THE INFLUENCE OF NEUREXIN 1 GENE VARIANTS ON COGNITIVE ABILITY IN MULTIPLEX SCHIZOPHRENIA FAMILIES

By

WANG KAH WEI

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ABSTRACT

THE INFLUENCE OF NEUREXIN 1 GENE VARIANTS ON COGNITIVE ABILITY IN MULTIPLEX SCHIZOPHRENIA FAMILIES

WANG KAH WEI

Brain synaptic dysfunction was suggested to play a key role in the cause and pathogenesis of schizophrenia. *NRXN1* gene encode for NRXN1 proteins that act as pre-synaptic neural adhesion molecules involve in synaptic transmission. Copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) of *NRXN1* confer high risk of schizophrenia. Multiplex families with more than one affected relatives were targeted in this study. The objective of this study is to determine the CNVs of *NRXN1* gene and its association with brain cognitive function of multiplex schizophrenia families and study the association of *NRXN1* SNPs with first-degree relatives of schizophrenia families. Thirteen schizophrenia families consisted of twenty eight patients and twelve healthy families which made up of twenty four healthy individuals were recruited in this study. Brain cognitive function of samples was studied by using Trail Making Test Part A (TMT-A) and part B (TMT-B). Seven sites of *NRXN1* gene were targeted and quantified with real time polymerase chain reaction (PCR) and copy number was measured by CopyCaller™ v2.0 software. Besides that, allele and genotype frequencies of five *NRXN1* SNPs were computed and linkage disequilibrium between SNPs was determined by using Haploview software. TMT-A ($p = 1.45\times10^{-4}$) and TMT-B ($p = 1.01\times10^{-5}$) scores made by multiplex schizophrenia families patients were statistically significant higher than healthy families controls. Patients also showed significantly ($p = 0.003$) higher TMT B−A score than controls. Twenty copy number

alterations (loss/ gain) were detected and 14 (70%) of these alterations occurred in isolated subjects from different families whereas 6 (30%) came from similar families. This suggests that the penetrance of CNVs at the targeted *NRXN1* regions may be relatively low in similar families. First-degree schizophrenia patients showed significant lower copy number mean than CN = 2.0 at Intron 6 (p = 0.003) and Intron 20 (p = 0.001). After false discovery rate (FDR) correction, copy number (CN) mean was found significantly different between controls and patients at three regions, Intron 6 (p = 0.010), Intron 7 – Exon 7 (p = 0.020) and Intron 20 (p = 0.019). The Spearman's rank correlation test was performed and no significant correlations were found between copy numbers of respective targeted *NRXN1* CNVs regions with the cognitive performance scores. Among five studied SNPs, rs2024513 allele (p = 0.005) and genotype (p = 0.002) frequencies were found significantly associated with schizophrenia. In this study, the results indicate that *NRXN1* gene variants as genetic factors that could contribute to susceptibility of schizophrenia.

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This dissertation entitled "**THE INFLUENCE OF NEUREXIN 1 GENE VARIANTS ON COGNITIVE ABILITY IN MULTIPLEX SCHIZOPHRENIA FAMILIES"** was prepared by WANG KAH WEI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Assistant Prof. Dr. TEE SHIAU FOON) Date: …….
Supervisor
Department of Chemical Engineering
Lee Kong Chian Faculty of Engineering and Science
Universiti Tunku Abdul Rahman

_____

(Associate Prof. Dr. TANG PEK YEE) Date: ……
Co-supervisor
Department of Mechatronics and BioMedical Engineering
Lee Kong Chian Faculty of Engineering and Science
Universiti Tunku Abdul Rahman

LEE KONG CHIAN FACULTY OF ENGINEERING AND SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that **WANG KAH WEI** (ID No: **14UEM07891**) has completed this dissertation entitled "**THE INFLUENCE OF NEUREXIN 1 GENE VARIANTS ON COGNITIVE ABILITY IN MULTIPLEX SCHIZOPHRENIA FAMILIES**" under the supervision of Dr. Tee Shiau Foon (Supervisor) from the Department of Chemical Engineering and Dr. Tang Pek Yee (Co-Supervisor) from the Department of Mechatronics and BioMedical Engineering, Lee Kong Chian Faculty of Engineering and Science.

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(WANG KAH WEI)

DECLARATION

I, WANG KAH WEI hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(WANG KAH WEI)

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LIST OF ABBREVIATIONS

CNV	Copy Number Variation
CN	Copy Number
SNP	Single Nucleotide Polymorphisms
NRXN1	*Neurexin1* gene
PCR	Polymerase Chain Reaction
WHO	World Health Organization
ICD	International Classification of Disease
DSM	Diagnostic and Statistical Manual
TMT	Trail Making Test
NRXN	Neurexin protein
NLGN	Neuroligin protein
PDZ	PSD-95/D1g/ZO-1 domain
EGF	Epidermal Growth Factor
CASK	Calcium/Calmodulin-dependent Serine protein Kinase
SHK	SRC Homology 3 domain
GUK	Guanylate-Kinase domain
Array-CGH	Array of Comparative Genome Hybridization
SV	Structural Variation
VNTR	Variable Number Tandem Repeat
NAHR	Nonallelic Homologous Recombination
FoSTeS	Fork Stalling and Template Switching
ASD	Autism Spectrum Disorder

GWAS	Genome Wide Association Studies
COMT	Catechol-o-methytransferase
dNTPs	Deoxyribonucleotide triphosphates
M.I.N.I	Mini-International Neuropsychiatric Interview
OR	Odds Ratio
CI	Confidence Interval
HWE	Hardy Weinberg Equilibrium
LD	Linkage Disequilibrium
Tm	Melting Temperature
HapMap	Haplotype Map
NCBI	National Center for Biotechnology Information
RefSeq	Reference Sequence Database
FDR	False Discovery Rate

CHAPTER 1

INTRODUCTION

Schizophrenia is a highly destructive mental disease with complicate etiology that had emerging as a serious health threats in both developed and developing nations (Krishnaswamy et al., 2009). In Malaysia, Ministry of Health has found that one out of hundred Malaysians suffers from schizophrenia (Lakshiny, 2015). 12% of Malaysians aged between 18 and 60 are suffering and affected by mental illness (Chun, 2012). Data gathered from different sources such as brain imaging, autopsy and epidemiological studies showed that schizophrenia is a neuronal developmental illness, and the brain functions were damaged progressively before clinical symptoms were fully developed (Walsh et al., 2008; Kirov et al., 2009).

Cognitive deficits are a core distinctive attribute of schizophrenia (Bowie and Harvey, 2006). 'Cognitive' refers to mental processes involved in perceiving, attending to, remembering, thinking (Moskowitz, 2004) and cognitive deficits can be range from mild to severe by affects multiple domains, including attention, verbal learning and memory as well as functions of executive. Moreover, 70% to 80% of schizophrenia patients were diagnosed with cognitive impairment (Holthausen et al., 2002; Reichenberg, 2009). Numerous studies have found deficits in brain executive function

in schizophrenic patients (Periáñez et al., 2007). Catherine et al. (2010) reported schizophrenic patients showed more indicators of left hemisphere damage.

One of the great remain challenges in psychiatry is to understanding the cause and pathogenesis of schizophrenia (Owen et al., 2005). In Malaysia, the commonness of mental sickness in the population of rural and urban area was 9.6% and 35% respectively. Schizophrenia had emerged as a serious health threat in our nation and it is equally serious forms of medical illness that should not be overlooked in a developed nation (Lakshiny, 2015).

Synaptic dysfunction was suggested act as a key role of schizophrenia. The genetic architecture of schizophrenia and the evidence for synaptic involvement was examined in different studies (Rees et al., 2011; Kavanagh et al., 2015). Impairment of glutamatergic signaling pathways take part in modulating synaptic plasticity could attribute to the etiology of schizophrenia (Pocklington et al., 2014). Neurexin1 (NRXN1) acts as an important synaptic neuronal adhesion molecule that interacts intimately with postsynaptic neuroligins in both glutamatergic and Gamma-Aminobutyric acid-ergic (GABAergic) signaling pathway (Todarello et al., 2014). Recently, many reports have suggested that chromosomal copy number deletions that affect the gene *Neurexin1* (*NRXN1*) increase the exposure of developing schizophrenia (Zahir et al., 2007).

Structural alteration of *NRXN1* gene in human genome had suggested as risk factor of causing schizophrenia. Many evidence shown additional copy number variation (CNVs) contributed to schizophrenia. Kirov et al. (2009) reported 19% cases versus 4% controls of *NRXN1* copy number deletion in case-control studies. Several groups have identified copy number deletions were disrupting the NRXN1 N-terminal encoding regions (Vrijenhoek et al., 2008; Rujescu et al., 2009). Moreover, reductions of *NRXN1* copy number were related to the mental retardation and delay of growth (Fredman et al., 2004).

Single Nucleotide polymorphisms (SNPs) markers in *NRXN1* gene region has been studied to further investigate the role of *NRXN1* in schizophrenia susceptibility. In a Asian population case-control study, significant differences were observed in frequencies of alleles between controls and patients at four SNPs (rs10490168, rs2024513, rs13382584, rs1558852) in *NRXN1* gene (Yue et al., 2011). Pharmacogenetic analysis showed notable outcome of *NRXN1* rs12467557 marker in response to neuroleptics drug treatment. Besides that, association also found between *NRXN1* rs10490162 marker with treatment of antipsychotic drug (Jenkins et al., 2014).

Objectives of this study are: (1) to study the copy number variations of *NRXN1* gene and its association with brain cognitive function and (2) to study the association of *NRXN1* single nucleotide polymorphisms with schizophrenia between the subjects of multiplex schizophrenia families and control families.

CHAPTER 2

LITERATURE REVIEW

2.1 Schizophrenia

National Institute of Mental Health reported schizophrenia has a worldwide incidence of approximately 1 in 4000 people (Sahoo et al., 2011) and it's characterized by psychotic symptoms, disorganized behavior, concern lacking and changed of emotional reactivity (Sullivan et al., 2008; Sahoo et al., 2011). Besides that, the onset of behavioral and cognitive symptoms usually exists since early of childhood although the more significant features usually diagnosed in early twenties or late teens. These symptoms may lead to impaired functioning in work, study, parenting and loss the ability of independent living (Owen et al., 2005).

Due to relative common of the associated deficits and often life-long impacts of the disorder, schizophrenia is one of the top ten important causes of disorder related disability around the world (Switaj et al., 2012). In Asian country such as Malaysia, there are rising in cases of schizophrenia patients that were newly diagnosed and proper psychiatric care and medical treatment been given (Krishnaswamy et al., 2009). Many patients loss the abilities of normal social functioning and cause distress to the patients themselves and also to the society (ZamZam et al., 2011). Previous studies concluded

that the quality of life of schizophrenia patients were affected due to higher burden of caregiver burden and their mental health problems (Chen and Greenberg, 2004; Maruish, 2004).

Schizophrenia patients are usually impotent, isolated and vulnerable peoples in society (Owen, 2012). Moreover, in addition to the core symptoms which result in impaired functioning, schizophrenic patients also noticed with increased risk of alcoholic and illegal use of drugs, violent victimization, post-traumatic stress disorder, instability of housing, depression and smoking-related illness. Exposure to these risks increase mortality rate of schizophrenia patients due to killing oneself (estimated at 5%), accidents, or illness related to heart lung diseases (Mueser and McGurk, 2004). National Academy of Sciences estimates the total financial costs of schizophrenia have been account for 2.3% of combined burdens within developed countries (Sahoo et al., 2011).

2.1.1 History of Schizophrenia

Schizophrenia was first characterized in 1893 by a German psychiatrist Emil Kraepelin, under the name dementia praecox. Kraepelin characterize dementia praecox by grouping the patients together according to previously diagnosed syndromes which included hebephrenia, catatonia and paranoid dementia (Ebert and Bär, 2010). Kraepelin found that dementia praecox usually began in late teens or early adulthood unlike other dementia such as Alzheimer's disease which occurred later in life. He also

described dementia praecox as a deteriorating psychotic disorder whose primary disturbance on cognition of patients rather than mood (Wing and Agrawal, 2003).

The belief of Kraepelin that dementia praecox was a deteriorating course and no chance of recovery was being objected by other psychiatrist (Ebert and Bär, 2010). At the early of the twenty century, dementia praecox was renamed by a Swiss psychiatrist Eugen Bleuler. Bleuler realised that some patients show improvement after treatment rather than continue to deteriorate as Kraepelin had suggested. Therefore, with other observations, Bleuler used the term schizophrenia, from the Greek schizen 'to split' and phren 'mind', to emphasise the feature that a serious fragmentation of thinking and personality resulted from this illness (Ashok et al., 2012).

2.1.2 Symptoms of Schizophrenia

Schizophrenia is characterized based on a diverse set of symptoms and signs which arise from almost all domains of brain including language, emotion, reasoning, motor activity and perception. The indications of schizophrenia can be categorized into negative, positive and cognitive features. Negative traits are related with normal emotions and behaviors disruptions (Maat et al., 2012) Patients will show reduced in expression of emotions via facial expression or voice tone, social withdrawal, disorganization and will reduce. Positive traits are psychotic actions that unable to be found in a normal individual (Hallak et al., 2013). The positive symptoms expressed by patients include disorders of perception (hallucinations), inferential thinking (delusions)

and involuntary actions (catatonia). Cognitive features include poor executive functioning, lacking for focusing abilities and deficit in memory (Green et al., 2015).

2.1.3 Diagnosis

A criterion-based approach for the diagnosis of schizophrenia was produced by the American Psychiatric Association and World Health Organization (Andreasen, 2010). Therefore, 10th International Classification of Disease (ICD) and 4^{th} version of American Psychiatric Association's Diagnostic and Statistical Manual (DSM-IV) had used as a guideline by physicians to diagnose the schizophrenia features and impairment traits (First, Spitzer and Gibbon, 2002).

The DSM-IV has been widely used clinically due to high reliability, and legitimate in diagnosis of schizophrenia (Tandon et al., 2009). Validity of DSM-IV widely supported by a set of precursor factors (environmental risk factors, familial aggregation) (Bromet et al., 2011), predictive (stability of diagnosis, response of treatment and illness course) and validity factors (Korver-Nieberg et al., 2011). DSM-IV offered reliable and stable diagnosis (Haahr et al., 2008), with more than 80% of individuals receiving an early diagnosis of remaining that diagnosis between one to ten years (Bromet et al., 2011).

Tandon and Carpenter (2012) concluded that DSM-V had retained the central of the DSM-IV diagnostic principle for schizophrenia. DSM-V was proposed mainly for addition of updated data regarding the nature of the disease gathered in last twenty years. Individuals who did (or did not) fulfill the DSM-IV traits for schizophrenia will proceed to fulfill (or not meet) the DSM-V requirements. The six features (A to F) for the schizophrenia diagnosis in DSM-IV remained with moderate changes made in criteria A and F (Table 2.1). In DSM-V, mood disorder symptoms such as mania and depression that exist during the period of psychotic illness were examined in order to further diagnose the present of schizoaffective disorder (Tandon and Carpenter, 2012).

Table 2.1: Proposed criteria for schizophrenia in DSM-V. The six criterions of DSM-IV retained in DSM-V with minor change in Criterion A and F (Tandon and Carpenter, 2012).

Proposed Criteria for Schizophrenia in DSM-V
Criterion A. Characteristic symptoms: (Minor change) Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated). At least one of these should include 1 – 3 1. Delusions 2. Hallucinations 3. Disorganized speech 4. Grossly disorganized or catatonic behavior 5. Negative symptoms (i.e., diminished emotional expression or a volition)
Criterion B. Social/ occupational dysfunction (No change)

Table 2.1, continued:

Criterion C. duration of 6 months (No change)
Criterion D. Schizoaffective and mood disorder exclusion (No change)
Criterion E. Substance/ general mood condition exclusion (No change)
Criterion F. Relationship to Global Developmental Delay or Autism Spectrum Disorder: (Minor change) If there is a history of autism spectrum disorder or other communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least 1 month (or less if successfully treated).

2.1.4 Brain Cognitive Test

Sharma and Antonova (2013) found there has been increase in recognition cognitive impairments diagnosis in schizophrenia over the last two decades. The impairment of neuronal cognitive is identified as a central part of schizophrenia. Hence, researchers indicate cognitive impairment as an important feature of the disease (Elvevag and Goldberg, 2000). The diminished of cognitive functions usually affect the ability to memorize and focus as well as information processing and executing functions.

