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Exploring mGluR5 dysregulation in schizophrenia: from gene to protein

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Exploring mGluR5 dysregulation in schizophrenia: from gene to protein

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By

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Certification

I, Natalie Matosin, declare that this thesis, submitted in entire fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is wholly my own work unless referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Statements

In accordance with the University of Wollongong thesis committee 'Guidelines for Higher Degree Research Candidates on the Preparation and Submission of Higher Degree Research Theses' and 'Higher Degree Research Thesis by Compilation Rules' (2014) this PhD is presented as a 'Thesis by Compilation'. It is comprised of a series of 4 original studies published in, or submitted to, peer-reviewed journals, of which I am the first author. I hereby declare that I am a designer of these studies, have carried out the experimental procedures (unless referenced or acknowledged), data analysis and manuscript preparation.

Natalie Matosin

I consent to the presentation of this PhD in 'Thesis by Compilation' and I acknowledge the above statement per-
o be correct.

Dr Kelly A Newell (Supervisor)

Publications

The following publications and presentations have arisen directly from work contained within this thesis.

Peer-Reviewed Publications

- **Matosin N,** Green MJ, Newell KA, Andrews JL, Fernandez-Enright F. Sex-specific associations of novel gene variants in metabotropic glutamate receptor 5 with schizophrenia and cognitive dysfunction. Submitted *American Journal of Psychiatry.*
- **Matosin N**, Lum JS, Fernandez-Enright F, Newell KA. Shifting towards a model of mGluR5 dysregulation in schizophrenia: consequences for future schizophrenia treatment. Special issue: "mGluRs: 5 years on", *Neuropharmacology*. Under review with Guest Editors.
- **Matosin N,** Fernandez-Enright F, Lum JS, Andrews JL, Engel M, Huang XF, Newell KA. Metabotropic glutamate receptor 5 and its trafficking molecules, Norbin and Tamalin, are increased in the CA1 hippocampal region of subjects with schizophrenia. Accepted, *Schizophrenia Research*.
- **Matosin N**, Fernandez-Enright F, Fung, SJ, Lum JS, Engel M, Andrews JL, Huang XF, Weickert CS, Newell KA. Alterations of mGluR5 and it endogenous regulators Norbin, Tamalin and Preso1 in schizophrenia: towards a novel model of mGluR5 dysregulation. *Acta Neuropathologica.* 2015. Epub ahead of print: doi: 10.1007/s00401-015-1411-6.
- **Matosin N,** Fernandez-Enright F, Frank E, Deng C, Wong J, Huang XF, Newell KA. Metabotropic glutamate receptors mGluR2/3 and mGluR5 binding in the anterior cingulate cortex in psychotic and non-psychotic depression, bipolar disorder and schizophrenia: implications for novel mGluR-based therapeutics. *Journal of Psychiatry & Neuroscience.* 2014. 39(6): 407-16.
- Newell KA, **Matosin N**. Rethinking metabotropic glutamate receptor 5 pathological findings in psychiatric disorders: implications for the future of novel therapeutics. *BMC Psychiatry.* 2014. 14:23.
- **Matosin N**, Newell KA. Metabotropic Glutamate Receptor 5 in the Pathology and Treatment of Schizophrenia. *Neuroscience & Biobehavioural Reviews.* 2013. 37(3): 256-268.

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- **Matosin N**, Green MJ, Andrews JL, Lum JS, Engel M, Huang XF, Fernandez-Enright F* and Newell KA*. mGluR5 dysregulation in schizophrenia and cognition: from gene to protein. 25th ISN-APSN Biennial Meeting in conjuction with ANS, Cairns, Australia August 23-27, 2015. Abstract submitted. *equal contribution
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- **Matosin N**, Fernandez F, Huang XF, Newell KA. mGluR5 and Its Modulation Is Disrupted in the Dorsolateral

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Conference Abstracts

- **Matosin N**, Engel M*, Fernandez-Enright F, Lum JS, Andrews JL, Huang XF, Newell KA. Alterations of mGluR5 and mGluR5 signaling partners in schizophrenia. Society for Neuroscience Meeting, Washington DC 15-19 November 2014.
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Additional Publications

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- Andrews JL, Goodfellow F, **Matosin N**, Snelling MK, Newell KA, Huang XF, Fernandez-Enright F. Alterations of Ubiquitin Related Proteins in the Pathology and Development of Schizophrenia: Evidence from Human and Animal Studies. Under review, *BBA Molecular Basis of Disease.*
- Andrews JL, Newell KA, **Matosin N**, Huang XF, Fernandez-Enright F. Alteration of p75 neurotrophin receptor and myelin transcription factor 1 in the hippocampus of perinatal phencyclidine treated rats. Under review, *Progress in Neuro-Psychopharmacology & Biological Psychiatry.*
- Wagner KV, Hartmann J, Labermaier C, Häusl AS, Zhao G, Harbich D, Schmid B, Wang XD, Santarelli S, Kohl C, Gassen NC, **Matosin N**, Schieven M, Webhofer C, Turck CW, Lindemann L, Jaschke G, Wettstein JG, Rein T, Müller MB, Schmidt MV. Homer1/mGluR5 signaling moderates vulnerability to chronic social stress. *Neuropsychopharmacology*. 2015. 40(5): 1222-1233.
- **Matosin N**, Frank E, Engel M, Lum JS, Newell KA. Negativity towards negative results: a discussion of the disconnect between scientific worth and scientific culture (editorial). *Disease Models and Mechanisms*. 2014. 7(2): 171-173.
- **Matosin N**, Frank E, Deng C, Huang XF, Newell KA. Metabotropic Glutamate Receptor 5 Binding and Protein Expression in Schizophrenia and Following Antipsychotic Drug Treatment. *Schizophrenia Research.* 2013. 146(1-3): 170-176.

Book chapter:

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- **Jimenez N**, Huang XF, Deng C, Newell KA. Metabotropic Glutamate Receptor Subtype 5 is Reduced in

Schizoaffective subjects of Major Depression Type and Unchanged Following Antipsychotic Drug Treatment (poster). *Australian Neuroscience Society 32nd Annual Meeting*, Queensland, Australia, January 29-February 1 2012.

Conference Abstracts:

- Newell KA, **Matosin N**, Fernandez-Enright F, Huang XF. Metabotropic Glutamate Receptor 5 dysregulation in schizophrenia (poster). Schizophrenia International Research Society Biennial Meeting, Florence Italy, April 5-9 2014.
- Newell KA, **Matosin N**, Geddes A, Frank E, Huang E. Metabotropic glutamate receptor 5 (mGluR5) shows differential associations with NMDAR NR2A and NR2B subunits in schizophrenia: implications for novel mGluR5 therapeutics (oral). *12th Biennial Australasian Schizophrenia Conference*, Melbourne, Australia, May 13-14 2013.
- Lum JS, Frank E, **Matosin N**, Huang XF and Newell KA. Subchronic Metabotropic Glutamate 5 Receptor Modulation in the Perinatal PCP Rodent Model of Schizophrenia (poster). *Australian Neuroscience Society 33rd Annual Meeting*, Melbourne, Australia, February 3-6 2013.
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- Newell KA, **Jimenez N (née)**, Geddes AE, Frank E, Du Bois TM, Safadi M. Huang XF. Diagnostic-specific reductions in NMDA and mGlu5 glutamatergic receptors in schizophrenia (poster). *Australian Neuroscience Society 32nd Annual Meeting*, Queensland, Australia, January 29-February 1 2012.

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[^{3}H]	Tritiated
ACC	Anterior cingulate cortex
Akt	Protein kinase B
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APD	Antipsychotic drug
BA	Brodmann's area
BDNF	Brain-derived neurotrophic factor
BP	Bipolar disorder
C-terminus	Carboxyl terminus
CA	Cornu ammonis
CaIN	Calcinuerin inhibitior
CD	Cognitive deficits
CREB	cAMP response element-binding
CS	Cognitively spared
CT	Control
DAG	Diacylglycerol
DLPFC	Dorsolateral prefrontal cortex
GKAP	Guanylate-kinase-associated protein
GoM	Grade of Membership
GPCR	G-protein coupled receptor
GRASP	GRP-1-associated scaffold protein or GRASP
GRK	G-protein regulatory kinase
HC	Healthy control
iGluR	Ionotropic glutamate receptor
IP_3	Intracellular inositol 1,4,5-triphosphate
LTD	Long term depression
LTP	Long term potentiation
MD	Major depression
MDNP	Major depression without psychosis
MDP	Major depression with psychosis
mGluR	Metabotropic glutamate receptor
MK-801	[5R,10S]-[+]-5-methyl-10,11- dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine
MPEP	2-Methyl-6-(phenylethynyl)pyridine
MTEP	3- [(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine
mTOR	Mammalian target of rapomycin
NMDAR	N-methyl-D-aspartate receptor
FRMPD4	FERM (4.1 protein, ezrin, radixin and moesin) and PDZ (post synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein) domain-containing protein 4
p.o.	Per oral
PAMS	Positive allosteric modulators
PCP	Phencyclidine
PFC	Prefrontal cortex
PI3K/PIKE	PI3 Kinase and its enhancer
PKC	Protein-kinase C
PLC	Phospholipase C
PP1	Protein phosphatase 1
PP2B/CaN	Protein phosphatase 2B/calcineurin
PSD	Postsynaptic density
PSD95	Post-synaptic density 95
Pyk2/CAKß	Proline-rich tyrosine kinase/cell adhesion kinase ß
Rs	Reference single nucleotide polymorphisms
S-SCAM	Membrane-associated guanylate kinase inverted-2
SHANK	SH_3 and multiple ankyrin repeat domains
s.c.	Subcutaneous
Siah1	Seven in absentia homolog 1.
SZ	Schizophrenia

