims of this study are: firstly, to characterize the DNA variations in the entire *ST8SIA2* gene in individuals with bipolar disorder; and

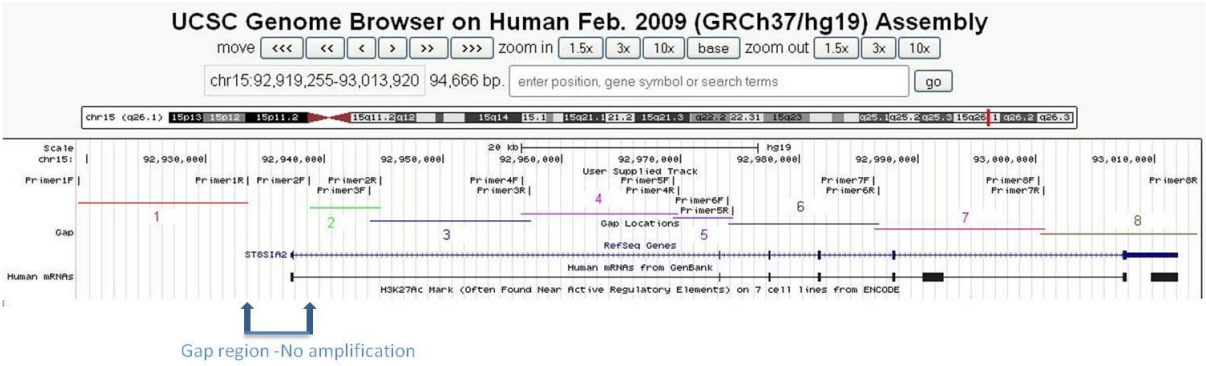
secondly, to investigate variation in the mRNA and protein levels in the DLPFC of available post-mortem brain tissue from controls and patients with psychotic mental illness.

5.2. Results

5.2.1. Genomic variation

Given that *ST8SIA2* has previously been associated with mental illness at the genetic level (Anney R, *et al.* 2010; Arai M, *et al.* 2006; Lee M T M, *et al.* 2011; McAuley E Z, *et al.* 2012), it is likely that individuals with mental illness may harbor one or more genetic variations in their DNA, which may influence *ST8SIA2* gene function. In order to characterise genetic variation in the *ST8SIA2* gene, I sequenced the entire gene and its 5'-upstream region (~95 kb in total) in forty-eight individuals affected with bipolar disorder. Given that bipolar disorder is genetically heterogeneous (at both allelic and locus levels), I wanted to increase the probability that the individuals selected for sequencing will have a functional genetic variant in *ST8SIA2*. Hence, I used a targeted sample selection strategy, to include twenty-eight individuals from families that contributed to the initial linkage signal observed in McAuley *et al* (2009) for sequencing. A detailed list of the individuals selected for resequencing is detailed in Chapter 2 (Table 2.5). Long range PCR was performed to amplify the DNA template in overlapping amplicons, followed by next generation sequencing on the Roche 454 platform for this experiment. Detailed steps for long-range PCR, information about primers used and steps for PCR clean-up are described in Chapter 2 (Section 2.3.1.2, 2.3.1.4 and Table 2.7).

Through long-range PCR, a 90kb region was amplified successfully, while a GC-rich region covering ~5kb of the sequence, containing the promoter, exon 1 and part of intron 1 failed to produce a robust long range PCR product. I tried to optimize several different primer sets in different combinations and amplicon sizes to cover this region, using different concentrations of Mg^{2+}, chemical compounds like betaine and DMSO, with no success. This non-amplifiable region is displayed as the 'Gap region' in Figure 5.1.



Amplicon No.	Primer F Sequence (5'-3')	Primer R Sequence (5'-3')	Amplicon length (bp)	Primer overlap (bp)
1	AGCGTCTTTTCAGGAGGTGA	CTACCCTGACCCAGCAACAT	14241	
2	CGCTTCCTGCTCTCATTTTC	GTTTCCTCCTTGCCATCGT	6067	0 (Gap region)
3	TCCCAGTGAAGAGCACAGTC	TTCCCATTGCCCTGAGTATC	13516	950
4	TAAGGTGGAGCTAGGGACCA	GGAGACCAAATGCCTTGAAA	13200	587
5	TCCCATCTGTGATTCCATGA	GGGCAGGATCTTCTTTCTCC	5045	405
6	AATGACAAGTGCCCCATAGC	ACTGCAAACTCATGCTGACAA	12719	439
7	ACCCACTTTTTCTCCCCAGT	TATGAGCACGGACAGCAATC	14331	337
8	ATCAGCCTTTCCCAACAGC	CCACTCCCTCTACCCAATTTC	13757	435

Figure 5.1: Long-range amplicon design covering whole *ST8SIA2* gene. Primers sequences used in long-range PCR with amplicon length and overlap with preceding amplicon, are shown as colour coded. GAP region is the region that did not amplify likely due to high GC content.

All the 48 samples were checked on gel for clean PCR bands and molar quantification relative to molecular weight marker, prior to performing equimolar pooling of all amplicons for each subject (Figure 5.2). Products were then purified and the purified products were pooled together, underwent fragmentation and multiplex barcoded linker ligation, and subjected to Roche 454 sequencing.

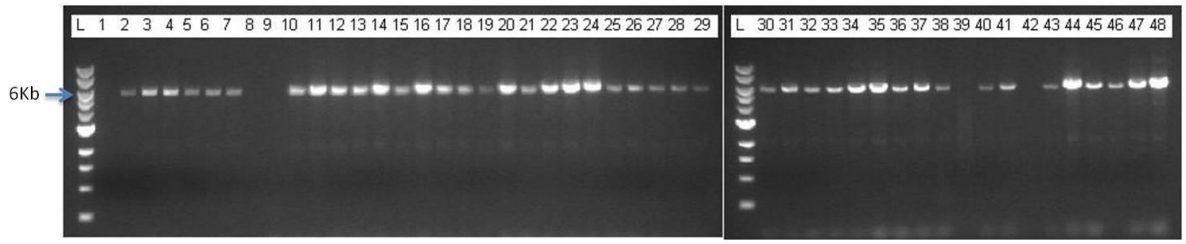


Figure 5.2: Representative gel image showing long range amplification for amplicon 2, for forty-eight samples. Samples with no product were repeated and then checked on gel before pooling them together with other seven amplicons, at apparent equimolar concentration, for each individual.

After successfully resequencing a region of approximately 90 kb in 48 individuals (i.e. total region ~ 4.5 Mb) with an average read depth of ~12, I started analyzing my data using the

Roche FLX GS Reference Mapper Software V2.5.3, designed for the analysis of long range amplicon sequencing data. Using the variant summary table from the GS Reference Mapper, I found 549 known variants (75%) and 182 novel variants (25%) in the 48 bipolar cases. However, I found that while the Reference Mapper software provided a summary of variants for the whole cohort, it was not designed to call the genotypes on an individual sample basis if multiple samples are included in the analysis run. I wanted to generate genotypes for each individual, as this is important for the identification of which variants which lie on the specific risk haplotype. So I then attempted to use GS Amplicon software, which is able to call genotypes at an individual level, but found that that the software could not analyze data generated from the long-range PCR. I had numerous meetings with the bioinformatic representatives from the Roche and the Ramacciotti Centre, and found that the advice I had been given by the Ramacciotti Centre to generate amplicons by long-range PCR was incorrect, given my original goal of wanting to assign genotypes to the individual samples, and the limitations of the software packages available commercially.

To obtain genotypes at an individual level, I analysed each of the 48 samples individually in GS Reference Mapper, and manually compared the sample sequences to the reference sequence to identify the novel SNPs and call genotypes based on the allele balance over multiple reads. But ideally, information is required across multiple individuals at each locus in order to accurately identify a SNP, which was not possible using GS Reference Mapper using a single individual in 48 separate analyses. As massively parallel sequencing comprises of multiple reads at each base position, the balance between reference and alternate alleles across multiple reads determine whether a SNP genotype should be called homozygote reference, heterozygote or homozygote alternate. However, as there was variation in sampling from each chromosome, due to PCR bias and sampling bias, the observed allele balance cannot be exactly as predicted (i.e. 50:50 balance for heterozygotes). Hence, genotypes were called as definite homozygotes if their allele balance was 95-100% for reference allele and 0-5% for alternative allele. A SNP genotype was called heterozygote if the allele balance was between 25-75% for both alleles. Potential homozygotes were called if allele balance for reference allele was between 85-95% or the

alternative allele was between 5-15%. Allele balance between 15-25% and 75-85% were called potential heterozygotes. I then compared my data to dbSNP130 (UCSChg18) to get information about known SNPs and identify novel variants, and identified 549 known variants (75%) and 182 novel variants (25%).

Later, Dr Alex Shaw (a research officer in our lab) analysed this 454 data using a Galaxy (Goecks J, *et al.* 2010) based analysis pipeline that he had developed (*manuscript in preparation*). Dr Shaw's analysis used a probabilistic genotype calling approach in GATK, which was able to assign genotypes at an individual level along with a probability score for that call being correct, and that incorporated read depth information and local base composition, in order to filter out potentially erroneous SNP calls. He identified 380 SNPs in the region studied, including 62 novel SNPs. None of the SNPs identified affected the protein coding sequence, although one previously known synonymous SNP (rs2305561, P186P) in exon 5, two SNPs in the 5'UTR of exon 1 (rs3743365, rs3743364) and 12 SNPs in the 3'UTR (rs139149207, rs2290492, rs12904773, rs8035760, rs1869774, rs115781738, rs116928729, rs17600420, rs117763930, rs145948851, novel SNP 60 and 61) were identified. Although the 62 novel SNPs generated via the probabilistic method were present in the list of SNPs that I generated through my analysis using GS Reference Mapper, my list however included additional SNPs which could be due to erroneous SNP calling and no data filtering (type I error) or they could be real and missed in data filtering (type II error). Since, Dr Shaw's data analysis method used a probabilistic method for more accurate and more conservative genotype calling; I used his data output to list the novel genetic variations shown in Table 5.1

SNP No.	bp position (hg19)	Variation	MAF	Count	SNP No.	bp position (hg19)	Variation	MAF	Count
1	92919540	Y (T/C)	0.011	0/1/47	34	92958793	M (C/A)	0.011	0/1/47
2	92920201	M (C/A)	0.011	0/1/47	35	92959424	R (A/G)	0.011	0/1/47
3	92921358	R (A/G)	0.011	0/1/47	36	92960031	W (A/T)	0.011	0/1/47
4	92921359	W (A/T)	0.011	0/1/47	37	92961050	R (A/G)	0.011	0/1/47
5	92921363	M (C/A)	0.011	0/1/47	38	92961385	R (A/G)	0.011	0/1/47
6	92921365	Y (T/C)	0.011	0/1/47	39	92961543	W (A/T)	0.011	0/1/47
7	92921366	W (A/T)	0.011	0/1/47	40	92962229	Y (C/T)	0.011	0/1/47
8	92921534	R (A/G)	0.011	0/1/47	41	92962920	K (G/T)	0.011	0/1/47
9	92921622	R (A/G)	0.011	0/1/47	42	92967421	Y (C/T)	0.021	0/2/45
10	92923096	R (A/G)	0.022	0/2/46	43	92967835	R (A/G)	0.011	0/1/47
11	92924017	R (A/G)	0.011	0/1/47	44	92967836	K (G/T)	0.011	0/1/47
12	92924023	R (A/G)	0.071	0/7/40	45	92967839	W (A/T)	0.011	0/1/47
13	92924024	R (A/G)	0.119	0/11/37	46	92967859	K (G/T)	0.011	0/1/47
14	92924025	R (A/G)	0.075	0/7/41	**EXON 2 (92973276-92973345)**				
15	92925842	R (A/G)	0.011	0/1/47	47	92973414	R (A/G)	0.011	0/1/47
16	92926056	R (A/G)	0.011	0/1/47	**EXON 3 (92977477-92977606)**				
17	92929229	S (G/C)	0.011	0/1/47	48	92979413	R (A/G)	0.011	0/1/47
18	92929614	S (G/C)	0.011	0/1/47	**EXON 4 (92981581-92981842)**				
19	92930822	Y (C/T)	0.011	0/1/47	49	92984673	R (A/G)	0.011	0/1/47
20	92933237	W (A/T)	0.011	0/1/47	50	92985963	S (C/G)	0.011	0/1/47
21	92933239	R (A/G)	0.011	0/1/47	**EXON 5 (92987866-92988161)**				
22	92933271	Y (C/T)	0.011	0/1/47	51	92991770	Y (C/T)	0.011	0/1/47
EXON 1 (92937141-92937394)					52	92992055	Y (C/T)	0.022	0/2/45
23	92942136	M (A/C)	0.011	0/1/47	53	92992177	K (G/T)	0.011	0/1/47
24	92944949	Y (C/T)	0.011	0/1/47	54	92993375	Y (C/T)	0.011	0/1/47
25	92947916	W (A/T)	0.011	0/1/47	55	92994449	R (G/A)	0.011	0/1/47
26	92947918	W (A/T)	0.011	0/1/47	56	93000069	Y (C/T)	0.011	0/1/47
27	92948064	M (A/C)	0.011	0/1/47	57	93002872	Y (C/T)	0.011	0/1/47
28	92950518	Y (C/T)	0.014	0/1/46	58	93007325	Y (C/T)	0.011	0/1/47
29	92951803	Y (C/T)	0.013	0/1/45	59	93007327	Y (C/T)	0.011	0/1/47
30	92951841	W (A/T)	0.011	0/1/47	**EXON 6 (93007331-93011965)**				
31	92956314	R (A/G)	0.011	0/1/47	60	93009004	W (A/T)	0.011	0/1/47
32	92956654	M (A/C)	0.011	0/1/47	61	93010789	K (G/T)	0.011	0/1/47
33	92958227	S (C/G)	0.011	0/1/47	62	93013520	M (A/C)	0.011	0/1/47

Table 5.1: The 62 novel SNPs identified in *ST8SIA2* in 48 bipolar disorder cases. Exon positions are shown as they are located on *ST8SIA2* (hg19). Variations are labeled R, Y, K, M, W and S for A/G, T/C, G/T,

A/C, A/T, G/C substitutions, respectively. Minor allele frequency (MAF) and allele count is also shown. SNP no. 60 and 61 were presented in 3' UTR region.