Trail Making Test (TMT) is a neuronal mentality test used to assess the brain cognitive function. It was initially formulated in military psychological test, and become widely used indicator of mental damage (Arbuthnott and Frank, 2000). According to different findings, TMT is useful in assess rapidity of brain processing, order alternation, flexibility of cognitive function (Ríos et al., 2004), visual search, executive functioning and execution of movement (Stuss et al., 2001).

The test has two sections (A and B) that should be carried out rapidly with fewer mistakes made. The outcome from each section represents the number of time needed to finish the task. Besides the two direct measure results, some researchers have suggested modified indexes to enhance the illustration of cognitive skills used to complete the test. The dissimilarity score (B – A) is aim to reduce the speed factor from the test evaluation (Salthouse et al., 2000). The B/A ratio score serve as a measurement of executive control ability referring to the correlation with task-switching function (Periáñez et al., 2007).

Some neuronal psychological finding shows the presence of association between brain frontal lobe damage resulted from traumatic injury and weak TMT performance. In a TMT assessment made by Stuss et al. (2001), patients with brain frontal lobe damaged were classified into separated groups in order to highlight the relations between brain frontal lobes and specific behavior (Hashimoto et al., 2006). The dorsolateral frontal

areas damaged patients were found out had more weakened in TMT-B performance compared with patients who having damage at inferior medial area of frontal lobes.

2.1.5 Epidemiology of Schizophrenia

In developed nation, schizophrenia is usually life-long threatening and approximately one in hundred persons have or prone to develop schizophrenia during their duration of life. Rates of schizophrenia are very identical between Asian and Western countries with minor regional variations (Schizophrenia Research Institute, 2013). Moreover, the lifetime relative risk of developing schizophrenia ranges from 0.3 to 2% with an average recurrence frequency of 0.7% (Saha et al., 2007).

The National Health and Morbidity Survey which conducted by Ministry of Health, Malaysia shown 20% Malaysians adolescence and adulthood are suffering from different kinds of psychiatric disorder. In Malaysia, there were more than 7000 schizophrenia cases registered from 2003 to 2005 under Malaysia National Mental Health Registry (Aziz, 2008). The distribution of schizophrenic patient's ethnic group in Malaysia were 54% Malays, 28% Chinese, 9% Indians and 9% others. This figure is also constantly matched with the ethnic group distribution in our country.

In developed countries, schizophrenia is a leading cause of early death (Lewis et al., 2003; Krishnaswamy et al., 2009) and the lifetime prevalence of suicide is 10% among patients (Siris, 2001). There are more than half of schizophrenia patients attempted to kill own self and 5% had caused their own death. The mortality rate of people with schizophrenia is almost three times higher than the public population, and life state is reduced by more than 15 years (Schizophrenia Research Institute, 2013). According to the registered schizophrenia cases in Malaysia, more than 60% were males. Most of the patients were developed the illness at the age of 30 and symptoms appeared earlier in male as compared to female (Ministry of Health Malaysia, 2009).

2.1.5.1 Environmental Risk Factors

Environmental factors might associate with increased incidence of schizophrenia and they include migrant status, urbanity and economic status (Cantor-Graae et al., 2003). Substantial evidence has been indicate that individuals live in an urban environment are more presumably to develop the disorder compare with those who living in rural areas (Boydell et al., 2003). Migration has also been related with an increased risk of schizophrenia. Other environmental factors include viruses exposure or malnutrition before birth and potentially socioeconomic and psychosocial factors. These may play a role in early death of patients who smoking or illicit drug uses, heart failure and diabetes mellitus (Saha et al., 2007; Brown et al., 2005).

Other known associated environment factors are maternal malnutrition during pregnancy, maternal infection in uterus (Saha et al., 2007) and obstetrical complications such as prematurity, low birth weight and complications of delivery (Cannon et al., 2003). Another potential cause is the effect of early childhood trauma which involves a range of severe negative experiences, including physical, mentally and sexually abuse (Boydell et al., 2003). The environment pressure could influence the way of a gene changes over a lifetime. The epigenetic progress could be thought of as a turn on and off of functional genes at different stage of life. More general environmental risk factors include lower social class, social stress, low IQ score and illicit drug abuse (Cantor-Graae et al., 2003; Saha et al., 2007).

2.1.5.2 Genetic Risk Factors

In the search of unravel the patho-etiology of schizophrenia, genetics candidates identified in Human Genome Project providing explanatory models that could strongly support identified environmental factors (Pickers, 2005). The outcomes from clinical studies indicated that the risk of developing the disorder is largely attributable to strong genetic risk factors. The problems of genetic causing to this disease may arise from multiple genes involved in neuronal pathway regulations (Owen et al., 2005; Stefansson et al., 2008). The heritability of schizophrenia within family has been hard to trace because of great changes in environment factors and separating genetic cause (Owen, 2012). A research done by Gottesman and Shields were able to show that schizophrenia runs in families (Gottesman, 1991). The observed chance of developing schizophrenia

was found to be at a higher prevalence in the relatives of affected individuals. The risk of schizophrenia developed in general population is approximately ~1% throughout the lifetime. This contrasts with the average risk to siblings (9%) and offspring (13%) of affected probands. Furthermore, an identical (monozygotic) twin who shares 100% of their genes with a proband has a risk of almost 50% (Figure 2.1). Twins were found that with a higher heritability rate, and implicate that gene factors play a key role of the disease (Owen, O'Donovan and Harrison, 2005).

Adoption studies reveal that schizophrenia patients first-degree relatives have 10-fold increased risk of having the disorder as compared to public population (Hoffman and McGlashan, 2001). Additionally, offspring born to mothers with schizophrenia, who are adopted out, still have a significantly elevated chance of encounter the disorder. When two parents have schizophrenia, there is a 27.3% risk their offspring will develop the disorder (International Schizophrenia Consortium, 2008).

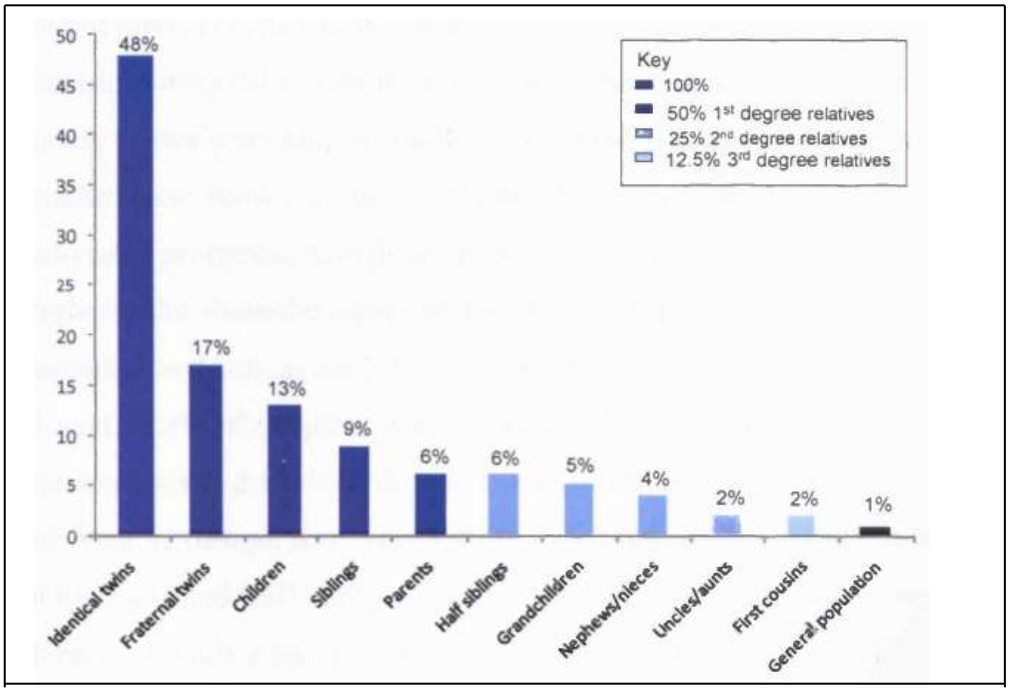

Figure 2.1: Risk of developing schizophrenia, for relatives of schizophrenia probands compared to public. The percentages indicated in the key refer to the proportion of genes shared (Klug et al., 2009).

The idea of genetic causation suggests that schizophrenia is a disorder with complex and complicated inheritance. Vrijenhoek et al. (2008) found that genes involved in coding mechanisms of dopaminergic pathway prevalent to schizophrenia. There is also evidence which showed that unusual linkage between several gene networks happened frequently in schizophrenic patients (Manji, Gottesman and Gould, 2003). Subsequently, research has focused on the detection of the duplications or deletions of DNA sequences of genes that are involved for synapse connectivity or development of brain (Walsh et al., 2008).

2.1.6 Multiplex Schizophrenia Families

Study design is a crucial component in the research of human genetics. The major approaches are case–control studies and pedigree-based studies. Besides that, the presence of other affected relatives of patients could be focused through the approach of multiplex families study (Sullivan, Daly and O'Donovan, 2012). 'Multiplex' refers to multiple individuals that are affected by a specific disease within families (Sanders, 2013). According to Sullivan, Daly and O'Donovan (2012), multiplex families were focused in human genetics studies under the assumption that these families are enriched for causal genetic risk factors with greater penetrance.

Myles-Worsley et al (2007) had figured out the recurrence risk of schizophrenia in offspring through multiplex families study. The recurrence rate was found to be 23.4% in the offspring with one affected parent, 6.4% in offspring with one affected aunt/uncle, and 15.0% in offspring with two or more affected aunts/ uncles. In the association study of de novo CNVs with schizophrenia, Wang et al (2013) reported that multiplex families study could accommodate the dependence among family members and the genetic variation in each family could be further quantified. Besides that, Shedlack et al (1996) found that schizophrenic siblings were significantly more impaired in cognitive tests and familial effect was noticed during the reading ability and attention tests. Moreover, Huepe et al (2012) suggested that multiplex families study was useful in highlighted the degree of cognitive impairments between schizophrenic patients and their first degree relatives from respective family.

2.2 Synaptic Transmission and Schizophrenia

The sensory inputs are integrated and processed by brain to produce motor outputs which important for organism to sustain their life. Cascades of synapses in nervous system responsible for information transformation and the cascades assembled in overlapping of multiple neural circuits. Synapses take part in all information processing of brain, and most of the malfunctions of brain function could caused from synaptic function abnormalities either in direct or indirect effect (Koester and Sakmann, 2000).

Synapse-related deficits appear to be widespread in schizophrenia. Deterioration of synapses could affect several cortical and subcortical region of brain, such as GABAergic and glutamatergic transmission pathways (Hashimoto et al., 2006; Shatz, 2009). Schizophrenia could be developed due to abnormal neuronal development and malfunction of synaptic flexibility and its transmission (Stellwagen and Malenka, 2006). Structural and functional synaptic impairment are actively contributing elements to the schizophrenia development and its pathology (Faludi and Mirnics, 2011).

2.2.1 Synapses

Synapses are group of confined intercellular junctions exclusively in transmission of electrical or chemical signal from a neuron to another (Figure 2.2). Synapses are asymmetrically inherited, transferring information with fast mechanism, and are highly flexible (Chung and Barres, 2011). Synapses have various characteristics that are categorized by both the pre-transmission synaptic neurons and postsynaptic neurons.

Information transmission of synapses is rapid, accurate, efficient and closely regulated. When presynaptic terminal invaded by an action potential, calcium ion channels unlocked and inflowing of calcium ion stimulates the fusion between presynaptic plasma membrane and synaptic vesicles. Neurotransmitters in the vesicles released into the synaptic cleft (Missler et al., 2003). Information transfer process completed when neurotransmitters bind with receptors presence on postsynaptic neurons. The vesicle fusion occurred in presynaptic neurons and receiving of signals at postsynaptic neurons are the main steps in the overall process are happened incredibly rapid. The time course of synaptic transmission initiating in less than 1 millisecond as measured electrophysiologically (Faludi and Mirnics, 2011).

From the composition, synapses are distinguished by intracellular layer present on surface of the presynaptic plasma layer and postsynaptic membrane of plasma. Presynaptic neuron’s plasma membrane always aligned in intact structure with postsynaptic neurons, and the membrane are isolated apart by a cleft of synapse

approximately 20 nanometers (Hashimoto et al., 2006). Synaptic cleft consist proteinaceous substances in between and serve as a medium for synaptic cell adhesive molecules such as neuroligins (NLGNs) and NRXNs (Iida et al., 2004; Meyer et al., 2004).

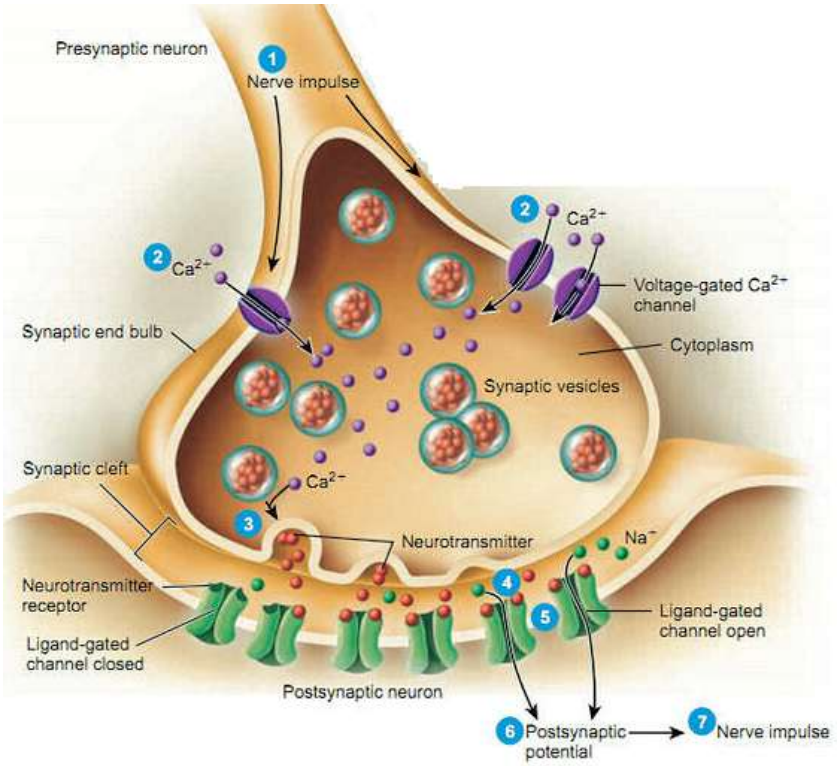

Figure 2.2: Synaptic transmission begins with the arrived of nerve impulse (Cowan et al., 2001).

2.2.2 Synapse Formation and Specialization

Synapses existed between neurons in the nervous system through the process of synaptogenesis. Configuration and specification of synaptic variety are closely connected and relies mainly to mechanisms of synaptic molecules which involve in adhesion of cells (Iida et al., 2004; Craig and Kang, 2007). The wide ranges of synapses are because of dissimilarity in the components of released neurotransmitter and receptor machineries. Moreover, synapse formation mainly based on differences of machineries organization. Synapse alignment and specification probably take part in three steps: neural growth cone by early stage recognition of the target cell, forming junctions of synaptic with involvement of synaptic elements, and readily of synaptic junctions with specified localized circuit (Cowan et al., 2001).

More efforts have to be made to understand the biological mechanisms involved synapse development and the characterization of synapse diversity and processes facilitating the assembly of synapses into circuits of nervous systems (Chung and Barres, 2011). For accurate and rapid neuronal pathway function, the development and synapses characterization is crucial. The information receiving and response properties remain intact in a neuronal circuit by synaptic connectivity and the multiple roles of individual synapses in the circuit (Dean and Dresbach, 2006). All individual synapses comprising the circuit play an important role in the pattern of circuit connectivity. Alteration in synaptic strength and synaptic flexibility can absolutely modify the

corresponding attributions of separated synapses in a neuronal pathway (Faludi and Mirnics, 2011).

2.2.3 Synaptic Cell-Adhesion Molecules

Vertebrate NRXNs and NLGNs are conceivably the most well-studied synaptic molecules in adhesion of neuronal cells (Dean and Dresbach, 2006) and they are the only synaptic molecules that well characterized and a specifically synaptic role has been discovered (Figure 2.3) (Craig and Kang, 2007). NRXNs and NLGNs function as molecules involved in adhesive of synapses and these molecules are essential for operative of synapse (Missler et al., 2003). They are mainly affecting trans-synaptic activation of synaptic transfer of information (Varoqueaux et al., 2006). Dysfunction of NRXNs and NLGNs disrupts the intactness of synapses and obstruct neural networks without fully eradicate synaptic transmission (Sheckler et al., 2006).

