Exploration of the *NRG-ErbB* Genetic Pathway for Biomarkers of Clozapine Mediated Symptom Remission and Symptom Severity in Treatment-Resistant Schizophrenia

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A thesis submitted in fulfillment of the requirements of the degree of

Doctor of Philosophy

December, 2017

Melbourne Neuropsychiatry Centre, Department of Psychiatry

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Dedicated to the almighty Allah- the most beneficial, the most merciful

Declaration

This thesis is submitted to the University of Melbourne in fulfillment of the requirements for the degree of Doctor of Philosophy. I certify that, this thesis comprises my original work and due acknowledgments have been made in the text to all other assistance received and sources used. I wrote the first draft of all the chapters and then improved the work following comments from my supervisors Dr. Chad Bousman, Prof. Ian Everall, and Prof. Suresh Sundram.

The thesis is less than 100,000 words in length, exclusive of tables, figure legends, appendices, and bibliography. Upon submission, all three results chapters have been published as original research articles. Part of the 'Introduction' chapter has been published as a 'Review article'.

In addition to the statement above, where I am not the corresponding author of a published item, permission has been granted from the corresponding author to include them in the thesis.

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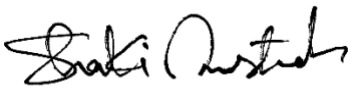
Signature:  **Date:** 18 December 2017

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Supervisor Name: Chad A Bousman

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Preface

This thesis was part of the Cooperative Research Centre (CRC) for Mental Health - Psychosis study with an aim to identify peripheral biomarkers of clozapine mediated symptom remission and symptom severity in treatment-resistant schizophrenia (TRS). The investigation was led by Prof. Ian Everall, Prof. Christos Pantelis, and Dr. Chad Bousman along with other scientists and collaborators. The study was funded by the Cooperative Research Centres programme -an Australian Government initiative. This thesis contains multi-authored publications. Study design and part of the statistical analyses in Chapter 2, was conducted with the assistance of Dr. Serafino Mancuso. The study design for chapter 3 and 4 was done by the author and supervisors (CB, IE and SS) with help from Dr. Gursharan Chana and Dr. Avril Pereira. The lab experiments were done by the author with assistance from Ms. Ting Ting Lee and Dr. Gursharan Chana. All other data pre-processing and analyses were conducted by the author of the thesis. The original research articles (Chapter 2, 3, and 4) were drafted by the author and edited primarily by Dr. Chad Bousman. Remaining contributing authors reviewed and provided important suggestions on the articles. The author's contribution to these papers was of the order of 80 percent. A portion of chapter 1 (sections 6-9) represents part of a published review article, written primarily by the author of the thesis. All the sections were drafted by the author as part of this thesis.

The author of this thesis was generously funded by MIFRS-MIRS scholarships provided by the University of Melbourne along with CRC for mental health top-up scholarship.

Publications and presentations

First author articles resulting from this thesis:

1. **Mostaid, M. S.**, D. Lloyd, B. Liberg, S. Sundram, A. Pereira, C. Pantelis, T. Karl, C. S. Weickert, I. P. Everall and C. A. Bousman (2016). "Neuregulin-1 and schizophrenia in the genome-wide association study era." Neurosci Biobehav Rev 68: 387-409.
2. **Mostaid, M. S.**, S. G. Mancuso, C. Liu, S. Sundram, C. Pantelis, I. P. Everall and C. A. Bousman (2017). "Meta-analysis reveals associations between genetic variation in the 5' and 3' regions of Neuregulin-1 and schizophrenia." Transl Psychiatry 7(1): e1004.
3. **Mostaid, M. S**., T. T. Lee, G. Chana, S. Sundram, C.S. Weickert, C. Pantelis, I. P. Everall and C. A. Bousman (2017). "Elevated peripheral expression of neuregulin-1 (NRG1) mRNA isoforms in clozapine-treated schizophrenia patients." Transl Psychiatry (accepted on: 23rd September, 2017).
4. **Mostaid, M. S**., T. T. Lee, G. Chana, S. Sundram, C.S. Weickert, C. Pantelis, I. P. Everall and C. A. Bousman (2017) Peripheral transcription of NRG-ErbB Pathway genes are upregulated in treatment-resistant schizophrenia. Front. Psychiatry 8:225. doi: 10.3389/fpsyt.2017.00225

Co-authored articles applying knowledge expertise gained from this thesis:

1. Saini, S. M., S. G. Mancuso, **M. S. Mostaid**, C. Liu, C. Pantelis, I. P. Everall and C. A. Bousman (2017). "Meta-analysis supports GWAS-implicated link between GRM3 and schizophrenia risk." Transl Psychiatry 7(8): e1196.
2. Bousman, C. A., V. Cropley, P. Klauser, J. L. Hess, A. Pereira, R. Idrizi, J. Bruggemann, **M. S. Mostaid**, R. Lenroot, T. W. Weickert, S. J. Glatt, I. P. Everall, S. Sundram, A. Zalesky, C. S. Weickert and C. Pantelis (2017). "Neuregulin-1 (NRG1) polymorphisms linked with psychosis transition are associated with enlarged lateral ventricles and white matter disruption in schizophrenia." Psychol Med: 1-9.
3. Bousman, C. A., T. Y. Lee, M. Kim, J. Lee, **M. S. Mostaid**, M. Bang, S. K. An, I. P. Everall, C. Pantelis and J. S. Kwon (2017). "Genetic variation in cytokine genes and risk for transition to psychosis among individuals at ultra-high risk." Schizophr Res.

Presentations

1. **Mostaid, M. S**., T. T. Lee, G. Chana, S. Sundram, C.S. Weickert, C. Pantelis, I. P. Everall and C. A. Bousman. "Elevated peripheral expression of neuregulin-1 (NRG1) mRNA isoforms in patients with treatment-resistant schizophrenia (2017)." World Congress of Psychiatric Genetics Conference in Orlando, Florida, USA. (poster presentation).
2. **Mostaid, M. S.**, S. G. Mancuso, C. Liu, S. Sundram, C. Pantelis, I. P. Everall and C. A. Bousman. "Meta-analysis reveals associations between genetic variation in the 5' and 3' regions of Neuregulin-1 and schizophrenia (2016)." Biological Psychiatry Australia (BPA) meeting in Newcastle, NSW, Australia (poster presentation).

Acknowledgements

I would like to express my deepest gratitude to my supervisor Dr. Chad Bousman for his tremendous help, support, and mentorship throughout my PhD journey. I have no words to express how grateful I am for all that you have done for me, starting from accepting me as a PhD student from Bangladesh and then guiding me all the way to the finish line. Thank you for always being available, answering my questions, motivating me when I got depressed and encouraging me to work harder and push myself to achieve great things. This has been a wonderful experience and I thank you for all the skills that I have learned along the way. I feel extremely lucky to have Prof. Ian Everall and Prof. Suresh Sundram as my co-supervisors. Ian, I would like to thank you for all the expert opinion and feedback that you have provided in our lab meetings. Despite being very busy, you gave me the opportunity to meet with you to discuss various research topics which helped a lot to design the study and prepare this thesis. My sincere thanks to Dr. Gursharan Chana and Ms. Ting Ting Lee to teach me all the essential laboratory techniques and guiding me in my experiments. Gursh, I could not have done the lab experiments without you and you were always there to help and support which I am ever grateful for. Besides research, I enjoyed the conversations with you regarding football and other topics.

I thank everyone at Melbourne Neuropsychiatry Centre for providing a friendly work atmosphere. This is the place where I spent most of my time, made great friends, met brilliant minds who were a source of inspiration and support. I must mention Chenxing, Deborah, Maria, and Stefanos with whom I enjoyed their friendship. Special thanks to Barbara and Lorraine for their kind words and support during the toughest part of my PhD. Thanks to Chester for all the technical support. I would also like to express my sincere gratitude to all my lab mates at the Centre for Nueral Engineering. Working with them was a great experience and the lab became a place of research with great joy and fun.

I extend my gratitude to all the collaborators particularly Dr. Carlos Martinez, Dr. Avril Pereira, Dr. Sandra Luza and Dr. Dali Kang. Thank you all for training me in the lab. Special thanks to Prof. Christos Pantelis and Prof. Cynthia Shannon Weickert for providing valuable comments and feedback which vastly improved my research articles and the thesis. Thank you to all the research participants, their families, research coordinators and CRC for mental health.

Thanks to all my Bangladeshi friends here in Melbourne with whom I have made a friendship that I hope will last for life. We proudly call ourselves 'Melbourne family' and I feel lucky to be a part of it. You were always there when I missed my family and inspired me to carry on my research. Special thanks to Mahmud, Alamgir, Bulbul, Ripa, Taufique, Baki, and Shahin.

I am forever indebted to my family for always being there for me and supporting me to fulfil my dreams. My mother (Mrs. Mamataz Begum) is the sole inspiration of my life and I cannot thank her enough for all the sacrifice that she made for me. If it was not for her tremendous support and selfless sacrifice, I would have never made it this far. I hope my dad (Mr. Akbar Ali) is happy watching me finishing my PhD from heaven. Thanks to my sister Labiba Laila and brother-in-law Ahmed Zakir-al-Mamun for all the support, inspiration, and kind words. I also thank my extended family of aunts (Lucy, Lovely, Josna, Shova, Lily, Lina), uncles (Prince, Rashed, Nasir), cousins (Maria, Era, Farabi, Mridu, Ariyan, Aliza), and friends for always believing in me and supporting me all the way.

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Abbreviations

5-HT	5 hydroxy tryptamine receptors
ABC	adenosine triphosphate binding casette
ATP	adenosine triphosphate
BA	Brodmann's area
BBB	blood brain barrier
BCA	bicinchoninic acid assay
BDNF	blood derived neurotrophic factor
BH	Benjamini-Hochberg
BSA	bovine serum albumin
CATIE	clinical antipsychotic trials of intervention effectiveness
cDNA	complementary dna
CI	confidence intervals
CLZ	clozapine
CO_2	carbon dioxide
COS	childhood onset schizophrenia
CUtLASS	cost utility of the latest antipsychotic drugs in schizophrenia study
CNS	central nervous system
CYP450	cytochrome p450
D2	dopamine receptor 2
DLPFC	dorsolateral prefrontal cortex
DNA	deoxyribonucleic acid
DSM	diagnostic and statistical manual of mental disorders
ECT	electroconvulsive therapy
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
eQTL	expression quantitative trait loci
GABA	gamma-aminobutyric acid
GLM	general linear model
GWAS	genome wide association study
Hap	haplotype

HC	hippocampus
Ig	immunoglobulin-g
IL	interleukins
INV	in-vitro
IPAP	international pharmacological algorithmic project
IQR	interquartile range
LD	linkage disequilibrium
LDH	lactate dehydrogenase
MALDI-TOF	mass assisted laser desorption ionization-time of flight
MINI	mini international neuropsychiatric interview
mRNA	messenger RNA
MS	microsatellite
NRG1	neuregulin-1
NRG2	neuregulin-2
NS	not significant
PANSS	positive and negative syndrome scale
PBMC	peripheral blood mononuclear cells
PFC	prefrontal cortex
PPI	prepulse inhibition
RIN	RNA integrity number
RSWG	remission in schizophrenia working group
RT-qPCR	real time quantitative polymerase chain reaction
SD	standard deviation
SEM	standard error mean
SNP	single nucleotide polymorphism
SZ	schizophrenia
TMD	transmembrane domain
TNFα	tumor necrosis factor alpha
TRS	treatment-resistant schizophrenia
USA	United States of America
VIC	Victoria
WB	whole blood
WBC	white blood cells

Abstract

Schizophrenia is a disabling mental health disorder that is characterized by positive symptoms (delusions, hallucinations etc.), negative symptoms (apathy, social withdrawal, emotional blunting etc.) and cognitive deficits (impaired memory, lack of attention etc.). Current pharmacological treatment includes typical and atypical antipsychotics but 20-30% of patients do not adequately respond to these treatments and are thus defined as treatment-resistant.

Clozapine is indicated for the treatment of treatment-resistant schizophrenia (TRS). However, biomarkers of clozapine mediated symptom remission and symptom severity in TRS have yet to be identified. One promising biomarker is neuregulin 1 (*NRG1*), a growth factors that activates *ErbB* receptor tyrosine kinases and initiates the *NRG-ErbB* signalling pathway, which plays a key role in neurodevelopment. Genomic, transcriptomic, and proteomic abnormalities in *NRG-ErbB* pathway have been linked to schizophrenia and clozapine has been shown to modulate *NRG1* gene and protein expression. Thus, *NRG-ErbB* pathway gene and protein expression profiles, as well as genetic variation, may serve as biomarkers for clozapine mediated symptom remission and symptom severity. In this thesis, we will present our investigation of the peripheral gene and protein expression levels of *NRG-ErbB* pathway genes in TRS patients and healthy controls and how they relate to clozapine mediated symptom remission as well as symptom severity. In addition, we will discuss the role genetic polymorphisms in *NRG1* play in regulating its gene and protein expression. Finally, we will present results from healthy peripheral blood mononuclear cells exposed in vitro to clozapine for 24 hours and seven days and discuss the effects of clozapine on *NRG-ErbB* pathway gene and protein expression.

Chapter 1 contains systematic review of scientific literatures and justifies the main 3 goals of the thesis. Chapter 2 of this thesis aimed at investigation of the candidate SNPs and microsatellites within the NRG1 gene among 16,720 patients, 20,449 controls, and 2,157 family trios via a meta-

analytic procedure. We found significant association for three polymorphisms at the 5' end (rs62510682, rs35753505, and 478B14-848) and two (rs2954041 and rs10503929) at the 3' end of the *NRG1* with schizophrenia. We could not find association for haplotypes.

Chapter 3 aimed to assess the peripheral expression pattern of major *NRG1* mRNA isoforms in whole blood and NRG1-β1 protein in serum in patients with TRS to find clinically useful biomarkers of clozapine mediated symptom severity and symptom remission. Using RT-qPCR we found upregulation of three *NRG1* mRNA isoforms (*NRG1* EGFα, *NRG1* EGFβ, *NRG1* type$I_{(Ig2)}$) in whole blood in TRS patients. However, protein assay via ELISA showed lower level of serum NRG1-β1 in TRS patients but it was confounded by smoking. Expression of *NRG1* EGFα, *NRG1* EGFβ was also negatively correlated with age of illness onset.

In Chapter 4, we continued to examine the peripheral mRNA expression pattern of the major *NRG-ErbB* pathway downstream signaling genes in TRS patients and controls to see if increased expression in ligands leads to overexpression of receptors and subsequent upregulation of the full pathway in treatment-resistant schizophrenia. We found that five mRNA transcripts (*ErbB3, PIK3CD, AKT1, P70S6K, eIF4EBP1*) were upregulated in TRS patients, although only one (*P70S6K*) survived after correction for multiple comparisons. Moreover, investigation of the clinical factors revealed that expression of *ErbB2, PIK3CD, PIK3R3, AKT1, mTOR, and P70S6K* were negatively correlated with duration of illness.

Chapter 5 summarises the main findings of the thesis, its relevance to previous literature, advancement of knowledge, implications and future steps in investigation of the *NRG-ErbB* genetic pathway for suitable biomarkers in schizophrenia, more specifically treatment-resistant schizophrenia.

Chapter 1
Introduction

1. Schizophrenia

Schizophrenia affects approximately 1% of the world's population (Rossler, Salize, van Os, & Riecher-Rossler, 2005) and is a complex, chronic neuropsychiatric syndrome characterized by positive (e.g., hallucinations, delusions and thought disorders), negative (e.g., blunted affect and emotion, apathy, social withdrawal) (Mei & Xiong, 2008) and cognitive (e.g. impaired memory, attention, executive functioning) symptoms. The typical onset of symptoms occurs between the age of 15 to 24 (American Psychiatric Association., 2000) with a symptom profile that can vary greatly from individual to individual (Liddle, 1987; Strauss, Carpenter, & Bartko, 1974). This inter-individual variation in symptoms is one of the major hurdles for early identification and treatment of schizophrenia.

1.1 Etiology of Schizophrenia

In spite of more than a century of research, the exact cause of schizophrenia is still unknown. It is widely considered as a heterogeneous group of disorders manifesting from a complex interaction of numerous genes along with epigenetic and environmental factors (DiPiro et al., 2014; Rapoport, Addington, Frangou, & Psych, 2005; Siever & Davis, 2004).

Scientific evidence suggests that genetic factors play an important role in the development of schizophrenia (DiPiro et al., 2014). In fact, the risk of developing this schizophrenia is 10% for a first degree relatives and 3% for a second degree relatives of an affected individual (McDonald & Murphy, 2003). Among monozygotic (identical) twins, the risk is nearly 50% if one twin has the disorder, while for the dizygotic twins it is 12-18% (McDonald & Murphy, 2003). Collectively, it has been estimated that 80% of the variation in the trait of schizophrenia is attributed to genetic variation (Sullivan, Kendler, & Neale, 2003). Genome-wide-association studies (GWASs), have

reported several genes linked to increased risk of developing schizophrenia (Stefansson et al., 2009; Williams et al., 2011) and suggest genetic risk is a combination of many common genetic alterations, each with a small effect, as well as, less common variants (e.g. copy number variants) with larger impact (Doherty, O'Donovan, & Owen, 2012).

To find out the probable effects of environmental factors on the development of schizophrenia, studies have been carried out with adopted children of schizophrenia affected parents. But results show that changes in environment do not reduce the risk for the child (Beck, 2009; Jentsch & Roth, 1999). The genetic basis for schizophrenia is also supported by the fact that the age of onset is same for schizophrenic siblings (DiPiro et al., 2014).

One possible hypothesis is that a number of maternal factors, such as pregnancy and birth complications, play crucial roles in the development of schizophrenia. There are findings showing that this disorder begins in utero (Jentsch & Roth, 1999). Based on data from prospective population studies, the major risk factors are: gestational diabetes, low birth weight, asphyxia, bleeding during pregnancy, congenital malformations, reduced head circumference, emergency cesarean section, rhesus incompatibility, pre-eclampsia and uterine atony(Cannon, Jones, & Murray, 2002; DiPiro et al., 2014).

Prenatal deprivation of nutrition is another plausible biological risk factor of schizophrenia. Maternal starvation due to the famine of World War II demonstrated a relative increased risk for schizophrenia and schizophrenia spectrum personality disorders for those individuals in utero at that moment (Susser & Lin, 1992). Catastrophic famine in China during the Cultural Revolution had the same effect on the offspring born to the nutrition deprived mothers (St Clair et al., 2005). Increased levels of homocysteine (a marker of folate metabolism) in the third

trimester of pregnancy (Brown et al., 2007) and low levels of vitamin D during early life are suggested as risk factors for schizophrenia (J. McGrath, Eyles, Mowry, Yolken, & Buka, 2003; J. McGrath, Saari, et al., 2004). Prenatal infections with influenza (Brown et al., 2004), rubella (Brown, Cohen, Greenwald, & Susser, 2000) and *Toxoplasma gondii* (Brown et al., 2005; Mortensen et al., 2007) increases the risk for schizophrenia but herpes simplex virus type 2 gave ambiguous results (Brown, Schaefer, Quesenberry, Shen, & Susser, 2006; Buka et al., 2001).

Several postmortem and imaging studies have revealed that structural and functional abnormalities of superior temporal gyrus occur in adults in chronic phase as well as in adolescents with childhood onset schizophrenia (Molina et al., 2006; Rajarethinam, DeQuardo, Nalepa, & Tandon, 2000; Sumich et al., 2002). Irrespective of age, volumetric reductions in multiple regions of the brain have been shown in schizophrenia at first diagnosis, especially in temporal lobe (Rapoport et al., 2005). Neurodevelopmental process disruptions during adolescence may be responsible for such volumetric reductions in gray matter (Shaw, Gogtay, & Rapoport, 2010).

There is robust evidence of environmental and social factors playing a significant role in the development of schizophrenia. Environmental stress factors such as childhood trauma especially sexual or physical abuse (Bebbington et al., 2004; Morgan & Fisher, 2007; Read, van Os, Morrison, & Ross, 2005; Scott, Chant, Andrews, Martin, & McGrath, 2007), ethnic minority and migration (Cantor-Graae & Selten, 2005; J. McGrath, Saha, et al., 2004), birthplace (metropolitan vs. rural areas) (Marcelis, Navarro-Mateu, Murray, Selten, & Van Os, 1998; Mortensen et al., 1999), and social isolation (J. J. McGrath, 2005) are found to be correlated with schizophrenia. Moreover, social stressors such as economic adversity and racial discrimination (J. J. McGrath, 2005) as well as use of cannabis (Murray, Morrison, Henquet, & Di Forti, 2007) may predispose individuals to delusional and paranoid thinking.

1.2 Pathophysiology of Schizophrenia

1.2.1 Dopamine Hypothesis

Several neurotransmitters are linked to the pathophysiology of schizophrenia and dopaminergic transmission is the one of the most heavily linked systems. Altered dopaminergic neurotransmission is primarily driven by the fact that most antipsychotics are dopamine D2 receptor antagonists or partial agonists, capable of alleviating positive symptoms (Carlsson, Carlsson, & Nilsson, 2004; Kinon & Lieberman, 1996). This hypothesis is based on the pharmacodynamics of drugs like amphetamine, cocaine and methamphetamine (METH) as they increase dopaminergic neurotransmission as well as produce psychosis (C. K. Chen et al., 2003; Janowsky & Risch, 1979). But this theory is challenged by drugs such as ketamine, phencyclidine (PCP), and lysergic acid diethylamide (LSD) as they have limited effect on dopamine neurotransmission but induce psychosis. Many studies reported insignificant D2 receptor density difference between patients and controls (Dean, Pavey, & Opeskin, 1997; Kornhuber et al., 1989; Mackay et al., 1982; Reynolds, Riederer, Jellinger, & Gabriel, 1981). There has been a revision in this hypothesis to account for both positive and negative symptoms as well as cortical hypofrontality from dopamine imbalance. It states that cortical hypoglutamatergia enhances subcortical hyperdopaminergia and cortical hypodopaminergia (Laruelle, Kegeles, & Abi-Dargham, 2003; S. Miyamoto et al., 2003). Four dopaminergic pathways have been implicated in schizophrenia (Jentsch & Roth, 1999; Stahl, 2008). They are the nigrostriatal pathway, mesolimbic pathway, mesocortical pathway, and tuberoinfundibular pathway (Mueser & Jeste, 2008). The extrapyramidal motor disturbances are thought to be generated by low dopamine level in the nigrostriatal system (Mueser & Jeste, 2008) while excessive dopamine levels in the mesolimbic pathway may be responsible for the positive symptoms (Mueser & Jeste, 2008). In contrast, the negative symptoms and cognitive deficits are hypothesized to occur because of low mesocortical dopamine levels and blockade of tuberoinfundibular dopamine results in increased level of

prolactin causing galactorrhea, amenorrhea and reduced libido.

1.2.2. Glutamate Hypothesis

Disturbances in glutamatergic neurotransmission may explain some of the deficits observed in schizophrenia (Carlsson et al., 2004; Goto, Otani, & Grace, 2007; Laruelle et al., 2003; Ross, Margolis, Reading, Pletnikov, & Coyle, 2006; G. Tsai & Coyle, 2002; Winterer & Weinberger, 2004). Glutamate is the primary excitatory neurotransmitter in the brain. Drugs like ketamine and phencyclidine are noncompetitive antagonists of NMDA receptors and induce schizophrenia like symptoms in healthy individuals and exacerbate these symptoms in patients (Luby, Gottlieb, Cohen, Rosenbaum, & Domino, 1962; Pomarol-Clotet et al., 2006; Vollenweider & Geyer, 2001). This finding led to the NMDA hypofunction/ glutamate hypothesis of schizophrenia (Olney & Farber, 1995) which points to a possibility that NMDA receptors are incapable of maintaining normal regulation of mesocortical dopamine neurons and thus leads to the positive, negative as well as cognitive symptoms in schizophrenia patients (Stahl, 2008). However, phencyclidine is also a partial antagonist of D2-type dopamine receptors and so interferes with dopaminergic transmission which may lead to the explanation of the effects observed (Seeman, 2009). One possible extension of the glutamate hypothesis is that there occurs a selective reduction of NMDA receptor activity in the GABAergic interneurons(Belforte et al., 2010; Benes & Berretta, 2001; Gonzalez-Burgos & Lewis, 2008; Lewis, Hashimoto, & Volk, 2005; Olney, Newcomer, & Farber, 1999). As a result, the GABAergic inhibitory neurons in the cortex and hippocampus are less stimulated. It prevents the glutamatergic efferent neurons from inhibition and releases excessive glutamine (Javitt & Zukin, 1991; Krystal et al., 1999; Lisman et al., 2008). Finally, elevated glutamate causes impairment of dopaminergic neurotransmission (Lisman et al., 2008). Although the majority of genetic and pharmacological findings point to the NMDA receptors but the effect of AMPA receptors should also be considered as they are closely related to each other. AMPA receptors are involved in membrane

depolarization which causes expulsion of the voltage gated magnesium blockade of the NMDA receptors and so anomalies in each of the receptors and their functions contribute to glutamate hypofunction.

1.2.3. GABA Hypothesis

Dysregulation of GABAergic interneurons has been implicated in schizophrenia pathophysiology following several post-mortem studies (Lewis et al., 2005). The most prominent is the reduction of the number of cells expressing the 67 kDa isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) within the prefrontal cortex (Akbarian & Huang, 2006; Akbarian et al., 1995; Guidotti et al., 2000; Veldic, Guidotti, Maloku, Davis, & Costa, 2005; Volk, Austin, Pierri, Sampson, & Lewis, 2000), temporal cortex (Akbarian & Huang, 2006; Heckers et al., 2002; Impagnatiello et al., 1998) as well as in hippocampus (Thompson Ray, Weickert, Wyatt, & Webster, 2011). The GAD67 is the major of all GAD isoforms and is also responsible for more than 90% of GABA synthesis (Asada et al., 1997; Fukuda, Aika, Heizmann, & Kosaka, 1998). Another probable link for GABA's involvement in psychosis is the reduction in GAD67 mRNA within the prefrontal cortex in both schizophrenia and bipolar disorder patients, while there is collateral increase in the cortical DNA-methyltransferase (DNMT1) (Veldic et al., 2005). DNMT1 is involved in downregulation of promoter functioning within the interneurons that secrete GABA (Veldic et al., 2005). The consequence is the functional deficit in GABA synthesis, which causes disturbance in impulse-dependent release. Evidence show the possibility of a compensatory mechanism as post-synaptic GABA receptors are upregulated in the same brain regions (Volk et al., 2002). Reports also suggest that there is reduced expression of the subunits making the $GABA_A$ receptor in schizophrenia, which supports the notion that GABAergic functioning is reduced in in patients (Charych, Liu, Moss, & Brandon, 2009). As GABAergic deficiencies in prefrontal cortex are widely replicated in schizophrenia and psychotic patients, there is an assumption that antipsychotics may work by

facilitating GABA synthesis (Wlodarczyk, Szarmach, Cubala, & Wiglusz, 2017).

1.2.4. Cholinergic System Hypothesis

Xanomeline, a partial agonist of muscarinic cholinergic receptor 1 (CHRM1), is capable of reducing psychotic symptoms in Alzheimer's disease patients (Bymaster et al., 1997), prompting the idea that the cholinergic system may be involved in schizophrenia pathophysiology. Numerous post-mortem studies have implicated cholinergic muscarinic receptors (CHRM) in having a possible pathophysiological role in schizophrenia and mood disorders (Crook, Tomaskovic-Crook, Copolov, & Dean, 2000; Gibbons, Scarr, McLean, Sundram, & Dean, 2009). The most significant is the involvement of CHRM1. Evidences from post mortem studies of the cortex in schizophrenic patients show that there occurs decrease in the number of M1 receptors with concomitant loss of cortical and sub-cortical CHRM1 protein expression (Gibbons et al., 2013; Scarr et al., 2009). It is also supported by a functional neuroimaging study which reveals the widespread reductions in muscarinic receptors in the brains of non-medicated schizophrenia patients (Raedler et al., 2003). Moreover, cognitive deficits and depression in mice are reported when treated with acetylcholinesterase (AChE) inhibitors (Kaufer, Friedman, Seidman, & Soreq, 1998). A recent study demonstrated that anxiety and depression symptoms, caused by the administration of an AChE inhibitor physostigmine, can be successfully reversed by antagonists of nicotinic and muscarinic receptors. All these studies point to possibility of using nicotinic muscarinic receptors as drugs in schizophrenia to minimize the negative symptoms to some extent. As the reductions of muscarinic receptors are also found in bipolar disorder and major depressive disorder (Gibbons et al., 2009) so it may be regarded as a common system deficit in major psychiatric disorders.

1.2.5. Serotonin Hypothesis

The serotonin hypothesis of schizophrenia emerged from the observation that lysergic acid

diethylamide (LSD) increases the effects of serotonin in the brain (Mueser & Jeste, 2008). Moreover, atypical antipsychotic clozapine has a high affinity for serotoninergic receptors, especially $5HT_{2A}$ receptors (Meltzer, 1992a). Many postmortem and functional neuroimaging studies report down regulation of $5HT_{2A}$ receptors in different brain regions (Burnet, Eastwood, & Harrison, 1996; Dean et al., 1996; Kouzmenko et al., 1997; Ngan, Yatham, Ruth, & Liddle, 2000; Rasmussen et al., 2010). Contrary to this, there is evidence of increases in $5HT_{2A}$ receptors within the prefrontal cortex, with a concomitant decrease in mGluR2, in the case of untreated schizophrenia patients (Gonzalez-Maeso et al., 2008), as well as in first-episode neuroleptic naïve subjects (Erritzoe et al., 2008). Thus, the serotonin hypothesis is controversial and requires further investigation.

In summary, the pathophysiology of schizophrenia is still unknown. Pharmacological treatment of schizophrenia mainly involves the use of typical and atypical antipsychotics that work on the dopamine and glutamate receptors and reduce the positive symptoms of schizophrenia.

2. Pharmacological Treatment of Schizophrenia

2.1. Typical and Atypical Antipsychotics

Effective drugs for treating schizophrenia were nonexistent until the early 1950s when clinicians began using chlorpromazine (Julien, 2001). Since the introduction of chlorpromazine, over 60 antipsychotic drugs have been developed and are often classified as either typical or atypical antipsychotics.

Chlorpromazine, a drug in the phenothiazine class, was the first antipsychotic drug that was remarkably effective in alleviating the positive symptoms of psychosis. Other drugs in the phenothiazine class included trifluperazine, prochlorperazine, fluphenazine. The use of alternative

drug classes such thioxanthenes (thiothixene, chlorprothixene), butyrophenones (haloperidol, droperidol) and miscellaneous drugs (loxapine, pimozide) followed but none of these drug classes were superior to phenothiazines, although butyrophenones (haloperidol, droperidol) serve as alternative agents for patients intolerant to phenothiazines (Coyle, 1982; Julien, 2001). Collectively, these drug classes are commonly known as "typical", "classical" or "traditional" antipsychotics. Typical antipsychotics effectively block dopamine D2 receptors (Coyle, 2008; Julien, 2001). This D2 blockade is key to their mitigating effects on positive symptoms but conversely can result in undesirable side-effects most notably extrapyramidal motor symptoms (Coyle, 1982). The extent of D2 binding often dictates the efficacy, daily dosing, and likelihood of extrapyramidal side-effects (Richelson, 1996). Studies show that therapeutic effectiveness begins by approximately 70% of D2 receptor occupancy while higher percentage gives rise to extrapyramidal side-effects. For this reason, these drugs are termed typical agents because of inseparable therapeutic and extrapyramidal effects. In case of haloperidol extrapyramidal side-effects are highest and this drug along with ziprasidone and lurasidone has less weight gain profiles compared to other antipsychotics.

Atypical antipsychotics also effectively block dopamine D2 receptors but can also block 5HT-2A, α1 adrenergic, H1 histaminergic and/or M1 muscarinic receptors. Blockade of 5HT2A receptors is thought to be the reason for their greater tolerability (X. Miyamoto & Wolfgang, 2012). In fact, the property of 5HT2A receptor antagonism was used to design several of the second-generation antipsychotics such as risperidone, quetiapine, olanzapine and ziprasidone. The receptor profile of olanzapine most closely mimics clozapine as it blocks α1 adrenergic, H1 histeminergic and M1 muscarinic receptors. But like clozapine is not devoid of side-effects such as weight gain and hyperlipidemia and metabolic syndromes (Meltzer, 2005). With the exception of amisulpride, sertindole, iloperidone and paliperidone all other antipsychotics are more sedating

than placebo. Moreover, prolactin elevation is highest with risperidone and paliperidone (Leucht et al., 2013).

Although both atypical and typical antipsychotics have similar response rates (Leucht et al., 2013), clozapine is the most effective of all the antipsychotic drugs and is the treatment of choice for treatment resistant schizophrenia (Coyle, 2008; Johnstone, Crow, Frith, Carney, & Price, 1978; Kapur & Remington, 2001). However, life threatening side-effects such as agranulocytosis (Coyle, 2008; Meltzer, 2004) have relegated clozapine as a tertiary treatment for schizophrenia despite its superior efficacy as well as its ability to reduce symptoms of tardive dyskinesia, suicidal ideation, smoking, and substance abuse (Meltzer, 2004).

2.2. Antipsychotic Mediated Symptom Response and Remission

Historically, schizophrenia was regarded as a chronic and debilitating syndrome with an inevitable deterioration course (Frese, Knight, & Saks, 2009). However, with the advent of antipsychotics it is now well documented that symptom response and remission are achievable (De Hert et al., 2007).

Response can be described as a meaningful clinical improvement in psychopathology (Leucht, Davis, Engel, Kissling, & Kane, 2009). Measuring this improvement has typically involved the longitudinal collection of symptom severity using one of the several symptom-rating scales from which the percentage reduction in symptom severity from baseline to endpoint is calculated. Response is typically defined as a 20% to 50% reduction in symptom severity with specific focus usually given to positive symptoms.

Remission, until recently, was loosely defined with no accepted criteria. In 2005, the Remission in Schizophrenia Working Group (RSWG) proposed criteria (Andreasen et al., 2005)

using a two-dimension approach: (1) symptom severity and (2) duration of sustained mild symptoms or absence of symptoms. Using these dimensions, they proposed remission was achieved when key positive (delusions, conceptual disorganization, hallucinations), disorganized (mannerism, unusual thought content), and negative symptoms (blunted affect, social withdrawal, lack of spontaneity) did not exceed mild severity for the duration of six months or more. However, in this project positive symptom remission was defined using the consensus positive symptom remission criteria with a PANSS (positive and negative syndrome scale) score of ≤ 3 on delusions, hallucinations, grandiosity and unusual thought content (Wallwork, Fortgang, Hashimoto, Weinberger, & Dickinson, 2012). PANSS is a standardized clinical interview that is used to rate the presence and severity of positive symptoms, negative symptoms, and general psychopathology in those with schizophrenia. There are 30 items in the PANSS scale and symptom severity for each item is rated on a 7-point severity scale ranging from absent (score = 1) to extreme (score = 7) (Kay, Fiszbein, & Opler, 1987).

3. Treatment-resistant Schizophrenia

Recently, a consensus definition of treatment-resistant schizophrenia (TRS) has been established by Treatment Response and Resistance in Psychosis (TRRIP) working group. It proposed that a schizophrenia patient may be considered as 'treatment-resistant' if he/she fails to respond to two or more trials of antipsychotics, have poor functioning and show persistent symptoms (Howes et al., 2017). However, previously there was no consensus definition several attempts were made conceptualize and define TRS (Suzuki et al., 2011). One of the earliest attempts defined TRS as: "at least three periods of treatment with antipsychotics from at least two different classes at adequate doses for an adequate period of time with no relief and no period of good functioning over the last five years" (Kane, Honigfeld, Singer, & Meltzer, 1988). Since its debut, several papers have

supported the basic tenets of this definition (Citrome, Bilder, & Volavka, 2002; Conley & Kelly, 2001; Pantelis & Lambert, 2003; Peuskens, 1999; Siegfried, Fleischhaker, & Lieberman, 2012). However, some have argued that there is a reliance on positive symptoms in defining TRS and inclusion of negative and cognitive symptoms as well as ability to return to the best premorbid level of functioning should be considered (Meltzer, 1990; Sheitman & Lieberman, 1998) (Suzuki et al., 2011; Tsang, Leung, Chung, Bell, & Cheung, 2010). In addition, others have warned against giving the label of TRS before confounding factors such as poor compliance, incorrect dose schedule, lack of family support, poor therapeutic alliance, physical comorbidity, substance abuse, and intolerable side-effects are adequately ruled out (Elkis, 2007).

Despite the lack of a consensus definition, a number of different treatment guidelines are available to help in the clinical decision making of TRS, for example, the American Psychiatric Association (Lehman, Lieberman, et al., 2004) guidelines and the Schizophrenia Patients Outcome Research Team guidelines (Lehman, Kreyenbuhl, et al., 2004). Moreover, there are algorithm definitions of TRS provided by the International Pharmacological Algorithm Project (IPAP: www.ipap.org) as well as the Texas Medication Algorithm Project (Miller et al., 2004). According to IPAP a patient can be considered TRS if he/she shows no signs of response to two trials of 4 to 6 weeks' duration using monotherapy with two different atypical antipsychotics (or two trials with typical antipsychotics, in case of unavailability of atypical antipsychotics) and is eligible to be treated with clozapine, for a six-month trial period with maximum dose of up to 900mg per day. Approximately 30% (range 10-45%) of all schizophrenic patients meet these criteria (Meltzer, 1997).

Although nearly one-third of individuals with schizophrenia will meet criteria for TRS, biological markers to distinguish these individuals from their non-TRS counterparts are sparse.

The few studies that have sought to discover such markers have revealed that genetic polymorphism of candidate SNPs in *DRD1, DRD2, DRD3*, and *COMT* were not associated with clinical symptoms in patients with TRS of Caucasian ethnicity (Terzic, Kastelic, Dolzan, & Plesnicar, 2016). Genetic association study of several other genes (*DISC1, HTR2A, HTR3A, HTR4, and ABCB1*) between TRS and non-TRS patients of Japanese ethnicity also produced negative results (Hotta et al., 2011; Ji, Takahashi, Saito, et al., 2008; Takao et al., 2006). However, one haplotype spanning the *UCP4* gene was under represented in Caucasian TRS patients compared to healthy controls (Mouaffak et al., 2011) but an in/del polymorphism in the *HTR3B* gene was more frequent in Japanese TRS patients compared to non-TRS patients (Ji, Takahashi, Branko, et al., 2008). Compared to genetic association studies, gene and protein expression studies are limited in treatment-resistant schizophrenia. Moretti et al. found that peripheral mRNA expression of *DICER1* and *AKT1* were elevated in patients with treatment-resistant schizophrenia compared to healthy controls but no difference was observed between TRS and non-TRS patients (Moretti et al., 2018). Increased serum level of IL-2, IL-8, and IL-10 was reported in Chinese and Caucasian TRS patients (Maes et al., 2002; Y. Tan et al., 2015). No difference was found for plasma BDNF level but MMP-9 was upregulated in Japanese TRS patients compared to healthy controls (Yamamori et al., 2013). More recently, it was shown that a downregulation of *TNFα* mRNA expression in whole blood in patients with treatment-resistant schizophrenia relative to controls (Mostaid, 2018) and a positive correlation between *SELENBP1* mRNA levels in blood and duration of illness in TRS (Chau et al., 2018).

3.1. Pharmacological Treatment of Treatment-resistant Schizophrenia

Clozapine is the most widely used drug in TRS, is recommended in current TRS clinical practice guidelines (Howes et al., 2017; Warnez & Alessi-Severini, 2014), and has unambiguous support from the large-scale Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) and Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS) trials (Jones et al., 2006;

Lieberman et al., 2005). However, polypharmacy remains a common approach despite lack of evidence of its superiority over clozapine monotherapy (Ananth, 2005; Barbui et al., 2006; McCue, Waheed, & Urcuyo, 2003; Suzuki et al., 2004). A recent review demonstrated limited evidence of superiority of combination therapy over monotherapy in improving clinical response, particularly for clozapine (Ortiz-Orendain et al., 2017).

Approximately 30% of TRS patients treated with clozapine will respond inadequately requiring additional and/or alternative interventions (Elkis, 2007; Meltzer, 1992b). This subgroup of TRS are often referred to as "super-refractory" or "clozapine resistant" and are commonly treated with a combination of pharmacological therapy (two antipsychotics concomitantly or one antipsychotic with another psychotropic drug of a different class), psychological treatment and electroconvulsive therapy (ECT) (Sinclair & Adams, 2014) despite lack of proper evidence of their effectiveness (Miller, McEvoy, & Jeste, 2006). This subgroup of TRS patients represents a great challenge for treatment with concomitant economic burden (Buckley et al., 2001). Thus, biomarkers that could assist in early identification and/or predict treatment response would be of substantial benefit.

4. Genetic markers of Clozapine Response

Predicting the clinical response to clozapine is absolutely vital to ensure maximum clinical effectiveness as well as minimizing adverse events (Krivoy, Gaughran, Weizman, Breen, & MacCabe, 2015). To date, a number of studies have examined genetic variation in genes involved in the pharmacokinetic and pharmacodynamics of clozapine (Kohlrausch, 2013). However, a biomarker for clozapine response is still undiscovered.

4.1. Pharmacokinetic Markers of Clozapine Response

Pharmacokinetics refers to how a drug is absorbed, distributed, metabolized, and excreted by the body (Meibohm & Derendorf, 1997). Clozapine is primarily metabolized by the liver into N-desmethylclozapine (via demethylation) and clozapine N-oxide (via oxidation). The main enzymes involved are *CYP1A2* and *CYP3A4*, whereas *CYP2D6, FMO3* also play a minor role (Pirmohamed, Williams, Madden, Templeton, & Park, 1995). The majority of drugs that act on the central nervous system (CNS) are metabolized by the cytochrome P450 enzymes and phenotypes from different CYP450 isoenzymes can influence medication response and sensitivity (Kawanishi, Tachikawa, & Suzuki, 2000; Ozaki, 2004; Poolsup, Li Wan Po, & Knight, 2000). Variations in the genes coding for CYP enzymes may result in absent, deficient, normal or increased activity of the drug. Studies on CYP450 genetic variation and clozapine response lack replication and often show contradictory results (Kohlrausch, 2013; Krivoy et al., 2015). One study reports that clozapine non-responders carried *CYP1A2 *1F/*1F* genotype and showed lower level of clozapine in blood (Eap et al., 2004). However, evidence also exists that there is no significant difference between mean clozapine ratios and daily doses among patients with different *CYP1A2* genotypes (van der Weide, Steijns, & van Weelden, 2003).

Studies on clozapine response and *CYP2D6* genetic variation have also produced unsatisfactory results and found no significant association (M. J. Arranz et al., 1995; Jaquenoud Sirot et al., 2009; Kohlrausch et al., 2009; Lee et al., 2012). Moreover, genetic variations in *CYP3A4* were also found to have no significant association with plasma level of clozapine and treatment response (Jaquenoud Sirot et al., 2009; Lee et al., 2012).

Interestingly, in a study comprising 27 candidate genes and clozapine treatment response, researchers found a significant association with clozapine treatment response in two SNPs of the

ATP binding cassette subfamily B member 1 (*ABCB1*) gene in a Korean population (Lee et al., 2012). However, no association was found between UDP glucuronosyltransferase family 1 member A1 (*UGT1A1*) (Lee et al., 2012) or flavin containing monooxygenase (*FMO3*) genetic polymorphism (Sachse et al., 1999) and clozapine treatment response.

Table 1. Candidate Pharmacokinetic Genes for Clozapine Response

Gene	Significant association with clozapine response	Ethnicity	Reference
ABCB1	Yes	Caucasian, Korean	(Consoli et al., 2009; Jaquenoud Sirot et al., 2009; Lee et al., 2012)
CYP1A2	Yes	Caucasian	(Eap et al., 2004)
	No	Korean	(Lee et al., 2012)
CYP3A4	No	Korean	(Lee et al., 2012)
CYP2D6	No	Korean	(Lee et al., 2012)
UGT1A1	No	Korean	(Lee et al., 2012)
FMO3	No	Caucasian	(Sachse et al., 1999)

In short, genetic variations in pharmacokinetic genes have largely been unsuccessful in predicting clozapine response and as such focus has centered on pharmacodynamic genes and their polymorphisms.

4.2. Pharmacodynamics Markers of Clozapine Response

Pharmacodynamics refers to the relationship between drug concentration and the biochemical and physiological effects of the drug at the site of action (e.g. receptors) (Meibohm & Derendorf, 1997). Clozapine is a high-affinity antagonist of the dopamine receptors (*DRD 1-5*), and so several studies examined the relationships between polymorphisms within dopamine receptor genes and treatment response (Kohlrausch, 2013). Despite a few significant associations (Hwang et al., 2012; Zhao et al., 2005) most of the studies found no association between *DRD4* gene polymorphisms and clozapine treatment response (Kaiser et al., 2000; Kerwin et al., 1994; Kohn et al., 1997; Rao et al., 1994; Rietschel et al., 1996; Shaikh et al., 1995). Contrary to *DRD4*, studies on *DRD3* polymorphisms

and clozapine response found that Ser9Gly polymorphism in *DRD3* was associated with clozapine response and Ser/Ser genotype was predominant among non-responders (Scharfetter et al., 1999; Shaikh et al., 1996) which was later confirmed by meta-analysis (Jonsson et al., 2003). But many studies failed to replicate this result (Arranz, Munro, et al., 2000; Barlas et al., 2009; Gaitonde et al., 1996; Hwang et al., 2011; Hwang et al., 2010; Malhotra et al., 1998; Staddon, Arranz, Mancama, Mata, & Kerwin, 2002) and a more recent meta-analysis reported a negative but consistent trend of Ser/Ser genotype and poor treatment response to clozapine (Hwang et al., 2010). For *DRD2* genetic variation, studies have reported mixed results. While some evidence exists for positive association (Hwang et al., 2005; Hwang et al., 2006; Malhotra et al., 1999), reports for negative result are also available (Hwang et al., 2011; Reynolds, Yao, Zhang, Sun, & Zhang, 2005). A relatively recent publication reported a significant association between a haplotype combination in the dopamine transporter gene (*DAT* or *SLC6A3*) and clozapine response (M. Xu et al., 2010) but the same outcome was not observed in a previous investigation (Szekeres et al., 2004). Two studies found significant relationships between *DRD1* polymorphism and clozapine response in Caucasian (Hwang et al., 2007) and African American populations (Potkin et al., 2003) but another investigation reported no significant association (Hwang et al., 2011). Studies on *DRD5* gene polymorphism and clozapine response also found no association between them (Hwang et al., 2012).

Among all the seven types of serotonin receptors (*5HT1-7*), *5HT2* is the most extensively studied in relation to clozapine response (Kohlrausch, 2013). Studies of *5HT2A* polymorphisms and clozapine treatment response have been contradictory (M. Arranz et al., 1995; Malhotra, Goldman, Ozaki, Breier, et al., 1996; Masellis et al., 1995; Nimgaonkar, Zhang, Brar, DeLeo, & Ganguli, 1996; Nothen et al., 1995; Yu et al., 2001). The same phenomenon was observed when the *5HTR2C* gene was taken into consideration regarding possible association with clozapine response (Malhotra,

Goldman, Ozaki, Rooney, et al., 1996; Masellis et al., 1998; Rietschel et al., 1997; Sodhi et al., 1995).

Results were unclear in the case of *5HTR3A* polymorphisms and clozapine response (Gutierrez et al., 2002; Rajkumar et al., 2012; Souza, de Luca, Meltzer, Lieberman, & Kennedy, 2010a), while no association was found for *5HTR5A* (Birkett et al., 2000) and *5HTR3B* (Gutierrez et al., 2002). Contradictory results for clozapine response were also found for *5HTR6* gene polymorphisms (Masellis et al., 2001; Yu et al., 1999). Investigations of the serotonin transporter gene (*HTT* or *SLC6A4*) length polymorphisms (HTTLPR) and clozapine response also produced contradictory results in Caucasian and Brazilian populations (Arranz, Bolonna, et al., 2000; Kaiser et al., 2001; Kohlrausch et al., 2010; S. J. Tsai et al., 2000).

A brief summary of the Pharmacodynamic genes and their association with clozapine response is given below:

Table 2. Candidate Pharmacodynamic Genes for Clozapine Response

Gene	Significant Association with Clozapine Response	Ethnicity	References
DRD1	Yes	African-American, Caucasian	(Hwang et al., 2005; Potkin et al., 2003)
	No	African-American, Caucasian	(Hwang et al., 2011)
DRD2	Yes	Caucasian, African-American	(Hwang et al., 2005; Hwang et al., 2006; Malhotra et al., 1999)
	No	Caucasian, African-American	(Hwang et al., 2011; Reynolds et al., 2005)
DRD3	Yes	Caucasian, Pakistani	(Jonsson et al., 2003; Scharfetter et al., 1999; Shaikh et al., 1996)
	No	Turkish, Caucasian, African-American	(Barlas et al., 2009; Hwang et al., 2011; Hwang et al., 2010; Staddon et al., 2002)
DRD4	Yes	Caucasian, African-American, Chinese	(Hwang et al., 2012; Zhao et al., 2005)

	No	German, Israeli	(Kaiser et al., 2000; Kohn et al., 1997; Rietschel et al., 1996)
DRD5	No	Korean	(Hwang et al., 2012)
5HTR2A	Yes	Caucasian, African-American, Chinese	(M. Arranz et al., 1995; Yu et al., 2001)
	No	Caucasian, African-American, Asian, German	(Malhotra, Goldman, Ozaki, Breier, et al., 1996; Masellis et al., 1995; Nimgaonkar et al., 1996; Nothen et al., 1995)
5HTR2C	Yes	Caucasian	(Sodhi et al., 1995)
	No	Caucasian, African-American, Asian, German	(Malhotra, Goldman, Ozaki, Rooney, et al., 1996; Masellis et al., 1998; Rietschel et al., 1997)
5HTR3B	No	Caucasian (British)	(Gutierrez et al., 2002)
5HTR5A	No	Caucasian (British)	(Birkett et al., 2000)
GNB3	Yes	Brazilian, Caucasian	(Kohlrausch et al., 2008; Muller et al., 2005)
COMT	Yes	Caucasian, African-American	(Woodward, Jayathilake, & Meltzer, 2007)
DTNBP1	Yes	Caucasian, African-American	(Zuo et al., 2009)
GFRA2	Yes	Caucasian	(Souza, Romano-Silva, et al., 2010)
NRXN1	Yes	Caucasian	(Lett et al., 2011)
OXT	Yes	Caucasian, African-American	(Souza, de Luca, Meltzer, Lieberman, & Kennedy, 2010b)
ADRA2A	No	Caucasian, Chinese	(Bolonna et al., 2000; S. J. Tsai et al., 2001)
BDNF	No	Chinese	(Hong, Yu, Lin, & Tsai, 2003)
GPX1	No	Caucasian, African-American	(Souza et al., 2009)
GRIN1	No	Korean	(Hwang et al., 2011)
GRIN2A	No	Korean	(Hwang et al., 2011)
GRIN2B	No	Chinese, Korean	(Hong, Yu, Lin, Cheng, & Tsai, 2001; Hwang et al., 2011)
HRH1	No	Caucasian (British)	(Mancama et al., 2002)

HRH2	No	Caucasian (British)	(Mancama et al., 2002)
MNSOD	No	Caucasian, African-American	(Souza et al., 2009)
TNF	Yes	Caucasian, African-American	(Zai et al., 2006)
	No	Chinese	(S. J. Tsai, Hong, Yu, Lin, & Liu, 2003)
SL6A3	Yes	Chinese	(M. Xu et al., 2010)
SL6A4	Yes	Caucasian, Brazilian	(Arranz, Bolonna, et al., 2000; Kohlrausch et al., 2010)

The majority of genetic studies of clozapine response have focused on genetic variation in genes related to known receptor targets of clozapine and other antipsychotics but have had limited success. Thus, investigation of genes and biological pathways beyond clozapine's primary receptor targets are warranted.

Clozapine has prominent effects on Neuregulin 1-*ErbB* receptor tyrosine kinase (*NRG1-ErbB*) signaling and several studies (Chana et al., 2009; Wang, Su, Guo, Yang, & Si, 2008) have demonstrated that expression of *NRG1-ErbB* pathway genes is altered due to clozapine administration (Pan, Huang, & Deng, 2011). *NRG1* plays important roles in nervous system development and plasticity (Mei & Nave, 2014). Evidence exists for abnormal *NRG1-ErbB* signaling and its potential role in schizophrenia pathophysiology (Chong et al., 2008; Deng, Pan, Engel, & Huang, 2013; Pan et al., 2011; Parlapani et al., 2010). The pathological mechanism connecting abnormal *NRG1-ErbB* signaling and schizophrenia is still not clear but studies on animal models showed that mice lacking one copy of the *nrg1, erbb2, erbb3*, or *erbb4* gene showed behavioral deficits (e.g. impairment in pre-pulse inhibition, reduced working memory, hyperactivity in an open field, abnormal social behavior etc.) relevant to schizophrenia (Mei & Nave, 2014). Moreover, clozapine can reverse abnormal behaviors in animals with deficient *NRG1-ErbB* signaling (Pan et al., 2011). In addition, a recent study showed that non-medicated high-risk individuals that converted to psychosis had a decrease in *NRG1* expression compared to non-converters (Kiss, Kelemen, &

Keri, 2012). Thus, *NRG1-ErbB* pathway genes have potential to serve as biomarkers for treatment response and symptom severity in TRS patients on clozapine therapy.

5. *NRG1-ErbB* Signaling and Clozapine

Several studies suggest a link between schizophrenia and abnormal *NRG1-ErbB* signaling (Mei & Nave, 2014) and there is some evidence to suggest *NRG1* genetic variation is associated with variable response to typical antipsychotic drugs (Kampman et al., 2004). A recent GWAS study reports that genetic variation in *NRG1* may have an effect on antidepressant treatment response and one SNP (rs10954808) located in the 5' promoter region of *NRG1* was found to be highly associated with treatment outcomes of selective serotonin reuptake inhibitors (*SSRIs*) (Biernacka et al., 2015). Interestingly, this SNP was found to be in strong linkage disequilibrium (LD) with rs7014762 (located near the promoter of *NRG1* type II) (r^2=0.8) which was associated with excellent recovery between episodes in bipolar disorder patients (Georgieva et al., 2008) and in relatively strong LD (r^2=0.85) with rs4281084 which was related to transition to psychosis in at-risk individuals (Biernacka et al., 2015; Bousman et al., 2013) in Caucasian population. Moreover, *NRG1* is suggested as a potential biomarker for major depressive disorder due to elevated gene expression in the periphery in Caucasian population (Belzeaux et al., 2010). All these studies indicate that *NRG1-ErbB* pathway genes may be used as a biomarker for TRS patients under clozapine therapy for treatment response as well as symptom severity.

Clozapine affects mRNA expression and protein levels of *NRG1* and *ErbB4* receptors in both humans and animals (Pan et al., 2011). However, no systematic investigation of the effects of clozapine treatment on *NRG1-ErbB* signaling has been performed (Deng et al., 2013). As the *NRG1-ErbB* pathway is one of the most important pathways for neuronal development, changes in the

expression of *NRG1-ErbB* pathway genes due to clozapine administration may be the cause for its superior clinical efficacy in TRS patients. *NRG1* is also regarded as a potential therapeutic treatment for psychotic symptoms, although it is still in the animal model trial phase (Deng et al., 2013). Clozapine has profound effects on different types of *NRG1* isoform expression as well as *ErbB1-4* expression. So, the *NRG1-ErbB* pathway genes have potential to act as biomarkers for TRS patients on clozapine therapy in relation to treatment response and symptom severity. Yet, there have been limited *in vitro*, animal, and clinical investigations of clozapine effects on *NRG1-ErbB* signaling gene/protein expression and no investigations of the association between genetic variation in *NRG1-ErbB* signaling genes and clozapine response/remission.

6. *NRG1* structure and isoforms

NRG1 is located in the short arm of chromosome 8 (8p12-p21). This chromosomal region is implicated in schizophrenia along with other diseases such as abnormal cerebral development, breast cancer, and Werner syndrome (Imbert et al., 1996). *NRG1* spans 1.125 megabases and comprises more than 20 exons as well as several large introns from which over 30 splice isoforms can be produced that are grouped into six types (I – VI) (Figure 1). *NRG1* types I and II contain an immunoglobulin (Ig) like domain, encoded by exons E178 and E122 respectively. This Ig region is present in types IV and V and is commonly defined as Ig-*NRG1*. The Ig domain is linked to the epidermal growth factor (EGF) like domain with or without the spacer region. In *NRG1* type III, the Ig like domain is not present and the EGF like domain is situated directly downstream of the specific and unique amino-terminal region. The N terminal region of *NRG1* type III is unique as it contains a cysteine-rich domain (CRD) and an N terminal transmembrane domain (TMn). In *NRG1* type VI, the amino-terminal region is also directly attached to EGF like domain but it lacks the CRD as well as the TMn regions (Harrison & Law, 2006; Mei & Nave, 2014; Mei & Xiong, 2008). All *NRG1* types (I – VI) bind to and/or interact with their cognate transmembrane receptor tyrosine kinases

(i.e. *ErbB3* and *ErbB4*). However, *ErbB4* is the main receptor for *NRG1* and evidence in mice suggests *ErbB*4 is selectively localized on the largest subclass of interneurons, namely those that express the calcium-binding protein, parvalbumin (PV+ interneurons; (Abe, Namba, Kato, Iwakura, & Nawa, 2011; Bi et al., 2015; Fazzari et al., 2010; Garcia, Vasudevan, & Buonanno, 2000; Huang et al., 2000; Ma et al., 2003). However, *ErbB*4 is also expressed by other types of cortical neurons, including those that express cholecystokinin, neuronal nitric oxide synthase (Neddens & Buonanno, 2010) and somatostatin (Yau, Wang, Lai, & Liu, 2003) in mice. Binding of *NRG1* to *ErbB4* leads to activation of the receptor tyrosine kinase domain, which initiates several downstream signaling cascades, including the phosphoinositide 3-kinase (PI3K) pathway. Type III *NRG1* promotes Schwann cell proliferation via activation of the PI3K pathway during development of the peripheral nervous system in mouse (Limpert & Carter, 2010; Maurel & Salzer, 2000), and in a similar fashion *NRG1* may increase the density of PV+ interneurons (Kato et al., 2010) and enhance GABAergic transmission (Engel et al., 2015; Wen et al., 2010; Woo et al., 2007) in the central nervous system (for comprehensive reviews see: (Buonanno, 2010; Burden & Yarden, 1997; Fischbach & Rosen, 1997; Mei & Nave, 2014; Yarden & Sliwkowski, 2001).