In order to determine whether any of these SNPs might be functional, I performed bioinformatic predictions on each variant. I decided to focus only on the novel SNPs for my PhD studies, as the other variants were being assessed by Dr Alex Shaw, and therefore analyzed 62 SNPs (Table 5.1) to investigate if they have a functional role through the approaches described below.

Splice site predictions: To study the effect these novel SNPs might have on the mRNA splicing; I performed bioinformatic analyses to explore if these SNPs introduce or alter any new splice donor or acceptor sites. I used the BDGP software (The Berkeley Drosophila Genome Project; http://www.fruitfly.org/about/index.html), a program to calculate the possible 5' and 3' donor/acceptor site scores for each allele of each SNP and compared these scores against the other software, such as Human Splicing Finder (HSF) V2.4.1 (Desmet F O, *et al.* 2009) and MaxEntScan (Yeo G, Burge C B 2004) which are based on the 'Maximum Entropy Principle' to investigate the effect of different SNP alleles on the small sequence motifs that are important in correct RNA splicing. I identified 6 SNPs out of the 62 novel SNPs which introduce either donor or acceptor sites with a high BDGP score (Table 5.2).

SNP No.	SNP position	Location	Allele 1	Allele 2	Creation of	BDGP score	Sequence
9	92921622	Upstream Exon 1	G	A	Donor site (G Allele)	0.85	cagtggg**gt**gagaac
21	92933239	Upstream Exon 1	A	G	Acceptor site (G Allele)	0.7	ttattcttaaggtttttta**ag**gtctgaattgattgaaataa
31	92956314	Intron 1	A	G	Acceptor site (G Allele)	0.68	gcgtttgtttcatgtcccc**ag**atttctattttaagcacctt
35	92959424	Intron 1	A	G	Donor site (G Allele)	0.72	ccctcag**gt**gggcga
38	92961385	Intron 1	A	G	Donor site (G Allele)	0.84	gtcagaa**gt**aagaaa
47	92973414	Intron 2	G	A	Acceptor site (A Allele)	0.98	tctgcttctcttctttgct**ag**atggtgcccttcttacttag

Table 5.2: Six novel SNPs that creates a donor or acceptor site with high BDGP score. Novel SNP number, base pair position, location, and sequence is shown. Allele 2 is novel allele.

To interpret the meaning of the BDGP scores in Table 5.2, I compared the BDGP scores for these six novel SNPs with BDGP scores for SNPs which are known to cause splicing

alterations and possibly relate this event to a disease. I found a list of such SNPs (Faber K, *et al.* 2011), which are known to affect splicing of genes associated with diseases such as lung cancer (Liloglou T, *et al.* 2002), renal cell carcinoma (Kenck C, *et al.* 1996) and tuberous sclerosis (Jobert S, *et al.* 1997). I calculated the BDGP values for those SNPs (Table 5.3) and compared those values to the values for the novel SNPs and found that they were very similar (range 0.65-0.91 versus 0.68-0.98, respectively), suggesting that some of the novel variants detected in our 48 bipolar cases may indeed effect the mRNA splicing. *In vitro* functional tests confirming the functionality of these variants would be the next step of analysis, involving transfection of neuronal cells (e.g. SH-SY5Y) with expression vector containing wild-type and mutant DNA variants to see the outcome on mRNA splicing, although this was not performed for this thesis due to time constraints.

Protein	SNP	SNP position (hg19)	Change in Splice pattern	Change	Associated Disease	Creation of	BDGP score	Sequence
GSTM4	rs41283498	1:110200294	Exon skipping	A/G	Lung cancer	Donor site	0.91	aacctgtgtgagtgt
PCTK3	rs55957903	2:205499478	Exon skipping	A/G		Donor site	0.86	gaagacagtgagtac
VHL	rs5030815	3:10188322	Exon skipping	C/T	Renal Cell Carcinoma	Donor site	0.82	ctgccaggtactgac
TSC2	rs45517091	16:2098755	Exon skipping	A/G	Tuberous sclerosis	Donor site	0.82	actgagagtgagtga
NCAN	rs61222528	19:19356121	Exon skipping	G/T		Acceptor site	0.65	ccctgccccctattcctgcag caatttgagaactggcgaga

Table 5.3: BDGP scores of some well known SNPs which cause exon skipping (splicing). These SNPs have been shown by *in vitro* methods to affect mRNA splicing by introducing either donor or acceptor sites, with the allele responsible for change indicated in bold text. Some of the SNPs are associated with diseases such as lung cancer (Liloglou T, *et al.* 2002), renal cell carcinoma (Kenck C, *et al.* 1996) and tuberous sclerosis (Jobert S, *et al.* 1997). SNPs were identified from (Faber K, *et al.* 2011).

Transcription factor binding predictions: In this analysis, I wanted to explore if the novel DNA variants could potentially alter transcription factor binding efficiency. Hence, I performed bioinformatic predictions using JASPAR software (http://jaspar.genereg.net/). JASPAR is a transcription factor binding profile database, that bioinformatically predicts transcription factor binding efficiency based on a given DNA sequence. I analyzed both alleles for all novel SNPs and observed that most of these novel DNA variants had the potential to influence transcription factor binding efficiency. This was reflected as a change in score between the two alleles of a SNP, whereby one allele reduced the similarity to the binding recognition sequence of the factor compared to the other allele. The SNPs with most significant scores (more than 8.0), are highlighted in Table 5.4. High scores indicate a stronger match to the sequence binding matrix for that transcription factor, and hence greater possibility that a SNP would potentially alter transcription factor binding efficiency.

In total, I found that approximately 48% of the novel variants were predicted to change a transcription factor binding score by >8.0 units, indicating that each of those variants may have some functional impact on *ST8SIA2* gene function. However each of the novel variants were rare, mostly observed in only one or two individuals, hence functional follow-up using *in vitro* methods like EMSA was not performed due to the limited impact of each individual variant and the limited time constraints for this thesis.

SNP No.	Start Pos	End Pos	Causative	Model name	Change type	Allele score
1	92919539	92919548	T	MEF2A	Score change	13.759
1	92919539	92919548	C	MEF2A	Score change	9.868
2	92920200	92920209	A	MEF2A	New Factor	8.807
3,4,5,6,7	92921350	92921367	AACTA	NR3C1	New Factor	12.19
9	92921618	92921623	A	ZNF354C	Score change	8.723
10	92923096	92923102	A	SPI1	Score change	8.725
11,12,13,14	92923865	92923871	GGAG	SPI1	New Factor	8.328
11,12,13,14	92924162	92924171	GGAG	SP1	New Factor	8.232
12,13	92924019	92924025	AG	SPI1	New Factor	8.679
17	92929228	92929234	G	NFATC2	New Factor	8.594
17	92929221	92929235	G	STAT1	New Factor	8.149
19	92930817	92930826	C	SP1	New Factor	8.074
28	92950516	92950521	T	SOX10	Score change	8.91
28	92950513	92950519	T	SPIB	Score change	8.051
30	92951832	92951849	A	NR3C1	New Factor	9.662
34	92958789	92958808	A	RREB1	Score change	15.538
34	92958789	92958808	C	RREB1	Score change	14.841
34	92958792	92958811	C	RREB1	Score change	13.982
34	92958793	92958812	C	RREB1	Score change	13.172
34	92958793	92958812	A	RREB1	Score change	11.967
34	92958792	92958801	C	SP1	New Factor	9.582
34	92958785	92958796	A	FOXI1	New Factor	9.015
34	92958793	92958802	C	SP1	New Factor	8.976
39	92961925	92961936	T	FOXI1	Score change	9.281
43,44,45,46	92967838	92967851	AGTT	RORA_2	New Factor	8.828
43,44,45,46	92967835	92967846	AGTT	IRF1	New Factor	8.66
43,44,45,46	92967836	92967853	GTAG	NR3C1	New Factor	8.645
50	92985952	92985969	G	ESR2	New Factor	9.351
51,52,53	92992053	92992066	TTT	Pax6	Score change	11.453
51,52,53	92992053	92992064	TTT	HNF1B	New Factor	10.481
51,52,53	92992054	92992060	TTT	AP1	Score change	8.68
52	92992053	92992066	C	Pax6	Score change	12.472
53	92992168	92992177	G	SP1	New Factor	10.676
53	92992170	92992179	G	SP1	New Factor	8.882
53	92992169	92992178	G	SP1	New Factor	8.793
55	92994443	92994454	G	Myf	Score change	10.524
55	92994443	92994454	A	Myf	Score change	9.524
56	93000060	93000071	T	SRF	New Factor	9.921
58,59	93007322	93007328	TC	NFATC2	Score change	11.36
58,59	93007322	93007328	CT	NFATC2	Score change	9.49
58,59	93007321	93007335	CT	STAT1	New Factor	9.113
60	93008998	93009009	A	FOXI1	New Factor	10.613
61	93010778	93010789	G	TEAD1	Score change	12.357
61	93010778	93010789	T	TEAD1	Score change	10.727
61	93010783	93010794	T	FOXI1	Score change	10.098
61	93010785	93010796	G	FOXI1	Score change	9.935
61	93010784	93010797	T	FOXF2	New Factor	9.161
61	93010786	93010796	T	FOXA1	New Factor	8.639
62	93013516	93013522	A	NFATC2	Score change	11.36
62	93013519	93013524	C	SOX10	Score change	8.91

Table 5.4: Predicted transcription factor binding results from JASPAR for the 62 novel DNA variations. Only selected results are displayed with cutoff score being selected as 8.0. Table displays novel SNPs, their start and end chromosomal positions (hg19), causative allele for that change, model name, type of change and their respective scores. Continuous SNPs with combined effect are shown together.

5.2.2. Transcriptional variation

To study ST8SIA2 mRNA and protein expression levels, I opted to focus on the DLPFC region of the brain, as it is site in the human brain responsible for higher cognitive tasks, which are disturbed in the patients with mental illness (Mega M S, Cummings J L 1994; Rajkowska G, *et al.* 2001). Unfortunately brain tissue was not available for the individuals who were selected for the resequencing study, so I moved my studies to post-mortem brain cohorts. For mRNA studies, I used cDNA from the MB and TRC post-mortem brain cohorts (Chapter 2; Section 2.2.3). For protein studies, I used TRC post-mortem brain cohort (Chapter 2; Section 2.2.2). These cohorts were used because they represented collection of well-characterized tissues with high quality RNA, and the quantities of tissue available were suitable for the experiments I wanted to perform. The MB cohort included samples of different age groups across development, while the TRC cohort included schizophrenia and control groups.

5.2.2.1. Identification of novel splice isoforms

In this analysis, I focused on ST8SIA2 mRNA structure and its expression levels to investigate if there are any changes at the transcriptional level in this gene which could increase the risk to mental illness. Initial experiments were undertaken with cDNA extracted from commercial fetal brain RNA and detailed analysis used DLPFC cDNA from the developmental MB cohort brains (Chapter 2; Section 2.1.1.3 and 2.2.3).

Different exons of *ST8SIA2* encode specific catalytic domains after translation (Figure 5.3). In addition to the full length mRNA, the *ST8SIA2* gene is known to produce a Δ2 isoform with the exclusion of exon 2, which is observed in brain tissue (www.genecards.org).

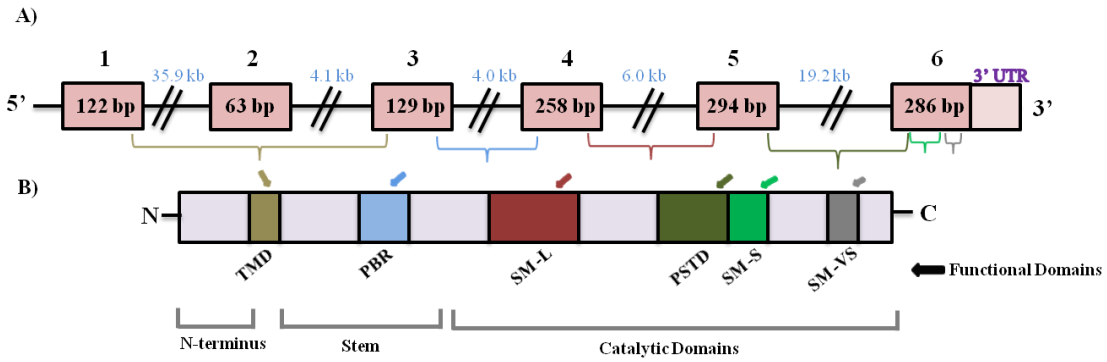


Figure 5.3: Schematic representation of *ST8SIA2* structure illustrating exons and their contribution to different functional domains. (A) Genomic structure of *ST8SIA2* has 6 exons, which are numbered and their lengths are shown inside boxes. Lengths of intron are shown in blue colour. (B) ST8SIA2 protein structure with their different functional domains such as, TMD = transmembrane domain helps in securing protein to cell membrane; PBR = polybasic region for acceptor substrate recognition; SM-L = long sialylmotif for substrate binding; PSTD = polysialyltransferase domain for catalytic activity; SM-S = short sialylmotif and SM-VS = very-short sialylmotif domain for substrate binding. N represents N-terminal cytosolic part and C represents C-terminal of catalytic domain.