Abstract

Metabotropic glutamate receptor subtype 5 (mGluR5)-targeting therapeutics display a favourable profile of effects in preclinical paradigms, particularly for the treatment of cognitive dysfunction. It however remains unclear whether mGluR5 plays a causal or epiphenomenal role in the pathophysiology of schizophrenia. It is important to understand the aspects of mGluR5 that might be altered within the pathological condition, as this could have implications for novel drugs targeted at these systems in the patient. This thesis therefore aimed to deconstruct [at least some] of the complexities of mGluR5 in schizophrenia, with a focus on potential dysregulation of mGluR5, predominately utilising human tissues (postmortem brain tissues and DNA samples derived from schizophrenia patients).

Considering many symptoms associated with schizophrenia overlap with other closely related neuropsychiatric disorders, the first study in this thesis (Chapter 2) aimed to address whether mGluR5 alterations were specific to a particular set of symptoms (such as depressive symptoms or psychotic symptoms, or a combination thereof). To do this, mGluR5 binding was performed in postmortem samples from the anterior cingulate cortex, a region that modulates cognitive and emotional processes, of subjects with schizophrenia, major depression (with or without psychosis) or bipolar disorder. The results revealed that mGluR5 binding was unaltered across all neuropsychiatric pathologies, providing preliminary evidence that mGluR5 is not affected, at least in this region. Binding studies provide valuable insight into the ability of a receptor to interact with ligands and are thus useful for determining the therapeutic utility of a receptor; however this method is unable to detect wider changes, such as altered regulation, which may be underlying the observed pathologies.

Building on from this work, Chapter 3 aimed to explore whether dysregulation was present within the mGluR5 system in schizophrenia. Postmortem samples from the dorsolateral prefrontal cortex (DLPFC; BA46 involved in higher executive and cognitive processes) of schizophrenia subjects were examined. mGluR5, and proteins known to regulate mGluR5 trafficking, endocytosis and signalling (Norbin, Tamalin and Preso1) were examined. This study was also expanded to include measures of mGluR5 mRNA levels in the same subjects (data contributed by the S. Fung and C.S. Weickert). Together, this study revealed that although mGluR5 mRNA levels were unaltered, mGluR5 protein levels were greatly increased in this brain region. Conversely, mGluR5 endogenous regulators (Norbin, Tamalin and Preso1) were significantly decreased in this region. Interestingly, mGluR5 protein levels were correlated with levels of mGluR5 mRNA and the measured mGluR5 endogenous regulators in control subjects, but this correlation was lost in schizophrenia subjects. These results strongly support the presence of mGluR5 dysregulation in schizophrenia, at least in this region.

The work in Chapter 4 aimed to determine whether these findings in the DLPFC were exclusive to this brain region, or whether they extended to the hippocampus. The hippocampus is another region involved in schizophrenia pathology, which is involved in learning and memory functions. Extensive animal and cell-based data suggests mGluR5 critically mediates synaptic plasticity specifically in the CA1 subregion of the hippocampus. The results from this study thus found, that similarly to the DLPFC, mGluR5 was increased in the CA1 region. In contrast to the DLPFC, however, levels of mGluR5 endogenous regulators were significantly increased in this region, and no correlations between mGluR5 and the mGluR5 endogenous regulators were observed. These findings suggest that whilst alterations to mGluR5 extend to the CA1 region, the function, signalling, or regulation of mGluR5 might be brain-region specific. This work nonetheless also supports that mGluR5 is dysregulated in schizophrenia.

In Chapters 3 and 4, the influence of commonly used antipsychotic drugs (APDs) on mGluR5 was examined. In this series of studies, adult male rats were treated with either haloperidol (a first generation APD) or olanzapine (a second generation APD) for short, medium and long-term durations (APD-treated rat brains contributed by X.F. Huang). The protein levels of mGluR5 and mGluR5 endogenous regulators were subsequently measured in regions corresponding to the human studies, the PFC and hippocampus. The results indicated that commonly used APDs do not influence the mGluR5 system, suggesting that the postmortem findings in Chapters 2, 3 and 4 are not confounded by premortem mediation history. These findings also suggest that novel drugs to correct potential changes in mGluR5 might be useful as an adjunct treatment with current APDs, to target the observed alterations in the postmortem human tissues that are not targeted by current APDs.

The knowledge regarding mGluR5 is becoming increasingly substantial. Nonetheless, very little is known about the role of the *GRM5* gene, encoding for mGluR5, in the genetic susceptibility to schizophrenia. Chapter 5 thus aimed to determine whether single nucleotide polymorphisms (SNPs) within the *GRM5* gene are associated with schizophrenia diagnosis in a large Caucasian case-control population. In light of my previous works from Chapters 3 and 4, indicating mGluR5 dysregulation at the protein level, SNPs located in the 3' untranslated region of *GRM5* were selected, as this is a genomic region that is highly involved in protein conformation and regulation of regions responsible for protein-protein interactions. Furthermore, due to the extensive evidence that mGluR5 underlies cognitive processes, effects of genetic variability within the chosen SNPs were analysed for their effects on various measures of cognitive function (data contributed by M.J. Green). As hypothesised, the measured SNPs within *GRM5* were differentially association with schizophrenia diagnosis. Further, genetic variability within *GRM5* affected and/or was able to significantly predict the level of cognitive dysfunction in schizophrenia patients. Interestingly in this study, all findings were sex-specific, suggest-

ing that *GRM5*/mGluR5 may be differently regulated in men and women with schizophrenia. Nonetheless, the association of SNPs located within the 3' untranslated region of *GRM5* contributes to the substantial evidence presented in this thesis that mGluR5 is dysregulated in the pathology of schizophrenia, and supports that mGluR5 has a modulatory effect on cognitive function in humans, not just animals.

Using a systematic approach to analyse mGluR5 from the gene-level to protein-level, and further, in human cognitive performance, the findings from this thesis provide the first compelling evidence that mGluR5 is dysregulated in schizophrenia. Although further studies are required to investigate the exact mechanisms responsible for this dysregulation, the findings presented here support the development of agents that modulate mGluR5 regulation and/or signalling to treat cognitive dysfunctions in patients with schizophrenia.

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brain
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Chapter One

This chapter is published in part:

- **Matosin N**, Lum JS, Fernandez-Enright F, Newell KA. Shifting towards a model of mGluR5 dysregulation in schizophrenia: consequences for future schizophrenia treatment. Special issue: "mGluRs: 5 years on", *Neuropharmacology*. Under review with Guest Editors.
- **Matosin N**, Newell KA. Metabotropic glutamate receptor 5 in the pathology and treatment of schizophrenia. *Neuroscience and Biobehavioural Reviews* 2013, 37(3).
- Newell KA, **Matosin N**. Rethinking metabotropic glutamate receptor 5 pathological findings in psychiatric disorders: implications for the future of novel therapeutics. *BMC Psychiatry*. 2014. 14(23).
- Newell KA, **Matosin N**, Lum J. Chapter 5: Metabotropic glutamate receptors in the pathophysiology and treatment of schizophrenia and major depression. *Metabotropic Glutamate Receptors: Molecular Mechanisms, Role in Neurological Disorders and Pharmacological Effects*. Nova Publishers. 2014.

1.1 Introduction

1.1.1 What is schizophrenia?

Schizophrenia is a severe neuropsychiatric disorder, characterised by disturbances of thought, emotion and behaviour. Despite decades of research, the exact causes remain unknown. Schizophrenia emerges primarily in early adulthood and affects approximately 1% of the general population (World Health Organization, 2006). Although there is a strong heritability of schizophrenia (~80%), studies have not identified one single genetic origin, but rather over 1500 single nucleotide polymorphisms (SNPs) have been associated with the disorder (Purcell et al., 2014; Ripke et al., 2013). Environmental influences, such as early-life stress and cannabis use, have also been linked to schizophrenia vulnerability (van Os et al., 2010). Accumulating evidence thus indicates that the aetiology of schizophrenia is polygenic, with both genetic and environmental factors involved (Altamura et al., 2011; Dickinson, 2014; Ehrenreich and Nave, 2014; Leask, 2004).