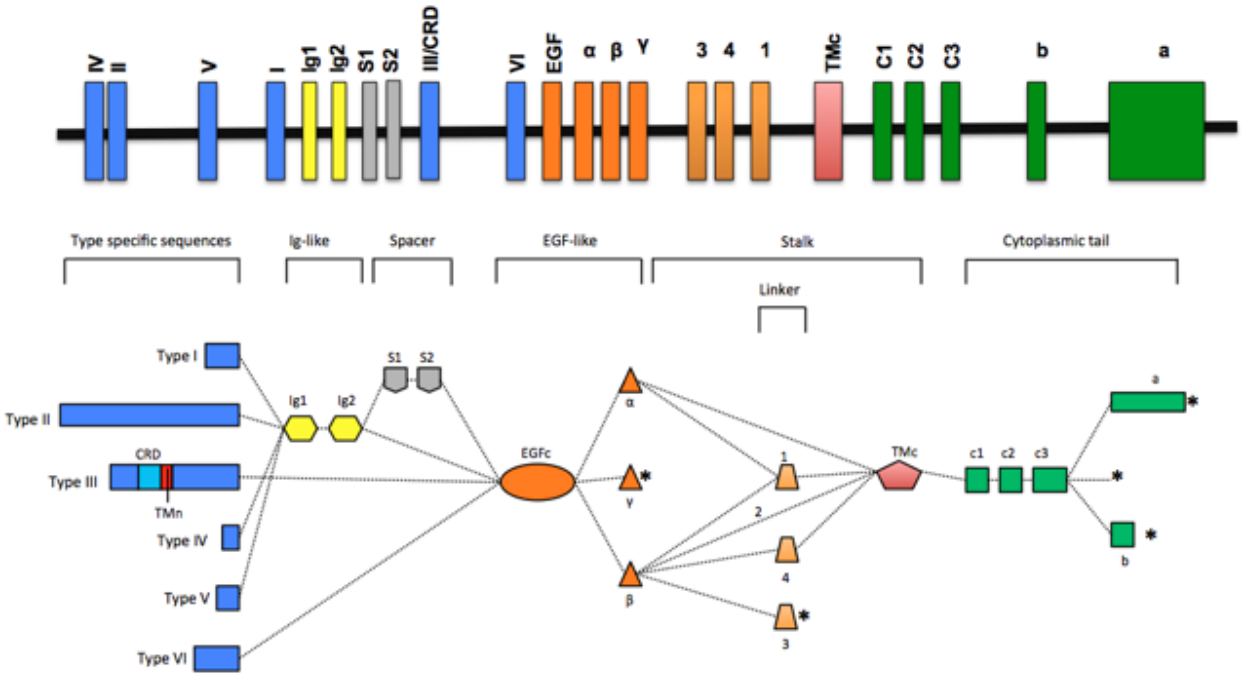

Fig 1. *NRG1* structure and isoforms. *NRG1*'s six known isoforms and their defining exons are depicted (blue). All *NRG1* isoforms have an epidermal growth factor (EGF)-like domain but only types I, II, IV, and V contain an immunoglobulin (Ig)-like domain (yellow). Type III is unique it that it contains a cysteine-rich domain (CRD) which includes a transmembrane domain (TMn). Splice variants can occur in the linker and C-terminal region. *Denotes stop codon. This figure is an update of that published by Mei and Xiong (Mei & Xiong, 2008).

6.1. *NRG1* genetic variation and schizophrenia

6.1.1. Family studies

The seminal family study in the Icelandic population linking a haplotype (Hap_{ICE}) in the 5' region of *NRG1* with SZ (Stefansson et al., 2002) stimulated numerous additional family studies of which seven were conducted in the past decade (Table 3, for details on family studies from 2002 to 2005 see: Harrison and Law 2006 (Harrison & Law, 2006). Unlike the family studies conducted soon after

the Icelandic study, the ancestral background of more recent studies has been diverse, covering populations beyond Northern and Central Europe. This diversity may have contributed to the mixed findings, with multiple regions of *NRG1* being linked to SZ (Figure 2).

In Asian populations, studies report haplotype and single marker associations in the 5' and 3' regions of *NRG1*, whereas studies of Caucasian populations have implicated the 5' region, albeit interrogation of the 3' region in Caucasians is sparse. Nonetheless, a majority of positively associated 5' single markers and haplotypes overlap or are near (within 200 kbp) the Hap_{ICE} region, regardless of Caucasian or Asian ancestry. In contrast, 3' single markers and haplotypes have predominantly surfaced in Asian populations but have yet to be uniformly replicated, prohibiting firm conclusions in other populations. Notably, two negative results have been reported in the past decade but these have been in a mixed-ethnic population (Yokley et al., 2012) or have examined psychosis more generally (e.g. inclusion of bipolar and depression psychosis) (Rosa et al., 2007), suggesting population stratification and phenotypic heterogeneity may have inhibited detection of an association, respectively.

Table 3. Summary of *NRG1* Family Studies in Schizophrenia (2005-2017)

Reference	Subjects	Ethnicity	SNP, MS	From	To	Association with SZ	Brief Result	Notes
Liu et al. 2005 (C. M. Liu et al., 2005)	52 schizophrenic families	TWN	0, 11	D8S1742	D8S1810	Yes	Narrow model,D8S1771 (p=0.01) D8S1222 (p=0.01); Broad model, D8S1771 (p=0.03),D8S1222 (p=0.003)	Non-parametric linkage analysis
Kim et al 2006 (J. W. Kim et al., 2006)	40 families, 140 individuals, 89 affected SZ patients	KOR	0,7	D8S258	D8S505	Yes	D8S1769; For Narrow phenotype class (p=0.036); Narrow with auditory hallucination (p=0.011)	
Walss-Bass et al. 2006a (Walss-Bass, Raventos, et al., 2006)	134 family trios	CVCR	4,2	rs73235619	420M9-1395	Yes	A novel 4 marker haplotype (G-T-218-274) was overrepresented in patients with psychosis (p=0.046).	4 marker haplotype: rs73235619, rs35753505, 478B14-848, 420M9-1395.
Walss-bass et al. 2006b (Walss-Bass, Liu, et al., 2006)	142 affected individuals and 236 unaffected family members	CVCR	1, 0	rs74942016	-	Yes	The minor (T) allele of rs74942016 was over-transmitted from parents to offspring with SZ (p=0.0191); patients with psychosis (p=0.0049)[a].	All the exons of *NRG1* were initially sequenced in a subsample of 12 affected individuals and rs74942016 (G>T; Val>Leu) was discovered in exon 11.
Turunen et al. 2007 (Turunen et al., 2007)	441 SZ family (865 affected individuals)	FIN	10,3	D8S1820	D8S1110	Yes	For SZ spectrum phenotype rs764059 (p=0.012); rs2919378 (p=0.030);	
Rosa et al. 2007 (Rosa et	151 families with 575 individuals	SP	8,0	rs763553	rs10503929	No[a]	-	-

al., 2007)								
Georgieva et al. 2008 (Georgieva et al., 2008)	634 SZ/SA parent offspring trios	92.3% BUL, 1.5% TURK, 3.2% ROM and 3% CAU	12,2	rs763551	420M9-1395	Yes	T allele of rs35753505 (p=0.013, corrected p=0.039); 2 marker haplotype (rs35753505 and rs7014762, T-A, p=0.006); (rs35753505 and rs62510682, C-T, p=0.001); (rs35753505 and rs6994992, C-G, p=0.001)	-
Zhang et al. 2009 (Zhang et al., 2009)	258 parent-proband trios	CHN	4,0	rs35753505	rs3924999	Yes	Allelic transmission of C in rs35753505 ($p<0.05$); A in rs113317778 ($p<0.05$); T in rs3924999 (p=0.001); Haplotype analysis: 3 marker haplotype (C-C-G, p= 0.026 and C-C-A, $p<0.05$); 4 marker haplotype: (C-C-G-T, p=0.001; C-C-A-C, p=0.006 and C-C-A-T, $p<0.05$);	3 marker haplotype: rs35753505, rs7820838, rs113317778; 4 marker haplotype: rs35753505, rs7820838, rs113317778, rs392499. This paper was published in Chinese.
Yokley et al. 2012 (Yokley et al., 2012)	419 (58 SZ/SAD & 361 unaffected relatives)	CAU, ME, AA	40,0	rs73235619	rs3735782	No	-	-

AA= African-American, BD=Bipolar disorder, BUL=Bulgarians, CAU=Caucasians, CVCR= Central Valley of Costa-Rica, CHN=Chinese, FBAT= Family Based Association Test, FIN=Finnish, KOR=Korean, ME=Mixed European, MS=microsatellite, ROM=Romans, SAD= Schizoaffective disorder, SP=Spanish, SNP=single nucleotide polymorphism, SZ=Schizophrenia, TURK=Turkish, TWN=Taiwanese.

rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006)

[a]Phenotype studied was psychosis

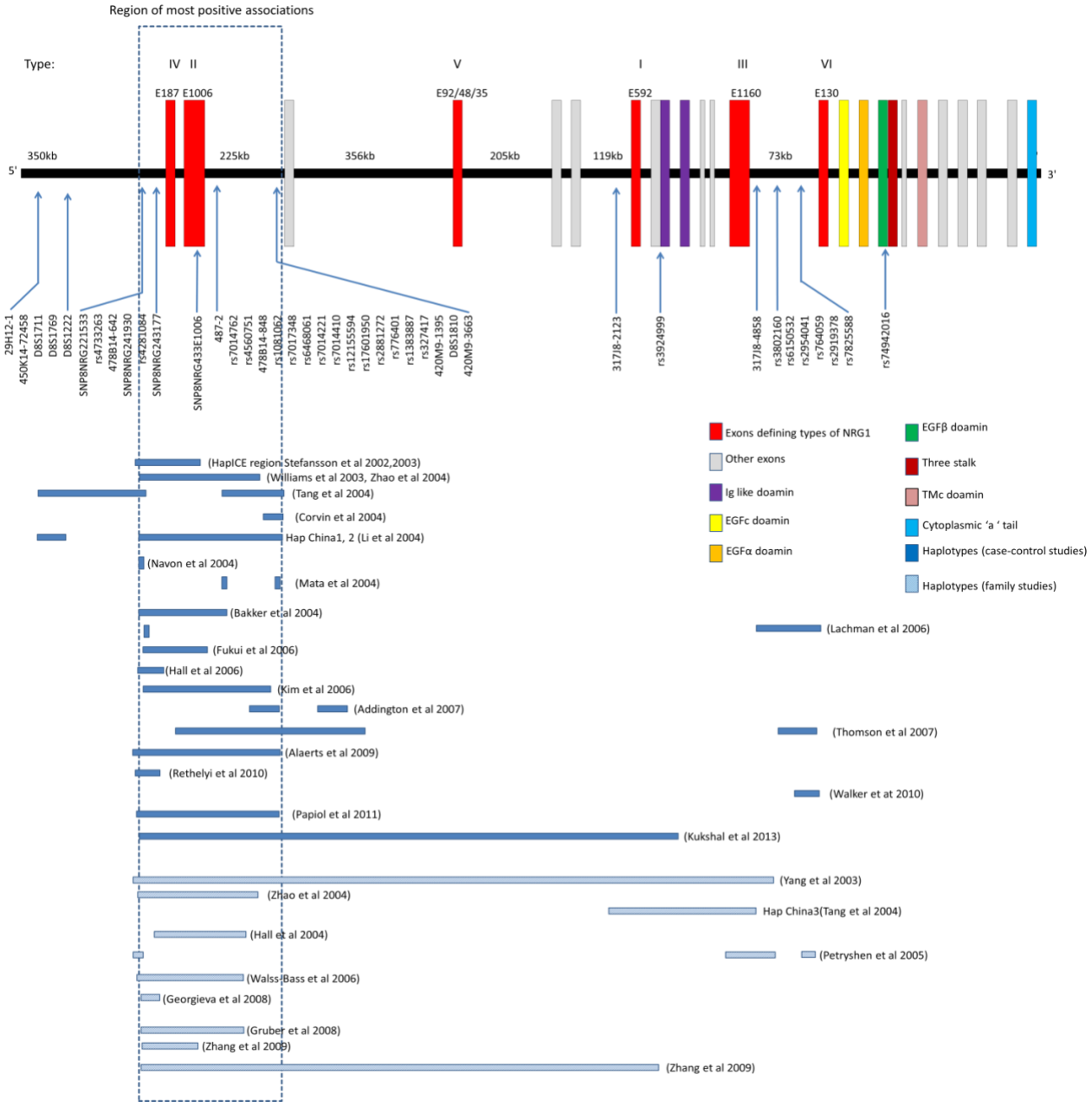

Fig. 2. Positively associated markers of the *NRG1* gene in schizophrenia in case-control studies and family studies. Exons are depicted as vertical bars with the *NRG1* type denoted above. Horizontal lines represent the coverage of each haplotype in case-control (dark blue lines) and family (light blue lines) studies. The dashed rectangle indicates the region where the most positive associations have been reported. This figure is an update of that published by Harrison and Law (Harrison & Law, 2006).

6.1.2 Case-control studies

Harrison and Law (Harrison & Law, 2006) reported in their original review of *NRG1* and SZ that among Caucasians the region of most significant association lay between the exons of *NRG1* type II and IV. A decade and 33 case-control studies later, this region remains the most implicated within the Caucasian population (Figure 2, Table 4). However, evidence of association for markers and haplotypes beyond the 5' region (near the exons for type III and VI) among Caucasians has emerged (Lachman et al., 2006; Thomson et al., 2007; Walker et al., 2010) and are aligned with markers and haplotypes reported in family studies conducted in Caucasian (Petryshen et al., 2005) as well as Han Chinese populations (T. Li et al., 2004; J. Z. Yang et al., 2003). Evidence for associations beyond the 5' region have also been reported in North Indian (Kukshal et al., 2013) and Pakistani (Naz, Riaz, & Saleem, 2011) populations. Importantly, very few single markers or haplotypes have been robustly replicated (see meta-analytic studies section), implying a high level of allelic heterogeneity. Thus, as the number of populations studied increases it is likely in the next decade we will witness an expansion of the 'region of most significant associations' and with this expansion see increased levels of allelic heterogeneity.

Table 4: Summary of *NRG1* Case-Control studies in schizophrenia

Reference	Case/Control Subjects	Ethnicity	SNP, MS	From	To	Association with SZ	Brief Result	Notes
Lachman et al 2006 (Lachman et al., 2006)	141/142+177/164	AA/CAU	1,3	rs35753505	rs10691392	Yes	In African-Americans: rs6150532 (p=0.02) and rs35753505 (p is not stated). 3 marker Haplotype (p<0.05)	3 marker haplotype: rs5890668, rs6150532, rs10691392
Fukui et al 2006 (Fukui, Muratake, Kaneko, Amagane, & Someya, 2006)	349/424	JP	4,0	rs1081062	rs6994992	Yes	rs62510682 (p=0.025), rs6994992 (p=0.041), rs1081062 (p=0.0023); 4 marker haplotype (p=0.026)	4 marker Haplotype: rs35753505, rs62510682, rs6994992, rs1081062
Kim et al. 2006 (J. W. Kim et al., 2006)	242/242	KOR	5,2	rs73235619	420M9-1395	Yes	rs62510682 (p=0.030) for patients with auditory hallucination. 3 marker haplotype (p=0.038); 5 marker haplotype: (p=0.012, total patients vs. controls) (p=0.028, patients with auditory hallucination vs.	3 marker haplotype: rs35753505, rs62510682, rs6994992; 5 marker haplotype: 3 marker haplotype + 478B14-848, 420M9-1395.

							controls)	
Ingason et al. 2006 (Ingason et al., 2006)	325/353	DN	3,2	rs35753505	420M9-1395	No	-	-
Addington et al. 2007 (Addington et al., 2007)	78 COS[b] patients and parents/165	Patients: 50% CAU, 28% AA, HS 7%, AS 5.5%, other 10%. Controls: 85% CAU.	54,2	rs2189145	rs6994992	Yes[a]	rs35753505 (p=0.040), rs2881272 (p=0.05), rs327417 (p=0.01), 420M9-1395 (p=0.01). 4 marker COS risk haplotype 1 (p= 0.007), 4 marker COS risk haplotype 2 (p=0.0004)	COS risk haplotype 1 : rs10503887, rs1354335, rs2881272, 420M9-1395 COS risk haplotype 2: rs1599677, rs7818326, rs10503899, rs11776959
Thomson et al. 2007 (Thomson et al., 2007)	386/478 (368 BD patients)	SC	40,0	rs4513929	rs4733140	Yes	[Region A]; 3-marker haplotype (p=0.00032); 7 marker haplotype (p=0.0025); alternate 7 marker haplotype (p=0.059) Combined SZ+BD cases (p=0.0017). [Region B]; 3 marker haplotype (p=0.00014). Combined SZ+BD cases	Region A: 3 marker haplotype: rs1503491, rs553950, rs327329; 7 marker haplotype: 3 marker haplotype + four of five HapICE SNPs (rs73235619, rs35753505, rs62510682, rs6994992) [g-c-g-t-t-t-t]; alternate 7 marker haplotype: same as above [g-t-g-g-t-t-t]. Region B: 3 marker haplotype: rs2919390, rs6988339, rs3757930.

							(p=0.000062)	
Benzel et al. 2007 (Benzel et al., 2007)	396/1342	CAU	365,0	rs7843578	rs3735782	Yes	11 SNPs were associated with SZ. The Most significant are rs776401 (p<0.01), rs1383887 (p<0.01), rsGSK8116812 (p<0.01), rs3924999 (p=0.0279)	365 SNPs in eight genes of *NRG-ErbB* family were tested & 42 were found significant. Among them 11 are on *NRG1*.
Hanninen et al. 2008 (Hanninen et al., 2008)	113/393	CAU (FIN)	1,0	rs35753505	-	No		
Viella et al 2008 (Vilella et al., 2008)	589/615	SP	19,0	rs35753505	rs764059	No		
Sanders et al. 2008 (Sanders et al., 2008)	1870/2002	CAU	217 SNPs and 159 tag SNPs in *NRG1*			No		789 SNPs in 14 candidate genes were analysed
Crowley et al. 2008 (Crowley et al., 2008)	738/733	Patients: 57% CAU, 29% AA, other 14%; Controls: 56% CAU, 30% AA and 14% other	1,0	rs6994992	-	No	-	No association was found between rs6994992 polymorphism and age at onset, WRAT-3 score (proxy for premorbid IQ) as well as neurocognition.
Shiota et al. 2008 (Shiota et	416/520	JP	11,2	rs35753505	rs2919381	No		rs73235619 and rs113317778 were excluded due to very

al., 2008)								low minor allele frequency.
Ikeda et al. 2008 (Ikeda et al., 2008)	First set screening analysis: 1126/1022. Confirmation analysis: 1262/1172, 166 trio samples	JP	60,0	rs12674974	rs17731664	No	3 marker haplotype (p=0.0244) in first-set screening sample but not replicated in confirmation sample	3 marker haplotype: rs10503917, rs10107065, rs6468118
Alaerts et al. 2009 (Alaerts et al., 2009)	486/514	CAU (SW)	37,2 (32 tag SNPs & 7 Hap$_{ICE}$ markers)	rs4268087	420M9-1395	Yes	rs7017348 (p=0.03), rs6468061 (p=0.03), rs7014221 (p=0.007), rs7014410 (p=0.008), rs17601950 (p=0.009).	Sliding window technique: six 2 SNPs, six 3-SNPs and seven 4 SNPs windows showed significant association. Block analysis: a 13 marker haplotype was associated with SZ (p=0.003)
Okochi et al. 2009 (Okochi et al., 2009)	184/534	JP	4,0	rs35753505	rs3924999	No[b]		
Nicodemus et al 2009 (Nicodemus et al., 2009)	296/365	CAU	1,0	rs7014762	-	Yes	Minor allele T of rs7014762 (p=0.031)	
Pedrosa et al 2009 (Pedrosa et al., 2009)	176/175	CAU (US)	1,0	rs7825588	-	Yes	AA for rs7825588 (p=0.03)	This SNP was not found to be associated with BD.
Jonsson et al. 2009 (Jonsson et al., 2009)	DN: 420/1004; NR: 162/177; SW: 255/292	CAU	12,0	rs35753505	rs10503929	No	-	

Rethelyi et al. 2010 (Rethelyi et al., 2010)	280/230	HN	4,0	rs73235619	rs6994992	Yes[c]	G allele of rs62510682 (p=0.04) in SZ-ND patients. Haplotype based analysis: Two 2 marker haplotypes (p=0.03) and (p=0.04)	For rs73235619 and rs62510682, p=0.03; for rs62510682 and rs6994992, p=0.04; 4 marker haplotype: No association. rs62510682 was associated with PANSS cognitive & hostility/excitability factors.
Squassina et al 2010 (Squassina et al., 2010)	171/349	ITA	5,0	rs73235619	rs6994992	No		Four Hap_{ICE} SNPs and SNP8NRG222662 were analysed.
Walker et al. 2010 (Walker et al., 2010)	Sample 1(SC): 386/455 (Thomson et al., 2007) & Sample 2 SC2: 303/307, GR: 396/397	SC/GER	6,0	Region A: rs1503491,rs553950, rs327329;	Region B: rs2919390, rs6988339, rs3757930	Yes	2 marker haplotype in region B (p=0.0037 for merged SC & p=0.0080 for BD+SZ sample)	2 marker haplotype: rs6988339 and rs3757930.
Nicodemus et al. 2010 (Nicodemus et al., 2010)	296/365	CAU	47,2	rs4513929	rs7005288	Yes	rs4560751 and rs3802160 (P=0.00020)	-
Naz et al. 2011 (Naz et al., 2011)	100/70	PK	2,0	rs3924999	rs2954041	Yes	rs3924999 (p=0.003)	-
Garcia-Barcelo et al. 2011 (Garcia-Barcelo et al., 2011)	270/270	CHN	1,0	rs74942016	-	No		-
Moon et al. 2011 (Moon	273/479	CVCR	3,0	rs6994992	rs74942016	No		

et al., 2011)								
Papiol et al. 2011 (Papiol et al., 2011)	1071 (835 SZ, 183 SAD & 53[d]) /1056	GER	4,4	D8S1810	rs6994992	Yes[e]	A 'protective' 5 marker haplotype was found to be less frequent in patients than in controls.	Less frequency of 'protective haplotype' in subgroup of SZ patients with early age of onset (<20 years) and more positive score on PANSS (>18). 5 marker Haplotype: rs35753505, rs62510682, rs6994992, MS487-2 and MS420M9-1395
Kim et al.2012 (J. H. Kim et al., 2012)	435/390	KOR	4,0	rs35753505	rs39249999	No		
Yang et al. 2012 (S. A. Yang, 2012)	221/359	KOR	3,0	rs7014762, rs11998176,	rs3924999 (Arg253Gln)	Yes	rs3924999 (p=0.022)[f] and (p=0.013)[g]	
Loh et al. 2013 (Loh et al., 2013)	417/419	MAL, CHN, IND	3,0	rs764059	rs3924999	Yes	rs2954041 (P=0.030) and rs3924999 (P=0.001) for Indians only	Both rs392499 and rs2954041 were not significant in Malays and Chinese, rs764059 was found monomorphic.
Kukshal et al. 2013 (Kukshal et al., 2013)	1007/1019	IND	35,3	Previously reported SNPs and MS of *NRG1* in SZ		Yes	rs35753505, rs4733263, rs6994992 and 420M9-1395 (p≤0.05). 6 marker haplotype (p=0.0004)	6 marker haplotype: rs6994992, rs1354336, rs10093107, rs3924999, rs11780123, rs35753505.
Gutierrez-Fernandez et al 2014	515 (215 SZ, 134 BD, 166[f])/650	SP	9,0	rs7005606	rs113317778	No	-	rs7005606, rs6468119, rs3802158, rs7834206 and 5SNPs of HapICE

(Gutierrez-Fernandez et al., 2014)								were analysed
Tosato et al. 2014 (Tosato et al., 2014)	461 (SC) & 439 (GR) patients only	SC/GER	216 htSNPs in *NRG1*			No[h]	-	-
Jajodia et al. 2015 (Jajodia et al., 2015)	436/401 (North India) & 351/385 (South India)	IND	1536 SNPs in 40 genes	N/A	N/A	Yes	rs17603876 (p=0.0126 in South Indian) and (p=0.00242 in North Indian)	rs17603876 is in linkage disequilibrium with rs12155594, associated with transition to psychosis (Bousman et al., 2013)

AA= African-American, AS= Asian, AUS=Australian, BD= Bipolar Disorder, CAU= Caucasians, CHN=Chinese, CVCR= Central Valley of Costa Rica, DN=Danish, FIN=Finnish, GER=German, HN=Hungarian, HS= Hispanic, IND=Indian, ITA=Italian, JP=Japanese, KOR=Korean, Leu= Leucine, MAL=Malaysian, ME=Mixed European, MS=Microsatellite, NR=Norwegian, PK=Pakistani, SAD= schizoaffective disorder, SC=Scottish, SNP= Single nucleotide polymorphism, SP=Spanish, SW=Swedish, SZ=Schizophrenia, SZ-D= Deficit-Schizophrenia, SZ-ND=Non-deficit Schizophrenia, US=United States of America, Val= Valine.

rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006)

[a]Childhood Onset Schizophrenia, [b]Methamphetamine Induced Psychosis, [c]Non-Deficit Schizophrenia, [d]Other psychotic disorder/Not confirmed, [e]Age of Onset and positive symptom severity, [f]Dominant model, [g]Log additive model, [h]psychopathological symptoms

6.1.3 Meta-analytic studies

Six meta-analytic studies have examined the association between *NRG1* genetic variation and SZ (Table 5). The Hap_{ICE} markers have been the main focus of these studies, although three other markers (rs10503929, rs3924999 and rs2954041) in the 3' region have also been subjected to meta-analysis. Results of these studies are mixed but suggest a significant association between 5' *NRG1* genetic variation and SZ. The largest and most recent analysis (Gong et al., 2009) showed support for three (SNP8NRG221132, 478B14-848, 420M9-1395) of the seven Hap_{ICE} markers when combining case-control and family studies across ancestry. While, Munafo and colleagues (Munafo, Thiselton, Clark, & Flint, 2006) (Munafo et al., 2008) showed support at the haplotype level using a combined p-value approach, others have argued that this approach should be extended to the gene level as this would address, in part, the allelic heterogeneity within *NRG1* between populations (Neale & Sham, 2004). However, to our knowledge a combined p-value approach at the gene-level has yet to be applied in a study of *NRG1* and SZ.

Table 5. Summary of *NRG1* Meta-Analysis Studies in Schizophrenia (2006-2017)

Reference	No of publications analysed	Years examined	Ethnicity	Case/Control Subjects	Markers	Association with SZ
Li et al. 2006 (D. Li, Collier, & He, 2006)	13	2002-2006	All	3947/4202; 708 parent offspring trios	rs73235619 rs35753505 rs62510682 rs6994992 rs113317778 478B14-848 420M9-1395	Yes Yes Yes Yes No Yes Yes
			CAU	2502/3074; 111 parent offspring trios	rs73235619 rs35753505 rs62510682 rs6994992 rs113317778 478B14-848 420M9-1395	Yes Yes Yes Yes No No No
			AS	1445/1128; 597 parent offspring trios	rs73235619 rs35753505 rs62510682 rs6994992 rs113317778 478B14-848 420M9-1395	Absence of allele A No No No No Yes Yes
Munafo et al. 2006 (Munafo et al., 2006)	13	2002-2006	All	8678/4423	rs35753505	No
			CAU	3555/2923	rs35753505	No
			EAS	4984/1372	rs35753505	No
			AF	139/135	rs35753505	No
Munafo et al. 2008 (Munafo,	8	2005- 2007	All	1476/1881	rs35753505	No

Attwood, & Flint, 2008)			CAU	1476/1881	rs35753505	No
			Mixed	Data not available	rs35753505	No
Gong et al. 2009 (Gong et al., 2009)	26	2002-2009	All[a]	8049/ 8869, 1515 families	SNP8*NRG1*03492 rs73235619 rs35753505 rs62510682 rs6994992 rs113317778 478B14-848 420M9-1395	No Yes No No No No Yes Yes
Loh et al. 2013 (Loh et al., 2013)	5 for rs2954041; 9 for rs3924999	2004-2012	All[b]	3916/5737 (for rs2954041) 6080/7839 (for rs3924999)	rs764059 rs3924999 rs2954041	No No No
			MAL	153/150	rs764059 rs3924999 rs2954041	Monomorphic No No
			CHN	183/179	rs764059 rs3924999 rs2954041	Monomorphic No No
			IND	81/100	rs764059 rs3924999 rs2954041	Monomorphic Yes Yes
SZ gene database (access date: 28 Sep. 2015)	5	2007-2009	CAU, Others/Mixed	3256/4183	rs10503929	Yes

AF= African, AS=Asians, CAU=Caucasians, CHN=Chinese, EAS= East Asians, IND=Indian, MAL=Malaysian

rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006)

[a]The meta-analysis study includes mixed population (Caucasians: 4689/5620, 917 families; Asians: 3219/3107, 598 families; Africans: 141/142, 0 families). Association with schizophrenia based on ethnicity was not reported.

[b]The combined meta-analysis contains people from Caucasian, Japanese, Malay, Chinese and Indian ethnicity.

7. NRG1 gene expression studies

7.1 Human post-mortem brain studies

Seven *NRG1* gene expression studies using human post-mortem brain tissue have been published, six in the past decade (Table 6). Four of these studies have used case-control and the other three controls only designs. Among the case-control studies, the first was conducted in 2004 (Hashimoto et al., 2004) using dorsolateral prefrontal cortex (DLPFC) tissue (20 SZ, 19 controls) and noted an increase in type I *NRG1* gene expression in SZ. This finding has since been replicated in hippocampus (Law et al., 2006) but not DLPFC. In fact, others have shown type I expression is decreased (Parlapani et al., 2010) or unchanged (Weickert, Tiwari, Schofield, Mowry, & Fullerton, 2012) in DLPFC and one study showed it was unchanged in hippocampus (Nicodemus et al., 2009). Other *NRG1* isoforms as well as Pan-*NRG1* have not been shown to be differentially expressed between SZ and healthy controls. One exception is type II *NRG1*, which was increased by 193% in Brodmann area 10 of SZ patients compared to controls (Parlapani et al., 2010).

Three healthy control only, post-mortem brain studies have provided insights into *NRG1* gene expression profiles across the lifespan. In PFC, type I *NRG1* expression has been shown to gradually decrease and type III expression increase during the gestational period (0-39 weeks); whereas in the post-natal period (0-84 years) type I isoform expression remained stable but type II and type III isoform expression decreased (Paterson, Wang, Kleinman, & Law, 2014). In early adulthood, type I expression appears to decrease in the PFC (Colantuoni et al., 2008), albeit some have reported that type I reaches a plateau during adolescence (a critical time for schizophrenia pathophysiology) with only subtle changes thereafter (Harris et al., 2009). In addition to these findings, results from the Human Brain Transcriptome atlas (hbatlas.org) suggests pan-*NRG1* expression peaks during early-mid fetal development (10-19 weeks post-conception) across six

brain regions and 11 areas of the neocortex (Figure S1) (Johnson et al., 2009; Kang et al., 2011; Pletikos et al., 2014).

Table 6 Summary of *NRG1* human post-mortem gene expression studies

Reference	Case/Control Subjects	Tissue analysed	Platform used	Ethnicity	mRNA analysed	Gene expression relative to controls
Case-control studies						
Hashimoto et al. 2004 (Hashimoto et al., 2004)	20/19	DLPFC	qRT-PCR	CAU, AA	*NRG1* type I	Increased
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
Law et al. 2006 (Law et al., 2006)	38/53	HC	qRT-PCR	CAU, AA, HSP, AS	*NRG1* type I	Increased
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
					NRG1 type IV	Unchanged
					Pan *NRG1*	Unchanged
Nicodemus et al. 2009 (Nicodemus et al., 2009)	44/84	HC	qRT-PCR	CAU, AA, HSP, AS	*NRG1* type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Decreased
					NRG1 type IV	Unchanged
Parlapani et al. 2010 (Parlapani et al., 2010)	10/7	PFC (BA9)	qRT-PCR	GER	*NRG1* type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
	11/8	PFC	qRT-PCR	GER	*NRG1* type I	Decreased

		(BA10)			*NRG1* type II	Increased
					NRG1 type III	Unchanged
	7/5	HC	qRT-PCR	GER	*NRG1* type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
Weickert et al. 2012 (Weickert et al., 2012)	37/37	DLPFC	qRT-PCR	AUS	*NRG1* type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Increased
					NRG1 type IV	Beyond limit of detection
					Pan *NRG1*	Unchanged
					NRG1 EGFα	Beyond limit of detection
					NRG1 EGFβ	Unchanged
Control only studies						
Colantuoni et al. 2008 (Colantuoni et al., 2008)	0/72	PFC	Custom DNA microarrays and qPCR	60% AA, 35% CAU, 4% HSP and 1% AS	*NRG1* and multiple other genes	-
Harris et al. 2009 (Harris et al., 2009)	0/48	PFC	Affymetrix U133 and qRT-PCR	-	Whole genome microarray analysis (approx. 2000 genes)	-
Paterson et al. 2014 (Paterson et al., 2014)	0/41 foetal brain samples and 195 control samples of age range: 0-83 years	PFC	qRT-PCR	AA, CAU, HSP, AS	*NRG1* type I, II, III, IV, *NRG1* IVNV	-

AA=African-American, AUS=Australian, AS=Asian, BA= Brodmann area, CAU=Caucasians, CHN=Chinese, DLPFC=Dorsolateral prefrontal cortex, GER=German, HC=Hippocampus, HSP=Hispanic, JP=Japanese, PFC=Prefrontal Cortex

rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006)

7.2 Human peripheral blood studies

Differential *NRG1* isoform expression is also found in blood of individuals living with SZ. Type II β3 *NRG1* and type III *NRG1* expression was found to be higher in peripheral leukocytes in those with SZ of Portuguese ethnicity (Petryshen et al., 2005). However in a Japanese cohort, type I, II, III and IV *NRG1* transcripts amplified from immortalized lymphocytes were not significantly different between patients and controls, although the expression of type I, III and IV *NRG1* were beyond the limit of detection (Yamamori et al., 2011). Further, decreased expression of pan-*NRG1* in peripheral blood lymphocytes was detected in Han Chinese SZ patients (Zhang et al., 2011; Zhang et al., 2008).

7.3 Expression quantitative trait loci (eQTL) studies

The ability of *NRG1* genetic variation to regulate *NRG1* gene expression in SZ was first reported by Law et al (Law et al., 2006) using hippocampal tissue from 84 SZ and 44 control subjects. They reported an increased expression of type I *NRG1* was associated with the rare A allele of SNP8NRG221132 (rs73235619) among controls but not those with SZ and an increase in type IV expression associated with the T allele of SNP8NRG243177 (rs6994992) in both those with SZ and controls (Law et al., 2006; Moon et al., 2011; W. Tan et al., 2007). The latter association has been replicated *in vitro* (Tan et al., 2007) and the SNP8NRG243177 T allele has also been linked to lower levels of Ig-*NRG1* immunoreactivity in serum (Shibuya et al., 2010) but replication of Law et al's (Law et al., 2006) initial eQTL findings in independent human brain tissue has yet to be reported. More recent studies have shown decreased expression of hippocampal type I *NRG1* and increased expression of type II among those with SZ carrying the SNP8NRG221533 (rs35753505) C allele (Parlapani et al., 2010). Moreover, there is evidence of increased DLPFC expression of type II and III *NRG1* in SZ among those with one or more copies of the five-marker Hap_{ICE} risk haplotype as well as a novel five-marker haplotype in intron 1, respectively- (Weickert et al., 2012).

To our knowledge, examination of distant (trans) *NRG1* eQTLs in SZ has been confined to a post-mortem human brain study (Mathew et al., 2007) and human plasma study (Marballi et al., 2010). In the post-mortem study, α7 nicotinic acetylcholine receptor expression in DLPFC but not hippocampal tissue from those with SZ was decreased among SNP8NRG243177 (rs6994992) T allele carriers as well as SNP8NRG221132 (rs73235619) G allele carriers. They further showed that haplotypes containing these alleles were associated with lower expression of the α7 nicotinic acetylcholine receptor in DLPFC followed by decrease in binding capacity of the receptors that may result in abnormal signaling in the brain in those with schizophrenia (Mathew et al., 2007).

In the plasma study, levels of 25 autoimmune markers including five cytokines (e.g. IL-6, TNF-α) were increased among rs74942016 T allele carriers compared to G/G genotype carriers. This was corroborated by *in vitro* analysis of transformed B-cells from G/T and G/G genotype carriers (T/T not examined) that showed increases in protein secretion levels of IL-6, TNF-α, and IL-8 in T-allele carriers compared with G/G genotype carriers; suggesting a link between *NRG1* genetic variation and immune dysregulation (Marballi et al., 2010).

8. NRG1 protein expression studies

Studies of *NRG1* protein levels in SZ have been hampered by the lack of specific antibodies for a majority of the *NRG1* isoforms and methodological variations across studies. Despite these limitations, six post-mortem brain, one serum, and one plasma study in humans have been published. Two studies have reported on *NRG1*α, one reporting a decrease in PFC white matter (Bertram et al., 2007) and the other no difference in Brodmann's area 46 (frontal cortex) (Boer, Berk, & Dean, 2009). By contrast, *NRG1*-ICD (intracellular cleavage domain) levels were found to be increased by 20% in the PFC (Chong et al., 2008) and *NRG1*-CTF (C-terminal fragment) levels decreased by 50% in the premotor frontal cortex (Brodmann's area 6) (Barakat, Dean, Scarr, &

Evin, 2010) in SZ compared to controls. While, Hahn et al (Hahn et al., 2006) found no difference in *NRG1*-GST (a combination of α and β EGF domains) levels in the PFC. In DLPFC (Brodmann's area 9), the ratio of type III to full length *NRG1* was found to be upregulated but in anterior prefrontal cortex (Brodmann's area 10) the *NRG1* intracellular domain (50kDa fragment) was lower in SZ patients (Marballi, Cruz, Thompson, & Walss-Bass, 2012).

In the only two peripheral protein studies to date, serum levels of I_g-*NRG1* (Shibuya et al., 2010) and plasma levels of *NRG1*-β1 (R. Wang et al., 2015) were decreased in SZ compared to controls. Given that neither of these isoforms has been examined in brain tissue, the concordance between central and peripheral levels remains unknown.

9. NRG1 and antipsychotic treatment

9.1. NRG1 and treatment response

The effect of *NRG1* genetic variation on antipsychotic response was first reported in a study of 94 Finnish individuals with SZ taking typical antipsychotics for a minimum of four weeks (Kampman et al., 2004). In this study, homozygotes of the 'non-risk' T allele at the SNP8NRG221533 (rs35753505, located in the 5' region, intronic SNP) locus were over-represented in the non-responder group. Interestingly, a decade has passed since this study but to our knowledge, only two additional studies examining *NRG1* genetic variation and antipsychotic treatment response have been published. Among a sample of 339 high symptom severity SZ patients (i.e. clinical global impression–severity scale > 3) from Indian populations of Indo-European and Dravidian ancestry, the A alleles for *NRG1* SNPs rs13250975 and rs17716295 were associated with non-response to antipsychotic (typical and atypical) medication (Jajodia et al., 2016). Neither of these SNPs have previously been described in the literature and both sit in the intronic region between the exons defining type V and type I *NRG1,* however it is not clear if these SNPs are functional or if they are

tagging for functional variants. Using a haplotype tagging genotyping approach, Terzic and colleagues (2015) examined four intronic *NRG1* tag-SNPs located in the 3' region (rs3735781, rs3735782, rs10503929, rs3924999) for their association with treatment responsiveness in a cohort of 138 SZ outpatients in Slovenia treated with typical or atypical antipsychotics, though results did not support an association between any of the *NRG1* SNPs examined and treatment responsiveness (Terzic, Kastelic, Dolzan, & Plesnicar, 2015). Collectively demonstrate the need for further *NRG1* pharmacogenetic studies, particularly studies capable of examining multiple variants and haplotypes within *NRG1* and possibly its cognate receptor *ERBB*4, which was shown to be associated with paliperidone response (D. Wang et al., 2015).

9.2 Antipsychotic effects on NRG1

A comprehensive review of *NRG1* expression in the context of antipsychotic treatment concluded that short-term (up to 4 weeks) treatment increases mRNA and protein expression of *NRG1* whereas longer-term treatment decreases this expression (Deng et al., 2013). This conclusion is primarily supported by animal work. Human studies have been conducted but results to date have been inconsistent. *NRG1* type II expression in immortalized lymphocytes was decreased following 48 hours of olanzapine stimulation in controls but not those with SZ (Chagnon, Roy, Bureau, Merette, & Maziade, 2008); whereas, two-weeks of risperidone and quetiapine treatment resulted in an increase in *NRG1* expression in peripheral blood lymphocytes (Zhang et al., 2011). Likewise, three-weeks of clozapine treatment increased *NRG1* expression in post mortem human fetal brain tissue, while no effect on expression was observed following haloperidol treatment (Chana et al., 2009).

10. *NRG-ErbB* pathway gene expression and schizophrenia

NRG-ErbB signaling is vital for the assembly of neuronal circuitry (Fazzari et al., 2010), myelination of axonal processes (Nave, 2010; Snaidero et al., 2014), neurotransmission and synaptic plasticity (Y. J. Chen et al., 2010; B. Li, Woo, Mei, & Malinow, 2007; Mei & Xiong, 2008). According to the KEGG database (Kanehisa & Goto, 2000), both *NRG1* and *NRG2* can bind with *ErbB4* or *ErbB3* and mediate downstream signaling via the *PI3K-AKT* signaling pathway. This results in activation of the *mTOR* which leads to protein synthesis via the *mTOR* signaling pathway (Figure 3). Abnormal *NRG-ErbB* signaling impairs brain function and has been linked to schizophrenia (Mei & Nave, 2014).

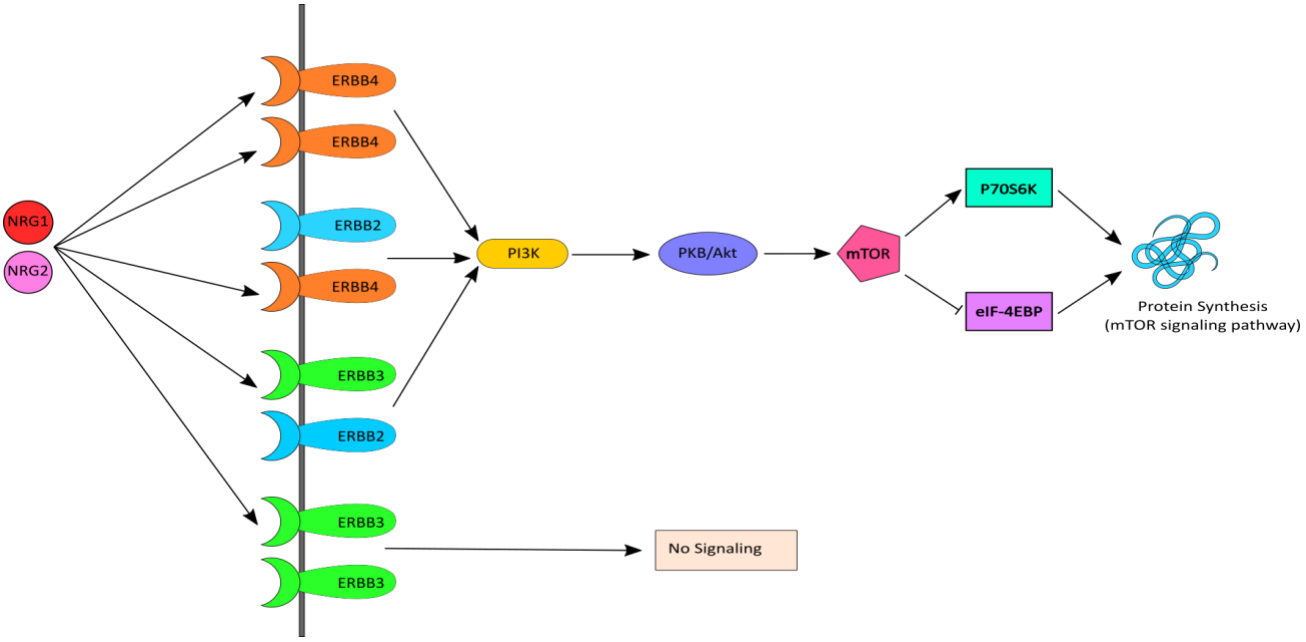

Figure 3: *NRG-ErbB* signaling pathway. *NRG1* and *NRG2* bind to *ErbB3* and/or *ErbB4,* which in turn undergoes homo or heterodimerization and activates *PI3K*. *PI3K* then activates AKT and subsequently *mTOR* causing initiation of protein synthesis via the *mTOR* signaling pathway. mTOR phosphorylates and activates *P70S6K* which facilitates phosphorylation of small ribosomal protein 6 (*S6*) and eukaryotic translation initiation factor 4B (*eIF4B*) and leads to initiation of protein synthesis. Activated *mTOR* also causes phosphorylation and inactivation of *eIF4EBP1*, which release *eIF4E* and facilitates translation.

NRG1 and *ErbB4* are the two most well-characterized molecules in the *NRG-ErbB* signaling pathway. Linkage and association studies conducted in various ethnic groups have identified schizophrenia associated single nucleotide polymorphism in *NRG1*, NRG2 as well as in all *ErbB* genes, i.e., *ErbB1, ErbB2, ErbB3, and ErbB4* (Benzel et al., 2007; Iwakura & Nawa, 2013; D. Li, Feng, & He, 2009; Mei & Xiong, 2008).

Several post-mortem human brain studies reported increased *NRG1* mRNA in DLPFC and hippocampus in those with schizophrenia (Hashimoto et al., 2004; Law et al., 2006) but reports of decreased or unchanged mRNA levels are also available (Nicodemus et al., 2009; Parlapani et al., 2010). Bertram et al. (2007) reported increased NRG1 protein level in prefrontal cortex (PFC), while Chong et al. ((Bertram et al., 2007; Chong et al., 2008) showed that NRG1 is decreased in the same brain region. Aston et al. (2004) showed *ErbB3* mRNA level is downregulated in the middle temporal gyrus in schizophrenia patients (Aston, Jiang, & Sokolov, 2004) but ErbB1 protein is upregulated in the fore-brain regions (Futamura et al., 2002). Elevated expression of *ErbB4* mRNA isoforms and protein are also reported in the PFC in those with schizophrenia (Joshi, Fullerton, & Weickert, 2014; Law et al., 2012).

Similar evidence of upregulation of *NRG1* mRNA but downregulation of protein level has been reported in the periphery in those with schizophrenia (Petryshen et al., 2005; Shibuya et al., 2010). Upregulation of *PI3K* and *AKT* mRNA isoforms in peripheral tissue of schizophrenia patients is also available, although limited in number (L. Liu et al., 2016; Y. Xu et al., 2016).

NRG2 is the closest homolog of *NRG1* and a recent study showed that *NRG2* knockout mice exhibited disturbances in dopamine regulation in the dorsal striatum and medial prefrontal cortex and showed abnormal behavioural phenotypes linked to schizophrenia (Yan et al., 2017). While a

few studies investigated the role of *ErbB3, ErbB1, PI3K,* and *AKT* in schizophrenia (Aston et al., 2004; Futamura et al., 2002; Law et al., 2012) but through investigation of mRNA expression level all the major *NRG-ErbB* downstream signaling genes have not yet been performed.

Overall, these studies suggest dysregulation of *NRG-ErbB* pathway genes mRNA level in both brain and blood in schizophrenia and leads to the idea of using peripheral blood as a proxy for dysregulation of gene expression in brain (Harris et al., 2012). Moreover, the impact of specific clinical subgroups (e.g. treatment-resistant), medication, lifestyle (e.g. smoking), and/or symptom severity may have on mRNA expression is yet to be explored.

Aims and Hypothesis

The overarching aim of this thesis was to determine if *NRG1* and/or molecules within the *NRG-ErbB* pathway could serve as suitable biomarkers for clozapine mediate symptom remission and symptom severity in treatment-resistant schizophrenia (TRS). To begin, a systematic review of the *NRG1* literature in schizophrenia was completed followed by a meta-analysis of *NRG1* genetic variation studies in schizophrenia. *NRG1* genetic variants identified in the meta-analysis were then further interrogated for their association with peripheral *NRG1* mRNA and protein expression using an expression quantitative trait loci analysis. *NRG1* peripheral mRNA and protein expression were then further examined to determine whether an association with clozapine mediated positive symptom remission could be detected. However, given all participants examined in the thesis were taking clozapine, effects of clozapine exposure on *NRG1* mRNA and protein expression were also investigated via a series of in-vitro experiments using peripheral blood mononuclear cells from health individuals. Finally, to explore molecules downstream of *NRG1* and determine if an overall pathway expression pattern could be detected, peripheral mRNA expression of the *NRG-ErbB* pathway were measured and assessed for associations with treatment-resistant schizophrenia as well as clozapine mediated positive symptom remission.

Aims

1. To conduct a meta-analysis on *NRG1* genetic variations and risk of schizophrenia from existing case-control and family studies to reconcile conflicting findings in the literature. (Chapter 2)
2. To examine peripheral expression of specific *NRG1* isoforms and *NRG1*-β1 protein among remitted and non-remitted TRS patients as well as healthy controls to identify expression profiles associated with symptom severity/remission status. (Chapter 3)

3. To examine the cis-regulatory effects of *NRG1* genetic variation on gene expression. (Chapter 3)
4. To examine the *in vitro* effect of clozapine exposure on gene expression of specific *NRG1* mRNA isoforms and *NRG1*-β1 protein in peripheral blood mononuclear cells (PBMC) from healthy controls. (Chapter 3)
5. To explore the peripheral expression pattern of *NRG-ErbB* pathway genes in TRS patients and controls and their association with symptom severity and remission status. (Chapter 4)

Hypotheses

1. Genetic variations (SNPs and microsatellites) within the *NRG1* gene will be associated with schizophrenia risk among populations from different ethnicity. (Chapter 2)
2. Gene expression of *NRG1* mRNA isoforms and *NRG1*-β1 protein will: (a) differentiate TRS patients in symptom remission from those who have not remitted and healthy controls, and (b) correlate with positive and negative symptom severity. (Chapter 3)
3. Genetic variation within the *NRG1* gene will have cis-acting effects on gene expression (chapter 3).
4. *In vitro* clozapine exposure will regulate gene and protein expression of *NRG1* in a relatively similar pattern observed *in vivo*. (Chapter 3)
5. Peripheral gene expression pattern of *NRG-ErbB* pathway genes will show a similar pattern with all the *NRG1* mRNA isoform ligands and will (a) differentiate TRS patients in symptom remission from those who have not remitted and healthy controls, and (b) correlate with positive and negative symptom severity. (Chapter 4)

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Chapter 2

Meta-analysis reveals associations between genetic variation in the 5'and 3' regions of Neuregulin-1 and schizophrenia

OPEN

Citation: Transl Psychiatry (2017) 7, e1004; doi:10.1038/tp.2016.279

www.nature.com/tp

ORIGINAL ARTICLE

Meta-analysis reveals associations between genetic variation in the 5′ and 3′ regions of Neuregulin-1 and schizophrenia

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Genetic, post-mortem and neuroimaging studies repeatedly implicate neuregulin-1 (*NRG1*) as a critical component in the pathophysiology of schizophrenia. Although a number of risk haplotypes along with several genetic polymorphisms in the 5′ and 3′ regions of *NRG1* have been linked with schizophrenia, results have been mixed. To reconcile these conflicting findings, we conducted a meta-analysis examining 22 polymorphisms and two haplotypes in *NRG1* among 16 720 cases, 20 449 controls and 2157 family trios. We found significant associations for three polymorphisms (rs62510682, rs35753505 and 478B14-848) at the 5′-end and two (rs2954041 and rs10503929) near the 3′-end of *NRG1*. Population stratification effects were found for the rs35753505 and 478B14-848(4) polymorphisms. There was evidence of heterogeneity for all significant markers and the findings were robust to publication bias. No significant haplotype associations were found. Our results suggest genetic variation at the 5′ and 3′ ends of *NRG1* are associated with schizophrenia and provide renewed justification for further investigation of *NRG1*'s role in the pathophysiology of schizophrenia.

Translational Psychiatry (2017) **7,** e1004; doi:10.1038/tp.2016.279; published online 17 January 2017

INTRODUCTION

Neuregulin-1 (NRG1) is a pleiotropic growth factor involved in circuitry generation, axon ensheathment, neuronal migration, synaptic plasticity, myelination and neurotransmission.[1–4] Thus, it is centrally involved in neurodevelopment and signalling in the mature central nervous system, where it exerts its actions through binding to its cognate receptor tyrosine kinases, ErbB3 and ErbB4, members of the epidermal growth factor system. The gene encoding *NRG1* is large, spanning ~1.2 Mb and contains >23 000 single-nucleotide polymorphisms (SNPs) among which ~40 have been associated with schizophrenia.[5] Genome-wide association studies have generally, however, only provided modest support with the most recent study implicating rs986110 ($P=1.5\times10^{-4}$) with the disorder.[6] This may in part be due to genome-wide association study to date focussing exclusively on SNP variation and consequently underestimating the importance of genes, such as *NRG1*, for which haplotype and microsatellite variation has been demonstrated. Thus, arguably a more thorough evaluation of *NRG1*'s association with schizophrenia requires examination of variation beyond SNPs.

Putative genetic/haplotypic variants in *NRG1* primarily sit within untranslated or intronic regions at the 5′ and 3′ ends of the gene. Yet, research to date has focused on the 5′-region of *NRG1*. This 5′-bias has been driven by the landmark study in 2002 conducted by Stefansson *et al.*,[7] who identified a seven-marker schizophrenia-associated haplotype in the Icelandic population (HapICE) consisting of five SNPs and two microsatellites (478B14-848 and 420M9-1395) in the 5′-region of *NRG1*. As this milestone study, additional 5′-schizophrenia-associated haplotypes in the Irish (HapIRE)[8] and Chinese (HapChina1-3)[9] populations have been identified. However, the most recent meta-analysis conducted in 2008 (ref.10) only showed significant support for three (rs73235619, 478B14-848 and 420M9-1395) of the seven HapICE markers. Eight years have now passed since that meta-analysis and >20 case–control and family-based genetic association studies have been conducted. Moreover, the data required to conduct meta-analyses on genetic variation in the 3′-region of *NRG1* is now available. Thus, we have conducted an updated comprehensive meta-analysis of the association between *NRG1* genetic variation and schizophrenia, including single markers across the entire gene as well as haplotypes.

MATERIALS AND METHODS

Search strategy

The 2015 PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) checklist[11] was followed in reporting this meta-analysis. Studies were identified independently by two of the authors (MSM and CL) by searching three electronic databases: PubMed, PsychInfo and Medline (Ovid), using the search terms 'neuregulin 1', 'neuregulin-1', 'neuregulin1', 'schizophrenia' and 'association', and the abbreviation of the gene 'NRG1' and 'NRG 1' with no language restrictions. Bibliographies of all research articles were hand searched for additional references not indexed by MEDLINE. In cases where genotype data were not available in the published research articles or Supplementary Materials, we attempted to contact authors and request the required data. We also used the SZGene database (www.szgene.org) as a resource for collecting genotype data. All

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Received 20 November 2016; accepted 27 November 2016

publications published from January 2002 through February 2016 were assessed for inclusion.

Study selection and data extraction

For a study to be included in the meta-analysis, the following criteria were required: (a) a case–control or family-based genetic association studies investigating one or more SNPs and/or microsatellites of *NRG1*; (b) published in peer-reviewed journal containing original data; (c) included clinically diagnosed schizophrenia patients using an accepted classification system (for example, DSM and ICD); and (d) provided sufficient genotype or allelic data for calculation of an odds ratio (OR). Based on these criteria, 48 (40 case–control and 8 family-based) studies were included (Supplementary Figure S1; Table S1).

From each case–control and family study, the following data were extracted: (a) author(s) and publication year; (b) number of cases and controls or family sample size; (c) country of origin or ethnicity; (d) diagnostic criteria used; (e) SNP reference sequence number or marker identifier; (f) the publication identification number (for example, PubMed ID); (g) genotype counts and/or allele counts in cases and controls or family samples; and (h) haplotype frequencies in cases and controls (where available). Extracted data for all selected studies can be found in Supplementary File 2.

Data synthesis and statistical analysis

Data from each case–control study were used to create 2×2 tables and data from each family study were used to create 1×2 tables. Classifications of the subjects were based on diagnostic category and type of allele they carried.

Data were analysed using *R* version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria). The meta[12] and metafor[13] packages were used to conduct the meta-analyses. The OR with 95% confidence intervals (CIs) was used as the effect size estimator. The method proposed by Kazeem and Farrall[14] was used to calculate the effect size for transmission disequilibrium test studies, where the ORs were estimated from the number of transmissions versus non-transmissions of the designated high-risk allele to schizophrenia cases from heterozygous parents. For case–control studies, ORs were estimated by contrasting the ratio of counts of the high-risk versus low-risk alleles in schizophrenia cases versus non-clinical controls. For those polymorphisms in which the previous literature provided an indication of the risk-inducing allele, one-tailed *P*-values were reported. In the absence of prior data, two-tailed *P*-values were reported and were indicated accordingly in the text. All statistical tests (except for the *Q*-statistic) were considered statistically significant at $P < 0.05$.

Because of the differences in study design and sample characteristics, considerable heterogeneity was expected between the studies. Therefore, the pooled OR was calculated using the random-effects models with the DerSimonian–Laird estimator,[15] which is based on a normal distribution. The standard error estimates were adjusted using the Hartung–Knapp–Sidik–Jonkman[16,17] correction, which then calculates the corresponding 95% CI based on the *t*-distribution. The Hartung–Knapp–Sidik–Jonkman method generally outperforms the DerSimonian–Laird approach on type-I error rates when there is heterogeneity and the number of studies in the meta-analysis is small.[18,19]

Outliers and influential studies were identified according to the recommendations of Viechtbauer and Cheung.[20] Studies with observed effects that are well separated from the rest of the data are considered outliers. Such studies were identified using studentised deleted residuals, with absolute values > 1.96 indicative of outliers. An influential study leads to considerable changes to the fitted model and a range of case deletion diagnostics adapted from linear regression can be used to identify these studies, including the DFFITS, DFBETAS and COVRATIO statistics (see Viechtbauer and Cheung[20] for more information). Potential outliers and influential studies were omitted and the analyses were then re-run to determine their influence on the pooled effect size.

Heterogeneity in effect sizes across studies was tested using the *Q*-statistic (with $P < 0.10$ indicating significant heterogeneity) and its magnitude was quantified using the I^2 statistic, which is an index that describes the proportion of total variation in study effect size estimates that is due to heterogeneity and is independent of the number of studies included in the meta-analysis and the metric of effect sizes.[21] As the *Q*-statistic has low power when the number of studies is small,[22] 95% prediction intervals were calculated to quantify the extent of heterogeneity in the distribution of effect sizes.[23] The prediction interval is an estimation of the range within which 95% of the true effect sizes are expected to fall.

Publication bias was assessed using funnel plots and the trim-and-fill procedure,[24] which estimates the number of studies missing from the funnel plot and imputes these missing studies to make the funnel plot symmetrical, and then calculates an estimate of the effect size adjusted for publication bias.[25] Following the recommendations of Sterne *et al.*,[26] a test for funnel plot asymmetry was only conducted if the number of studies was 10 or greater. The regression test proposed by Harbord *et al.*[27] was used to quantify the bias captured by the funnel plot and tested whether it was statistically significant. In addition, cumulative meta-analyses sorted by the sampling variance of the respective studies were conducted to examine the relationship between imprecise samples and effect sizes.[28] This visualises the effect that small imprecise study samples have on the estimations of the pooled effect size.