NLGNs consists a huge domain presence outer membrane that share sequence homology with acetylcholinesterase and that is important for NRXNs attachments and synpatogenic events. As molecules involved in bioadhesive of cells, NRXNs and NLGNs mostly react by attach to one another via interaction with intracellular organic compounds particularly the PSD-95/Dlg/ZO-1 (PDZ) domain (Dean and Dresbach, 2006). Upon binding NRXNs, NLGNs facilitate synapse signaling, recruit neurotransmitter receptors, and mediate neural networks and synaptic works. A severe marked deficit and disrupted in synaptic information transferring of mice deficient of

NRXNs or NLGNs were shows their importance properties in synaptic function (Varoqueaux et al., 2006).

The feature of both bioadhesive molecules in synaptic function practically determines them for a function in cognitive disorders, such as schizophrenia and bipolar disorder; the detailed molecular reactions in the mechanisms remained to be discovered (Walsh et al., 2008). Understanding cognitive diseases could remain challenge as they may arise from delicately complex and rapidly changes in synapses of a neural circuit rather than an overall damaged of all synapses in entire circuits (Jamain et al., 2003). Mental disorder such as schizophrenia may have the same molecular changes but produce multiple circuit alteration and neurological symptoms (Walsh et al., 2008).

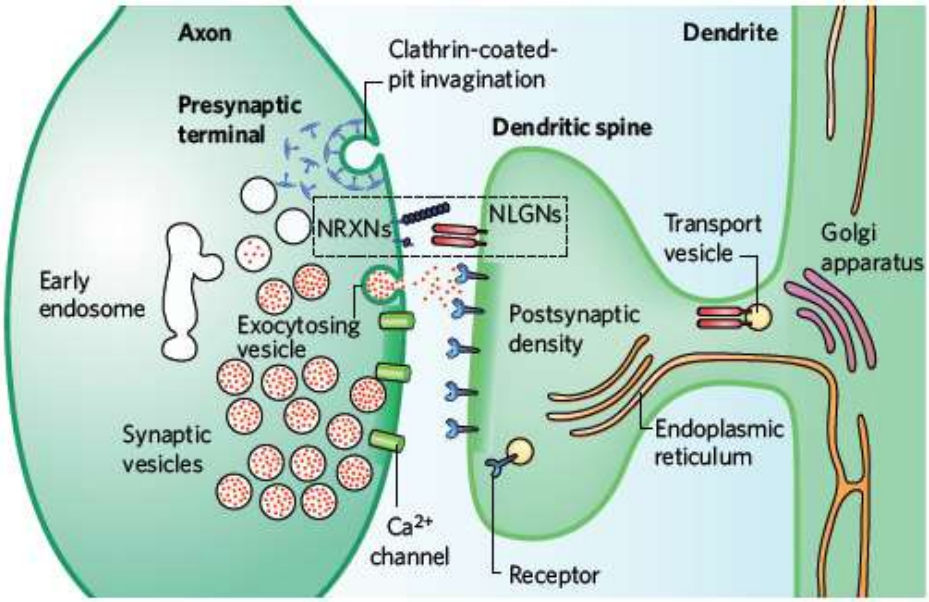

Figure 2.3: The illustration of synaptic molecules interaction and the assumed destinations of synaptic NRXNs and NLGNs. Dendritic structure with receptors for neurotransmitter indicated on the right while axons with vesicles of synapses showed on the left (Südhof, 2008).

Recently, various studies have determined genes mutations encoding NRXNs and NLGNs to be the root of distinct cognitive disorders such as schizophrenia (Feng et al., 2006; Kim et al., 2008; Zahir et al., 2007). Thus, NRXNs and NLGNs act as essential molecules for synaptic cell adhesion that induces signaling between presynaptic and postsynaptic components (Marshall, 2008). The information transfer is core to the brain's capability in processing and is a main cause of cognitive disorders (Yan et al., 2008). Mutations in NRXNs and NLGNs have been identified to cause malfunctions in synaptic transmission of neural circuits and result in cognitive diseases, especially schizophrenia and ASDs (Kirov et al., 2009; Südhof, 2008).

2.3 Neurexin (NRXN)

The poison released by black-widow spider made up of a vertebrate targeted toxin known as α-latrotoxin. This toxin acts as an organic compound that adheres to presynaptic receptors and mediates a large amount generate of neurotransmitters (Ushkaryov et al., 1992). NRXNs were found as α-latrotoxin receptors. NRXNs are type I transmembrane organic compounds with one path transmembrane helix (TM) that isolates cytosolic intracellular domains and extracellular amino-terminal. It can be categorized as two kinds: α-NRXNs and β-NRXNs. α-NRXNs are sizable than β-NRXNs. These two kinds of NRXNs made up of unalike amino end extracellular chain but with interchangeable carboxyl end transmembrane areas and cytoplasmic tails (Figure 2.4). Structurally, α-NRXNs have six laminin/ neurexin/ sex-hormone-binding (LNS) globulin domains with three epidermal growth factor (EGF)-like domains in between, whereas β-NRXNs consist only one LNS domain (Ullrich et al., 1995; Jenkins et al., 2014).

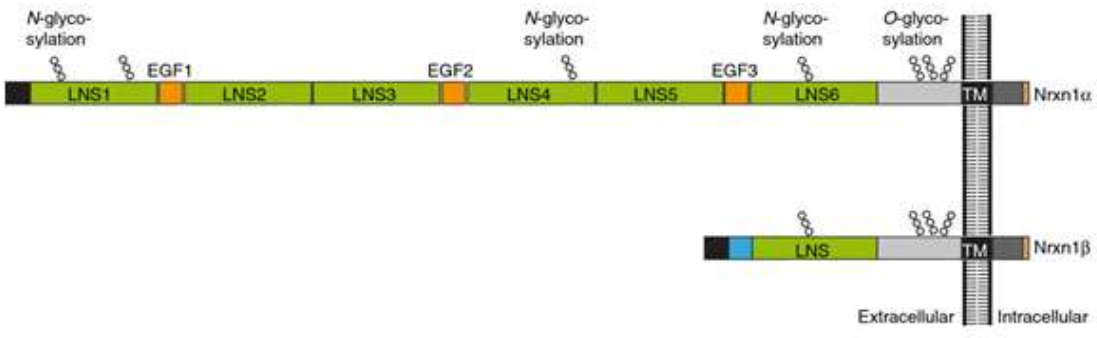

Figure 2.4: Domain orientations of α-NRXN and β-NRXN (Reissner et al., 2008).

Neurexin is a sequence of LNS-EGF-LNS with three times replicated in α-neurexin whereas β-neurexin consists of one exon that encodes unique 37 histidine-rich residues and a signal peptide (SP), the rest of its structure is similar to α-neurexin initiate from the last LNS domain (Reissner et al., 2008).

The genome of mammal consists three *NRXN* genes (*NRXN1*, *NRXN2* and *NRXN3*), every one of them making a lengthy mRNA translated to form α-NRXNs and a short mRNA forming β-NRXNs after transcription from independent promoters (Tabuchi and Südhof, 2002). Moreover, by depending on different sensory inputs, substantial selectively joining between encoded organic compounds at distinct canonical junctions could produce variety kinds of NRXN isoforms (Ullrich et al., 1995) (Figure 2.5). Therefore, specific NRXNs isoforms standing for independent 'code' were formed during synapse transmission.

NRXNs selectively splice reaction is modulated apart and modified through neuronal events (Ullrich et al., 1995; Tabuchi and Südhof, 2002). α-NRXNs contain five canonical splicing junction while β-NRXNs only contain two. The first splice region at C-terminal next to the EGF-parallel region placed at first of sequence. The following splice junctions are at identical places in another three LNS domains (Figure 2.5). The last splice site is present in between the transmembrane region and the glycosylated carboxyl sequence.

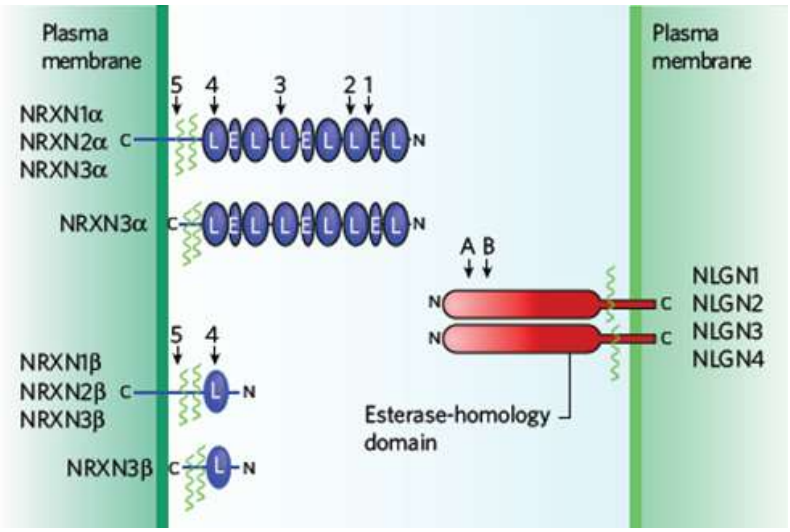

Figure 2.5: Selective splicing of NRXNs and NLGNs (Südhof, 2008).

By molecular hybridizations technique, mRNAs encoding various α-NRXNs and β-NRXNs were found have undergo coexpression and differentially allocated between multiple classes of neuron (Ullrich et al., 1995). High sensitivity fluorescence studies, molecular chromatography studies and the response of NRXNs as receptors towards α-latrotoxin showed the NRXNs most likely situated on terminal of presynaptic neurons (Reissner et al., 2008). The removal of genes involved in formulating α-NRXNs could have negative postsynaptic effects (Südhof, 2008).

The architecture of NRXNs and their situation at the synapse suggest they act as receptors on cellular surface or micro-adhesion molecules. Cell adhesion is crucial in regulating the synaptic mechanisms of brain (Garner et al., 2002) and the formation, regulation and alteration of synapses are having a major role in the process of studying, memorizing, and cognition (Dean and Dresbach, 2006).

2.3.1 Structural and Role of *Neurexin1* (*NRXN1)*

NRXN1, situated at chromosome 2p16.3. It contains 24 exons that stretch across 1.1 megabases of located chromosome with very lengthy introns (Tabuchi and Südhof, 2002). The gene annotations of *NRXN1* in National Center Biotechnology Information (NCBI) Reference Sequence (RefSeq) and Ensembl bioinformatics database are showed in Figure 2.6 (Ching et al., 2010). Exons 2 to 8, exons 9 to 14 and exons 14 to 20 encode for proximal, intermediate and distal region of NRXN1 respectively. *NRXN1* Exon 1 encodes for NRXN1 N-terminal signal peptide whereas the cytoplasmic tails and O-glycosylation sequence at C-terminal encoded by remaining exons (Figure 2.6).

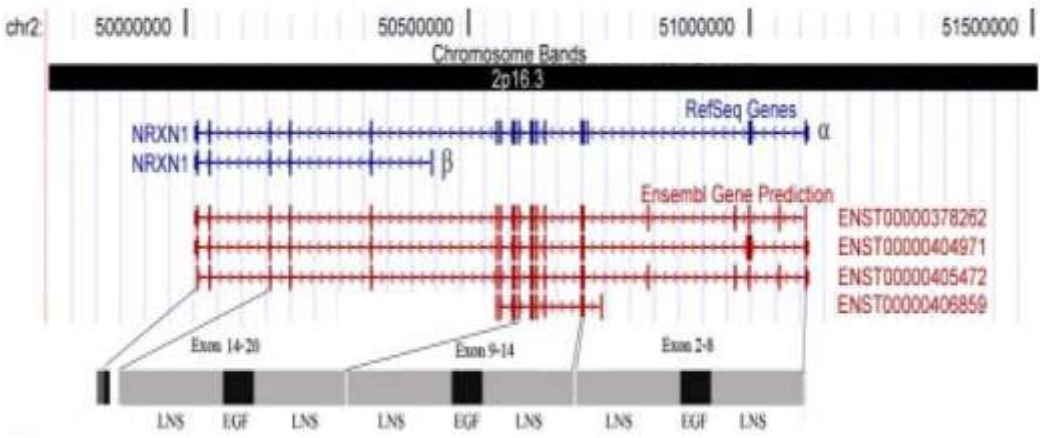

Figure 2.6: The black panel on top indicates the selected band of chromosome 2p16.3. The blue tracks show the *α-NRXN1* and *β-NRXN1* gene annotations in NCBI RefSeq whereas the red tracks indicate 3 other *NRXN1* isoforms annotated in the Ensembl bioinformatics database. α-NRXN1 protein domain showed at the bottom panel (Ching et al., 2010).

NRXN1 was subjected to large scale alternative splicing, which regulated by regional neuronal action potential via calcium/calmodulin-dependent kinase IV signaling cascade either temporally or spatially (Rujescu et al., 2009). *NRXN1* is among the sizable studied human genes and it can be influenced by gene alterations including point mutation, rearrangement, loss of entire gene, and copy number duplications (Kirov et al., 2009).

Close and rapid interaction was found between α-NRXN1 and isoforms of neuroligin and neurexin-attach organic compounds called neurexophilins. This protein located presynaptically and is needed for calcium-triggered neurotransmitter releasing (Zhang et al., 2005) and involved in mechanism of electric potential difference calcium channels at brainstem and neocortex synaptic junction (Dudanova et al., 2007). Mice knockoffs of the three *α-NRXNs* cause severe impaired of synaptic function while no severe malfunctions of axonal path finding in the process of development was demonstrated (Dudanova et al., 2007). Mice with single removes of *α-NRXN* genes have unexceptional reduced in postnatal mortality. Mice with double knockout encounter high reduction of postnatal viability rate. Triple knockout of *α-NRXNs* in mice cause the subjects could not sustain oneself past the very early stage of living (Missler et al., 2003).

β-NRXN1 is much smaller than α-NRXN1. β-NRXN1 has been found interacting with the cell adhesion organic molecules of neuroligin family (Sugita et al., 2001) and dystroglycans (Tabuchi and Südhof, 2002; Chen et al., 2008). Moreover, there was no animal studies with β-NRXN1 knockouts, either single or multiple knockouts involved α-NRXN1, have yet been carried out (Südhof, 2008).

2.3.2 Synaptic Neurexin –Neuroligin Complex

A trans synapses composite structure was configured by NRXNs with NLGNs formed at the middle of the synaptic knob, with the C-terminus amino acid chain protruding outside the complex in contrary directions (Figure 2.7). The fluorescence stained organic compound of interaction at membrane layer across synaptic cleft able to be seen under high resolution electron microscopy. The interaction layers of presynaptic neurons are differentiated apart from postsynaptic neurons by the glycosyl group attachment which exists at NLGNs and NRXNs of exterior part of membrane (Südhof, 2008). These glycosylated chains act as an interaction zone that generates a gap between the membrane of plasma and the interaction layer. It facilitates the outer cellular chemical interaction to be placed at synaptic cleft apart of the membrane (Missler et al., 2003).

The cytoplasmic chain of NRXNs consist a C-terminal attaching place for calcium/calmodulin-dependent serine protein kinase (CASK) proteins. CASK proteins include calcium/calmodulin-dependent kinase (CAMK) domain, PSD-95/D1g/ZO-1 (PDZ) domain, guanylate-kinase (GUK) domain and SRC Homology 3 (SH3) domain.

CASK proteins react with actin localized on the NRXN cytoplasmic chain by concurrently attach with other cell surface molecular compounds (Biederer and Südhof, 2001). Removal of *CASK* gene through sequences deletion in mice resulted in a fatal phenotype such as synaptic malfunctions, showing that CASK is a crucial molecule (Pang et al., 2010). CASK is mostly an organic compound of a signal transmission mechanism that transduces outer cellular signals of cellular outer membrane compounds into an intra-layer feedback by facilitating of phosphorylating target proteins and actin cytoskeleton (Tanaka et al., 2011).