In order to detect the mRNA splice isoforms of ST8SIA2 in the DLPFC, I performed a PCR covering ST8SIA2 exons 1-6 (external PCR). As the expression of ST8SIA2 in adult tissues is low, I designed a nested or internal PCR to allow the robust amplification of ST8SIA2 mRNA in all the samples (Figure 5.4).

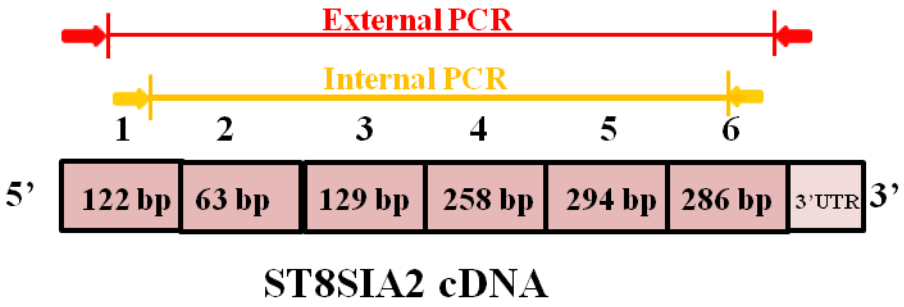


Figure 5.4: Schematic diagram showing positions of internal and external PCR primers and region covered by them. External PCR product was 1044 bp, while the internal PCR product was 983 bp.

After the first round of PCR, samples were purified and the product was used as a template in 'nested' PCR. Samples were run on 3% agarose gel and the bands that were smaller or larger than 983 bp (main product) were dissected and subjected to Sanger sequencing. The sequence data was then compared against the human genome using 'BLAT' (http://genome.ucsc.edu/) and the sequence traces were assessed manually to confirm its accuracy. Steps and primer sequences are described in detail in (Chapter 2; Section 2.3.1 to 2.3.2 and 2.4.2).

During these experiments, I identified 5 different mRNA splicing isoforms in the human DLPFC, including four previously unreported isoforms. These were:

i) ST8SIA2_*Intron inclusion*: This isoform represents an inclusion of 126bp of intronic sequence between exon 1 and exon 2 (hg19: 92956595-92956717), as shown in Figure 5.5. The isoform was initially identified in the toddler brain samples from MB cohort. The functional impact of this isoform is that the translated intronic sequence introduces inframe stop codons before exon 2 (Figure 5.6), which would stop the process of protein synthesis. This truncated isoform could be later targeted for degradation, by cellular machinery.

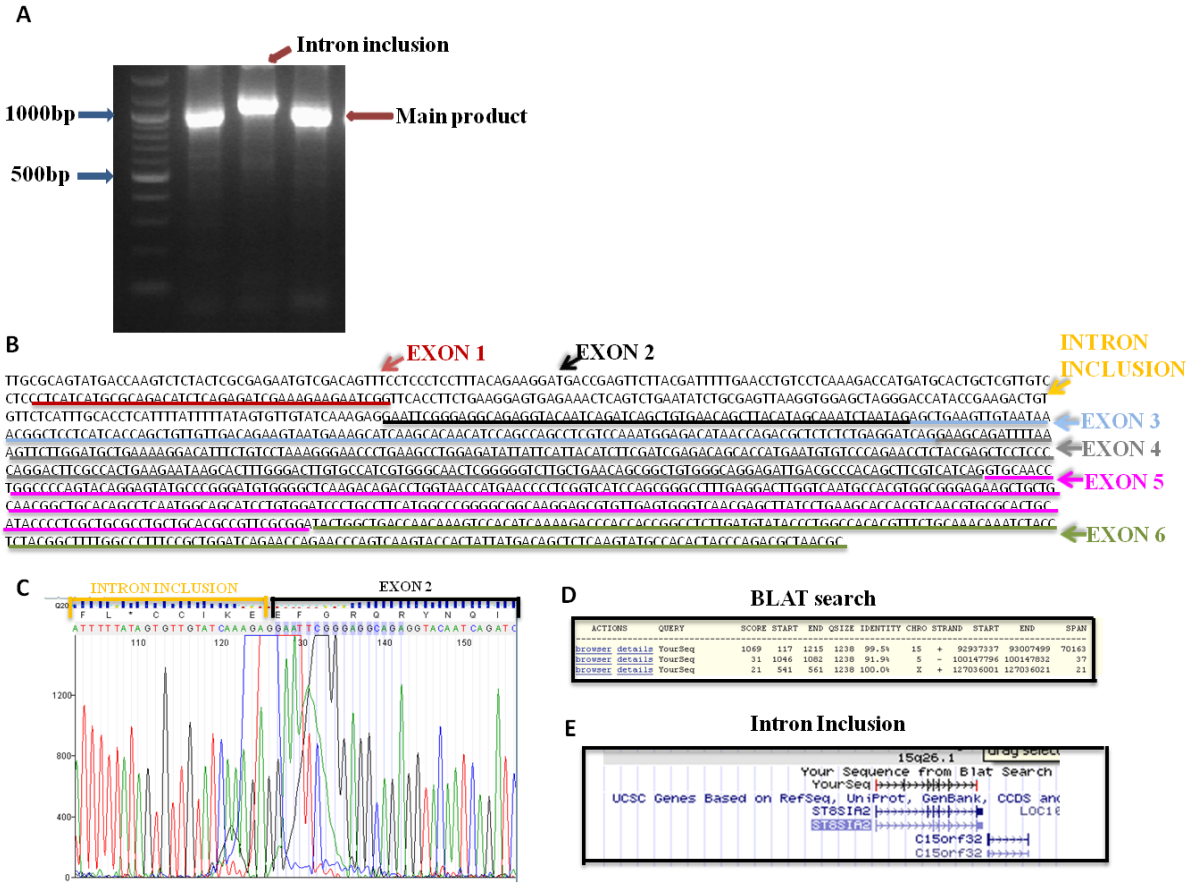


Figure 5.5: Identification of the intron inclusion splice isoform. After end-point 'nested' PCR any novel bands (A) were dissected and subjected to Sanger sequencing. Sequence traces were analyzed for its accuracy (C) and alignment to reference sequence (B) and then entered in UCSC BLAT search to confirm its location within human genome (D and E).

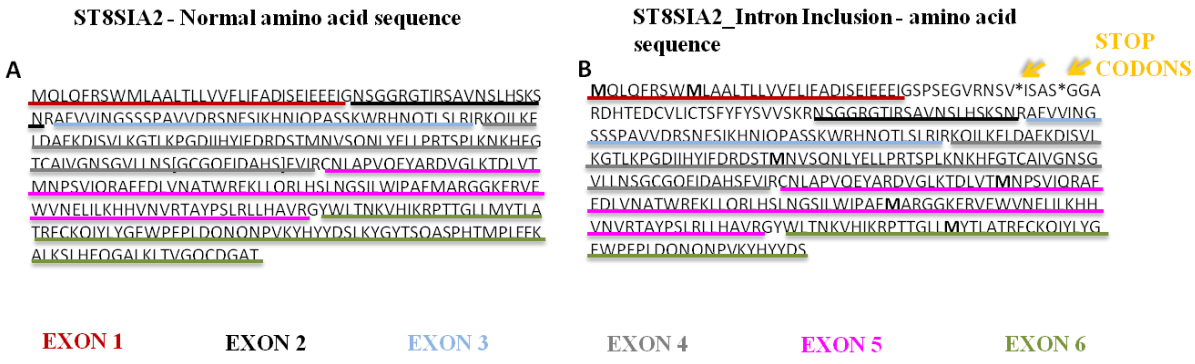


Figure 5.6: Difference between translated amino acid sequence of normal ST8SIA2 protein (A) and ST8SIA2 protein with intron inclusion (B). Sequence (B) indicates the presence of stop codons represented as "*".

ii) ST8SIA2_Δ5 *isoform*: This isoform results from the deletion of exon 5 (and as a result exon 4 and exon 6 are joined together) without changing the reading frame of the resulting protein (Figure 5.7). This isoform was initially identified in both the fetal brain and neonate samples from MB cohort. The functional impact of this isoform is the deletion of a part of the functional catalytic domains sialylmotif-L and PSTD. Loss of PSTD would mean that this isoform cannot perform polysialylation of target proteins. This isoform, if not degraded by cellular machinery, could potentially compete with the full-length ST8SIA2 isoform to bind to the NCAM's Ig domains.

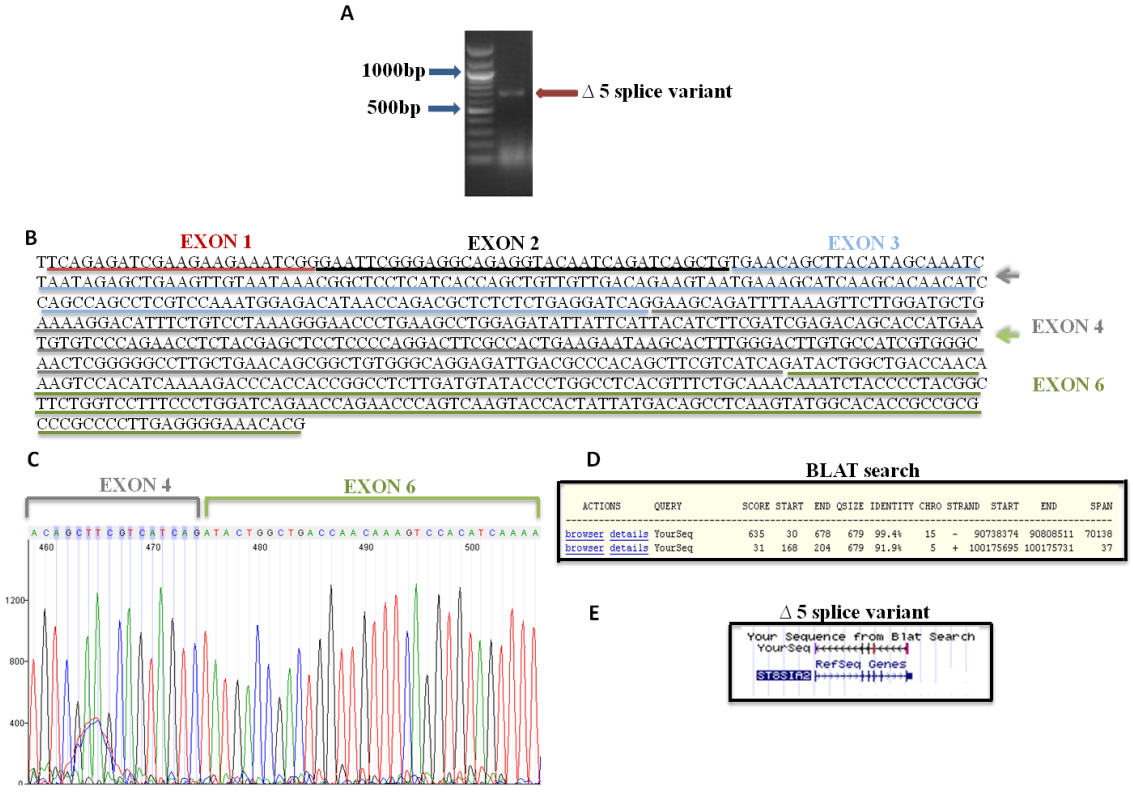


Figure 5.7: Identification of Δ5 splice isoform. After end-point 'nested' PCR any novel bands (A) were dissected and subjected to Sanger sequencing. Sequence traces were analyzed for its quality (C) and alignment to reference sequence (B) and then entered in UCSC BLAT search to confirm its location within human genome (D and E).

iii) ST8SIA2_Δ2 *isoform:* This isoform was previously known in the literature, and arises from the deletion of exon 2 (and as a result exon 1 and exon 3 are joined

together) without changing the reading frame of the protein (Figure 5.8). This isoform was initially identified in neonate and infant brain samples from MB cohort. The functional impact of this isoform is the deletion of a part of the TMD, which would likely be disruptive to protein function, although interfered adhesion to the cell membrane may result in no competitive inhibition between this isoform and wild-type protein.