The generalised linear mixed model method (that is, logistic regression) detailed in Bagos[29] was used for the haplotype meta-analyses to avoid the inflation of the type-I error rate that is observed in the traditional approach of comparing a haplotype against the remaining ones.[29]

Moderator analyses for study design, diagnostic criteria and ancestry were conducted using mixed-effects meta-analyses. For this method, studies within potential moderator groups were pooled with the random-effects model, whereas tests for significant differences between the groups were conducted with the fixed-effects model. The Hartung–Knapp–Sidik–Jonkman adjustment was used if there were at least three studies in each group, otherwise the unadjusted DerSimonian–Laird method was used.

RESULTS

Meta-analysis

A total of 22 single markers and two haplotypes that appeared in three or more studies were examined (Figure 1). Significant associations were found for three (rs62510682, 478B14-848(0) and rs2954041) of the 22 single markers but neither of the two haplotypes examined (Table 1; Supplementary Figures S2–S4).

Heterogeneity, outlier and publication bias analysis

Across the three significant single markers, heterogeneity was low to moderate ($I^2 = 18.5$–54.3%). The funnel plots are presented in Supplementary Figures S5–S7. The regression tests for funnel plot asymmetry were not statistically significant (Supplementary Table S2). Although the trim-and-fill method imputed two studies for rs62510682 and 478B14-848 (0), respectively, and three studies for rs2954041, the effect size adjusted for publication bias was comparable to the unadjusted effect size (Supplementary Table S2). The cumulative forest plots (Supplementary Figures S5–S10) also show that the point estimate stabilises with the inclusion of studies with smaller sampling variances. Taken together, this pattern of results suggests that the findings for the three significant single markers are likely robust to publication bias. Removal of potential outlier (that is, influential) studies in each of the meta-analyses produced small-to-moderate reductions in heterogeneity with minimal impact on the odd ratio (Supplementary Table S3). One exception was rs10503929, which after removal of an outlier study showed a significant association with schizophrenia ($k = 5$, OR = 1.14, 95% CI = 1.10–1.18, $P \leqslant 0.001$).

Moderator analysis

Differential effects by study design, diagnostic criteria or ancestry were identified for two markers (Supplementary Table S3). The 4 allele of the 478B14-848 microsatellite had a 'risk' association among Asian studies ($k = 2$, OR = 1.18, 95% CI = 1.01–1.38, $P = 0.021$) and conversely a 'protective' association among European studies ($k = 3$, OR = 0.83, 95% CI = 0.69–1.00, $P = 0.025$; Supplementary Figure S11). Likewise, the rs35753505 (SNP8NRG221533) C-allele was associated with schizophrenia among Asian ($k = 12$, OR = 1.11, 95% CI = 1.01–1.23, $P = 0.018$) but not European ($k = 22$, OR = 1.01, 95% CI = 0.94–1.09, $P = 0.376$) studies (Supplementary Figure S12).

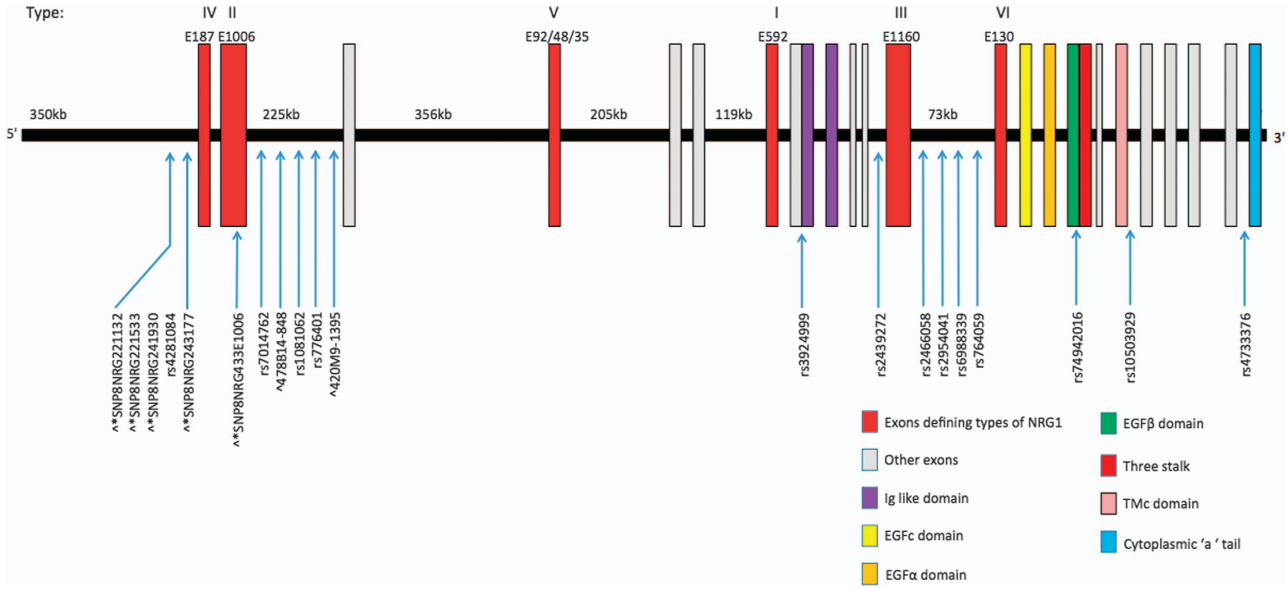

Figure 1. Location of NRG1 genetic variants included in the meta-analysis. *SNPs forming core 'at-risk' five-marker HapICE haplotype. ^Microsatellites in seven-marker HapICE haplotype. #Markers shown to be significant in the current meta-analysis. HapICE, haplotype in the Icelandic population; SNP, single-nucleotide polymorphism.

Table 1. Summary of single marker and haplotype meta-analyses

NRG1 markers/haplotypes	*Risk*	k	*Case/control (family trios)*	*Meta-analysis*				*Heterogeneity*			
				OR	*95% CI*	*90% CI*[a]	P	Q/ τ^2	P	I^2	*95% PI*
Single markers											
rs73235619[b,c]	G	13	6145/6607 (262)	1.18	0.81, 1.71	0.87, 1.60	0.180	80.6	< 0.001	85.1	0.47, 2.94
rs35753505[b,c]	C	35	12 708/14 302 (1601)	1.04	0.97, 1.11	0.98, 1.10	0.120	85.4	< 0.001	60.2	0.79, 1.36
rs62510682[b,c]	G	25	10 791/11 986 (1248)	1.10	1.01, 1.20	1.02, 1.18	**0.018**	54.3	< 0.001	55.8	0.84, 1.44
rs4281084	A	3	2217/2919	1.03	0.98, 1.08	1.00, 1.06	0.060	0.1	0.96	0.0	0.90, 1.18
rs6994992[b,c]	T	27	11 848/14 106 (1097)	1.00	0.96, 1.05	0.97, 1.04	0.440	36	0.09	27.8	0.88, 1.14
rs113317778[b,c]	G	10	4586/4935 (1003)	0.81	0.52, 1.25	0.57, 1.15	0.150	146.4	< 0.001	93.9	0.17, 3.76
rs7014762	A	4	2128/2398 (634)	1.05	0.98, 1.12	1.00, 1.10	0.060	0.7	0.87	0.0	0.96, 1.15
478B14-848 (0)[c]	-	11	1071/1056 (111)	1.11	1.02, 1.20	—	**0.008**	12.3	0.27	18.5	0.97, 1.27
478B14-848 (4)	-	5	1015/894 (463)	0.98	0.74, 1.29	—	0.410	9.3	0.05	57.2	0.54, 1.78
rs1081062	C	4	2635/2946 (634)	0.99	0.83, 1.19	0.87, 1.14	0.460	3.6	0.31	16.1	0.71, 1.39
rs776401	C	3	3103/4817	0.97	0.63, 1.48	0.73, 1.29	0.390	10.3	0.006	80.6	0.12, 7.68
420M9-1395 (0)[c]	-	10	4777/4567 (111)	1.01	0.81, 1.25	—	0.460	36.5	< 0.001	75.4	0.63, 1.63
420M9-1395 (-2)	-	7	1313/1130 (647)	1.05	0.96, 1.15	—	0.100	3.6	0.73	0.0	0.96, 1.15
rs3924999	A	16	6725/8551 (725 +15bios)	1.02	0.90, 1.16	—	0.370	50.3	< 0.001	70.2	0.72, 1.46
rs2439272	A	5	3003/4106 (111)	0.87	0.61, 1.23	0.67, 1.14	0.170	20.3	< 0.001	80.3	0.42, 1.81
rs2466058	T	4	1863/1784 (111)	1.08	0.67, 1.74	0.76, 1.54	0.330	8.1	0.05	62.8	0.34, 3.39
rs2954041	T	7	3906/5527 (246)	1.21	0.97, 1.52	1.02, 1.45	**0.038**	10.6	0.10	43.3	0.76, 1.94
rs6988339	G	4	1113/2104 (111)	0.99	0.72, 1.37	0.78, 1.26	0.470	9.6	0.023	68.7	0.43, 2.27
rs764059	G	3	910/857 (111)	0.97	0.74, 1.28	0.81, 1.17	0.350	1.0	0.60	0.0	0.43, 2.18
rs74942016	T	3	1380/2222	1.11	0.77, 1.61	0.87, 1.43	0.170	0.3	0.87	0.0	0.38, 3.29
rs10503929	T	6	3399/4635 (151)	1.54	0.65, 3.65	0.78, 3.03	0.128	60.9	< 0.001	91.8	0.36, 6.53
rs4733376	G	4	1843/2606 (111)	1.12	0.88, 1.42	0.94, 1.34	0.110	4.2	0.25	27.8	0.69, 1.81
Haplotypes											
Five-marker HapICE haplotype	GCGTG	5	2501/2283 (111)	1.16	0.91, 1.48	—	0.180	0.02[d]	0.07	53.1	0.67, 1.99
Seven-marker HapICE haplotype	GCGTG00	5	2501/2283 (111)	1.29	0.77, 2.13	—	0.250	0.12[d]	0.01	68.5	0.37, 4.39

Abbreviations: CI, confidence interval; HapICE, haplotype in the Icelandic population; OR, odds ratio; PI, prediction interval. SNP8NRG221132 = rs73235619, SNP8NRG221533 = rs35753505, SNP8NRG241930 = rs62510682, SNP8NRG243177 = rs6994992, SNP8NRG433E1006 = rs113317778. $P < 0.05$ are bold faced. [a]90% CI for one-sided test. [b]Markers forming five-marker HapICE haplotype. [c]Markers forming seven-marker HapICE haplotype. [d]Tau squared (τ^2) values.

DISCUSSION

Three of the seven HapICE markers (rs62510682, rs35753505 and 478B14-848) at the 5′-end as well as two SNPs (rs2954041 and rs10503929) near the 3′-end of *NRG1* showed significant associations with schizophrenia. Our results concur with previous meta-analyses of *NRG1* that have reported associations for one or more of these markers (SZGene.org.),[10,30–33] with the exception of the 3′ SNP rs2954041. To our knowledge, this is the first meta-analysis to identify an association between schizophrenia and rs2954041.

The rs2954041 SNP is located in the fifth intron of *NRG1*, ~ 18 kb from the type III (SMDF) promoter, the most brain abundant isoform of *NRG1*.[34] To our knowledge, rs2954041 has not been assessed as expression quantitative trait loci for type III expression. However, given its proximal location to the type III promoter and preclinical evidence suggesting disruption of type III results in phenotypes commonly associated with schizophrenia (for example, enlarged ventricles and prepulse inhibition deficits),[35] rs2954041 could have a functional role in the pathophysiology of schizophrenia. In addition, others have shown this SNP interacts with rs7424835 in *ERBB4*, the cognate receptor for *NRG1* (ref. 36) further highlighting a need to interrogate more comprehensively the 3′-end of *NRG1* in the context of schizophrenia. In fact, our results also showed the missense rs10503929 SNP, situated in exon 11 of the 3′-region, was associated with schizophrenia, although only after removal of an outlying family study.[37] Importantly, our findings replicate those available in the SZGene database (www.szgene.org) and are based exclusively on studies within populations of European descent. This is notable because the rs10503929 'risk' allele (T) is the major allele and is carried by all East Asians, 99% of Africans and 94% of South Asians relative to 81% of Europeans (http://browser.1000genomes.org/index.html). Thus, future studies in Asian and/or African populations may not be relevant or will require extremely large sample sizes.

Our findings from the 5′-end of *NRG1* that associate rs62510682, rs35753505 and 478B14-848 with schizophrenia have previously been identified in other meta-analyses. The rs35753505 is the most studied and the first *NRG1* marker to receive meta-analytic support for an association with schizophrenia.[30] However, in three subsequent meta-analyses, this association was not detected.[10,31,32] In the current meta-analysis, we have revived this association but only among Asians, which is contrary to the original meta-analytic association for rs35753505 that was found only among Caucasians.[30] This finding is perhaps not surprising given evidence of population stratification at the *NRG1* locus.[10] In fact, we also found that the 4 allele of the 478B14-848 microsatellite is a marker of 'risk' among Asians but 'protection' among Caucasians. This aligns with knowledge that the 0 allele in Asian populations is low[38,39] compared with the 4 allele, which is quite prevalent and forms in part the HapCHINA schizophrenia risk haplotype.[38–40] However, no other markers we investigated were moderated by ancestry, including the three omnibus markers (rs62510682, 478B14-848(0) and rs2954041) associated with schizophrenia, albeit the number of non-Caucasian studies available for many of the markers hinders firm conclusions.

The rs62510682 (SNP8NRG241930) is the second most frequently studied *NRG1* marker but previous meta-analyses have been mixed. Li *et al.* showed in a meta-analysis of eight studies that carriers of the G allele had greater odds of a schizophrenia diagnosis, particularly among individuals of European descent; but in a subsequent meta-analysis of 14 studies by Gong *et al*., this association was not upheld. Our meta-analysis of rs62510682 included 25 studies, a near doubling of the most recent meta-analysis, and reproduced the finding reported by Li *et al.* that suggests the G allele of rs62510682 is associated with schizophrenia. Our moderator analysis showed that this association did not differ by ancestry, although stratification analysis did suggest that this association might be stronger among individuals of European descent.

Although studied less frequently than other HapICE markers, the 0 'risk' allele of the microsatellite 478B14-848 has been linked to schizophrenia in two previous meta-analyses,[10,30] although Li *et al.* combined carriers of the 0 and 4 alleles in their meta-analysis —an approach that has important implications with interpretation given our finding that the 4 allele can confer a 'risk' or 'protective' effect depending on ancestry. Nevertheless, our meta-analysis results uphold the meta-analytic association between the 0 allele and schizophrenia reported by Gong *et al.* and support further study of this potentially important microsatellite.

Our results, however, do not support an association between either the five- or seven-marker HapICE haplotypes and schizophrenia. To our knowledge, this is the first meta-analysis to examine the five- and seven-marker HapICE haplotypes. Although previous meta-analysis have showed positive associations for both five- and seven-marker haplotypes in schizophrenia,[10] they pooled the results for non-identical five- and seven-marker haplotypes. Thus, their results do not reflect the overall association of the HapICE haplotype block in schizophrenia. Furthermore, most of the included studies were conducted in populations of European ancestry, which is not surprising given the frequency of the alleles that constitutes the HapICE risk haplotype is relatively low in Asian populations. In fact, most Asian studies do not look at the full HapICE haplotype but rather select SNPs and microsatellites forming the HapCHINA haplotype.

In conclusion, we have replicated and identified novel strong positive associations among polymorphisms situated at the 5′ and 3′ ends of *NRG1*. Although support for an association between the five- or seven-marker HapICE haplotypes and schizophrenia was not found, three of the markers within these haplotypes had robust associations. Our results highlight the importance of genetic variation at both the 5′ and 3′ ends of *NRG1* and provide justification for further investigation of NRG1's role in the pathophysiology of schizophrenia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

MSM was supported by a Cooperative Research Centre for Mental Health Top-up Scholarship. SS was supported by One-in-Five Association Incorporated. CAB was supported by a University of Melbourne Research Fellowship as well as a Brain and Behavior Research Foundation (NARSAD) Young Investigator Award (20526). None of the Funding Sources played any role in the study design; collection, analysis or interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

SUPPLEMENTARY MATERIAL

Meta-analysis reveals associations between genetic variation in the 5' and 3' regions of neuregulin-1 and schizophrenia

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Supplementary Tables

Supplementary Figures

Supplementary Table S1: Characteristics of included samples in the meta-analysis.

Author	Case/Control (family trios)	Ancestry	Method	Criteria
Stefansson, Sigurdsson [1]	478/394	Icelandic	Case-control	DSM-III-R
Stefansson, Sarginson [2]	609/618	Scottish	Case-control	DSM-III-R
Yang, Si [3]	246	Chinese	Family-based	ICD-10
Williams, Preece [4]	573/618	Irish (UK)	Case-control	DSM-IV
Iwata, Suzuki [5]	607/515	Japanese	Case-control	DSM-IV
Bakker, Hoogendoorn [6]	282/585	Dutch	Case-control	DSM-IV
Hong, Huo [7]	228/269	Chinese	Case-control	DSM-IV
Hong, Huo [7]	221 (15 bios)	Chinese	Family-based	DSM-IV
Corvin, Morris [8]	243/222	Irish	Case-control	DSM-IV
Li, Stefansson [9]	298/336	Chinese	Case-control	Mixed
Li, Stefansson [9]	184	Chinese	Family-based	Mixed
Kampman, Anttila [10]	94/395	Finnish	Case-control	DSM-IV
Zhao, Shi [11]	369/299	Chinese	Case-control	DSM-III-R
Zhao, Shi [11]	352	Chinese	Family-based	DSM-III-R
Petryshen, Middleton [12]	321/242	Portuguese	Case-control	DSM-IV
Petryshen, Middleton [12]	111	Portuguese	Family-based	DSM-IV
Lachman, Pedrosa [13]	141/142	African-American (USA)	Case-control	Mixed
Lachman, Pedrosa [13]	177/164	USA (Caucasian)	Case-control	Mixed
Fukui, Muratake [14]	349/424	Japanese	Case-control	DSM-IV
Ingason, Soeby [15]	325/353	Danish	Case-control	ICD-10
Kim, Lee [16]	242/242	Korean	Case-control	DSM-IV
Benzel, Bansal [17]	396/1342	Caucasian (UK)	Case-control	OPCRIT
Rosa, Gardner [18]	151	Spanish	Family-based	DSM-IV
Jungerius, Hoogendoorn [19]	310/880	Netherlands	case-control	DSM-IV
Georgieva, Dimitrova [20]	634	Bulgarian	Family-based	DSM-IV
Crowley, Keefe [21]	738/733	USA (Mixed)	Case-control	DSM-IV
Hanninen, Katila [22]	113/393	Finnish	Case-control	DSM-IV
Vilella, Costas [23]	589/615	Spanish	Case-control	DSM-IV
Ikeda, Takahashi [24]	1126/1022	Japanese	Case-control	DSM-IV
Shiota, Tochigi [25]	416/520	Japanese	Case-control	DSM-IV
Sanders, Duan [26]	1870/2002	USA, Australia	Case-control	DSM-IV
Bramon, Dempster [27]	64/35	UK	Case-control	DSM-IV
Hong, Wonodi [28]	244/186	USA (Mixed)	Case-control	DSM-IV
Alaerts, Ceulemans [29]	486/514	Swedish	Case-control	DSM-IV
Pedrosa, Nolan [30]	176/175	USA (Caucasian)	Case-control	Mixed
Zhang, Li [31]	258	Chinese	Family-based	CCMD-3
Jonsson, Saetre [32]	837/1473	Caucasian (Denmark, Norway, Sweden)	Case-control	Mixed
So, Fong [33]	489/519	Chinese	Case-control	DSM-IV
Haraldsson, Ettinger [34]	113/106	Icelandic	Case-control	RDC-SADS(L)
Rethelyi, Bakker [35]	280/230	Hungarian	Case-control	DSM-IV
Squassina, Piccardi [36]	171/349	Italian	Case-control	DSM-IV

Author	Case/Control (family trios)	Ancestry	Method	Criteria
(continued)				
Garcia-Barcelo, Miao [37]	270/270	Chinese	Case-control	DSM-IV
Naz, Riaz [38]	100/70	Pakistani	Case-control	Unknown
Moon, Rollins [39]	273/479	Central Valley of Costa-Rica	Case-control	DSM-IV
Papiol, Begemann [40]	1071/1056	German (Caucasisan)	Case-control	DSM-IV-TR
Mohamad Shariati, Behmanesh [41]	95/95	Iranian	Case-control	DSM-IV
Yang [42]	221/359	Korean	Case-control	DSM-IV
Kang, Yang [43]	287/120	Chinese	Case-control	DSM-IV
Crisafulli, Chiesa [44]	221/170	Korean	Case-control	DSM-IV
Weickert, Tiwari [45]	37/37	Australian (Caucasian)	Case-control	DSM-IV
Gutierrez-Fernandez, Palomino [46]	215/650	Spanish	Case-control	DSM-IV
Thirunavukkarasu, Vijayakumari [47]	38/37	Indian	Case-control	DSM-IV
Terzic, Kastelic [48]	138/94	Slovenian	Case-control	DSM-IV

Supplementary Table S2: Publication bias analyses.

Polymorphism	Risk	Meta-analysis					Regression Test		Trim-and-Fill				
		k	OR	(95% CI)	*t*	*p*	*t*	*p*	$k_{Imputed}$	OR	(95% CI)	*t*	*p*
rs62510682	G	27	1.00	(0.96, 1.05)	0.17	0.027	1.53	0.140	2	1.06	(0.95, 1.18)	1.13	0.027
478B14-848 (0)	0	10	1.08	(1.01, 1.16)	2.53	0.016	0.55	0.597	2	1.12	(1.04, 1.21)	3.21	.008
rs2954041[a]	T	6	1.13	(1.00, 1.27)	2.68	0.022	-	-	3	1.33	(1.10, 1.62)	3.31	.009

[a] Regression test not run due to k < 10.

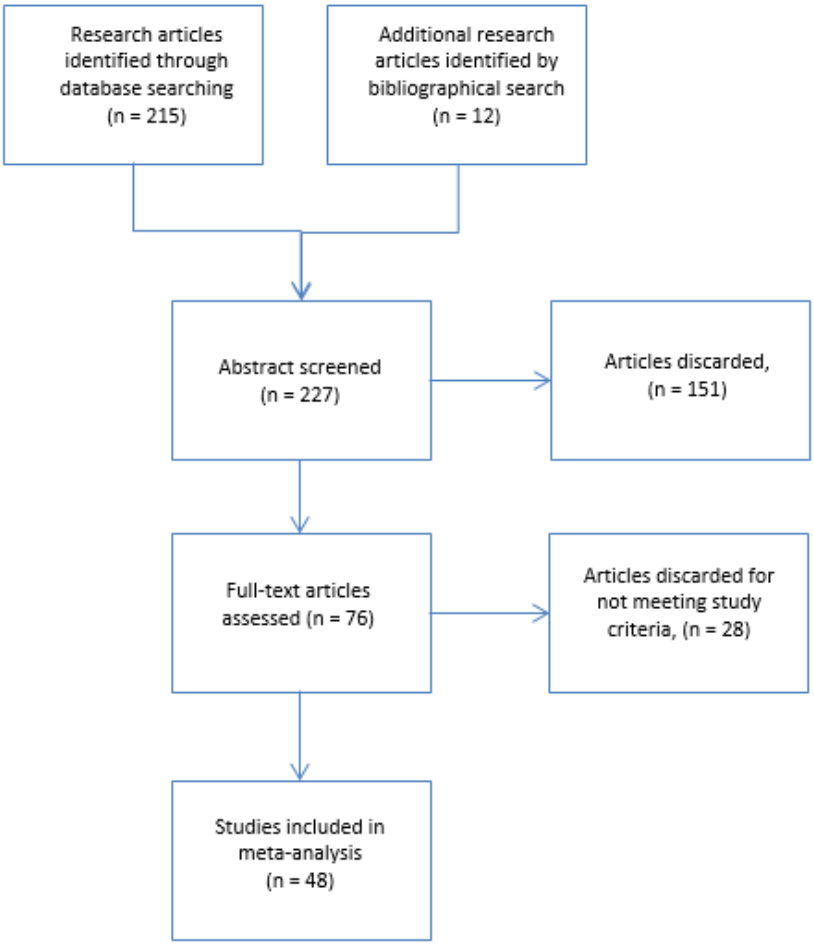
Supplementary Table S3: Removal of potential outlier studies in each marker and subsequent meta-analysis.

Polymorphism	Risk	Meta-analysis					Heterogeneity			Study removed
		k	OR	(95% CI)	*t*	*p*	*Q*	*p*	I^2	
rs73235619	G	12	1.02	(0.91, 1.15)	0.46	0.327	13.4	0.268	17.9	Rosa, Gardner [18]
rs35753505	C	34	1.02	(0.97, 1.09)	0.84	0.202	66.7	<0.001	50.6	Zhang, Li [31]
rs62510682	G	27	1.00	(0.96, 1.05)	0.17	0.027	36.0	0.092	27.8	Rosa, Gardner [18]
rs6994992	T	26	0.99	(0.95, 1.03)	0.47	0.321	25.9	0.411	3.6	Stefansson, Sarginson [2]
rs113317778	G	9	0.98	(0.80, 1.20)	0.19	0.428	15.1	0.058	0.0	Zhang, Li [31]
478B14-848 (0)	0	10	1.08	(1.01, 1.16)	2.53	0.016	7.4	0.593	0.0	Iwata, Suzuki [5]
rs2439272	A	4	0.95	(0.87, 1.03)	2.17	0.059	1.4	0.701	0.0	Petryshen, Middleton [12]
rs4733376	G	3	1.05	(0.96, 1.15)	2.38	0.070	0.2	0.890	0.0	Benzel, Bansal [17]
rs2954041	T	6	1.13	(1.00, 1.27)	2.68	0.022	2.0	0.847	0.0	Yang, Si [3]
rs10503929	T	5	1.14	(1.10, 1.18)	10.11	**<0.001**	0.3	0.987	0.0	Rosa, Gardner et al. (2007)
rs7014762	A	3	1.03	(0.95, 1.12)	1.76	0.110	0.3	0.866	0.0	Georgieva, Dimitrova [20]

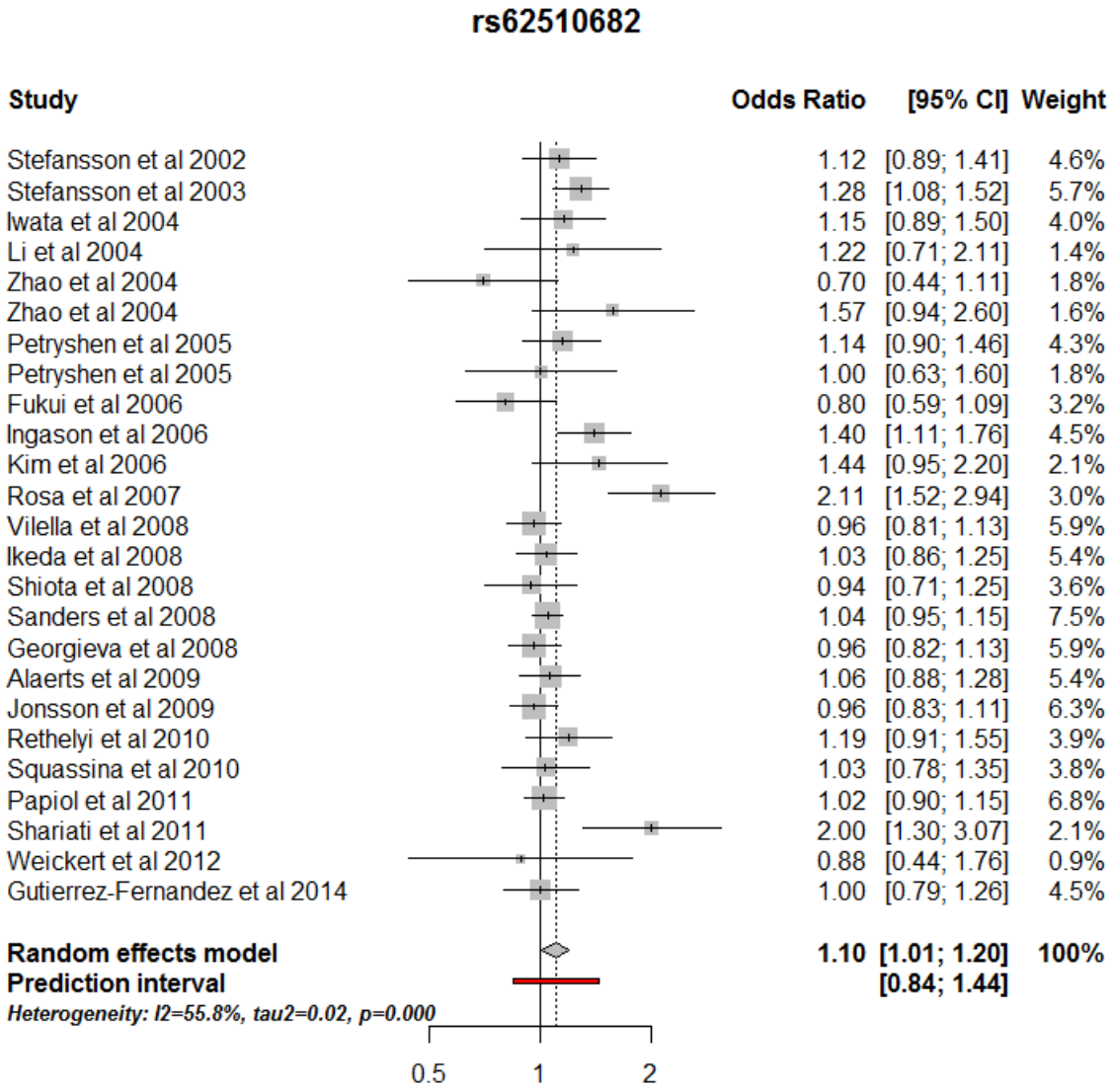
Supplementary Table S4: Moderator analyses of NRG1 markers.

Polymorphism	Risk	Meta-analysis					Heterogeneity			Moderator	
		k	OR	(95% CI)	*t/z*	*p*	*Q*	*p*	I^2	Q_M	*p*
rs73235619	G										
Design										0.3	0.599
Case-control		11	1.03	(0.95, 1.12)	0.74	0.230	9.1	0.522	0.0		
TDT		2	2.21	(0.17, 28.40)	0.61	0.272	52.6	0.000	98.1		
rs35753505	C										
Design										0.7	0.397
Case-control		30	1.02	(0.97, 1.08)	0.92	0.178	50.7	0.008	42.8		
TDT		5	1.18	(0.85, 1.64)	1.00	0.158	33.8	0.000	88.2		
Criteria										0.0	0.939
DSM		28	1.04	(0.98, 1.10)	1.37	0.086	49.2	0.006	45.1		
Other		7	1.03	(0.81, 1.31)	0.23	0.410	36.2	0.000	83.4		
Ancestry										2.4	0.124
Asian		12	1.11	(1.01, 1.23)	2.11	0.018	25.7	0.007	57.3		
European		22	1.01	(0.94, 1.09)	0.32	0.376	49.4	0.000	57.5		
rs62510682	G										
Design										0.9	0.347
Case-control		21	1.07	(1.01, 1.15)	2.18	0.015	34.0	0.026	41.1		
TDT		4	1.32	(0.86, 2.04)	1.27	0.101	19.5	0.000	84.6		
Criteria										0.1	0.720
DSM		22	1.09	(1.01, 1.18)	2.29	0.011	46.8	0.001	55.1		
Other		3	1.15	(0.86, 1.55)	0.96	0.168	7.5	0.023	73.5		
Ancestry										0.3	0.578
Asian		8	1.04	(0.89, 1.22)	0.52	0.301	11.7	0.109	40.4		
European		16	1.09	(1.01, 1.18)	2.31	0.011	34.1	0.003	56.0		
rs6994992	T										
Design										0.2	0.625
Case-control		24	1.00	(0.95, 1.15)	0.05	0.481	35.1	0.051	34.5		
TDT		3	1.01	(0.91, 1.17)	0.54	0.294	0.6	0.724	0.0		
Criteria										2.8	0.096
DSM		22	1.02	(0.97, 1.07)	0.86	0.195	27.0	0.170	22.3		
Other		5	0.93	(0.84, 1.03)	1.44	0.076	5.0	0.285	20.4		
Ancestry										0.1	0.712
Asian		9	1.00	(0.94, 1.07)	0.13	0.447	8.2	0.413	2.6		
European		17	1.02	(0.96, 1.08)	0.71	0.240	23.4	0.103	31.7		
rs113317778	G										
Design										0.6	0.448
Case-control		7	0.98	(0.81, 1.18)	0.25	0.400	14.0	0.030	57.1		
TDT		3	0.59	(0.16, 2.17)	0.80	0.211	49.3	0.000	95.9		
Ancestry										1.1	0.302
Asian		2	0.41	(0.08, 2.15)	-1.06	0.145	15.2	0.000	93.4		
European		8	0.98	(0.82, 1.17)	-0.22	0.414	15.1	0.035	53.5		

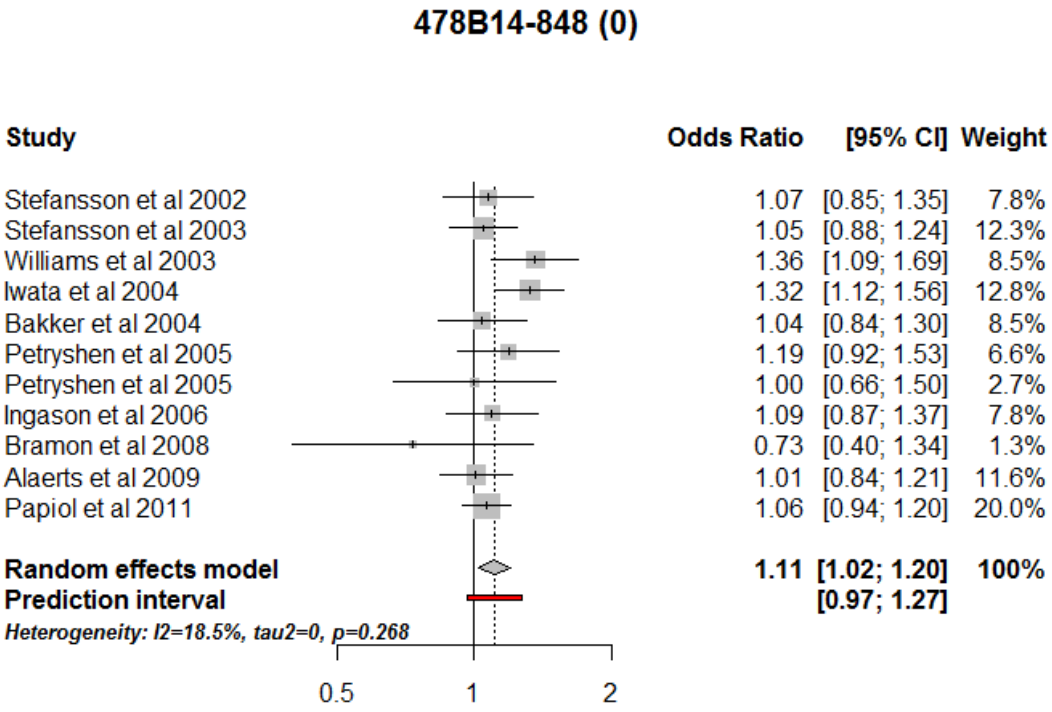
Polymorphism	Risk	Meta-analysis					Heterogeneity			Moderator	
		k	OR	(95% CI)	*t*/*z*	*p*	*Q*	*p*	I^2	Q_M	*p*
(continued)											
rs3924999	A										
Design										0.1	0.753
Case-control		3	1.09	(0.65, 1.83)	0.33	0.370	23.0	0.000	91.3		
TDT		13	1.00	(0.92, 1.09)	0.08	0.469	27.0	0.008	55.6		
Criteria										0.0	0.894
DSM		11	1.02	(0.94, 1.12)	0.53	0.297	17.5	0.064	42.8		
Other		5	1.04	(0.79, 1.38)	0.31	0.379	32.1	0.000	87.5		
Ancestry										0.2	0.632
Asian		8	1.07	(0.86, 1.33)	0.59	0.279	39.9	0.000	82.4		
European		7	1.01	(0.93, 1.09)	0.20	0.421	9.5	0.149	36.6		
420M9-1395 (-2)	(-2)										
Design										1.0	0.318
Case-control		4	1.02	(0.89, 1.16)	0.39	0.360	1.3	0.741	0.0		
TDT		3	1.09	(0.84, 1.43)	1.47	0.140	1.7	0.427	0.0		
Ancestry										0.6	0.457
Asian		4	1.07	(0.95, 1.21)	1.73	0.091	1.7	0.648	0.0		
European		3	1.00	(0.69, 1.44)	-0.02	0.491	1.5	0.462	0.0		
478B14-848 (4)	4										
Design										9.4	0.053
Case-control		3	0.98	(0.78, 1.23)	0.17	0.434	4.4	0.111	54.6		
TDT		2	0.93	(0.55, 1.56)	0.29	0.387	4.9	0.027	79.6		
Ancestry										8.0	**0.005**
Asian		2	1.18	(1.01, 1.38)	2.04	0.021	0.0	0.996	0.0		
European		3	0.83	(0.69, 1.00)	-1.97	0.025	1.4	0.503	0.0		
rs2466058	T										
Ancestry										1.5	0.221
Asian		2	0.95	(0.70, 1.29)	-0.32	0.377	3.1	0.079	67.7		
European		2	1.37	(0.84, 2.24)	1.24	0.107	1.7	0.190	41.9		
rs1081062	C										
Ancestry										1.2	0.266
Asian		2	1.12	(0.86, 1.45)	0.84	0.202	1.6	0.201	38.9		
European		2	0.95	(0.87, 1.05)	-0.94	0.173	0.1	0.744	0.0		
rs2954041	T										
Criteria										10.6	0.102
DSM		3	1.17	(1.01, 1.36)	2.03	0.022	0.3	0.840	0.0		
Other		4	1.24	(0.77, 1.99)	0.87	0.192	8.8	0.032	66.0		
Ancestry										1.8	0.174
Asian		4	1.33	(1.02, 1.73)	2.12	0.017	7.1	0.069	57.8		
European		3	1.03	(0.80, 1.33)	0.23	0.408	0.9	0.634	0.0		



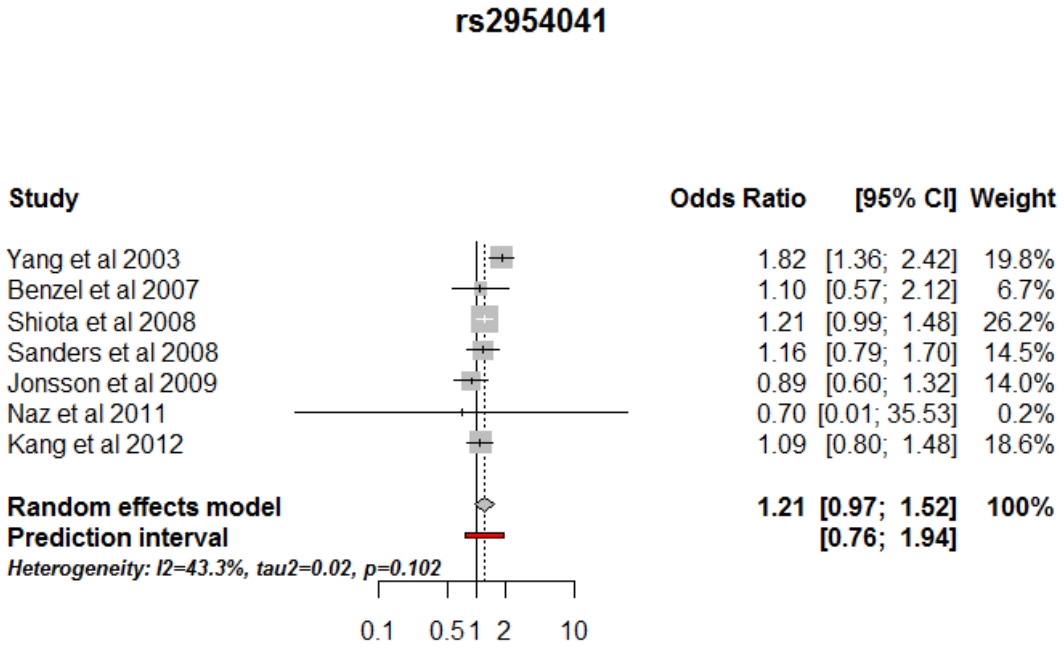
Supplementary Figure S1: Overview of the literature search and article screening procedure



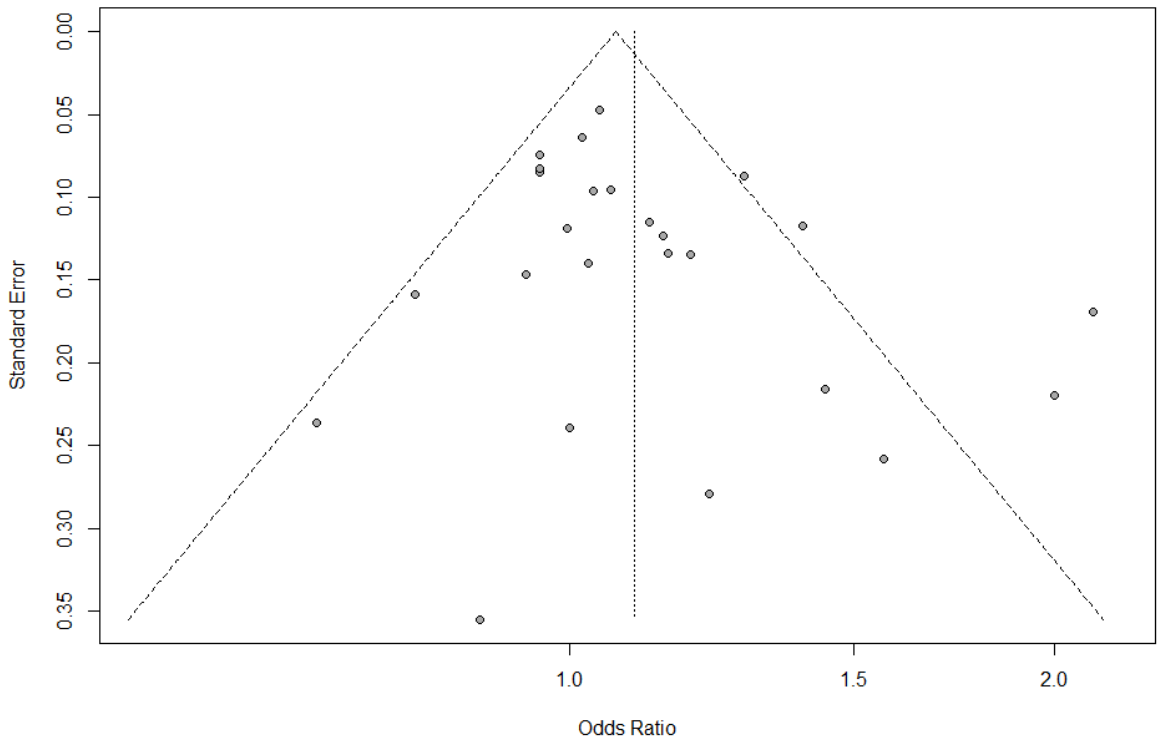
Supplementary Figure S2: Forest plot of rs62510682 meta-analysis.



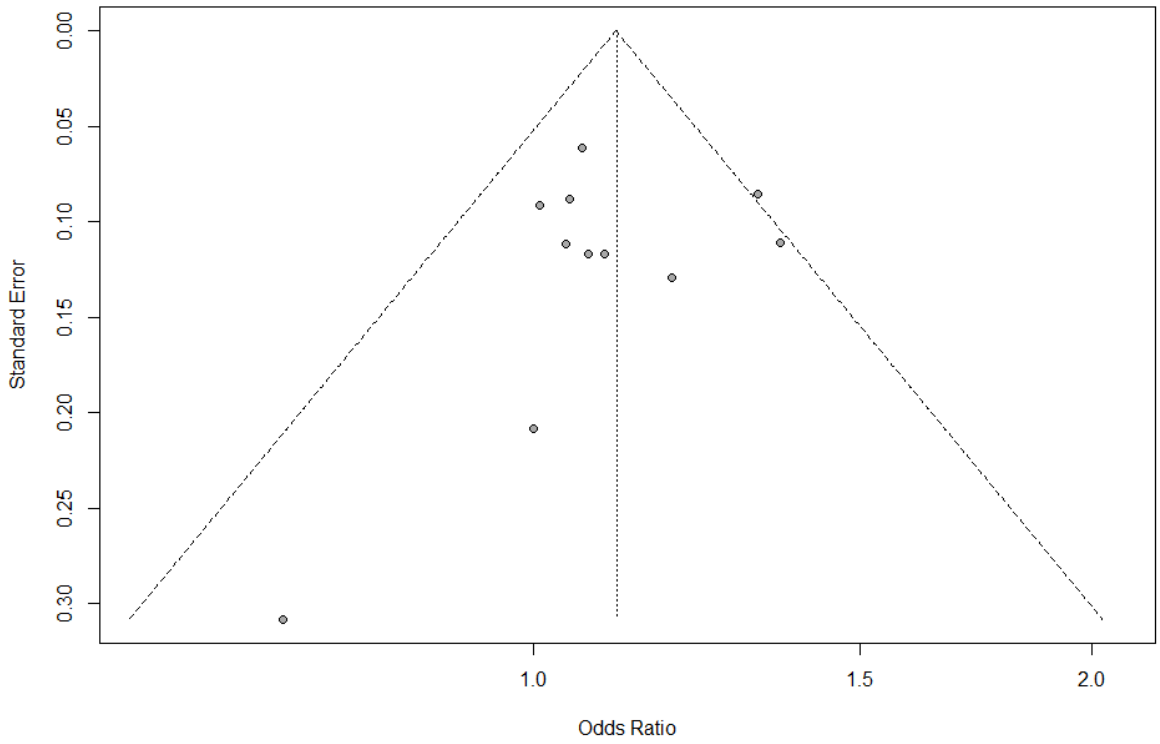
Supplementary Figure S3: Forest plot of microsatellite 478B14-848(0) meta-analysis.



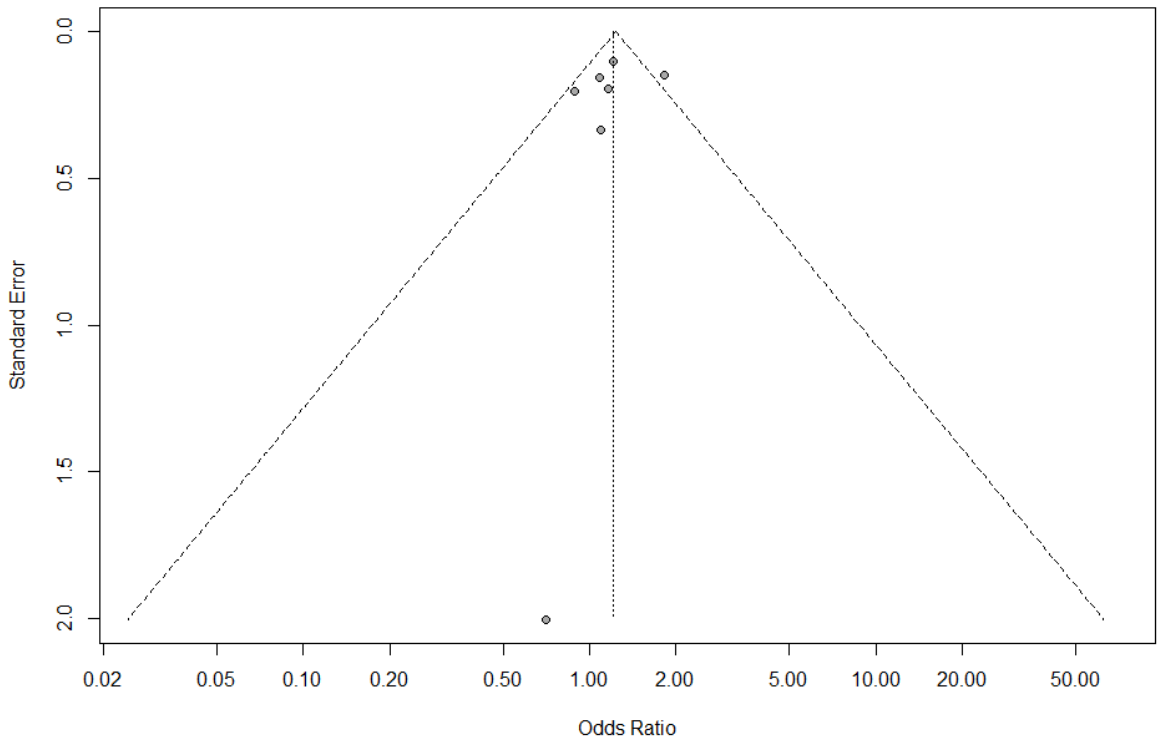
Supplementary Figure S4: Forest plot of rs2954041 meta-analysis



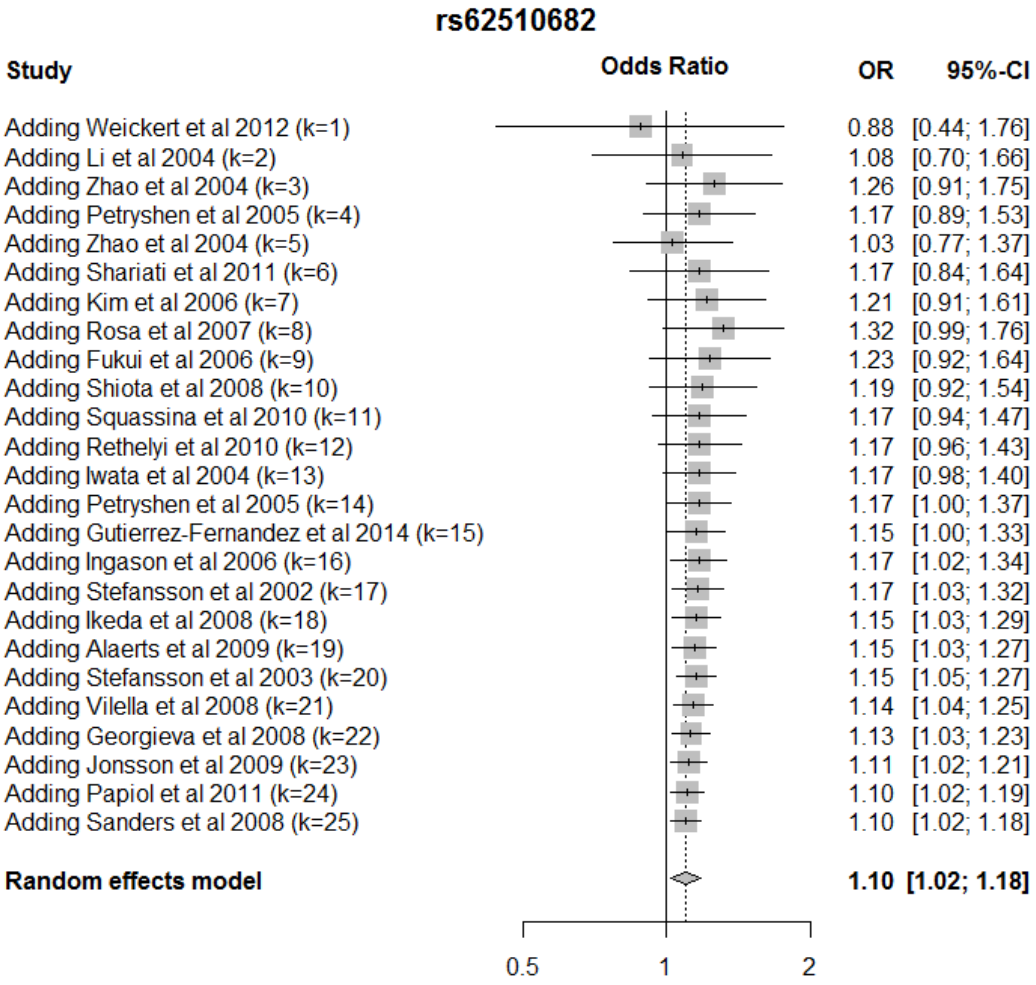
Supplementary Figure S5: Funnel plot of rs62510682 meta-analysis.



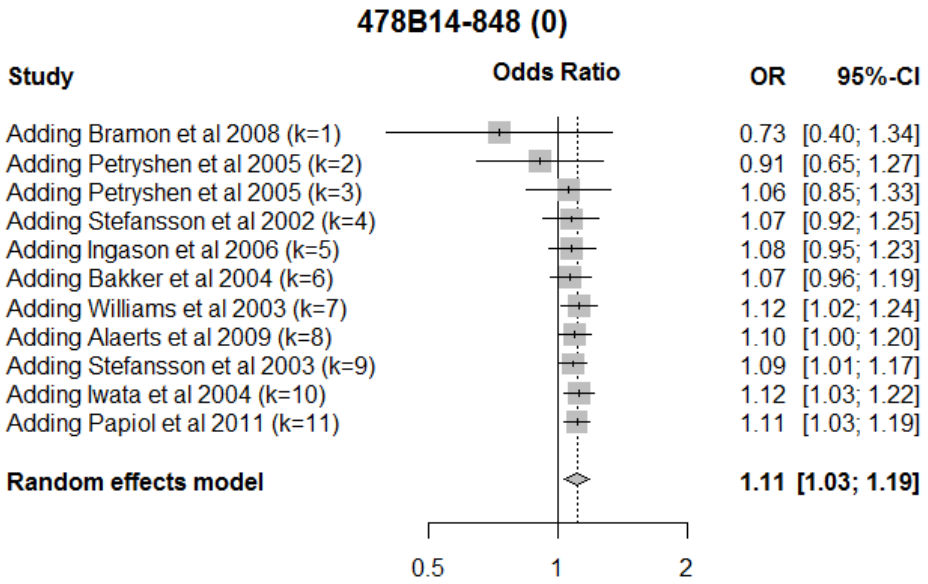
Supplementary Figure S6: Funnel plot of rs478B14-848 (0) meta-analysis.



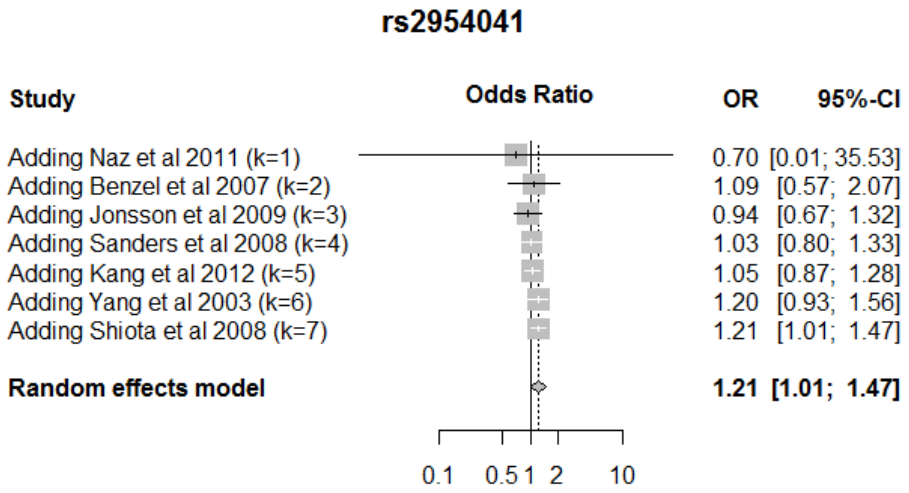
Supplementary Figure S7: Funnel plot of rs2954041 meta-analysis.



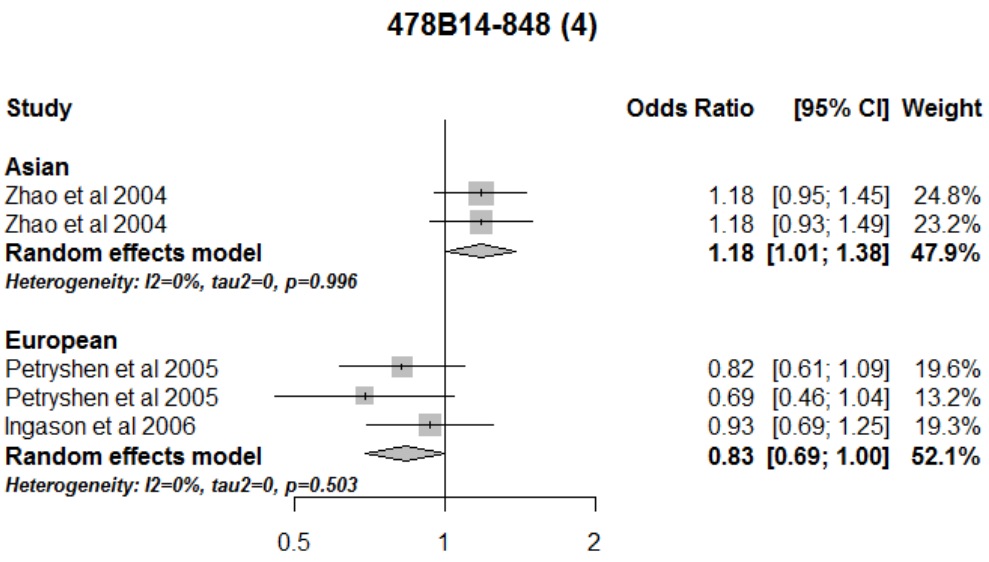
Supplementary Figure S8: Cumulative forest plot of rs62510682 meta-analysis.