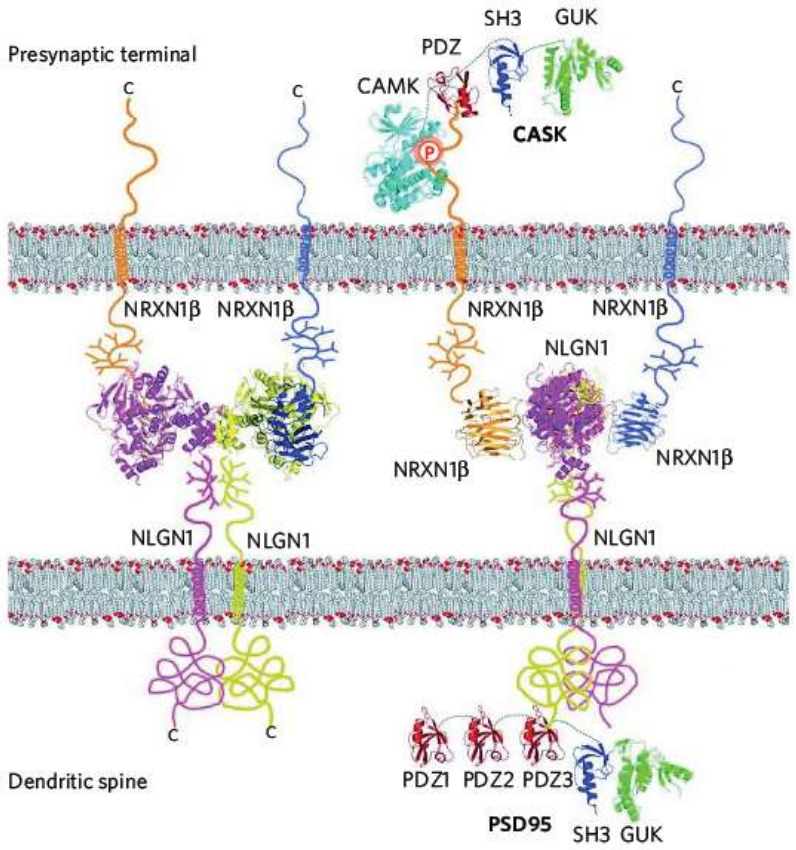

Figure 2.7: Molecular illustration structure of the synapse interaction exists between NLGN1 with β-NRXN1 (Südhof, 2008).

At the dendritic spine site, postsynaptic density protein 95 (PSD95) domain binds to intracellular adaptor proteins which then included postsynaptic sites recruitment of glutamate receptors (Biederer and Südhof, 2001). Postsynaptic adaptor proteins attached to synaptic junctions through these multiple domains interaction. As the outcome of the attachment to PDZ domain compounds, the space created by NLGNs and NRXNs maintain regulated and resembles the structure of close regulated junctions (Missler et al., 2003).

2.3.3 *NRXN1* and Neuropsychiatric Disorder

There are many facts that *NRXN1* deletions (Glessner et al., 2009), point mutations (Yan et al., 2008), and disruptions (Kim et al., 2008) are present of association with psychiatric disorder. *NRXN1* gene was early implicated and related to autism spectrum disorders due to reported cases of heterozygous inactivation. The gene was found silence resulting from de novo deletion and balanced translocation (Szatmari et al., 2007) and, subsequently, by numerous copy-number variants (Yan et al., 2008) and single nucleotides mutations (Zahir et al., 2007).

NRXN1 copy number loss subsequently had been linked to schizophrenia (International Schizophrenia Consortium, 2008; Kirov et al., 2009; Walsh et al., 2008; Glessner et al., 2009; Levinson et al., 2011). Besides that, deletions of *NRXN1* have been highly associated with numerous other conditions including language delay, intellectual impairment, attention deficit disorder and epilepsy (Ching et al., 2010; Schaaf et al., 2012). These alteration mainly because of deletions confer genetic risk in a wide range of neuropsychiatric and/or neurodevelopmental disease. On top of such variable indication, cases of penetrance incompletely and heterozygosity deletions of *NRXN1* have also been discovered (Harrison et al., 2011).

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Disparate from repeated deletions at flanking terminal repeats, *NRXN1* deletions could different in their length and position (Harrison et al., 2011). The observation of regular and rapid rearrangement of *NRXN1* suggests that it is usually susceptible to mutational molecular level mechanisms that lead to copy number imbalance and expose the individuals to risk of neuropsychiatric disorders (Ikeda et al., 2008). Some findings have revealed that disruption of the *NRXN1* by submicroscopic chromosomal deletions rising the risk of developing schizophrenia. Array of comparative genome hybridization (array-CGH) been used to analyze the targeted genomes of schizophrenia subjects with approximately 35000 probes for chromosomal copy number variantions (CNVs) and deletion of *NRXN1* been identified stretch across the promoter and the exon of the gene (Kirov et al., 2009).

2.4 Identifying Copy Number Variations (CNVs)

Copy Number Variations (CNVs) are the unbalanced and quantitative alterations in normal diploid genomic loci. Feuk et al. (2006) defined copy number variations as changes that more than one kilobase in DNA segment in order to differentiate smaller variations, such as, segmental duplications (SDs), Variable number tandem repeats (VNTRs), micro- and mini-satellite repeats etc. CNVs are either deletions, insertion or tandem duplications which cause quantitatively changes in normal diploid state. Gene rich regions have been found to have higher frequencies of CNVs as compared to conserved regions and this is indicating that structural variation is a property of the functional genome (Szatmari et al., 2007).

In the continuous identification for genetic causes of schizophrenia, most of the effort has been focus on alterations in deoxyribonucleic acid (DNA) sequence that may rising the risk for the disorder. Moreover, genomic copy number changes are progressively identified as a cause lead to risk for human diseases (Sahoo et al., 2011). Therefore, variation in copy number of susceptibility genes which confer higher risk to schizophrenia was identified (Ching et al., 2010). This in turn will hopefully lead to an understanding of the biological pathways involved in schizophrenia etiology and as a result, successful treatment (Kirov et al., 2009). There was fact showed that CNVs occurred in *NRXN1* are associated with cognitive capability (Rujescu et al., 2009), language development disorders (Magri et al., 2010), autism (Ikeda et al., 2008), and psychiatric disorders (Dabell et al., 2013).

2.4.1 Genomic Effects of CNVs

CNVs are often reported to be flanked by closely similar blocks of sequence, such as low-copy repeats, segmental duplications (SDs), and LINE repetitive elements etc. CNVs also found to be abundant in the centromeric and telomeric regions in most organisms (Cooper et al., 2007).

Formation of CNVs facilitated by repeat sequences (Weischenfeldt et al., 2013) through nonallelic homologous recombination (NAHR) due to unequal crossing over and misalignment in the DNA strands (Figure 2.8). NAHR occurred when recombination between lengthy and highly alike low-copy-number repeats. Besides that, novel

genomic insertion can resulted from mobile DNA segments insertion of transposable elements through retrotransposition. The third mechanism is termed, Fork Stalling and Template Switching (FoSTeS). FoSTeS is found to facilitate mistake in DNA replication mechanism resulting in CNVs (Stankiewicz and Lupski, 2002). Non-homologous end joining (NHEJ) is another recombination based occurrence of CNV formation. It is a procedure of DNA double-strand breaking repairment without large scale sequence homology and is usually occurred simultaneously with addition or deletion of several nucleotides. NHEJ occurs when broken DNA strands are bridged, modified, and ligated incorrectly (Lee et al., 2007).

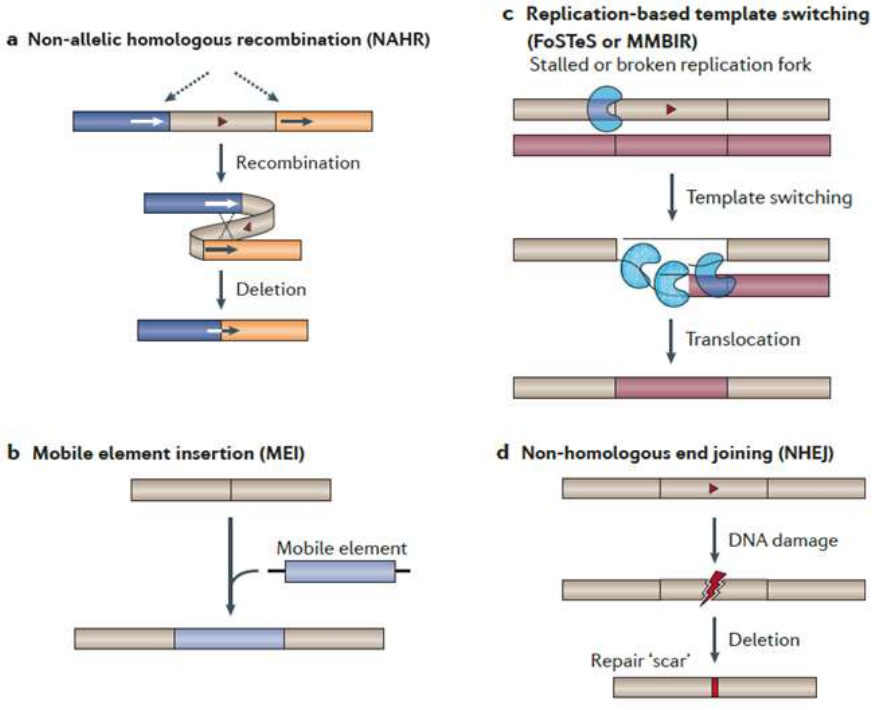

Figure 2.8: Structural variants involved different classes and formation mechanisms (Weischenfeldt et al., 2013).

CNVs are a key source of genetic variation resulted in phenotypic diversity and evolution, conversely also lead to disease pathogenesis, as observed in strikingly high amount of CNVs per genome in cancer-prone persons with neuroblastoma and Li-Fraumeni syndrome (Schneider et al., 2013). These observations have prompted investigations into occurrence of copy number polymorphism as an indication of genomic instability leading to disease pathogenesis. Abnormal frequencies or change of copy number during transmission between generations (Levinson et al., 2011) have been compromised with autism spectrum disorders (ASD) (Stankiewicz and Lupski, 2002).

2.4.2 Functional Effects of CNVs

The most common ways CNVs affect phenotypic variability is by alteration in transcription of genes that are sensitive to dosage affects. In the case of trisomy 21, chromosome 21 hypothesized as critical region containing a subset of dosage-sensitive genes determine the disease phenotype. Trisomy 21 patients identified to have amyloid precursor protein duplication result in cerebral amyloid angioplasty phenotype (Rovelet-Lecrux et al., 2006).

In normal diploid genome, offspring often inherit one copy of gene from each parent (Figure 2.9). Heterozygous and homozygous copy number (CN) loss result in not functioning and disruption of gene expression, and are linked with various human disorders (Lupski and Stankiewicz, 2005). Gain in CN in the genomic locus causing

increase in gene copy numbers and also deregulations of gene expression (Sharp et al., 2005). CNVs confer phenotypes through other mechanisms in genomic disorders such as disruption of gene, fusions of gene at the junction, position effects in which the rearrangement changes the mechanisms of a nearby gene, and revealed of recessive mutations.

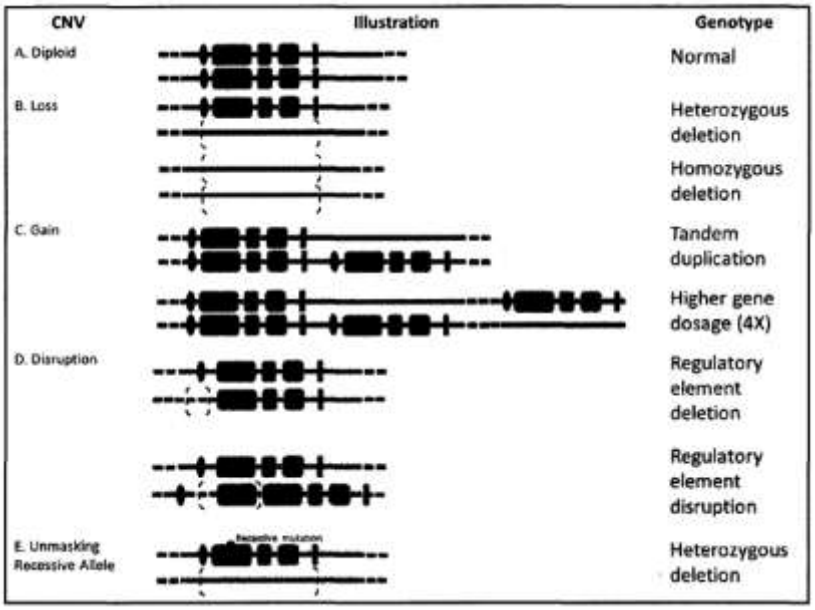

Figure 2.9: Illustration of CNVs in the genome and examples of outcome on phenotypic variability (Hooli, 2011).

Recent studies also report that CNVs overlapping locus control region include genes involved mainly in sensory perception (Marshall et al., 2008). These genes are familiar with dosage sensitive and might affect neurocognitive skills/deficits, personality determinants, behavioral abnormalities, and psychiatric disorders. In line with this, several psychiatric disorders, including mood and anxiety disorders, have been associated with CNVs (Lee et al., 2007).

CNVs confer phenotypes through several mechanisms in genomic disorders. The increase or decrease in the transcribed protein in a dose dependent manner due to changes in gene copy number leads to pathogenesis of diseases (Hooli, 2011). In case of autism and schizophrenia, where the phenotypes in the patients are heterogeneous, rare complex rearrangements in multiple genomic loci are reported (Stranger et al., 2007). These include gene dosage effect at the transcription and translational levels or functional single nucleotide polymorphisms (SNPs) on the existing allele (Stankiewicz and Lupski, 2002). CNV analysis could hence reveal novel genes and pathways of functional significance that may not have been obvious from studies of nucleotide level alterations.

2.5 Single Nucleotide Polymorphisms (SNPs)

With the increasing availability of human genome data, it has become clear that the differences from one human genome to other often due to single nucleotide base changes, termed as single nucleotide polymorphisms (SNPs) (McCarthy et al., 2008). Many SNPs have been identified through genome-wide association studies (GWAS) – studies that examine frequent variants in huge numbers of individuals in order to determine whether any association exists between a particular variant and a specific trait (Manolio, 2008). The SNPs which are found to be associated with a trait are often merely markers for genomic locations and are selected for their ability to adequately tag the genome (Kraft and Hunter, 2009).

2.5.1 Linkage Disequilibrium and Haplotype

Linkage disequilibrium (LD) is the specific interrelation of alleles at various loci. It is a sensitive measurement of the population genetic forces within structure of a genome (Fallin and Schork, 2000). Due to many existing procedures for determine variation of genetic with a sensitive scale, human geneticists and evolutionary scientists are gradually utilizing disequilibrium of linkage to study the confluence evolution of associated sets of genes and mapping the genes that are linked with hereditary diseases and personal characters (Barrett et al., 2005).

LD at each region of genomes reviews the evolutional natural selection, transmutation of genes, nonsense alteration and some another factors which manipulate through the occurrence rate of gene evolution. Local recombination rates affect the linkage disequilibrium in a genome. Besides that, the inheritance of selective sites of loci by relies on rates of local recombination (Schaffner et al., 2005).

Most of the time, a single SNP marker does not reveal much information. Hence, multiple SNPs have to be identified across the DNA strand in order to figure out the association between SNPs and certain diseases. For example, a chromosome region with three SNPs could result in eight different SNPs combination. Statistically, haplotype is the set of SNPs combination on chromosome that tend to associated together. It is a way of determining these statistical associations and several alleles of a specific haplotype across the sequence. This technique further allows the identification

of all other possible polymorphic sites that are located nearby on same chromosome. This collective of scientific data is important for identifying the roots of genetics in most disorders (International HapMap Consortium, 2005).

Each blocks of haplotype in humans different in length from a few kb to more than 100 kb. However, some studies of haplotype found that linkage disequilibrium normally extended over a much further chromosomal lengths (Barrett et al., 2005). Testing on SNPs present in haplotype block of selective population for association with certain disorder might be helpful in figure out the significant nucleotides. Therefore, the number of studied SNPs within haplotype block is important when tested the haplotype in case–control studies of disease association (Fallin and Schork, 2000).

2.5.2 SNPs and Schizophrenia

Genome-wide association studies had discovered many susceptible SNPs were associated with schizophrenia. Previous study had investigated the relationship of gene encode for a protein constituent of dystrophin-associated protein complex (DPC) of with mental illness (Numakawa et al., 2004). A study revealed the identified SNPs markers (rs2619528 and rs2619522) were positively related with schizophrenia in the Asian people typically in population of Japanese (Numakawa et al., 2004). In contradictory of the result, the two SNPs were showed no significant correlation in another study done by Tochigi et al. (2006). However, the result may vary due to

different targeted geographical isolated population as the finding in Korea showed consistent result for rs2619522 (Joo et al., 2006).