The symptoms of schizophrenia are broadly characterised into positive, negative and cognitive categories (Eaton et al., 1995). Each patient displays a combination of these symptoms, with differing severities that vary according to the stage of disease progression (Tandon et al., 2008; Tandon et al., 2009). Positive symptoms (such as hallucinations and delusions) emerge gradually at the onset of disease, marking the first psychotic episode (Tandon et al., 2009). Whilst positive symptoms are the most striking indications of schizophrenia, negative (depressive-like symptoms) and cognitive symp-

toms (deficits in attention, concentration, learning, memory, language, psychomotor speed and higher order thinking) cause greater debilitation, as they are strongly linked with the individual's functional outcome and ability to live independently (Green, 2006). Negative and cognitive aspects of schizophrenia often develop prior to the onset of the first episode, and are less successfully treated with antipsychotic drug (APD) therapy (Schenkel and Silverstein, 2004; Tandon et al., 2010).

In addition to the unique combination of symptoms in every case of schizophrenia, patients commonly exhibit characteristics that overlap with symptoms of other closely related neuropsychiatric disorders, including bipolar disorder and major depression (Rothschild, 2013). This overlap includes symptoms such as psychotic features, mood dysregulation and cognitive deficits (Lee, SH and the Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013), although the disorders also have distinct molecular underpinnings, consequently resulting in some phenotypic distinctions and separate diagnostic classification and treatment in the clinic (Buckley et al., 2009; Rothschild, 2013). Yet the sometimes-blurred diagnostic boundaries, in combination with the heterogeneous and dynamic symptom profile, render schizophrenia a complex disorder to characterise and ultimately treat. It is thus fundamental to decode which molecular alterations underpin each specific symptom, as this has critical implications for advising the use of pharmacotherapies (DeRubeis et al., 2014; Mirzakhanian et al., 2014).

1.1.2 The genetic underpinnings of schizophrenia

Although several common genetic variations have been associated with the aetiology schizophrenia, the type and number of genes involved remain unclear (Ripke et al., 2013). A large number of genetic studies have been undertaken in schizophrenia case-control populations, including candidate gene approaches, gene expression analyses, linkage analyses and genome-wide association studies (e.g. Andreassen et al., 2014; Crowley et al., 2013; McCarroll et al., 2014; Modinos et al., 2013; Ripke et al., 2013). It has been particularly challenging to define a clear number of genes and markers involved in schizophrenia, mainly owing to the heterogeneity of the schizophrenia patients in the tested populations. Nonetheless, the contribution of genetic variation in the emergence of schizophrenia has been well established and is widely accepted.

The strongest evidence in support of a genetic susceptibility to schizophrenia stems from the first familial studies (Kendler and Diehl, 1993). Approximately 10% of patients with schizophrenia have a first-degree relative also affected by the disorder (Cardno and Gottesman, 2000; Gottesman et al., 1976), with the prevalence being 10 times higher in biological relatives compared to adoptees with schizophrenia (Kety, 1987; Kety et al., 1994). Reports from twin studies suggest that the incidence of schizophrenia is significantly higher in monozygotic (31%) compared to dizygotic (7%) twins, and the concordance rate in monozygotic twins is approximately 75-91% (Kendler and Robinette, 1983).

Over twenty genome-wide association studies have investigated the location of genes that may be involved in the emergence of schizophrenia. Meta-analyses of these investigations have suggested the consistent involvement of particular loci, with several candidate genes identified through systematic fine mapping, linkage analysis, and SNP analysis of linkage peaks (Badner and Gershon, 2002; Lewis et al., 2003). A multitude of loci have been implicated, including 1q, 2, 3p, 5q, 6p, 8p, 11q, 13q, 14q, 20p and 22q, with over 1500 SNPs in these regions being associated with the disorder (Ripke et al., 2013).

As highlighted by these studies, schizophrenia cannot be defined by one sole genetic origin, which is reflected by the inherent complexity of the disorder. Attempts to identify associated genes have been limited, due to factors including variable diagnostic boundaries, heterogeneity, comorbidity and polygenic non-Mendelian inheritance patterns (Cloninger, 1994). However, molecular genetics remain an important component in classifying the spectrum of the clinical phenotype (Ayalew et al., 2012), and may also have an important impact on psychopharmacology (Harrison, 2013). Some clinical features can already be correlated with specific genetic variance or mutations, and therefore genetic studies provide a way to improve nosology and move towards individualised treatment approaches (Ayalew et al., 2012; Harrison, 2013).

1.1.3 The molecular neurobiology of schizophrenia

Whilst it has been established that multiple genetic factors are involved in the emergence of schizophrenia, a consistent understanding of how genetic variations may lead to specific symptoms is lacking. However with evidence from behavioural animal and human studies, it has become clear that altered psychiatric-like behaviours (e.g. hyperlocomotion, prepulse inhibition, social interaction in animals, and hallucinations, delusions, social withdrawal in humans) are reflective of alterations in many neurotransmitter systems, such as the dopaminergic, glutamatergic, GABAergic, and serotoninergic systems, among more (Deng and Dean, 2013). It is likely that core alterations to one system causes a cascade of events that lead to globally altered neurotransmission in the majority (if not all) neurotransmitter systems, attributable to the close interplay of neurotransmitter systems in both time and space. Researchers have therefore searched for the cause of this "domino effect". Of these many studies, it has been repeatedly hypothesised that core alterations might originate within dopamine and glutamate signalling. Signalling via these neurotransmitters have been strongly linked with the pathophysiology of schizophrenia, and are arguably amongst the leading theories defining the underlying molecular neurobiology of schizophrenia and manifestation of the related phenotype (Deng and Dean, 2013).

The dopaminergic system was the first to be implicated in the pathology of schizophrenia, and an imbalance in dopaminergic neurotransmission has been well documented (Toda and Abi-Dargham, 2007). Dopamine is a monoamine neurotransmitter that controls fast glutamate and gamma-aminobutyric acid (GABA) neurotransmission via G-protein coupled dopamine receptors (Toda and Abi-Dargham, 2007). Accumulated evidence for a role of dopamine in schizophrenia includes the ability of dopamine reuptake inhibitors to induce hallucinations and delusions analogous to the positive symptom profile of schizophrenia (Matthysse, 1974), and therapeutic efficacy of dopamine D_2 receptor antagonists against hallucinations and delusions (Andersson et al., 1998). Yet, dysfunctions of the dopaminergic system do not absolutely account for cognitive symptoms which are arguably the more discreet and debilitating aspects of the disorder. Alterations to glutamatergic signalling has become increasingly implicated in the emergence of these symptoms, particularly cognitive deficits (Moghaddam and Javitt, 2011). Hence, in recent years, the glutamatergic theory of schizophrenia has gained traction, and many researchers are focusing efforts on the development of drugs that target the glutamate system, with a focus on addressing the cognitive deficits (Moghaddam and Javitt, 2011).

1.1.3.1 Glutamate and the glutamatergic hypothesis of schizophrenia

Glutamate is the principal excitatory neurotransmitter present in the majority of synapses in the brain. It exerts its excitatory effects through a number of receptors classified into two groups: ionotropic and metabotropic receptors. Ionotropic glutamate receptors (iGluRs), which include *N*-methyl-D-aspartate receptors (NMDAR), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) and kainate receptors, are ligand-gated ion channels (Goff and Wine, 1997). Conversely, metabotropic glutamate receptors (mGluR) are G-protein-coupled receptors (GPCRs). Eight mGluRs (mGluR1-8) have been discovered and cloned thus far, and are grouped into three categories: Group 1 (mGluR1 and 5), Group 2 (mGluR2 and 3) and Group 3 (mGluR4, 6, 7 and 8) according to sequence homology, pharmacological properties and downstream activations (Nicoletti et al., 2011).

Glutamate is vital to neurodevelopment, as well as synapse organisation and sensorimotor gating in adults (Arnold et al., 2005). Glutamate has been linked to many processes involved in cognition, memory, pain and perception (Bleakman et al., 2006; Robbins and Murphy, 2006). Almost every excitatory neuron in the brain is glutamatergic, which is extremely important considering excessive concentrations of glutamate are neurotoxic, and understimulation of glutamatergic receptors can disrupt a multitude of processes in the brain responsible for cell survival and plasticity. It is thus imperative that the glutamate system is properly regulated, as altered modulation can lead to cell death or neuronal dysfunction, and ultimately neuropsychiatric diseases such as schizophrenia (Moghaddam and Javitt, 2011; Sanacora et al., 2012).