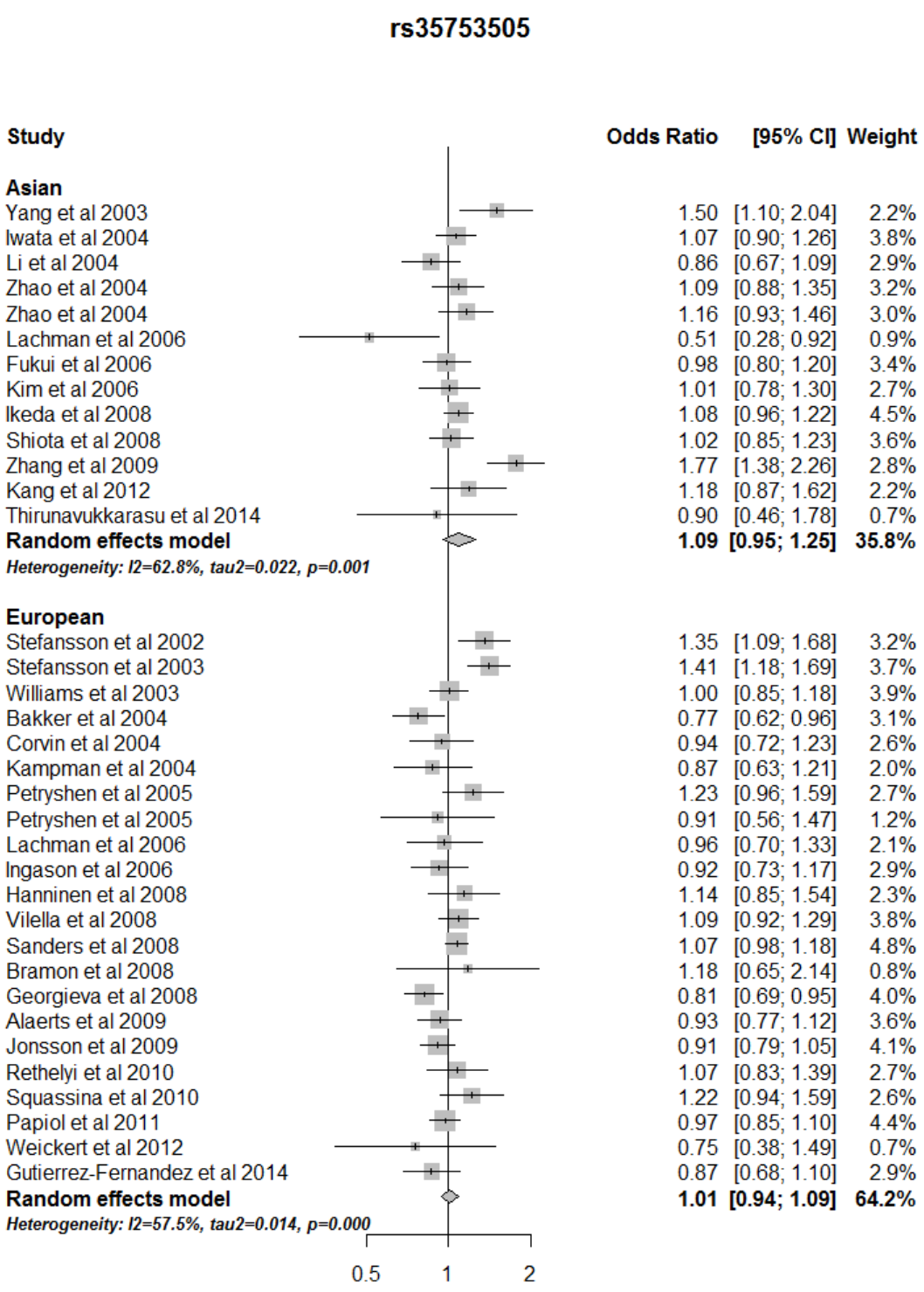
Supplementary Figure S9: Cumulative forest plot of rs478B14-848 (0) meta-analysis.



Supplementary Figure S10: Cumulative forest plot of rs2954041 meta-analysis.



Supplementary Figure S11: Forest plot of microsatellite 478B14-848 (4) by ancestry.



Supplementary Figure S12: Forest plot of rs35753505 by ancestry.

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Chapter 3

Elevated peripheral expression of neuregulin-1 (*NRG1*) mRNA isoforms in clozapine-treated schizophrenia patients

Mostaid et al. Translational Psychiatry (2017)7:1280
DOI 10.1038/s41398-017-0041-2

ARTICLE Open Access

Elevated peripheral expression of neuregulin-1 (*NRG1*) mRNA isoforms in clozapine-treated schizophrenia patients

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Abstract

Differential expression of neuregulin-1 (*NRG1*) mRNA isoforms and proteins has been reported in schizophrenia, primarily in post-mortem brain tissue. In this study, we examined 12 *NRG1* SNPs, eight *NRG1* mRNA isoforms (type I, type $I_{(Ig2)}$, type II, type III, type IV, EGFα, EGFβ, pan-NRG1) in whole blood, and NRG1-β1 protein in serum of clozapine-treated schizophrenia patients ($N = 71$) and healthy controls ($N = 57$). In addition, using cultured peripheral blood mononuclear cells (PBMC) from 15 healthy individuals, we examined the effect of clozapine on *NRG1* mRNA isoform and protein expression. We found elevated levels of *NRG1* mRNA, specifically the EGFα ($P = 0.0175$), EGFβ ($P = 0.002$) and type $I_{(Ig2)}$ ($P = 0.023$) containing transcripts, but lower NRG1-β1 serum protein levels ($P = 0.019$) in schizophrenia patients compared to healthy controls. However, adjusting for smoking status attenuated the difference in NRG1-β1 serum levels ($P = 0.050$). Examination of clinical factors showed *NRG1* EGFα ($P = 0.02$) and EGFβ ($P = 0.02$) isoform expression was negatively correlated with age of onset. However, we found limited evidence that *NRG1* mRNA isoform or protein expression was associated with current chlorpromazine equivalent dose or clozapine plasma levels, the latter corroborated by our PBMC clozapine exposure experiment. Our SNP analysis found no robust expression quantitative trait loci. Our results represent the first comprehensive investigation of *NRG1* isoforms and protein expression in the blood of clozapine-treated schizophrenia patients and suggest levels of some *NRG1* transcripts are upregulated in those with schizophrenia.

Introduction

Neuregulin-1 (*NRG1*) is vital for neurodevelopment and plasticity[1], making it an appealing gene to examine in schizophrenia. This appeal has been weakened by genome-wide association study results that have failed to identify it as a top schizophrenia "risk" gene[2]; questioning the relevance of *NRG1* in schizophrenia[3]. However, the relevance of any gene to schizophrenia should not be determined exclusively on whether sequence variations within the gene meet genome-wide significance but rather on the compendium of knowledge available for that gene. A recent meta-analysis[4] and systematic review[3] have showed a number of *NRG1* genetic variants as well as mRNA and protein levels associated with schizophrenia in specific populations or in certain contexts, which could, in part, be attributed to the complex and highly interactive nature of the NRG-ErbB signaling pathway[1,5]. Nevertheless, the bulk of the evidence to date suggests *NRG1* remains an important target for schizophrenia research.

Post-mortem human brain studies in schizophrenia have shown differential expression of *NRG1* mRNA and protein in various brain regions, most notably in dorsolateral prefrontal cortex and hippocampus[6–9], although other studies of both regions have been negative[10–14]. Similar evidence of differential gene and protein expression in the peripheral tissue of schizophrenia patients is

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Table 1 Demographic data and clinical characteristics of participants

Characteristic	Schizophrenia (*n* = 71)	Controls (*n* = 57)	*P*-value
Age, mean (sd) years	40 (10)	40 (11)	0.702[a]
Gender, *n* (%) males	53 (75)	35 (61)	0.108[b]
RNA integrity number, mean (sd)	8.4 (0.9)	8.7 (0.3)	0.006[a]*
Ancestry, *n* (%) CEU	62 (90)	50 (88)	0.742[b]
Substance use in past three months, *n* (%)			
Tobacco (smoked)	33 (47)	12 (21)	0.003[b]*
Alcohol	59 (83)	55 (97)	0.016[b]*
Cannabis	11 (15)	7 (12)	0.385[b]
Amphetamine	4 (6)	2 (4)	0.439[b]
Cocaine	0 (0)	2 (4)	0.137[b]
Opiates	1 (1)	1 (2)	0.990[b]
Clozapine plasma level, mean (sd) μg/L	432 (234)	—	—
Chlorpromazine equivalent (excluding clozapine) dosage mean (sd) mg/day	142 (286)	—	—
Age of onset, mean (sd) years	22.5 (6)	—	—
Duration of illness, mean (sd) years	17 (8)	—	—
PANSS scores, mean (sd)			
Positive	10 (6)	—	—
Negative	15 (5)	—	—
Disorganized	8 (3)	—	—
Excitement	6 (2)	—	—
Depression	6 (3)	—	—
Total	62 (14)	—	—

CEU Northern and Western European ancestry, *TRS* treatment-resistant schizophrenia, *RIN* RNA integrity number, *PANSS* Positive and Negative Syndrome Scale, *mg* milligram
[a]Independent sample *t*-test
[b]Chi-square(χ^2) test
*$P < 0.05$

also available. *NRG1* mRNA expression, specifically type II β3 and *NRG1* type III isoforms, have also been shown to be increased in peripheral leukocytes in Portuguese schizophrenia patients[15] and pan-*NRG1* was shown to be decreased in Chinese schizophrenia patients compared to healthy controls[16,17]. Furthermore, the only two protein studies of *NRG1* in peripheral samples found decreased plasma *NRG1*-β1[18] and serum Ig-*NRG1* levels[19] in people with schizophrenia relative to healthy controls. Collectively, these studies suggest *NRG1* may be dysregulated in brain and blood at both the mRNA and protein level in schizophrenia and flag peripheral blood as a potential surrogate for brain *NRG1* dysregulation[20]. However, the number of peripheral blood studies is limited and the influence specific clinical subgroups (e.g., treatment-resistant), genetic variation, medication, lifestyle (e.g., smoking), and/or symptom severity may have on *NRG1* mRNA and protein expression is not clear.

The aim of this study was to address these gaps in the current literature by investigating peripheral mRNA and protein levels of *NRG1* in schizophrenia, as peripheral measures have the potential to serve as biomarkers in the clinical setting. We particularly focused on patients being treated with clozapine. Clozapine is the drug of choice for a subgroup of schizophrenia patients who do not respond to other antipsychotics, referred to as treatment-resistant schizophrenia[21]. Herein, we examined in whole blood, several *NRG1* mRNA isoforms, and *NRG1*-β1 protein levels in serum within those with schizophrenia compared to healthy controls. We also explore how these expression levels relate to symptom severity, age of onset, duration of illness, and *NRG1* genetic variation as well as examine clozapine's effect on *NRG1* mRNA and protein expression in peripheral blood mononuclear cells (PBMCs) from healthy control subjects.

Materials and methods

Participants

Clinical samples

Seventy-one individuals with schizophrenia were recruited from inpatient and outpatient clinics located in Melbourne, Australia. Inclusion criteria included: (1) diagnosis of schizophrenia, (2) currently prescribed and taking clozapine, and (3) aged between 18–65 years. Fifty-seven unrelated healthy controls matched for age and sex with similar socio-economic backgrounds were recruited from the general community. Controls with a first-degree family history of psychiatric illness, neurological disease, head injury, seizures, prior or current use of antipsychotic medication, impaired thyroid function and/or substance abuse/dependence were excluded from the study. Participant characteristics are shown in Table 1.

All participants were administered the Mini International Neuropsychiatric Interview (MINI)[22] to confirm the diagnosis of schizophrenia and to rule out current or past psychiatric illness in healthy controls. Clinical symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS)[23] and scored in accordance with the consensus five-factor (i.e., positive, negative, depressed,

excited, disorganized/concrete) PANSS model[24]. Tobacco, alcohol, and illicit drug use in the past 3-months was collected using a substance use questionnaire. Blood was collected after overnight fasting and processed according to standardized blood collection and processing protocol (see Supplementary Methods for more details). Clozapine plasma level was measured and current chlorpromazine equivalent dosage (except clozapine) was calculated in all patients by following standard guidelines[25,26]. The study was approved by the Melbourne Health Human Research Ethics Committee (MHREC ID 2012.069), and all participants provided written informed consent prior to participation.

In vitro clozapine exposure samples

Fresh frozen human PBMCs were obtained from 15 healthy donors (eight males and seven females) of Caucasian ethnicity with a mean age of 35 (sd = 13.5; range 20–54 years) from STEMCELL™ Technologies Inc. (Vancouver, British Columbia, Canada). One-third ($n = 5$) of the PBMC donors were current smokers. All the donors tested negative for HIV-1, HIV-2, Hepatitis B, and Hepatitis C. Sample size calculations showed 15 samples were sufficient to detect a large effect (Cohen's $d = 0.80$) between exposed and unexposed conditions at $\alpha = 0.05$ and power = 0.80.

PBMCs were isolated from peripheral blood and were supplied as vials of 100 million cells. PBMCs were seeded at a concentration of 2 million cells per well (1×10^6 cells/mL) in triplicate in six-well plates and incubated in RPMI-1640 medium (Sigma-Aldrich; St. Louis, Missouri, USA) supplemented with L-glutamine (0.3 g/L) and sodium bicarbonate (2 g/L), penicillin (100units/mL), streptomycin (100 µg/mL), 10% fetal bovine serum for 24 h. PBMCs were then exposed to clozapine (Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration of 1.2 µM (control wells exposed to vehicle only, see Supplementary Methods for details) and incubated at 37 °C in 5% CO_2. Absolute ethanol was used to dissolve clozapine and media was used for dilution. The concentration of clozapine used was determined from the mean plasma concentration of clozapine found in the first 48 recruited clinical samples (1.2 µM or 384 ng/mL). Toxicity assays (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega Corporation, Madison, Wisconsin, USA) were performed at baseline, 24 h and 7-day time points to measure the production of lactate dehydrogenase within the media (see Supplementary Fig. S1 for more details).

SNP selection, DNA extraction, and genotyping

Fourteen *NRG1* single-nucleotide polymorphisms (SNPs) were selected based on their previously reported associations with schizophrenia (for review see refs. [3,4]) along with 60 unlinked ancestry-informative markers (Supplementary Table S1) representing the three HapMap phase III populations (Northern/Western European, Han Chinese, and Yoruba in Nigeria)[27]. DNA extraction and quantification were performed using standard procedures described in detail in the Supplementary Methods. SNPs were genotyped at the Australian Genome Research Facility (Brisbane, Australia) with the Sequenom MassARRAY MALDI-TOF genotyping system using Sequenom iPLEX Gold chemistries according to manufacturer's instructions (Sequenom, Inc., San Diego, CA). Two (rs113317778 and rs6150532) of the 14 *NRG1* SNPs assessed failed (0% call rate) but call rates for all remaining SNPs including the 60 ancestry markers were > 96% (Supplementary Table S1).

RNA extraction and gene expression analysis

RNA extraction and quantification for both clinical and in vitro samples were performed using PureLink RNA Mini Kit (ThermoFisher scientific, Waltham, MA, USA) per the standard manufacturer's instructions. Total RNA from both clinical and in vitro samples was reverse transcribed to cDNA using SuperScript® IV First-Strand Synthesis System (Invitrogen, Foster city, CA, USA) using random hexamers. cDNA (10.25 ng) was used as a template for quantitative reverse transcriptase (RT-qPCR) using master-mix and gene specific validated Taqman assays from Applied Biosystems, Foster City, California, USA. Custom designed primer and probe combinations were used for *NRG1* isoforms (type $I_{(Ig2)}$, type II and type IV) previously investigated[9,14,28], while inventoried assays (TaqMan®, aInvitrogen, USA) were used for all other *NRG1* isoforms (type III, Pan-*NRG1*, type I, EGFα and EGFβ) and four reference genes (beta-actin, *ACTB*; ubiquitin C, *UBC*; glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; and TATA box-binding protein, *TBP*). *NRG1* mRNA isoforms were selected based on reported associations in previous gene expression experiments using post-mortem brain tissue or peripheral blood from schizophrenia patients[9,14,15,29,30]. See Supplementary Table S2 and Fig. S2 for a list and genomic locations of each of the *NRG1* probes and primers.

Gene expression levels were determined in duplicate using FAM-MGB TaqMan® gene expression probes (Invitrogen, Foster city, CA, USA) in 192 × 24 Dynamic Arrays IFC in Fluidigm® BioMark™ HD system (South San Francisco, CA, USA) at the Monash Health Translation Precinct Medical Genomics Facility (Hudson Institute of Medical Research, Clayton, VIC, Australia). In addition, no reverse transcriptase controls and no template controls were included to rule out genomic DNA

contamination and reagent contamination, respectively. Adhering to minimum information for publication of RT-qPCR (MIQE) guidelines[31], normalized relative quantities (NRQ, i.e., $2^{-\Delta Ct}$ where $\Delta C_t = Ct_{(candidate\ gene)} - Ct_{(geometric\ mean\ of\ reference\ genes)}$) of each *NRG1* mRNA isoform was calculated using the geometric mean expression of two reference genes (ACTB and UBC) that did not differ between groups in either the clinical or in vitro cohorts, with the exception of the 24-hour in vitro time point for which no reference gene was stable. GAPDH and TBP were not used as reference genes because their expression differed significantly by group in both the clinical and in vitro cohorts (Supplementary Figs. S3–S5).

Protein quantification

Clinical samples

Human NRG1-β1 ELISA kits (Catalog number: EHNRG1; ThermoFisher Scientific™, Life Technologies®, Waltham, MA, USA) were used to measure NRG1-β1 levels in serum according to the manufacturer's protocol (see Supplementary Methods for details). In brief, 100 ul of serum or standard *NRG1*-β1 (0 pg/mL-20,000 pg/mL) was added to the wells in duplicate. The ELISA kits have a sensitivity of 50 pg/mL. Absorbance was measured on a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, LLC; Sunnyvale, CA, USA) at 450 nm and 550 nm wavelength. The 550 nm values were subtracted from the 450 nm values to correct for optical imperfections in the microplate. A standard curve was generated for each assay by plotting mean absorbance for each standard concentration vs. the corresponding *NRG1*-β1 concentration. The standard curve ($r^2 \geq 0.99$) was generated with a four-parameter logistic curve fit. The concentration of *NRG1*-β1 in the serum samples was obtained by interpolating the absorbance values using the standard curve in GraphPad Prism 6.

In vitro samples

The same *NRG1*-β1 ELISA kit used for the clinical samples was also used for the in vitro samples. Prior to ELISA, protein lysates were prepared from both the 24-hour clozapine exposed and control cells using RIPA buffer (Sigma-Aldrich®, Saint Louis, Missouri, USA). Due to limited baseline quantity of cells, protein lysates at 7 days were not available. The amount of total protein was quantified from each cell lysate using the Thermo-Scientific™ Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA). Absorbance was measured at 562 nm using SpectraMax® M3 microplate reader. A standard curve ($r^2 \geq 0.99$) was generated by plotting the absorbance value at 562 nm for each bovine serum albumin (BSA) standard vs its concentration (μg/mL). The total protein concentration of each unknown sample was determined using the standard curve. Five microgram of total cell lysate samples were mixed with appropriate amount 1x assay diluent B to be used in the ELISA system. One-hundred microliters of total cell lysate samples (0.05 μg/μL) or standard NRG1-β1 (0 pg/mL-20,000 pg/mL) was added to the wells in duplicate. Assay diluent B was used to prepare standards and served as the zero standards (0 pg/mL).

Statistical analysis

Two-tailed tests were used for all statistical analyses. Quantile–quantile (Q–Q) plots and the Shapiro–Wilk test were used to assess normality of variable distributions. Student's *t*-tests were used to test differences for continuous variables between schizophrenia patients and healthy controls, while chi-squared (χ^2) tests were used for categorical variables. The Benjamini and Hochberg (B–H) step-up procedure[32] was used to adjust for multiple comparisons for all analyses. Effect sizes were calculated using the Hedges' *g* method[33].

NRG1 isoform/protein analysis

Prior to analysis, the normalized relative quantity data for all the *NRG1* isoforms and the *NRG1*-β1 data were checked for normality using Q–Q plots (Supplementary Fig. S4) and as required were log10 transformed for subsequent analysis. The log-transformed values were compared among groups using a general linear model (GLM), with the group as a fixed factor and age, gender, RNA integrity number ((RIN) (isoforms only)), and current smoking status as covariates. Despite differences in alcohol use between the schizophrenia and control groups, alcohol was not included as a covariate because it had no effect on *NRG1* isoform or protein expression (see Supplementary Table S3). For protein analysis, we used the generalized linear model, as *NRG1*-β1 levels were not normally distributed (see Supplementary Fig. S6). Outliers were identified using the Grubbs' test for outliers and removed from further analysis.

Within the schizophrenia group, Pearson correlations between *NRG1* isoform/protein levels and symptom severity, age of onset, illness duration, current chlorpromazine equivalent dose, and clozapine plasma levels were assessed. In addition, *NRG1* isoform/protein levels between participants in positive symptom remission and non-remission were assessed using a *t*-test. Positive symptom remission was defined as a score of ≤ 3 on four PANSS items (delusions, hallucinations, grandiosity, and unusual thought content)[24].

SNP and haplotype analysis

NRG1 SNPs were mapped using the GRCh38/hg19 human genome reference assembly. Linkage disequilibrium (LD) between SNPs was examined in Haploview and haplotype blocks determined using the solid

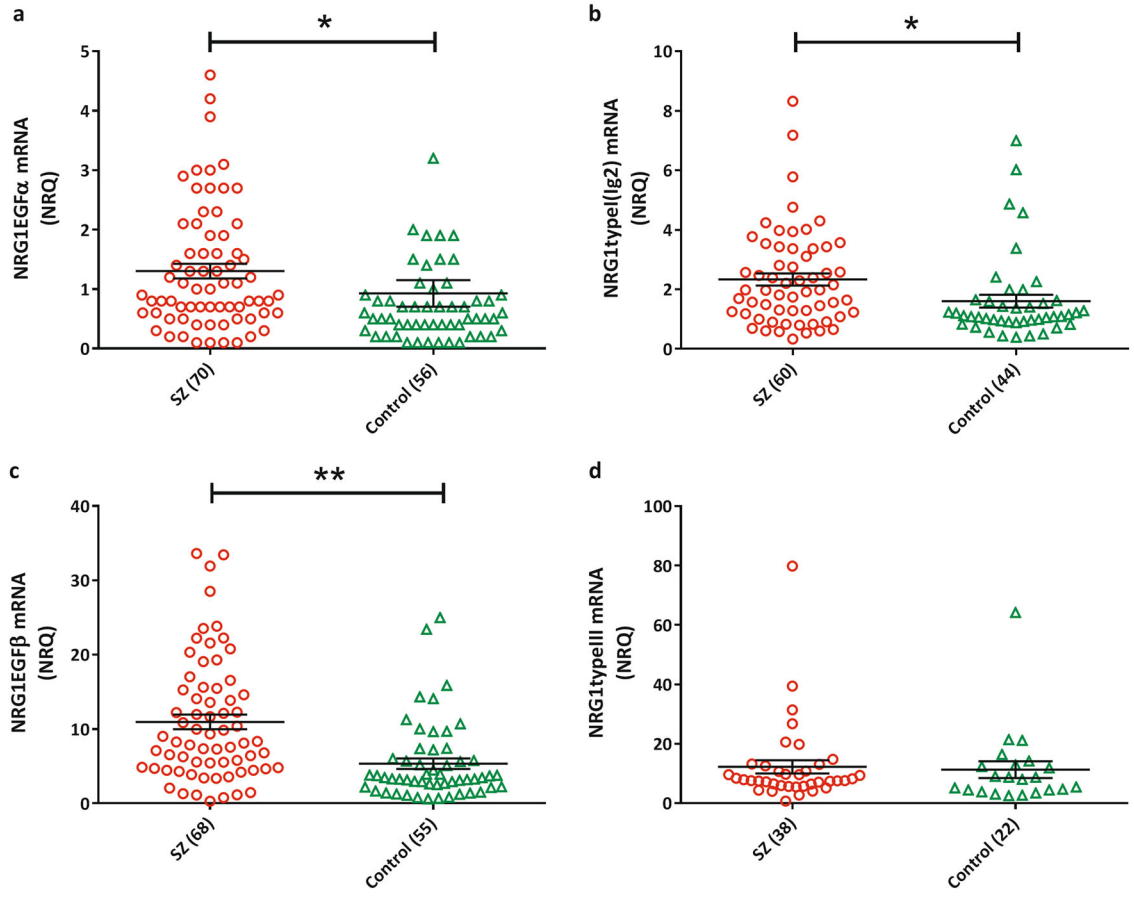

Fig. 1 Normalized relative quantities (NRQ) of NRG1 mRNA isoforms. **a** *NRG1* EGFα (schizophrenia: 1.30 ± 0.12, controls: 0.93 ± 0.22; $F_{1,125} = 7.56$, $P = 0.0175$, Hedges' $g = 0.33$); **b** *NRG1* type $I_{(Ig2)}$ (schizophrenia: 2.23 ± 0.21, controls: 1.61 ± 0.22; $F_{1,103} = 6.261$, $P = 0.023$, $g = 0.70$); **c** *NRG1* EGFβ (schizophrenia: 10.95 ± 0.99, controls: 7.30 ± 2.08; $F_{1,122} = 13.14$, $P = 0.002$, $g = 0.66$); and **d** *NRG1* type III (schizophrenia: 12.22 ± 2.23, controls: 11.3 ± 2.80; $F_{1,59} = 4.23E^{-10}$, $P = 1.0$, $g = 0.02$). Error bars represent mean ± s.e.m. Benjamini-Hochberg adjusted *P*-values are shown. *$P < 0.05$ and **$P < 0.01$

spine method[34]. For each individual, haplotypes were determined based on the best posterior probability procedure implemented in PLINK 1.07[35]. GLMs were used to explore cis-regulatory effects of *NRG1* SNPs and haplotypes on isoforms and protein expression. Each GLM included genotype/haplotype, case status, genotype/haplotype x case status as well as other relevant covariates (age, gender, RIN). Significant genotype/haplotype x case status interactions were analyzed post hoc by case status stratification analyses.

In vitro clozapine exposure analysis

Linear mixed models were used to determine the differences in gene expression over two-time points. In this model, the difference in transcript levels was the outcome variable and was adjusted for age, gender, and RIN. Due to non-normal distributions, Wilcoxon matched pair *t*-test was used to measure the difference in gene expression between clozapine exposed and unexposed cells at each time point.

Results

NRG1 mRNA expression

Among the eight *NRG1* mRNA isoforms interrogated, four of them (type I, type II, pan-*NRG1*, and type IV) were not detectable in more than 60% of the full cohort and so were removed from further analysis. Rates of non-detection were evenly distributed between cases and controls, with the exception of *NRG1* type II, which had a greater non-detect rate in controls ($P = 0.00016$, Supplementary Table S4). Among the remaining four *NRG1* isoforms, levels of EGFα, EGFβ, and type $I_{(Ig2)}$ mRNA were significantly elevated and type III did not differ in schizophrenia patients compared to healthy controls after adjustment for covariates and correction for multiple testing (Fig. 1). Importantly, gene expression levels of *NRG1* isoforms were not correlated with clozapine plasma

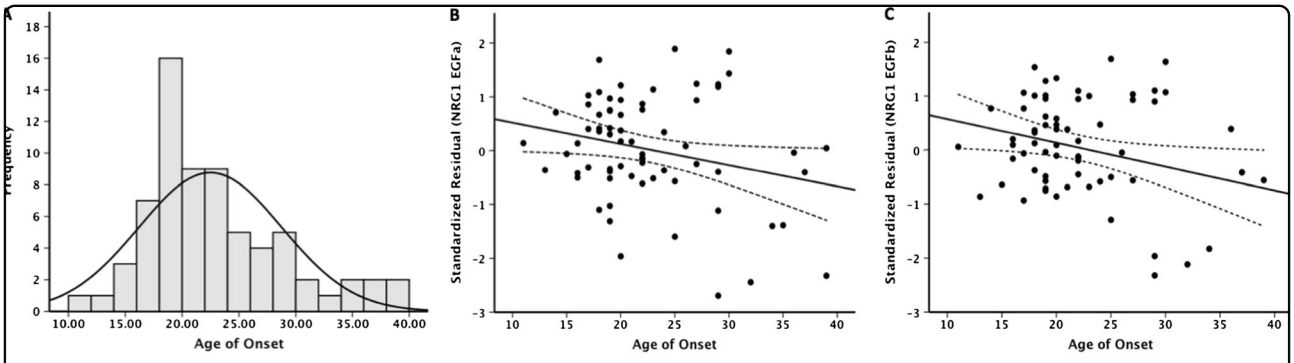

Fig. 2 Distribution of age of onset at first diagnosis **a** and the correlation between age of onset and *NRG1* EGFα ($r = -0.37$, $P_{raw} = 0.002$, $P_{B\text{-}H} = 0.02$) **b** and *NRG1* EGFβ ($r = -0.36$, $P_{raw} = 0.001$, $P_{B\text{-}H} = 0.02$) **c** expression. *NRG1* isoform expression is represented as the standardized residual from a linear regression model after adjusting for the significant effect of age on expression. Dotted lines represented the 95% confidence intervals for the linear regression line (solid black line)

levels or chlorpromazine equivalent antipsychotic exposure (excluding clozapine) (Supplementary Table S5), which was further corroborated by our in vitro analysis that showed no difference in mRNA levels of detectable isoforms (EGFα, EGFβ, and type II) in clozapine exposed compared to unexposed PBMCs (Supplementary Fig. S7). Furthermore, within the patients with schizophrenia significant negative correlations between age of onset and *NRG1* EGFα ($r = -0.37$, $P_{raw} = 0.002$, $P_{B\text{-}H} = 0.02$) and EGFβ ($r = -0.36$, $P_{raw} = 0.001$, $P_{B\text{-}H} = 0.02$) expression were detected (Fig. 2, Supplementary Table S5). No significant correlations were observed between *NRG1* isoforms and duration of illness after adjustment for multiple testing, although a trend-level negative correlation was found between *NRG1* type III expression and duration of illness ($r = -0.36$, $P_{raw} = 0.027$, $P_{B\text{-}H} = 0.167$).

NRG1 protein expression

In contrast to the increase in NRG1 mRNAs, we found that NRG1-β1 protein levels were lower in schizophrenia patients compared to healthy controls ($P = 0.019$) but after adjustment for smoking status, this finding was attenuated ($P = 0.050$; Fig. 3a). Current smokers had lower NRG1-β1 protein levels compared to non-smokers in the full cohort ($P = 0.033$, Fig. 3b) and schizophrenia participants were more likely to be current smokers as compared to controls (46.5% vs. 21.1%, $P = 0.002$, Table 1). However, clozapine plasma levels were not associated with NRG1-β1 expression ($r = -0.023$, $P = 0.85$), and there was no difference in NRG1-β1 protein levels between clozapine exposed and unexposed cells (exposed: median 2.31 log10 pg/mL, unexposed: median 2.2 log10 pg/mL; $P = 0.191$; Supplementary Fig. S8). We found no association between NRG1-β1 protein levels and chlorpromazine equivalent antipsychotic exposure, age of onset, or illness duration (Supplementary Table S5).

NRG1 isoforms/protein expression and symptomatology

Significant negative correlations between *NRG1* mRNA isoform EGFα expression and depression severity score ($r = -0.241$, $P_{raw} = 0.045$, $P_{B\text{-}H} = 0.270$) as well as type III expression and positive symptom severity score ($r = -0.377$, $P_{raw} = 0.020$, $P_{B\text{-}H} = 0.120$) were observed but did not survive correction for multiple comparisons (Supplementary Table S6). An exploratory examination of schizophrenia patients in positive symptom remission vs. non-remission revealed no statistically significant differences in levels of any of the *NRG1* isoforms or NRG1-β1 serum protein after correction for multiple comparisons, although a trend ($P_{raw} = 0.013$, $P_{B\text{-}H} = 0.065$) toward greater *NRG1* type III expression in remitters vs. non-remitters was observed (Supplementary Table S7).

Genotype and haplotype effects on NRG1 isoforms/protein expression

LD analysis revealed two haplotype blocks among the 12 successfully genotyped SNPs (Supplementary Fig. S9). Block 1 contained four of the Icelandic schizophrenia-risk haplotype (Hap_{ICE}) SNPs (rs73235619, rs35753505, rs62510682, rs6994992) along with two other SNPs (rs4281084 & rs7014762) in the 5'-region and Block 2 included four SNPs (rs3924999, rs2439272, rs2954041, rs74942016) in the 3'-region of *NRG1*. Examination of these haplotypes, as well as each SNP, independently revealed several nominal *NRG1* isoforms and protein expression quantitative trait loci but none survived correction for multiple comparisons (Supplementary Table S8).

Discussion

Among the four detectable *NRG1* isoforms in whole blood three (EGFα, EGFβ, and type $I_{(Ig2)}$) were elevated and one (type III) did not differ between clozapine-treated

schizophrenia patients and healthy controls. Importantly, we could not attribute these overall increases in *NRG1* mRNA levels to demographic characteristics and did not find a correlation with clozapine blood levels, suggesting that elevated *NRG1* mRNA in whole blood may not be a direct consequence of age, sex, smoking, or exposure to clozapine; the latter supported by our in vitro experiments. However, age of illness onset was negatively correlated with expression of *NRG1* EGFα and EGFβ containing trancripts, suggesting increased expression of these isoforms are assocated with an earlier age of illness onset.

To our knowledge, we are the first to report elevated levels of *NRG1* EGFα, EGFβ, and type $I_{(Ig2)}$ in schizophrenia, specifically in those with treatment-resistant schizophrenia. Previous peripheral expression studies have not measured these isoforms[15–17,36], although a previous study examining peripheral expression of two other *NRG1* isoforms (ndf43a and ndf43b) covered by the *NRG1* EGFβ probe reported no difference between schizophrenia and control participants[15]. Furthermore, one post-mortem study, which specifically measured the EGF domain containing mRNAs reported no difference in *NRG1* EGFβ levels and was unable to reliably detect *NRG1* EGFα or type $I_{(Ig2)}$ in the dorsolateral prefrontal cortex of schizophrenia and control participants[14]. This suggests our findings, if extended, may contribute to a unique *NRG1* mRNA alteration (signature) in the blood of individuals with schizophrenia or more specifically treatment-resistant schizophrenia. However, in the current study we were unable to compare individuals with and without treatment-resistant schizophrenia and as such the ability of these *NRG1* isoforms to identify treatment-resistant schizophrenia patients remains to be confirmed.

We also found a novel and robust negative correlation between age of onset and expression of EGFα and EGFβ isoform levels, suggesting elevated levels of these isoforms were more frequently detected in those with an earlier age of illness onset. Interestingly, a previous post-mortem brain study[14], showed that brain abundant *NRG1* type III expression was negatively correlated with age of onset. These findings across two different cohorts and from two different cell populations, suggest a relationship between age of onset and *NRG1* gene expression in both brain and blood such that higher gene expression of *NRG1* may accelerate or serve to precipitate transition to full blown symptoms. While, these studies suggest that the distinct *NRG1* isoforms may monitor clinically meaningful events in blood as compared to brain, and suggest the possibility that blood measures of *NRG1* could serve as surrogate markers for *NRG1* in brain. Future longitudinal studies are needed to explore whether elevated *NRG1* gene expression is a precipitating factor and/or a consequence of an earlier age of onset.

Our analyses showed no difference in the expression of *NRG1* type III between schizophrenia patients and controls, which do not concur with a previous study that reported increased expression of *NRG1* type III in peripheral leukocytes of schizophrenia patients[15]. *NRG1* type III is the most abundant of all *NRG1* isoforms in the human brain and increased expression of this isoform was found to be associated with genetic variation in the *NRG1* Hap_{ICE} region[9,14]. However, we were unable to replicate the increase *NRG1* type III in whole blood. We did however, find trend-level negative correlations between *NRG1* type III expression and duration of illness and positive symptom severity as well as elevated expression in remitters, providing preliminary evidence that down-regulation of this isoform in blood may occur with disease progression but this in turn is associated with greater positive symptom severity and lower likelihood of achieving positive symptom remission. To our knowledge no other blood-based study of *NRG1* type III expression has examined these associations and as such it is not clear if they are unique to treatment-resistant schizophrenia or are generalizable to all those with a schizophrenia diagnosis.

We could not detect *NRG1* type IV mRNA in any sample and pan-*NRG1*, type I, and type II mRNAs were not detectable in greater than 60% of our cohort. Our failure to detect *NRG1* type IV and type I are in alignment with a previous study that failed to detect these isoforms in immortalized lymphocytes[36]. However, pan-*NRG1* was shown to be decreased in peripheral lymphocytes[16,17] and type II β3 increased in peripheral leukocytes[15], suggesting detection of these isoforms in the periphery may depend on the cell populations examined. We did observe, however, a significantly lower frequency of type II non-detects among our schizophrenia group compared to controls (55% vs. 86%, $P < 0.01$, Supplementary Table S4), indicating that there may be elevated type II expression in schizophrenia. Still, our low detection of pan-*NRG1* was unexpected given that the probes for this transcript targeted both the Ig1 and Ig2 regions of the *NRG1* gene, which all isoforms we measured contain (see Supplementary Fig. S2 for the regions of amplification for each *NRG1* isofrom). However, for all the isoforms with low detection the probes we used targeted the Ig1 region, suggesting this region of *NRG1* may be downregulated in whole blood and resulted in lower amplification. Although, these probes and primers have been succesfully employed by our group in postmortem human brain, to our knowledge this is the first time they have been used in whole blood. Future studies using whole blood should consider alternative probes for these *NRG1* isoforms.

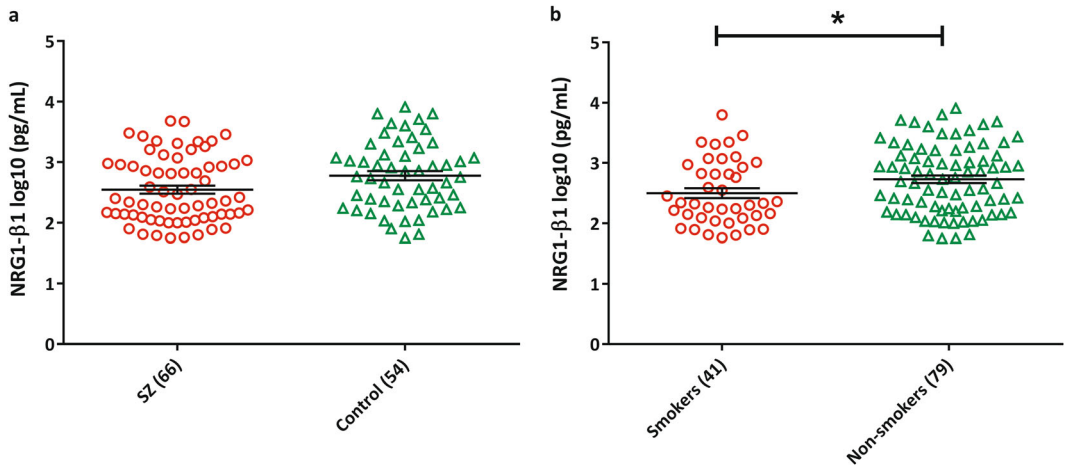

Fig. 3 NRG1-β1 protein expression between **a** schizophrenia and controls (schizophrenia: 2.55 ± 0.067 pg/mL, controls: 2.78 ± 0.077 pg/mL; $P_{unadjusted} = 0.019$, $P_{adjusted\ for\ smoking} = 0.050$, Hedges' $g = 0.42$) and **b** current smokers and non-smokers (smokers: 2.49 ± 0.083 pg/mL, non-smokers: 2.73 ± 0.064 pg/mL; $t = -2.153$, df = 118, $P = 0.033$, $g = 0.43$). Error bars represent mean ± s.e.m. *$P < 0.05$

In contrast to our mRNA findings, NRG1-β1 protein abundance was lower in clozapine-treated schizophrenia patients relative to controls. This finding did not appear to be related to demographic characteristics, genetic variation, or clozapine dose or clozapine blood levels (confirmed by our in vitro clozapine exposure experiment) but was attenuated after adjustment for smoking status. To our knowledge, previous studies measuring peripheral or central NRG1 protein levels have not accounted for smoking status as a potential confound. Our results suggest smokers, regardless of case status, have lower peripheral NRG1-β1 protein levels than non-smokers. Given that smoking prevalence rates are known to be significantly higher among individuals with schizophrenia compared to the general population[37], it is possible that previously reported differences in NRG1 protein levels between schizophrenia and control participants may have also been confounded by smoking. We are aware of two previous studies that have examined peripheral NRG1 protein levels in schizophrenia. The first reported lower Ig-NRG1 levels in serum[38] and the second reported lower NRG1-β1 protein in plasma from schizophrenia patients[18]. However, neither study adjusted for smoking status in their analyses. It is not clear if smoking status would have similar effects on brain NRG1 protein levels reported in post-mortem studies[8,11,39], as none of these studies examined this potential effect and it remains uncertain whether NRG1 protein levels in the brain concur with levels in the periphery. Nevertheless, our findings provide reasonable evidence for inclusion of smoking as a potential confound when measuring and interpreting NRG1 protein levels in groups with known differences in smoking prevalence and support future preclinical experiments assessing the effect of smoking on NRG1 protein levels in blood and brain.

Several limitations should be noted. First, the size of the cohort only allowed for detection of moderate to large differences in mRNA and protein abundances between groups. Second, the study employed a cross-sectional design, inhibiting examination of temporal expression patterns and how these patterns map on to clinical trajectories. Third, measurement of mRNA and protein expression occurred in whole blood and serum, respectively. Although both of these tissues are clinically accessible and commonly used in biomarker research, it is not fully clear how well our findings will generalize to other peripheral (e.g., plasma, lymphocytes) or central (e.g., brain) tissues despite some support for their applicability in schizophrenia[20]. In addition, the generalizability of our findings beyond those with treatment-resistant schizophrenia is not clear. Future studies comparing *NRG1* gene and protein expression between treatment-resistant and non-resistance schizophrenia patients, including treatment-naive patients, are warranted. Finally, our in vitro clozapine exposure experiments examined a single clozapine concentration (1.2 μM) guided by pilot data from our study population. A previous study used higher clozapine concentrations (2 μM) for three weeks in cultured post-mortem human fetal brain tissue and showed an upregulation of *NRG1* protein[40]. As such, future work with PBMCs should examine multiple concentrations that reflect the range of clozapine blood levels observed in the clinic. Future in vitro clozapine experiments with PBMCs should also screen a greater number of genes to identify more suitable

references, particularly genes that are stable during acute clozapine exposure.

Despite these limitations, the current study represents the first comprehensive investigation of *NRG1* isoforms and protein expression in whole blood of clozapine-treated schizophrenia patients. In general, our results support the notion posed by previous peripheral blood and post-mortem brain studies that *NRG1* transcription is dysregulated in schizophrenia and perhaps more specifically treatment-resistant schizophrenia. However, we have also expanded on this by showing *NRG1* mRNA isoforms EGFα, EGFβ, and type $I_{(Ig2)}$ are elevated in whole blood of clozapine-treated schizophrenia patients. Our findings further suggest that *NRG1* expression is associated with age of onset, particularly *NRG1* EGFα and EGFβ isoforms and that *NRG1* type III expression may vary by disease progression. As such our results suggest that *NRG1* overexpression may not be restricted to the brain of those with schizophrenia, and blood-based *NRG1* transcription may serve, in part, as a suitable biomarker for schizophrenia and perhaps treatment-resistant schizophrenia.

Acknowledgements
We acknowledge the financial support of the CRC for Mental Health. The Cooperative Research Center (CRC) program is an Australian Government Initiative. We also wish to acknowledge the CRC Scientific Advisory Committee, in addition to the contributions of study participants, clinicians at recruitment services, staff at the Murdoch Children's Research Institute, staff at the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging, and research staff at the Melbourne Neuropsychiatry Center, including coordinators Phassouliotis, C., Merritt, A., and research assistants, Burnside, A., Cross, H., Gale, S., and Tahtalian, S. Participants for this study were sourced, in part, through the Australian Schizophrenia Research Bank (ASRB), which is supported by the National Health and Medical Research Council of Australia (Enabling Grant N. 386500), the Pratt Foundation, Ramsay Health Care, the Viertel Charitable Foundation and the Schizophrenia Research Institute. We thank the Chief Investigators and ASRB Manager: Carr, V., Schall, U., Scott, R., Jablensky, A., Mowry, B., Michie, P., Catts, S., Henskens, F., Pantelis, C., Loughland, C. We acknowledge the help of Jason Bridge for ASRB database queries. M.M. was supported by a Cooperative Research Center for Mental Health Top-up Scholarship. S.S. was supported by One-in-Five Association Incorporated. C.S.W. is supported by Schizophrenia Research Institute (utilizing infrastructure funding from the NSW Ministry of Health and the Macquarie Group Foundation), the University of New South Wales, and Neuroscience Research Australia. C.S.W. is a recipient of a National Health and Medical Research Council (Australia) Principal Research Fellowship (PRF) (#1117079). C.P. was supported by an NHMRC Senior Principal Research Fellowship (628386 & 1105825), and a Brain and Behavior Research Foundation (NARSAD) Distinguished Investigator Award. C.B. was supported by a NHMRC Career Development Fellowship (1127700) and Brain and Behavior Research Foundation (NARSAD) Young Investigator Award (20526). None of the funding sources played any role in the study design; collection, analysis or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

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Competing interests
The authors declare that they have no competing financial interests.

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Supplementary information
The online version of this article (doi:10.1038/s41398-017-0041-2) contains supplementary material.

Received: 1 May 2017 Revised: 13 July 2017 Accepted: 23 September 2017
Published online: 11 December 2017

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SUPPLEMENTARY MATERIAL

Elevated peripheral expression of neuregulin-1 (NRG1) mRNA isoforms in clozapine-treated schizophrenia patients

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Supplementary methods

Blood sampling method

Blood was collected after overnight fasting and processed according to the Australian Imaging, Biomarker and Lifestyle (AIBL) blood collection and processing protocol. A trained nurse or scientist undertook the blood draw at existing facilities within the local hospital and pathology services. The blood sample was then transported to the AIBL laboratory at the Mental Health Research Institute for further processing. Briefly, blood samples were collected in EDTA tubes (for DNA), PAXgene® Blood RNA tubes (for mRNA) and in SST tube (for the collection of serum). Blood processing was done within 2 hours of venipuncture under sterile conditions. The gel serum tubes were allowed to clot before processing (at least 20 minutes following venipuncture). After the tubes were filled with blood, they were inverted 8-10 times for mixing. They were kept at ambient temperature until processed. The PAXgene tubes were allowed to stand upright at room temperature for 24 hours in the dark and then overnight at -20°C. The EDTA and SST tubes were centrifuged for 15 minutes at 1500 x g at 20°C. The serum samples were collected in Nunc™ Coded Cryobank Vial Systems (ThermoFisher Scientific™, Waltham, MA, USA). All the tubes were stored at -80°C until further processing.

Cell culture method

The clozapine used in this experiment was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The concentration of clozapine used in this experiment was determined from the mean plasma concentration of clozapine found in the cohort of treatment-resistant schizophrenia patients (1.2µM or 384ng/mL). Clozapine induces PBMC death at higher concentrations ($5x10^{-6}$ to $2.5x10^{-5}$ M) dosage [1]. In spite of this LDH toxicity assay was performed at baseline, 24 hours and 7 day time periods.

PBMCs were incubated in RPMI-1640 medium (Sigma-Aldrich; St. Louis, Missouri, USA) supplemented with L-glutamine (0.3g/L) and sodium bicarbonate (2g/L), penicillin (100units/mL), streptomycin

(100µg/mL), 10% fetal bovine serum and 1.2µM of clozapine at 37°C in 5% CO_2. They were seeded at a concentration of 2 million cells per well ($1x10^6$ cells/mL) in triplicate in six-well plates and treated with 1.2µM of clozapine for 24 hours and 7 days. Absolute ethanol was used to prepare 10mM clozapine solution and it was diluted with media to prepare 0.48mM clozapine solution. From this 5µL was added to each well so that each well is exposed with 1.2µM of clozapine. Each control well was exposed with media only. Cells were exposed to clozapine for 24 hours and 7 days. Total RNA was extracted at both time points but protein lysates were extracted at 24-hour time point only. A separate six-well plate with 2 million cells per well ($1x10^6$ cells/mL) was set up for DNA extraction.

DNA extraction and quantification

Clinical samples. DNA was recovered from approx. 2 ml EDTA-anti-coagulated blood samples using the "NucleoSpin® Blood L" silica matrix binding system (Macherey-Nagel GmbH & Co. KG, 52355 Düren, Germany) system according to manufacturer's instructions. DNA eluates were quantified by fluorimetry (QuantiFluor™ dsDNA System; Promega Corporation, Madison, Wisconsin, USA) in conjunction with a Gemini™ Spectramax3 microplate reader (Molecular Devices, LLC; Sunnyvale, CA, USA). DNA stocks were adjusted to a working concentration of between 10 and 50 ng ul-1 for subsequent genotyping. Concentration and purity of DNA were checked by NanoDrop 2000 UV-Vis Spectrophotometer (Thermoscientific®, MA, USA).

In-vitro samples. Genomic DNA was extracted from the cultured cells of each individual by AccuPrep® Genomic DNA Extraction Kit from Bioneer Corporation® (Daejeon, Republic of Korea) using the standard protocol for cultured cells. Concentration and purity of DNA were checked by NanoDrop 2000 UV-Vis Spectrophotometer (Thermoscientific®, MA, USA).

RNA extraction from clinical cohort and quality control

For RNA extraction blood was collected in PAXgene® Blood RNA tubes (Qiagen, Hilden, Germany). Intracellular RNA from whole blood was extracted using PAXgene® Blood RNA kit using "The Manual PAXgene Blood RNA procedure" (www.qiagen.com/at/resources/resourcedetail?id=6e32307d-7e54-4767-8f25-4d9e6b9e0d15&lang=en). The quality of extracted RNA was checked for RIN and concentration using the Agilent® RNA 6000 Nano kit on the Agilent® 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). The lowest RIN was found to be 6.6 for one sample but for rest of samples, it was above 7 (range 7.7 – 9.3).

RNA extraction from cultured PBMCs and quality control

Total RNA was extracted from 24 hours and 7 day time periods from both clozapine exposed and control cells using PureLink™ RNA Mini Kit (Life technologies®, ThermoFisher Scientific™, Waltham, MA, USA) using a standard protocol. Briefly, cells were lysed with using lysis buffer containing 1% 2-mercaptoethanol. After the pellets were dispersed and cells appear lysed they were homogenized by passing the lysate 5-10 times through an 18-gauzge syringe needle. Next, RNA purification was performed using wash buffer I and II and RNA was eluted in 30µL of RNase-free water. All the RNA tubes were stored at -80°C until further use. The quality of extracted RNA was checked for RIN and concentration using the Agilent® RNA ScreenTape assay with the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA).

Reverse Transcription of mRNA

After extraction, RNA was reverse transcribed to cDNA. Then, cDNA was used as a template for RT-qPCR using master-mix and gene specific validated Taqman assays from Applied Biosystems, Foster City, California, USA. For cDNA synthesis, total RNA (200 ng) was denatured for 5 min at 65°C, and then

reverse transcribed using the SuperScript® IV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) in a 20µL reaction volume containing 1× Reverse Transcription SSIV buffer, 10mM dNTP mixture, 50µM random hexamers, 100mM DTT, 40 U/µl RNase OUT™ Recombinant RNase Inhibitor and 200 U/µl SuperScript® IV Reverse Transcriptase. The reaction (40 cycles) will be incubated at 23°C for 10 min, followed by 55°C for 10 min, 80°C for 10 min. To remove RNA, 1 µl of *E.coli* RNase H was added to each reaction mixture and incubated at 37°C for 20min and finally held at 4°C. All cDNA samples were stored at -20°C until qPCR analysis. In each plate, one no template control and one RT negative were used for quality control purposes.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Gene expression was performed using FAM-MGB TaqMan® gene expression probes (Invitrogen, Foster city, CA, USA) in 192.24 Dynamic Arrays IFC in Fluidigm® BioMark™ HD system (South San Francisco, CA, USA) at the MHTP Medical Genomics Facility (Monash Health Translation Precinct, Hudson Institute of Medical Research, Clayton, VIC, Australia). All the samples were run in duplicates in two independent 192.24 BioMark IFC arrays to ensure there was no technical variability. Prior to gene expression, quality control was performed. All the samples qualified except one and that was excluded from the further experiment. The RT negatives showed a very high Ct value indication absence or low genomic DNA contamination and the 'no template' control did not show any amplification.

The FAM-MGB, TaqMan gene expression assays are provided as 20x forward and reverse primer and probe mixes. Each primer is at a concentration of 18µM and the probe is at a concentration of 4µM. The TaqMan assays were selected from the Single Cell Genomics Taqman Library at the Single Cell Genomics Centre (MHTP Medical Genomics Facility, Monash Health Translation Precinct, Clayton, VIC, Australia). Custom designed primer and probe combinations were used for specific NRG1 isoforms previously investigated [2-4], while inventoried assays (TaqMan®, Invitrogen, Foster city, CA, USA) were

used for all other NRG1 isoforms and housekeeping genes. Supplementary table (2) contains the list of the probes and primers.

Pre-amplification was done to increase the number of copies of each gene to detectable levels as detailed in Gene Expression Preamp with Fluidigm® Preamp Master Mix and TaqMan® Assays Quick Reference PN 100-5876B1. To reduce bias, the pre-amplification procedure takes probes and primers of all genes of interest and makes a probe-primer pool and then all the samples get the equal amount of the mixture for amplification. Taqman assays were firstly pooled by combining 4µL of each of the 24 20X TaqMan assays and 304µL C1 DNA suspension buffer for a final volume of 400µL. The final concentration of each assay was 0.2X (180nM).

Pre-amplification allows multiplex amplification. 3.75µL of Sample Pre-Mix (Life Technologies TaqMan® PreAmp Master Mix and Pooled Taqman assays) was combined with 1.25µL of each of the cDNA samples, RT-negative samples, and 'no template' water controls for a final reaction volume of 5µL per sample. An additional no template control (by the gene expression facility) was also included and all samples were pre-amplified for 14 cycles. Following pre-amplification, reaction products were diluted 1:5 by adding 20µL C1 DNA suspension buffer to the final 5µL reaction volume for a total volume of 25µL.

Assays and Samples were combined in a 192.24 Dynamic array IFC according to Fluidigm® 192.24 Real-Time PCR Workflow Quick Reference PN 100-6170. Briefly, 3µL of each assay at a final concentration of 10X was added to each assay inlet port and 3µL of diluted sample to each sample inlet port according to the Chip Pipetting Map. For unused sample inlets, 2.2uL of sample premix and 1.8uL of water per inlet were used. The data were analyzed with Fluidigm Real-Time PCR analysis software (V4.1.1).

Normalized relative quantities of different NRG1 isoforms (mRNA) were calculated relative to the geometric mean of two reference genes, beta actin (ACTB) and ubiquitin-c (UBC). The NRG1

isoforms and reference genes were selected based on a previously reported gene expression experiment conducted on post-mortem brain tissue in schizophrenia patients and controls [5] The relative quantities of ACTB and UBC were not significantly different between the groups in both clinical cohort and in-vitro experiment. Two of the four reference genes (GAPDH and TBP) were found to significantly different in the clinical cohort and therefore was not used as reference genes for normalization.

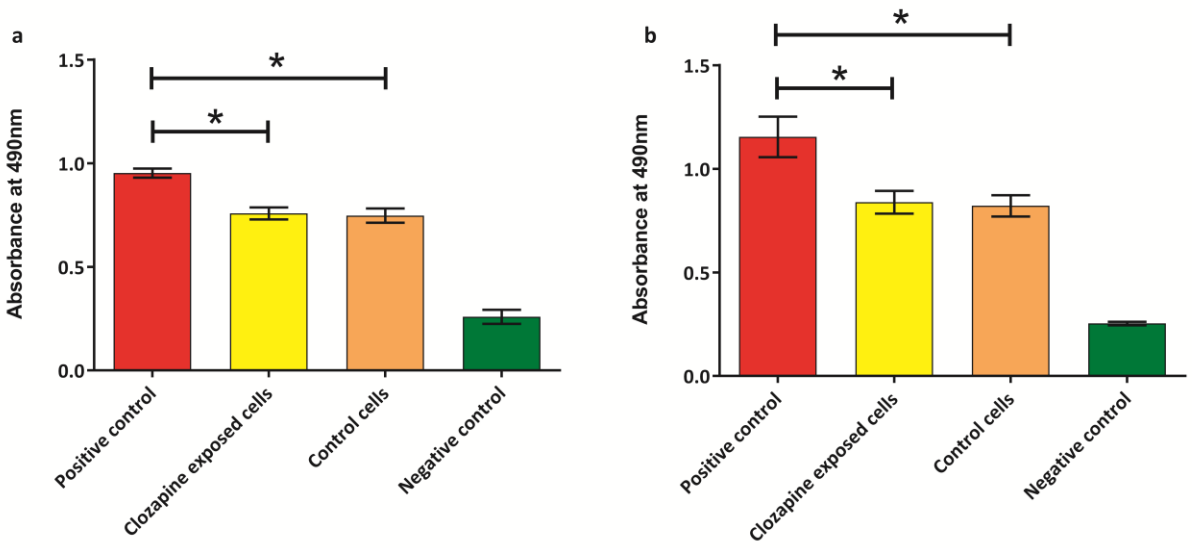
Quantitation of total protein

The amount of total protein was quantified from each cell lysate using the ThermoScientific™ Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA). A set of diluted protein (BSA) standards (0-1000µg/mL) were prepared using the supplied albumin standard ampule (2mg/mL). The total cell lysate samples were prepared in 1:20 dilution with Milli-Q® ultrapure water (Merck Millipore™, Massachusetts, USA). BCA working reagent was prepared by diluting copper (II) sulfate solution in bicinchoninic acid (BCA) solution (1:50). 25µL standards and diluted cell lysate protein samples were mixed with 200µL of BCA working reagent (sample to working reagent ratio=1:8) in a clear flat bottom 96-well plate in triplicates. The plate was incubated at 37°C for 30 mins. Absorbance was measured at 562nm using SpectraMax® M3 microplate reader (Molecular Devices, LLC; Sunnyvale, CA, USA). A standard curve was generated by plotting the absorbance value at 562nm for each BSA standard vs its concentration (µg/mL). The total protein concentration of each unknown sample was determined using the standard curve.