Some studies have reported a positive association of *AKT1* gene with schizophrenia in European (Karege et al., 2012), and Japanese population (Ikeda et al., 2004). Another studied SNP (rs2494732) of *AKT1* gene was found associated with schizophrenia in a Malaysian case-control study (Loh et al., 2013). Dysregulation in the dopaminergic system has been suggested to play a crucial part in the pathophysiology of schizophrenia (Utsunomiya et al., 2008). On contrary, Tee, Tang and Loh (2011) found no significant association of catechol-o-methytransferase (*COMT*) gene SNPs (rs165656) with schizophrenia in Malay population of Malaysia.

Shah et al. (2010) genotype the rs2287235 SNP marker, which is approximately 3700 bases apart from the translation starting site of *NRXN1* gene through case control study in European population. The finding showed rs2287235 does not involve in schizophrenia pathogenesis but it suggested that rs2287235 might play an important role of potential allele-specific differences in transcription factor attachment. Another *NRXN1* SNP marker, rs1045881 variant was found associated with brain frontal lobe white matter volume, where homozygous C allele carriers showed reduction of frontal white matter volumes in schizophrenia subjects compared to homozygous or heterozygous 'T' allele carriers (Voineskos et al., 2011).

2.5.3 *NRXN1* SNPs

Previously, a study on genetic variants of *NRXN1* single nucleotide modifications (rs10490162 and rs12467557) with response to treatment of neuroleptic drug in more than 50 selected schizophrenia patients was conducted by Jenkins et al. (2014). Significant associations of rs12467557and rs10490162 with drug response were revealed in the trial composed of patients and controls with included of placebo in this pharmacogenetic analysis. They also found that individuals who were carried homozygous A allele in either rs10490162 and rs12467557 showed observable improve in terms of overall mental pathology and feeling disturbance. On the other hand, patients who either homozygous or heterozygous G allele carrier showed no significant response to the treatment (Jenkins et al., 2014).

Besides that, Souza et al (2010) was reported clinical evidence on association of polymorphisms rs10490162 and rs12467557 markers with medical treatment response in schizophrenia patients. Carriers who carry homozygous A alleles at either markers were found more tentatively in respond to a drug known as clozapine which classified as atypical antipsychotic medication (Souza et al., 2010). Clinical pharmacogenetic study showed *NRXN1* SNPs have been associated with treatment using clozapine in schizophrenia subjects (Lett et al, 2011) as well as dependence on nicotine (Bierut et al, 2007) in healthy individuals (Nussbaum et al, 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 Samples Collection

The study sample consisted of twenty eight schizophrenia patients (17 males and 11 females; mean age: 40.75±10.20 years) and twenty four healthy controls (12 males and 12 females; mean age: 38.42±13.21 years). The subjects were ethnically matched and subsequently divided into three different ethnic groups. The distributions of recruited patient's ethnic group were 50% Malays, 36% Chinese and 14% Indians while control's ethnic group made up of 58% Chinese, 33% Malays and 9% Indians. The twenty eight schizophrenic patients were originated from thirteen different families. Patients consisted of nine sibling pairs, two sibling trios and two parent-offspring pairs. Twenty four healthy controls are from twelve different families. Controls included nine sibling pairs and three parent-offspring pairs.

The patients were recruited from Psychiatry Department of Hospital Permai Johor Bharu, Johor, Malaysia. All hospitalized patients were met the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) diagnostic measurement scale. They were gone through interview session by experienced psychiatrists utilized the Mini International Neuropsychiatric Interview (MINI)

(Appendix A). MINI is a specific mentality oriented diagnoses examination in Diagnostic and Statistical Handbook of Mental Disorders-Fourth Edition (DSM-IV) and International Classification of Diseases-Tenth Edition (ICD 10) mental illness. Control subjects were required absence of recreational drugs abuse or a family history of neurodegenerative or psychiatric disorders, extended including first-degree relatives. Exclusion criteria from being recruited as controls were: subjects with psychiatric comorbidities, generalized anxiety disorder, antecedent history of illicit substance abuse, history of mental retardation, neurological disease, or any clinical condition that could affect cognitive performance. This study was approved under University Malaya Medical Centre (UMMC) Medical Ethics Committee Institutional Review Board, Malaysia. The research purposes and procedures of the study were explained to all participants and written informed consent (Appendix B) was obtained from all participants.

3.2 Trail Making Test (TMT)

The Trail Making Test composed of duo sections: Part A and B. Each part composed of 25 circles spread over on paper. TMT was performed followed the rules formulated by Strauss, Sherman and Spreen (2006). The circles have to be connected as fast as possible, with absence of remove the pencil or pen away from work sheet. The measure begins to count once the test was started. The direct score of parts A and B are constituted by the time of completion of the tasks. Total duration measured in seconds for both sections was documented, representing the TMT Part A (TMT-A) and TMT

Part B (TMT-B) direct measure scores. Alternatively, another two scores were derived and calculated: differential score (B−A) and score of ratio (B: A).

3.2.1 TMT Part A

TMT-A is used to determine the visual search, screening and cognitive processing speed of subjects (Tombaugh, 2004; Ríos et al., 2004). In Part A, the circles that numbered from 1 to 25 and distributed thoroughly on a sheet of paper. Lines have to be drawn to link up all the figures in ascending order. First, the subject was given a hard copy Trail Making Test Part A (Appendix C) writing sheet and a pencil. Next, the subject was timed as he or she begins to connect the numbers in ascending order. If an error made, the mistake was pointed out to subject immediately and has he/she corrected it. The time to correct error was included in completion time for task. Scoring method of the test was determined by the seconds used to complete the test.

3.2.2 TMT Part B

Besides determine the visual search and processing speed, Part B of TMT plays an important role in examine the working memory, mental flexibility and task switching ability of subjects (Tombaugh, 2004; Ríos et al., 2004). In section B, the circles include both numeral and alphabetical characters. There are 13 numerals (1 to 13) and 12 alphabetical letters (A to L) in TMT Part B. All circles have to be connected with lines in ascending manner, but with the additional undertaking of alternate switching

between the numbers and letters (i.e., 1-A-2-B-3-C, etc.). The subject was given a hardcopy worksheet of the Trail Making Test Part B (Appendix D) and a pencil. Time was start to record when subject begin connect alternately between numbers and letters sequentially. When error happened, the mistake was pointed out and corrected by subjects immediately. The time to correct error was not excluded and the duration used to finish the test was documented.

3.2.3 TMT Derived Score

Furthermore, on top of direct scores, the B−A dissimilarity result and the B: A ratio were calculated. Both derived scores had been practically used for clinical assessment purposes as the indicators of cognitive functions or direct markers of brain damage (Sanchez-Cubillo et al., 2009). B − A minimized visuoperceptual and demand of working memory, providing a relatively pure measurement index of executive functioning abilities (Sanchez-Cubillo et al., 2009). Alternatively, the B:A score was used to examine the divided attention control of subjects (Ríos et al., 2004).

3.2.4 Statistical Analysis

Statistical analysis was conducted by using IBM SPSS Statistics (version 20.0, SPSS Inc., Chicago IL). The TMT direct and derived results conferred as mean and deviation from standard. Additionally, Mann-Whitney U test was applied to compare differences between the TMT scores made by controls and patients. Next, Spearman's correlation

(r_s) was employed to measure the strength and association that exists between the variables of direct and derived TMT scores.

3.3 DNA Extraction from Blood

For the purpose of DNA extraction, a peripheral blood sample (10 ml) was acquired from every subject and gathered in ethylene diamine tetraacetic acid (EDTA) anticoagulant tubes (BD Vacutainer®, U.S.A) to prevent clotting. Extraction of DNA was conducted using the QIAamp DNA Blood Mini Kit (QIAGEN®, Germany) by centrifugation technique.

Firstly, 20 µl of QIAGEN Protease (proteinase K) was pipetted into the lowest part of a 1.5 ml microcentrifuge tube, followed by 200 µl of whole blood specimen was added to the microcentrifuge tube. After that, 200 µl of Buffer AL was added to the tube and mixed up completely by pulse-vortexing for 15 seconds to generate a well-mixed and homogenous solution. The tube was incubated at 56 °C for 10 minutes in order to have a maximum DNA yield after break down of blood cells.

The microcentrifuge tube was momentarily centrifuge to get rid of drops from the inner of the lid. Sample was added with 200 µl of absolute ethanol (98%) and mixed again by quick pulse-vortexing for 15 seconds. After put the ingredients together, the microcentrifuge tube was briefly centrifuge to take off drop from the inner of the lid.

Next, the mixed up solutions was carefully pipetted to QIAamp Mini spin column (in a 2 ml collection tube). The QIAamp Mini spin column was centrifuged for 1 minute at 8000 rpm. After that, the filtrate was disposed.

The QIAamp Mini spin column was pipetted with with 500 µl Buffer AW1. The column was centrifuged for 1 minute at 8000 rpm. After centrifugation, the filtrate was discarded again. Then, QIAamp Mini spin column was add up with 500 µl Buffer AW2 and was further centrifuged with full speed at 14,000 rpm for 3 minutes.

Once centrifugation was done, the filtrate was discarded. Finally, the spin column was add up with 200 µl Buffer AE and was keep in order at room temperature (15 – 25 °C) for 5 minutes and subsequently undergo centrifugation at 8000 rpm for exact 1 minute. The eluted DNA was then stored at –20 °C.

3.4 Quantification of DNA Samples

Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, U.S.A) was used to determine the DNA concentration and purity ratios. Firstly, blank measurement was performed by loading 1µl deionized water. As blank measurement was completed, 1 µl of DNA sample was pipetted directly onto the measurement pedestal. DNA concentration was measured and 260 /280 nm, 260/230 nm purity ratios were calculated.

The concentrations of DNA samples were standardized. Each DNA sample was diluted to 5 ng/ µl by using 1x TE buffer (pH 8.0). The pH was adjusted to pH 8.0 and diluted DNA was then stored at –20 °C.

3.5 Copy Number Assays

3.5.1 Genotyping Master Mix

The TaqMan® genotyping master mix contains AmpliTaq Gold® DNA Polymerase, ROX™ Passive Reference, deoxyribonucleotide triphosphates (dNTPs), and buffer components. AmpliTaq Gold® DNA Polymerase used as a key ingredient in Real Time PCR. ROX™ Passive Reference was used to normalize the well-to-well differences and gives an internal reference to which the signal of reporter-dye can be normalized during analysis of data.

3.5.2 Copy Number Reference Assay

TaqMan® Copy Number Reference Assay RNase P was used as the typical reference assay in analysis copy number of *NRXN1*. This assay diagnoses the Ribonuclease P RNA component H1 (H1RNA) gene (*RPPH1*) located on cytoband 14q11.2 of chromosome 14 (chr.14:20811565). It has an 87 bp amplicon that maps within the single exon *RPPH1* gene. The reference assays consisted of 4,7,2-trichloro-7-phenyl-6-carboxyfluorescein (VIC) dye–labeled TAMRA™ probe and reference sequence–specific forward and reverse primers.

3.5.3 *NRXN1* Gene Copy Number Assay

Introns and intron-exon boundaries were targeted for *NRXN1* CNV genotyping in this study. Intron consists of regulatory elements which guide the spliceosome whereas intron-exon junctions act as crucial regions to be recognized by splicing machinery mechanisms for precise ligated of coding regions prior translated into amino acid sequence (Ward and Cooper, 2010).

According to *NRXN1* deletions detected by Schaaf et al (2012), 30% and 10% of intronic deletions were found at Intron 6 and Intron 20 respectively. Based on the outcome of array comparative genomic hybridization (aCGH) done by Dabell et al (2012), 7.5% *NRXN1* deletion was found at region Exon 6 – Exon 8. Besides that, *NRXN1* Exon 9 to Exon 17 was found deleted in 12.5% of deletion samples (Dabell et al., 2012). Ching et al (2010) reported 25% of *NRXN1* deleted samples were found absence of regions from Exon 6 to Exon 17. Hence, *NRXN1* deletions at Intron 6 (Schaaf et al., 2012), Intron 7 – Exon 7, Intron 9 – Exon 10, Intron 12 – Exon 13, Intron 15 – Exon 15, Intron 16–Exon 17 (Ching et al., 2010; Dabell et al., 2012) and Intron 20 (Schaaf et al., 2012) were reported to be associated with schizophrenia. These seven *NRXN1* regions were chosen for CNV genotyping in this study and listed in Table 3.1.

Each *NRXN1* CNV genotyping assay contained targeted region sequence specific forward and reverse primers and 6-carboxy-flurescine (FAM)^{TM} dye-labeled Minor Groove Binding (MGB) probe. All assays listed in Table 3.1 were specifically designed for selected targeted *NRXN1* copy number alterations regions in the human genome.

Table 3.1: Details of *NRXN1* copy number assays.

Assay ID	Region	Location on Chromosome 2	Probe	Amplicon Size (bp)
Hs04655378_cn	Intron 6	50916609	NFQ-MGB	108
Hs01321892_cn	Intron 7 – Exon 7	50850463	NFQ-MGB	102
Hs00349838_cn	Intron 9 – Exon 10	50780123	NFQ-MGB	109
Hs01745979_cn	Intron 12 – Exon 13	50755753	NFQ-MGB	99
Hs02921447_cn	Intron 15 – Exon 15	50724514	NFQ-MGB	103
Hs02861396_cn	Intron 16 – Exon 17	50699549	NFQ-MGB	109
Hs04692971_cn	Intron 20	50428586	NFQ-MGB	85

3.5.4 Preparation of Reaction Mixture

Reaction mixture was prepared according to Table 3.2 and loaded into each well of MicroAmp® 384-well plate (Thermo Fisher, U.S.A). The reaction mixture was mixed up thoroughly. After that, the 384-well plate was sealed with MicroAmp® Optical Adhesive Film (Thermo Fisher, U.S.A). The standard 10 µl PCR reaction was carried out in triplicate for each sample according to manufacturer's protocol.

Table 3.2: Volume per well of components in reaction mixture for copy number detection.

Components	Volume per well (µl) 384-well plate
TaqMan® Genotyping Master Mix	5.0
NRXN1 Copy Number Assay	0.5
TaqMan® Copy Number Reference Assay	0.5
Nuclease-free water	2.0
DNA sample	2.0
Total volume per well	10.0

3.5.5 Real Time PCR

Amplification of *NRXN1* variants was done by polymerase chain reaction (PCR) and the process was monitored in real time. First, the reaction was initiated with a starting denaturation at 95°C for 600 seconds. This was come after by exact 40 cycles of denaturation at 95°C for 15 seconds. Next, the reaction was reduced in temperature for annealing at 60°C for 60 seconds. QuantStudio™ 12K Flex PCR System (Thermo Fisher, U.S.A) was used to quantify the amplification of the *NRXN1* and *RPPH1* copy number variants.

3.5.6 Copy Number Quantification

The copy number (CN) of each targeted *NRXN1* variant measured by Applied Biosystems CopyCaller® Software (Version 2.1). Confidence metric and absolute *z*-score metric were calculated by the software. Copy number cut-off values were calculated by the gained or losses of sequences larger than 25% of corresponding p or q arm (Capizzi et al., 2011). The detected copy number with a cut-off value of less than 1.78 copies designated as CN loss, while the copy number with marginal cut-off value greater than 2.28 are showed as CN gained. Each sample was categorized as normal CN, CN gain or CN loss.

3.5.7 Copy Number Quality Control

In order to perform quality control on collected data, three steps were performed to filter the copy number of each probe from real time PCR. In the initial phase, the data from individual real time PCR runs were tested by the software. The following factors were used to exclude samples that not meet standard requirement from further analysis. First, exclusion made on VIC Ct value > 32 which due to failure of internal target gene amplification, FAM Ct value > 40 and any probe with $\Delta Ct > 4.0$ resulted from targeted probes unable to be amplified. In second steps, the mean ΔCt was calculated. The outliers of mean ΔCt were excluded using ± 3 standard deviations as cutoff point.

In the third step of copy number quality control, the software statistical formula $\Delta\Delta Ct$ was implied in software statistical formula to measure the copy number of each probe. A standardized *z* score and confidence value were established based on copy number data. Samples that have absolute value of *z* score > 2.62 and confidence value < 0.9 which will implied greater variation were excluded from further analysis.