Glutamatergic hypofunction in schizophrenia was first proposed in 1980 by Kim and colleagues as a result of low levels of glutamate cerebrospinal fluid of individuals with schizophrenia (Kim et al., 1980). After several decades of further investigations, the resulting glutamate hypothesis of schizophrenia provides a useful framework to delineate the complexities of schizophrenia, as it largely accounts for the neurotransmitter alterations that have been reported in schizophrenia, and subsequent symptom profiles (positive, negative and cognitive) (Moghaddam and Javitt, 2011). Specifically, hypofunction of glutamatergic NMDARs results in a cascade of molecular alterations that contribute to psychosis and other aspects of schizophrenia symptomatology. This is supported by the action of NMDAR antagonists, which precipitate positive, negative and cognitive schizophrenia-like symptoms in humans and animals (Chartoff et al., 2005; Olney et al., 1999), and exacerbates pre-existing symptoms in schizophrenia subjects (Javitt and Zukin, 1991). In addition, adjunct treatment of schizophrenia patients with glycine or cycloserine (co-agonists of the NMDAR) has been shown by some studies to improve schizophrenia symptoms, including the negative and cognitive deficits (Lin et al., 2011; Singh and Singh, 2011). Extensive molecular evidence of NMDAR hypofunction has also been reported in by many groups utilising the postmortem schizophrenia brain, although nearly as many have detected no change (Hu et al., 2014). While it cannot be concluded from these studies that a primary deficit in the NMDAR is solely responsible for the aetiology of schizophrenia, these studies highlight that dysfunction of glutamatergic signalling, likely via NMDAR dysfunction, greatly contributes to the pathology of schizophrenia (Kantrowitz and Javitt, 2010).

1.1.4 Metabotropic glutamate receptor 5

1.1.4.1 mGluR5 distribution and structure

Although several lines of evidence support the involvement of the NMDAR in glutamatergic dysfunction associated with schizophrenia, it is unlikely that it is the solitary aetiological factor. Several studies have reported no changes in NMDARs in the schizophrenia brain (see Hu et al., 2014) and glycine agonists have been unsuccessful in some populations (Singh and Singh, 2011), suggesting NMDAR hypofunction may be specific to a population of schizophrenia subjects. mGluR networks dynamically modulate fast-signalling iGluRs in synchronisation with signalling demands. This modulation is critical for efficient glutamate signalling and the prevention of excitotoxicity. It is thus possible that dysfunction within this aspect of the glutamate system may also exist, which contributes to the glutamatergic dysfunction observed in the disorder (Newell et al., 2014). In particular, Groups 1 and 2 mGluRs are currently under scrutiny in relation to schizophrenia, although unique interconnections between the NMDAR and mGluR5 cause this glutamate receptor subtype to be of special interest (see section 1.1.4.2).

Unlike Group 2 mGluRs, which are predominately expressed on the presynaptic membrane and modulate glutamate release, Group 1 mGluRs are most prevalently expressed on the post- and peri-

synaptic neuronal membrane (Lujan et al., 1996) where they encircle and regulate a core of fast-signalling NMDARs (Nusser et al., 1994). Although mGluR1 shares similar sequence homology and protein interactions with mGluR5, the latter is of particular interest. mGluR5 is abundantly expressed on cortical, hippocampal and striatal neurons (Abe et al., 1992; Masu et al., 1991; Prezeau et al., 1994). The increased distribution of mGluR5 in these particular brain regions (which are highly involved in schizophrenia pathophysiology) is notable: firstly, this may be indicative of involvement of mGluR5 in the pathophysiology of schizophrenia, considering mGluR5 is highly expressed in regions vastly reported to have altered function in the disorder such as the PFC and hippocampal regions; secondly, it renders mGluR5 a particularly valuable target with regards to the treatment of schizophrenia, as it allows more specified correction of glutamatergic dysfunction rather than a global effect which currently limits many preclinical glutamatergic-based drugs (Newell, 2013). mGluR5 also has a favourably distinct interaction with the NMDAR (Pietraszek et al., 2005), and is able to modulate the response of other neurotransmitters including dopamine and GABA (Bordi and Ugolini, 1999; Mohn et al., 1999; Olney and Farber, 1995).

mGluR5 consists of a large N-terminal containing an agonist-binding Venus-fly-trap domain, joined to the 7-transmembrane domain by a conserved cysteine rich chain (Francesconi and Duvoisin, 1998; Rondard et al., 2006) (Figure 1.1). The 7-transmembrane domain is also partly responsible for activation of the coupled G-protein (Niswender and Conn, 2010). The cytoplasmic tail of mGluR5 extends intracellularly, and contains binding motifs for many interacting proteins that regulate its function and localisation. This region is also important for G-protein coupling (Mary et al., 1998; Prezeau et al., 1996), which ultimately regulates intracellular second messengers.

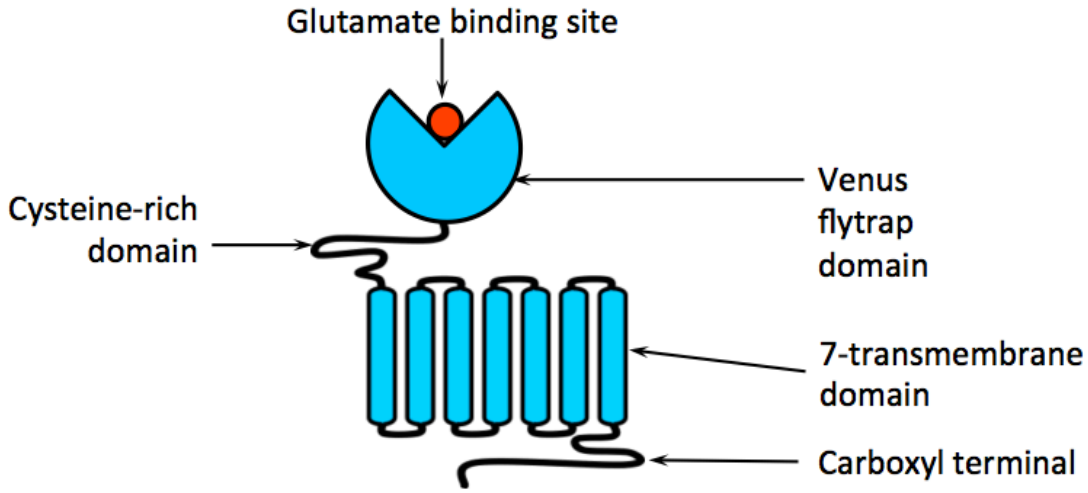


Figure 1.1 mGluR5 structure. mGluR5 consists of an extracellular venus fly trap domain, linked to a seven transmembrane domain by a highly conserved cysteine rich domain, and a long cytoplasmic carboxyl tail.

The Venus fly trap domain (or N-terminus) is the site of agonist binding, as well as having dimer-forming properties (Parnot and Kobilka, 2004). Accumulating evidence supports the existence and importance of class C GPCR homo and heterodimers, joined at the N-termini by disulphide bonds (Doumazane et al., 2011; Figure 1.2a). Dimerisation is hypothesised to play a key role in mGluR5 activation, and dimer arrangement may be critical to mGluR5/G-protein coupling (Kniazeff et al., 2004; Tateyama and Kubo, 2007). Regarding dimer synthesis, it is hypothesised that GPCR dimers are formed intracellularly soon after biosynthesis, and trafficked or internalised as a dimeric complex; however, it is unclear whether these dimers are permanent structures (Parnot and Kobilka, 2004), if they are dynamically regulated (break apart and reform) at the cell-surface (Milligan, 2004), or which molecules are responsible for the synthesis or regulation of these dimers. Considering mGluR5 is reported to only be functional in a dimer complex (El Moustaine et al., 2012; Romano et al., 1996), dynamic regulation of the dimer might act as a strategy to reduce mGluR5 function in situations of over-stimulation. Thus, the overall knowledge regarding mGluR5 dimers is limited, and additional "chaperoning"-type molecules, that may play a role in mGluR5 dimer regulation, are unknown.

Beyond extracellular activation, mGluR5 endogenous interactions predominately take place at the intracellular cytoplasmic tail, which is distinctive between the three mGluR5 splice variants (Figure 1.2b). mGluR5a is highly expressed in the postnatal rat brain and therefore thought to be involved in early development (Minakami et al., 1995). mGluR5b differs to mGluR5a as it contains a 32 amino acid insertion (Romano et al., 1996). Conversely, mGluR5d, with a 267 amino acid shorter C-terminus that mGluR5a (Malherbe et al., 2002). Insofar, our knowledge regarding the functional differences in these mGluR5 splice variants is limited. mGluR5b is reportedly more highly expressed than mGluR5a in the adult brain (Malherbe et al., 2002); however, mGluR5 splicing may vary in specific cell types, such as in the case of mGluR1 (Pin et al., 1992). Although the pharmacological profiles of mGluR5a and mGluR5b do not appear to differ (Mion et al., 2001), mGluR5a and mGluR5b were reported to have opposing functions in one study: mGluR5a was shown to supress neuronal maturation, while mGluR5b encouraged neurite growth and expansion in a neuroblastoma cell line (Mion et al., 2001). This suggests that synchronised function of these splices is essential for healthy brain development. There are no reports of how mGluR5d splice function differs to mGluR5a/b or its role during development. However, the shorter C-terminus suggests differential downstream interactions and effectors (Table 1), but this remains to be experimentally confirmed. Notably, due to the close sequence homology it is currently not possible to experimentally distinguish between protein expression of the mGluR5 splice variants, thus limiting the ability to determine functional/expressional differences of these splices at the protein level.