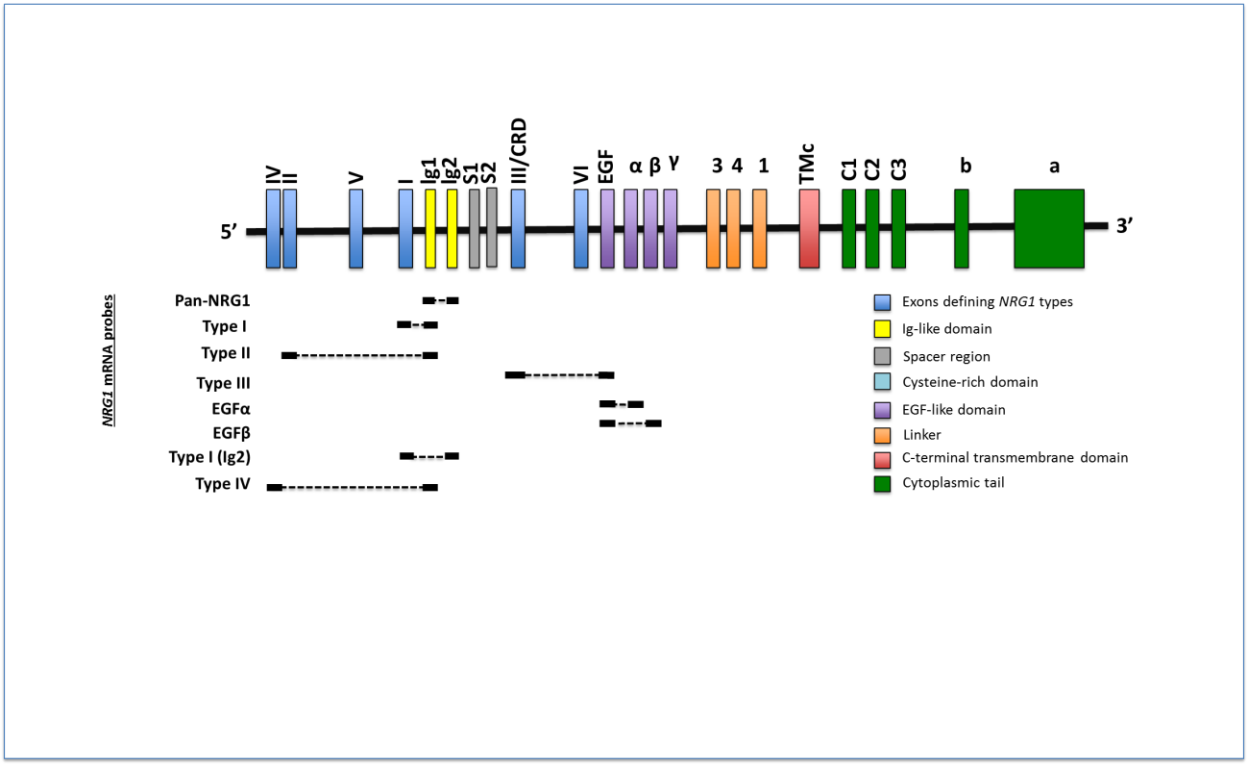
ELISA for clinical cohort

Human NRG1-β1 ELISA kits (Catalog number: EHNRG1; ThermoFisher Scientific™, Life Technologies®, Waltham, MA, USA) were used to measure NRG1-β1 levels in serum according to manufacturer protocol. The kit comes with an ELISA plate pre-coated with the anti-human NRG1β1

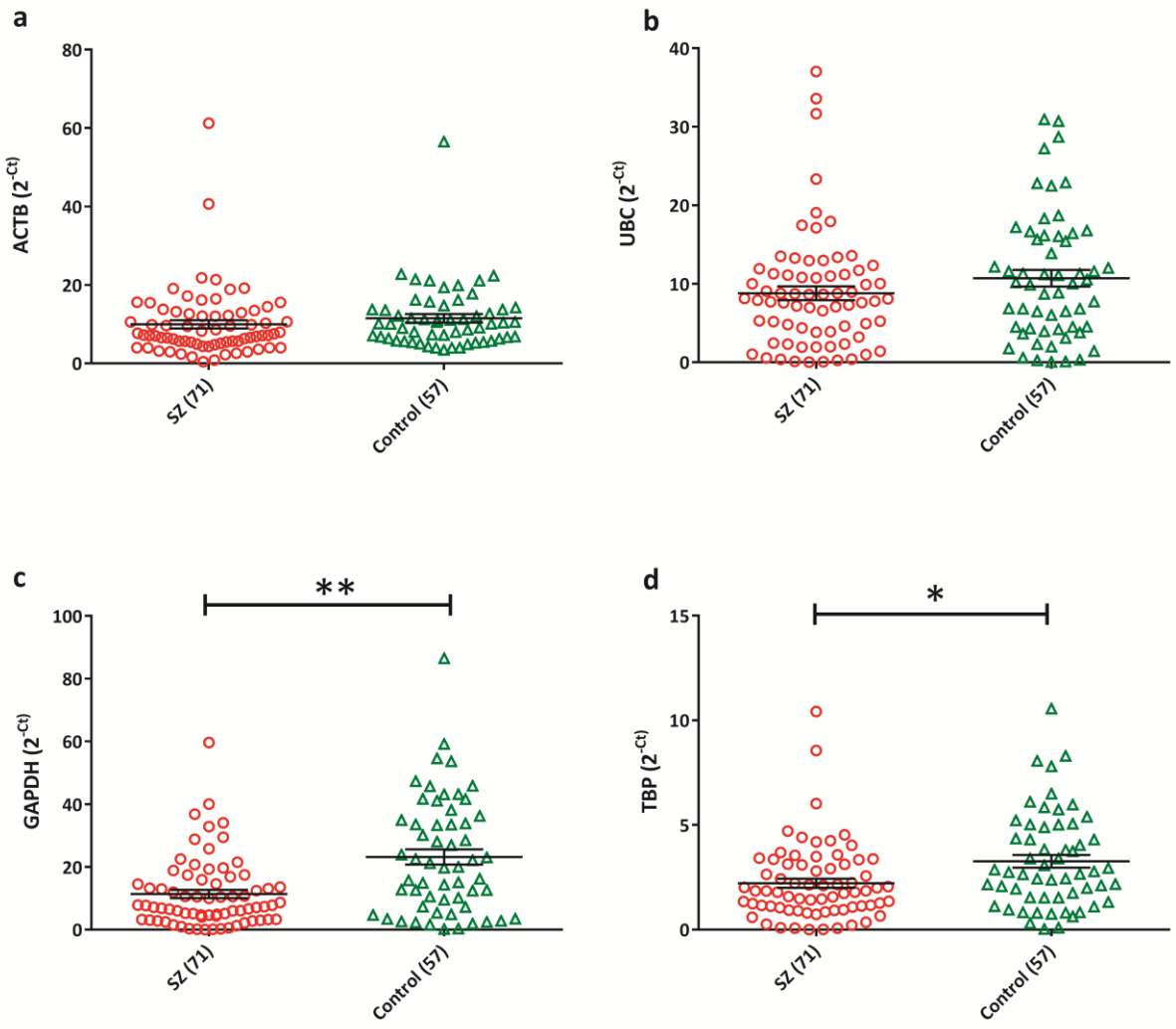
antibody (NRG1 antibody 147705, Invitrogen®, ThermoFisher Scientific™, Waltham, MA, USA) and other components to perform the assay. The serum samples along with the reagents were thawed at room temperature before use in the assay. In brief, 100ul of serum and standard NRG1-β1 (0 pg/ml-20,000 pg/ml) was added to the wells in duplicate. Assay diluent A was used to prepare standards and it serves as the zero standards (0 pg/ml). The plate was incubated overnight at 4°C with gentle shaking. After four washing, 100ul of 1X prepared Biotinylated Antibody reagent was added to the wells and incubated for 1 hour at room temperature. Next, Streptavidin-HRP reagents (100ul) were added to each well followed by incubation for 45 minutes at room temperature. The plate was again washed four times and 100ul of TMB substrate solutions was loaded to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. The reactions were stopped by adding 50ul of Stop solution to each well. Absorbance was measured on a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, LLC; Sunnyvale, CA, USA) at 450nm and 550nm wavelength. The 550nm values were subtracted from the 450nm values to correct for optical imperfections in the microplate. A standard curve was generated for each assay by plotting mean absorbance for each standard concentration versus the corresponding NRG1-β1 concentration. The standard curve was generated with a four-parameter logistic curve fit. The concentration of NRG1-β1 in the serum samples was obtained by interpolating the absorbance values using the standard curve in GraphPad Prism 6. According to the manufacturer, this ELISA kit has <10% of Intra-assay and <12% of Inter-assay coefficients of variation and has no cross-reactivity with BDNF, BLC, ENA-78, FGF- 4, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN-gamma, Leptin (OB), MCP-1, MCP-2, MCP-3, MDC, MIP-1 alpha, MIP-1 beta, MIP-1 delta, PARC, PDGF, RANTES, SCF, TARC, TGF-beta, TIMP-1, TIMP-2, TNF-alpha, TNF-beta, TPO, VEGF.



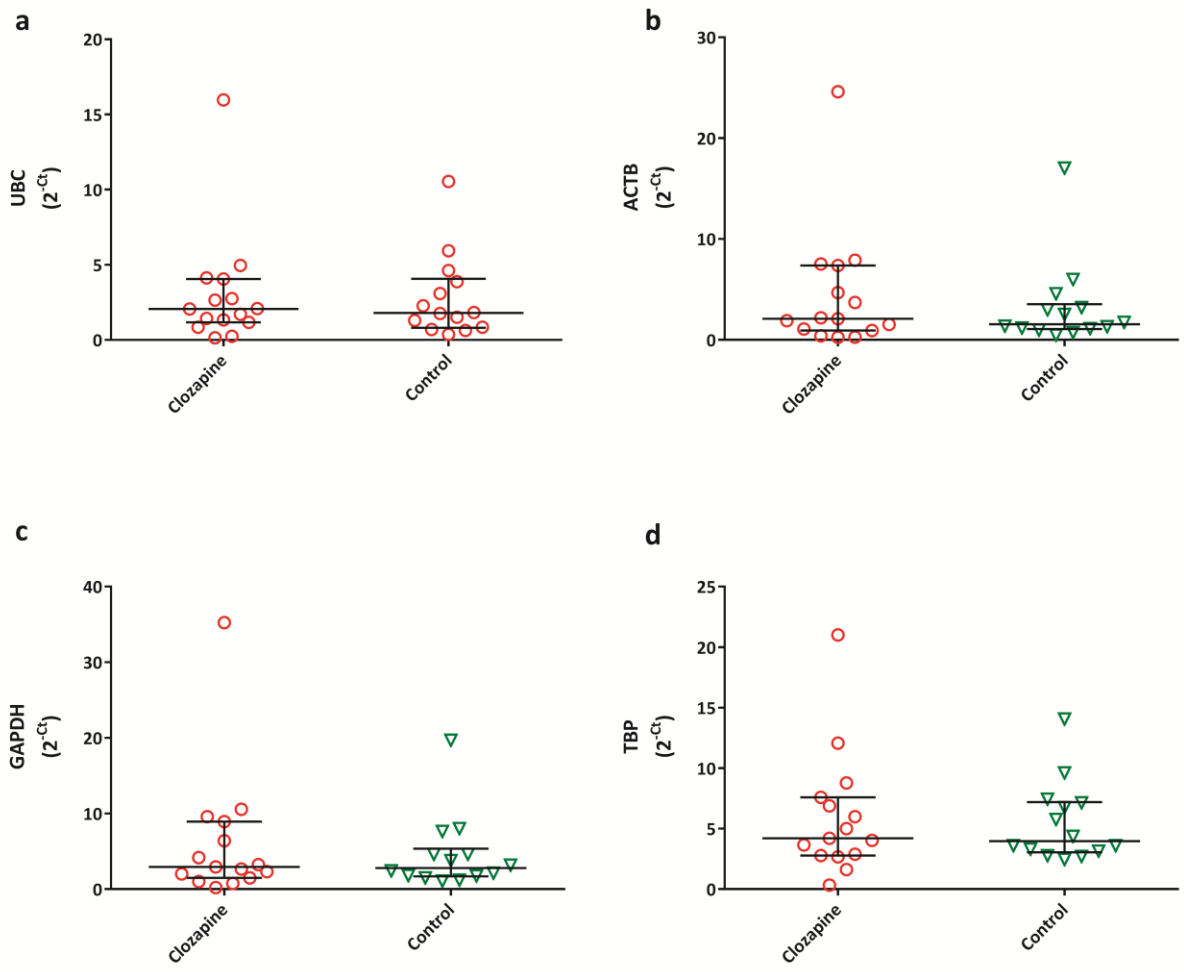
Supplementary Figure S1: LDH toxicity assay results in cultured PBMCs, (a) 24 hour post exposure and (b) 7-days post exposure. *P=0.001



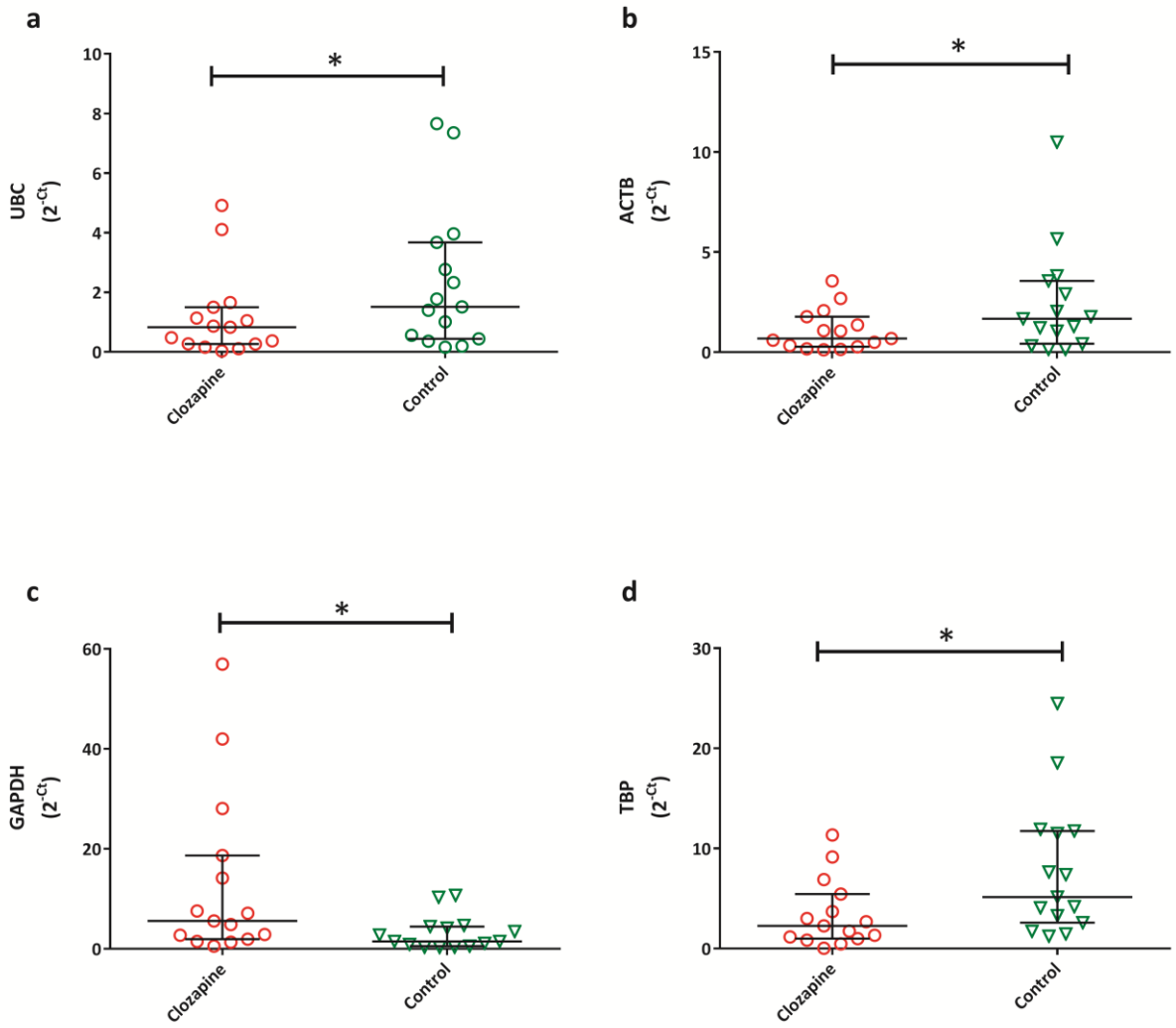
Supplementary Figure S2: *NRG1* mRNA probes and their region of amplification on the *NRG1* gene.



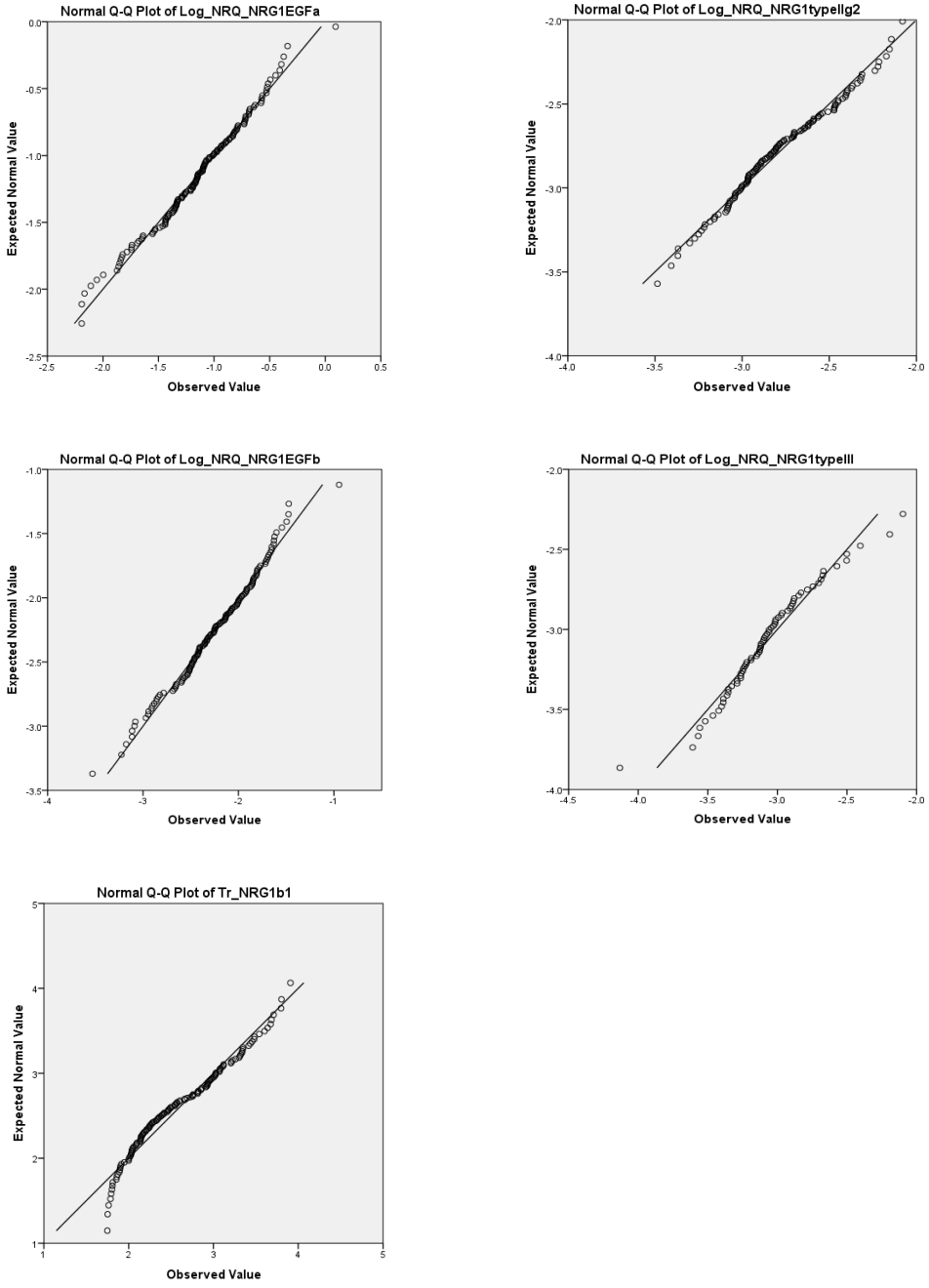
Supplementary Figure S3: Expression of reference genes in schizophrenia patients vs healthy controls; (**a**) ACTB (t=-1.027, df=126, P=0.306), (**b**) UBC (t=0.170, df=126, P=0.163), (**c**) GAPDH (t=-4.259, df=87.383, P=0.000052), (**d**) TBP (t=-2.810, df=105.563, P=0.006). Error bars represent mean ± s.e.m. *P<0.05, **P<0.001.



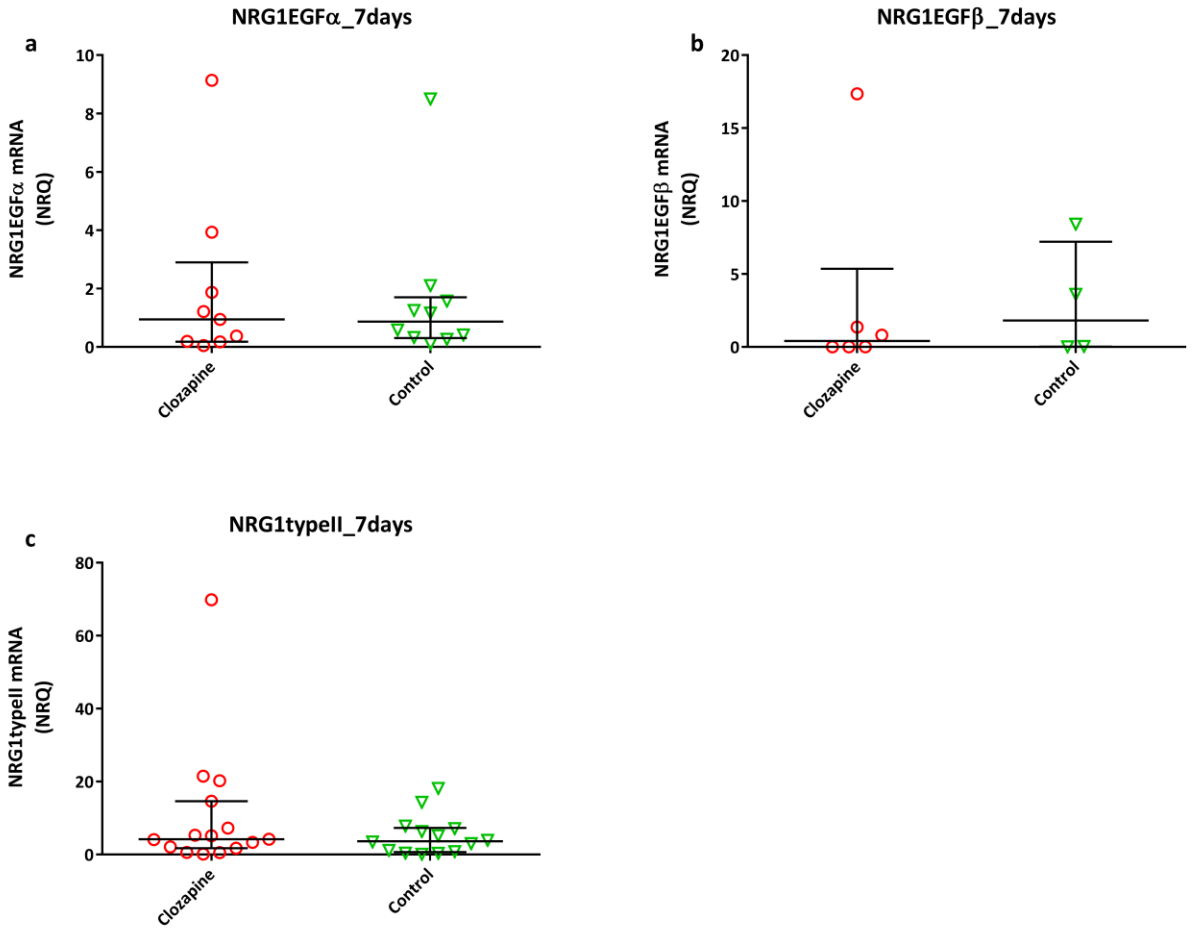
Supplementary Figure S4: Expression of reference genes after 7 days clozapine exposure. Wilcoxon signed rank test (matched pair, α=0.05, N=15) was used to measure the difference in reference gene expression between clozapine exposed and control cells; (**a**) UBC (W=-0.722, P=0.47), (**b**) ACTB (W=-0.722, P=0.470), (**c**) GAPDH (W=-1.287, P=0.198), (**d**) TBP (W=-0.909, P=0.363). Error bars represent median ± interquartile range.



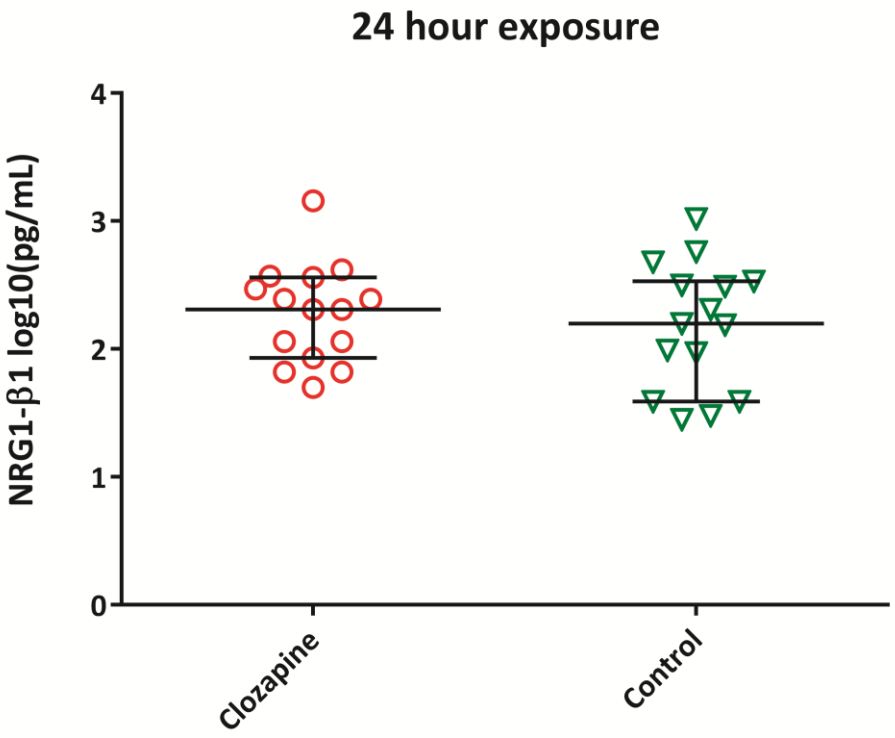
Supplementary Figure S5: Expression of reference genes after 24 hour clozapine exposure ; Wilcoxon signed rank test (matched pair, α=0.05, N=15) was used to measure the difference in reference gene expression between clozapine exposed and control cells; (**a**) UBC (W=3.408, P=0.001), (**b**) ACTB (W=3.408, P=0.001), (**c**) GAPDH (W=3.408, P=0.001), (**d**) TBP (W=3.408, P=0.001). Error bars represent median ± interquartile range. *P=0.001.



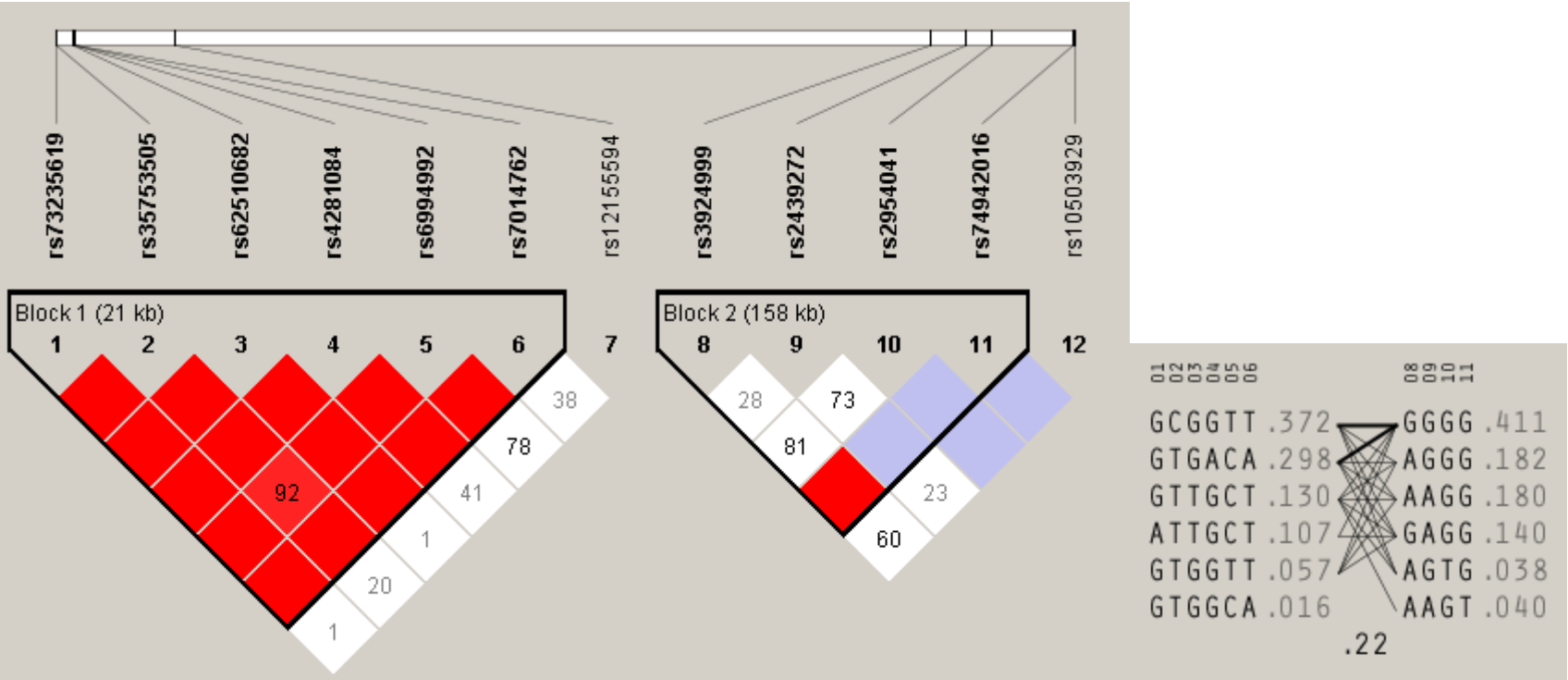
Supplementary Figure S6: Quantile-quantile plots of Log10 transformed NRQ values for (a) NRG1EGFα (SW=0.989, df=145, P=0.325), (b) NRG1 typeI$_{(Ig2)}$ (SW=0.986, df=120, P=0.23), (c) NRG1EGFβ (SW=0.99, df=141, P=0.401), (d) NRG1 typeIII (SW=0.968, df=68, P=0.076) and (e) NRG1-β1 (SW=0.957, df=137, P=0.000248). SW=Shapiro-Wilk test.



Supplementary Figure S7: Expression of detectable NRG1 isoforms after 7 days clozapine exposure. Wilcoxon signed rank test (matched pair) was used to measure the difference in the normalized quantity of NRG1 isoforms between clozapine exposed and control cells; (**a**) NRG1EGFα (W=-0.105, P=0.917), (**b**) NRG1EGFβ (W=-0.365, P=0.715), (**c**) NRG1typeII (W=-1.538, P=0.124). Error bars represent median ± interquartile range.



Supplementary Figure S8: Expression of NRG1-β1 after 24-hour exposure. Wilcoxon signed rank test (matched pair) was used to measure the difference in NRG1-β1 protein between clozapine exposed and control (W=-1.306, P=0.191). Error bars represent median ± interquartile range.



Supplementary Figure S9: Linkage disequilibrium (D') between SNPs in the *NRG1* gene. The first block represents strong LD (D'=1.0, r^2=0.055) between four HapICE SNPs with two other SNPs (rs4281084 and rs7041762) in the 5' region. The second block represents the LD (D'=1.0, r^2=0.052) between four SNPs in the 3' region of the NRG1 gene. The most frequent haplotype in the first block contains each of the risk alleles from the four HapICE SNPs. The most frequent haplotype in the second block contains none of the risk alleles for the corresponding SNPs.

		Ancestry Informative markers					
		CEU		CHB		YRI	
NRG1 SNPs	Call rate, %	dbSNP	Call rate, %	dbSNP	Call rate, %	dbSNP	Call rate, %
rs73235619	100	rs1402851	99	rs10488619	100	rs1368928	100
rs35753505	99	rs16877243	100	rs11098964	100	rs1446959	100
rs62510682	100	rs1698042	100	rs11184898	100	rs1494962	100
rs4281084	100	rs2930125	100	rs11203006	100	rs1563382	100
rs6994992	99	rs2934193	100	rs1347201	100	rs2388511	100
rs7014762	100	rs3912537	100	rs1488299	100	rs4241398	99
rs113317778	Fail	rs4721415	100	rs1519260	100	rs7158302	100
rs12155594	99	rs6510332	100	rs1538956	96	rs10933921	100
rs2954041	100	rs1001484	100	rs315280	99	rs1444893	99
rs6150532	Fail	rs6552216	100	rs36110	100	rs1716167	100
rs3924999	100	rs1002587	100	rs5753625	100	rs1811510	100
rs2439272	100	rs10879311	100	rs6595142	100	rs1823778	100
rs74942016	100	rs1227647	100	rs12595448	100	rs1827950	100
rs10503929	100	rs12678324	100	rs12644851	100	rs1894450	100
		rs2759281	100	rs2416504	100	rs2220128	100
		rs326626	100	rs2927385	99	rs2416791	96
		rs6141319	100	rs4240793	100	rs2948905	100
		rs679832	100	rs4265409	100	rs4737761	98
		rs10420077	100	rs590614	100	rs6785846	100
		rs2102727	100	rs4841401	100	rs692713	100

Supplementary Table S1: NRG1 SNPs with ancestry informative markers and their corresponding call rates. CEU, Northern and Western European; CHB, Han Chinese, Beijing; YRI, Yoruba in Ibadan, Nigeria

Gene	Probe name	Isoforms detected	Inventoried assay	Forward	Reverse	Probe (FAM-MGB)
NRG1	Type $I_{(Ig2)}$	Ig2 and s1 domains (excluding GGF-2, HRG-β1d, HRG-β3b, HRG-γ3, SMDF)	-	GCCAATATCACCATCGTGGAA	CCTTCAGTTGAGGCTGGCATA	CAAACGAGATCATCACTG
NRG1	Type II	GGF-2, HRGβ-1d, HRG-β3b, HRG-γ3	-	GAATCAAACGCTACATCTACATCCA	CCTTCTCCGCACATTTTACAAGA	CACTGGGACAAGCC
NRG1	Type IV	HRG-β1b, HRG-β1c & HRG-β1d	-	GCTCCGGCAGCAGCAT	GAACCTGCAGCCGATTCCT	ACCACAGCCTTGCCT
NRG1	EGFα	HRG-α, ndf43 and ndf43c	Hs01103794_m1	-	-	-
NRG1	EGFβ	SMDF, GGF, GGF2, ndf43a, ndf43b and all HRG-β isoforms	Hs00247624_m1	-	-	-
NRG1	Type III	SMDF	Hs01103792_m1	-	-	-
NRG1	pan-NRG1	all isoforms (excluding SMDF, ndf43c)	Hs00247620_m1	-	-	-
NRG1	Type I	HRG-β3b and HRG-γ3 (excluding HRG-β1b, HRG-β1c)	Hs01108479_m1	-	-	-
UBC	-	ubiquitin C	Hs00824723_m1	-	-	-
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	-	-	-
ACTB	-	beta-actin	Hs99999903_m1	-	-	-
TBP	-	TATA box binding protein	Hs00427620_m1	-	-	-

Supplementary Table S2: TaqMan probes and primer sequence for quantification and normalization of different NRG1 isoform expression. Custom designed probe and primer combinations were used to target specific NRG1 isoforms previously investigated [2]. For all the other isoforms, inventoried assays were used (Applied Biosystems, Foster City, CA, USA). The geometric mean of ACTB and UBC were used for transcript normalization.

NRG1 isoforms/ protein	Alcohol Use N; mean (se)	No Alcohol Use N; mean (se)	P value
NRG1EGFα	113; 1.12 (0.13)	13; 1.25 (0.25)	0.75
NRG1 typeI$_{(Ig2)}$	93; 2.10 (0.17)	11; 1.61 (0.24)	0.39
NRG1EGFβ	111; 9.17 (1.18)	12; 10.74 (2.02)	0.67
NRG1 typeIII	52; 12.48 (1.98)	08; 8.01 (1.33)	0.39
NRG1-β1 log10(pg/mL)	108; 2.65 (0.05)	12; 2.63 (0.20)	0.89

Supplementary Table S3: Normalized relative quantities (NRQ) of detectable *NRG1* mRNA isoforms and NRG1-β1 by alcohol use (past 3-months).

	Non-detects, n (%)			
Gene	**Total (128)**	**Schizophrenia (71)**	**Controls (57)**	**P value**
NRG1 EGFα	2 (1.6)	1 (1.4)	1 (1)	0.87
NRG1 EGFβ	5 (3.9)	3 (4.2)	2 (3.5)	0.83
NRG1 type $I_{(Ig2)}$	24 (18.8)	11 (15.5)	13 (22.8)	0.29
NRG1 typeIII	68 (53.1)	33 (46.5)	35 (61.4)	0.09
Pan NRG1	100 (78.1)	51 (71.8)	49 (86)	0.06
NRG1 type I	109 (85.2)	57 (80.3)	52 (91.2)	0.08
NRG1 type II	88 (68.8)	39 (54.9)	49 (86)	0.00016*
NRG1 type IV	128 (100)	71 (100)	57 (100)	0

Supplementary Table S4: Number of non-detects n (%) for all *NRG1* isoforms assessed. Z-test was performed to find differences between schizophrenia and controls. *P<0.05

Variables	**NRG1 EGFα (n=70)**	**NRG1 type$I_{(Ig2)}$ (n=60)**	**NRG1 EGFβ (n=68)**	**NRG1 typeIII (n=38)**	**NRG1-β1 (n=70)**
Clozapine plasma level (µg/L)	0.08 (0.513)	0.08 (0.538)	0.19 (0.134)	-0.02 (0.929)	-0.02 (0.851)
Chlorpromazine equivalent antipsychotic exposure	-0.03 (0.806)	0.08 (0.570)	-0.06 (0.647)	-0.05 (0.768)	-0.01 (0.925)
Age of onset, years	-0.37 (**0.002, 0.02**)	-0.05 (0.708)	-0.38 (**0.001**, **0.02**)	0.19 (0.250)	0.18 (0.142)
Duration of illness, years	0.01 (0.964)	-0.22 (0.094)	0.05 (0.696)	-0.36 (**0.027**, 0.167)	-0.11 (0.375)

Supplementary Table S5: Pearson's correlation (raw P-value, Benjamini-Hochberg adjusted P- value) between different NRG1 isoforms and NRG1-β1 with clozapine plasma level and chlorpromazine equivalent antipsychotic exposure (excluding clozapine).

Transcript	Positive score	Negative score	Disorganized score	Excitement score	Depression score	Total score
NRG1EGFα (n=70)	-0.02 (0.903)	0.020 (0.867)	-0.012 (0.921)	0.160 (0.185)	0.241 (**0.045**, 0.270)	-0.014 (0.908)
NRG1type$I_{(Ig2)}$ (n=60)	-0.169 (0.197)	0.112 (0.393)	-0.067 (0.612)	-0.014 (0.918)	-0.057 (0.664)	-0.015 (0.909)
NRG1EGFβ (n=68)	-0.057 (0.643)	0.085 (0.490)	-0.043 (0.726)	0.127 (0.303)	-0.227 (0.063)	-0019 (0.881)
NRG1typeIII (n=38)	-0.377 (**0.020**, 0.120)	0.257 (0.119)	-0.129 (0.441)	-0.130 (0.436)	-0.182 (0.275)	-0.133 (0.427)
NRG1-β1 (n=70)	-0.01 (0.942)	0.11 (0.348)	-0.15 (0.213)	-0.10 (0.399)	0.19 (0.111)	0.05 (0.689)

Supplementary Table S6: Pearson's correlation (raw P-value, Benjamini-Hochberg adjusted P- value) between NRG1 isoforms and NRG1-β1 protein level with PANSS positive, negative, disorganized, excited, depression score and total score.

NRG1 isoforms/ protein	Remission N; mean (se)	Non-remission N; mean (se)	Raw P value	BH P value*
NRG1EGFα	31; 1.35 (0.23)	39; 1.27 (0.13)	0.525	
NRG1 typeI$_{(Ig2)}$	26; 2.70 (0.37)	34; 1.70 (0.23)	0.303	
NRG1EGFβ	29; 12.4 (1.89)	39; 9.9 (1.01)	0.511	
NRG1 typeIII	13; 18.7 (5.56)	25; 8.82 (1.47)	**0.013**	0.065
NRG1-β1 log10(pg/mL)	31; 2.57(0.11)	39; 2.39(0.10)	0.239	

Supplementary Table S7: Normalized relative quantities (NRQ) of detectable *NRG1* mRNA isoforms and NRG1-β1 by positive symptom remission status. Positive symptom remission was defined as a score of ≤3 on four PANSS items (delusions, hallucinations, grandiosity and unusual thought content) [6]. *Benjamini-Hochberg adjusted P-value

SNP	NRG1EGFα (n=126)	NRG1typeI$_{(Ig2)}$ (n=104)	NRG1EGFβ (n=123)	NRG1TypeIII (n=60)	NRG1-β1 (n=120)
rs73235619	5.278 (0.207)	0.006 (0.964)	1.661 (0.523)	3.633 (0.295)	0.191 (0.867)
rs35753505	1.614 (0.522)	0.212 (0.861)	0.271 (0.861)	0.431 (0.849)	4.855 (0.207)
rs62510682	2.814 (0.330)	0.240 (0.861)	2.470 (0.377)	3.488 (0.299)	5.198 (0.207)
rs4281084	0.330 (0.861)	0.452 (0.849)	1.412 (0.529)	0.033 (0.957)	SZ: 0.626 (0.764) Con: 6.475 (0.207)
rs6994992	1.507 (0.522)	0.876 (0.686)	0.002 (0.967)	2.800 (0.331)	8.757 (P=0.152)
rs7014762	0.004 (0.964)	1.205 (0.579)	0.789 (0.696)	0.080 (0.910)	SZ: 0.814 (0.697) Con: 7.749(0.203)
rs12155594	SZ: 0.213 (0.861) Con: 4.557(0.211)	0.706 (0.729)	0.024 (0.963)	0.004 (0.963)	0.067 (0.916)
rs3924999	8.641 (0.152)	0.008 (0.964)	2.801 (0.330)	0.110 (0.899)	0.238 (0.861)
rs2439272	0.238 (0.861)	0.179 (0.867)	1.082 (0.616)	0.338 (0.861)	0.083 (0.910)
rs2954041	3.029 (0.331)	0.128 (0.898)	2.854 (0.330)	0.016 (0.964)	SZ: 3.368 (0.299) Con: 4.487(0.212)
rs74942016	0.039 (0.956)	0.257 (0.861)	1.476 (0.523)	2.210 (0.403)	2.173 (0.403)
rs10503929	SZ: 4.986 (0.207) Con: 4.610 (0.211)	0.106 (0.899)	SZ: 5.876 (0.207) Con: 2.414 (0.386)	0.229 (0.861)	1.536 (0.523)
Hap GCGT	1.210 (0.579)	0.484 (0.843)	0.129 (0.898)	0.947 (0.67)	5.142 (0.207)
Hap ATTC	5.598 (0.207)	0.004 (0.964)	1.813 (0.491)	3.633 (0.295)	0.236 (0.861)

Supplementary Table S8: Expression quantitative trait loci (eQTL) analysis of putative NRG1 SNPs with NRG1 isoforms and NRG1-β1 protein expression. F value (Benjamini-Hochberg adjusted P-value) is shown for each SNP. In cases where a significant SNP x group interaction was detected, F values (p-values) are presented for schizophrenia and control groups separately.

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Chapter 4

Peripheral Transcription of *NRG-ErbB* Pathway Genes Are Upregulated in Treatment-resistant Schizophrenia

frontiers in Psychiatry

ORIGINAL RESEARCH
published: 06 November 2017
doi: 10.3389/fpsyt.2017.00225

Peripheral Transcription of *NRG-ErbB* Pathway Genes Are Upregulated in Treatment-Resistant Schizophrenia

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Reviewed by:
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Specialty section:
This article was submitted to Schizophrenia, a section of the journal Frontiers in Psychiatry

Received: *05 September 2017*
Accepted: *23 October 2017*
Published: *06 November 2017*

Citation:
Mostaid MS, Lee TT, Chana G, Sundram S, Shannon Weickert C, Pantelis C, Everall I and Bousman C (2017) Peripheral Transcription of NRG-ErbB Pathway Genes Are Upregulated in Treatment-Resistant Schizophrenia. Front. Psychiatry 8:225. doi: 10.3389/fpsyt.2017.00225

Investigation of peripheral gene expression patterns of transcripts within the *NRG–ErbB* signaling pathway, other than neuregulin-1 (*NRG1*), among patients with schizophrenia and more specifically treatment-resistant schizophrenia (TRS) is limited. The present study built on our previous work demonstrating elevated levels of *NRG1* EGFα, EGFβ, and type $I_{(Ig2)}$ containing transcripts in TRS by investigating 11 *NRG–ErbB* signaling pathway mRNA transcripts (*NRG2*, *ErbB1*, *ErbB2*, *ErbB3*, *ErbB4*, *PIK3CD*, *PIK3R3*, *AKT1*, *mTOR*, *P70S6K*, *eIF4EBP1*) in whole blood of TRS patients ($N = 71$) and healthy controls ($N = 57$). We also examined the effect of clozapine exposure on transcript levels using cultured peripheral blood mononuclear cells (PBMCs) from 15 healthy individuals. Five transcripts (*ErbB3*, *PIK3CD*, *AKT1*, *P70S6K*, *eIF4EBP1*) were significantly elevated in TRS patients compared to healthy controls but only expression of *P70S6K* ($P_{corrected} = 0.018$), a protein kinase linked to protein synthesis, cell growth, and cell proliferation, survived correction for multiple testing using the Benjamini–Hochberg method. Investigation of clinical factors revealed that *ErbB2*, *PIK3CD*, *PIK3R3*, *AKT1*, *mTOR*, and *P70S6K* expression were negatively correlated with duration of illness. However, no transcript was associated with chlorpromazine equivalent dose or clozapine plasma levels, the latter supported by our *in vitro* PBMC clozapine exposure experiment. Taken together with previously published *NRG1* results, our findings suggest an overall upregulation of transcripts within the *NRG–ErbB* signaling pathway among individuals with schizophrenia some of which attenuate over duration of illness. Follow-up studies are needed to determine if the observed peripheral upregulation of transcripts within the *NRG–ErbB* signaling pathway are specific to TRS or are a general blood-based marker of schizophrenia.

Keywords: treatment-resistant schizophrenia, *NRG–ErbB* pathway, gene expression, symptom severity, schizophrenia

INTRODUCTION

Intracellular signaling initiated by neuregulins (NRGs) and their cognate receptors (ErbBs) are vital for the assembly of neuronal circuitry (1, 2), including myelination of axonal processes (3, 4), neurotransmission (5), and synaptic plasticity (6–8). Abnormalities in *NRG–ErbB* signaling have been implicated in schizophrenia, with the majority of evidence linked to neuregulin-1 (*NRG1*) and *ErbB4* (5, 9–11).

Neuregulin-1 and *ErbB4*, together, initiate signaling *via* the *PI3K-AKT* signaling pathway, which results in activation of *mTOR* and in turn stimulates protein synthesis (**Figure 1**). Several human postmortem brain studies have shown dysregulation of gene expression of *NRG1*, ErbB4 or down-stream targets among individuals with schizophrenia (12–17). Likewise, evidence of dysregulated gene expression of *NRG1* (18–20), *ErbB1*/*ErbB4* (21), and *PI3K*/AKT (22, 23) in peripheral tissues [i.e., whole blood, peripheral blood mononuclear cells (PBMCs), monocytes] in schizophrenia has also been shown in people with chronic schizophrenia. Treatment-resistant schizophrenia (TRS) patients represent a considerable subgroup who have significant increases in multiple *NRG1* splice variants in peripheral blood (24). Thus, we may expect the biological interactors (receptors) and mediators (kinase) of this pathway to also be changed. However, peripheral examination of gene expression within this pathway among individuals with TRS has yet to be completed. Moreover, the impact of medication, lifestyle (e.g., smoking, alcohol use), and/or symptom severity on *NRG1*-related mRNA expression is largely unknown.

The present investigation, therefore, quantiatively compared (i) whole blood mRNA levels of 11 *NRG–ErbB* signaling receptors and pathway genes (*NRG2*, *ErbB1*, *ErbB2*, *ErbB3*, *ErbB4*, *PIK3CD*, *PIK3R3*, *AKT1*, *mTOR*, *P70S6K*, *eIF4EBP1*) among individuals with TRS and healthy controls, (ii) associations between mRNA levels and symptom severity, age of onset, duration of illness, clozapine plasma level, and chlorpromazine equivalent dosage, and (iii) the effect of clozapine exposure on mRNA expression in PBMCs from healthy controls. We expected that there would be multiple molecular changes in TRS compared to controls that may contribute to the amplification of *NRG1* signaling in perhiperal blood in support of a widespread gain of function model of *NRG1* in the pathophysiology of schizophrenia.

MATERIALS AND METHODS

Participants

Clinical Samples

Seventy-one participants aged 18–65 with schizophrenia who were treated with clozapine were recruited from inpatient and outpatient clinics in Melbourne, Australia. As these individuals failed to respond to two or more previous trials of antipsychotics, had poor functioning, and persistent symptoms, they were considered "treatment-resistant," consistent with current criteria

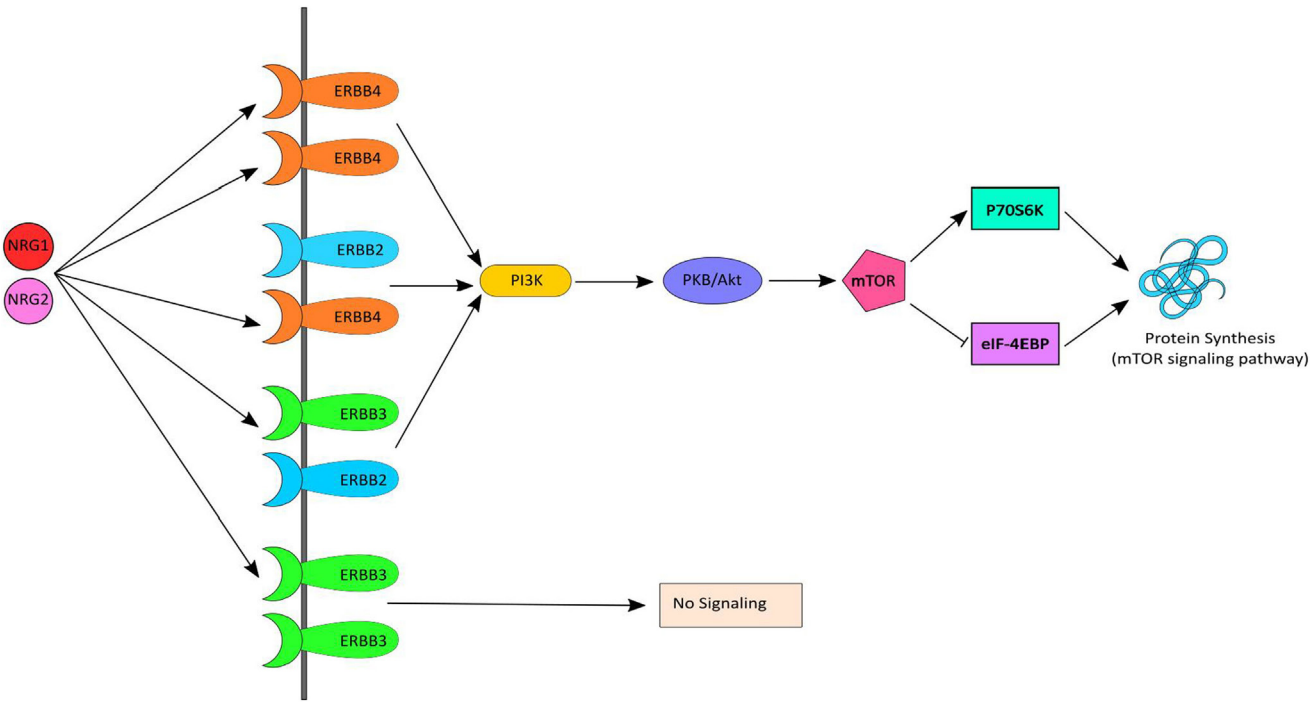

FIGURE 1 | *NRG–ErbB* signaling pathway. Neuregulin-1 (*NRG1*) and *NRG2* bind to *ErbB3* and/or *ErbB4,* which in turn undergoes homo or heterodimerization and activates *PI3K*. *PI3K* then activates AKT and subsequently *mTOR* causing initiation of protein synthesis *via* the *mTOR* signaling pathway. mTOR phosphorylates and activates *P70S6K* which facilitates phosphorylation of small ribosomal protein 6 (*S6*) and eukaryotic translation initiation factor 4B (*eIF4B*) and leads to initiation of protein synthesis. Activated *mTOR* also causes phosphorylation and inactivation of *eIF4EBP1*, which release *eIF4E* and facilitates translation.

(25). In addition, 57 age-, sex-, and socioeconomic-matched unrelated healthy controls were recruited from the general community. Controls with a first-degree family history of psychiatric illness, prior or current use of antipsychotic medication, head injury, seizure, neurological disease, impaired thyroid function, and/or substance abuse/dependence were excluded. Detailed demographic characteristics of all participants are presented in **Table 1**.

Mini International Neuropsychiatric Interview (26) was administered to all participants to confirm the diagnosis of schizophrenia as well as to rule out the presence of psychiatric disorders in healthy controls. The Positive and Negative Syndrome Scale (PANSS) (27) was used to assess the clinical symptoms and the patients were scored in accordance with the consensus five-factor (i.e., positive, negative, disorganized/concrete, excited, depressed) PANSS model (28). Information on tobacco, alcohol, and illicit drug use in the past 3 months was collected using a substance use questionnaire. Whole blood samples were collected after overnight fasting and processed according to standardized blood collection and processing protocol (see supplementary methods for more details). Plasma levels of clozapine were measured and chlorpromazine equivalent dosage (excluding clozapine) were calculated for the 31% ($n = 22$) of participants with schizophrenia who were taking concomitant antipsychotic medication in accordance with published guidelines (29, 30). All the participants provided written informed consent and the study protocol was approved by the Melbourne Health Human Research Ethics Committee (MHREC ID 2012.069). The study complied with the Declaration of Helsinki and its subsequent revisions (31).

TABLE 1 | Demographic data and clinical characteristics of participants.

Characteristic	Schizophrenia ($n = 71$)	Controls ($n = 57$)	*P*-value
Age, mean (SD) years	40 (10)	40 (11)	0.702[a]
Gender, *n* (%) males	53 (75)	35 (61)	0.108[b]
RIN, mean (SD)	8.4 (0.9)	8.7 (0.3)	0.006[a*]
Ancestry, *n* (%) CEU	62 (90)	50 (88)	0.742[b]
Substance use in past 3 months, *n* (%)			
Tobacco (smoked)	33 (47)	12 (21)	0.003[b*]
Alcohol	59 (83)	55 (97)	0.016[b*]
Cannabis	11 (15)	7 (12)	0.385[b]
Amphetamine	4 (6)	2 (4)	0.439[b]
Cocaine	0 (0)	2 (4)	0.137[b]
Opiates	1 (1)	1 (2)	0.990[b]
Clozapine plasma level, mean (SD) μg/L	432 (234)	–	–
Chlorpromazine equivalent (excluding clozapine) dosage mean (SD) mg/day	142 (286)	–	–
Age of onset, mean (SD) years	22.5 (6)	–	–
Duration of illness, mean (SD) years	17 (8)	–	–
PANSS scores, mean (SD)			
Positive	10 (6)	–	–
Negative	15 (5)	–	–
Disorganized	8 (3)	–	–
Excitement	6 (2)	–	–
Depression	6 (3)	–	–
Total	62 (14)	–	–

CEU, Northern and Western European ancestry; TRS, treatment-resistant schizophrenia; RIN, RNA integrity number; PANSS, Positive and Negative Syndrome Scale.

[a]*Independent sample t-test.*

[b]*Chi-square (χ^2) test.*

**P < 0.05.*

In Vitro Clozapine Exposure Samples

To assess the effect of clozapine exposure on gene expression of our candidate transcripts, fresh frozen PBMCs from 15 healthy individuals (8 males and 7 females) of European ancestry with a mean age of 35 (SD = 13.5; range 20–54 years) were purchased from STEMCELL™ Technologies, Inc. (Vancouver, BC, Canada). A sample size of 15 was sufficient to detect a large effect (Cohen's $d = 0.80$) between exposed and unexposed conditions at $\alpha = 0.05$ and power $(1 - \beta) = 0.80$. The percentage of current smokers among the donors was 33.3% ($n = 5$). All the donors were tested for HIV-1, HIV-2, hepatitis B and hepatitis C prior to blood collection.

Peripheral blood mononuclear cells isolated from whole blood were supplied as vials containing 100 million cells. PBMCs were rapid-thawed from liquid nitrogen and seeded in six-well plates in triplicates at a concentration of 2 million cells per well (1×10^6 cells/mL) in RPMI-1640 medium (Sigma-Aldrich; St. Louis, MO, USA) supplemented with L-glutamine (0.3 g/L) and sodium bicarbonate (2 g/L), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum for 24 h. Cells were then exposed to clozapine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h and 7 days, at a concentration of 1.2 μM (control cells were exposed to vehicle only, see supplementary methods for details) and incubated at 37°C in 5% CO_2. Clozapine was initially dissolved in absolute ethanol and media was used for dilution. The final concentration of ethanol on each well was 1 in 8,000. The concentration of clozapine used was determined from the mean plasma concentration of clozapine found in the first 48 recruited clinical samples (1.2 μM or 384 ng/mL). Toxicity assays (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega Corporation, Madison, WI, USA) were performed at baseline, 24 h and 7-day time points after clozapine exposure to measure the production of lactate dehydrogenase within the media (see Figure S1 in Supplementary Material for more details).

RNA Extraction, Complementary DNA (cDNA) Synthesis, and Quantitative Real-time PCR

PureLink RNA Mini Kit (ThermoFisher scientific, Waltham, MA, USA) was used to extract total RNA from both clinical and *in vitro* samples following standard manufacturer's instructions. The RNA integrity number (RIN) range was 3.60–9.50 (mean = 8.59, SD = 0.79). Total RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript® IV First-Strand Synthesis System (Invitrogen, Foster city, CA, USA) using random hexamers. cDNA (10.25 ng) was used as a template for real-time PCR (RT-qPCR) using master-mix and gene specific validated Taqman assays from Applied Biosystems, Foster City, CA, USA. Inventoried assays (TaqMan®, Invitrogen, USA) were used for all the genes of interest as well as for four reference genes (beta-actin, ACTB; ubiquitin C, UBC; ABL proto-oncogene 1,

ABL1; Succinate Dehydrogenase Complex Flavoprotein Subunit A, SDHA). See Table S1 in Supplementary Material for a list of each of the probes and primers.

Complementary DNA (10.25 ng) was subjected to quantitative real-time PCR in duplicate using FAM-MGB TaqMan® gene expression probes (Invitrogen, Foster city, CA, USA) in 192 × 24 Dynamic Arrays IFC in Fluidigm® BioMark™ HD system (South San Francisco, CA, USA) at the Monash Health Translation Precinct Medical Genomics Facility (Hudson Institute of Medical Research, Clayton, VIC, Australia). In addition, no reverse transcriptase controls and no template controls were included to rule out genomic DNA contamination and reagent contamination, respectively. Adhering to minimum information for publication of RT-qPCR (MIQE) guidelines (32), normalized relative quantities (NRQ), i.e., $2^{-\Delta Ct}$ where $\Delta C_t = [C_{t(\text{candidate gene})} - C_{t\ (\text{geometric mean of reference genes})}]$ of each mRNA isoform was calculated using the geometric mean expression of two reference genes (UBC and ACTB) that did not differ between groups in the clinical cohort. ABL-1 and SDHA were not used as reference genes because their expression differed significantly by group in the clinical cohort (Figures S2–S4 in Supplementary Material). In the *in vitro* cohort only, ABL-1 was stable after 24 h clozapine exposure and ACTB was stable after 7 days clozapine exposure and were used for normalization and subsequent analysis at specific time points.