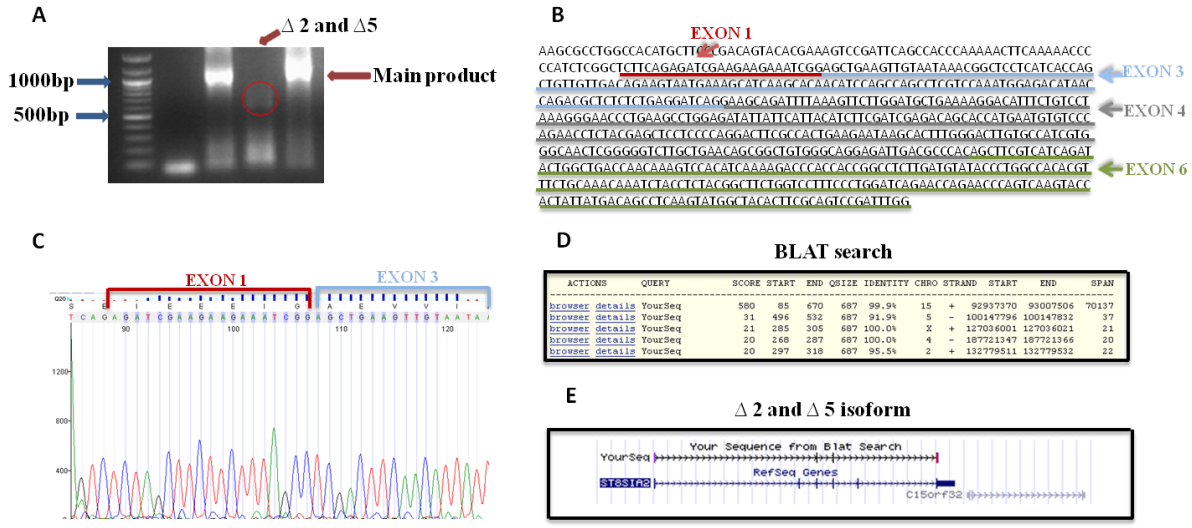


Figure 5.8: Identification of Δ2 and Δ5 splice isoform. After end-point 'nested' PCR any novel bands (A) were dissected and subjected to Sanger sequencing. Sequence traces were analyzed for its quality (C) and alignment to reference sequence (B) and then entered in UCSC BLAT search to confirm its location within human genome (D and E).

iv) ST8SIA2_Δ*2345 isoform*: This isoform arises from the deletion of exons 2, 3, 4 and 5; and as a result exon 1 and exon 6 are joined together, with no change in the reading frame of the resultant protein (Figure 5.9). This isoform was initially identified in neonate brain samples from the MB cohort. The functional impact of this isoform is the deletion of all major catalytic domains including TMD, Sialylmotif-L, PSTD and Sialylmotif-S. Given the absence of any main functional domains, this transcript is unlikely to result in competitive inhibition of full length transcript and may be targeted for mRNA degradation.

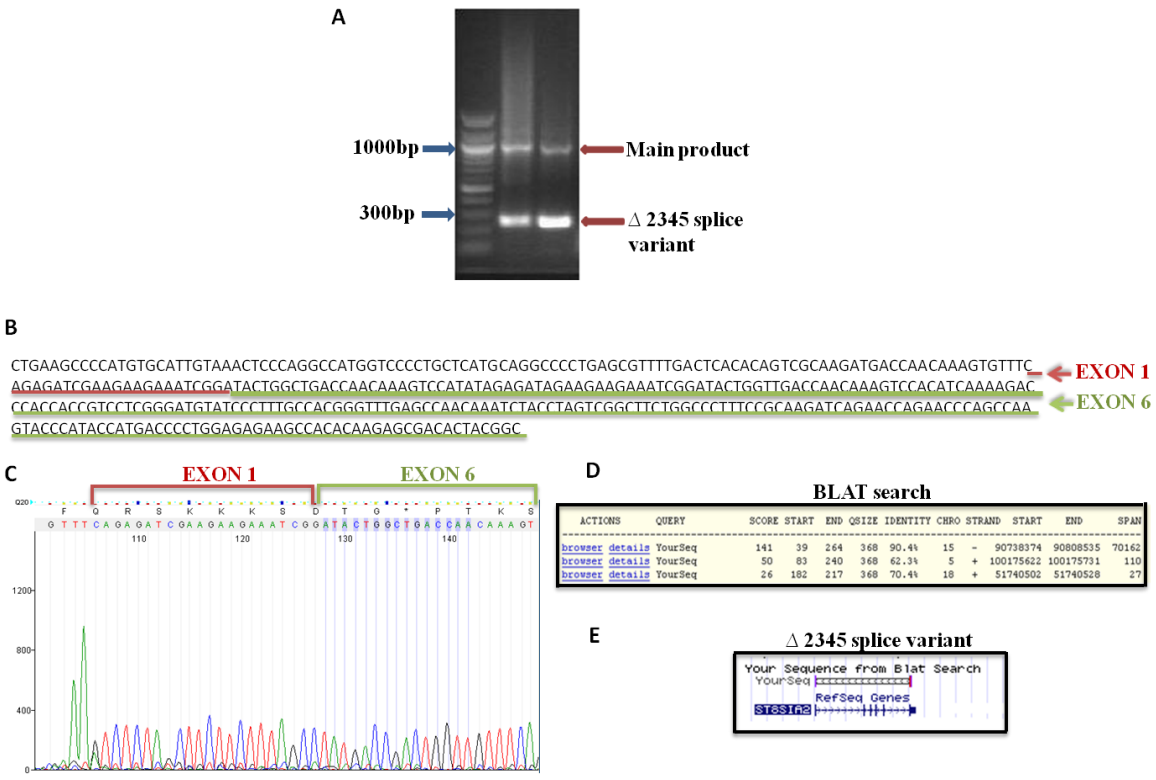


Figure 5.9: Identification of Δ2345 splice isoform. After end-point 'nested' PCR any novel bands (A) were dissected and subjected to Sanger sequencing. Sequence traces were analyzed for its quality (C) and alignment to reference sequence (B) and then entered in UCSC BLAT search to confirm its location within human genome (D and E).

v) ST8SIA2_Δ*234 isoform*: This isoform comprises a deletion of exons 2, 3 and 4 and as a result exon 1 and exon 5 are joined together, with no change in the reading frame of the resultant protein (Figure 5.10). This isoform was initially identified in neonate brain samples from MB cohort. The functional impact of this isoform is the deletion of three functional domains: the TMD, polybasic region and Sialylmotif-L. This isoform still possesses a PSTD so is potentially capable of polysialylation, if not degraded by cellular machinery, although is unlikely to compete with the full-length ST8SIA2 isoform due to lacking many of the other functionally important protein domains.

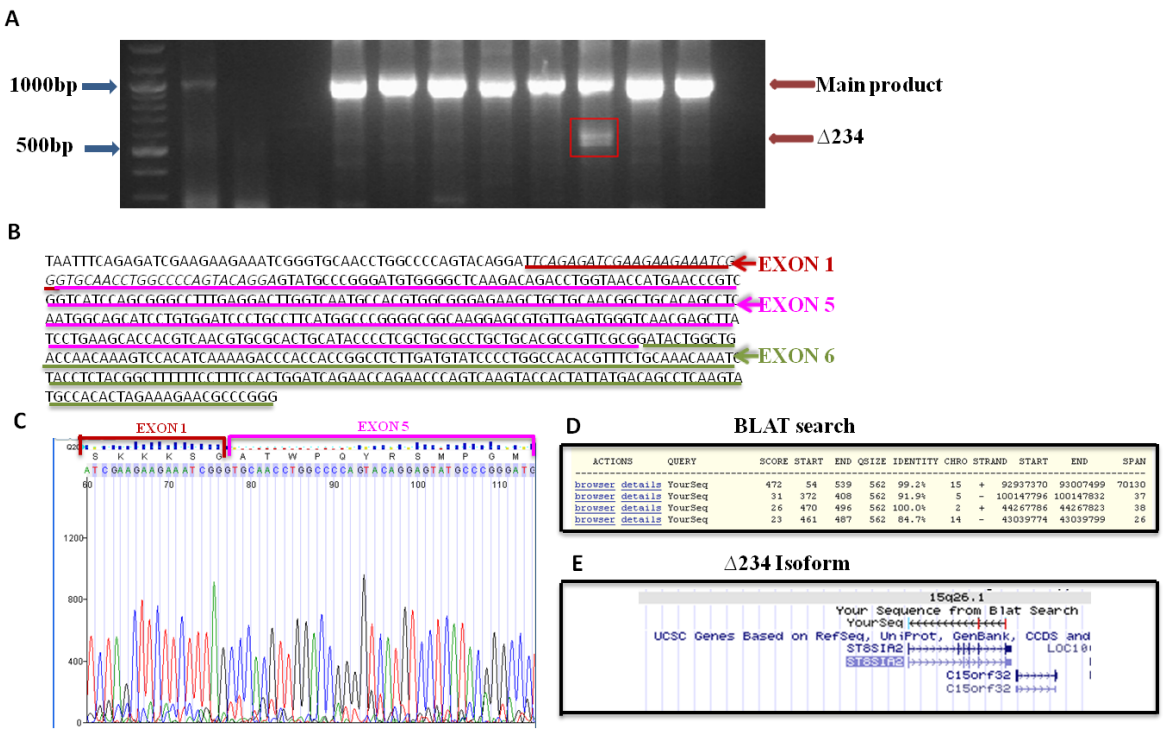


Figure 5.10: Steps involved in identification of Δ234 splice isoform. After end-point 'nested' PCR any novel bands (A) were dissected and subjected to Sanger sequencing. Sequence traces were analyzed for its quality (C) and alignment to reference sequence (B) and then entered in UCSC BLAT search to confirm its location within human genome (D and E).

In the next analysis, I wanted to see if these novel splice isoforms appear in a temporal pattern across human development. Hence, I performed nested end-point PCR (with internal and external primers) across MB cohort, which includes individuals across seven age groups from neonate to adult. As this is an endpoint PCR not a quantitative PCR, the objective was to identify subjects in which the mRNA isoform was present or absent, rather than quantifying the abundance of transcript present. None of the detected isoforms seemed to be specific to any developmental stages, rather they appeared at similar rates across all age groups (Table 5.5). Hence, it appears that formation of these isoforms is not developmentally regulated although further experiments which follow splicing quantitatively and temporally (at multiple time-points in the same individuals across different ages) would be required to confirm this. Interestingly though, the novel splicing

isoforms appear to be reasonably common in the DLPFC of individuals of unknown psychiatric condition.

	MB COHORT - AGE GROUPS							
Splice Isoform	**Neonates (n = 10)**	**Infants (n = 13)**	**Toddlers (n = 9)**	**School Age (n = 8)**	**Teenage (n = 8)**	**Young Adults (n = 8)**	**Adults (n = 7)**	**Total (n = 63)**
Δ5	1	2	2	2	1	4	2	14 (22%)
Δ2345	2	3	1	1	2	2	2	13 (20%)
Δ2	4	3	2	1	4	1	3	18 (29%)

Table 5.5: Preliminary endpoint PCR results for MB cohort. Numbers in the table represent the number of novel isoforms identified in different age groups. Age group represents: neonates (39days – 89 days), infants (118 days – 332 days), toddler (1 year– 4 year), school age (5 year– 12 year), teenage (15 year – 17 year), young adults (20 year– 23 years), adults (35 year – 49 year).

After identifying these alternate splice isoforms, I wanted to explore if there is any other evidence for the occurrence of these isoforms in publically available data. I found evidence supporting my Δ5 and intron 1 inclusion discoveries in RNA-Seq data from ENCODE/Caltech, particularly in the myocyte and HSMM (skeletal muscle) cell lines (Figure 5.11). This data also indicates that these isoforms are not specific to brain tissue.

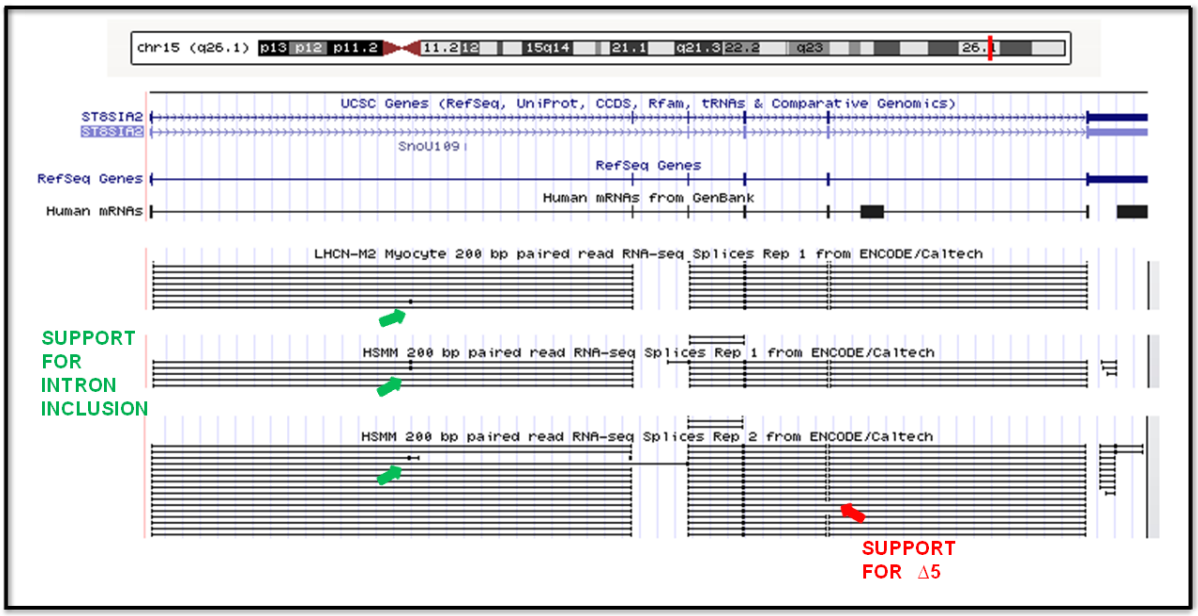


Figure 5.11: ENCODE/Caltech support for the novel alternative splice isoforms through RNA-SEQ data. Intron inclusion indicated by black dots between exon 1 and exon 2 (marked by green arrows) observed in Myocyte and HSMM cell line. Third arrow shows presence of stop codon after intron inclusion. Isoform Δ5, indicated by no dots at the corresponding position of exon 5 (marked by red arrow), was observed in HSMM cell lines.