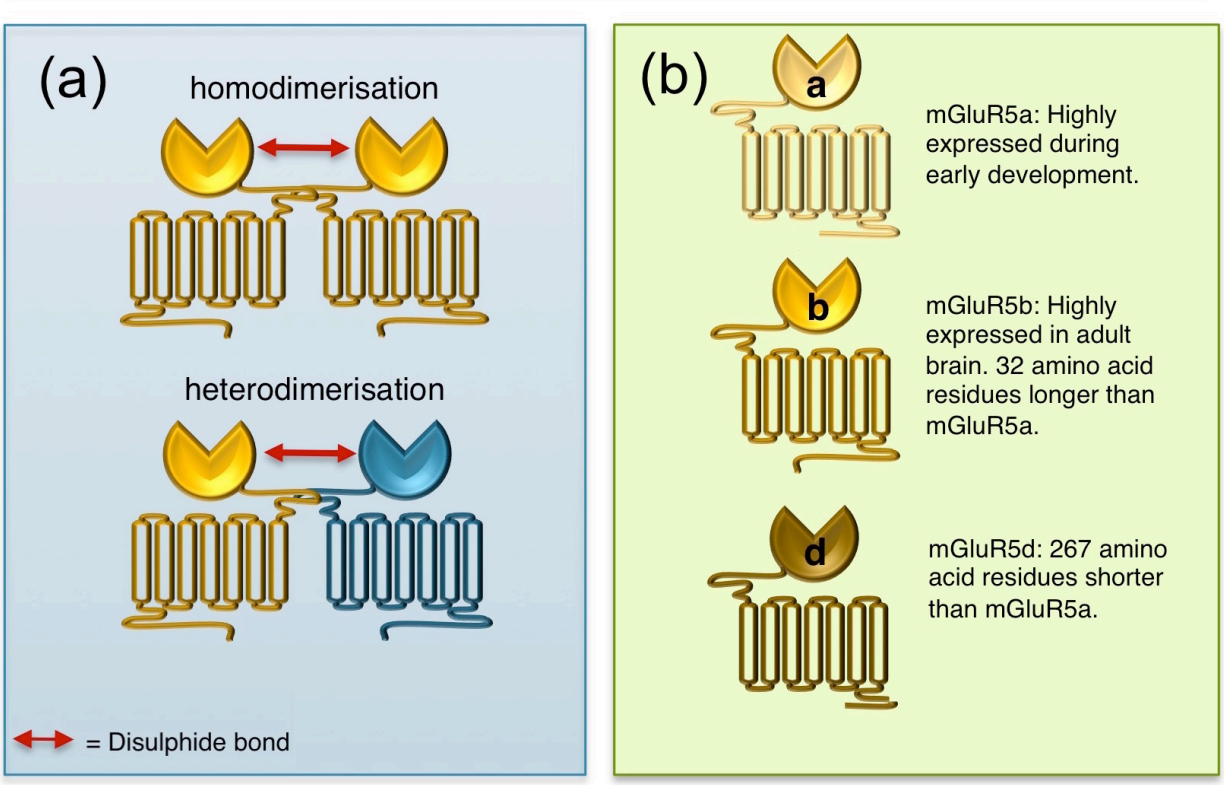


Figure 1.2 Dimerisation and splice variants of mGluR5. (a) Schematic representation of mGluR5 dimerisation. mGluR5 is reported to form homodimer and heterodimer complexes, by the formation of disulphide bonds between the extracellular VFTDs with other mGluR5s, or structurally similar GPCR such as mGluR51a, adenosine or GABA-B receptors (Doumazane et al., 2011; Fuxe et al., 2008; Romano et al., 1996). (b) Schematic representation of the three known mGluR5 splice variants, mGluR5a, mGluR5b and mGluR5d, which have different length c-termini.

1.1.4.2 mGluR5 signalling pathways

Studies spanning over 25 years have elucidated numerous details regarding mGluR5 pathways, effectors and interactions. The mGluR5 pathway is summarised in Figure 1.3.

mGluR5 and NMDAR are physically linked by the scaffolding proteins Homer, SH_3 and multiple ankyrin repeat domains (SHANK), guanylate-kinase-associated protein (GKAP, also known as SAPAP) and post-synaptic density 95 (PSD-95)(Tu et al., 1999). This link is modulated by the multiscaffolding proteins Tamalin and Preso1 (Hu et al., 2012; Kitano et al., 2002). These scaffolds also connect mGluR5 to intracellular inositol 1,4,5-triphosphate (IP_3) receptors expressed on the endoplasmic reticulum, and couple mGluR5 to the mammalian target of rapomycin (mTOR) pathway via protein kinase B (Akt) and PI3 Kinase and its enhancer (PI3K/PIKE) (Page et al., 2006). Binding of glutamate to mGluR5 activates the G-protein Gq, subsequently activating phospholipase C (PLC); PLC initiates conversion of phosphoinositide (PI) to IP_3 as well as activation of diacylglycerol (DAG). IP_3 also causes release of intracellular calcium from the endoplasmic reticulum. The combination of DAG with intracellular calcium release (which also occurs due to NMDAR activation), initiates phosphorylation of protein-kinase C (PKC) (Skeberdis et al., 2001). PKC signalling results in downstream activation of

cAMP response element-binding (CREB), brain-derived neurotrophic factor (BDNF), proline-rich tyrosine kinase/cell adhesion kinase ß (Pyk2/CAKß) and Src protein; in turn, Src directly potentiates the NMDAR and calcium dependant protein phosphatase 2B/calcineurin (PP2B/CaN) (Ali and Salter, 2001; Groveman et al., 2012). Lastly, Norbin modulates mGluR5 signalling and cell surface expression, and potentiates the release of intracellular calcium downstream (Wang et al., 2009).

mGluR5 downstream signalling molecules play an important role in cell homeostasis and regulation of neuronal function. For example, (i) CREB binds to DNA sequences to induce or reduce transcription factors for many genes such as BDNF (Carlezon Jr et al., 2005), (ii) the neurotrophic factor BDNF promotes the survival, growth and differentiation of new neurons (Acheson et al., 1994), and (iii) mTOR modulates cell growth, proliferation, motility, survival, as well as protein synthesis and transcription (Hay and Sonenberg, 2004). Via activation of these signalling molecules, mGluR5 is also involved in regulating many processes involving synaptic plasticity (Vinson and Conn, 2012), such as memory, cognition, pain and movement (Ayala et al., 2009; Ménard and Quirion, 2012; Radulovic and Tronson, 2012; Samadi et al., 2008). In accordance, mGluR5 pathways are implicated in many pathological states where alterations to these downstream pathways has been reported, such as schizophrenia, depression, bipolar disorder, Alzheimer's disease, Parkinson's disease, and pain syndromes (Angelucci et al., 2005; Carlezon Jr et al., 2005; Gururajan and Buuse, 2014).