Statistical Analysis

Two-sided tests were used for all statistical analyses. Shapiro–Wilk test and quantile–quantile (Q–Q) plots were used to assess normality of variable distributions. Student's *t*-tests were used to test differences for continuous variables between schizophrenia patients and healthy controls, while chi-squared (χ^2) tests were used for categorical variables. The Benjamini and Hochberg (B–H) step-up procedure (33) was used to adjust for multiple comparisons for all analyses. Effect sizes were calculated using the Hedges' *g* method (34).

Prior to analysis, the NRQ values for all the mRNA transcripts were checked for normality using Q–Q plots (Figure S5 in Supplementary Material) and as required were $\log_{10}$ transformed for subsequent analysis. In addition, we assessed the following variables as potential confounders: age, sex, RIN, alcohol use, and smoking status. A variable was considered a confounder and included in our statistical models only when it was significantly different between groups ($P < 0.05$) and was significantly associated with gene expression. The log-transformed NRQ values were compared among groups using general or generalized linear models based on their distribution and adjusted for appropriate covariates. Outliers were identified using the Grubbs' test for outliers and removed from further analysis.

Within the schizophrenia group, Pearson or Spearman correlations, depending on data distribution, were calculated between gene transcript levels and symptom severity, age of onset, illness duration, current chlorpromazine equivalent dose, and clozapine plasma levels. In addition, mRNA transcript levels between participants in positive symptom remission and non-remission were assessed using a *t*-test or Mann–Whitney *U* test. Positive symptom remission was defined as a PANSS score of ≤3 on delusions, hallucinations, grandiosity, and unusual thought content (28).

To assess differences in gene expression between clozapine exposed and unexposed PBMCs at both time points (24 h and 7 days), Wilcoxon matched paired *t*-test were used, adjusting for age, gender, and RIN.

RESULTS

NRG–ErbB Signaling Pathway Transcripts Are Upregulated in TRS

Two (*ErbB1*, *ErbB4*) of the 11 *NRG–ErbB* pathway mRNA transcripts interrogated, were not detectable in more than 80% of the full cohort and so were removed from further analysis. The rates of non-detects were not significantly different between groups (*ErbB1*: case 95%, control: 97%; *ErbB4*: case 81%, control 85%). Analysis on the remaining nine transcripts showed significantly elevated levels of five transcripts: *ErbB3* ($P = 0.046$), *PIK3CD* ($P_{\text{raw}} = 0.035$), *AKT1* ($P_{\text{raw}} = 0.018$), *P70S6K* ($P_{\text{raw}} = 0.002$), and *eIF4EBP1* ($P_{\text{raw}} = 0.013$) in TRS patients compared to healthy controls after adjustment for covariates. However, only *P70S6K* ($P_{\text{B–H}} = 0.018$) remained significant after correction for multiple comparisons (**Figure 2**). Importantly, transcript levels were not correlated with clozapine plasma levels or chlorpromazine equivalent antipsychotic exposure (excluding clozapine) (Table S2 in Supplementary Material). The lack of relationship between mRNA levels and clozapine levels were further corroborated by our *in vitro* analysis that showed no difference in mRNA levels of detectable transcripts ($n = 9$) in clozapine exposed compared to unexposed PBMCs, except *mTOR* mRNA which showed decreased expression levels in clozapine exposed cells at both 24 h ($P = 0.001$) and 7-day ($P = 0.05$) time points (Figures S6 and S7 in Supplementary Material).

NRG–ErbB Signaling Pathway Transcripts Are Associated with Duration of Illness but Not Age of Onset or Symptom Severity

Among individuals with TRS, significant negative correlations between duration of illness and *ErbB2* ($r = -0.293$, $P_{\text{raw}} = 0.016$, $P_{\text{B-H}} = 0.031$), *PIK3CD* ($r = -0.303$, $P_{\text{raw}} = 0.013$, $P_{\text{B-H}} = 0.031$), *PIK3R3* ($r = -0.275$, $P_{\text{raw}} = 0.025$, $P_{\text{B-H}} = 0.038$), *AKT1* ($r = -0.290$, $P_{\text{raw}} = 0.017$, $P_{\text{B-H}} = 0.031$), *mTOR* ($r = -0.339$, $P_{\text{raw}} = 0.005$, $P_{\text{B-H}} = 0.023$), and *P70S6K* ($r = -0.347$, $P_{\text{raw}} = 0.005$, $P_{\text{B-H}} = 0.023$) expression were detected (**Figure 3**). None of the reference genes were significantly correlated with duration of illness, *UBC* ($r = -0.139$, $P_{\text{raw}} = 0.263$), *ACTB* ($r = 0.232$, $P_{\text{raw}} = 0.59$). No significant correlations were observed between any of the transcripts and age of onset (Table S2 in Supplementary Material).

A significant positive correlation between *ErbB2* expression and PANSS excitement score ($r = 0.289$, $P_{\text{raw}} = 0.014$, $P_{\text{B–H}} = 0.667$) was observed but did not survive correction for multiple comparisons (Table S3 in Supplementary Material). An exploratory examination of TRS patients in positive symptom remission versus non-remission revealed no statistically significant differences in levels of any of the gene mRNA

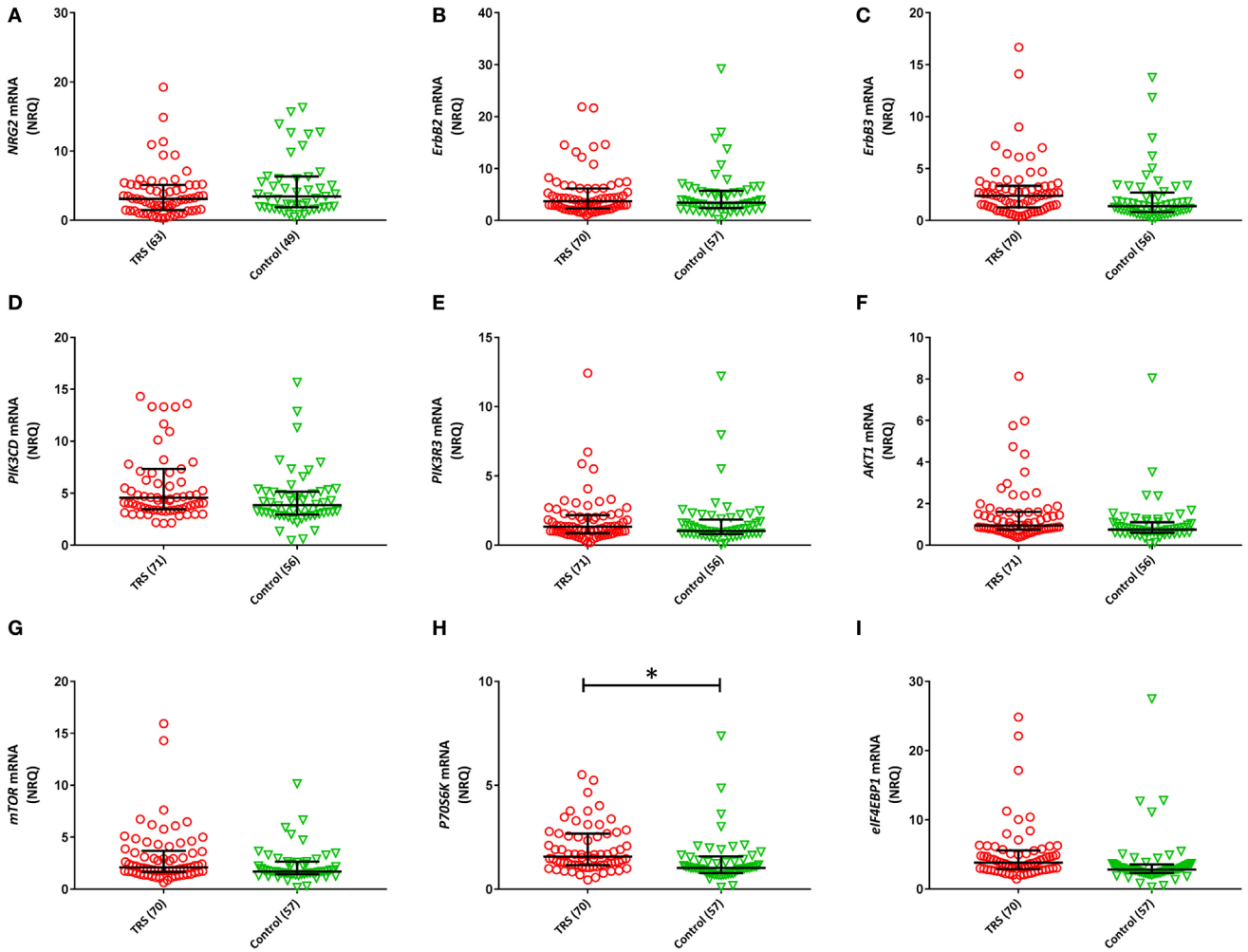

FIGURE 2 | Normalized relative quantities (NRQ) of the gene transcripts: **(A)** *NRG2* [treatment-resistant schizophrenia (TRS): 3.11, interquartile range (IQR) = 1.5–5.12, controls: 3.44, IQR = 1.89–6.34; $F_{1,111} = 0.524$, $P = 0.113$]; **(B)** *ErbB2* (TRS: 3.72, IQR = 2.39–6.19, controls: 3.44, IQR = 2.42–5.73; Wald $\chi^2 = 0.029$, $P = 0.864$); **(C)** *ErbB3* (TRS: 2.39, IQR = 1.26–3.35, controls: 1.38, IQR = 0.82–2.70; $F_{1,126} = 4.071$, $P = 0.083$); **(D)** *PIK3CD* (TRS: 4.57, IRQ = 3.45–7.34, controls: 3.86, IQR = 2.94–5.16; Wald $\chi^2 = 4.464$, $P = 0.079$); **(E)** *PIK3R3* (TRS: 1.34, IQR = 0.86–2.17, controls: 1.02, IQR = 0.8–1.85; Wald $\chi^2 = 0.104$, $P = 0.84$); **(F)** *AKT1* (TRS: 0.94, IQR = 0.75–1.61, controls: 0.75, IQR = 0.59–1.11; Wald $\chi^2 = 5.605$, $P = 0.054$); **(G)** *mTOR* (TRS: 2.10, IQR = 1.66–3.69, controls: 1.44, IQR = 1.44–2.65; Wald $\chi^2 = 4.746$, $P = 0.20$); **(H)** *P70S6K* (TRS: 1.57, IQR = 1.16–2.68, controls: 1.02, IQR = 0.77–1.58; Wald $\chi^2 = 13.90$, $P = 0.018$); **(I)** *eIF4EBP1* (TRS: 3.81, IQR = 2.88–5.58, controls: 2.80, IQR = 2.30–3.55; Wald $\chi^2 = 8.71$, $P = 0.054$). Error bars represent median with interquartile range. Benjamini–Hochberg adjusted *P*-values are shown (*$P < 0.05$).

transcripts after correction for multiple comparisons (Table S4 in Supplementary Material).

DISCUSSION

Our findings suggest transcription in the *NRG–ErbB* signaling pathway is upregulated in the whole blood of individuals with TRS and is negatively correlated with duration of illness. Among the nine detectable *NRG–ErbB* pathway transcripts we examined, five (*ErbB3*, *PIK3CD*, *AKT1*, *P70S6K*, and *eIF4EBP1*) were elevated and, of these, *P70S6K* survived correction for multiple comparisons. Importantly, we could not attribute this upregulation of peripheral transcription in the *NRG–ErbB* pathway to age, sex, or medication. In fact, results from our *in vitro* clozapine exposure experiment suggested clozapine might reduce rather than increase transcription of genes within the *NRG–ErbB* signaling pathway, particularly *mTOR* expression. Overall, our findings support our hypothesis that there is a generalized increase in *NRG1* signaling in people with TRS.

Previous findings by us and others support the notion of increased transcription of genes within the *NRG–ErbB* signaling pathway in schizophrenia. We recently showed in the same cohort used in the current study, an increased expression of three *NRG1* transcripts [i.e., *NRG1*-EGFα, *NRG1*-EGFβ, and *NRG1*-type$I_{(Ig2)}$] in TRS compared to controls (24). In addition, several studies by others have reported increased expression of specific isoforms of *NRG1* (18) and mRNA of down-stream signaling molecules, including *PIK3CD*, *PIK3CB* (16, 22), and *AKT1*

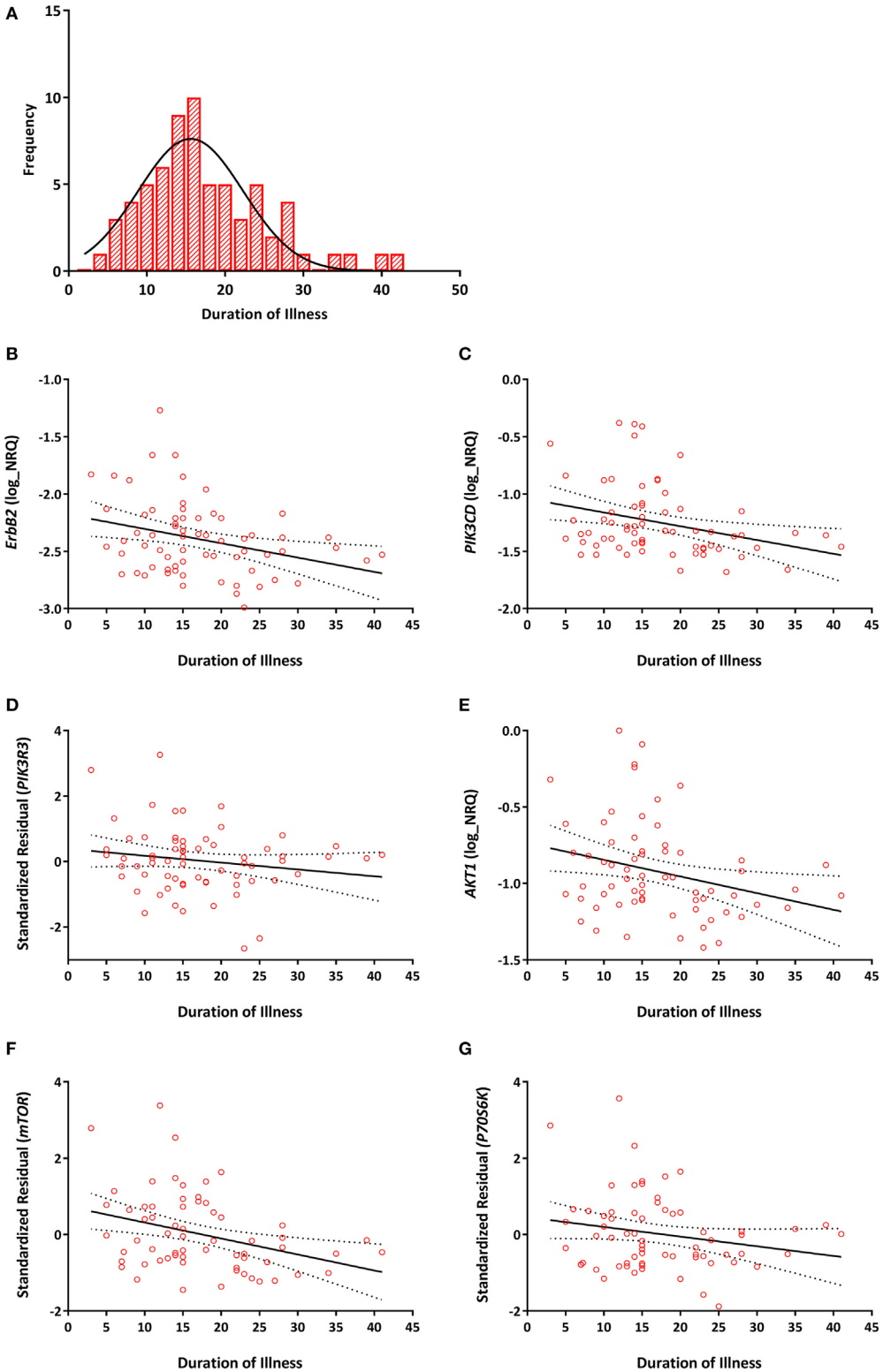

FIGURE 3 | **(A)** Distribution of duration of illness in years (mean = 17, SD = 8). Correlations between duration of illness and **(B)** *ErbB2* ($r = -0.293$, $P_{B\text{-}H} = 0.031$); **(C)** *PIK3CD* ($r = -0.303$, $P_{B\text{-}H} = 0.031$); **(D)** *PIK3R3* ($r = -0.275$, $P_{B\text{-}H} = 0.038$); **(E)** *AKT1* ($r = -0.290$, $P_{B\text{-}H} = 0.031$); **(F)** *mTOR* ($r = -0.339$, $P_{B\text{-}H} = 0.023$); **(G)** *P70S6K* ($r = -0.347$, $P_{B\text{-}H} = 0.023$) mRNA expression. Expression of *PIK3R3*, *mTOR*, and *P70S6K* are represented as the standardized residual from a linear regression model after adjusting for potential confounds [i.e., age for *PIK3R3*, RNA integrity number (RIN) and smoking for *mTOR*, age, RIN and smoking for *P70S6K*]. Solid lines represent the line of best fit and dotted lines represents 95% confidence intervals for the line of best fit.

(22, 23) in schizophrenia patients. Furthermore, other downstream signaling molecules, such as *mTOR*, *P70S6K*, and *eIF4B*, have been shown to be increased in major depressive disorder (35). However, as we are not aware of any human studies that have interrogated *P70S6K*, in schizophrenia, we are the first to report increased mRNA of *P70S6K* in TRS.

P70S6K encodes for a vital kinase in the mTOR signaling pathway (36–38) that when phosphorylated by mTOR results in phosphorylation and activation of translation elongation factors *eIF4B* and *eEF2K*, thereby promoting protein translation (39, 40). Our findings suggest upregulation of *P70S6K*, in part, may result from an increase in transcription of several genes upstream of *P70S6K* within the *NRG–ErbB* signaling pathway. However, other genes (i.e., *BDNF*, *DISC1*) as well as neurotransmitters (i.e., glutamate, serotonin) and hormones (e.g., insulin) have also been shown to activate the *PI3K–AKT–mTOR* signaling pathway (41–43) and as such may contribute or confound the increase in *P70S6K* expression we have observed. However, most studies find decreased BDNF levels in the blood of people with schizophrenia (44) and suggest some degree of insulin resistance in clozapine-treated patients (45). Future investigations should attempt to account for these other signaling factors and the potential confounders of metabolic changes in people with schizophrenia being treated with clozapine, as doing so will further elucidate the suitability of *P70S6K* as a peripheral biomarker of over-activity in the *NRG1* pathway in schizophrenia.

We also detected trend-level increases in three transcripts (*ErbB3*, *PIK3CD*, and *AKT1)* upstream of *mTOR*, within the *NRG–ErbB* signaling pathway among those with TRS. These increases in whole blood expression are, in part, supported by previous studies that have shown an increased *AKT1* mRNA expression in PBMCs from individuals with early-onset (23) and treatment-naïve schizophrenia (46), suggesting peripheral upregulation of *NRG–ErbB* pathway transcripts may not be specific to the stage of illness and may occur during the first phases of schizophrenia and continue during the chronic phases. However, six of the mRNA transcripts (*ErbB2*, *PIK3CD*, *PIK3R3*, *AKT1*, *mTOR*, and *P70S6K*) we examined were negatively correlated with duration of illness, suggesting that as the illness progresses the upregulation of transcription within the *NRG–ErbB* signaling pathway might become less apparent. However, it is not clear whether this correlation represents a potential disease process and/or a compensatory response in an effort to maintain signaling homeostasis. Studies examining patterns of *NRG–ErbB* signaling pathway transcripts over the course of the illness are required to confirm this notion and determine the underlying mechanism.

We did not find differences in the peripheral expression of *NRG2* between TRS patients and controls. To our knowledge, we are the first to examine *NRG2* mRNA in the blood in schizophrenia or other psychiatric disorder. However, a recent study showed that ablation of *NRG2* in the adult mouse brain mimicked dopaminergic imbalance seen in schizophrenia (i.e., high subcortical dopamine, low cortical dopamine) and resulted in severe behavioral phenotypes relevant to psychiatric disorders (47). Thus, *NRG2* may play a role in the pathophysiology of schizophrenia but based on our results seems less likely to serve as a peripheral marker of neurobiological changes found in schizophrenia. Likewise, *ErbB2* mRNA expression seems an unlikely peripheral marker of schizophrenia based on our null findings as well as findings from others that reported no difference in *ErbB2* mRNA expression in monocytes of first-episode, drug-naive patients with schizophrenia compared to healthy controls (48). However, this same study suggested that there may be an exaggerated *NRG1* stimulated cytokine response from PBMC in people with schizophrenia compared to controls (48), suggesting a link between overactive *NRG1* signaling and inflammation.

Our study has notable limitations. First, we were unable to compare affected individuals with and without TRS and as such the specificity of our results to TRS patients remains to be confirmed. Second, we analyzed cross-sectional data, which makes it complicated to predict how gene expression patterns might change with disease progression and their possible relation to clinical symptoms. Third, we measured gene expression in whole blood, as this tissue is clinically accessible and commonly used in biomarker research. However, it is unclear how our findings will relate to other peripheral (PBMCs or lymphocytes) or central tissues (e.g., brain) despite some suggestion for their relevance in schizophrenia (49). Fourth, we did not investigate all transcripts within the *NRG–ErbB* pathway (i.e., *PIK3CA-B*, *PIK3R1-2*, *eIF4B*, *eEF2*, and *eIF4E*). We instead, chose transcripts based on evidence from the current literature in schizophrenia. Furthermore, we only interrogated mRNA levels of our candidate genes within the *NRG–ErbB* pathway and as such cannot rule out the potential that genetic, protein, and/or epigenetic markers in this pathway may differ in those with schizophrenia. Fifth, our sample size was relatively small and as such requires independent validation. Finally, our *in vitro* clozapine exposure experiments examined a single clozapine concentration (1.2 μM) that was guided by pilot data from our study population. While this concentration of clozapine does reflect steady state plasma concentrations (50–52), future work with PBMCs should examine multiple concentrations that reflect the range of clozapine blood levels observed in the clinic together with interrogating a greater number of candidates at both genetic, gene expression and protein levels.

In summary, our results provide the first peripheral gene expression profile of the major *NRG–ErbB* pathway genes among individuals with TRS. We detected an overall upregulation of *NRG–ErbB* pathway transcripts among those with TRS, most robustly for *P70S6K*. We further showed that most of the transcripts we examined were negatively correlated with duration of illness, suggesting the upregulation of *NRG–ErbB* pathway transcripts we observed in the current chronic schizophrenia cohort may be more easily detectable among individuals at earlier stages of the illness relative to healthy individuals. If this notion is substantiated by future research, *NRG–ErbB* pathway gene expression may serve, in part, as a useful peripheral biomarker for staging of the illness and possibly assist in the identification of those at greatest risk for TRS.

ETHICS STATEMENT

All the participants provided written informed consent and the study protocol was approved by the Melbourne Health Human

Research Ethics Committee (MHREC ID 2012.069). The study complied with the Declaration of Helsinki and its subsequent revisions.

AUTHOR CONTRIBUTIONS

MSM, CB, IE, GC, and SS designed the study and wrote the protocol. MM, TL, and GC conducted the lab experiments. MM managed the literature searches and analyses and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

FUNDING

The authors acknowledge the financial support of the CRC for Mental Health. The Cooperative Research Centre (CRC) programme is an Australian Government Initiative. The authors also wish to acknowledge the CRC Scientific Advisory Committee, in addition to the contributions of study participants, clinicians at recruitment services, staff at the Murdoch Children's Research Institute, staff at the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging, and research staff at the Melbourne Neuropsychiatry Centre, including coordinators, C. Phassouliotis, A. Merritt, and research assistants, A. Burnside, H. Cross, S. Gale, and S. Tahtalian. Participants for this study were sourced, in part, through the Australian Schizophrenia Research Bank (ASRB), which is supported by the National Health and Medical Research Council of Australia (Enabling Grant N. 386500), the Pratt Foundation, Ramsay Health Care, the Viertel Charitable Foundation, and the Schizophrenia Research Institute. The authors thank the Chief Investigators and ASRB Manager, V. Carr, U. Schall, R. Scott, A. Jablensky, B. Mowry, P. Michie, S. Catts, F. Henskens, C. Pantelis, C. Loughland. The authors acknowledge the help of Jason Bridge for ASRB database queries. MM was supported by a Cooperative Research Centre for Mental Health Top-up Scholarship. SS was supported by One-in-Five Association Incorporated. CSW is supported by Schizophrenia Research Institute (utilizing infrastructure funding from the NSW Ministry of Health and the Macquarie Group Foundation), the University of New South Wales, and Neuroscience Research Australia. CSW is a recipient of a National Health and Medical Research Council (Australia) Principal Research Fellowship (PRF) (#1117079). CP was supported by an NHMRC Senior Principal Research Fellowship (628386 and 1105825), and a Brain and Behavior Research Foundation (NARSAD) Distinguished Investigator Award. CB was supported by a NHMRC Career Development Fellowship (1127700) and Brain and Behavior Research Foundation (NARSAD) Young Investigator Award (20526). None of the funding sources played any role in the study design; collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fpsyt.2017.00225/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

Title: Peripheral Transcription of *NRG-ErbB* Pathway Genes is Upregulated in Treatment-Resistant Schizophrenia

SUPPLEMENTARY METHODS

Blood sampling method

Blood was collected after overnight fasting and processed according to the Australian Imaging, Biomarker and Lifestyle (AIBL) blood collection and processing protocol. A trained nurse or scientist undertook the blood draw at existing facilities within the local hospital and pathology services. The blood sample was then transported to the AIBL laboratory at the Mental Health Research Institute for further processing. Briefly, blood samples were collected in EDTA tubes (for DNA), PAXgene® Blood RNA tubes for total RNA extraction. Blood processing was done within 2 hours of venipuncture under sterile conditions. The gel serum tubes were allowed to clot before processing (at least 20 minutes following venipuncture). After the tubes were filled with blood, they were inverted 8-10 times for mixing. They were kept at ambient temperature until processed. The PAXgene tubes were allowed to stand upright at room temperature for 24 hours in the dark and then overnight at -20°C. The EDTA tubes were centrifuged for 15 minutes at 1500 x g at 20°C. All the tubes were stored at -80°C until further processing.

Cell culture method

The clozapine used in this experiment was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The concentration of clozapine used in this experiment was determined from the mean plasma concentration of clozapine found in the cohort of treatment-resistant schizophrenia patients (1.2µM or 384ng/mL). Clozapine induces PBMC death at higher concentrations ($5x10^{-6}$ to $2.5x10^{-5}$ M) dosage (1). In spite of this LDH toxicity assay was performed at baseline, 24 hours and 7 day time periods.

PBMCs were incubated in RPMI-1640 medium (Sigma-Aldrich; St. Louis, Missouri, USA) supplemented with L-glutamine (0.3g/L) and sodium bicarbonate (2g/L), penicillin (100units/mL), streptomycin (100µg/mL), 10% fetal bovine serum and 1.2µM of clozapine at 37°C in 5% CO_2. They were seeded at a concentration of 2 million cells per well ($1x10^6$ cells/mL) in triplicate in six-well plates and treated with 1.2µM of clozapine for 24 hours and 7 days. Absolute ethanol was used to prepare 10mM clozapine solution and it was diluted with media to prepare 0.48mM clozapine solution. From this 5µL was added to each well so that each well is exposed with 1.2µM of clozapine. Each control well was exposed with media only. Cells were exposed to clozapine for 24 hours and 7 days. Total RNA was extracted at both time points.

RNA extraction from cultured PBMCs and quality control

Total RNA was extracted from 24 hours and 7 day time periods from both clozapine exposed and control cells using PureLink™ RNA Mini Kit (Life Technologies®, ThermoFisher Scientific™, Waltham, MA, USA) using a standard protocol. Briefly, cells were lysed with using lysis buffer containing 1% 2-mercaptoethanol. After the pellets were dispersed and cells appear lysed they were homogenized by passing the lysate 5-10 times through an 18-gauzge syringe needle. Next, RNA purification was performed using wash buffer I and II and RNA was eluted in 30µL of RNase-free water. All the RNA tubes were stored at -80°C until further use. The quality of extracted RNA was checked for RIN and concentration using the Agilent® RNA ScreenTape assay with the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA).

Reverse Transcription of mRNA

After extraction, RNA was reverse transcribed to cDNA. Then, cDNA was used as a template for RT-qPCR using master-mix and gene specific validated Taqman assays from Applied Biosystems, Foster City, California, USA. For cDNA synthesis, total RNA (200 ng) was denatured for 5 min at 65°C, and then reverse transcribed using the SuperScript® IV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) in a 20µL reaction volume containing 1× Reverse Transcription SSIV buffer, 10mM dNTP mixture, 50µM random hexamers, 100mM DTT, 40 U/µl RNase OUT™ Recombinant RNase Inhibitor and 200 U/µl SuperScript® IV Reverse Transcriptase. The reaction (40 cycles) will be incubated at 23°C for 10 min, followed by 55°C for 10 min, 80°C for 10 min. To remove RNA, 1 µl of *E.coli* RNase H was added to each reaction mixture and incubated at 37°C for 20min and finally held at 4°C. All cDNA samples were stored at -20°C until qPCR analysis. In each plate, one no template control and one RT negative were used for quality control purposes.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Gene expression was performed using FAM-MGB TaqMan® gene expression probes (Invitrogen, Foster city, CA, USA) in 192.24 Dynamic Arrays IFC in Fluidigm® BioMark™ HD system (South San Francisco, CA, USA) at the MHTP Medical Genomics Facility (Monash Health Translation Precinct, Hudson Institute of Medical Research, Clayton, VIC, Australia). All the samples were run in duplicates in two independent 192.24 BioMark IFC arrays to ensure there was no technical variability. Prior to gene expression, quality control was performed. All the samples qualified except one and that was excluded from the further experiment. The RT negatives showed a very high Ct value indication absence or low genomic DNA contamination and the 'no template' control did not show any amplification.

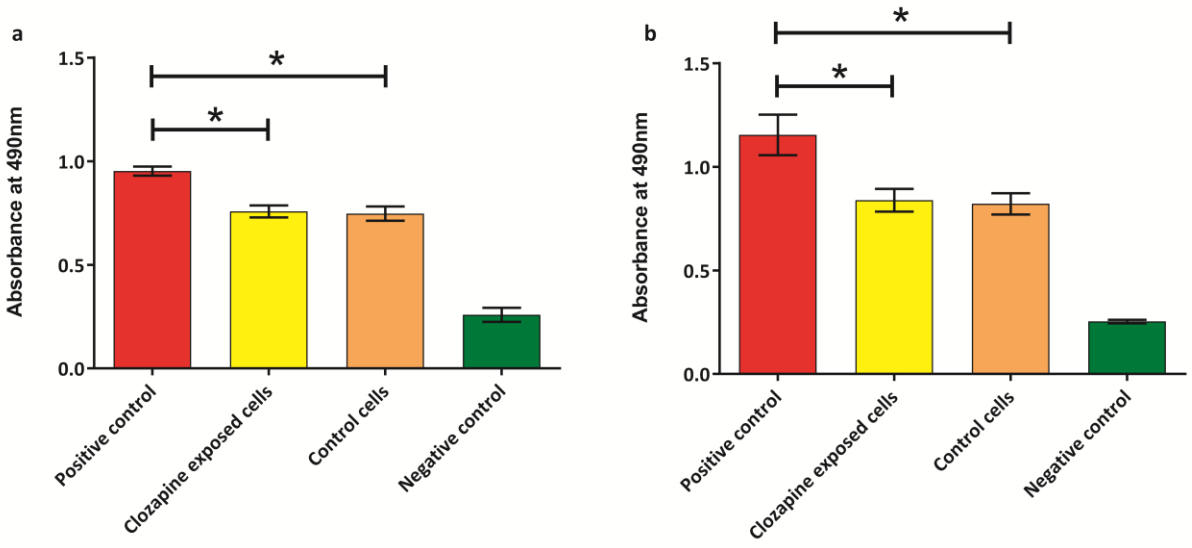
The FAM-MGB, TaqMan gene expression assays are provided as 20x forward and reverse primer and probe mixes. Each primer is at a concentration of 18µM and the probe is at a concentration of 4µM. The TaqMan assays were selected from the Single Cell Genomics Taqman Library at the Single Cell Genomics Centre (MHTP Medical Genomics Facility, Monash Health Translation Precinct, Clayton, VIC, Australia). Inventoried assays (TaqMan®, Invitrogen, Foster city, CA, USA) were used for all genes of interest and reference (housekeeping) genes. Supplementary table (2) contains the list of the probes and primers.

Pre-amplification was done to increase the number of copies of each gene to detectable levels as detailed in Gene Expression Preamp with Fluidigm® Preamp Master Mix and TaqMan® Assays Quick Reference PN 100-5876B1. To reduce bias, the pre-amplification procedure takes probes and primers of all genes of interest and makes a probe-primer pool and then all the samples get the equal amount of the mixture for amplification. Taqman assays were firstly pooled by combining 4µL of each of the 24 20X TaqMan assays and 304µL C1 DNA suspension buffer for a final volume of 400µL. The final concentration of each assay was 0.2X (180nM).

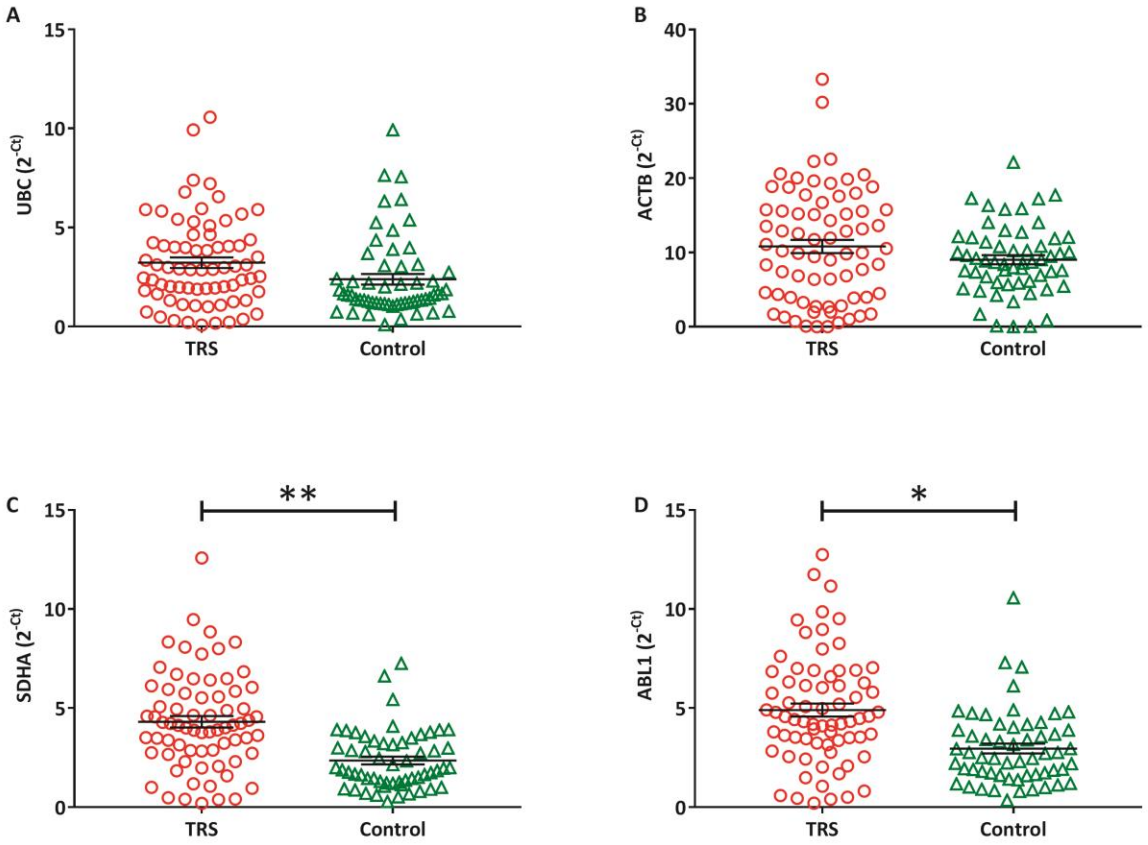
Pre-amplification allows multiplex amplification. 3.75µL of Sample Pre-Mix (Life Technologies TaqMan® PreAmp Master Mix and Pooled Taqman assays) was combined with 1.25µL of each of the cDNA samples, RT-negative samples, and 'no template' water controls for a final reaction volume of 5µL per sample. An additional no template control (by the gene expression facility) was also included and all samples were pre-amplified for 14 cycles. Following pre-amplification, reaction products were diluted 1:5 by adding 20µL C1 DNA suspension buffer to the final 5µL reaction volume for a total volume of 25µL.

Assays and Samples were combined in a 192.24 Dynamic Array IFC according to Fluidigm® 192.24 Real-Time PCR Workflow Quick Reference PN 100-6170. Briefly, 3µL of each assay at a final concentration of 10X was added to each assay inlet port and 3µL of diluted sample to each sample inlet port according to the Chip Pipetting Map. For unused sample inlets, 2.2uL of sample premix and 1.8uL of water per inlet were used. The data were analyzed with Fluidigm Real-Time PCR analysis software (V4.1.1).

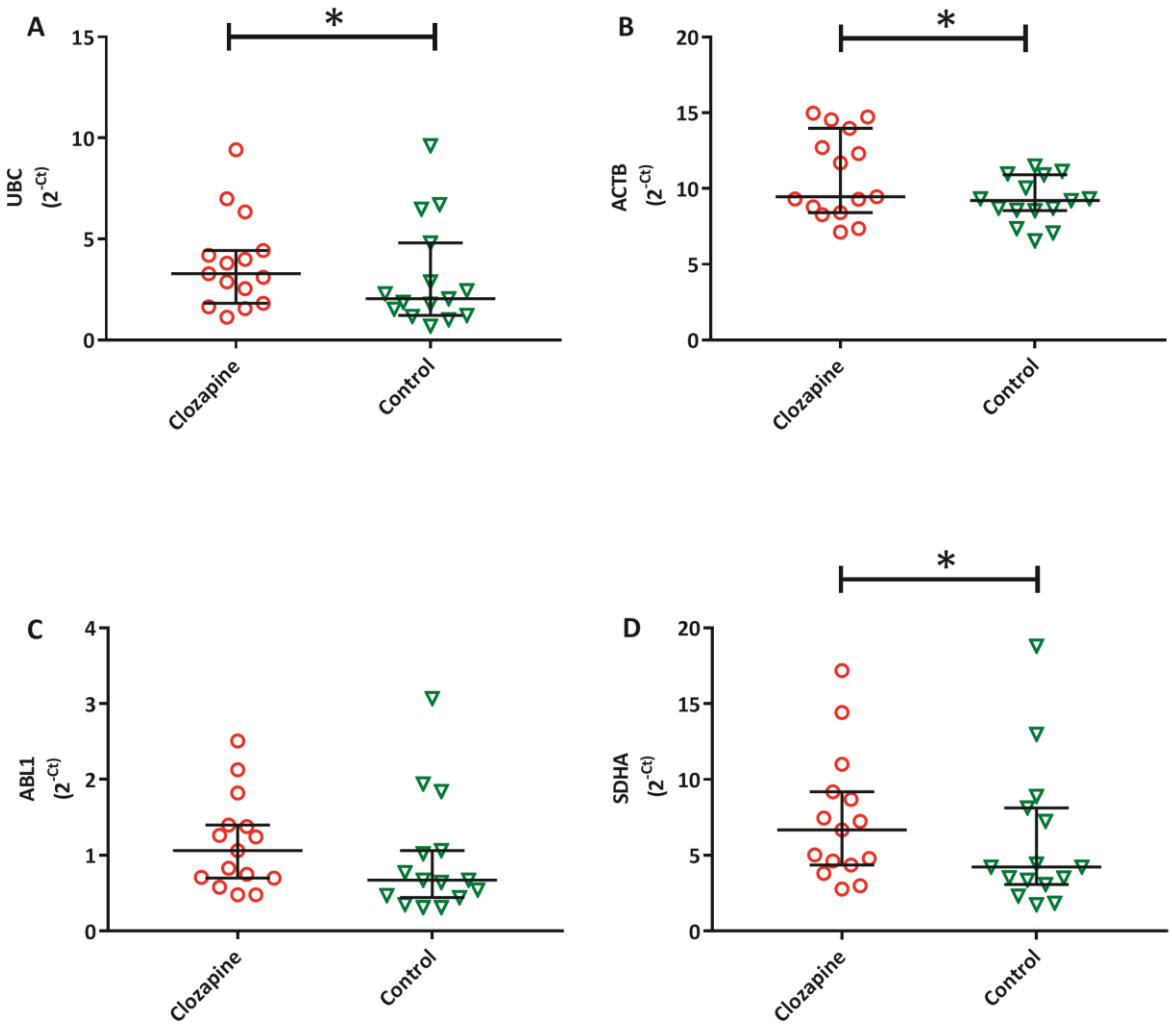
Normalized relative quantities of different gene isoforms (mRNA) were calculated relative to the geometric mean of two reference genes, beta actin (*ACTB*) and ubiquitin-c (*UBC*). The reference genes were selected based on a previously reported gene expression experiment conducted on post-mortem brain tissue in schizophrenia patients and controls (2). The relative quantities of *ACTB* and *UBC* were not significantly different between the groups in the clinical cohort. Two of the four reference genes (*ABL-1* and *SDHA*) were found to significantly differ in the clinical cohort and therefore was not used as reference genes for normalization. For the *in-vitro* cohort, *ABL-1* was stable at 24hours and *ACTB* was stable at 7days clozapine post exposure and so were used for normalization at specific time points.



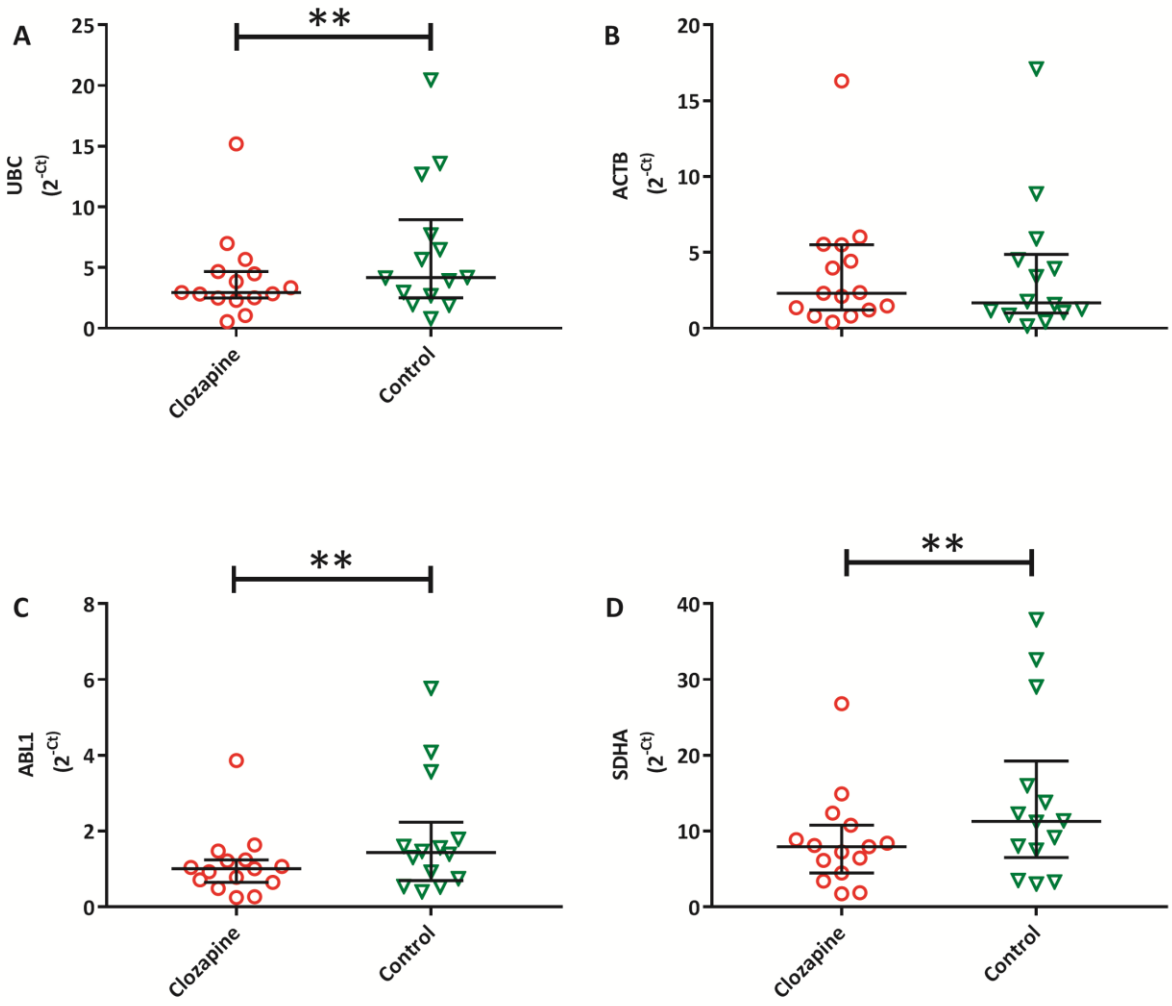
Supplementary Figure S1: LDH toxicity assay results in cultured PBMCs, (a) 24 hour post exposure and (b) 7-days post exposure. *P=0.001



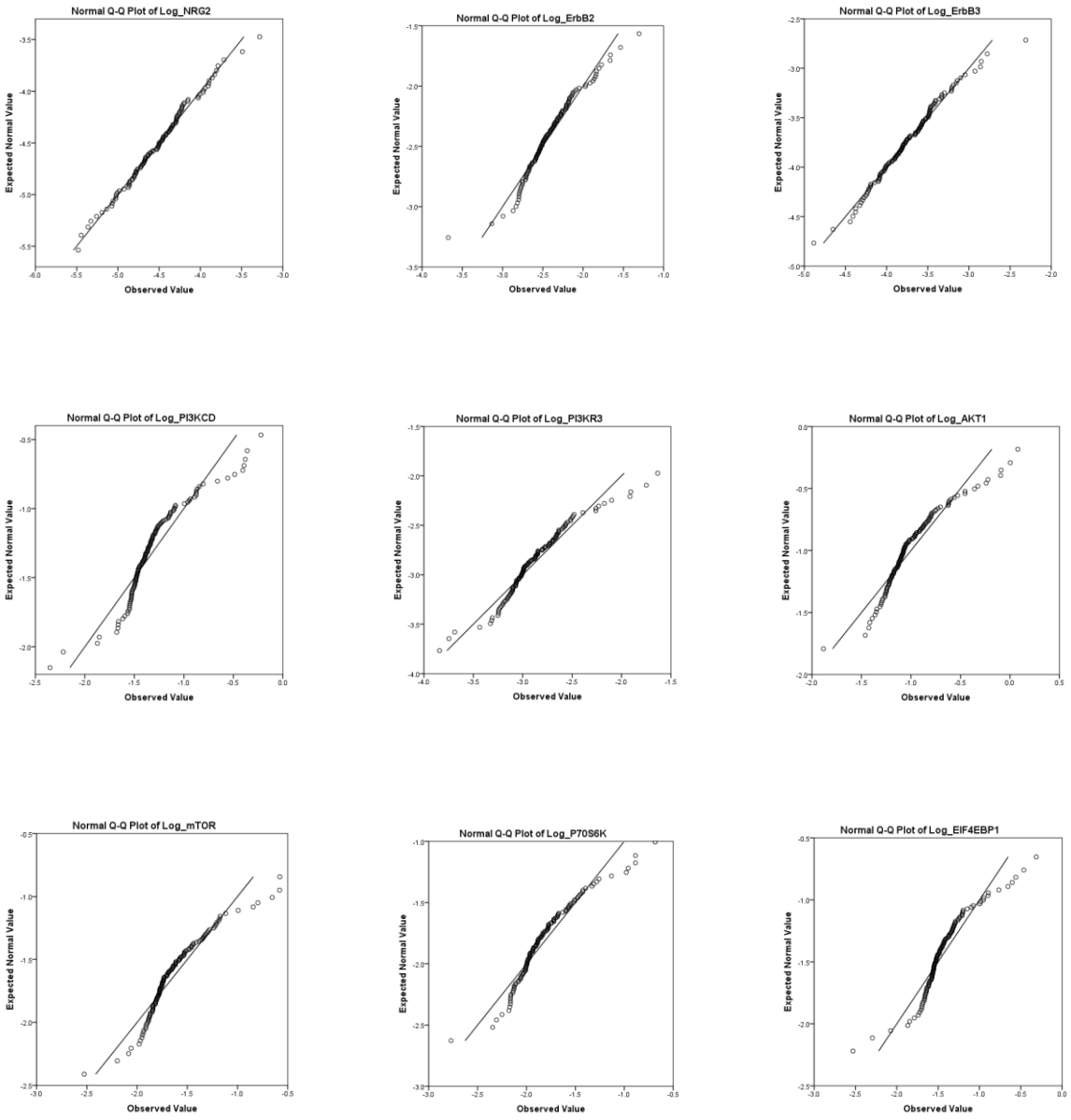
Supplementary Figure S2: Expression of reference genes in treatment-resistant schizophrenia (TRS) patients vs healthy controls; (**A**) *UBC* (t=-1.877, df =126, P=0.063), (**B**) *ACTB* (t=0.140, df=126, P=0.889), (**C**) *SDHA* (t=-4.20, df=87.383, P=0.000051), (**D**) *ABL-1* (t=-3.466, df=105.563, P=0.001). Error bars represent mean ± s.e.m. *P<0.05, **P<0.001.



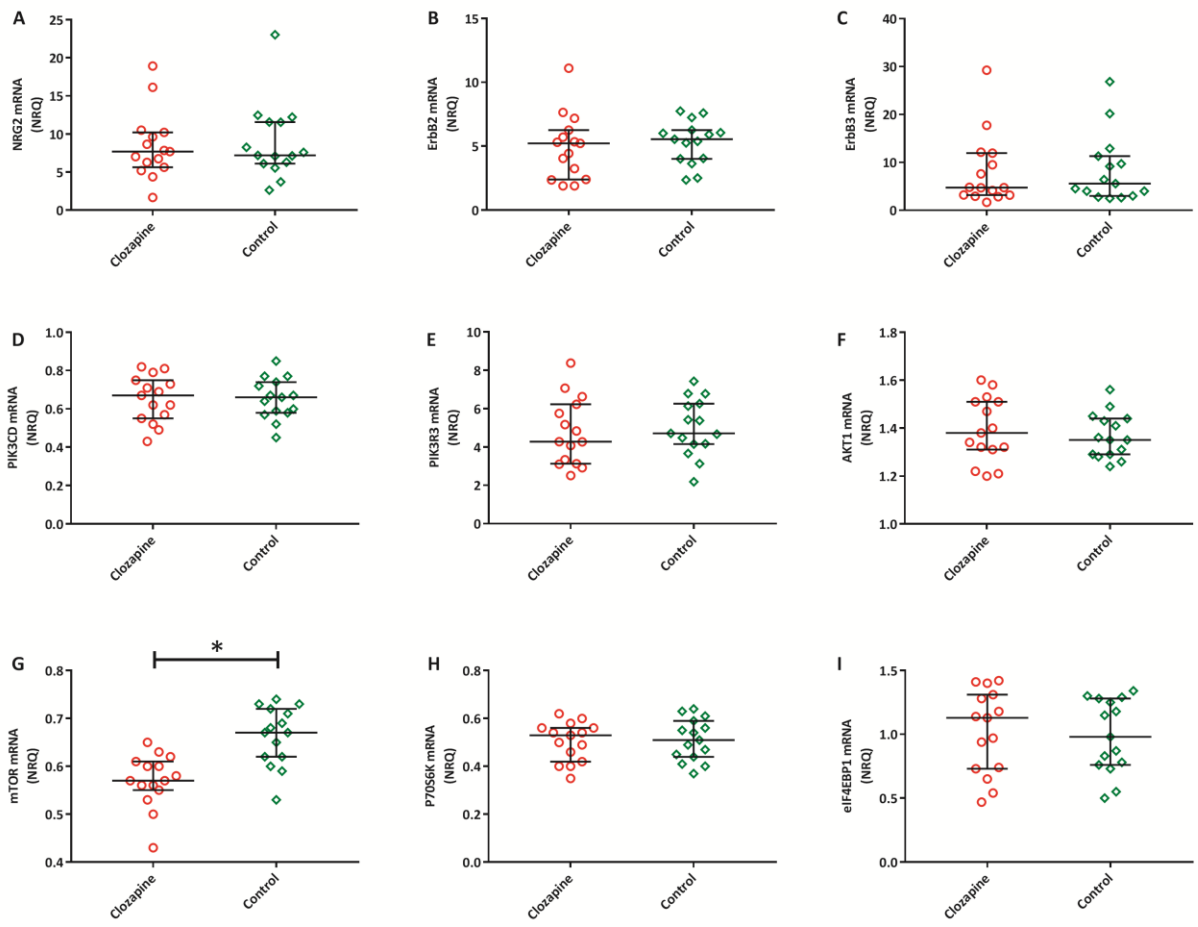
Supplementary Figure S3: Expression of reference genes after 24hours clozapine exposure. Wilcoxon signed rank test (matched pair, α=0.05, N=15) was used to measure the difference in reference gene expression between clozapine exposed and control cells; (**a**) *UBC* (W=-2.385, P=0.017), (**b**) *ACTB* (W=2.499, P=0.012), (**c**) *ABL-1* (W=-1.931, P=0.053), (**d**) *SDHA* (W=-2.158, P=0.031). Error bars represent median ± interquartile range. *P<0.05.



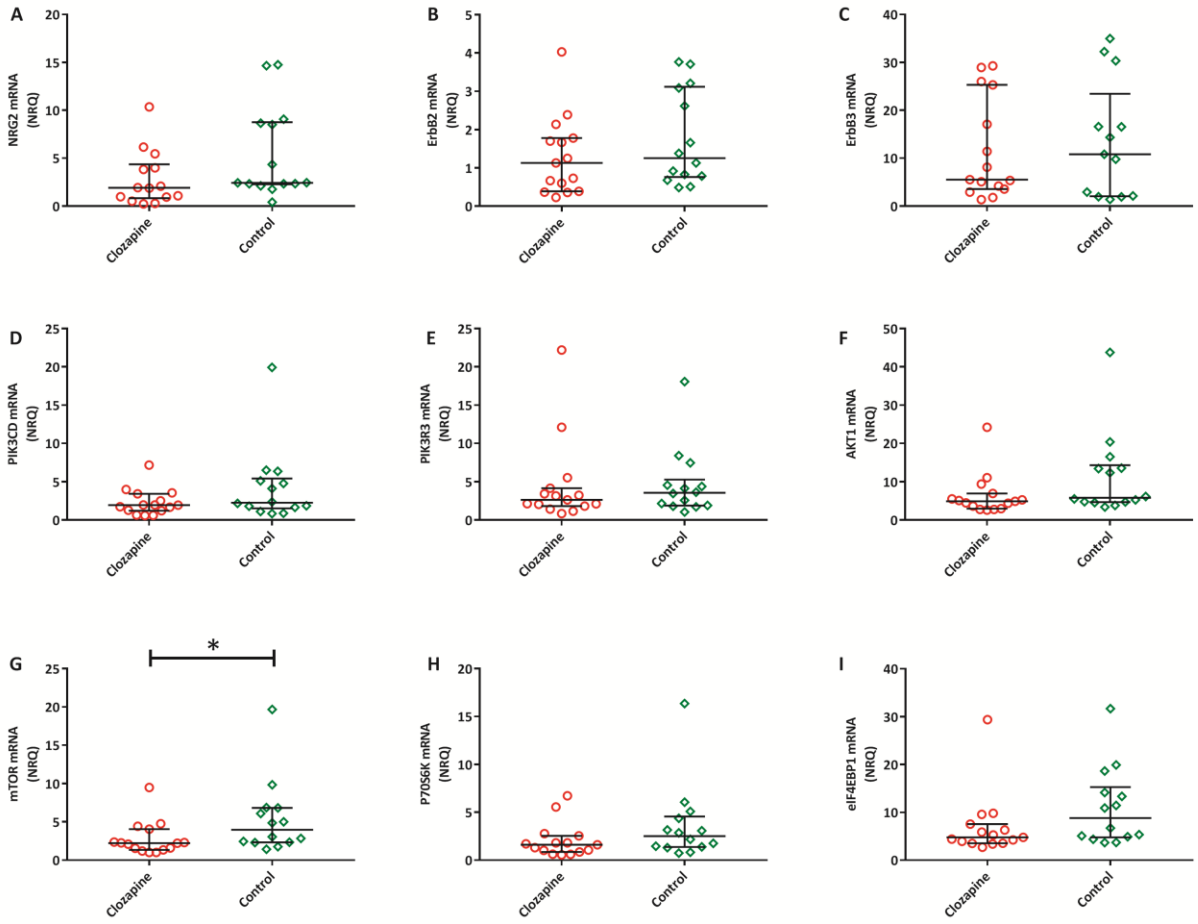
Supplementary Figure S4: Expression of reference genes after 7 days clozapine exposure. Wilcoxon signed rank test (matched pair, α=0.05, N=15) was used to measure the difference in reference gene expression between clozapine exposed and control cells; (**a**) *UBC* (W=3.045, P=0.002), (**b**) *ACTB* (W=-0.722, P=0.470), (**c**) *ABL-1* (W=3.233, P=0.001), (**d**) *SDHA* (W=3.233, P=0.001). Error bars represent median ± interquartile range. **P<0.01.



Supplementary Figure S5: Quantile-quantile plots of Log10 transformed NRQ values for (A) *NRG2* (SW=0.992, df=112, P=0.789), (B) *ErbB2* (SW=0.961, df=127, P=0.001), (C) *ErbB3* (SW=0.987, df=126, P=0.279), (D) *PIK3CD* (SW=0.887, df=128, P<0.001) and (E) *PIK3R3* (SW=0.941, df=127, P<0.001), (F) *AKT1* (SW=0.910, df=127, P<0.001), (G) *mTOR* (SW=0.908, df=127, P<0.001), (H) *P70S6K* (SW=0.925, df=127, P<0.001), (I) *eIF4EBP1* (SW=0.889, df=127, P<0.001). SW=Shapiro-Wilk test.



Supplementary Figure S6: Expression of detectable gene transcripts after 24hours clozapine exposure. Wilcoxon signed rank test (matched pair) was used to measure the difference in the normalized quantity of transcripts between clozapine exposed and control cells; (**A**) *NRG2* (W=0.341, P=0.733), (**B**) *ErbB2* (W=1.874, P=0.061), (**C**) *ErbB3* (W=0.966, P=0.334), (**D**) *PIK3CD* (W= 0.001, P=1.0), (**E**) *PIK3R3* (W= 1.193, P=0.233), (**F**) *AKT1* (W=-1.079, P=0.281), (**G**) *mTOR* (W=3.408, P=0.001), (**H**) *P70S6K* (W=1.59, P=0.112), (**I**) *eIF4EBP1* (W=-0.341, P=0.733). Error bars represent median ± interquartile range. *P<0.01.