5.2.2.2. Further validation of novel splice isoforms

In order to further validate these novel splice isoforms, I designed specific PCR assays for each of the main splice isoforms, namely; Δ2, Δ5, and Δ2345. Primers were designed such that the forward primer is placed on the junction of two adjacent exons, while the reverse primer sits on the later exon (Table 5.6).

Primers	Sequence
Δ2 F	5'- CGAAGAAGAAATCGGAGCTG -3'
Δ2 R	5'- GAGCGTCTGGTTATGTCTCCA -3'
Δ5 F	5'- CAGCTTCGTCATCAGATACTGG -3'
Δ5R	5'- CCTGGGAGGTGTAGCCATACT -3'
Δ2345 F	5'- CGAAGAAGAAATCGGATACTGG -3'
Δ2345 R	5'- CCTGGGAGGTGTAGCCATACT -3'

Exon 1
Exon 3
Exon 4
Exon 6

Table 5.6: Design of specific primers targeting a particular alternative splicing isoforms. Different exon regions are underlined with their corresponding colors, showing the specificity of primers for individual splice isoforms.

For example, Δ2 specific primers had forward primer located on the exon boundaries of exon 1 and exon 3, while the reverse primer was located towards 3' end of exon 3. Using these specific assays for the novel splice isoforms in the DLPFC of the developmental cohort, I confirmed that these isoforms are expressed in the neonate brain cDNA samples in which the isoforms were first detected (Figure 5.12).

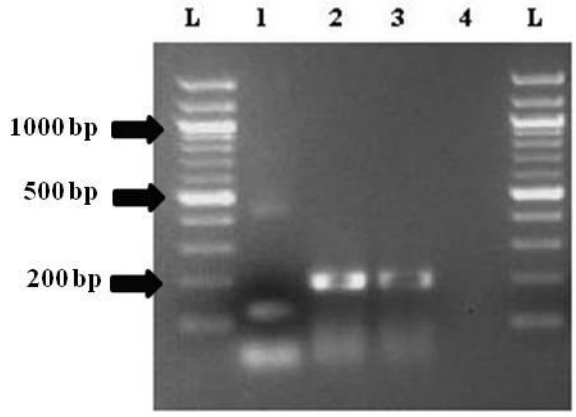


Figure 5.12: Representative figure showing three main splice isoforms Δ2, Δ5, and Δ2345 observed after using specific primers targeting a particular splicing isoform. Neonate brain cDNA from MB cohort was used as the template. L is 100bp marker. Lanes 1, 2, 3 and 4 contain Δ2 (~130 bp), Δ5 (~200 bp), Δ2345 (~200 bp) and negative control, respectively.

Optimization of these assays then allowed me to perform endpoint PCR on a pre-amplified template, to determine if these isoforms are more commonly observed in individuals with mental illness.

5.2.2.3. Role of novel splice isoforms in mental illness

To investigate if the novel splice isoforms are more common in the disease state, specific primers targeting particular alternative splicing isoform were used (Table 5.6). Screening was performed in TRC schizophrenia cohort and Stanley cohort (Supplementary material) to investigate if the presence of these splice isoforms are more common in disease state versus controls. In addition to the end-point PCR which used a pre-amplified nested PCR product as template, a specific qPCR probe was designed to quantify the Δ5 isoform concentrated cDNA direct from reverse transcriptase synthesis in TRC cohort. Isoform Δ5 is of particular interest because it is found in multiple individuals in the developmental cohort and it is likely to produce a protein which may competitively inhibit the full length isoform, as it includes most vital structural domains. The qPCR probe was located on the boundaries of exon 4 (3' end) and 6 (5' end) with the context sequence 5'-TCGTCATCAGATACTGGCTGACCAA'-3' and an amplicon size of 61 base pairs. Data for the nested endpoint PCR and qPCR was compared and combined for Δ5 detection (presence/absence comparison). Student's t-test did not confirm any significant effect of any splice isoform on the disease status, Δ5 (p = 0.244, t = 1.17, df = 72), Δ2345 (p = 0.092, t = 1.10, df = 72) and Δ2 (p = 0.087, t = -1.73, df = 72).

Considering that DNA variations could affect splice donor/acceptor site recognition, I wanted to assess the DNA variation in *ST8SIA2* to identify variations which may contribute to Δ5 mRNA formation. I examined the region around exon 5, and found a SNP (rs11637874) located only 4 basepairs after the 3' end of exon 5, and splicing prediction analysis showed a high BDGP score for the minor T allele (MAF = 0.19; BDGP score = 0.95), but with a reduction in score for the major C allele (BDGP score = 0.81). I selected SNP rs11637874 for genotyping of the TRC cohort to see if genotype related to formation of the Δ5 isoform. However, there was no significant effect of rs11637874 genotype (CC vs

CT or TT) on ST8SIA2 Δ5 isoform production (p = 0.501, F = 0.457, n = 72, df = 1). Nor was there an effect of genotype × diagnosis (p = 0.223, F = 1.514, n = 70, df = 1).

5.2.2.4. Global ST8SIA2 mRNA expression

Next, I studied the global mRNA expression of ST8SIA2 to analyze if there is any difference in expression of this gene between schizophrenia cases and normal controls. Previously, our group had used exon 5-6 as a pan probe, but after discovering the prevalence of the Δ5 isoform, the use of the exon 5-6 "pan" probe was no longer appropriate. However, there were limited options on what could be chosen as a "pan" as I had discovered that all exons are alternatively spliced in the DLPFC (see Chapter 5.2.2.1). As the Δ5 and Δ2 isoforms are more likely to have direct functional effects, I therefore selected an exon 3-4 Taqman probe (Hs00195648_m1; Invitrogen) to study ST8SIA2 mRNA expression.

First, ST8SIA2 mRNA expression was analysed to see if it was influenced by brain cohort demographic variables. These quantitative variables comprised of age at death (Age), the time between death and brain harvesting and preservation i.e. post-mortem interval (PMI), the quality of RNA after extraction from brain tissue i.e. RNA integrity (RIN), brain pH, total RNA yield, age at disease onset (age of onset), duration of the illness in the cases (illness duration), levels of drugs as chlorpromazine equivalents (daily, last and life time levels).

ST8SIA2 mRNA expression was found to be significantly correlated with pH (p = 0.000649, r = 0.400, N = 69) and age (p = 0.000085, r = ¯0.455, N = 69). The negative correlation with age is consistent with previous findings that ST8SIA2 expression declines with age. Significant demographic variables were included as covariates in all ANCOVA calculations. Notably, no significant group difference was observed between ST8SIA2 mRNA expression and disease status ANCOVA results [F (1, 65) = 5.15, p = 0.475; Using age and pH as co-variates].

Next analysis was performed to see if global expression of ST8SIA2 was associated with the specific risk haplotype previously identified by our group (McAuley E Z, *et al.* 2012). However, I did not see any effect of the risk [F (1, 62) = 0.148, p = 0.701] or protective [F (1, 62) = 0.178, p = 0.186] haplotype on global ST8SIA2 mRNA expression in the DLPFC.

5.2.3. Protein variation

These experiments were performed to test if the levels of ST8SIA2 protein varied according to psychiatric diagnosis. Western blotting was performed by using the proteins extracted from TRC brain tissue. Housekeeper β-actin was used to check the protein loading (Figure 5.13). However, the final data was not normalized to β-actin because the quantification range for β-actin was far lower (at 0-10 µg, Figure 4.5) than the ST8SIA2 protein loaded on the gel (20 µg). Therefore, I used the percentage internal control method to normalize the western blot data. Average values between three rounds of replicate experiments were used for the final data analysis and the values outside twice the standard deviation of the average means were excluded to reduce outlier effects. Average correlation values for the three repeated rounds of quantitation were 0.505 (ST8SIA2) and 0.645 (PSA-NCAM). Detailed steps and information regarding the use of different antibodies are described in (Table 2.10). Data was normally distributed and therefore parametric statistics were applied.

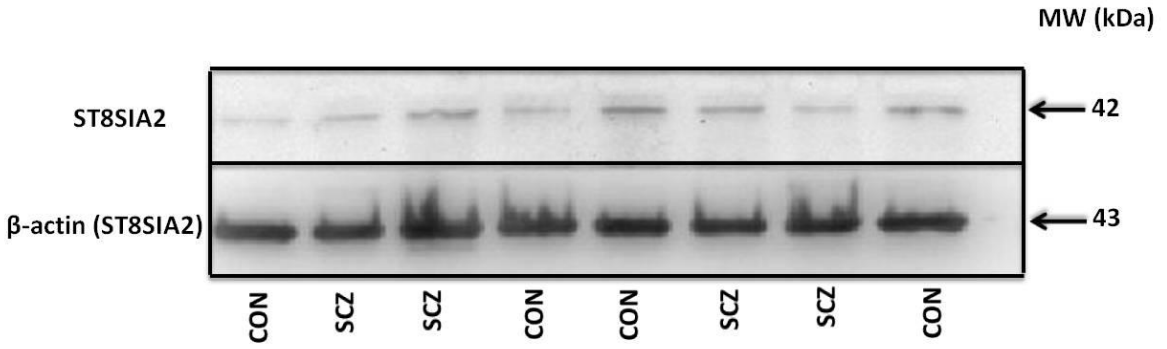


Figure 5.13: Representative Western blot image showing ST8SIA2 protein and β-actin bands detected in DLPFC. Total 20 µg of protein was loaded in each lane of the gel. Linear range for quantification was determined from five point standard curve.

Analysis of the demographic variables was performed to investigate if the ST8SIA2 protein expression levels were influenced by the brain cohort demographic and clinical variables. Variables included age, PMI, brain pH, age of disease onset, illness duration and levels of drugs as chlorpromazine equivalents (daily, last and life time levels). A significant correlation was observed with PMI (r = 0.269, p = 0.020, N = 74). As a result, PMI was included as a covariate in the ANCOVA analysis.

Investigating ST8SIA2 expression and diagnosis, a significant effect was observed by Student's t-test (Figure 5.14), where ST8SIA2 protein levels were increased by about 30% in schizophrenia cases relative to controls (p = 0.05, t = 1.98, df = 72).

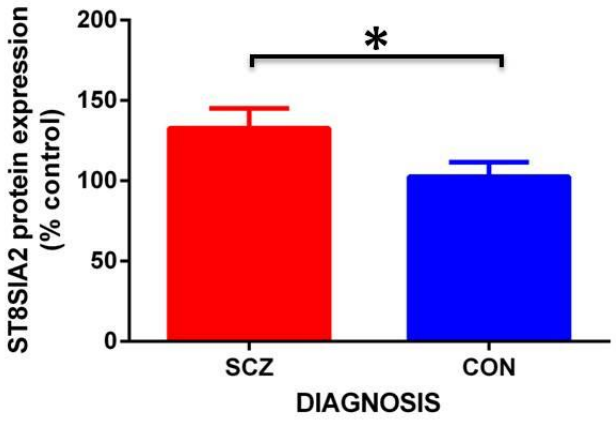


Figure 5.14: Significant increase in the ST8SIA2 protein levels in schizophrenia cases. Student's t-test results were **(p = 0.05, t = 1.98, df =72)**. Group means 132.8±12.37 for schizophrenia (SCZ) cases and 102.7 ±8.857 for unaffected controls (CON). Numbers of individuals were 37 in each group. Bars denote standard error of the mean (SEM).

No significant effect of the risk or protective haplotype was observed on ST8SIA2 protein expression, either by itself or in conjunction with the diagnosis (all p > 0.600). However, a significant ~25% increase was observed in the ratio of ST8SIA2/PSA-NCAM in schizophrenia cases by Student's t-test (p = 0.050, t = 1.985, df = 72) (Figure 5.15).

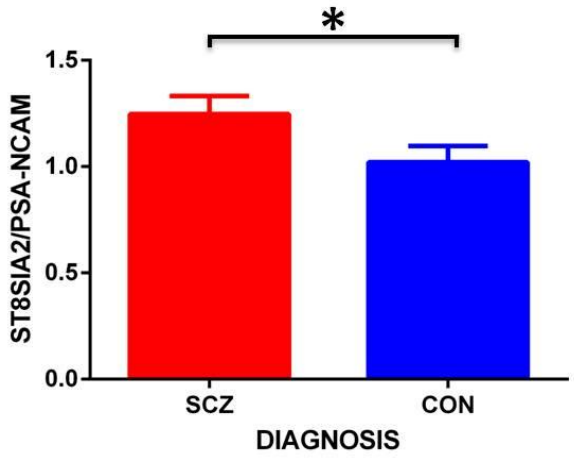


Figure 5.15: Significant increase in the ratio of ST8SIA2/PSA-NCAM in schizophrenia cases compared to controls. Student's t-test results were (p = 0.050, t = 1.985, df =72). Group means 1.247±0.085 for schizophrenia (SCZ) cases and 1.021 ±0.0755 for unaffected controls (CON). Numbers of individuals were 37 in each group. Bars denote SEM.