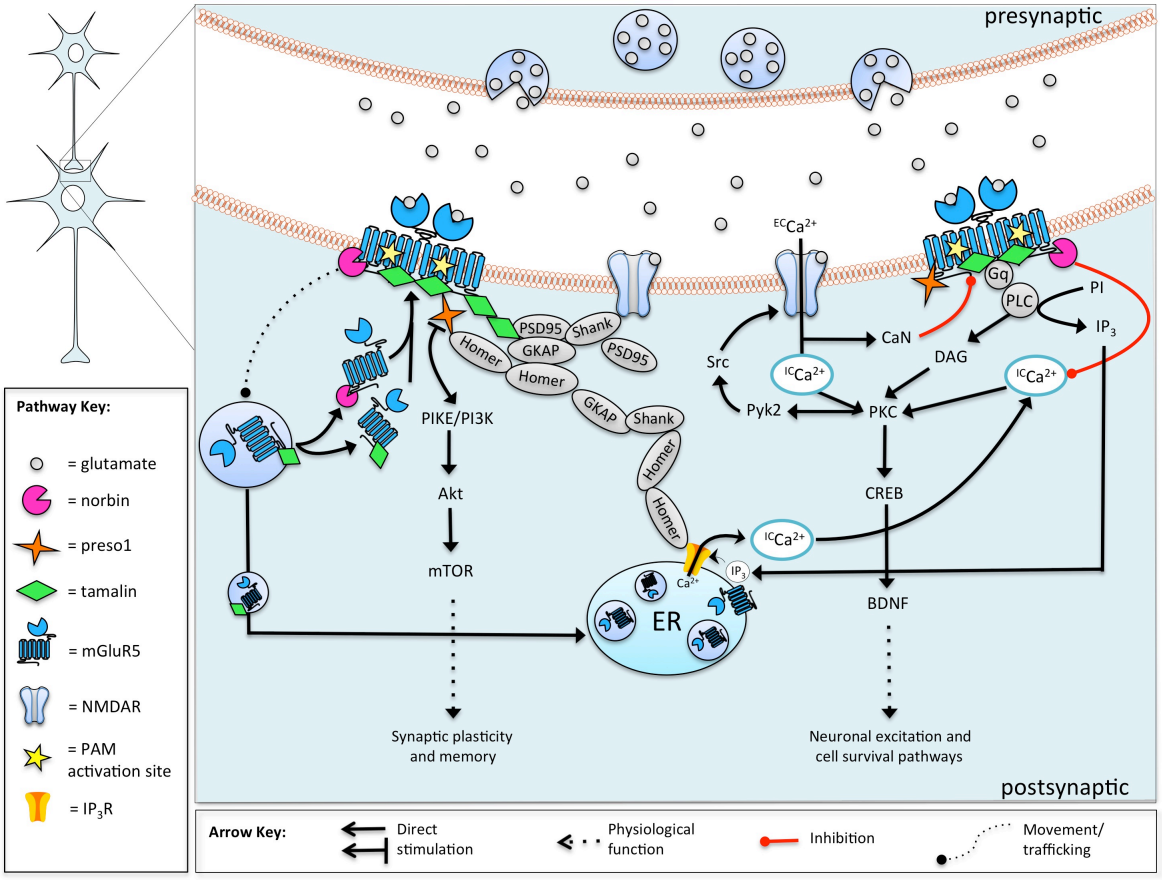


Figure 1.3 Schematic of mGluR5 signalling pathway, in the context of the NMDAR. Glutamate-containing vesicles are exocytosed into the synaptic cleft, and glutamate binds to both mGluR5 and NMDAR. Extra-cellular calcium ion influx occurs through phosphorylated NMDAR ion channel and triggers further release of calcium from intracellular stores. Binding of glutamate to mGluR5 activates Gq, subsequently activating PLC. PLC initiates conversion of phosphoinositide to IP_3 and subsequent activation of DAG, and release of intracellular calcium from the ER. Scaffold proteins (Homer, SHANK, GKAP and PSD95) physically link mGluR5 and NMDAR, modulated by Tamalin and Preso1, which also connect mGluR5 to intracellular IP_3 receptors expressed on the endoplasmic reticulum. mGluR5 signals via PKC and mTOR pathways. Lastly, Norbin binds directly to the C-terminus of mGluR5 where it positively modulates mGluR5 signalling and cell surface expression, and inhibits the release of intracellular calcium downstream.
Abbreviations: Akt: protein kinase B, BDNF: brain derived neurotrophic factor, CaN: calcineurin, $^{ec}Ca^{2+}$: extracellular calcium, $^{ic}Ca^{2+}$: intracellular calcium, CREB: cAMP response element binding protein, DAG: diacyl glycerol, ER: endoplasmic reticulum, GKAP: guanylate-kinase-associated protein, Gq: guanosine nucleotide-binding protein q, IP_3: inositol triphosphate, mTOR: mammalian target of rapamycin, Norbin: neurochondrin, PIKE: PI3K enhancer, PI: inositol monophosphate, PI3K: PI3 kinase, PKC: protein kinase C, PLC: protein lipase C, Preso1: FRMPD4, PSD95: post-synaptic density 95, Pyk2: protein tyrosine kinase 2, SHANK: SH_3 and multiple ankyrin repeat domains, Src: Proto-oncogene tyrosine-protein kinase, Tamalin: GRASP1

1.1.4.3 mGluR5 G-protein independent pathways and endogenous regulation

Like most GPCRs, mGluR5 activation is modulated by phosphorylation. Enzymatic activity at the mGluR5 phosphorylation sites is a major process in regulation of mGluR5 signalling, localisation and function (Mao et al., 2008). However, in addition to enzymatic modulators, G-protein independent pathways also modulate the mGluR5 cellular response (Heuss et al., 1999). mGluR5 intracellular domains are coupled to a multitude of endogenous molecules that modulate mGluR5 trafficking, cell-surface expression, protein-protein couplings and internalisation (Table 1.1). These endogenous molecules may also have additional chaperoning roles, involving post-translational protein folding, trafficking between cellular machinery, and tagging mutant proteins for degradation (Ellis and Van der Vies, 1991; Hartl, 1996); however, virtually nothing is known regarding these processes in relation to mGluR5.

Table 1.1 Summary of endogenous molecules that interact with the intracellular tail of mGluR5 (Adapted from Enz, 2007).

Modulating protein	mGluR5 isoform	Interacting Region	Reference
CaIN	mGluR5a	657-727, distal C-terminus (exact residue unknown)	(Ferreira et al., 2009)
Calcineurin	mGluR5	Unknown if binds to C-terminus	(Alagarsamy et al., 2005)
Calmodulin	region1: mGluR5a/b/d; region 2: mGluR5a/b	region 1: 842-869; region 2: 890-918	(Minakami et al., 1997)
GRK 2/3	mGluR5a/b/d	840	(Sorensen and Conn, 2003)
Homer1a	mGluR5a/b/d	1171-1180	(Brakeman et al., 1997)
Homer1b/c	mGluR5a/b	1133-1138	(Brakeman et al., 1997; Tu et al., 1999)
Norbin	region 1: mGluR5a/b/d, region 2: mGluR5a/b only	region 1: 857-867; region 2: 893-903	(Wang et al., 2009)

Optineurin	mGluR5a	828-1180	(Anborgh et al., 2005)
PP1 gamma 1	mGluR5a/b	881-885	(Croci et al., 2003)
Preso1	mGluR5a/b	986-974	(Hu et al., 2012)
Siah-1A	mGluR5a/b	893-919	(Ishikawa et al., 1999)
Siah-1A	mGluR1a/b	region 1: 905-932 region 2: 893-919	(Ishikawa et al., 1999)
Tamalin	mGluR5a/b/d	1176-1180	(Kitano et al., 2002)

Abbreviations: CaIN, calcinuerin inhibitior protein; GRK, G-protein regulatory kinase; PP1, protein phosphatase 1; Siah1, seven in absentia homolog 1.

The Homer1 scaffold protein was the first discovered modulator of mGluR5 (Brakeman et al., 1997). Homer1 proteins plays a critical role in organisation of the postsynaptic density and are reported as altered in patients with schizophrenia (total isoforms; Engmann et al., 2011). Additionally, genetic variation in the *HOMER1* gene has been significantly associated with schizophrenia (Spellman et al., 2011). In support, genetic abolition of Homer1 (total isoforms) produces strong schizophrenia phenotypical behaviours in mice (Jaubert et al., 2007). Disruption to Homer1/mGluR5 linkages have also been recently demonstrated in an acute and chronic stress-induced model of psychiatric disorder (Wagner et al., 2013; Wagner et al., 2015). A role of Homer1 in the modulation of mGluR5 and schizophrenia has thus been established; however, Homer is only one of many mGluR5-interacting molecules.

The importance, role and function of other mGluR5 endogenous interacting partners has gained traction with many basic science researchers, such as the Enz group (Enz, 2007). Through their work and others, it is clear that certain molecules are modulators of mGluR5 activity and localisation. In some instances, this has been experimentally confirmed, as alterations to the expression of these modulators in animals can induce schizophrenia-like behaviours (e.g. Homer1: Szumlinski et al., 2005; Norbin: Wang et al., 2009). Considering that mGluR5 activity and/or signalling might be impaired should these signalling partners be altered, it is crucial to consider these mGluR5-interacting proteins in the context of schizophrenia and other pathological states. However to date, many of these molecules remain unstudied in animal models, and virtually unstudied in human tissues of pathological conditions, including neuropsychiatric disorder.

Of the endogenous proteins that modulate mGluR5 (Table 1.1), Norbin (neurochondrin-1) (Wang et al., 2009), Tamalin (GRP-1-associated scaffold protein or GRASP) (Kitano et al., 2002) and Preso1 (FERM and PDZ domain-containing protein 4 or FRMPD4) (Hu et al., 2012) are particularly distinctive modulators of mGluR5 signalling, desensitisation/internalisation and trafficking/localisation. Despite that some information regarding their biological roles has been uncovered, they remain unstudied in the postmortem schizophrenia brain, and largely unstudied in any schizophrenia-relevant paradigm.

1.1.4.3.1 mGluR5 modulation by Norbin

Norbin, also known as neurochondrin-1, is a 75kDa cytoplasmic protein expressed mainly in the central nervous system. The overexpression of Norbin in neuronal cultures resulted in neurite outgrowth, suggesting Norbin plays an important role in neuronal plasticity (Mochizuki et al., 1999; Shinozaki et al., 1997). Total deletion of Norbin leads to embryonic death 3.5 to 6.5 days following conception, suggesting Norbin plays a crucial role in neuronal development (Mochizuki et al., 1999). Norbin has been found to interact with a number of membrane-bound proteins, such as transmembrane semaphorin Sema4C, melanin-concentrating hormone receptor 1, and group I mGluRs (Wang et al., 2010). Notably, these receptors modulate calcium influx, suggesting that Norbin plays a critical role in modulating neuronal excitability and signalling.