Supplementary Figure S7: Expression of detectable gene transcripts after 7 days clozapine exposure. Wilcoxon signed rank test (matched pair) was used to measure the difference in the normalized quantity of transcripts between clozapine exposed and control cells; (**A**) *NRG2* (W=1.922, P=0.055), (**B**) *ErbB2* (W=1.601, P=0.109), (**C**) *ErbB3* (W=1.915, P=0.056), (**D**) *PIK3CD* (W=1.664, P=0.096), (**E**) *PIK3R3* (W=1.161, P= 0.245), (**F**) *AKT1* (W=1.726, P=0.084), (**G**) *mTOR* (W=2.80, P=0.005), (**H**) *P70S6K* (W=1.664, P=0.096), (**I**) *eIF4EBP1* (W=1.726, P=0.084). Error bars represent median ± interquartile range. *P<0.01.

No.	Gene	Isoforms detected	Inventoried assay
1	*NRG2*	DON1, HRG2, NTAK	Hs00171706_m1
2	*ErbB1/EGFR*	HER1, NISBD2, PIG61,mENA	Hs01076090_m1
3	*ErbB2*	CD340, HER-2, HER-2/neu, HER2, MLN 19, NEU, NGL, TKR1	HS01001580_m1
4	*ErbB3*	HER3, LCCS2, MDA-BF-1, c-erbB-3, c-*ErbB3*, *ErbB3*-S, p180-*ErbB3*, p45-s*ErbB3*, p85-s*ErbB3*	Hs00176538_m1
5	*ErbB4*	ALS19, HER4, p180*ErbB4*	Hs00955525_m1
6	*PIK3CD*	APDS, IMD14, P110DELTA, PI3K, p110D	Hs00908666_m1
7	*PIK3R3*	p55, p55-GAMMA, p55PIK	Hs01103591_m1
8	*AKT1*	AKT, CWS6, PKB, PKB-ALPHA, PRKBA, RAC, RAC-ALPHA	Hs00178289_m1
9	*mTOR*	FRAP, FRAP1, FRAP2, RAFT1, RAPT1, SKS	Hs00234508_m1
10	*P70S6K*	PS6K, S6K, S6K-beta-1, S6K1, STK14A, p70 S6KA, p70(S6K)-alpha, p70-S6K, p70-alpha	Hs00356367_m1
11	*eIF4EBP1*	4E-BP1, 4EBP1, BP-1, PHAS-I	Hs00607050_m1
12	*ABL-1*	ABL proto-oncogene 1	Hs01104728_m1
13	*SDHA*	Succinate Dehydrogenase Complex Flavoprotein Subunit A	Hs00188166_m1
14	*ACTB*	beta-actin	Hs99999903_m1
15	*UBC*	ubiquitin C	Hs00824723_m1

Supplementary Table S1: Inventoried TaqMan assays (Applied Biosystems, Foster City, CA, USA) for quantification and normalization of NRG-ErbB pathway genes of interest expression. The geometric mean of *UBC* and *ACTB* were used for transcript normalization.

Transcripts	Age of Onset, years	Clozapine Plasma level (µg/L)	Chlorpromazine equivalent antipsychotic exposure (excluding clozapine)
NRG2	0.130 (0.315)	0.117 (0.360)	0.010 (0.936)
ErbB2	0.019 (0.879)	-0.014 (0.911)	0.083 (0.493)
ErbB3	-0.051 (0.679)	0.094 (0.444)	-0.020 (0.869)
PIK3CD	0.033 (0.791)	-0.016 (0.893)	0.082 (0.500)
PIK3R3	-0.063 (0.607)	-0.024 (0.847)	0.057 (0.639)
AKT1	0.003 (0.983)	0.023 (0.853)	0.076 (0.532)
mTOR	0.003 (0.981)	0.033 (0.785)	0.012 (0.922)
P70S6K	-0.004 (0.977)	0.018 (0.880)	0.017 (0.891)
eIF4EBP1	-0.198 (0.104)	0.022 (0.855)	0.071 (0.558)

Supplementary Table S2: Spearman's correlation (raw P-value) between different gene transcripts level with age of onset, clozapine plasma level and chlorpromazine equivalent antipsychotic exposure (excluding clozapine).

Transcripts	Positive score	Negative score	Disorganized score	Excitement score	Depression score	Total score
NRG2	-0.138 (0.667)	0.010 (0.964)	0.117 (0.667)	0.077 (0.784)	-0.125 (0.667)	-0.086 (0.778)
ErbB2	-0.069 (0.784)	0.161 (0.667)	0.051 (0.828)	0.289 (**0.014**, 0.667)	-0.073 (0.784)	0.097 (0.687)
ErbB3	-0.066 (0.787)	0.212 (0.667)	0.044 (0.834)	0.196 (0.667)	-0.013 (0.964)	0.132 (0.667)
PIK3CD	0.108 (0.667)	0.070 (0.784)	0.146 (0.667)	0.061 (0.787)	0.042 (0.834)	0.124 (0.667)
PIK3R3	0.006 (0.964)	0.103 (0.667)	0.037 (0.852)	0.230 (0.667)	-0.141 (0.667)	0.061 (0.787)
AKT1	0.117 (0.667)	0.119 (0.667)	0.183 (0.667)	0.142 (0.667)	0.005 (0.964)	0.160 (0.667)
mTOR	0.045 (0.834)	0.105 (0.667)	0.166 (0.667)	0.103 (0.667)	-0.107 (0.667)	0.121 (0.667)
P70S6K	0.093 (0.895)	-0.026 (0.667)	0.121 (0.667)	0.180 (0.667)	0.142 (0.667)	-0.124 (0.701)
eIF4EBP1	0.032 (0.874)	0.176 (0.667)	0.222 (0.667)	0.183 (0.667)	-0.051 (0.828)	0.178 (0.667)

Supplementary Table S3: Spearman's correlation (raw P-value, Benjamini-Hochberg adjusted P- value) between gene transcripts level with PANSS positive, negative, disorganized, excited, depression score and total score.

Gene transcripts	Remission N; median (IQR)	Non-remission N; median (IQR)	Raw P value	BH P value*
NRG2	28; 3.82 (1.52-5.88)	35; 3.01 (1.41-4.40)	0.261	-
ErbB2	31; 3.85 (2.55-6.78)	39; 3.68 (2.20-6.01)	0.727	-
ErbB3	31; 2.58 (1.45-3.79)	39; 2.10 (0.93-3.24)	0.158	-
PIK3CD	31; 4.02 (3.45-7.34)	40; 4.82 (3.43-7.62)	0.557	-
PIK3R3	31; 1.34 (0.77-2.04)	40; 1.33 (0.97-2.20)	0.581	-
AKT1	31; 0.86 (0.73-1.61)	40; 1.02 (0.79-1.72)	0.523	-
mTOR	31; 1.99 (1.66-4.31)	39; 2.29 (1.70-3.60)	0.837	-
P70S6K	31; 1.47 (1.25-2.68)	39; 1.67 (1.10-2.72)	0.982	-
eIF4EBP1	31; 3.70 (2.77-5.69)	39; 3.99 (2.92-5.61)	0.502	-

Supplementary Table S4: Normalized relative quantities (NRQ) of detectable gene transcripts by positive symptom remission status. Positive symptom remission was defined as a score of ≤3 on four PANSS items (delusions, hallucinations, grandiosity and unusual thought content) (3). *Benjamini-Hochberg adjusted P-value

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Chapter 5
Discussion

5.1 Overall findings

The overall aim of this thesis was to examine if gene transcripts (mRNA) and protein expression variations within the *NRG1-ErbB* pathway genes could be used as peripheral biomarkers for symptom severity and symptom remission in treatment-resistant schizophrenia (TRS) with a close focus on the *NRG1* gene. Moreover, we looked at genetic variations in the *NRG1* gene to see which SNPs and microsatellites may be acting as susceptible loci for schizophrenia using a meta-analytic approach. Chapter 2 showed that two SNPs and one microsatellite within the *NRG1* gene were robustly associated with schizophrenia (Mostaid et al., 2017) despite previous GWAS studies that have failed to identify *NRG1* variation as a risk for the disorder (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Thus, our meta-analytic findings resurrect *NRG1* as a susceptible gene for schizophrenia and suggests further close investigation is warranted. The results presented in Chapter 3 showed an up-regulation of specific *NRG1* transcripts (*NRG1 EGFα, NRG1 EGFβ, NRG1 type* $I_{(Ig2)}$) in the whole blood of patients with treatment-resistant schizophrenia and that *NRG1 EGFα* and *NRG1 EGFβ* expression were negatively correlated with age of onset. Contrary to the increase in mRNA expression, serum NRG1-β1 protein expression was lower in patients but we also showed that this decrease in protein was likely confounded by smoking. Moreover, the *NRG1* SNPs and haplotypes investigated in this study showed no cis-regulatory effects on mRNA and protein expression. To explore beyond *NRG1*, in Chapter 4 the peripheral expression pattern of all the major *NRG-ErbB* pathway genes along with *NRG2* in whole blood in the same cohort were examined. Consistent with our results in Chapter 3, we found an overall upregulation of *NRG-ErbB* pathway transcripts in treatment-resistant schizophrenia and this upregulation was negatively correlated with duration of illness.

5.2 Genetic variants in the 5' and 3' region of *NRG1* are associated with schizophrenia

NRG1 has been one of the most heavily studied genes in schizophrenia but recent GWAS studies have failed to identify it as a schizophrenia 'risk' gene (Schizophrenia Working Group of the Psychiatric Genomics, 2014). GWAS studies are extremely important to find susceptible markers for complex mental health disorders but they focus exclusively on SNPs, ignoring other types of variation such as microsatellites and haplotypes. Thus, the importance of a gene to schizophrenia may be overlooked if conclusions are drawn based on GWAS studies only. Several linkage and association studies along with gene and protein expression studies have reported an association between *NRG1* gene and schizophrenia (Mostaid et al., 2016). In Chapter 2, we have shown that two SNPs (rs62510682, rs2954041) and one microsatellite marker (478B14-848) of *NRG1* gene have a robust association with schizophrenia. An important finding of this study was the association between rs2954041, a 3' region SNP, and schizophrenia, which is supported by a previous genetic association study in Han Chinese population (Yang et al., 2003). The location of this SNP is near (~18 kb) the promoter region for the most brain abundant *NRG1* isoform, *NRG1* type III (X. Liu et al., 2011). Thus, rs2954041 may act as an eQTL for *NRG1* type III. Transgenic mice with *Nrg1typeIII* overexpression have shown schizophrenia related phenotypes such as decreased pre-pulse inhibition, impairment of fear-associated memory, and lack of social preference (Olaya et al., 2017). Future studies should consider if this SNP has any pathological link to schizophrenia and whether it serves as an eQTL for type III expression or any other *NRG1* isoform or protein expression in post-mortem human brain tissue. In chapter 3, we did not find evidence to suggest this SNP served as an eQTL for *NRG1* type III or any other of the tested *NRG1* isoforms in whole blood nor did we detect a significant correlation with serum NRG1-β1 protein expression. However, the correlation between central and peripheral expression of *NRG1* is unclear and as such the absence of an eQTL in blood does not rule out its presence in the brain. Concurrent analysis with post-mortem human brain

tissue and periphery are needed to investigate the similarities in expression profile of *NRG1* type III and if rs2954041 act as an eQTL for mRNA expression in both tissue type.

Our meta-analysis also reported the association of three 5' markers (rs62510682, rs35753505 and 478B14-848) of *NRG1* with schizophrenia. All three are part of the original 7-marker HapICE haplotype reported by Stefansson et al. (Stefansson et al., 2002) and 478B14-848. had been shown to be associated with schizophrenia in two earlier meta-analyses (Gong et al., 2009; Li, Collier, & He, 2006). However, our results in Chapter 2 showed for the first time the likely presence of population stratification. The '0' allele of 478B14-848 was the risk allele for schizophrenia in Caucasian population, while the '4' allele served as the risk allele among Asians. Moreover, the '0' allele is extremely rare in Asian populations (Tang et al., 2004; Zhao et al., 2004) and the '4' allele was 'protective' among Caucasians (Mostaid et al., 2017). Likewise, we found an association of rs35753505 in Asians only, whereas a previous meta-analysis (Li et al., 2006) reported the association in Caucasians only. However, the exact mechanism of how these population specific findings confer schizophrenia risk or protection is not clear and warrants further investigation.

5.3 Elevated expression of *NRG1* mRNA isoforms in whole blood may serve as biomarkers for treatment-resistant schizophrenia

Identification of suitable biomarkers for diagnosis and prediction of treatment response in schizophrenia is extremely important towards making personalized treatment a reality (Lai et al., 2016; Weickert, Weickert, Pillai, & Buckley, 2013). In Chapter 3, we interrogated eight major *NRG1* mRNA transcripts (*NRG1* type I, II, III, IV, EGFα, EGFβ, type $I_{(Ig2)}$, *and* pan-*NRG1*) in whole blood as well as NRG1-β1 protein in serum from treatment-resistant schizophrenia (TRS) patients and controls. We found higher expression of *NRG1* EGFα, EGFβ, type $I_{(Ig2)}$ in whole blood of TRS patients but no difference in expression for *NRG1* type III. Our findings are the first report of upregulation of

these specific *NRG1* mRNA isoforms in TRS patients. A previous study reported no difference in peripheral expression of two *NRG1* isoforms (ndf43a and b) covered by *NRG1* EGFβ probe (Petryshen et al., 2005) and another post-mortem study reported no difference in expression of *NRG1* EGFβ in DLPFC of schizophrenia patients (Weickert, Tiwari, Schofield, Mowry, & Fullerton, 2012). Our findings add to this research suggesting that upregulation of specific *NRG1* mRNA isoforms may be a unique feature of treatment-resistant schizophrenia. However, an obvious limitation is that we did not include schizophrenia patients without TRS. Future studies are thus required to investigate if these *NRG1* isoforms are specific markers for TRS or schizophrenia more generally.

Results of our mRNA findings did not translate to protein. Lower rather than higher expression of NRG1-β1 protein was detected in TRS patients compared to healthy controls. After adjusting for smoking status this result did not survive, suggesting that smoking is a potential confounder for protein levels in serum. Smokers, in general, had a lower protein level compared to non-smokers regardless of case status. Two previous peripheral protein expression studies reported decreased NRG1 protein levels in plasma (Wang et al., 2015) and serum (Shibuya et al., 2010) but neither study adjusted for smoking status in their analyses. It is not clear how smoking affects the protein expression level in the periphery and whether similar effects are observable in central tissue. A recent study by Gupta et al. demonstrated that *NRG1* mRNA expression levels in occasional smokers resemble that of non-smokers compared with daily smokers (Gupta et al., 2017). Collectively, these findings advocate for careful measurement of smoking status in studies investigating NRG1 protein level in serum and perhaps plasma as well as post-mortem brain tissue. However, our results would. Preclinical studies are also warranted to determine the mechanism by which smoking effects central and peripheral NRG1 protein expression.

In summary, the results of chapter 3 showed peripheral expression of specific *NRG1* mRNA isoforms (NRG1 *EGFα,* NRG1 *EGFβ,* NRG1 *type I* $_{(Ig2)}$) have potential to be used as clinical biomarkers for treatment-resistant schizophrenia but needs to be replicated and validated in other independent cohorts. Our second hypothesis is thus partially fulfilled as upregulation of some *NRG1* mRNA isoforms were observed in TRS patients but could not differentiate the TRS patients from non-TRS patients. Moreover, they are not associated with symptom severity or remission status suggesting examination of other genetic and/or environmental factors are warranted. Our third hypothesis was not supported by our experiment results as examination of SNPs and haplotypes could not detect any eQTLs for mRNA and protein expression suggesting that expression of *NRG1* may be regulated by distant SNPs in other genes and needs further investigation in larger cohorts. *In-vitro* clozapine exposure does not cause significant changes in *NRG1* mRNA or protein expression in the periphery. This result did not support our fourth hypothesis and confirmed that elevation of gene expression in the periphery may not be due to clozapine therapy rather a signature of treatment-resistant schizophrenia. Finally, we found that smoking is associated with lower NRG1 protein expression in serum in TRS patients which implies smoking needs to be taken into consideration in future proteomic studies aimed at clinically suitable biomarkers for treatment-resistant schizophrenia.

5.4 *NRG1* mRNA isoforms are associated with age of illness onset but not symptom severity or remission status in treatment-resistant schizophrenia

In Chapter 3, we showed that expression of *NRG1* EGFα and *NRG1* EGFβ were negatively correlated with age of illness onset. These findings suggest that upregulation of *NRG1* isoforms may not be clinically useful in all patients but rather could be specific to those with an earlier stage of illness onset. A post-mortem gene expression study by Weickert et al. reported that expression of *NRG1* type III was negatively correlated to the age of illness onset (Weickert et al., 2012). However,

in our study, we found no such correlation for *NRG1* type III. Together, these results suggest a relationship between *NRG1* mRNA and age of illness onset but this relationship appears to be tissue and isoform specific. Nonetheless, future longitudinal data is needed to investigate if the higher expression of specific *NRG1* mRNA isoforms is a contributing factor or consequence of an earlier age of illness onset.

We found limited evidence suggesting *NRG1* mRNA or protein levels were associated with symptom severity. This suggests that other genetic or environmental factors may be responsible for symptom severity where *NRG1* may be involved indirectly in the complex mechanism. Moreover, there was no difference in mRNA and protein expression between patients under positive symptom remission and non-remission which leads to the idea that *NRG1* expression levels may not be unique to any sub-group of TRS patients. We did detect trend level negative association between *NRG1* EGFα expression with depression severity score and *NRG1* type III expression with positive symptom severity score. Overall this suggests that a larger sample size with greater variance in symptom severity may be able to detect a significant signal in TRS patients. However, the effect sizes (correlation coefficients) were modest and as such even if they reached statistical significance, the clinical relevance would be debatable. Furthermore, our ability to detect differences by remission status were hindered by the number of patients meeting remission criteria. Future longitudinal studies using serial measurements starting at the initiation of clozapine therapy would be better suited for investigating *NRG1* mRNA and protein levels as makers of clozapine response and remission.

5.5 Increased peripheral transcription of *NRG-ErbB* pathway genes may be a signature of treatment-resistant schizophrenia

Chapter 4 aimed to investigate whether upregulation of the *NRG1* isoforms reported in Chapter 3 would lead to an upregulation of downstream signaling genes in the NRG-ErbB pathway. Among the eleven transcripts (*NRG2, ErbB1, ErbB2, ErbB3, ErbB4, PIK3CD, PIK3R3, AKT1, mTOR, P70S6K, eIF4EBP1*) examined, *ErbB1* and *ErbB4* were not detectable in whole blood. *ErbB1* and *ErbB4* are found in all cell populations related to schizophrenia pathophysiology such as GABAergic neurons, glial cells and dopaminergic neurons (Iwakura & Nawa, 2013) but both were beyond the limit of detection in whole blood. This suggests that *NRG-ErbB* signaling in the periphery may be activated by binding of specific *NRG1* isoforms (ligands) to the *ErbB3* rather than *ErbB1* or *ErbB4* receptors. Interestingly, our analysis showed that five transcripts (*ErbB3, PIK3CD, AKT1, P70S6K, eIF4EBP1*) were upregulated in whole blood in TRS patients although only one i.e. *P70S6K* survived correction for multiple comparisons. These findings supported our hypothesis that increased expression of *NRG1* transcripts would result in elevated expression of downstream signaling molecules in whole blood and generalized upregulation of *NRG-ErbB* signaling in schizophrenia, more specifically treatment-resistant schizophrenia. Our findings are supported by previous studies where they reported increased expression of several *NRG-ErbB* pathway downstream signalling molecules such as *PIK3CD*, *PIK3CB* (Law et al., 2012; L. Liu et al., 2016) and *AKT1* (L. Liu et al., 2016; Xu et al., 2016) in the peripheral blood from patients with schizophrenia. Increased expression of *mTOR, P70S6K,* and *eIF4B* are also found in patients with major depressive disorder (Jernigan et al., 2011). Notably, we are the first to report an increase in *P70S6K* in schizophrenia and as such the suitability of *P70S6K* as a biomarker of treatment-resistant schizophrenia remains to be determined.

Aligned with our NRG1 findings in Chapter 3, we also found that expression of *ErbB2, PIK3CD, PIK3R3, AKT1, mTOR,* and *P70S6K* were negatively correlated with duration of illness.

Together, these findings suggest that an increase in *NRG-ErbB* pathway gene expression is more prominent in a subgroup of treatment-resistant schizophrenia patients who have made their transition to treatment-resistance from a typical schizophrenia state in a quick time span. Thus, a relatively shorter time for the transition to full-blown symptoms may be responsible for this overall increased expression. Moreover, we speculate that, with more illness duration, a schizophrenia patients body has more time to adjust and compensate for the abnormalities via some other alternative pathway because we know that many molecules other than *NRG1* such as *NRG6, AR, BTC, TGFα, EGF* can bind with *ErbB* molecules to activate the downstream signaling pathway (Iwakura & Nawa, 2013). Future studies need to investigate if specific *NRG1* isoforms compete with other molecules to bind and activate the *PI3K-AKT* signaling pathway.

In summary, the results of chapter 4 showed that peripheral transcription of genes within the *NRG-ErbB* pathway showed a similar pattern of elevated expression in the periphery, which partially supports our hypothesis. However, we could not find any relationship of mRNA expression with symptom severity or remission status suggesting the mRNA levels of the *NRG-ErbB* pathway genes we examined may not be directly involved with symptom severity or have modest effects that will only be detectable in larger cohorts.

5.6 Future studies

The results of this thesis reveal a number of opportunities for future research. First, it is not clear how generalizable our findings are to other ethnic populations and as such future studies are need to confirm our findings of upregulation of specific *NRG1* mRNA transcripts in whole blood among patients of non-European background with treatment-resistant schizophrenia. Second, detection of associations between SNPs and gene/protein expression in this thesis was likely inhibited by sample size. As such, future studies in larger cohorts are required to further explore possible eQTLs for *NRG1* mRNA and protein expression. Third, our findings suggest smoking status interacts with

NRG1 mRNA and protein expression. However, the mechanisms by which this interaction occurs is unknown and requires further in-depth analysis. Preclinical studies with animal models may be particularly useful for disentangling the effect of smoking on NRG1 protein expression. Fourth, additional studies are needed to determine if the general upregulation of peripheral mRNA among genes within the *NRG-ErbB* pathway can be observed at the protein level and whether this pathway could severe as a target for novel therapies. Finally, longitudinal studies and/or cross-sectional studies across the different stages (prodromal, first-episode, TRS) of schizophrenia are required to determine if the effects observed in this thesis are specific to TRS, a general trait of the illness regardless of stage, or dynamically changing throughout the course of the illness.

5.7 Conclusion

Results from this thesis suggest associations between *NRG1* and schizophrenia and/or TRS are present at the sequence, transcript, and protein level. At the sequence level, we found several genetic variants at both the 5' and 3' ends of the gene that are associated with schizophrenia but are likely population specific. At the transcript level, *NRG1* mRNA transcripts as well as transcripts in the *NRG-ErbB* pathway were upregulated in the whole blood of individuals with TRS and negatively correlated with age of illness onset. Whereas at the protein level, NRG1-β1 levels in serum were lower in TRS patients but this was presumably due to the confounding effect of smoking. Collectively, these results suggest *NRG1* genetic variation, mRNA and protein expression, and *NRG-ErbB* pathway gene expression are relevant to schizophrenia. Given the paucity of candidate biomarkers for schizophrenia and/or TRS, these results empirically support *NRG1* and the *NRG-ErbB* pathway as promising markers that warrant further investigation.

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Appendix

Neuroscience and Biobehavioral Reviews 68 (2016) 387–409

Contents lists available at ScienceDirect

Neuroscience and Biobehavioral Reviews

journal homepage: www.elsevier.com/locate/neubiorev

Review article

Neuregulin-1 and schizophrenia in the genome-wide association study era

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ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form 30 May 2016
Accepted 3 June 2016
Available online 6 June 2016

Keywords:
Neuregulin 1
Schizophrenia
Psychosis
Excitatory neurotransmission
Inhibitory neurotransmission
Neuroimaging
Genotype
Transcript

ABSTRACT

Clinical and pre-clinical evidence has implicated neuregulin 1 (*NRG1*) as a critical component in the pathophysiology of schizophrenia. However, the arrival of the genome-wide association study (GWAS) era has yielded results that challenge the relevance of *NRG1* in schizophrenia due to the absence of a genome-wide significant *NRG1* variant associated with schizophrenia. To assess *NRG1*'s relevance to schizophrenia in the GWAS era, we provide a targeted review of recent preclinical evidence on *NRG1's* role in regulating several aspects of excitatory/inhibitory neurotransmission and in turn schizophrenia risk. We also present a systematic review of the last decade of clinical research examining *NRG1* in the context of schizophrenia. We include concise summaries of genotypic variation, gene-expression, protein expression, structural and functional neuroimaging as well as cognitive studies conducted during this time period. We conclude with recommendations for future clinical and preclinical work that we hope will help prioritize a strategy forward to further advance our understanding of the relationship between *NRG1* and schizophrenia.

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http://dx.doi.org/10.1016/j.neubiorev.2016.06.001
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1. Introduction

Neuregulin-1 (*NRG1*) is one of the most extensively studied genes in schizophrenia. The genomic region surrounding *NRG1* first attracted attention following linkage analyses (Brzustowicz et al., 1999; Gurling et al., 2001; Kendler et al., 1996; Pulver et al., 1995) implicating a locus on chromosome 8p21-22 with the disorder. Subsequent fine mapping and linkage disequilibrium analysis in Icelandic patients led to the discovery of a haplotype (HAP_{ICE}) associated with schizophrenia and a parallel preclinical study showed *NRG1* hypomorphic (*i.e.* reduced gene expression) mice had a reduction in *N*-methyl-D-aspartate receptors (NMDAR) in forebrain areas and deficits in prepulse inhibition of the startle response compared to wild-type mice (Stefansson et al., 2002). This seminal work generated a tremendous amount of enthusiasm when published and remains one of the cornerstones of *NRG1* research in schizophrenia to date. However, no polymorphism has reached genome-wide significance ($p = 5 \times 10^{-8}$) in any genome-wide association study (GWAS) in schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014), despite the inclusion of 400 or more *NRG1* SNPs and gene coverage within the upper 10% of all protein-coding genes included on conventional GWAS platforms (Lehne et al., 2011). This suggests that the absence of a *NRG1* signal in GWAS is unlikely to be a result of poor SNP coverage but rather a result of allelic heterogeneity at the *NRG1* locus (Weickert et al., 2012), which in large samples is likely to result in a loss of a genetic signal. Nevertheless, this absence of GWAS support has attenuated enthusiasm for future *NRG1* research and has questioned the relevance of *NRG1* in schizophrenia.

Relevance of a gene to a particular disorder however, should not be determined exclusively on whether nucleotide variation within it meets genome-wide significance but rather on the whole of the evidence base. As such, we have completed a comprehensive assessment of the last decade of clinical research into the relationship between *NRG1* and schizophrenia (for a review of the research prior to 2006 see: Harrison and Law, 2006). In addition, we provide a targeted review of preclinical studies that have examined *NRG1*'s effects on excitatory and inhibitory neurotransmission as recent preclinical reviews (Lisman, 2012; Mei and Nave, 2014) have not covered this topic thoroughly and an imbalance of excitatory/inhibitory transmission is one of several putative neurobiological mechanisms of schizophrenia (Chana et al., 2013). We conclude with recommendations for future research that we hope will help prioritize a strategy forward to further advance our understanding of the relationship between *NRG1* and schizophrenia.

2. *NRG1* structure and isoforms

NRG1 spans 1.125 megabases and comprises more than 20 exons as well as several large introns from which over 30 splice isoforms can be produced that are grouped into six types (I–VI) (Fig. 1). *NRG1* types I and II contain an immunoglobulin (Ig) like domain, encoded by exons E178 and E122 respectively. This Ig region is also present in types IV and V and, together (I, II, IV and V) are commonly defined as Ig-*NRG1*. The Ig domain is linked to the epidermal growth factor (EGF) like domain with or without the spacer region. In *NRG1* type III, the Ig like domain is not present and the EGF like domain is situated directly downstream of the specific and unique amino terminal region. The N terminal region of *NRG1* type III is unique as it contains a cysteine rich domain (CRD) and an N terminal transmembrane domain (TMn). In *NRG1* type VI, the amino terminal region is also directly attached to EGF like domain but this *NRG1* isoform lacks the CRD as well as the TMn regions (Harrison and Law, 2006; Mei and Nave, 2014; Mei and Xiong, 2008). *NRG1* protein is functionally activated by proteolytic cleavage of its membrane-bound precursor, known as pro-*NRG1*, by one of three type I transmembrane domain proteases (*i.e. BACE1*, *ADAM17* and *ADAM19*) that release a bioactive extracellular *NRG1* fragment, except in the case of *NRG1* type III, which acts as a membrane-bound signalling molecule due to the presence of two transmembrane domains. All *NRG1* types (I–VI) bind to and/or interact with their cognate transmembrane receptor tyrosine kinases (*i.e. ErbB3* and *ErbB4*). However, *ErbB4* is the main receptor for *NRG1* and evidence in mice suggests ErbB4 may be selectively localised on the largest subclass of interneurons, namely those that express the calcium-binding protein, parvalbumin [PV+ interneurons; (Abe et al., 2011; Bi et al., 2015; Fazzari et al., 2010; Garcia et al., 2000; Huang et al., 2000; Ma et al., 2003)]. However, mice studies have showed ErbB4 is also expressed by other types of cortical neurons, including those that express cholecystokinin, neuronal nitric oxide synthase (Neddens and Buonanno, 2010) and somatostatin (Yau et al., 2003) and in primates ErbB4 immunoreactivity on pyramidal neurons has been reported (Thompson et al., 2007). Furthermore, mouse and rat studies suggest ErbB3 and ErbB4 are also expressed by oligodendrocytes and dopaminergic neurons (Roy et al., 2007; Steiner et al., 1999) implicating possible points of intersection with dopamine and white matter dysfunction hypotheses of schizophrenia. Binding of *NRG1* to *ErbB4* leads to activation of the receptor tyrosine kinase domain, which initiates several downstream signalling cascades, implicating *NRG1* in distinct neurobiological processes, including peripheral myelination and central neurotransmission.

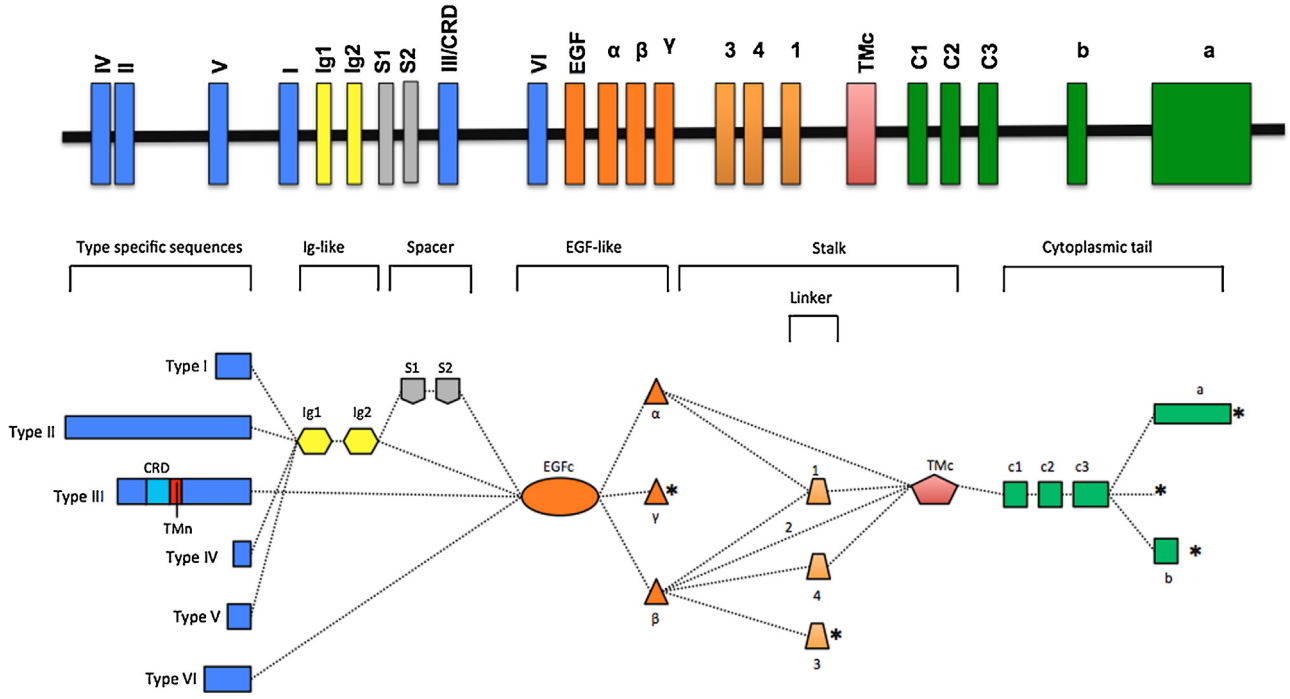

Fig 1. *NRG1* structure and isoforms. NRG1's six known isoforms and their defining exons are depicted (blue). All NRG1 isoforms have an epidermal growth factor (EGF)-like domain but only types I, II, IV, and V contain an immugloblin (Ig)-like domain (yellow). Type III is unique it that it contains a cysteine-rich domain (CRD) which includes a transmembrane domain (TMn). Splice variants can occur in the linker and C-terminal region. *Denotes stop codon. This figure is an update of that published by Mei and Xiong (2008).

3. *NRG1* and excitatory/inhibitory neurotransmission

A putative imbalance of excitatory/inhibitory transmission in the cortex, striatum and hippocampus is one likely neurobiological underpinning of schizophrenia (Carlsson and Carlsson, 1990). Current theories suggest that in schizophrenia excitatory activity of pyramidal neurons may be blunted in frontal brain regions (Stone et al., 2007) responsible for attention, memory and other executive functions, but overactive in the hippocampus (Grace, 2012; Schobel et al., 2013) and in certain subcortical areas involved in reward processing, stimulus salience and emotion. While evidence of widespread regional changes do not necessarily agree, there is evidence that the overall activity state of any given brain region may vary with clinical course, mood state (*e.g.* psychotic episode), severity of illness, cognitive ability and environmental context, such as task demands (Mathalon et al., 2001). Perturbations to the normal excitatory-inhibitory balance in the brain is thought to contribute to the positive and negative symptoms of schizophrenia, perhaps by alteration of cortical control over subcortical dopamine synthesis (Grace, 2012; Meyer-Lindenberg et al., 2002), although it is noteworthy that antipsychotic medications, which are antagonists of dopamine receptors, appear to only alleviate positive symptoms suggesting that they make act on systems down stream of cortical changes. While the causes of disrupted cortical function in schizophrenia have been shown to relate to both genetic and environmental risk factors (Bayer et al., 1999; Caspi and Moffitt, 2006), here we provide a review of recent transgenic mouse studies that have investigated the effects of *Nrg1* on the balance of excitatory and inhibitory transmission in the brain.

3.1. *NRG1* and excitatory neurotransmission

Glutamate is the predominant excitatory neurotransmitter in the brain (Meldrum, 2000) and acts post-synaptically on several ionotropic and metabotropic receptors, including NMDAR. NMDAR activity is a useful index for excitatory tone in the brain due to the high affinity of this receptor for glutamate and other excitatory neurotransmitters, and high levels of expression throughout the brain (Coyle, 1996). The NMDAR is a heterotetramer comprised of four protein subunits (denoted GluN1/2 or NR1/2) and rather than directly measure NMDAR activity, surrogate markers of function are often used such as NMDAR binding, subunit expression levels, and phosphorylation status. NMDAR hypofunction is thought to be central to the brain pathology in schizophrenia, with lower expression levels of the NMDAR1 subunit found in the brains of people with schizophrenia (Catts et al., 2015; Weickert et al., 2013). Therefore, it is important to consider how *NRG1* potentially confers risk for schizophrenia *via* modulation of NMDARs or *via* neurobiological mechanisms mediated by NMDARs (see Table 1 for a summary of "excitatory" studies covered in this review).

The seminal human association study on *Nrg1* and schizophrenia by Stefansson et al. (Stefansson et al., 2002) was carried out alongside animal studies, which using MK-801 binding showed that NMDARs are decreased in homogenate samples of forebrain areas in adult (20–28wks), type I *Nrg1* hypomorphic (*i.e.* reduced gene expression) mice. Subsequently, several studies have attempted to replicate this finding, but confirmatory evidence has been limited (Bjarnadottir et al., 2007; Long et al., 2015; Newell et al., 2013). Some studies have indicated that the effects of reduced *Nrg1* on NMDAR expression vary dependent on age and brain region analysed. For example, Newell et al. (Newell et al., 2013) showed that 21 week-old, but not 14 week-old, *Nrg1* transmembrane domain heterozygote knockout mice (TM Nrg1, the same mice used by Stefansson et al., 2002) had reduced NMDAR binding in the thalamus, but a trend for increased NMDAR binding in the nucleus accumbens in younger TM Nrg1 mice. Both younger and older mice also showed modest increases in NMDAR binding in sensory, motor and cingulate cortices. These findings contrast with two other studies that used the same *Nrg1* model, but found no genotype related differences in NMDAR binding in adult (20–27 weeks) mice (Dean

Table 1
Summary of recent studies investigating the effects of *Nrg1* on excitatory neurotransmission.

Reference	Age	Brain region	*Nrg1* model	Brief result (Nrg1 vs WT)	Notes
Stefansson et al. (2002)	–	Forebrain homegenate	Type I *Nrg1* Heterozygote "knockout"	1. Reduced NMDA receptor binding	
Newell et al. (2013)	All ages	Prefrontal cortex Hippocampus Nucleus accumbens Thalamus Globus pallidus Caudate putamen Substantia nigra Cingulate cortex Motor cortex Sensory cortex	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. Increased NMDA receptor binding in cingulate, sensory and motor cortices	Male only cohort Minimal environmental enrichment Mice previously tested in open field and light/dark tests
	14 wks			1. Increased NMDA receptor binding in nucleus accumbens	
	20 wks			1. Reduced NMDA receptor binding in thalamus	
Dean et al. (2008)	20–24 wks	Cortex (layers I–III) Striatum	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. No difference in NMDA receptor binding	Male only cohort Minimal environmental enrichment
Long et al. (2012)	21–27 wks	Hippocampus Cingulate cortex Caudate putamen Dorsolateral septum Retrosplenial granular cortex	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. No difference in NMDA receptor binding	Male only cohort Minimal environmental enrichment 3-week vehicle treatment (i.p. injections) prior to molecular analysis
Bjarnadottir et al. (2007)	36 wks	Hippocampus	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. Reduced level of phosphyorylated NR2 B	Reversed with antipsychotic clozapine Equivalent finding with acute NRG1 (extracellular domain peptide) treatment *in vitro*
Long et al. (2015)	14–18 wks	Prelimbic cortex	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. Reduced level of phosphyorylated NR2 B	Female only cohort Acute vehicle injection prior to molecular analysis
Kato et al. (2010)	8–12 wks	Hippocampus Prefrontal cortex Striatum	Type I *Nrg1* overexpressing	1. No difference in levels of NR1, NR2A/B	
Nawa et al. (2014)	>12 wks	Basal ganglia	EGF-like domain overexpressing	1. No difference in levels of NR1/2, GluR1 (AMPA)	
Yin et al. (2013)	–	Prefrontal cortex Hippocampus	Type I *Nrg1* overexpressing (selective expression in forebrain pyramidal neurons)	1. No difference in levels of NR2A/B, PSD95, GluR1/2	
Luo et al. (2014)	12 wks	Hippocampus	Type I *Nrg1* overexpressing	1. Decreased levels of NR1, NR2A/B	Overexpression of secreted N-terminal fragment only
Agarwal et al. (2014)	60 wks	Hippocampus	CRD (type III), Ig, and c-terminal product Nrg1 heterozygote "knockout"	1. No difference in levels of NR1, NR2B, GluR1 (AMPA), ErbB4, or PSD-95 2. Decreased glutamate transmission (sEPSC amplitude) Reduced STP and LTP	Acute MK-801 treatment at 12–16 wks and 52 wks
O'Tuathaigh et al. (2010)	60–72 wks	Cerebral cortex	CRD (type III) *Nrg1* overexpressing	1. No difference in levels of NR1, NR2B, GluR1 (AMPA), ErbB4, or PSD-95 2. Unaltered glutamate transmission (sEPSC amplitude and frequency) 3. Reduced STP and LTP	
	20–28 wks	Hippocampus Frontal cortex Striatum Cerebellum	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. No difference in levels of extracellular *N*-acetylaspartate or glutamate	Male only cohort
Pei et al. (2014)	12–16 wks	Hippocampus	*Nrg1* Transmembrane-domain heterozygote "knockout"	1. Reduced levels of GAD67 and parvalbumin	Finding detected in males, but not females
Jiang et al. (2013)	2–3 wks	Amygdala	Type III *Nrg1* Heterozygote "knockout"	Decreased glutamate transmission (faster signal decay; no change in signal amplitude or frequency) Reduced LTP	LTP deficits in type III *Nrg1* mutants not reversed by nicotine

et al., 2008; Long et al., 2012), although variable levels of environmental enrichment, which has been found to affect NMDAR subunit expression (Guilarte et al., 2003), may have been a factor in these studies. Two further studies employing the TM *Nrg1* model found reduced NMDAR subunit phosphorylation in both hippocampus (Bjarnadottir et al., 2007) and prelimbic (*i.e.* prefrontal) cortex (Long et al., 2015). Hence, there has been considerable inconsistency between findings, which render the effects of the TM *Nrg1* model on the NMDAR quite ambiguous. Originally, this mutant *Nrg1* mouse was considered to have reduced

gene expression across all *Nrg1* isoforms that contain the transmembrane domain, as was shown in (Stefansson et al., 2002) who found a 50% reduction in Nrg1 mRNA in the hippocampus. Nevertheless, recent work suggests that these TM *Nrg1* mice may not be a simple loss-of-function model, as exons upstream of the genetic deletions appear to have normal levels of expression (Long et al., 2015) and theoretically this model may still generate functionally mature Nrg1 (*i.e.* cleaved fragment), although it is not clear how the absence of proteolytic processing may effect Nrg1 neurobiological activity. The current findings suggest that *Nrg1* expression may dynamically influence expression of NMDARs as a function of age, brain region and cohort differences, consistent with the varying patterns of *Nrg1* expression across the lifespan (Long et al., 2015).

Several investigations of *Nrg1* over-expression in mice have been carried out in response to evidence of elevated *NRG1* expression (sometimes linked to genetic change in *NRG1*) in post-mortem brains of patients with schizophrenia and bipolar disorder (Georgieva et al., 2008; Hashimoto et al., 2004; Weickert et al., 2012). Many animal studies have focused on the type I *Nrg1* isoform explored in the original study by Stefansson (Stefansson et al., 2002), and suggest that NMDAR subunit phosphorylation (NR1/2) is not altered by type I over-expression in mice, at least not in early adulthood (8–12wks) (Kato et al., 2010; Yin et al., 2013). Interestingly, another study reported reduced NR2A/B phosphorylation in an *Nrg1* model that over-expressed only the secreted fragment of the type I *Nrg1* gene at a similar age (Luo et al., 2014). This may suggest that extracellular *Nrg1* alone has distinct neurobiological effects from over-expression of uncleaved or pro-Nrg1 (*i.e.* uncleaved). Indeed, some studies suggest that pro-Nrg1 may serve as a receptor to ecto-ErbB4, the extracellular portion of the Nrg1 receptor, ErbB4, in a reverse-signalling fashion leading to cleavage of the intracellular domain of Nrg1, which then regulates intracellular transcriptional activity (Bao et al., 2003; Chen et al., 2010a,b). The findings of Luo et al. (2014) indicate that type I *Nrg1* forward-signalling (*i.e.* the release of extracellular *Nrg1* that binds ErbB4) may alter NMDAR phosphorylation, which may then have downstream effects on excitatory and inhibitory networks. Nevertheless, further studies are needed to determine the neurobiological effects of the gain or loss of pro-*Nrg1* function, particularly in regard to the most brain-abundant, type III isoform, and how the balance between different isoforms may also influence *Nrg1* activity and to determine which putative changes in NMDAR may be occurring.

Recent evidence suggests that *Nrg1* follows an inverted "U" dose-response pattern of activity, such that too much or too little *Nrg1* may be detrimental to brain function (Agarwal et al., 2014; Law, 2014; Role and Talmage, 2007). In *Nrg1* hypomorphic rodents, miniature and spontaneous excitatory post synaptic currents (m/sEPSC) were decreased in the CA1 subregion of the hippocampus (Agarwal et al., 2014), but long-term potentiation (LTP) appeared normal according to input-output curves at Schaffer collateral-CA1 synapses in the hippocampus (Agarwal et al., 2014). In another study, type III *Nrg1* heterozygote "knockout" mice showed a faster time constant of decay, but no change in amplitude or frequency, of mEPSCs in amygdala compared with wild-type (WT) mice (Jiang et al., 2013). Type III *Nrg1* hypomorphs also had lower AMPA/NMDA ratios, indicative of LTP and neural plasticity, in response to evoked electrical currents compared to WT (Jiang et al., 2013). Similarly, type I (Yin et al., 2013), but not type III (Agarwal et al., 2014), *Nrg1* over-expressing mice showed reduced m/sEPSC in prefrontal cortex and hippocampus, and both type I/III *Nrg1* over-expressing mice showed impaired synaptic plasticity in the hippocampus, demonstrated by a downward shift in the input-output curve (Agarwal et al., 2014; Yin et al., 2013). Together, these studies suggest that both too much, and too little, *Nrg1* expression may contribute to complex alterations in firing patterns and impaired plasticity of excitatory neurons in mice, and such neuronal changes are known to relate to learning and memory performance in mice, as well as humans.

3.2. NRG1 and inhibitory neurotransmission

Inhibitory activity is central to the "computational architecture" of excitatory networks (Huang et al., 2007), and is primarily controlled by gamma-aminobutyric acid (GABA), the predominant inhibitory neurotransmitter in the brain (Huang et al., 2007). GABA is released by presynaptic inhibitory interneurons, which make up approximately 20% of all neurons in the cortex and constitute the dominant inhibitory network (Lehmann et al., 2012; Mody et al., 1994). Interneurons are unique in that they branch in a web-like fashion, and can form synaptic terminals with up to 1200 different pyramidal neurons for each inhibitory neuron (Kann et al., 2014). In this way, relatively few interneurons regulate large excitatory networks throughout the brain (Huang et al., 2007), and the inhibitory network as a whole is capable of regulating a range of cognitive processes, from attention and impulse-control to sensorimotor behaviour. Inhibitory neurons can also synapse on other inhibitory neurons to indirectly control the level of inhibitory control over pyramidal neuronal excitation (Huang et al., 2007). The function of inhibitory networks in key brain regions including the prefrontal cortex, hippocampus and amygdala is thought to be implicated in the neurobiological abnormalities putatively underpinning both positive and negative symptoms in schizophrenia, including psychotic episodes and poor cognitive control (Lewis et al., 2012; Lisman, 2012). Indeed, neurodevelopmental theories of schizophrenia suggest that deficits in inhibitory control may be a primary neuropathology (Catts et al., 2013) beginning during tangential migration of inhibitory neurons in the embryo and including final maturation of interneuron processes that are regulated by several schizophrenia susceptibility genes such as *NRG1*, *DISC1* and *ErbB4* (Jaaro-Peled et al., 2009; Seshadri et al., 2015). Several reports have shown that levels of various inhibitory interneuron markers are reduced in prefrontal cortex and hippocampus in schizophrenia patients (Fung et al., 2010; Konradi et al., 2011), alterations that have been linked to changes in NRG1-ErbB4 (Chung et al., 2015; Joshi et al., 2014). Tied in with this evidence is the fact that Nrg1 has been shown to effect immunity, namely pro-inflammatory markers such as IL-6 and IL-8 (Marballi et al., 2010), which in turn may dampen PV+ interneuron activity (Behrens and Sejnowski, 2009). Hence the direct and indirect effects of *Nrg1* on inhibitory neurons and their networks spanning prefrontal cortex, hippocampus, striatum, and amygdala are also highly relevant to the understanding of how this candidate gene may contribute to schizophrenia risk (see Tables 2 and 3 for a summary of *in vitro* and *in vivo* "inhibitory" studies covered in this review).

In vitro studies suggest that *Nrg1* plays an important role in regulating basal GABAergic transmission. Reducing endogenous *Nrg1* levels *via* excess administration of ecto-ErbB4, which binds and sequesters extracellular *Nrg1*, causes a decrease in miniature ("spontaneous") inhibitory post-synaptic currents (mIPSC) in basolateral amygdala in mouse (Bi et al., 2015). Conversely, in a study in which healthy rodents were categorised as having low or high levels of *Nrg1* mRNA and protein in the brain, adding the *bace1*-cleaved epidermal growth factor-like domain of Nrg1 (Nrg1β) *in vitro* to "low *Nrg1*" mice was shown to increase mIPSC frequency in the amygdala (Bi et al., 2015). Interestingly, *in vitro* Nrg1β did not enhance basal GABAergic transmission in mice with normal levels of *Nrg1* across a range of brain regions, including prefrontal cortex (Li et al., 2012; Woo et al., 2007), hippocampus (Li et al., 2012), and amygdala (Bi et al., 2015; Mody et al., 1994). Mody and colleagues (Mody et al., 1994) suggested that $GABA_A$ receptors are naturally low in number and may become saturated by spontaneous GABAergic currents under normal neurobiological conditions, which could

Table 2
Summary of recent *in vitro* studies investigating the effects of *Nrg1* on inhibitory neurotransmission.

Reference	Age	Brain region	*Nrg1* model	Brief result (Nrg1 vs WT)	Notes
Woo et al. (2007)	6–7 wks	Prefrontal cortex	NRG1β EGF-like domain added *in vitro*	1. Increased mean amplitude of depolarisation-evoked IPSCs 2. No effect on frequency or amplitude of basal mIPSCs	Effect blocked by ErbB4 knockout genotype and ErbB4 (AG1478), but not ErbB2 (AG879), antagonist.
Wen et al. (2010)	6–8 wks	Prefrontal cortex	NRG1β EGF-like domain added *in vitro* to slices from PV-specific ErbB4 $^{+/+}$ and $^{-/-}$ mice	1. Increased mean amplitude of depolarisation-evoked IPSCs ($^{+/+}$, but not $^{-/-}$)	Effect blocked by PV-specific ErbB4 knockout genotype.
Chen et al. (2010a,b)	5–8 wks	Hippocampus	NRG1β EGF-like domain added *in vitro*	1. Increased mean amplitude of depolarisation-evoked IPSCs	Effect blocked by ErbB4 antagonist AG1478
Abe et al. (2011)	2–3 wks	Neocortex (layer VI)	NRG1β1 EGF-like domain added *in vitro*	1. Increased mean amplitude of depolarisation-evoked IPSCs 2. No effect on frequency or amplitude basal mIPSCs	Polysynaptic, but not monosynaptic, currents
Bi et al. (2015)	10–12 wks	Basolateral amygdala	NRG1β EGF-like domain added *in vitro*	1. Increased mean amplitude of depolarisation-evoked IPSCs 2. Decreased amplitude of depolarisation-evoked IPSCs with acute eco-erbB4 and AG1478 (ErbB4 antagonist)	[1]Only in mice categorised as 'low *Nrg1*, but not high *Nrg1*, expression'
Hou et al. (2014)	3 wks	Prefrontal cortex	NRG1β EGF-like domain added *in vitro* and in vivo	1. Increased synchrony between paired inhibitory and excitatory currents *(in vitro)* 2. Increased kainate-induced gamma oscillations *(in vivo)*	Male only cohort Blocked by ErbB4$^{-/-1,2}$ and $GABA_A$ antagonist[1]
Li et al. (2012)	4 wks	NeocortexHippocampus	NRG1β EGF-like domain added *in vitro*	1. Increased frequency of PV^+ interneuron-mediated currents	Blocked by ErbB4 antagonist (AG1478), but not by $GABA_A$, NMDA, AMPA, or kainate receptor antagonists

limit the efficacy of exogenous agents that potentially enhance GABAergic activity. It may be for this reason that only a reduction in endogenous *Nrg1* or reversal of deficits in *Nrg1* expression has been found to enhance certain components of GABAergic transmission, including the frequency of inhibitory currents. With this in mind, these results are consistent with the notion that *Nrg1* is important for maintenance of GABAergic tone at an optimal level for the functioning of cortical/subcortical networks in support of learning and behaviour.

Nrg1 treatment also enhances evoked GABAergic transmission *in vitro*. Acute application of Nrg1β in tissue derived from prefrontal cortex, hippocampus and amygdala increases the amplitude of depolarisation-induced inhibitory currents (Abe et al., 2011; Bi et al., 2015; Chen et al., 2010a,b; Li et al., 2012; Wen et al., 2010; Woo et al., 2007), and in some cases it also enhances current frequency (Li et al., 2012). This effect is abolished in mice lacking the Nrg1 receptor, ErbB4 receptor (Wen et al., 2010; Woo et al., 2007), or when brain tissue is pre-treated with ErbB4 receptor antagonists (Chen et al., 2010a,b; Li et al., 2012; Woo et al., 2007), suggesting that Nrg1-ErbB4 signalling is directly responsible for the enhancement of GABAergic transmission. It is possible that *Nrg1* increases the synchrony of polysynaptic GABAergic events (Hou et al., 2014), facilitating the summation of multiple sub-threshold currents toward the formation of an action potential (Mody et al., 1994). Other findings indicate that *Nrg1* drives the phosphorylation of presynaptic potassium channels, prolonging their functional activity (Li et al., 2012), and enabling more rapid recovery of GABAergic interneurons from post-synaptically driven suppression of inhibition (Du et al., 2013). What is clear from this preclinical evidence is that the function of inhibitory networks relies at least partially on *Nrg1*-induced enhancement of GABAergic transmission, as is also suggested by post-mortem studies in humans (Chung et al., 2015; Joshi et al., 2014).

Several *in vivo* mouse studies suggest that *Nrg1* deficiency leads to increased neural "noise" (*i.e.* baseline activity) and decreased neural responsivity to actual events in the environment (*i.e.* evoked activity) when measuring gamma oscillations in the cortex. This effect is proposed as neurophysiological phenotype of schizophrenia and is thought to be regulated by inhibitory PV+ interneuron networks (Gandal et al., 2012). Gamma oscillations occur between 25 and 100 Hz and reflect the synchronous activity of distinct neuronal populations producing brain states that are thought to contribute to conscious awareness and attention, and some authors have likened the elevation in baseline gamma oscillations in diseases such as schizophrenia to neural "noise" brought about by dysfunction of the GABAergic system (Gandal et al., 2012). Putative neural "noise" in the form of baseline gamma oscillations is increased in the parietal cortex in TM *Nrg1* mice (Long et al., 2015), and also in the prefrontal cortex in ErbB4 hypomorphic mice (Hou et al., 2014) when compared with control mice. Similarly, baseline synaptic firing of somatosensory neurons in EGF-like domain "knockout" mice is significantly above control levels. In contrast, these same *Nrg1* models show deficits in evoked gamma oscillations induced by whisker stimulation (Barz et al., 2016), auditory cues – albeit all *Nrg1* mutant mice are known to have auditory abnormalities (Jin et al., 2011; Kato et al., 2015; Stankovic et al., 2004; Tang et al., 2015) – and the psychotomimetic, ketamine (Long et al., 2015) whilst addition of *Nrg1* in wild-type mice has been shown to enhance kainic-acid evoked gamma oscillations in prefrontal cortex (Hou et al., 2014) and hippocampus (Fisahn et al., 2009), the former *in vivo* and the latter *in vitro*. One study also suggests that excess Nrg1 may lead to similar impairments, as peak frequency, but not amplitude, of gamma oscillations were reduced in type I *Nrg1* over-expressing mice (Deakin et al., 2012), which also supports the inverted "U" model of *Nrg1* function (Agarwal et al., 2014; Law, 2014; Talmage, 2008). Gamma oscillations are linked to cognitive performance in humans (Engel et al., 2015; Heermann et al., 2011; Jensen et al., 2007) and mice with abnormal *Nrg1* expression which have the aforementioned deficits in evoked gamma oscillations, also have cognitive deficits (Agarwal et al., 2014; Barz et al., 2016; Chen et al., 2008; Chesworth et al., 2012; Duffy et al., 2010; Yin et al., 2013). Therefore, maladaptive gamma oscillations, putatively stemming from *Nrg1*-induced GABAergic dysfunction, may be a key downstream

Table 3
Summary of recent *in vivo* studies investigating the effects of Nrg1 on inhibitory neurotransmission.

Reference	Age	Brain region	*Nrg1* model	Brief result (Nrg1 vs WT)	Notes
Long et al. (2012)	18–24 wks	Prelimbic cortex Cingulate cortex Caudate putamen Dorsolateral septum Retrosplenial granular cortex	*Nrg1* transmembrane-domain heterozygote "knockout"	1. No difference in $GABA_A$ receptor binding in vehicle-treated mice	Male only cohort Minimal environmental enrichment
Long et al. (2015)	12–16 wks	Parietal cortex	*Nrg1* transmembrane-domain heterozygote "knockout"	1. Increased ongoing and reduced auditory- and ketamine-evoked gamma oscillations	Female only cohort
Barz et al. (2016)	16–24wks	Primary somatosensory cortex	*Nrg1* EGF-like domain heterozygote "knockout"	1. Increased baseline and sensory-evoked synaptic firing rate 2. Reduced gamma oscillations evoked by whisker stimulation	Male only cohort
Deakin et al. (2012)	12–24 wks	Hippocampus	Type I *Nrg1* overexpressing	1. Normal basal synaptic firing 2. Decreased peak frequency, but not power, of carbachol-induced gamma oscillations	
Kato et al. (2010)	8–12 wks	Frontal cortex Hippocampus Striatum	Type I *Nrg1* overexpressing	1. Increased levels of parvalbumin protein in frontal cortex, but not other regions 2. No difference in GABAergic markers GAD65/67 in any region	
Nawa et al. (2014)	>12 wks	–	Epidermal growth factor-like domain overexpressing	1. No difference in levels of parvalbumin, GAD65/67	
Yin et al. (2013)	16 wks	Prefrontal cortex Hippocampus	Type I *Nrg1* overexpressing (selective expression in forebrain pyramidal neurons)	1. Decreased $GABA_A$ α1 subunit 2. Decreased GABA transmission (basal mIPSC amplitude, but not frequency)	Changes able to be "switched off" with adult doxycycline treatment suggesting a lack of permanent neurobiological alteration in the cortex
Luo et al. (2014)	12 wks	Hippocampus	Type I *Nrg1* overexpressing (extracellular fragment only)	1. No difference in levels of $GABA_A$ α1, parvalbumin, or GAP43	Overexpression of secreted N-terminal fragment only
Agarwal et al. (2014)	12 wks	Hippocampus	HRGα (type I) *Nrg1* homozygous knockout	1. Altered basal GABAergic transmission (mIPSC amplitude increased, mIPSC frequency decreased)	Acute MK-801 treatment at 12–16 wks and 52 wks months
O'Tuathaigh et al. (2010)	60–72 wks	Hippocampus	Type III *Nrg1* overexpressing	1. Increased basal GABAergic transmission (mIPSC amplitude, but not frequency) 2. No difference in parvalbumin or GAD67 protein levels	
	20–28 wks	Hippocampus Frontal cortex Striatum Cerebellum	*Nrg1* Transmembrane-domain Heterozygote "knockout"	1. No difference in levels of extracellular GABA (males) 2. Reduced ventricular and olfactory bulb volume (females)	
Hou et al. (2014)	–	Prefrontal cortex	Exogenous *NRG1β* EGF-like domain peptide added *in vivo*	1. Increased kainic-acid induced gamma oscillations 2. Reduced kainic-acid induced gamma oscillations in separate ErbB4 homozygous knockout line	Male only cohort
Pei et al. (2014)	12–16 wks	Hippocampus	*Nrg1* Transmembrane-domain Heterozygote "knockout"	1. Decreased parvalbumin and GAD67, but not calretinin, protein levels	Male only cohort Reduced GAD67 rescued by chronic valproate
	12–16 wks	Hippocampus	1. No difference parvalbumin and GAD67 or calretinin protein levels	Female only cohort	

neurobiological consequence of abnormal *Nrg1* expression, mediating risk for schizophrenia.