In Chapter 4, I observed a suggestive decrease in PSA-NCAM protein expression in female schizophrenia cases (Figure 4.10). Therefore, results from analysis of ST8SIA2 enzyme levels suggest that an increase in ST8SIA2 enzyme is not contributing towards increasing PSA-NCAM levels, but may in fact represent a compensatory increase of protein synthesis in the presence of variations which reduce functionality of existing protein.

5.3. Discussion

The overall goal of this chapter was to investigate how variations in *ST8SIA2* at genetic level and its expression at mRNA and protein level, could be associated with mental illness. At the genetic level, 62 novel DNA variations were observed in people with bipolar disorder after the resequencing study. Around half of these novel variations were bioinformatically predicted to alter transcription factor binding sites. In addition, six SNPs were predicted to create splice donor/acceptor sites with score changes which were in-line with those previously described to cause splicing changes resulted in increased disease risk. At the mRNA level, I found 4 new splice variants in the human DLPFC, in addition to the already known Δ2 isoform. I could not relate these novel splice variants to SNP variation identified in the genomic variation chapter (5.2.1), as that study used a different set of

subjects. However, I did examine genotype effects of a SNP on the exon 5 boundary, which seemed not to relate to formation of the Δ5 isoform. I did not observe any significant association between global ST8SIA2 mRNA expression and disease state, but a significant effect was observed at the protein level, where I found an increase in the expression of ST8SIA2 protein in schizophrenia cases relative to controls. Subsequently, I also observed an increase in the ratio of ST8SIA2/PSA-NCAM in the schizophrenia cases. These results need to be verified in a larger cohort.

With particular focus on entire *ST8SIA2* genomic structure and focusing on the DNA variations in the disease state, I provided results from 454 next generation sequencing performed on bipolar disorder cases, in this chapter. In resequencing a region of ~95 kb in 48 individuals (i.e. total region ~ 4.5 Mb), a total of 380 SNPs were identified, including 62 novel SNPs. None of the SNPs identified affected the protein coding sequence, although one previously known synonymous SNP (rs2305561, P186P) in exon 5, two known SNPs in the 5'UTR of exon 1, and 10 SNPs in the 3'UTR were identified. The number of novel SNPs was higher in 5'-upstream region (22/62) and intron 1 region (23/62), suggesting high genetic variation in this area. Most of the novel SNPs were also predicted to alter transcription factor binding, suggesting a functional nature of these SNPs. Interestingly, this region lies in the LD (linkage disequilibrium) block with SNPs that showed significant association with bipolar disorder (McAuley E Z, *et al.* 2012) and autism (Anney R, *et al.* 2010).

In eukaryotes, genes undergo several post-transcriptional changes; with alternative splicing occurring in 92-94% of all human pre-mRNA transcripts. As a result of this process, several mRNA isoforms are produced, which later give rise to a number of protein isoforms to regulate a diverse range of specific functions (Grigoryev Y A, *et al.* 2009; Papanastasiou A D, *et al.* 2012). In the past, several SNPs have been reported by other groups to be involved in erroneous splicing events that eventually lead to a disease, such as cancer and tuberous sclerosis (Faber K, *et al.* 2011; Jobert S, *et al.* 1997; Kenck C, *et al.* 1996; Liloglou T, *et al.* 2002). Hypothesizing that DNA variations identified in *ST8SIA2* in mental illness cases could be functional and contribute towards the disease, bioinformatic

predictions were performed to see if these variations play a role in differential mRNA splicing. Using the same software, the scores of 6 novel DNA variations were predicted to create splicing donor/acceptor sites, with scores which were comparable to the scores of well known DNA variations that caused splicing related diseases (Faber K, *et al.* 2011; Jobert S, *et al.* 1997; Kenck C, *et al.* 1996). Therefore, novel SNPs in *ST8SIA2* could cause differential splicing to produce different mRNA isoforms of ST8SIA2, although direct testing of this was not performed due to the rarity of the individual novel SNPs, the unavailability of an appropriate mRNA source for subjects in which the novel variation was observed, and time constraints within this thesis.

The 3'-UTR region of a gene plays a very important role in mRNA localization, stability and its conversion into protein [reviewed in Chatterjee *et al* (2009)]. Polymorphisms in this region have been shown to effect stability and binding properties of mRNA (Al Rayyan N, *et al.* 2013). A study by Gong *et al* (2013) has shown that altered miRNA binding to mRNA could increase risk to mental illness. In my resequencing study, a number of SNPs were identified in the 3'-UTR region. A future study to test their functional capability to effect mRNA binding and stability of the primary transcript would be very exciting and is currently underway by other researchers in my laboratory.

To carry out crucial functions including recognition and binding of donor and acceptor substrates, ST8SIA2 has different functional domains known as polybasic region (PBR), large (L), small (S) and very small (VS) sialylmotifs (Datta A K, Paulson J C 1995; Datta A K, *et al.* 1998; Geremia R A, *et al.* 1997; Harduin-Lepers A, *et al.* 2001). In addition, ST8SIA2 has a characteristic sequence of 32 amino acids, known as polysialyltransferase domain (PSTD), which is essential for its catalytic activity. It is situated just upstream of sialylmotif S and differentiates polySTs (ST8SIA2 and ST8SIA4) from the other sialyltransferases. Loss of catalytic activity has been reported as a result of PSTD removal (Nakata D, *et al.* 2006). Considering the importance of different motifs in ST8SIA2 functioning, I hypothesized that alternative splicing isoforms of ST8SIA2 may impact function and increase risk of mental illness. My endpoint PCR results confirmed the presence of several new isoforms of ST8SIA2. Isoform Δ5 is of particular interest as it

deletes parts of both the sialylmotif L and PSTD. This isoform could potentially compete with the full-length ST8SIA2 isoform for NCAM1 binding, as it retains TMD, PBR and most of SM, and so could potentially competitively inhibit full-length protein, and reduce formation of PSA-NCAM. In contrast, the Δ2 isoform would lose its capacity to bind the golgi membrane and therefore is less likely to competitively inhibit full-length protein, with potentially a smaller impact on PSA-NCAM formation. These results are exciting and identify ST8SIA2 to be transcriptionally more complicated than initially thought. Knowledge of these novel isoforms will be important when investigating the function of ST8SIA2 in future.

For ST8SIA2, most of the expression-based studies are conducted in animal models, likely due to the difficulties in studying ST8SIA2 expression in adult tissues, and with human studies limited to post-mortem brain samples where subjects are typically of more advanced age at the time of death. ST8SIA2 expression is reduced in the mature brain (McAuley E Z, *et al.* 2012), where it is constrained to the areas undergoing a regular synaptic reorganization (Hildebrandt H, *et al.* 1998). Previous published findings have reported ST8SIA2 over-expression and under-expression defects in animal model (Hildebrandt H, *et al.* 2009) and cell culture studies (Close B E, Colley K J 1998; Horstkorte R, *et al.* 1999). ST8SIA2 expression is crucial for correct hippocampus formation (Brocco M A, Frasch A C 2006) and its deficit is linked to defective mossy fibers tracts, synapse formation (Angata K, *et al.* 2004). Furthermore, complete absence of *ST8SIA2* gene is linked to severe brain abnormalities (Hildebrandt H, *et al.* 2009). Nevertheless, precise control of ST8SIA2 expression during development is very important because its over-expression is linked to distorted adhesion to substrate, causing cell death (Horstkorte R, *et al.* 1999) and cancerous metastatic invasion (Falconer R A, *et al.* 2012). ST8SIA2 over-expression has also been reported to interfere with cell viability (Brocco M, *et al.* 2003). In my mRNA studies, I did not observe any significant effect of ST8SIA2 global expression on disease status or specific risk haplotype, but in protein expression studies, I noticed a significant 30% increase in ST8SIA2 protein level in DLPFC in schizophrenia, although this did not reflect in an overall increase in PSA-NCAM, suggesting a quantitative upregulation of ST8SIA2

to compensate for a functional deficit. Results again highlighted discordant mRNA and protein expression in this cohort, but such inconsistent results have been reported before (Chen G, *et al.* 2002). In a systematic proteomic and transcriptomic analysis, it has been predicted that correlations between mRNA and protein expression levels could be as low as 40% (Vogel C, Marcotte E M 2012). Possible reasons could be involvement of different complex post-transcriptional mechanisms between mRNA and protein synthesis, *in vivo* half-lives of protein isoform and potential errors and noise values in both mRNA and protein quantification (Greenbaum D, *et al.* 2003). However, my results are investigatory and the replication studies should be performed in a larger cohort to confirm them.

There have been reports previously of valproic acid, a drug commonly used to treat bipolar disorder, effecting the expression of ST8SIA2 mRNA (Beecken *et al*., 2005), however I found no significant effect of antipsychotic use (chlorpromazine equivalent) and ST8SIA2 protein or mRNA expression. There have also been reports that cocaine increases ST8SIA2 mRNA expression and PSA-NCAM formation (Mackowiak M, *et al.* 2011), and there is a possibility that the increase in ST8SIA2 expression I have observed could be due to substance abuse, a common problem in schizophrenia cases (Soyka M, *et al.* 1993). However, I had no data on cocaine use in TRC cohort to perform this correlation. Notably, I did not notice a subsequent increase in PSA-NCAM levels as had been observed in the study of Mackowiak *et al* (2011); rather I detected a suggestive increase in ratio of ST8SIA2/PSA-NCAM, suggesting that the expression pattern of ST8SIA2 and PSA-NCAM is not unidirectional for schizophrenia.

This study has some limitations such as schizophrenia and normal control samples were not included in 454 sequencing, which was due to limited funding availability. For this reason, correlations between the SNPs predicted to alter splicing and splicing expression studies could not be established. Also, protein and mRNA expression experiments could not be performed in the Stanley Medical Research Institute post-mortem brain cohort, which includes both bipolar disorder and schizophrenia cases, as the studies would have required abundant RNA and protein from this very limited international resource, which was not possible. Now that more targeted questions could be asked with regards to ST8SIA2

function, future experiments could involve working with the Stanley Medical Research Institute cohort so that genetic, mRNA, and protein studies could be performed in samples with schizophrenia, bipolar disorder and control individuals.

In conclusion, the findings from this study indicate that variations exist in the genetic structure of ST8SIA2 that could affect its transcription and mRNA splicing, eventually leading to dysregulation of its protein expression and possibly associating this gene with psychotic mental illness. Further work is required to confirm this hypothesis.

Chapter 6

General Discussion

6.1 General Discussion

This thesis involved the examination of three target genes at the genetic, RNA and protein levels to identify difference in their structure and any alteration in their normal levels of expression or activity that could have a potential functional impact on the disease status. The results from this thesis have provided a distinctive contribution to the knowledge about variations present in these candidate genes and their possible relation to mental illness. My results require replication in other cohorts, but they provide underpinning to perform further functional studies of these interesting functional candidate genes.

Studying NRG1 (Chapter 3), I performed targeted sequencing of upstream regulatory sequences of alternative promoters of this gene and identified 26 novel DNA variations, out of which 11 were found only in people affected with schizophrenia. I observed an increased novel variant load in schizophrenia cases. Further bioinformatic prediction results indicated that seventy-five percent of these novel SNPs were capable of altering transcription factor binding and through *in-vitro* experiments, I showed that novel SNPs could actually reduce protein binding efficiency compared to the wild-type allele. Results further identified that schizophrenia cases had more nucleotide diversity in their genetic structure and identified nucleotide diversity was found to be increased in the HAPICE region, a schizophrenia-associated-risk haplotype identified in Icelandic population (Stefansson H, *et al.* 2003; Stefansson H, *et al.* 2002). The main finding of my study was the identification of a new mechanism through which HAPICE haplotype could be increasing disease risk: *via* increasing the expression of NRG1 type III mRNA isoform. NRG1 type III is the most brain abundant NRG1-subtype and the only membrane bound isoform of NRG1, which is capable of back-signaling, as well as forward signaling through interaction with ErbB4 receptors (Bao J, *et al.* 2003). Overall, the findings suggest that individuals with HAPICE risk alleles contribute in part to an earlier age of disease onset through elevated NRG1 type III levels.