Specifically, Norbin was reported to closely modulate mGluR5 signalling and cell-surface localisation (Wang et al., 2009). Overexpression of Norbin in primary cortical neurons was shown to increase mGluR5 cell-surface expression, whilst in cultures with depleted Norbin, mGluR5 cell-surface expression was reduced (Wang et al., 2009). Conditional cortical Norbin knockout mice also display schizophrenia-like phenotypic behaviours, such as altered sensorimotor gating, psychotomimetic-induced hyperlocomotion, deficits in hippocampal synaptic plasticity, as well as reduced mGluR5 cell surface expression (Wang et al., 2009). The behavioural profile of cortical Norbin knockout mice strongly suggests an important role for Norbin in the context of schizophrenia; however since the Norbin/mGluR5 interaction was reported in 2010, it has not been followed-up in a schizophrenia relevant paradigm.

1.1.4.3.2 mGluR5 modulation by Tamalin

Tamalin (also known as GRIP-associated protein-1 or GRASP1) is a 95kDa PSD-95 binding scaffold abundant in the post-synaptic density (Kitano et al., 2002). As a scaffold protein, its main cellular function is to facilitate macromolecular protein complexes by binding signalling partners through its multiple binding motifs. However, Tamalin was also shown to critically coordinate endosomal pathways of neurotransmitter receptor recycling, which is a process crucial to synaptic function and plasticity (Hoogenraad et al., 2010). Therefore, it is likely that Tamalin also plays an important role in learning and memory processes.

Tamalin has been specifically shown to regulate the cell surface expression and dendritic targeting of mGluR5, by binding directly to the C-terminus of mGluR5 (Kitano et al., 2002). Tamalin also forms complexes with membrane-associated guanylate kinase inverted-2 (S-SCAM) molecules, which are responsible for subcellular trafficking, and it has thus been hypothesised that Tamalin may be key to proper cellular localisation and trafficking of mGluR5 (Kitano et al., 2003). Since both mGluR5 and

Tamalin are reported to dimerise, it has also been suggested that the Tamalin may facilitate mGluR5 dimerisation, but this remains to be experimentally confirmed (Sugi et al., 2007). Considering that mGluR5 can only be activated by an agonist when in a dimerised state (Kniazeff et al., 2004), Tamalin may critically modulate mGluR5 signalling. Furthermore, as Tamalin binds also to other scaffold proteins such as PSD-95 and GKAP (Kitano et al., 2002; Kitano et al., 2003), this presents the possibility of Tamalin's involvement in a variety of important protein-protein interactions, including protein clustering in the PSD, and facilitation of mGluR5/NMDAR signalling. Despite that the role of Tamalin in schizophrenia is largely unknown, one study reported that a frameshift within *GRM5* in one schizophrenia pedigree resulted in disrupted mGluR5/Tamalin linkages (discussed in section 1.5.1; Timms et al., 2013), providing motive to further study the role of Tamalin in schizophrenia pathology.

1.1.4.3.3 mGluR5 modulation by Preso1

Preso1, encoded by the *FRMPD4* gene, is a scaffold protein that crucially coordinates dendritic spine morphogenesis (Mo et al., 2012). Preso1 is comprised of WW, PDZ, and FERM domains, and is reported to bind to PSD95 (Lee et al., 2008). More recently, in a search for proteins that bind Homer1, the multi-scaffolding protein Preso1 was identified in addition to its ability to bind group I mGluRs (Hu et al., 2012). Preso1 was shown to potentiate binding of protein-directed kinases (CDK5 and ERK) to mGluR5, and phosphorylate the Homer binding motif of mGluR5. This Preso1-dependent phosphorylation increases mGluR5/Homer binding to reduce mGluR downstream signalling. In accordance, activation of the mGluR5/Preso1 complex up-regulated mGluR5 linkages with long Homer isoforms (Hu et al., 2012). Thus, Preso1 fundamentally modulates mGluR5/Homer linkages and mGluR5 signalling. As mGluR5 linkages to Homer are critical for mGluR5/NMDAR synchronisation, Preso1 is an attractive novel therapeutic target to correct potential alterations in mGluR5 signalling and protein couplings. However the involvement of Preso1 in the molecular biology or behavioural phenotype of schizophrenia is unclear, as to date, Preso1 has only been studied in the context of pain (Hu et al., 2012; Radulovic and Tronson, 2012).

1.1.5 Pathological evidence of mGluR5 involvement in schizophrenia

1.1.5.1 Evidence from rodent studies

Investigations of mGluR5 knockout mice support the involvement of mGluR5 in the aetiology of schizophrenia. Deletion of *GRM5* gene causes behaviours that are analogous to behavioural manifestations induced by glutamate hypofunction models as well as observed schizophrenia phenotypes, such as decreased sensorimotor gating, decreased short-term spatial memory and sensitivity to locomotor deficits induced by NMDAR antagonists (Brody et al., 2003; Chiamulera et al., 2001; Gray et al., 2009; Lu et al., 1997). Furthermore, conditional cortical knockout of mGluR5 specifically modulates locomotor reactivity in response to a novel environment, but does not affect sensorimotor gat-

ing, anxiety, motor coordination, and social interactions, which are likely attributable to subcortical and/or other brain structure mGluR5 activity (Jew et al., 2013).

In line with what has been observed in the mGluR5 knockout studies, adult rats treated with mGluR5 selective negative allosteric modulators, MPEP (2-methyl-6-(phenylethynyl)-pyridine) or MTEP (3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine), displayed social interaction deficits, impaired working memory, reduced instrumental learning, as well as intensification of the detrimental effects induced by NMDAR antagonists (Campbell et al., 2004; Henry et al., 2002; Homayoun et al., 2004; Pietraszek et al., 2005; Vales et al., 2010; Vollenweider et al., 1998; Zou et al., 2007). Homayoun and Moghaddam (2007) further observed cortical neurons in the prefrontal cortex (PFC) of awake rats, where MPEP decreased spontaneous burst activity of these neurons and augmented the effects of the non-competitive NMDAR antagonist, MK-801, on spontaneous neuronal activity.

Another collection of studies in rodents indicates mGluR5 is crucial for proper cognitive function such as learning and memory, which are processes disrupted in many schizophrenia patients (Harvey, 2013). Long-term depression (LTD) and long-term potentiation (LTP) are two opposing neuronal processes to do with the formation and dissociation of synapses, underlying synaptic plasticity in the brain. When these processes are imbalanced, this can cause deficits in memory and cognition. Disruption to mGluR5 signalling can lead to imbalance of LTP and LTD (Mukherjee and Manahan-Vaughan, 2013). For example, mGluR5 knockout mice have reduced performance in NMDAR-mediated memory tasks due to deficits in hippocampal NMDAR-induced LTP; these deficits can be rescued by stimulation of PKC (Jia et al., 1998). Furthermore, LTP induced by theta-burst stimulation in hippocampal slices are impaired by treatment with MPEP (Francesconi et al., 2004; Shalin et al., 2006), and accordingly antagonism of mGluR5 in rats impacts on spatial learning performance and synaptic plasticity, specifically inhibition of LTP in CA1 and the dentate gyrus (Manahan-Vaughan and Braunewell, 2005). Lastly, the Group I mGluR agonist DHPG induces LTD, which is blocked only by an mGluR5 antagonist, and is not present in mGluR5 knockout mice (Faas et al., 2002; Gasparini et al., 1999; Huber et al., 2001). Notably, a recent study has reported that the cellular location of mGluR5 may impact on the exact role it plays in synaptic plasticity, with cell-surface expressed mGluR5 regulating both LTP and LTD, whilst intracellularly expressed mGluR5 modulate LTD only (Purgert et al., 2014). These studies nonetheless collectively support a role for mGluR5 in the modulation of cognitive functions, such as learning and memory.

1.1.5.2 Evidence from genetic studies

GRM5 (coding for mGluR5; located at chromosomal region 11q14.3) is a large gene containing 8 exons (coding gene region) and 7 introns (non-coding gene region) that span over 49,000 base pairs (Devon et al., 2001; Figure 5). A long-range restriction map covering the *GRM5* locus was previously

linked to schizophrenia in a large Scottish pedigree (Millar et al., 1998). A novel intragenic microsatellite was characterised in intron 6 of *GRM5* (Figure 1.4), and was then analysed by microsatellite typing in a case-control Scottish population consisting of patients with schizophrenia (n=231) and bipolar disorder (n=149), matched with control subjects (n=421). A significant difference in allelic frequency distribution was reported between schizophrenia cases and controls, but this association was not apparent in subjects with bipolar disorder (Devon et al., 2001). Although this intronic repeat might suggest alternative splicing or uncontrolled mRNA stability, the authors further suggested that the microsatellite was likely to be in linkage disequilibrium with a causative mutation, and further studies would be required to detect functional polymorphisms associated with the minor allele at 197bp, which was present only in the schizophrenia cases in this study (Devon et al., 2001).