3.5.8 Statistical Analysis

IBM SPSS Statistics (version 20.0, SPSS Inc., Chicago IL) was used to analyze the copy number results of controls and patients. The difference of copy number mean between patients and controls were compared by independent *t* test. According to outcome of CNVs identified by quantitative PCR as reported by Wang et al (2009), CN > 2 had been classified as increase of copy number whereas CN < 2 was categorized as decrease in copy number. Quantitative PCR results of CN with diploid number (CN = 2.0) indicated as no copy number change (Wang et al., 2009). Hence, the detected CN mean of patients and controls group at each targeted *NRXN1* regions of this study was compared with CN = 2.0 through one sample *t*- test. Spearman's rank correlation (r_s) analysis was used to explore the possible effect of *NRXN1* copy number detected at targeted regions and four measures of cognitive function performances (TMT-A, TMT-B, TMT B−A and TMT B: A).

3.6 SNP Assays

3.6.1 *NRXN1* SNP Genotyping Assays

According to SNPs TaqMan® genotyping outcomes reported by Yue et al (2011), *NRXN1* SNP markers rs10490168, rs2024513 and rs13382584 were found significantly associated with schizophrenia. Jenkins et al (2014) had conducted an association study of *NRXN1* polymorphisms (rs12467557 and rs10490162) with neuroleptic drug treatment response. Both SNPs markers revealed a significant association between schizophrenia and administered antipsychotic drug response. Hence, *NRXN1* single nucleotide alterations of markers rs10490168, rs2024513, rs13382584 (Yue et al., 2010), rs12467557 and rs10490162 (Jenkins et al., 2014) were reported to be associated with schizophrenia. These five SNPs markers were selected for SNPs genotyping in this study and listed in Table 3.3.

Each tube of SNP genotyping assay mix was contained *NRXN1* single nucleotide polymorphisms sequence clearly defined forward and reverse primers with two TaqMan® MGB probes. VIC® dye labeled probe discover the Allele 1 sequence while another probe labeled with FAM™ dye identify the sequence of Allele 2. The SNP assay IDs and respective location on chromosome 2p16.3 were listed in Table 3.3. The context sequences of each assay and both targeted alleles were listed in same table. Five SNPs located on intron 6 of *NRXN1* gene was targeted. All five assays listed were specifically designed for targeted *NRXN1* single nucleotide variation in human genome.

Table 3.3: Five *NRXN1* SNP assays and their respective context sequence.

SNPs	Position on Chromosome2	Location	Context Sequence ([VIC/FAM])
rs10490168	50908336	Intron 6	TGTGTGAATGTGTGTATGTATTTTT**[A/G]**TCAGTGTTACTGTTTTATCAAT
rs2024513	50924881	Intron 6	GAAGTGTTCTTTCTTAGATACATGA**[A/G]**GTCTTGGTAACCTTAATGGCTATTT
rs12467557	51015132	Intron 6	TTAAATTTTAAACCAACAGTTCTCA**[A/G]**TGTCCTCATTGTTTCAAATTCTGTT
rs13382584	51020156	Intron 6	TCTTTACAAATGTAACCACCACCCA**[C/T]**ATCATGCCCAATGCTCCATTGTTTT
rs10490162	51020519	Intron 6	GGACGTTGTGAATGCTAATAGTATT**[A/G]**TAAAAATGTGAATCATATTTCT

3.6.2 Reaction Mixture Preparation

Reaction mixture was prepared according to Table 3.4 and loaded into each well of MicroAmp® 384-well plate (Thermo Fisher, US). The reaction mixture was mixed up thoroughly. After that, 384-well plate was sealed up with MicroAmp® Optical Adhesive Film (Thermo Fisher, US).

Table 3.4: Volume per well of reaction mixture components for SNPs genotyping.

Components	Volume per well (µl) 384-well plate
TaqMan® Genotyping Master Mix	2.50
NRXN1 SNP Genotyping Assay	0.25
DNA Samples	2.75
Total volume per well	5.50

3.6.3 Real Time PCR

Real time PCR was used to amplify targeted *NRXN1* variants. An initial denaturation at 95°C for 600 seconds aimed to denature the DNA and activate *Taq* DNA polymerase. Next, denaturing of DNA for 40 cycles at 92°C for 15 seconds, followed by recombine DNA into double stranded at 60°C for 60 seconds were done. An endpoint plate reading was determined through QuantStudio™ 12K Flex PCR System (Thermo Fisher, U.S.A). Fluorescence (Rn) values plot that contained the signal from each well was used to determine the specific alleles of each sample for respective SNP marker.

3.6.4 Statistical Analysis

Fisher's exact test was used as a statistical significance test to analyze the allele and genotype frequencies between patients and controls by IBM SPSS Statistics (version 20.0, SPSS Inc., Chicago IL). Arlequin version 3.11 was used to perform the testing of Hardy-Weinberg Equilibrium (HWE) (Excoffier et al., 2005). Microsoft Excel was used to generate Haploview data format from SNP raw data. To further analyze the haplotype structure of samples, pairwise linkage disequilibrium (LD) for all possible combination of five SNPs were computed using D' and r^2 values. The linkage disequilibrium (LD) value were obtained using Haploview version 4.2 (Broad Insititute of MIT and Harvard, Cambridge) (Barrett et al., 2005).

3.7 False Discovery Rate (FDR)

The statistical outcomes of cognitive ability test, *NRXN1* CNVs and SNPs genotyping were further analyzed with multiple testing corrections via false discovery rate (FDR) estimation. FDR correction was done by followed Benjamini-Hochberg procedure with α-level set at 0.05 (Benjamini and Hochberg, 1995). According to Glickman, Rao and Schultz (2014), FDR is a recommended alternative to Bonferroni-type adjustments in health studies by controlling the false positive rate. The chance of logical inconsistencies could be minimized in multiplicity test of health studies (Glickman, Rao and Schultz, 2014). The obtained *p*-values were sorted in ascending order, and each observed *p*-value was divided by its percentile rank respectively (Noble, 2009). IBM SPSS Statistics (version 20.0, SPSS Inc., Chicago IL) was used as an interface and the SPSS syntax format of Benjamini-Hochberg FDR estimation was generated according to the procedure described by Jakab et al (2013).

CHAPTER 4

RESULTS

4.1 TMT Direct and Derived Scores

4.1.1 Descriptive Analysis

Descriptive statistics of TMT direct measured and derivative scores of healthy controls and schizophrenia patients were calculated (Table 4.1). Mean TMT-A score was 30.3 $\pm$ 12.39 in controls and 60.53 $\pm$ 53.31 in patients, showing a significant difference between two groups ($p = 1.45\times10^{-4}$). TMT-B performance score was 61.56 $\pm$ 34.12 in controls while patient's score was 141.81 $\pm$ 94.58. A significant difference of TMT-B mean score was found between two groups ($p = 1.01\times10^{-5}$). The mean outcome measured by deducting the TMT-A from the TMT-B score (B – A outcome) was 31.53 $\pm$ 28.23 in controls and 81.28 $\pm$ 78.45 in patients group. Significant difference of B – A performance shown between two groups ($p = 0.003$). These results were remains significant after FDR estimation. The mean of B: A ratio of control was 2.11 $\pm$ 0.75 while patients mean score was 2.64 $\pm$ 1.28. No significant difference found between two groups ($p = 0.192$).

Table 4.1: Mean of TMT scores between healthy controls and schizophrenia patients.

	Mean ± standard deviation		
	Controls	**Patients**	***p*-value**
TMT-A	30.03 ± 12.39	60.53 ± 53.31	1.45×10^{-4} *
TMT-B	61.56 ± 34.12	141.81 ± 94.58	1.01×10^{-5} *
B−A	31.53 ± 28.23	81.28 ± 78.45	0.003*
B: A	2.11 ± 0.75	2.64 ± 1.28	0.192

*Result remains significant after FDR correction.

4.1.2 Correlation

Spearman's correlation matrixes between TMT scores of controls group were shown in Table 4.2. There was presence of positive and significant correlation (r_s = 0.805, p = 2.37×10^{-7}) between TMT-A and TMT-B in control group. Control's TMT-B was positively correlated with B – A, and found statistically significant (r_s = 0.787, p = 6.70×10^{-7}). Besides that, control's TMT-B was positively correlated with B: A ratio, which was significant (r_s = 0.595, p = 0.001). Positive correlation was found significantly between two derived score (r_s = 0.923, p = 2.81×10^{-12}). On the other hand, no significant positive correlations present between TMT-A with B – A, and TMT-A with B: A after FDR correction.

According to patient's TMT result (Table 4.2), TMT-A was found positively correlated with TMT-B, which was statistically significant (r_s = 0.585, p = 0.001). Patient's TMT-B showed significant correlation with B – A (r_s = 0.747, p = 5.06×10^{-6}). Besides that, TMT-B of patients showed significant positive correlated with B: A (r_s = 0.505, p = 0.006). Significant positive correlation was found between two derived score (r_s = 0.863, p = 3.41×10^{-9}) of patients whereas no difference between patients TMT-A score with both derived scores.

Table 4.2: Correlation matrixes of TMT scores for controls and patients.

	Spearman's correlation, r_s (*p*-value)			
	TMT-A	TMT-B	B–A	B: A
Healthy Controls				
TMT-A		0.805 (2.37×10^{-7})	0.379 (0.047)	0.150 (0.445)
TMT-B			0.787 (6.70×10^{-7})	0.592 (0.001)
B–A				0.923 (2.81×10^{-12})
Schizophrenia Patients				
TMT-A		0.585 (0.001)	0.136 (0.491)	−0.291 (0.133)
TMT-B			0.747 (5.06×10^{-6})	0.505 (0.006)
B–A				0.863 (3.41×10^{-9})

4.2 Concentration and Purity of DNA samples

Descriptive analysis of absorbance reading of healthy controls and schizophrenia patients were illustrated in Table 4.3. For A260/A280 absorbance reading, healthy controls have a mean of 1.82 ± 0.06 while schizophrenia patients have 1.84 ± 0.06. In the reading of A260/A230 absorbance, healthy controls have the mean of 1.93 ± 0.17 while the means of patients were 1.99 ± 0.15. Mean DNA concentration value of healthy controls (39.70 ± 6.35) and patients (39.14 ± 6.59) were close to each other.

Table 4.3: Mean ± standard deviation of A260/A280, A260/A230 and DNA concentration obtained from controls and patients.

Measurements	Healthy Controls (N= 24)	Schizophrenia Patients (N= 28)
A260/A280	1.82 ± 0.06	1.84 ± 0.06
A260/A230	1.93 ± 0.17	1.99 ± 0.15
DNA Concentration	39.70 ± 6.35	39.14 ± 6.59

4.3 *NRXN1* CNVs

4.3.1 Distribution of *NRXN1* Copy Number

The copy number of each sample at seven targeted *NRXN1* regions were measured and determined by CopyCaller® Software v 2.1 (Appendix E). Table 4.4 shows the distribution of *NRXN1* gene copy number in controls and patients across seven targeted regions. Within Intron 6, there was 1 control (4.2%) with CN loss and 1 control (4.2%) with CN gain and other 22 controls were normal (median: 2.00; range, 1.70 to 2.33), while for patients, 4 (14.3%) had CN loss and other patients were normal (median: 1.90; range, 1.44 to 2.22). At Intron 7 – Exon 7, in controls group, 1 (4.2%) with *NRXN1* CN gain and the other 23 were found normal (median: 2.06; range, 1.80 to 2.43), while in patients, 5 patients (17.8%) with CN loss, 1 (3.7%) with CN gain and 22 patients had normal CN (median: 1.98; range, 1.59 to 2.29).

At Intron 9 – Exon 10, all controls were having normal CN (median: 2.03; range, 1.79 to 2.22), while in patients group, one (3.7%) detected with CN loss and the other 27 patients found CN normal (median: 1.99; range, 1.70 to 2.20). At Intron 12 –Exon 13 and Intron 16 – Exon 17, all 24 healthy controls and 28 schizophrenia patients had shown normal *NRXN1* CN. At Intron 15 – Exon 15, 1 controls (4.2%) with CN gain, others were found CN normal (median: 2.03; range, 1.81 to 2.26). At Intron 20, 1 control (4.2%) had CN loss, 23 controls were found CN normal (median: 2.01; range, 1.65 to 2.20) while in patients group, 4 patients (14.3%) with CN loss and 24 patients CN were normal (median: 1.90; range: 1.67 to 2.21).

Table 4.4: Distribution of *NRXN1* gene copy number in healthy controls and schizophrenia patients.

***NRXN1* Target Region**	***NRXN1* NCBI Location**	**Copy Number**	**Healthy Controls *N* = 24 (%)**	**Schizophrenia Patients *N* = 28 (%)**
Within Intron 6	Chr2 50916609	Cont.*	2.00/ 0.20 (1.70, 2.33)	1.92/ 0.20 (1.44, 2.22)
		Loss	1 (4.2)	4 (14.3)
		Normal	22 (91.6)	24 (85.7)
		Gain	1 (4.2)	0 (0.0)
Overlaps Intron 7 – Exon 7	Ch2 50850463	Cont.*	2.06/ 0.26 (1.80, 2.43)	1.98/ 0.29 (1.59, 2.29)
		Loss	0 (0.0)	5 (17.8)
		Normal	23 (95.8)	22 (78.5)
		Gain	1 (4.2)	1 (3.7)
Overlaps Intron 9 – Exon 10	Chr2 50780123	Cont.*	2.03/ 0.19 (1.79, 2.22)	1.99/ 0.15 (1.70, 2.20)
		Loss	0 (0.0)	1 (3.7)
		Normal	24 (100.0)	27 (96.3)
		Gain	0 (0.0)	0 (0.0)
Overlaps Intron 12– Exon 13	Chr2 50755753	Cont.*	2.03/ 0.22 (1.81, 2.21)	2.00/ 0.12 (1.90, 2.20)
		Loss	0 (0.0)	0 (0.0)
		Normal	24 (100.0)	28 (100.0)
		Gain	0 (0.0)	0 (0.0)
Overlaps Intron 15 – Exon 15	Chr2 50724514	Cont.*	2.03/ 0.21 (1.81, 2.26)	2.00/ 0.20 (1.81, 2.17)
		Loss	0 (0.0)	0 (0.0)
		Normal	23 (95.8)	28 (100.0)
		Gain	1 (4.2)	0 (0.0)
Overlaps Intron 16 – Exon 17	Chr2 50699549	Cont.*	2.04/ 0.20 (1.80, 2.21)	2.00/ 0.10 (1.88, 2.18)
		Loss	0 (0.0)	0 (0.0)
		Normal	24 (100.0)	28 (100.0)
		Gain	0 (0.0)	0 (0.0)
Within Intron 20	Chr2 50428586	Cont.*	2.01/ 0.19 (1.65, 2.20)	1.90/ 0.18 (1.67, 2.21)
		Loss	1 (4.2)	4 (14.3)
		Normal	23 (95.8)	24 (85.7)
		Gain	0 (0.0)	0 (0.0)

***The continuous copies were reported by Median/Interquartile Range (IQR) (min, max).**

In total, 20 copy number alterations (CN loss / CN gain) were detected in controls and patients (Table 4.4). Five CNVs (3 gains and 2 losses) were found within control's group. All occurred exceptional in distinct healthy sibling pairs at different targeted sites of *NRXN1* gene. In patient's group, 15 CNVs (14 losses and 1 gain) were detected. Eight alterations occurred in isolated subjects from different families across seven *NRXN1* targeted sites. Six variations occurred within full siblings of 3 separated first-degree schizophrenia families. Two CN losses found in a sibling pair at Intron 20 while another 4 CN losses were occurred in two independent sibling trio pairs at Intron 6 of *NRXN1*.

In these 20 CN alterations, 14 (70%) CNVs were detected in separated subjects from different families versus 6 (30%) found in full siblings of similar families. Besides that, 16 (80%) CN losses found whereas 4 (20%) CN gains were detected in the finding.

4.3.2 Copy Number Mean of Samples

The CN mean of healthy control and schizophrenic patients at seven targeted *NRXN1* site were showed in Table 4.5. After FDR correction, significant differences were found at three targeted CNV regions, Intron 6 (p = 0.010), Intron 7 – Exon 7 (p = 0.020) and Intron 20 (p = 0.019). At Intron 9 – Exon 10, the CN mean of patients (1.97 ± 0.11) was lower than controls (2.03 ± 0.11), it was not significant (p = 0.076). The CN mean of controls and patients were close to each other and no statistical difference was found at Intron 12 – Exon 13 (p = 0.389), Intron 15 – Exon 15 (p = 0.293) and Intron 16 – Exon 17 (p = 0.150).