Studying *NCAM1* (Chapter 4), I tried to replicate the main association findings of Arai *et al* (2004) and Atz *et al* (2007) with mental illness, in an Australian bipolar disorder cohort. However, I observed only very weak association of any SNP with mental illness. Looking

at DNA variation within this gene, I focused only on its functional motifs important for its glycosylation and interactions with polySTs (Foley D A, *et al.* 2009). Through a resequencing study, a total of 21 SNPs were genotyped, including 5 novel SNPs. I also observed that the mRNA expressions of different NCAM1 isoforms were altered in brains of individuals with mental illness. Results showed a significant increase in expression of NCAM-180 mRNA, but the affect was specific to right brain hemisphere, a region which has been previously associated with schizophrenia due to errors in inter-hemispheric communication (Corradi-Dell'Acqua C, *et al.* 2012). However, this effect was not repeated at the protein level in my studies, where I observed a significant increase in protein levels of NCAM120 isoform in schizophrenia cases compared to normal controls. I also observed some gender specific effects (particularly in females), including an increased expression in the ratio of NCAM120/Total NCAM protein, a decreased expression in the ratio of main glycosylatable NCAM1 isoforms (i.e. NCAM140+180+D) and a corresponding decrease in PSA-NCAM protein, this effect was however not significant. A decrease in the glycosylatable NCAM isoforms in schizophrenia cases may result in a reduction in available substrate (glycosylatable NCAM) that enzyme (ST8SIA2) could modify into product (PSA-NCAM). A suggestive indication in reduction in PSA-NCAM in female schizophrenia subjects is in agreement with Barbeau *et al* (1995) and Gilabert-Juan *et al* (2012) findings as they also noticed a decrease in PSA-NCAM expression in schizophrenia. Decrease in PSA-NCAM expression could affect neuronal plasticity, as PSA-NCAM is the main controller of synaptic plasticity, and decreases signaling through NMDA GluN2B receptors (Kochlamazashvili G, *et al.* 2010). My findings suggest that in psychotic illness, an increase in non-glycosylatable NCAM1 isoforms relative to glycosylatable isoforms could cause a decrease in PSA-NCAM formation, a requirement for maintaining synaptic plasticity (Muller D, *et al.* 1996; Senkov O, *et al.* 2012), although this hypothesis requires further verification.

Studying *ST8SIA2* (Chapter 5), I focused my resequencing study on bipolar disorder cases only, including those who contributed to a bipolar linkage peak at 15q25-26, identified by our group (McAuley E Z, *et al.* 2009). The results identified 380 DNA variations overall

within *ST8SIA2* gene, including 62 novel DNA variations, however no non-synonymous SNPs were found in the coding region of *ST8SIA2* gene. Interestingly, most of the novel variations were predicted to alter transcription factor binding, and 6 novel SNPs were found to have a putative role in RNA splicing by either creating a donor or acceptor splice site. It must be noted that these are bioinformatic predictions and functional confirmations were not performed to verify those putative functions. I also reported four new splice isoforms in ST8SIA2 mRNA structure: namely the Δ5, Δ2345 and Δ234 exon deletions, and an intron inclusion; together with the already known Δ2 isoform. Although, there was no significant change in ST8SIA2 mRNA expression in the brains of individuals with psychotic mental illness and control group, I observed a significant increase in the amount of ST8SIA2 protein in people affected with mental illness, and an increase in the ratio of ST8SIA2/PSA-NCAM, despite observing a decrease in PSA-NCAM in female schizophrenia patients. Interestingly, Brocco *et al* (2003) identified that over-expression of ST8SIA2 protein on the neuronal surface could interfere with cell viability, therefore an increase in ST8SIA2 enzyme could be responsible for neuronal death and a reduction in PSA-NCAM. Another interpretation could be that the cell is producing more ST8SIA2 protein because the ST8SIA2 protein already available is not efficient or capable in carrying out its activities, and this could be because splice isoforms like Δ5 that lack the main polysialyltransferase domain.

Overall, my results are somewhat in agreement with some previously known findings in peer-reviewed journals, for example dysregulation of NCAM isoforms at mRNA and protein level and decrease in the level of PSA-NCAM protein in patients with psychotic illness. In addition, my results provide explanations to some unclear concepts relating to both bipolar disorder and schizophrenia, for example the mechanism through which HAPICE risk haplotype presents risk to mental illness. It highlights the importance of looking at the effect of brain hemisphere and gender on disease status and the identification of novel splice isoforms of gene. My approach to normalize western blot data, using a % control method, for normalizing proteins which are expressed at very low or very high levels relative to standard protein housekeepers, offers an alternative and reliable method to

use where standard normalization is unreliable. Replication studies in larger cohorts with equivalent number cases and controls matched for gender and brain hemisphere would further confirm my preliminary findings.

6.4 Cell signaling and mental illness

The three selected candidate genes play a very important role in cell signaling, which is a process that involves flow of information from extracellular sources into the cells for suitable implementation of the system and communication between cell membrane, nucleus and cytoskeleton. The nerve cells or neurons are constantly exposed to various stimuli in a biological system, such that a continuous flow of signals from one cell to another is often required to regulate gene expression and other brain functions. It is mediated by signaling molecules (ligands), specific receptors, and machinery for transducing signals (Singh A B, Harris R C 2005).

NRG1 is a growth factor that signals by activating membrane-associated tyrosine kinases, especially the ErbB4 receptor kinases (Falls D L 2003). Interestingly, my results showed an association between the HAPICE risk haplotype and increased NRG1 type III mRNA expression levels in the human frontal cortex and specifically point to over expression of type III isoform as a novel mechanism of disease risk. An increase in type III NRG1 would mean increase in contact dependent or juxtacrine signaling (Bao J, *et al.* 2004; Bao J, *et al.* 2003; Hancock M L, *et al.* 2008; Wang J Y, *et al.* 2001). Furthermore, Type III NRG1's proficiency in back signaling to the nucleus by proteolytic activity and secretion of an intracytoplasmic domain could induce transcriptional related changes (Bao J, *et al.* 2003) and could activate P3K signaling pathway and promote the insertion of α7* nicotinic acetylcholine receptors into axonal membranes (Hancock M L, *et al.* 2008). Type III NRG1 back-signaling could change the release amount of neurotransmitter acetylcholine (Jones I W, Wonnacott S 2004; MacDermott A B, *et al.* 1999) and is capable of contributing to both physiological as well as pathological changes in the brain (Bermingham-McDonogh O, *et al.* 1997; Hancock M L, *et al.* 2008; Kerber G, *et al.* 2003). Therefore, my results offer additional confirmation that dysregulation of different NRG1 isoform expression in DLPFC may be related to the pathophysiology of schizophrenia.

Some molecules perform signaling by adhering to the other molecules and a good example is NCAM1, which is involved in both Ca^{2+}- independent homophilic (NCAM binding to NCAM) and heterophilic (NCAM binding to other molecule) manner. NCAM's adhesive properties are restricted by the addition of PSA chain. My studies identified a suggestive decrease in PSA-NCAM expression, although the effect was gender specific (observed only in female schizophrenia patients) and not significant. The results from Barbeau *et al* (1995) have previously reported decreased immunoreactivity of PSA-NCAM in post-mortem hippocampal neurons in schizophrenia. Recently, Gilabert-Juan *et al* (2012) also confirmed this decrease in deeper layers of DLPFC. Presence of PSA-free NCAM, in absence of polySTs, is coupled with severe brain defects due to NCAM gain of function (Hildebrandt H, *et al.* 2009). PSA-NCAM is involved in neurotrophin signaling through high-affinity-receptor Trk, while PSA-free NCAM enhances low-affinity-receptor p75 signaling (Gascon E, *et al.* 2007a). Gascon *et al* (2007a) have proposed that PSA-NCAM promotes cell-survival through controlled expression of p75 receptor in developing neurons, as receptor p75 plays a role in cell-apoptosis (Kenchappa R S, *et al.* 2010; Yan C, *et al.* 2005). Decreased expression of PSA-NCAM could not only increase p75 receptor expression, but also could cause unblocking of GluN2B-containing glutamate receptors. This unblocking could activate Ras-GRF1-p38 MAPK signaling cascade, leading to faulty synaptogenesis, impaired cognitive tasks and thus increased risk of developing schizophrenia and other mental disorders (Kochlamazashvili G, *et al.* 2012; Kochlamazashvili G, *et al.* 2010; Senkov O, *et al.* 2012). Interestingly, L1CAM, a heterophilic binding partner of NCAM1 (Heiland P C, *et al.* 1998; Horstkorte R, *et al.* 1993), binds ErbB receptors through Ig-like domains coupling cell adhesion and neuregulin signaling (Donier E, *et al.* 2012). Anton *et al* (2004) study found ErbB4 receptors in a migrating neuroblast subpopulation that were PSA-NCAM positive (Figure 6.1).

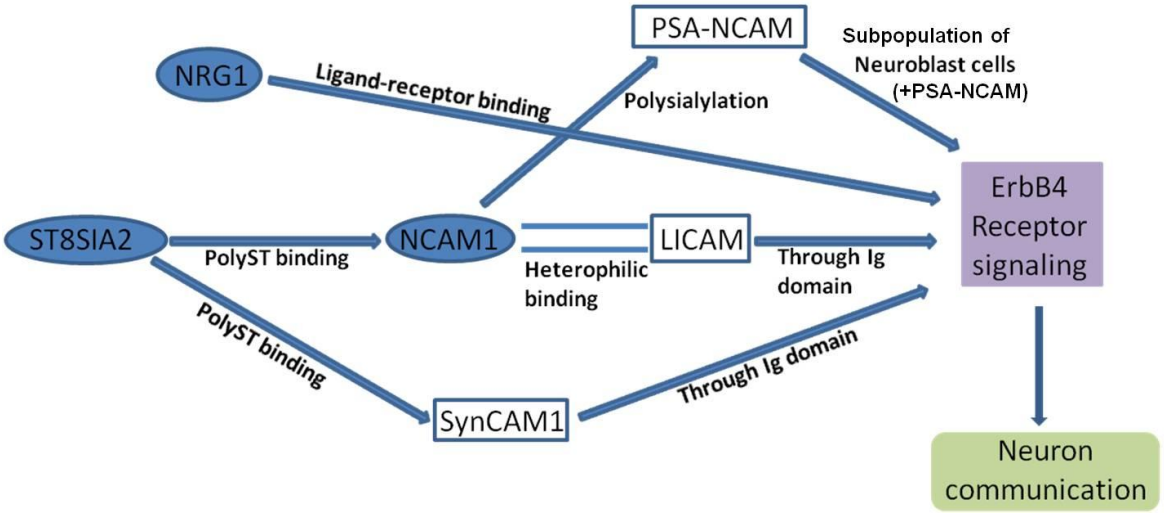


Figure 6.1: Figure showing how three candidate genes *ST8SIA2*, *NRG1*, *NCAM1* are connected in the process of cell-signaling and contributing towards neuron communication which is very important for brain development and its proper functioning.

Similarly, ST8SIA2 glycosylates other members of Ig superfamily such as SynCAM1 (synaptic cell adhesion molecule 1) (Rollenhagen M, *et al.* 2012), which is involved in erbB4 receptor mediated signaling (Sandau U S, *et al.* 2011a; Sandau U S, *et al.* 2011b) and plays an important role in neuron communication (Biederer T, *et al.* 2002). Neuron communication plays a very important role in the proper development of brain structures and defects in this communication and coordination may result in variety of disconnection problems, as observed in psychotic mental illness (Ford J M, Mathalon D H 2008).

6.5 Synaptic plasticity and mental illness

The three candidate genes studied in this thesis also play an important role in synaptic plasticity in the adult brain, which is defined as flexibility of the brain through adjustment of the connectivity between neurons and neuronal connections (Marsden W N 2013). The process of synaptic plasticity involves the modification of signaling systems near synaptic connections and engages equivalent alterations to the cellular structure within the synaptic connection (Marsden W N 2013). Vogt *et al* (2012) reported that accurate regulation of PSA-NCAM is critical for proper synaptic targeting, such that its alteration could result in faulty synaptic circuit formation. In addition, both NRG1 and PSA-NCAM are capable of

modifying the integrity of synapse, PSA-NCAM through blocking GluN2B-containing NMDA receptors (Kochlamazashvili G, *et al.* 2010), and NRG1 type III through back signaling (Bao J, *et al.* 2003). Efficient synaptic plasticity depends on right balance between synaptic remodeling and stabilization. NCAM1 being highly adhesive helps in proper orientation, positioning and arrangement of synaptic proteins, while PSA-NCAM with its anti-binding properties could help in synaptic modification by shifting *cis*-, homophilic or heterophilic communications (Gascon E, *et al.* 2007b). Therefore, precise control of NRG1, NCAM1 and PSA-NCAM is very essential for normal brain functioning. In my studies, I noticed dysregulation of different isoforms of these genes, which could disturb the integrity of synapse and therefore leading to faulty brain circuits.