In vivo mouse models also suggest that one mechanism by which *Nrg1* expression may enhance GABAergic transmission in the brain is through an increased density of PV+ interneurons in distinct cortical and subcortical regions. For example, levels of parvalbumin are elevated in the frontal cortex in type I *Nrg1* overexpressing mice compared to wild type mice (Kato et al., 2010). In contrast, the TM *Nrg1* model is associated with reduced parvalbumin in the hippocampus for male, but not female, mice (Pei et al., 2014). Also, genetic ablation of the *Nrg1* receptor tyrosine kinase, ErbB4, downregulates the number of PV^+ interneurons in the same brain region by 30% from normal levels (Fisahn et al., 2009). These findings suggest that *Nrg1* may be a key factor mediating the altered levels of interneuron markers, especially parvalbumin, observed in schizophrenia, and are consistent with human studies in which the parvalbumin, as well as somatostatin, interneuron subtypes are robustly and reproducibly diminished interneuron markers in

patients with schizophrenia (Chung et al., 2015; Fung et al., 2010; Hashimoto et al., 2008a,b, 2003; Joshi et al., 2015; Joshi et al., 2014).

The majority of studies in mice indicate that GABA markers (*e.g.* synthesis enzyme GAD67) are not affected by altered *Nrg1* expression in the brain. For example, TM *Nrg1* mice displayed normal levels of extracellular GABA (O'Tuathaigh et al., 2010) and $GABA_A$ (Long et al., 2012) binding in areas including hippocampus, striatum and prefrontal cortex. Similarly, levels of GAD67, the principal precursor enzyme for GABA, were unaltered in Type I (Kato et al., 2010), Type III (Agarwal et al., 2014), and EGF (Nawa et al., 2014) *Nrg1* overexpressing mice in the same brain regions. Moreover, overexpression of the secreted fragment of type I *Nrg1* was not associated with altered levels of the α1 $GABA_A$ receptor subunit in hippocampus compared to wild type mice (Luo et al., 2014). In contrast, a number of human postmortem studies have found a reduction in GAD67 mRNA expression. (Duncan et al., 2010; Guidotti et al., 2000; Hashimoto et al., 2008a,b, 2003) and other transcriptional markers of GABA, such as mRNA expression of the α1, α5 and δ subunits of the $GABA_A$ receptor (Duncan et al., 2010; Hashimoto et al., 2008a,b) in the brain of patients with schizophrenia. In agreement with this human postmortem finding, a study by Pep and group (Pei et al., 2014) found abnormally low levels of GAD67 in male, but not female, type I *Nrg1* "knockout" mice in the hippocampus, and another study by (Yin et al., 2013) observed elevated α1 in type I *Nrg1* overexpressing mice relative to wild type mice in frontal cortex and hippocampus. In light of the human postmortem findings, it may be that *Nrg1* leads to small and often undetectable changes in GAD67 and other markers of GABAergic activity through its more potent effects on PV+ interneurons, but it is only when combined with the effects of several other risk genes and environmental stressors in patients with schizophrenia, that broader aberrations in GABA occur.

Several conclusions can be drawn based on current preclinical evidence for the effects of *Nrg1* expression on excitatory/inhibitory balance throughout the brain. First, there is consistent evidence that acute exposure to *Nrg1* enhances GABAergic transmission; especially within PV+ interneurons and that this *Nrg1* effect is mediated *via* the ErbB4 receptor tyrosine kinase. Second, *Nrg1*, presumably *via* the upregulation of GABAergic activity, dampens neural "noise" in the brain, which improves sensory-evoked gamma oscillations and possibly enhances cognition through a more optimal cortical or hippocampal signal/noise ratio in a learning context. Third, long-term rather than acute changes in *Nrg1* lead to broader variation in neurobiological function, and recent evidence suggests that *Nrg1* function may follow an inverted "U" curve, such that too much or too little *Nrg1* over a long period of time is detrimental to brain development and adult brain function.

The last decade of preclinical research has seen much progress in our basic understanding of the neurobiological effects of *Nrg1* in cortical and subcortical brain regions and as outlined above this has contributed considerably to our understanding of how *Nrg1* may confer risk for schizophrenia and similar psychiatric illnesses. In parallel, clinical studies in a wide variety of patient cohorts have continued efforts to localise the exact region of genetic interest for *NRG1*, as well as linking *NRG1* genotypes to more in-depth phenotypes beyond the overall diagnosis of schizophrenia, and these clinical investigations have yielded several interesting findings that are reviewed below.

4. *NRG1* genetic variation and schizophrenia

4.1. Family studies

The original family study in the Icelandic population linking a haplotype (HAP_{ICE}) in the 5′ region of *NRG1* with schizophrenia (Stefansson et al., 2002) stimulated numerous additional family studies of which seven were conducted in the past decade (Table 4; for details on family studies from 2002 to 2005 see: Harrison and Law, 2006). Unlike the family studies conducted soon after the Icelandic study, the ancestral backgrounds of cohorts in more recent studies have been diverse, covering populations beyond Northern and Central Europe. This diversity may have contributed to the mixed findings, with multiple regions spaning the entire length of *NRG1* being linked to schizophrenia (Fig. 2).

In Asian populations, studies report haplotype and single marker associations in both the 5′ and 3′ regions of *NRG1*, whereas studies of Caucasian populations have implicated the 5′ region, albeit interrogation of the 3′ region in Caucasians is limited. Nonetheless, a majority of positively associated 5′ single markers and haplotypes overlap or are near (within 200 kbp) the HAP_{ICE} region, regardless of Caucasian or Asian ancestry. In contrast, 3′ single markers and haplotypes have predominantly been identified in Asian populations but have yet to be uniformly replicated, prohibiting firm conclusions in other populations. Notably, two negative results have been reported in the past decade but these have been in a mixed-ethnic population (Yokley et al., 2012) or have examined psychosis more generally (*e.g.* inclusion of bipolar and depression psychosis) (Rosa et al., 2007), suggesting population stratification and phenotypic heterogeneity may have constrained detection of an association, respectively.

4.2. Case-control studies

Harrison and Law (2006) reported in their original review of *NRG1* and schizophrenia that among Caucasians the region of most significant association lay between the exons of *NRG1* type II and IV. A decade and 33 case-control studies later, this upstream region of the *NRG1* gene remains the most implicated within the Caucasian population (Fig. 2, Supplementary Table S1). However, evidence of association for markers and haplotypes beyond the 5′ region (near the exons for type III and VI) among Caucasians has emerged (Lachman et al., 2006; Thomson et al., 2007; Walker et al., 2010) and are aligned with markers and haplotypes reported in family studies conducted in Caucasian (Petryshen et al., 2005) as well as Han Chinese populations (Li et al., 2004; Yang et al., 2003). Evidence for associations beyond the 5′ region has also been reported in North Indian (Kukshal et al., 2013) and Pakistani (Naz et al., 2011) populations. Importantly, very few single markers or haplotypes have been robustly replicated (see meta-analytic studies section), implying a high level of allelic heterogeneity. This is somewhat similar to the preclinical studies that suggested the context in which *NRG1* changes were studied may impact the neurobiological outcome, and we suggest that in the case of the clinical work, this context could include other genes or environmental differences across various ethnic populations, countries and cultures. Thus, as the number of populations studied increases it is likely in the next decade we will witness an expansion of the 'region of most significant associations' within *NRG1* and with this expansion see increased levels of allelic heterogeneity.

4.3. Meta-analytic studies

Six meta-analytic studies have examined the association between *NRG1* genetic variation and schizophrenia (Table 5). The HAP_{ICE} markers have been the main focus of these studies, although three other markers (rs10503929, rs3924999 and rs2954041) in the 3′ region have also been subjected to meta-analysis. Results of these studies are mixed but suggest a significant association between 5′ *NRG1* genetic variation and schizophrenia. The largest and most recent analysis (Gong et al., 2009) showed support for three (SNP8NRG221132, 478B14-848, 420M9-1395) of the seven

Table 4
Summary of NRG1 family studies in schizophrenia (2005–2015).

Reference	Subjects	Ethnicity	SNP, MS	From	To	Association with SZ	Brief Result	Notes
Liu et al. (2005)	52 schizophrenic families	TWN	0, 11	D8S1742	D8S1810	Yes	Narrow moel,D8S1771 (p = 0.01) D8S1222 (p = 0.01); Broad model, D8S1771 (p = 0.03), D8S1222 (p = 0.003)	Non parametric linkage analysis
Kim et al. (2006)	40 families, 140 individuals, 89 affected SZ patients	KOR	0, 7	D8S258	D8S505	Yes	D8S1769; For Narrow phenotype class (p = 0.036); Narrow with auditory hallucination (p = 0.011)	
Walss-Bass et al. (2006a)	134 family trios	CVCR	4, 2	rs73235619	420M9-1395	Yes	A novel 4 marker haplotype (G-T-218-274) was overrepresented in patients with psychosis (p = 0.046).	4 marker haplotype: rs73235619, rs35753505, 478B14-848, 420M9-1395.
Walss-Bass et al. (2006b)	142 affected individuals and 236 unaffected family members	CVCR	1, 0	rs74942016	–	Yes	The minor (T) allele of rs74942016 was over-transmitted from parents to offspring with SZ (p = 0.0191); patients with psychosis (p = 0.0049)[a].	All the exons of NRG1 were initially sequenced in a subsample of 12 affected individuals and rs74942016 (G > T; Val > Leu) was discovered in exon 11.
Turunen et al. (2007)	441 SZ family (865 affected individuals)	FIN	10, 3	D8S1820	D8S1110	Yes	For SZ spectrum phenotype rs764059 (p = 0.012); rs2919378 (p = 0.030);	
Rosa et al. (2007)	151 families with 575 individuals	SP	8, 0	rs763553	rs10503929	No[a]	–	–
Georgieva et al. (2008)	634 SZ/SA parent offspring trios	92.3% BUL, 1.5% TURK, 3.2% ROM and 3% CAU	12, 2	rs763551	420M9-1395	Yes	T allele of rs35753505 (p = 0.013, corrected p = 0.039); 2 marker haplotype (rs35753505 and rs7014762, T-A, p = 0.006); (rs35753505 and rs62510682, C-T, p = 0.001); (rs35753505 and rs6994992, C-G, p = 0.001)	–
Zhang et al. (2009)	258 parent-proband trios	CHN	4, 0	rs35753505	rs3924999	Yes	Allelic transmission of C in rs35753505 (p < 0.05); A in rs113317778 (p < 0.05); T in rs3924999 (p = 0.001); Haplotype analysis: 3 marker haplotype (C-C-G, p = 0.026 and C-C-A, p < 0.05); 4 marker haplotype: (C-C-G-T, p = 0.001; C-C-A-C, p = 0.006 and C-C-A-T, p < 0.05);	3 marker haplotype: rs35753505, rs7820838, rs113317778; 4 marker haplotype: rs35753505, rs7820838, rs113317778, rs392499. This paper was published in Chinese.
Yokley et al. (2012)	419 (58 SZ/SAD & 361 unaffected relatives)	CAU, ME, AA	40, 0	rs73235619	rs3735782	No	–	–

AA = African-American, BD = Bipolar disorder, BUL = Bulgarians, CAU = Caucasians, CVCR = Central Valley of Costa-Rica, CHN = Chinese, FBAT = Family Based Association Test, FIN = Finnish, KOR = Korean, ME = Mixed European, MS = microsatellite, ROM = Romans, SAD = Schizoaffective disorder, SP = Spanish, SNP = single nucleotide polymorphism, SZ = Schizophrenia, TURK = Turkish, TWN = Taiwanese.
rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006).

[a] Phenotype studied was psychosis.

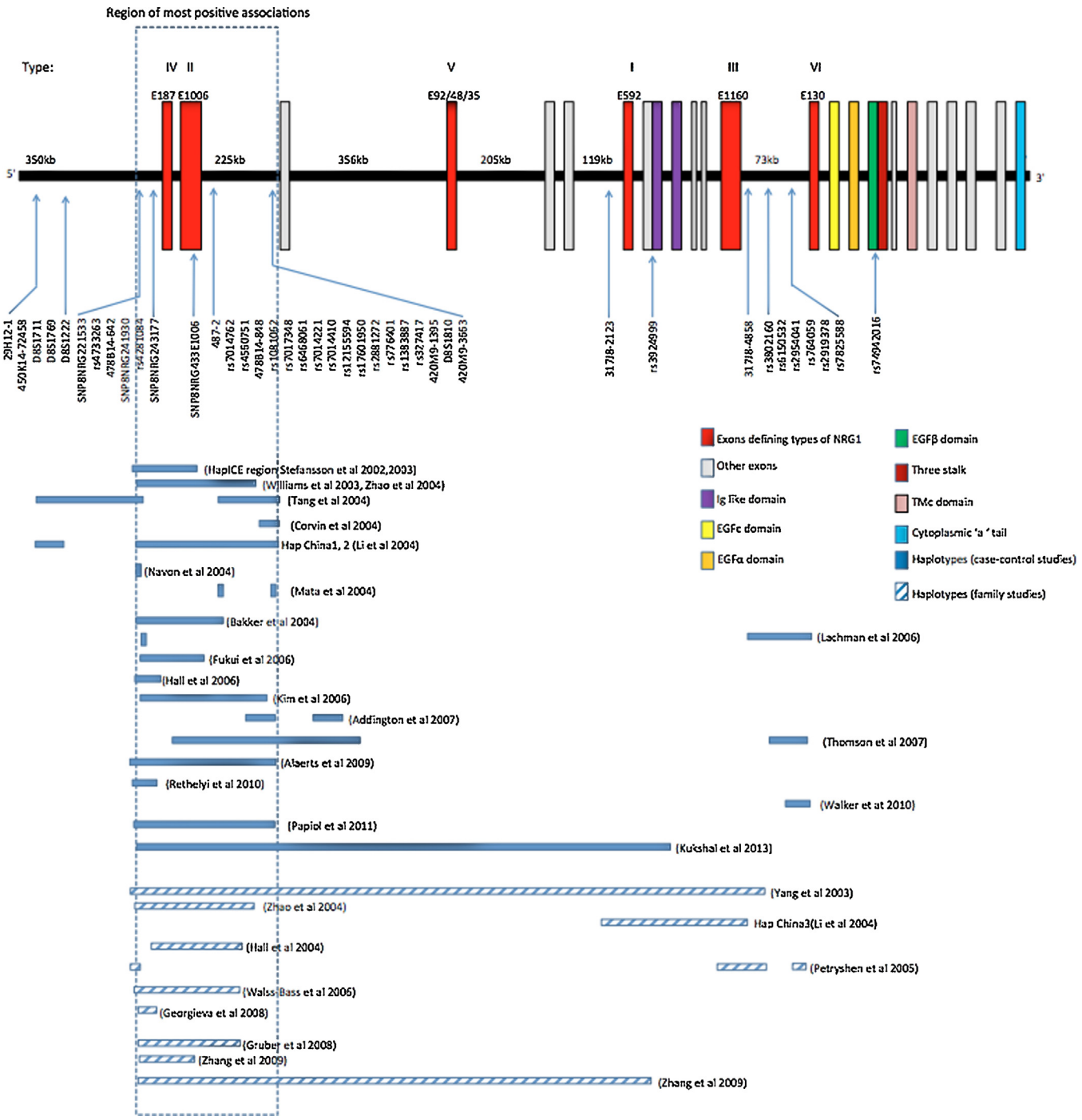

Fig. 2. Positively associated markers of the *NRG1* gene in schizophrenia in case-control studies and family studies. Exons are depicted as vertical bars with the NRG1 type denoted above. Horizontal lines represent the coverage of each haplotype in case-control (solid lines) and family (hashed lines) studies. The dashed rectangle indicates the region where the most positive associations have been reported. This figure is an update of that published by Harrison and Law (2006).

HAP_{ICE} markers when combining case-control and family studies across ancestry. While, Munafo et al. (2006, 2008) showed support at the haplotype level using a combined p-value approach, others have argued that this approach should be extended to the gene level as this would address, in part, the allelic heterogeneity within *NRG1* between populations (Neale and Sham 2004). However, to our knowledge a combined p-value approach at the gene-level has yet to be applied in a study of *NRG1* and schizophrenia.

To date, the majority of *NRG1* studies in schizophrenia have focused on examining associations between genetic variation and the categorical diagnosis of schizophrenia. However, the past decade has witnessed a sharp increase in the number of studies looking wider and deeper at *NRG1*'s role in schizophrenia. This expansion has included studies on *NRG1*'s association with psychosis onset, gene and protein expression, brain structure and function, cognition, as well as its interaction with other genes and the environment. In the sections below, we review this body of research.

4.4. At-risk mental state studies

Recent results from three independent prodromal cohorts suggest that *NRG1*, specifically the HAP_{ICE} region, may harbor markers capable of identifying high-risk individuals with the greatest

Table 5
Summary of NRG1 meta-analysis studies in schizophrenia (2006–2015).

Reference	No of publications analysed	Years examined	Ethnicity	Case/Control Subjects	Markers	Association with SZ
Li et al. (2006)	13	2002–2006	All	3947/4202; 708 parent offspring trios	rs73235619	Yes
					rs35753505	Yes
					rs62510682	Yes
					rs6994992	Yes
					rs113317778	No
					478B14-848	Yes
					420M9-1395	Yes
			CAU	2502/3074; 111 parent offspring trios	rs73235619	Yes
					rs35753505	Yes
					rs62510682	Yes
					rs6994992	Yes
					rs113317778	No
					478B14-848	No
					420M9-1395	No
			AS	1445/1128; 597 parent offspring trios	rs73235619	Absence of allele A
					rs35753505	No
					rs62510682	No
					rs6994992	No
					rs113317778	No
					478B14-848	Yes
					420M9-1395	Yes
Munafo et al. (2006)	13	2002–2006	All	8678/4423	rs35753505	No
			CAU	3555/2923	rs35753505	No
			EAS	4984/1372	rs35753505	No
			AF	139/135	rs35753505	No
Munafo et al. (2008)	8	2005–2007	All	1476/1881	rs35753505	No
			CAU	1476/1881	rs35753505	No
			Mixed	Data not available	rs35753505	No
Gong et al. (2009)	26	2002–2009	All[a]	8049/8869, 1515 families	SNP8NRG103492	No
					rs73235619	Yes
					rs35753505	No
					rs62510682	No
					rs6994992	No
					rs113317778	No
					478B14-848	Yes
					420M9-1395	Yes
Loh et al. (2013)	5 for rs2954041; 9 for rs3924999	2004–2012	All[b]	3916/5737 (for rs2954041) 6080/7839 (for rs3924999)	rs764059	No
					rs3924999	No
					rs2954041	No
			MAL	153/150	rs764059	Monomorphic
					rs3924999	No
					rs2954041	No
			CHN	183/179	rs764059	Monomorphic
					rs3924999	No
					rs2954041	No
			IND	81/100	rs764059	Monomorphic
					rs3924999	Yes
					rs2954041	Yes
SZ gene database (access date: 28 Sep. 2015)	5	2007–2009	CAU, Others/Mixed	3256/4183	rs10503929	Yes

AF = African, AS = Asians, CAU = Caucasians, CHN = Chinese, EAS = East Asians, IND = Indian, MAL = Malaysian.
rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006).
[a] The meta-analysis study includes mixed population (Caucasians: 4689/5620, 917 families; Asians: 3219/3107, 598 families; Africans: 141/142, 0 families). Association with schizophrenia based on ethnicity was not reported.
[b] The combined meta-analysis contains people from Caucasian, Japanese, Malay, Chinese and Indian ethnicity.

likelihood for transition to psychosis (Bousman et al., 2013; Hall et al., 2006; Keri et al., 2009). Hall et al. (2006) and Keri et al. (2009) reported that the T/T genotype of the HAP_{ICE} SNP8NRG243177 (rs6994992) polymorphism was associated with a 100% psychosis transition rate in their high-risk cohorts, comprising a genetic high-risk (*e.g.* family history of schizophrenia) cohort and a clinical high-risk (*e.g.* sub-threshold positive symptoms) cohort. Interestingly, the largest study to date of *NRG1* in a high-risk cohort did not replicate this association but did identify two other polymorphisms (rs4281084 and rs12155594) within the HAP_{ICE} region that independently and in combination increased the risk for psychosis transition more than five-fold (Bousman et al., 2013). Notably, only the Bousman et al. (2013) study examined markers in the 3′ region, although found no associations, and no *NRG1* prodromal studies have been reported among non-European populations. As such the generalizability of these findings to non-European populations and the predictive value of 3′ genetic variants remain unclear.

4.5. Gene-gene interaction (epistasis) studies

Only in the past decade have human *NRG1* epistasis studies in schizophrenia been published. In fact, in their 2006 review of *NRG1* and schizophrenia, Harrison and Law (2006) identified one published *NRG1* epistasis study showing an interaction

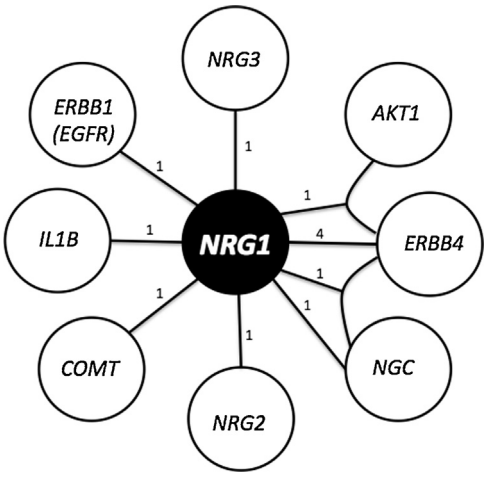

Fig. 3. Statistical epistatic network of *NRG1* in clinical studies of schizophrenia. Edges (lines) represent reported statistical interaction reported in the current clinical literature between two or more genes that include *NRG1*. Interactions including more than two genes are represented by a forked line. The number associated with each line denotes the number of studies that have reported the depicted interaction. *AKT1*, v-akt murine thymoma viral oncogene homolog 1; *COMT*, catechol-o-methyltransferase; *ERBB1 (EGFR)*, epidermal growth factor receptor; *ERBB4*, Erb-B2 receptor tyrosine kinase 4; *IL1B*, interleukin-1 beta; *NGC*, neuroglycan c; *NRG2*, neuregulin-2; *NRG3*, neuregulin-3.

between genetic variation in *NRG1* and ERRB4 (Norton et al., 2006). Since then, 11 gene-gene interaction studies involving *NRG1* in schizophrenia have been published (Supplementary Table S2). From these studies a number of positive statistical interactions associated with schizophrenia risk have been identified (Fig. 3). The strongest clinical support rests in the interaction between genetic variation in *NRG1* and ERBB4, which is also supported by *in vivo* animal and *in vitro* studies (Barbacci et al., 1995). Interestingly, three-way interactions [*NRG1*-ERBB4-AKT1 (So et al., 2010) and *NRG1*-ERRB4-NGC (Nicodemus et al., 2010)] have also been identified, supporting the notion that epistatic networks of complex disorders, such as schizophrenia, will be polygenic in nature (Phillips, 2008). However, epistatic studies to date are biased toward genes within the NRG1-ErbB signalling pathway and as such we have limited knowledge of NRG1's genome-wide interaction network. Future epistatic analysis of genome-wide data could considerably expand and provide greater resolution of *NRG1*'s interaction network.

4.6. *NRG1* x environment interaction studies

It is well accepted that schizophrenia has both genetic and environmental antecedents that interact and affect the presentation and course of the illness (Rethelyi et al., 2013). In the last decade, *NRG1* x environment interaction studies have predominantly been carried out in animal models with particular focus on environmental influences such as cannabis and chronic stress (Hida et al., 2013). However, analogous studies in clinical populations have yet to be conducted. In fact, we are aware of only two *NRG1* x environment studies in the clinical literature. The first human study examined 50 *NRG1* SNPs (5′ and 3′) for an interaction with obstetric complications in a family-based study of 116 trios but found no interactions (Nicodemus et al., 2008). The second study longitudinally examined the interaction between the HAP_{ICE} rs6994992 (SNP8NRG243177) marker and depressive symptom severity in 301 primary care patients with and without histories of psychotic symptoms. They showed depressive symptom severity had less of relationship to longitudinal occurrence of psychotic symptoms among minor allele homozygotes compared to major allele carriers (Bousman et al., 2014). Collectively, these two studies do not provide the evidence base required for sound conclusions to be drawn about *NRG1*'s interaction with environment factors in humans. As such, it remains unclear whether the gene-environment interactions reported in animal models are translatable to clinical populations.

5. *NRG1* gene expression studies

5.1. Human post-mortem brain mRNA studies

Seven *NRG1* gene expression studies using human post-mortem brain tissue have been published, six in the past decade (Table 6). Four of these studies have used case-control and the other three control only designs. Among the case-control studies, the first was conducted in 2004 (Hashimoto et al., 2004) using dorsolateral prefrontal cortex (DLPFC) tissue (20 schizophrenia, 19 controls) and noted an increase in type I *NRG1* gene expression in schizophrenia. This finding has since been replicated in hippocampus (Law et al., 2006) but not DLPFC. In fact, others have shown type I expression is decreased (Parlapani et al., 2010) or unchanged (Weickert et al., 2012) in DLPFC and one study showed NRG1 type I mRNA was unchanged in hippocampus (Nicodemus et al., 2009). Other *NRG1* isoforms as well as Pan-*NRG1* have not been shown to be consistently differentially expressed between schizophrenia and healthy controls. However type II *NRG1*, which was increased by 193% in Brodmann area 10 of schizophrenia patients compared to controls (Parlapani et al., 2010). Together, these findings suggest a complex *NRG1* expression profile in schizophrenia that at the very least differs by brain region and is isoform specific.

Three healthy control only, post-mortem brain studies have provided insights into *NRG1* gene expression profiles across the lifespan. In PFC, type I *NRG1* expression has been shown to gradually decrease and type III expression increase during the gestational period (0–39 weeks); whereas in the post-natal period (0–84 years) type I isoform expression remained stable but type II and type III isoform expression decreased (Paterson et al., 2014). In early adulthood, type I expression appears to decrease in the PFC (Colantuoni et al., 2008), albeit some have reported that type I reaches a plateau during adolescence with only subtle changes thereafter (Harris et al., 2009). In addition to these findings, results from the Human Brain Transcriptome atlas (hbatlas.org) suggests pan-*NRG1* expression peaks during early-mid fetal development (10–19 weeks post-conception) across six brain regions and 11 areas of the neocortex (Fig. S1) (Johnson et al., 2009; Kang et al., 2011; Pletikos et al., 2014). The implications of the temporal profile of *NRG1* expression in the human brain for schizophrenia and the potential impact of allelic variation remain to be determined.

5.2. Human peripheral blood studies

Differential *NRG1* isoform expression is also found in blood of individuals living with schizophrenia compared to controls. Type II β3 *NRG1* and type III *NRG1* expression was found to be higher in peripheral leukocytes in those with schizophrenia of Portuguese ethnicity (Petryshen et al., 2005). However in a Japanese cohort, *NRG1* transcripts amplified from immortalized lymphocytes were not significantly different between patients and controls, although the expression of type I, III and IV *NRG1* were below the limit of detection (Yamamori et al., 2011). Further, decreased expression of pan-*NRG1* in peripheral blood lymphocytes was detected in Han Chinese schizophrenia patients (Zhang et al., 2011, 2008). Due to the limited number as well as demographic and technical differences in the studies conducted to date, there is no clear peripheral *NRG1* gene expression profile in schizophrenia and it remains to be established if peripheral *NRG1* expression could serve as a surrogate

Table 6
Summary of NRG1 human post-mortem gene expression studies.

Reference	Case/Control Subjects	Tissue analysed	Platform used	Ethnicity	mRNA analysed	Gene expression relative to controls
Case-control studies						
Hashimoto et al. (2004)	20/19	DLPFC	qRT-PCR	CAU, AA	NRG1 type I	Increased
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
Law et al. (2006)	38/53	HC	qRT-PCR	CAU, AA, HSP, AS	NRG1 type I	Increased
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
					NRG1 type IV	Unchanged
					Pan NRG1	Unchanged
Nicodemus et al. (2009)	44/84	HC	qRT-PCR	CAU, AA, HSP, AS	NRG1 type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Decreased
					NRG1 type IV	Unchanged
Parlapani et al. (2010)	10/7	PFC (BA9)	qRT-PCR	GER	NRG1 type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
	11/8	PFC (BA10)	qRT-PCR	GER	NRG1 type I	Decreased
					NRG1 type II	Increased
					NRG1 type III	Unchanged
	7/5	HC	qRT-PCR	GER	NRG1 type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Unchanged
Weickert et al. (2012)	37/37	DLPFC	qRT-PCR	AUS	NRG1 type I	Unchanged
					NRG1 type II	Unchanged
					NRG1 type III	Increased
					NRG1 type IV	Beyond limit of detection
					Pan NRG1	Unchanged
					NRG1 EGFα	Beyond limit of detection
					NRG1 EGFβ	Unchanged
Control only studies						
Colantuoni et al. (2008)	0/72	PFC	Custom DNA microarrays and qPCR	60% AA, 35% CAU, 4% HSP and 1% AS	NRG1 and multiple other genes	–
Harris et al. (2009)	0/48	PFC	Affymetrix U133 and qRT-PCR	–	Whole genome microarray analysis (approx. 2000 genes)	–
Paterson et al. (2014)	0/41 foetal brain samples and 195 control samples of age range: 0–83 years	PFC	qRT-PCR	AA, CAU, HSP, AS	NRG1 type I, II, III, IV, NRG1 IVNV	–

AA = African-American, AUS = Australian, AS = Asian, BA = Brodmann area, CAU = Caucasians, CHN = Chinese, DLPFC = Dorsolateral prefrontal cortex, GER = German, HC = Hippocampus, HSP = Hispanic, JP = Japanese, PFC = Prefrontal Cortex.
rs73235619 (SNP8NRG221132), rs35753505 (SNP8NRG221533), rs62510682 (SNP8NRG241930), rs6994992 (SNP8NRG243177), rs113317778 (SNP8NRG433E1006).

for brain expression. In addition, a recent review reported short-term (up to 4 weeks) antipsychotic treatment increases mRNA and protein expression of *NRG1* whereas longer-term treatment decreases this expression (Deng et al., 2013), albeit most of these studies used animal models and did not examine specific *NRG1* isoforms. Thus, future *NRG1* pharmaco-transciptomic studies using human peripheral and central tissue are warranted.

5.3. Expression quantitative trait loci (eQTL) studies

The ability of *NRG1* genetic variation to regulate *NRG1* gene expression in schizophrenia was first tested by Hashimoto et al. (2004); but only first detected later by Law et al. (Law et al., 2006) using hippocampal tissue from 84 controls and 44 schizophrenia subjects. They reported an increased expression of type I *NRG1* was associated with the rare A allele of SNP8NRG221132 (rs73235619) among controls but not those with schizophrenia and an increase in type IV expression associated with the T allele of SNP8NRG243177 (rs6994992) in both those with schizophrenia and controls (Law et al., 2006; Moon et al., 2011; Tan et al., 2007). The latter association has been replicated *in vitro* (Tan et al., 2007) and the SNP8NRG243177 T allele has also been linked to lower levels of Ig-*NRG1* immunoreactivity in serum (Shibuya et al., 2010) but replication of Law et al.'s (2006) initial eQTL findings in independent human brain tissue has yet to be reported. More recent studies have shown decreased expression of hippocampal type I *NRG1* and increased expression of type II among those with schizophrenia carrying the SNP8NRG221533 (rs35753505) C allele (Parlapani et al., 2010). Moreover, there is evidence of increased DLPFC expression of type II and III *NRG1* in schizophrenia among those with one or more copies of the five-marker HAP_{ICE} risk haplotype as well as a novel five-marker haplotype in intron 1, respectively (Weickert et al., 2012). Collectively, these findings suggest a number of SNPs in the HAP_{ICE} risk haplotype serve as putative local eQTLs for *NRG1*.

To our knowledge, examination of distant (trans) *NRG1* eQTLs in schizophrenia has been confined to a post-mortem human brain study (Mathew et al., 2007) and human plasma study (Marballi et al., 2010). In the postmortem study, α7 nicotinic acetylcholine receptor expression in DLPFC but not hippocampal tissue from those with schizophrenia was decreased among SNP8NRG243177 (rs6994992) T allele carriers as well as SNP8NRG221132 (rs73235619) G allele carriers. They further showed that haplotypes containing these alleles were associated with lower expression of the α7 nicotinic acetylcholine receptor in DLPFC (Mathew et al., 2007). In the plasma study, levels of 25 autoimmune markers including five cytokines (*e.g.* IL-6, TNF-α) were increased among rs74942016 T allele carriers compared to G/G genotype carriers. This was corroborated by *in vitro* analysis of transformed B-cells from G/T and G/G genotype carriers (T/T not examined) that showed increases in protein secretion levels of IL-6, TNF-α, and IL-8 in T-allele carriers compared with G/G genotype carriers; suggesting a link between *NRG1* genetic variation and immune dysregulation (Marballi et al., 2010). This distant eQTL association between a *NRG1* SNP and immune markers aligns with a clinical study in Finland reporting an interaction between a *NRG1* SNP (SNP8NRG221533) and a SNP (rs16944) within the interleukin 1 beta gene, a pro-inflammatory cytokine (Hanninen et al., 2008) as well as a preclinical study that showed developmental stage-specific deficits in phentoypes related to schizophrenia (*i.e.* social behavior, spatial working memory, PPI) among TM *Nrg1* mice exposed to maternal immune activation (O'Leary et al., 2014). These clinical and preclinical findings are particularly relevant given evidence of an increase in pro-inflammatory cytokines in schizophrenia (Muller et al., 2015) and is further supported by reports that *NRG1* influences cell adhesion of immune cells (Kanakry et al., 2007) and decreases the release of free radicals from microglial cells (Dimayuga et al., 2003). Furthermore, a recent study (Sekar et al., 2016) has highlighted complement 4 gene within the major histocompatibility complex (MHC) playing a leading role in synaptic dysregulation and risk for schizophrenia and therefore Nrg1 may act in concert with complement 4 and other genes in the MHC region to enhance risk for schizophrenia, perhaps *via* stimulation of pro-inflammatory markers that contribute to autoimmune activation of complement in the brain.

6. *NRG1* protein expression studies

Studies of *NRG1* protein levels in schizophrenia have been hampered by the lack of specific antibodies for a majority of the *NRG1* isoforms and methodological variations across studies. Despite these limitations, six post-mortem brain, one serum, and one plasma study in humans have been published (Table 7). Two studies have reported on *NRG1α*, one reporting a decrease in PFC white matter (Bertram et al., 2007) and the other no difference in Brodmann's area 46 (frontal cortex) (Boer et al., 2009). By contrast, *NRG1*-ICD (intracellular cleavage domain, 53 kDa) levels were found to be increased by 20% in the PFC (Chong et al., 2008) and *NRG1*-CTF (C-terminal fragment, 55 kDa) levels decreased by 50% in the premotor frontal cortex (Brodmann's area 6) (Barakat et al., 2010) in schizophrenia compared to controls. While, Hahn et al. (Hahn et al., 2006) found no difference in *NRG1*-GST (a combination of α and β EGF domains) levels in the PFC. In DLPFC (Brodmann's area 9), the ratio of type III to full length *NRG1* was found to be up-regulated but in anterior prefrontal cortex (Brodmann's area 10) the *NRG1* intracellular domain (50 kDa fragment) was lower in schizophrenia patients (Marballi et al., 2012). In the only two peripheral protein studies to date, serum levels of I_g-NRG1 (Shibuya et al., 2010) and plasma levels of NRG1-β1 (Wang et al., 2015a,b) were decreased in schizophrenia compared to controls. Given that neither of these isoforms has been examined in brain tissue, the concordance between central and peripheral levels remains unknown.

7. *NRG1* and intermediate phenotypes

7.1. Structural neuroimaging studies

In the past decade, twelve studies have examined the association between *NRG1* genetic variation and brain structure (Table 8). These studies have in large part taken a region of interest approach and have almost exclusively selected polymorphisms within the HAP_{ICE}. The SNP8NRG221533 polymorphism has received the most attention (6 studies). The C 'risk' allele at this locus was associated with lower white matter volume (detected with voxel based morphometry) in the regions of the right uncinate fasciculus, right inferior longitudinal fasciculus and in the overlapping portions of the anterior thalamic radiations and the corticopontine tracts (Cannon et al., 2012) compared to TT-genotype carriers. Further, less gross white matter (Suarez-Pinilla et al., 2015) and longitudinal changes in lateral ventricular volumes were also noted in those with the C allele at SNP8NRG221533 (Suarez-Pinilla et al., 2015). However, one European study has reported negative associations with hippocampus and lateral ventricular volumes (Dutt et al., 2009) and the 'non-risk' T-allele was associated with decreased anterior cingulum fractional anisotropy compared to CC genotype carriers in a Han Chinese schizophrenia population (Wang et al., 2009), suggesting HAP_{ICE} 'risk' alleles may differ by ancestry.

Other polymorphisms in the HAP_{ICE} region have also been linked to variation in brain structure. The SNP8NRG243177 T 'risk' allele was associated with a 31% greater ventricular volume in schizophrenia patients compared to those homozygous for the

Table 7
Summary of NRG1 protein analysis studies in schizophrenia (2006–2015).

Reference	Case/Control Subjects	Ethnicity/Source	Tissue type	Result	Other Proteins Analysed	Notes
Hahn et al. (2006)	14/14	CAU and AA	Post-mortem brain tissue (PFC)	NRG1-GST level did not differ significantly between patients and controls	ErbB4	NRG1 stimulation suppresses NMDA hypofunction in human prefrontal cortex.
Bertram et al. (2007)	22/22	–	Post-mortem brain tissue (PFC)	NRG-1α was reduced in white matter of PFC of SZ patients.	–	In gray matter, the density of NRG-1α expressing neurons was reduced.
Chong et al. (2008)	15 SZ, 15 BD, 15 MDD patients/15 controls	CAU, AS and AA	Post-mortem brain tissue (PFC)	PFC cortical 53 kDa NRG1-ICD protein levels were significantly increased (∼20%) in schizophrenic patients ($p = 0.001$)	ErbB4 protein	No significant differences in the levels of the 48 or 42 kDa bands were detected in either cytoplasmic or nuclear fractions of the PFC
Boer et al. (2009)	20 SZ, 8 BD-I/20 controls	CAU, VBBN	Post mortem brain tissue (Brodmann's area 46)	NRG 1Iα level was unchanged	NRG3 and α-synuclein	Protein levels of both NRG1Iα and NRG3 were unchanged in schizophrenia
Barakat et al. (2010)	39/20 (20 MDRS SZ, 19 SZ with normal levels of CHRM1 and 20 healthy controls)	CAU, VBBN	Post mortem brain tissue (Brodmann's area 6 from the left hemisphere)	The level of NRG1-CTF was decreased by 50% in both SZ groups ($p < 0.0027$). The ratio of NRG1-CTF vs NRG1 precursor was decreased in both SZ groups compared to control group ($p = 0.051$).	BACE1	No statistically significant difference in levels of NRG1 full length precursor between the SZ groups and controls.
Shibuya et al. (2010)	40/59	JP	Human Sera	Mean I_g-NRG1 in the SZ group was 63.2% of that measured in controls	–	Patients with T allele (T/C or T/T) of rs6994992 exhibited lower levels of Ig-NRG1 immunoreactivity (for T/T, $p = 0.038$ and for T/C, $p = 0.016$)
Marballi et al. (2012)	6/6 (BA9) and 6/5 (BA10)	GRK	post mortem brain tissue (Brodmann's area 9 and 10)	In BA9: the ratio of NRG1 type III NTF to Full length was upregulated in SZ patients ($p = 0.011$). In BA10: lower levels of NRG1 50 kDa fragment ($p = 0.0018$).	ADAM17	ADAM 17 was negatively correlated with full length NRG1 levels in SZ cohort ($p = 0.008$).
Wang et al. (2015a,b)	166 SZ (80 FES), 60 BD1, 60 BDII, 60 MDD/82 controls	CHN	Human Plasma	NRG1-β1 was decreased in SZ but not in those BD-I, BD-II or MDD, relative to controls.	–	–

AA = African-Americans, AS = Asian, BD = Bipolar disorder, BD-I = Bipolar disorder I, BD-II = Bipolar disorder II, CHN = Chinese, CHRM1 = Cholinergic receptor, muscarinic 1, CAU = Caucasians, FES = first-episode schizophrenia, GRK = Greek, JP = Japanese, MDRS = Muscarinic receptor deficit schizophrenia, MDD = Major depressive disorder, NTF = N terminal fragment, PFC = Prefrontal Cortex, VNBB = Victoria Brain Bank Network (Australia), SZ = Schizophrenia.

C allele, an effect partially explained by an interaction between *NRG1* and *DISC1* (Mata et al., 2009). Furthermore, individuals with schizophrenia homozygous for the SNP8NRG222662C 'risk' allele had reduced left superior temporal gyrus grey and white matter volumes in comparison to those homozygous for the G allele. Furthermore, a longitudinal study of childhood-onset schizophrenia patients showed risk allele carriers at the microsatellite maker 420M9-1395, had greater total grey and white matter volume in childhood and a steeper rate of subsequent decline in frontal, temporal and parietal lobe volume into adolescence, compared to patients without the risk allele (Addington et al., 2007). Taken together, these studies suggest that genetic variation within *NRG1* may be linked with the well replicated finding of tissue reduction and lateral ventricle enlargement found in chronic patients; however evidence also suggests that impact of *NGR1* gene variation may be dynamic and change over time suggesting that longitudinal study designs may be informative.

7.2. Functional neuroimaging studies

Studies examining the effect of *NRG1* genetic variation on neural activation patterns in schizophrenia show heterogeneous results in terms of anatomical localization and degree of activation that, in part, can be attributed to heterogeneity in study design. Among these studies, the HAP_{ICE} markers SNP8NRG243177 and SNP8NRG221533 have gained the most attention. One study of subjects at high-risk of psychosis (Hall et al., 2006), found that homozygotes for the SNP8NRG243177 'risk' T allele had decreased activity in the medial frontal cortex and right temporo-occipital junction when compared with CC carriers during the Hayling's Sentence Completion Task (a covert verbal initiation task). However, healthy children (age range 10–12) with the TT genotype showed greater brain activation in the right posterior orbital gyrus during the Go/Nogo task (an impulse control task) (Mechelli et al., 2009) as well as greater brain activation in the left caudate nucleus, left precuneues, and left angular gyrus during a perceptual matching task (Mechelli et al., 2010). These studies again suggest that the relationship of the human *NRG1* gene variation may change with age.

In a study of individuals undergoing their first episode of psychosis (Kircher et al., 2009a,b), SNP8NRG221533C 'risk' allele carriers showed decreased activation in regions implicated in schizophrenia such as anterior cingulate cortex, hippocampus, precuneus, but also in the lingual gyrus, and cerebellum during a two-back working memory task compared to TT carriers. Similarly, Mechelli et al. (2008) showed C allele carriers had greater deactivation of the left precuneus during a verbal fluency task, compared to TT carriers in schizophrenia, bipolar, and control individuals but increased activation in right inferior frontal/insular cortex in schizophrenia patients only. Furthermore, the C allele was

Table 8
Summary of structural neuroimaging studies in schizophrenia.

Name	Cases/Control Subjects	Field-strength	Modality, Analysis	Ethnicity	SNP, MS	Significant markers	Association with
Addington et al. (2007)	59 COS/165	1.5 T	sMRI; ROI (GM, WM, frontal, temporal, parietal and occipital lobes, CB, CD)	50% CAU, 28% AA, 7% HSP, 5.5% AS and 10% from other or mixed ethnicities	56, 2	420M9-1395	GM and WM volume
Gruber et al. (2008)	30/52[f]	1.5 T	sMRI; ROI[b] (HP)	CAU	1, 2	3-marker haplotype (rS35753505, 478B14-848, 420M9-1395)	HP volume
Dutt et al. (2009)	128/194[f]/61	1.5 T	sMRI; ROI[b] (LV, HP)	EU	1, 2	–	–
Mata et al. (2009)	95[d]	1.5 T	sMRI; ROI[a] (LV, CSF, parietal, temporal, and frontal lobes)	SP	3, 0	rs6994992	LV
Wang et al. (2009)	31/36	1.5 T	DWI; ROI[b] (cingulate)	NHC	1, 0	rS35753505	FA (AC)
Haukvik et al. (2010)	54/53	1.5 T	sMRI; ROI[a,b] (GM, WM, CSF, venous blood, HP)	CAU	12, 0	rs2954041	HP volume
Mata et al. (2010)	91[d]/26	1.5 T	sMRI; ROI[a] (LV, CSF, CD, PU, TH)	SP	1, 0	rs6994992in NRG1 and rs2793092 in DISC1	LV
Cannon et al. (2012)	70 (37[c] + 33 BP)/119 controls (39 healthy controls, 40 with a relative with SZ and 40 with a relative with BP)	1.5 T	sMRI, VBM	UK	1, 0	rs35753505	WMV
Tosato et al. (2012)	27[c]	1.5 T	sMRI; ROI[b] (STG)	ITA	1, 0	rs4623364	STG (GM and WM volume)
Grimm et al. (2014)	54[f]/80	3.0 T	sMRI; VBM and ROI[a] (STR)	GER	1, 0	–	–
Thirunavukkarasu et al. (2014)	38[d,e]/37	3.0 T	sMRI; VBM	sIND	1, 0	–	–
Suarez-Pinilla et al. (2015)	59[c,d]/14	1.5 T	sMRI; ROI[a] (Br, GM, WM, CSF, LV, TH, CD)	SP	3, 0	rs35753505	LV and WM volume

AA = African-American, AC = Anterior cingulum, AS = Asian, BP = Bipolar disorder, Br = Brain, CAU = Caucasian, CB = Cerebellum, CD = Caudate, COS = Child onset schizophrenia, CSF = Cerebrospinal fluid, DWI = Diffusion weighted imaging, EU = European, FA = Fractional anisotropy, GER = German, GM = Grey matter, HP = Hippocampus, HSP = Hispanic, ITA = Italian, LV = lateral ventricle, NHC = Northern Han Chinese, PU = Putamen, ROI = Region of interest, sIND = South Indian, sMRI = Structural magnetic resonance imaging, SP = Spanish, STG = Superior temporal gyrus, STR = Striatum structures, TH = Thalamus, UK = United kingdom, VBM = Voxel-based morphometry, WM = White matter.
rs35753505 (SNP8NRG221533), rs4623364 (SNP8NRG222662), rs6994992 (SNP8NRG243177).

[a] Automated analysis.
[b] Manually traced.
[c] Schizophrenia, schizophreniform disorder, brief psychotic disorder, or psychosis not otherwise specified.
[d] First Episode.
[e] Paranoid schizophrenia, undifferentiated schizophrenia, schizophreniform disorder.
[f] First degree relatives to schizophrenia patients.

associated with hyperactivation of the superior frontal gyrus in healthy controls during a two-back version of the Continuous Performance Task (Krug et al., 2010). Similar to the preclinical work described in the first section of this review, the putative neurocircuitry impact of biological changes in *NRG1* appear to vary depending on brain region and task employed.

Importantly, other *NRG1* polymorphisms have also been studied and have shown associations with brain function in healthy subjects. Nicodemus et al. (2010) observed a novel interaction between the 5-prime rs4560751 and 3-prime rs3802160 in healthy controls that was associated with inefficient processing in the DLPFC during a working memory task. In addition, Grimm et al. (2014) recently showed the rs10503929 missense variant in the 3-prime region was associated with decreased ventral striatal activation during reward anticipation in first degree relatives of those with schizophrenia compared to controls without a family history of psychiatric illness. Thus, SNPs beyond the HAP_{ICE} risk haplotype including SNPs in the 3-prime region may serve as markers of brain function and warrant further investigation.

7.3. Cognitive studies

To date, the most comprehensive analysis of *NRG1* and cognition in schizophrenia examined 1536 SNPs in 94 functionally relevant candidate genes (including 117 *NRG1* SNPs) for association with 25 cognitive endophenotypes in 130 families comprising a schizophrenia proband, both parents, and at least one unaffected sibling (Greenwood et al., 2016, 2011). Thirty-nine of 126 *NRG1* SNPs examined were associated ($p < 0.05$) with at least one of 10 cognitive phenotypes related to prepulse inhibition, verbal learning, attention, abstraction, spatial processing, and sensorimotor dexterity, suggesting a high degree of pleiotropy and diffuse functional effects across the brain. Furthermore, two of the most significant findings were associations between spatial processing and rs4281084 ($p = 6.4 \times 10^{-6}$) and verbal learning and rs3924999 ($p = 5.6 \times 10^{-4}$). The latter SNP is a missense variant located near the 3-prime end of *NRG1* and has also been associated with two oculomotor endophenotypes (antisaccade and smooth pursuit eye movement) in healthy controls (Roussos et al., 2011), prepulse inhibition in military conscripts (Schmechtig et al., 2010), and P300 latency in schizophrenia and control subjects (Kang et al., 2012). On the other hand, rs4281084 is in the 5-prime region and was recently shown to be a risk marker for psychosis transition in an ultra-high risk population (Bousman et al., 2013).

Other *NRG1* polymorphisms, primarily in the HAP_{ICE} region, have also been linked to cognitive endophenotypes. The 'risk' T allele of rs6994992 (SNP8NRG243177) has been associated with reduced spatial working memory and prepulse inhibition in military conscripts (Roussos et al., 2011; Stefanis et al., 2007), reduced attention and general cognitive ability in those with schizophrenia (Cho et al., 2015; Kukshal et al., 2013), and lower IQ in the prodromal period (Keri et al., 2009). Approximately 21.4 kbp upstream of this variant, the 'risk' C allele of rs35753505 (SNP8NRG221533) has been linked to deficits in semantic verbal fluency and P300 latency (Bramon et al., 2008; Kircher et al., 2009a,b), supported in part by functional neuroimaging findings that C allele carriers show a deactivation of the left precuneus during a verbal fluency task (Mechelli et al., 2008). However, poor premorbid adjustment was associated with homozygotes of the 'non-risk' T genotype (Walshe et al., 2012). For the remaining HAP_{ICE} SNPs (SNP8NRG221132, SNP8NRG241930, SNP8NRG433E10006) and microsatellites (478B14-848, 420M9-1395) results on the relationship to cognitive deficits have been largely inconsistent or negative. Together with *NRG1* neuroimaging findings reviewed above, the current cognitive findings suggest *NRG1* genetic variation has pleiotropic effects on brain structure and function that may be age and/or context dependent.

8. *NRG1* and antipsychotic treatment response

The association between *NRG1* genetic variation on antipsychotic response was first reported in a study of 94 Finnish individuals with schizophrenia taking typical antipsychotics for a minimum of four weeks (Kampman et al., 2004). In this study, homozygotes of the 'non-risk' T allele at the SNP8NRG221533 (rs35753505) locus were over-represented in the non-responder group. Interestingly, a decade has passed since this study but to our knowledge, only two additional studies examining *NRG1* genetic variation and antipsychotic treatment response have been published. Among a sample of 339 high symptom severity schizophrenia patients (*i.e.* clinical global impression–severity scale > 3) from Indian populations of Indo-European and Dravidian ancestry, the A alleles for *NRG1* SNPs rs13250975 and rs17716295 were associated with non-response to antipsychotic (typical and atypical) medication (Jajodia et al., 2016). Neither of these SNPs (rs13250975 and rs17716295) have previously been described in the literature and both sit in the intronic region between the exons defining type V and type I forms of *NRG1,* however it is not clear if these SNPs are functional or if they are tagging functional variants. Using a haplotype tagging genotyping approach, Terzic and colleagues (2015) examined four *NRG1* tag-SNPs (rs3735781, rs3735782, rs10503929, rs3924999) for their association with treatment responsiveness in a cohort of 138 schizophrenia outpatients in Slovenia treated with typical or atypical antipsychotics, though results did not support an association between any of the *NRG1* SNPs examined and treatment responsiveness (Terzic et al., 2015). Collectively these studies demonstrate the need for further *NRG1* pharmacogenetic studies, particularly studies capable of simultaneously examining multiple variants and haplotypes within *NRG1* and possibly its cognate receptor ERBB4, which was recently shown to be associated with paliperidone response (Wang et al., 2015a,b).

9. Future directions

Throughout our review we have noted a number of gaps and limitations within the current *NRG1* literature that warrant future study. To guide this future clinical and preclinical work and assist in prioritizing a strategy forward we have generated the following recommendations:

1. Look beyond the central dogma. Human-based evidence related to the epigenetic (*e.g.* methylation), post-transcriptional (*e.g.* microRNA), or post-translational (*e.g.* phosphorylation, ubiquitination, cleavage) modification of *NRG1* is currently limited or absent from the literature. Uncovering how, when, and by what mechanisms the various isoforms of *NRG1* are regulated and/or modified is fundamental to our understanding of how variation of *NRG1* may make the human brain more vulnerable to developing schizophrenia. Furthermore, a deeper understanding of the functional differences between the various *NRG1* isoforms in the context of ErbB4 changes using clinical and preclinical approaches is warranted. Utilising tools such as human induced pluripotent stem cell derived neurons from schizophrenia patients would permit the study of cellular defects in cells with a genetic predisposition toward schizophrenia. Whereas, genomic editing techniques (*e.g.* CRISPR-Cas system) could be used in healthy tissue to mimic genetic risk variations in DNA. Similarly, transgenic rodent models offer an efficient way of simultaneously investigating cellular, regional and behavioral

neurobiological components associated with functional *Nrg1* variants; however age, brain region and gender needs to be considered when interpreting possible dynamic effects. These varied approaches may assist in dissecting the effects of *NRG1* modification on molecular signalling pathways or the mechanisms that underpin aberrant *NRG1* action.

2. Utilize multimodal neuroimaging approaches. Multimodal approaches have the greatest potential for improving our understanding of *NRG1*'s influence on the brain *via* cross verification within subjects in a single study. Importantly, only one study (Grimm et al., 2014) to date has adopted this approach and no study in humans has used molecular or metabolic neuroimaging methods (*i.e.*, positron emission tomography, single-photon emission tomography, or magnetic resonance spectroscopy) to investigate cerebral effects of *NRG1* and its structural variants.
3. Mind the environment. The interactions between *NRG1* and key beneficial (enrichment) and deleterious environments (*e.g.* prenatal infection, early life stress, cannabis, antipsychotics) are not well understood at the clinical level. More intensive evaluation of these interactions is needed to assist in untangling current heterogeneous findings and will be greatly aided by in-depth collection of study participants' environmental exposures across the lifespan. This in turn will support epigenetic studies of *NRG1*, which to date have not been undertaken in schizophrenia but have the potential to improve our understanding of the interplay between environmental factors and *NRG1* in the pathophysiology of the disorder. In addition, further development and use of mutant and transgenic rodent models of *Nrg1* and its isoforms to characterize the complexity of such gene-environment interactions and inform on the epistatic effects of *Nrg1* variants will be vital.
4. Maximize existing consortia resources. Shared data resources provide access to the sample sizes required to detect modest effects. The Psychiatric Genomics Consortium (http://med.unc.edu/pgc), the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) consortium (http://enigma.ini.usc.edu), European Network of National Networks studying Gene-Environment Interactions in Schizophrenia (EU-GEI) (van Os et al., 2014), psychENCODE (http://psychencode.org), UK Brain Expression Consortium (http://www.braineac.org), and the Genotype-Tissue Expression (GTEx) Portal Anon. (2013) are current examples of consortia providing opportunities to examine global associations between *NRG1* genetic variation, gene expression, brain structure and environmental factors. At the preclinical level, resources such as the International Mouse Phenotyping Consortium (IMPC; http://www.mousephenotype.org) and Mouse Genome Informatics (MGI; http://www.informatics.jax.org) allow researchers to keep track of new *Nrg1* phenotypes and develop new studies and transgenic models that build on international findings. However, consortiums may not be appropriate for addressing all the gaps in our knowledge related to *NRG1* and as such independent and context specific studies are still warranted.

10. Summary

The past decade of clinical research and recent preclinical findings clearly show that the link between *NRG1* and schizophrenia is complex and will require further concerted and collaborative efforts to be elucidated. At the preclinical level, there is strong evidence that *Nrg1* may disrupt normal excitatory/inhibitory neurotransmission via the ErbB4 receptor, potentially mediated by downstream effects such as gamma oscillations that then interfere with cognitive processes principally involving frontal cortex, hippocampus, and striatum, but the exact cellular mechanisms and neurocircuitry involved are not well understood and investigating these should be one of the primary directions of future research. At the clinical level, although there is reasonable support for an association between genetic variation in the 5-prime region of *NRG1* and schizophrenia, the subsequent transcriptional and translational mechanism(s) through which this variation is expressed and/or regulated is less clear. Likewise, our current understanding of the downstream effects of *NRG1* variation and/or expression on brain structure and function and on cognition processes is limited. However, relative to other current candidate genes for schizophrenia, *NRG1* is arguably one of the most extensively characterized. As such, future clinical and preclinical research should continue to build upon the empirical foundation that has been created over the past decade in an effort to broaden and deepen our understanding of *NRG1*'s role in the pathophysiology of schizophrenia.

Acknowledgements

MM was supported by a Cooperative Research Centre for Mental Health Top-up Scholarship. SS and AP were supported by One-in-Five Association Incorporated. CP was supported by an NHMRC Senior Principal Research Fellowship (#628386), and a Brain and Behavior Research Foundation (NARSAD) Distinguished Investigator Award. TK is supported by a Career Development Fellowship (Level 2: #1045643) and a project grant (#1102012) from the National Health and Medical Research Council (NHMRC), as well as the Rebecca L. Cooper Medical Research Foundation Ltd. CSW is supported by Schizophrenia Research Institute (utilizing infrastructure funding from the NSW Ministry of Health and the Macquarie Group Foundation), the University of New South Wales, and Neuroscience Research Australia. CSW is a recipient of a National Health and Medical Research Council (Australia) Senior Research Fellowship (#1021970). CAB was supported by a University of Melbourne Ronald Phillip Griffith Fellowship and Brain and Behavior Research Foundation (NARSAD) Young Investigator Award (#20526). None of the Funding Sources played any role in interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neubiorev.2016.06.001.

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