Table 4.5: Mean ± standard deviation of copy number (CN) at each *NRXN1* gene CNV targeted region.

***NRXN1* Gene Target CNV Region**	**CN ± standard deviation Healthy Controls**	**Schizophrenia Patients**	***p*-value**
Intron 6	2.00 ± 0.14	1.89 ± 0.18	0.010*
Intron 7 – Exon7	2.06 ± 0.16	1.94 ± 0.19	0.020*
Intron 9 – Exon 10	2.03 ± 0.11	1.97 ± 0.11	0.076
Intron 12 – Exon 13	2.03 ± 0.12	2.01 ± 0.08	0.389
Intron 15 – Exon 15	2.03 ± 0.12	2.00 ± 0.11	0.293
Intron 16 – Exon 17	2.05 ± 0.11	2.01 ± 0.08	0.150
Intron 20	2.00 ± 0.13	1.91 ± 0.14	0.019*

* Result significant after FDR estimation.

Table 4.6 shows the outcome of detected CN mean of control and patient groups compared with CN = 2.0 separately. Healthy controls showed no significant difference from CN = 2.0 in all seven targeted *NRXN1* sites after FDR correction. Intron 6 (p = 0.003) and Intron 20 (p = 0.002) of schizophrenia patients were found significantly difference from CN = 2.0. No significant different were found at Intron 7 – Exon 7 (p = 0.098), Intron 9 – Exon 10 (p = 0.172), Intron 12 – Exon 13 (p = 0.453), Intron 15 – Exon 15 (p = 0.818) and Intron 16 – Exon 17 (p = 0.415).

Table 4.6: Comparison of controls and patients CN mean with CN = 2.0.

***NRXN1* Gene Target CNV Region**	**Healthy Controls *p*- value**	**Schizophrenia Patients *p*- value**
Intron 6	0.781	0.003*
Intron 7 – Exon7	0.097	0.098
Intron 9 – Exon 10	0.252	0.172
Intron 12 – Exon 13	0.160	0.453
Intron 15 – Exon 15	0.252	0.818
Intron 16 – Exon 17	0.034	0.415
Intron 20	0.911	0.001*

* Result significant after FDR estimation.

4.4 *NRXN1* Copy Number and Brain Cognitive Function

Spearman's Rank Correlation Coefficients (r_s) of all the four cognitive performance measurements at each targeted *NRXN1* region were showed in Table 4.7. The correlations between four measures of cognitive performances at each *NRXN1* targeted regions were not significant ($p > 0.05$).

Negative correlation coefficient (r_s) values were found between *NRXN1* Intron 6, Intron 7 – Exon 7 and Intron 20 with all TMT direct scores (TMT-A and TMT-B) and TMT derived scores (TMT B–A and TMT B: A).

Spearman's rank correlation analysis shown positive correlation coefficient values between Intron 9 – Exon 10 with TMT-A whereas negative r_s found between Intron 9 – Exon 10 with TMT-B and TMT derived scores. The Intron 12 – Exon 13 showed positive r_s with all direct and TMT derived scores. Correlation coefficients values of Intron 15 – Exon 15 with both derived TMT scores were found positive. Moreover, Intron 16 – Exon 17 showed positive correlation coefficients values with TMT-A and TMT B: A. However, there were absence of significant correlations between all targeted *NRXN1* regions with four measures of cognitive performances in this study.

Table 4.7: Correlation between *NRXN1* target CNV regions and TMT results.

***NRXN1* Gene CNV Region**	**Spearman's Correlation coefficient, r_s (*p*- value)**			
	TMT-A	**TMT-B**	**B – A**	**B: A**
Intron 6	−0.126 (0.372)	−0.224 (0.111)	−0.203 (0.150)	−0.138 (0.329)
Intron 7 – Exon7	−0.205 (0.145)	−0.256 (0.067)	−0.100 (0.479)	−0.052 (0.716)
Intron 9 – Exon 10	0.024 (0.869)	−0.102 (0.471)	−0.133 (0.348)	−0.221 (0.116)
Intron 12 – Exon 13	0.165 (0.242)	0.035 (0.804)	0.028 (0.842)	-0.034 (0.813)
Intron 15 – Exon 15	−0.262 (0.06)	−0.036 (0.797)	0.125 (0.375)	0.219 (0.118)
Intron 16 – Exon 17	0.045 (0.753)	−0.034 (0.811)	−0.020 (0.886)	0.022 (0.875)
Intron 20	−0.168 (0.233)	−0.221 (0.155)	−0.127 (0.368)	−0.108 (0.448)

4.5 *NRXN1* SNPs

4.5.1 Allele and Genotype Frequencies

The frequencies of allele and genotype of five SNPs for subjects were indicated in Table 4.8. Patients were found deviated from HWE for rs2024513 and rs13382584 after tested with FDR. Control group of rs10490162 was discovered with HWE deviation. Among the five studied SNPs, the allele frequency (p = 0.005) and genotype frequency (p = 0.002) of rs2024513 (Appendix F) were showed significant association with schizophrenia after FDR correction. At rs2024513, patient found to have more minor G allele and higher homozygous recessive GG allele than control groups. The other four SNPs do not showed any significant difference of allele and genotype frequencies between healthy controls and patients group..

Table 4.8: Allele and genotype frequencies of five *NRXN1* SNPs.

	Allele N (Freq.)		Genotype N (Freq.)			HWE
rs10490168	A	G	AA	AG	GG	
Patients	16 (0.286)	40 (0.714)	3 (0.107)	10 (0.357)	15 (0.536)	0.508
Controls	14 (0.292)	34 (0.708)	2 (0.083)	10 (0.417)	12 (0.500)	0.967
p-value	0.805		0.606			
rs2024513	A	G	AA	AG	GG	
Patients	40 (0.714)	16 (0.286)	17 (0.607)	6 (0.214)	5 (0.179)	0.012*
Controls	42 (0.875)	6 (0.125)	19 (0.792)	4 (0.167)	1 (0.042)	0.243
p-value	0.005**		0.002**			
rs12467557	A	G	AA	AG	GG	
Patients	51 (0.911)	5 (0.089)	24 (0.857)	3 (0.107)	1 (0.036)	0.071
Controls	41 (0.854)	7 (0.146)	18 (0.750)	5 (0.208)	1 (0.042)	0.422
p-value	0.276		0.117			
rs13382584	C	T	CC	CT	TT	
Patients	7 (0.125)	49 (0.875)	2 (0.072)	3 (0.107)	23 (0.821)	0.007*
Controls	4 (0.083)	44 (0.917)	1 (0.042)	2 (0.084)	21 (0.874)	0.026
p-value	0.357		0.499			
rs10490162	A	G	AA	AG	GG	
Patients	44 (0.786)	12 (0.214)	19 (0.679)	6 (0.214)	3 (0.107)	0.054
Controls	39 (0.875)	9 (0.125)	18 (0.833)	3 (0.125)	3 (0.125)	0.004*
p-value	0.127		0.203			

* Result of HWE significant after FDR estimation

** Significant *p*-value after FDR estimation

4.5.2 Linkage Disequilibrium

In Table 4.9, D' values (recombination rate between two SNPs) displayed at the upper right diagonal while r^2 values (correlation strength between two SNPs) shown at the lower left diagonal. Absence of recombination occurred and weak correlation strength was noticed between *NRXN1* SNPs markers, rs10490168 and rs12467557 (*D*' = 1.0, r^2 = 0.053), rs2024513 and rs12467557 (*D*' = 1.0, r^2 = 0.035). Low recombination rate and weak strength of correlation was found between rs13382584 and rs10490162 (*D*' = 0.303, r^2 = 0.036) whereas absence of linkage was found between rs12467557 and rs10490162 (D' = 1.0, r^2 = 0.000).

Table 4.9: Pairwise linkage disequilibrium of five SNPs. The value of D' displayed at right diagonal while r^2 value showed at the left diagonal.

	rs10490168	rs2024513	rs12467557	rs13382584	rs10490162
rs10490168	–	0.076	1.000	0.034	0.073
rs2024513	0.001	–	1.000	0.636	0.112
rs12467557	0.053	0.035	–	0.220	0.000
rs13382584	0.000	0.016	0.044	–	1.000
rs10490162	0.003	0.012	0.000	0.036	–

4.5.3 Haplotype Analysis

To explore the potential association between SNPs, haplotype association analysis test was carried out using five *NRXN1* SNPs marker. A significant haplotype, the G – A (p = 0.045) was observed between rs2024513 and rs12467557 (Table 4.10). The association between minor G allele of rs2024513 and major A allele of rs12467557 tends to occur more frequent on patients (0.286). After FDR estimation, there was absence of any haplotypes showing significant association with schizophrenia.

Table 4.10: Haplotype associations of rs2024513 – rs12467557 at *NRXN1* gene.

Haplotype	Haplotype Frequency	Frequency		x^2	*p*- value
		Patient	Control		
A – A	0.673	0.729	0.625	1.275	0.259
G – A	0.212	0.125	0.286	4.003	0.045
A – G	0.115	0.146	0.089	0.810	0.368

CHAPTER 5

DISCUSSION

5.1 Brain Cognitive Test

TMT has been used in psychological mental study on various kind of psychiatric disorder typically in patients with post-traumatic stress disorder (PTSD), bipolar disorder and schizophrenia patient (Periáñez et al., 2007). Hashimoto et al (2006) found that schizophrenia patients were lacking of cognitive ability as diagnosed at the early beginning stage of the disease.

Schizophrenia multiplex families patients take longer executive time than healthy control families to complete TMT section A and B. The results were identical to the finding by Fujiki, et al. (2013). A later finding has also revealed low performance of patients on both TMT parts compared to healthy subjects (Kourtidou et al., 2015). Studies showed that lower performance on TMT-B is not caused merely by a slowed motor performance, but also due to reduced capability of shifting and switching between sets; a step which identified as an important composite of mental flexibility (Anderson et al., 2008). TMT section A and B scores were strong predictor for impairment of mobility, increased lower extremity function deterioration of subjects (Lezak et al., 2004).

Besides that, two derived scores had been proposed as optional indexes to further illustrate the conscious intellectual activity needed to complete the TMT. The difference score (B–A) is to reduce the factor of speed from the examined result (Lezak et al., 2004). Once the speed component is removed, results showed that schizophrenia patients took almost double the time used by controls to complete the test. This is similar to finding reported by Sánchez-Cubillo et al (2009) that patients with schizophrenia performed notable worse than normal subjects on differential derived score autonomously of their predominant indications. Watanabe and Kato (2004) reported the high efficiency of healthy subjects group in completing both section of TMT compared with schizophrenia patients.

TMT B: A also seems to be a good indicator of cognitive deterioration in certain cases. Felmingham et al. (2004) suggest the application of rational score (TMT B: A), due to deficits in TMT-B may appeared to underlie by slower performance on TMT-A. Slow information processing speed increase the difficulties of subjects when facing more complex cognitive tasks. Outcomes of this study did not show such observed differences in TMT B: A and this was similar to the finding by Kourtidou et al (2015). Significant correlation was found between TMT section A and B in both groups of subjects. This outcome agreed with the finding by Sánchez-Cubillo et al (2009) that general cognitive factors regulating and affecting both scores.

A series of correlations test confirmed the relationship between TMT section A and B direct measured scores in controls and patients group. This had supported the common speculation of common cognitive factors would adjust and modify both scores (Sanchez-Cubillo et al., 2009). All derived scores of samples were found correlated significantly with TMT-B. This had indicated that TMT-B was among the measurements that generated the largest percentage notable outcomes on subjects with schizophrenia subjects as compared to normal controls studies (Periáñez et al., 2007). Quiñones et al (2009) found a significant heritability estimates for TMT-A to TMT-B performance.

Neuroimaging and cognitive displayed has correlate the involvement of brain frontal lobe and weak executive function to schizophrenia (Demakis, 2004; Rodríguez-Sánchez et al., 2005). Studies by Demakis (2004) showed that schizophrenia subjects with destruction in brain frontal lobe preforming worse in TMT-A than non-frontal lobe damaged subjects. Furthermore, an above average consequence size for this differentiation revealed that TMT-A was less much different between frontal and non-frontal destruction. Thus, more studies should be conducted to consider frontal lobes as a function and operating unit of brain executive function (Demakis, 2004; Sánchez-Cubillo et al., 2009).

5.2 *NRXN1* CNVs

5.2.1 Intronic Deletion

The *NRXN1* intronic deletions have been found to be associated with schizophrenia (Ching et al., 2010; Schaaf et al., 2013). The finding on intronic deletion found by Curran et al (2013) was similar to the studies seen by Rujescu et al (2009). *NRXN1* intronic deletions were found in approximately two thirds of all patients who expressed neurodisability phenotypes (Curran et al, 2007). Deletions of *NRXN1* introns was pathogenic due to the losses of important regulatory substances in the gene, such as promoters, alternative enhancers, or regulatory segments take part in the mechanism splicing which produces different *NRXN1* mRNA isoforms. Losses of copy number on Exon 20 – Exon 21 disrupts the regular mechanisms consequences on the related RNA-binding proteins in the flanking introns and existence of AU-rich recognition segments for SAM68 (Iijima et al., 2011).

There are other deleterious intronic copy number losses in previous studies. Intronic copy number losses in the *SLC34A3* gene lead to hypercalciuria with hereditary hypophosphatemic rickets (Ichikawa et al., 2006), occurred of Rothmund-Thomson syndrome due to *PKD1* gene mutation (Wang et al., 2002), and patients who encountered 5-fluorouracil toxicity caused by dihydropyrimidine dehydrogenase gene mutation (Owen, 2012). The diseases could be triggered by alteration in mechanisms or biochemical pathway relatively (Sahoo et al., 2011), including removal of a specific

introns, mutation of an alternative promoter. Deleterious consequences on regulatory sequences such as those involved in gene splicing controls, resulted in constraining of intron size to below of normal. The gene might not function properly due to deletion of cis-acting elements affecting the proper splicing, exon skipping, intron retention, and turn-on of cryptic splice site (Curran et al., 2007).

The finding showed a much higher rate of *NRXN1* copy number loss was detected in schizophrenia patients compared with control populations. This agreed with Ching et al (2010) who found frequency of *NRXN1* deletion of control populations was much lower than patients group. Similar findings had been reported for schizophrenia patients group, where occurrences of *NRXN1* deletions among schizophrenia patients and healthy controls subjects are 0.19 versus 0.04 respectively (Kirov et al., 2009). It showed that the rate of schizophrenia patients have *NRXN1* copy number deletions approximately 5 times higher than mentally healthy subjects.

5.2.2 CNVs Susceptible Regions

Schizophrenia patients were found presence of copy number deletions at proximal region of *NRXN1* gene that encoding for N-terminal of NRXN1 isoforms (Ikeda et al., 2008; Vrijenhoek et al., 2008). Six heterozygous intragenic *NRXN1* deletions were detected in clinical samples, three of which had deletion at proximal and intermediate region of gene (Gregor et al., 2011).

Rowen et al. (2002) found that the *NRXN1* introns occupied large regions within the gene. Its sizable length resulted its high conservative degree throughout vertebrates include humans. It was suggested involved a critical function in the hereditary process of the *NRXN1* gene. Introns are thought to be existed and present throughout the human genome. It was speculated to fulfill many molecular functions, such as transporter of transcriptional regulatory elements or main compounds in selective splicing (Fedorova and Fedorov, 2003). Lengthy introns with more than 445 kb show a much more interspersed repetitive sequences form stable structures within the genome. It was suggested to gather splicing junctions near to each other and increase the efficiency of splicing (Shepard et al, 2009). Recent finding also shows that short inverted repeats are over expressed in the more than 50 targeted sites of *NRXN1*. The proximal region of *NRXN1* was identified as key segments of genomic instability and region of copy number losses hotspot in psychiatric and neurocognitive disorders (Chen et al, 2013).

At the proximal region of *NRXN1* gene, significant differences of copy number were found between control and patient groups at Intron 6 and Intron 7 – Exon 7. Intron 6 was the largest intron at proximal region of *NRXN1* (Tabuchi and Südhof, 2002). Intron 6 and Intron 7 – Exon 7 were found within site 1 and site 2 of alternative splicing sites. Site 1 involved in generating α-NRXN1 transcripts where exons 3, 4, and 5 alternatively spliced into 12 possible forms. Site 2 consists of two alternative 5' splices site to generate three possible isoforms (Rowen et al., 2002).