Synaptic plasticity also plays a very important role in the neural communication such as processing thoughts, forming memory and storing information (Martin S J, *et al.* 2000; Neves G, *et al.* 2008). The three selected candidate genes could play a role in synaptic plasticity during the neuronal development and its maintenance in the adult brain regions, this could involve; PSA-NCAM, to play a role in fasciculation of nerves, branching of axons, formation of synapse and process of pruning (Bonfanti L 2006; Butler A K, *et al.* 1998; Rutishauser U, Landmesser L 1996); ST8SIA2, to play role in outgrowth of neurite (Brocco M A, Frasch A C 2006); and NRG1, to play role in formation of synapse (Li B, *et al.* 2007), in addition to outgrowth of neurite (Bermingham-McDonogh O, *et al.* 1996). The harmony of plasticity related events is controlled by secretion of excitatory (glutamate, glycine) or inhibitory (GABA) neurotransmitters and availability of ionotropic receptors such as AMPA or NMDA at the post-synaptic location (Yuste R, Bonhoeffer T 2001). The normal brain activities involve equilibrium between excitation and inhibition to maintain the homeostatic control. A disturbance in this equilibrium such as excessive increase in glutamate availability (Javitt D C 2007), increased dopaminergic response (Abi-Dargham A, *et al.* 1998), hypoactivity of NMDA receptor activity (Kantrowitz J T, Javitt D C 2010; Kehrer C, *et al.* 2008; Lindsley C W, *et al.* 2006) have been reported in the pathologies of schizophrenia (Javitt D C 2007; Kantrowitz J T, Javitt D C 2010; Kehrer C, *et al.* 2008) and bipolar disorder (Chen G, *et al.* 2010; Cousins D A, *et al.* 2009). A study by Bokkon *et al*

(2011) has reported abnormal neurotransmission could cause disconnection between brain regions in schizophrenia resulting in abnormal control of NMDA-dependent synaptic plasticity. A different study on the interneurons by Shamir *et al* (2012) has shown that NRG1/ErbB4 pathway play a vital role in the maintenance of hippocampal synaptic plasticity and abnormalities could represent behavioral phenotypes associated with psychotic disorders. PSA-NCAM is also believed to play a role in controlling the amount of inhibitory (GABA-ergic) neurotransmission in primary neurons by preventing the detachment of inhibitory synapses (Varea E, *et al.* 2007). Therefore irregular NRG1 and PSA-NCAM expression could disturb synaptic harmony by affecting inhibitory neurotransmission which is very helpful in synchronization of synaptic circuits (Nacher J, *et al.* 2013).

Synaptic plasticity depends on proper formation of efficient neural networks or synapse, and appropriate timing of contact formation. Non-efficient synapses could cause impairments and therefore they are removed by a process called synaptic pruning (Paolicelli R C, *et al.* 2011). ST8SIA2, to play role in outgrowth of neurite (Brocco M A, Frasch A C 2006) and an excessive growth of neurite could lead to formation of non-efficient synapses. ST8SIA2 over-expression is linked to distorted adhesion to substrate (Horstkorte R, *et al.* 1999), which could cause inhibition to proper cell signaling and therefore could affect efficiency of synapse as substrates like NCAM1 play a vital role in cell to cell communication. ST8SIA2 overexpression could also affect neuronal connections by causing cancerous metastatic invasion (Falconer R A, *et al.* 2012) and cell death (Horstkorte R, *et al.* 1999). In this project, ST8SIA2 protein is reported to be *increased* in the brains of people with schizophrenia.

Synaptic plasticity in the hippocampus could be divided into short term plasticity (example synaptic depression and augmentation) and long term plasticity (e.g. long term potentiation/LTP, long term depression/LTD) (Abraham W C, *et al.* 2002; MacDougall M J, Howland J G 2012; Manahan-Vaughan D 1997). Studies have shown that much of brain's plasticity in form of LTP occurs at the excitatory postsynaptic connections and spines of dendrites (Gascon E, *et al.* 2007b; Yuste R, Bonhoeffer T 2001). PolySTs

(ST8SIA2 and ST8SIA4), as mentioned before, play a vital role in brain plasticity such that knock-down of these genes could result in; impaired LTP and LTD in hippocampal CA3-CA1 synapses, distorted fear conditioning, and flawed brain-wiring abnormalities (Senkov O, *et al.* 2012). NRG1 also has a role in depotentiation of LTP in CA1 glutamatergic synapses, in mature brain (Buonanno A, *et al.* 2008). Errors in the expression of these genes could lead to non-efficient synaptic plasticity, which is further linked to patients suffering from mental illness (Duman R S 2002; Friston K J 1999; Friston K J, Frith C D 1995; Stephan K E, *et al.* 2006).

Proper transcriptional and translational regulation is essential for long-lasting synaptic plasticity (Kelleher R J, 3rd, *et al.* 2004). Dysregulation of NCAM1 is associated with neuropsychiatric disorders (Vawter M P 2000) and is parallel with the synaptic irregularity that could be the basis of disconnectivity among different regions of the brain (Vawter M P 2000). In my studies, I found a significant increase in non-glycosialylatable NCAM-120 in schizophrenic cases compared to normal controls, a condition previously reported in cerebrospinal fluid (CSF) (Poltorak M, *et al.* 1996; Tanaka Y, *et al.* 2007) and prefrontal cortex, hippocampus and cingulate cortex area in schizophrenia patients (Tanaka Y, *et al.* 2007; Vawter M P, *et al.* 1998a). An increase in the ratio of NCAM-120 isoform relative to the glycosylatable forms would mean increase in PolySia-free form of NCAM, which could interfere with the proper formation of PSA-NCAM. This hypothesis of dysregulation of NCAM was further supported in female patients with schizophrenia, where I found a significant increase in NCAM120/Total NCAM ratio, and significant decreases in NCAM180, NCAM180+D, and NCAM180+140+D leading to decreased PSA-NCAM expression. Results should be replicated in a larger cohort, but gender differences are common in schizophrenia, where females have been linked to a discrete symptom profile, later age of disease onset, different cognitive deficits and brain structural irregularities (Canuso C M, Pandina G 2007; Hafner H 2003; Lindamer L A, *et al.* 1999).

Overall, abnormalities in the selected three candidate genes and their expression could disturb the integrity of synapse, leading to changes in plasticity and faulty brain connections, eventually increasing the risk of developing psychotic mental illness.

6.6 Limitations of this thesis

Although this research was planned carefully to achieve its aims, there were some inevitable limitations. First of all, the functional follow-up of SNPs identified after *ST8SIA2* resequencing could not be completed because the data filtering and analysis process of the Roche 454 next generation sequencing took longer than initially anticipated. Secondly, the ST8SIA2 splice isoform quantification for all identified isoforms in Stanley cohorts could not be performed, because the expression levels of these isoforms were very low and in order to accurately quantify the isoforms, a large amount of cDNA was required which was not available to my studies. Thirdly, the *NRG1* sequencing study could not be extended to the bipolar based cohort and the NCAM1 targeted resequencing of functional region could not be performed in unaffected controls or schizophrenia cohort due to funding limitations. Fourthly, inadequate sample numbers are a common issue in association studies. My study was appropriately powered to detect large genetic effects (OR>2) from SNPs with relatively small frequency (MAF<0.1), but was under-powered to detect smaller genetic effects with more common variation. In the table below (Table 6.1) I have performed power calculations to highlight the required sample numbers to achieve a range of odds ratios with differing minor allele frequencies. For example, to achieve an odds ratio of 1.1 with minor allele frequency of 0.1, a total of 17,595 cases and similar controls were required. I only had 353 bipolar disorder cases and 187 controls for my NCAM1 association studies.

Minor Allele Frequency (MAF)	Odds ratio (OR)				
	1.1	**1.3**	**1.5**	**2**	**5**
0.1	17597	2210	889	282	47
0.2	38923	4743	1870	566	83
0.3	66342	7999	3132	931	129

Table 6.1: Relationship between a range of odds ratios and minor allele frequencies. Power calculations were conducted using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/˜purcell/gpc/), and are based on a disease risk allele frequency of 0.1, number of subjects (blue text) given in table are based on 80% power at Alpha 0.05. D-prime between risk allele and marker is set at 0.80.

While performing the western blotting studies, I tried to normalize my data against the commonly used housekeepers like β-actin and α-tubulin, but both these housekeepers were unreliable to normalize the raw data, such that the percentage variance of normalized data was more than the non-normalized data. Therefore, standard normalization methods could not be used in my western blot studies. In addition to the difficulties in normalization of western blot data, other limitations were the variance in western blot values across gels, which were between 40-90%, largely due to the gel to gel variations or performing the western blotting experiments on two separate days.

There were other considerations such as, the ST8SIA2 expression level was very low in the adults; therefore, I had to perform nested PCRs to amplify enough template from cDNA for the subsequent experiments, resulting in various problems obtaining clean PCR products, and possibly leading to the artifactual results due to unequal amplification of all transcripts. In addition, during ST8SIA2 mRNA expression studies, a lot of cDNA was required for each experiment, which was often difficult to get, as the brain tissue is a limited resource. It is therefore vital to take these features into consideration. Another major limitation of the current study was the small and uneven sample numbers in the post-mortem brain cohort sub-groups after stratification by demographic variables. Although the TRC post-mortem brain cohort is quite large by international standards, the numbers of samples from right and left hemispheres and males and females in schizophrenia and control group were not equal. In addition to that, stratifying by sex or brain hemisphere in different analyses further

reduced sample numbers in each group. This made it difficult to be confident in the robustness of my results and compare my hemisphere and gender-specific findings with the other published studies. Given these cautions, there is continuing need for larger post-mortem brain case-control cohorts with comparable numbers of cases and controls along with equal individuals of both sexes.

6.7 Future work

The outcomes of this thesis have not only answered some important questions, but also it leaves the opportunity for interesting future work. Chapter 3 of this thesis represents result of upstream regulatory sequences of various promoter regions of NRG1, in schizophrenia case control cohort. A replication study in bipolar based cohort would be very informative and could prioritize some interesting SNPs for future functional studies. This study could also involve next-generation sequencing of the entire *NRG1* gene in SMRI cohort and investigate; firstly, if there is any coding variation in *NRG1* in schizophrenia or bipolar disorder; secondly, if there is a relationship between novel variation and transcript expression in bipolar disorder; third, if there is significant difference in HAPICE haplotype distribution between bipolar cases and controls, as observed in a UK based population (Green E K, *et al.* 2005); and fourth, if HAPICE haplotype is associated with increased NRG1 type III expression in bipolar based cohort.

Investigating DNA variations in *ST8SIA2* (Chapter 5), I performed next-generation sequencing of 48 bipolar disorder cases. However, due to funding limitations I could not sequence an equivalent control population to directly compare my resequencing results, but instead used the published 1000 genomes data for comparison. Resequencing of control population could again help my colleagues in prioritizing SNPs to target for other future studies, particularly when the 1000 genomes study has been done using a different platform and with smaller coverage depth than my targeted resequencing study. Also, SNPs that have shown significant scores to alter transcription factor binding, could be tested *in vitro* by EMSA. One of the most promising leads from my thesis would be to determine functional repercussions of newly identified ST8SIA2 splice variants, especially the Δ5

isoform that lacks part of the polysialyltransferase domain and therefore could be deficient in adding PSA chain on NCAM's Ig domain. The Δ5 isoform could also compete with the wildtype isoform for the attachment and interactions within NCAM's glycosidic region (5th and 6th glycosylation site and acidic patch). To investigate the function of Δ5, cDNA from individuals possessing wildtype and Δ5 splice isoforms could be cloned, and transfected into neuronal cell lines (example SH-SY5Y) along with a vector carrying NCAM1, to test for the effect of Δ5 splicing isoforms on NCAM1's FN-1 domain recognition, NCAM1 polysialylation and neuronal migration.

This thesis also reports western blotting results of ST8SIA2 and NCAM1 in schizophrenia based cohort, a similar experiment should be extended in bipolar and another schizophrenia based cohort, to replicate and compare present findings. SMRI cohort would be an ideal cohort as a recent immune-staining based study has reported alterations in the expression of PSA-NCAM and synaptic proteins in the DLPFC of psychiatric disorder patients, in the same cohort (Gilabert-Juan J, *et al.* 2012).

It is well documented that ST8SIA2 and NCAM1 interact at protein level (Hildebrandt H, *et al.* 2010), it would be interesting to see if these two genes also interact at DNA level. Therefore, a study of gene-gene interactions using gene variants from these two genes would be very exciting. Such a study could involve testing genotypes from *ST8SIA2* SNPs against the genotypes from *NCAM1* SNPs through epistasis analysis using PLINK software. However, more genotyping would be required in a larger cohort to leave margin for significant correlations after corrections for multiple testing. It would also be of interest to extend the *NCAM1* resequencing study to the entire *NCAM1* gene in ABC cohort and another mental illness case control cohort (for e.g. SMRI which involves schizophrenia and bipolar disorder cases and controls) to study if there are other DNA variations in this gene that could increase risk to psychotic mental illness.

6.8 Concluding remarks

This thesis provides evidence that the three selected candidate genes each have novel variations in their genetic and cDNA structure and their mRNA and protein levels are

altered in psychotic mental illness. These genes could be involved in increasing disease susceptibility through changes in neuron migration, synaptic plasticity and signaling, or leading to structural malformations in brain. Through my work, I illustrated that targeted sequencing can be an important step to aid in identifying novel risk variations in the DNA structure. My study also supports the view that human post-mortem brain studies can make an important contribution to understand pathology of mental disorders.

GWAS has its strength in identifying common variants with more than 5% of the allele frequency that contribute to common diseases. Most of the time, these common variants are present in the intronic regions and do not code for a specific protein. Also, GWAS studies require a huge sample size, which is not always available. That is where targeted sequencing approaches could be really helpful as they could help us in identifying rare mutations, present within introns or exons in a targeted region of small population, with strong effect. This thesis illustrates that while functional follow-up of genes that are strongly associated to psychotic illness through GWAS is essential, so is analysis of candidate genes like *NRG1, ST8SIA2* and *NCAM1* that do not have strong associations with psychotic illness through GWAS results, but may still could have a strong neurobiological role in disease pathogenesis, in specific individuals.

Finally, the functional follow-up studies of candidate genes, identified though GWAS association or based on neurobiology, are essential in understanding the impact of alterations in such genes on neuronal migration, brain development and other cell signaling process, to better understand etiology of complex mental disorder diseases, their diagnosis and treatment in future.

Chapter 7

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Appendices

Published papers