The CN loss of Intron 6 could be pathogenic as affecting the splicing of respective isoforms. Intron 6 deletions were found inherited and this may suggest that *NRXN1* Intron 6 could attribute much of the genetic variations and increased the risk for developmental and neuropsychiatric phenotypes (Schaaf et al., 2012). Among the identified deleted samples, 30% of intragenic deletions were found in Intron 6 (Schaaf et al., 2012).

The finding of structural variations influence the *NRXN1* Intron 6 (Schaaf et al., 2012) and Intron 7 – Exon 7 (Dabell et al., 2012) suggests that changed of expression in site 1 and site 2 of alternative splicing sites could interrupt α-NRXN1 transcripts formation. These identifications also reveal that more understated mutations of gene expression, such as disruption of intron region could involved in the disorder susceptibility of schizophrenia patients (Owen, Williams and O'Donovan, 2009).

At the distal region of *NRXN1* gene, Intron 20 copy number was found significantly different between control and patient groups. Intron 20 presents within site 4 of alternative splicing sites on *NRXN1* gene to generate both α-NRXN1 and β-NRXN1 transcripts (Rowen et al., 2002). Disruptions of Intron 20 adversely affect the regulatory elements on splicing sites and alter the formation of both α- and β-NRXN1 isoforms (Rowen et al., 2002). Deletions of *NRXN1* distal part could disrupt the gene region that encode for C-terminal of Neurexin1 isoforms. In the study done by Schaaf et al (2012), 10% of intronic deletion was found at Intron 20 among copy number deleted samples. One hypotheses that this is because of deletions at C-terminal affecting intensively on

Neurexin1 isoforms formation, considering the disruption of splicing alternatively, which has been found in *NRXN1* genes (Südhof, 2008). In the study done by Schaaf et al. (2012), nine out of seventeen patients affected with epilepsy were diagnosed with C-terminal intragenic deletions. Five out of seven patients with deletions at C-terminal were having macrocephaly. Harrison et al (2011) findings showed two sisters with *NRXN1* deletions, one disrupting the proximal site while another deletion occurred at distal part of gene. Both sisters had serious and early stage onset of epilepsy.

No significant differences were found at the intermediate region of *NRXN1* gene: Intron 9 – Exon 10, Intron 12 – Exon 13, Intron 15 – Exon 15 and Intron 16 – Exon 17. Results of this findings were similar with the results presented by Rujescu et al (2009) that no notable association of intermediate region of 2p16.3 genetic variants markers was detected within the huge patient-control study groups. Intron 9 – Exon 10, Intron 15 – Exon 15 and Intron 16 – Exon 17 were located outside of the alternative splicing sites whereas Intron 12 – Exon 13 was located within splicing sites 3. The intermediate region was highly conserved (Reissner, Runkel and Missler, 2013) compared with both proximal and distal region of *NRXN1* gene which encode for N-terminal and C-terminal of NRXN1 isoforms. Some selectively spliced exons in *NRXN1* are much conserved than other. Moreover, the inserted protein sequences between splicing site 2 and splicing site 4 are highly conserved (Reissner, Runkel and Missler, 2013).

The findings showed that the penetrance of CNVs within the similar families at targeted site of *NRXN1* gene were relatively low. Yang, Visscher and Wray (2010) found that schizophrenia is a polygenic inheritance disease with more expected isolated occurrences than familial cases. This was agreed with Xu et al (2008) who found de novo copy number mutations were more significantly linked with schizophrenia (p = 0.00078) in non-familial schizophrenia patients. Besides that, 15 de novo mutations which detected on separate individuals were found by Girald et al (2011) in eight schizophrenia families which is significantly higher than expected counting of previous study (Awadalla et al., 2010). In another study, 60% of the 258 recruited schizophrenia patients were identified without familial history of schizophrenia or any other related cognitive disorders (Awadalla et al., 2010). In this study, the detected CNVs could not be assured as de novo mutation due to patient's parents was not recruited and assessed clinically.

In this study, 10% CN losses and 15% CN gains were found in healthy subjects versus 70% CN losses and 5% CN gains were detected in patient group. The rate of *NRXN1* CN loss was shown 4 times higher than CN gain. Bassett et al (2010) reported that CN deletions show more severe phenotype expression and higher penetrance rate than CN duplications. Results reported by Rujescu et al (2009) showed a higher CN losses compared with CN gains on a subset of *NRXN1* gene disrupted individuals. Most studies suggest *NRXN1* deletions to be pathogenic while little is known about the prevalence and pathogenicity of NRXN1 duplications. The gain of *NRXN1* copy number was described in one such case (Wisniowiecka-Kowalnik et al., 2010).

In this study, CNVs were also detected in healthy subjects. This has suggested that copy number variations could exist in normal population, as discussed in several studies (Iafrate et al., 2004; Sebat et al., 2004; McCarroll et al., 2006; Redon et al., 2006). Wang et al (2009) reported that the copy number gains and losses in Asian normal study group were 14% and 86%. Moreover, 65% gains and 35% losses of copy numbers were found in Caucasian normal population.

5.2.3 CNVs and Cognitive Function

Schizophrenia was found associated with impaired cognitive performance (Stefansson et al., 2014). As reported by Kendall et al (2016), United Kingdom BioBank Project had discovered that carriers of pathogenic CNVs such as *NRXN1* deletion had impaired performances on seven cognitive tests (Pairs Matching, Reaction Time, Fluid Intelligence Score, Digit Span, Symbol Digit Substitution, TMT-A and TMT-B). Kendall et al (2016) compared the results of cognitive tests between schizophrenia CNV carriers with *NRXN1* deletion and healthy controls, significant differences were found in TMT-A (p = 0.008) and TMT-B ($p = 2.07\times 10^{-8}$). A study on the Icelandic population found reduced cognitive performance in *NRXN1* deletion carriers. Moreover, the *NRXN1* deletion was associated with impaired verbal IQ and deficits in verbal letter and category tests (Stefansson et al., 2014).

In this study, absence of significant correlations were found between targeted *NRXN1* CNVs regions with four cognitive measurements (TMT-A, TMT-B, TMT B−A, TMT B: A). This finding suggests that the effect of CNVs at targeted *NRXN1* regions on the TMT cognitive ability tests may be mediated by slightly different mechanisms that are not fully encompassed within the measurement of TMT direct and derived scores. Besides that, the effects on cognitive domains might vary from one CNV to another (Stefansson et al., 2014). Kendall et al (2016) found no significant correlations exist between *NRXN1* CNV with non-schizophrenia associated CNVs in TMT-A and TMT-B cognitive test, however significant correlations were found in Digit Spin and Reaction Time cognitive tests. Stefansson et al (2014) suggested that the effects of CNVs on manifestation of disease could be investigated through various cognitive function tests which target different cognitive domains.

The finding was similar to result demonstrated by McRae et al (2013), where no association was found between copy number called from the Illumina 610K SNP genotyping microarray and full-scale IQ assessment. These findings were consistent with previous published genome wide association studies of CNVs and cognitive performances, where no significant associations were found (Need et al., 2009). In spite of that, the result was contradictory with outcomes reported by Vangkilde et al (2016). The associations between cognition performances and copy number deletion at 22q11.2 region were studied with Spearman's correlation analysis and significant correlation was found between cognitive rating scales with 22q11.2 copy number deletion (Vangkilde et al., 2016).

There were other genes that reported with copy number alterations associated with brain cognitive deficit. Neuroblastoma breakpoint family *(NBPF)* gene copy number is proportionally linked with raised of cognitive function as measured by sum of IQ and aptitude scores of mathematics (Davis et al., 2003). Dosage differences involving *NBPF* gene have now been associated to expression in human, cognitive aptitude and autism severity, showing that such conditions may be present of relation between genetically and mechanistically factors. In another study, cognitive abilities were examined by using the Mini-Mental State Examination (MMSE) on 107 elderly women and mitochondrial DNA copy number was measured. MMSE scores were positively correlated with their copy number (Lee et al., 2010).

Schizophrenia patients often have reduced brain volumes (Haijma et al., 2013). Schizophrenia patients were found, intracranial and total volume of brain was significantly reduced by 2.0% and 2.6% respectively. Larger impact sizes were noticed for gray matter structures. Gray matter decreased was associated with illness that exists for longer duration and higher dose of antipsychotic medication at time of scanning. Haijma et al (2013) suggested that brain loss in schizophrenia may attribute to an overlap of early neurodevelopmental abnormalities which reflected in intracranial volume reduction as well as illness progression. Moreover, findings have revealed that there are direct relations between decline of cognitive functions and reduction in brain tissue volume (Sluimer et al., 2008; Cardenas et al., 2011)

5.3 *NRXN1* SNPs

There are many findings that *NRXN1* single nucleotide mutations (Feng et al., 2006; Yan et al., 2008) confer high susceptibility rate for schizophrenia. In a genome-wide association studies (GWAS) of SNPs markers, *NRXN1* has also been found to be linked with psychiatric disorder (Wang et al., 2009).

The genotype distribution for schizophrenia patients at rs2024513 and rs13382584 statistical deviated from Hardy-Weinberg expectations. The deviation observed in patients may be due to mutation which is the dominant source of variation in human genome. Mutation has the potential to happen in somatic cell line or germ line, it could occur due to point mutation or copy number replication error (Carlson et al., 2003). Deviation was also found in rs10490162 control group. The population bottlenecks effect may leads to unbalance selection of controls to maintain multiple alleles in a population (Przeworski et al., 2005). The deviation may indicate failure in non-random mating. Other reasons as population stratification and bias of selection are possible (Raymond and Rousset, 1995; Przeworski et al., 2005).

Absence of evidence of association between rs10490168 and schizophrenia was found. The G allele remains dominant among Caucasian and Asian populations (Patil et al., 2001). Genotypes and allele frequencies with schizophrenia were continuously observed as weak associations in European population through case control studies (Rujescu et al., 2009). The risk of NRXN1 isoform malfunction was significantly

higher in those with the A allele of rs10490168. Disruption in formation of NRXN1 isoforms will prevent the release of neurotransmitter triggered by calcium ions (Zhang et al., 2005) and causing the malfunction of electric potential difference at channels in the brainstem synapses (Dudanova et al., 2007).

Significant association of rs2024513 allele and genotype frequencies and schizophrenia was determined in our study group. The outcome was identical to the findings by Yue et al (2011) that shows significant difference in allele and genotype frequencies between patients and controls (allele p = 0.006, genotype p = 0.023) in Asian population. The rs2024513 allele frequency presented by Yue et al (2011) remained significant after the Bonferroni correction.

In the cytogenetic analysis of Indian population of schizophrenia patients (Arun et al., 2016), *NRNX1* SNP marker, rs2024513 was found significantly associated with schizophrenia (p = 0.017) and it remained significant after Bonferroni correction. Arun et al (2016) found that schizophrenia patients of Indian population have higher homozygous recessive GG allele than control groups. However, contradictory result was shown by Bentley et al. (2008) in which allele A remained as the dominant allele in European population. This may due to geography-based genetic differences that reflect different adaptive evolutionary processes by separated populations (Baye, Wike and Olivier, 2010). Evaluation of SNPs variation coverage in human genome carried out by

Li, Li and Guan (2008) revealed that selected SNPs for one population might not work well for another different population.

In this study, rs12467557 was found to have no association with schizophrenia. The data set is fairly similar with the lacking positive signals for associations of common variants in a huge study of Swedish population (Ripke et al., 2013). No association was found between rs10490162 and schizophrenia. These results indicated that neither A nor G allele was notably correlate with schizophrenia which is supported by the study of Nussbaum et al. (2008) on European and Africa population.

Specific marker analysis in the family-based subjects showed absence of evidence of association with rs10490162 ($p = 0.54$) and rs12467557 ($p = 0.33$). There were absence of association of these SNPs with schizophrenia in case-control study group (rs10490162, p = 0.959; rs12467557, $p = 0.74$) (Jenkins et al., 2014). The individuals who carried homozygous A alleles at both SNPs markers showed a responsive result to neuroleptic drug treatment. Subjects who are heterozygous (AG) and homozygous recessive (GG) at the similar variants do not showed any obvious positive outcome according to measurement of response to antipsychotic treatment (Jenkins et al., 2014).

No association was found between rs13382584 and schizophrenia. Allele C remains as recessive allele in Asian and Europe Population (Patil et al., 2001). Some finding showed that the specific site with presence of these SNPs play a part in the splicing or expression of the gene. The first NRXN transcripts go through complicate selective splicing which is then to produce many distinct isoforms (Rowen et al., 2002).

One haplotype at *NRXN1* gene was found to be associated with schizophrenia but no haplotype remain significant after FDR correction. The linkage between rs2024513 with rs12467557 showed in this study was well supported by previous report (Yue et al., 2011). The two introns located SNPs could be in present in linkage disequilibrium status while their effects have to additionally be determined. The linkage disequilibrium pattern may suggest high recombination rate in these SNPs that may be related to the point mutation at the site, through an unclear mechanism (Lupski and Stankiewicz, 2005; Lee et al., 2007). This form of haplotype diversity has great consequences for association mapping. The increase set of haplotype sharing among populations proposed that it might tend to form an efficient set of SNPs for investigate in many different populations (Carlson et al., 2003).

5.4 Future Studies

Recruitments of nuclear family which consist of parents and offspring remain a challenge in Malaysia. Many schizophrenia patients do not receive a proper health care and abandoned by their family (Krishnaswamy et al., 2011). In this study, only schizophrenia sibling pairs or parent-offspring pairs from nuclear families were enrolled rather than random samples. Some studies suggested that schizophrenia arise from multiple genes variation. In this study, only *NRXN1* was focused instead of several schizophrenia candidate genes because target neuron cascade or pathways of each gene are different.

Neuroimaging studies revealed that schizophrenia patients show neuronal vulnerability, most obviously in brain frontal lobe and their circuitry (Pettersson-Yeo et al., 2011). *NRXN1* gene copy number loss confers susceptibility to schizophrenia could influence the brain structure and function of cognitive (Voineskos et al., 2011). Structural Magnetic resonance imaging (MRI) discovered a reduced volume of temporal and frontal lobe, and reductions in pariental volumes and total brain volume within schizophrenia populations (Minshew and Keller, 2010). Moreover, a meta-analysis of over 15 separated studies assured a frontal lobe white matter deteriorations in patients with schizophrenia (Di et al., 2009). Furthermore, cognitive assessment also identify deficit of sensorimotor function in schizophrenia (Rajji and Mulsant, 2008; Welham et al., 2009). In future study, a high resolutions MRI could be used to scan all subjects, to assess total brain volume and cortical gray and white matter lobar volumes. Besides

that, a cognitive assessment known as finger tapping test will be used to assess sensorimotor function of each subject. Therefore, the influence of genetic variation (CNVs and SNPs) on cognitive and neural phenotypes could be further assessed.

CHAPTER 6

CONCLUSION

In this study, a significant correlation confirmed the relationship between TMT-A and TMT-B scores. Cognitive deficit in schizophrenia patients could modulate both direct scores. Besides that, TMT B−A derived scores act as a sensitive indicator of cognitive executive function and significant difference was detected between control and patient groups. The patients from multiplex schizophrenia families required more processing time in task switching and execute function compared with healthy families. The copy number losses at *NRXN1* Intron 6, Intron 7 – Exon 7 and Intron 20 were suspected to be associated with first-degree of schizophrenia families. The copy number mean of *NRXN1* Intron 6 and Intron 20 of first-degree schizophrenia families were found significantly lower than CN = 2.0. Low copy number at these regions of *NRXN1* may contribute to susceptibility of schizophrenia. There were absences of correlations found between targeted *NRXN1* CNVs regions with all the four measures of cognitive performances. However, study should be further conducted to determine the effect of *NRXN1* CNVs on cognitive performance. In this study, allele and genotype frequencies of rs2024513 were found associated with schizophrenia developed in first-degree families. It was suggested that *NRXN1* SNP rs2024513 may play a key role in schizophrenia. The linkage of haplotype rs2024513–rs12467557 should study further. A full understanding about the copy number variations and single nucleotides

polymorphisms of *NRXN1* are necessary and act as a milestone for clinicians to diagnose schizophrenia and come out with appropriate antipsychotic drugs as treatment or preventions.

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