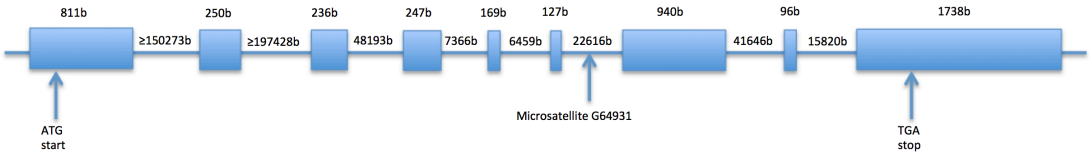


Figure 1.4. Genomic organisation of GRM5 located at 11q14.3. Exons are represented by boxes, introns are represented between (not to scale). Microsatellite G46931 within intron 7 was previously associated with schizophrenia in a case-control population (Adapted from Devon et al., 2001). **Abbreviations:** b=base pair.

More recently, *GRM5* was reported to be one of the top candidate genes in the aetiology of schizophrenia. Using a convergent functional genomics approach, data was integrated from genome-wide association studies with available previous studies from the postmortem human brain, human induced pluripotent stem cells, human blood gene expression data, linkage analyses, copy number variation/association, and relevant animal and cellular-based models (Ayalew et al., 2012). Three putative SNPs in *GRM5* were identified as being significantly associated with schizophrenia in three independent cohorts: the International Schizophrenia Consortium [rs992259, p=0.002], the Genetic Association Information Network (GAIN) European American cohort [rs170110, p=0.02] and the GAIN African American cohort [rs1846475, p=0.001] (Ayalew et al., 2012). However, no SNP associations have been reported in independent schizophrenia case-control populations to date.

In another study, a range of techniques (genome-wide array, comparative genomic hybridisation, linkage analysis and exome sequencing) was performed in multiplex families with schizophrenia, to identify genetic factors which may predispose families with affected members to schizophrenia (Timms et al., 2013). In one of the 5 pedigrees examined, both a missense (G369) and cis frameshift mutation (P1148fs) was reported in *GRM5*. The G369 missense, translating to the Venus flytrap domain of mGluR5 (corresponding to N-terminal extracellular domain), was hypothesised to affect glutamate binding to mGluR5. Alternatively, the cis P1148fs substitution was located to the Tamalin binding region on the mGluR5 C-terminus. Follow up coimmunoprecipitation experiments within cel-

lular assays demonstrated that the frameshift indeed disrupted Tamalin binding to mGluR5; interestingly, this appeared to be specific to Tamalin, as Homer binding was not affected even though Homer and Tamalin have overlapping binding sites on mGluR5. Furthermore, this disconnection from Tamalin caused decreased mGluR5 surface expression, increased mGluR5 internalisation and reduced agonist-induced activation of mGluR5 (Timms et al., 2013).

Whilst these genetic data directly implicate *GRM5* in schizophrenia aetiology, other genes that indirectly affect mGluR5 should be noted. For example, several studies have reported that *RGS4* is a susceptibility gene in schizophrenia (Large, 2007; Shao et al., 2010; Tarazi et al., 1996), which is a gene that negatively modulates Gq protein-mediated signalling, including that of mGluR5. Furthermore, polymorphisms in *HOMER1* have also been associated with schizophrenia in a Caucasian case-control population (Spellmann et al., 2011). In addition, a number of candidate genes differentially involved in excitatory glutamate signalling have been associated with schizophrenia, including *GRIN2B, DISC1*, *COMT*, *NRG1*, *RGS4*, and *GABA-A* (Alfimova et al., 2011, 1; Collier and Li, 2003; Hennah et al., 2003; Hodgkinson et al., 2004; Li and He, 2006; Liu et al., 2002a; Liu et al., 2002b; Petryshen et al., 2005a; Petryshen et al., 2005b; Shifman et al., 2002; Weickert et al., 2012). Together, these studies provide support for the possibility of genetic mutations affecting glutamatergic signalling in schizophrenia, including that of mGluR5.

1.1.5.3 Evidence from human postmortem studies

Whilst the cellular, animal and genetic studies presented implicate mGluR5 and/or its effectors in the development and pathology of schizophrenia, little alteration has been reported directly within the postmortem schizophrenia brain (Table 1.2). Several of these investigations have analysed mGluR5 mRNA, binding and protein within human postmortem tissues. mGluR5 gene expression was found to be unaltered in the thalamus, hippocampus, PFC and striatum of patients with schizophrenia (Fatemi et al., 2013; Gupta et al., 2005; Ohnuma et al., 2000a; Richardson-Burns et al., 2000; Volk et al., 2010). Conversely, Ohnuma et al. (1998) reported that mGluR5 mRNA expression and glutamate transporter protein were respectively increased and decreased in Brodmann's Area (BA) 11, pointing towards less functional glutamate and decreased glutamate transmission in this region. Furthermore, mGluR5 mRNA was reported to be decreased in the cerebellum, indicating that mGluR5 synthesis may be affected in this region (Fatemi et al., 2013). It should therefore be considered that alterations to mGluR5 gene expression are brain region-specific. However it should also be noted that many of these previous studies used small cohorts, which may limit the interpretation of these studies ($n<20$/group; Gupta et al., 2005; Ohnuma et al., 1998; Ohnuma et al., 2000b; Richardson-Burns et al., 2000).

The majority of previous investigations examining mGluR5 protein by immunoblot in postmortem brain samples from schizophrenia subjects identified no change in mGluR5 protein levels in the prefrontal cortex (PFC), specifically Brodmann's areas (BA) 9, 10, 11, 32 and 46 as well as the caudate, putamen and nucleus accumbens (Corti et al., 2011; Gupta et al., 2005; Matosin et al., 2013). In contrast to these findings, Fatemi and colleagues recently reported a large reduction in mGluR5 (monomer) protein levels in the BA9 and lateral cerebellum of schizophrenia subjects (Fatemi et al., 2013). Although these contradictions may be due to cohort-specific differences, these findings raise the strong probability that alterations to mGluR5 in schizophrenia are brain-region specific, highlighting that regional differences can occur even between adjacent brain regions, and thus it is important to explore all regions independently.

Few groups have examined mGluR5 binding in neuropsychiatric disorder, although this is an important approach as it is useful for quantifying binding sites of interest. We were recently the first to measure the MPEP binding site of mGluR5 in the dorsolateral prefrontal cortex (DLFPC) of schizophrenia subjects (Matosin et al., 2013). Whilst overall we found no change in the DLPFC, we did observe a reduction in binding in schizoaffective subjects of depressive subtype (unpublished observation, due to limited sample size n=3-4). In accordance, Deschwanden and colleagues (2011) previously reported region specific reductions in *in-vivo* mGluR5 binding (using [11C] ABP688) to the MPEP site in multiple regions of the PFC, insula, and parts of the parietal and temporal cortices in individuals with depression. mGluR5 binding also correlated with depressive symptom severity. It is therefore possible that mGluR5 may be reduced in patients displaying prominent negative (i.e. depressive) symptoms in schizophrenia, although further experimentation is required to confirm this notion. Nonetheless, considering many novel mGluR5 drugs target the MPEP site, our findings suggest that this binding site is accessible in these disorders, at least in the DLPFC.

Although there is strong evidence for a role of mGluR5 in the pathology of schizophrenia, the exact nature of its involvement is unclear. Indirect results from animal manipulation studies (i.e. mGluR5 knockout and mGluR5 antagonism) indicates involvement of mGluR5, however, some data from postmortem human studies suggest that mGluR5 is not altered in schizophrenia. These negative findings may be explained by alteration of mGluR5 function, rather than expression, or concealed due to heterogeneity within the examined cohorts. Alternatively, alterations in mGluR5 may be brain-region specific. No studies to date have explored whether there is altered regulation of mGluR5 in schizophrenia in the postmortem brain or in animal models, although this is critical to uncover, as alterations to mGluR5 may play a significant role in the pathophysiology of schizophrenia and might also impact on the efficiency of mGluR5-targeted therapeutics.

Table 1.2 Summary of mGluR5 investigations in postmortem human brain samples from

patients with schizophrenia, major depression or bipolar disorder. (Adapted from Newell et al., 2014).

Study	Subjects	Brain region	Analysis	Outcome
Matosin et al., 2014	15 SZ 15 MD 15 BP 15 CT 12 CT 12 MDNP 12 MDP	ACC	Receptor autoradiography ([^{3}H]MPEP) labelling of mGluR5.	No overall change in mGluR5 binding in SZ, MD, BP, but differential age associations were present. No overall change in mGluR5 binding in MDNP or MDP, but differential age associations were present.
Fatemi et al., 2013	20 SZ 19 BP 29 CT	PFC (BA9)	qRT-PCR of *GRM5*. Immunoblot analysis of mGluR5.	Reduction in mGluR5 mRNA in BP but not SZ or MD. Reduction in mGluR5 protein (monomer, but not dimer) in SZ and BP.
	15 SZ 14 MD 15 BP 14 CT	Lateral cerebella	qRT-PCR of *GRM5*. Immunoblot analysis of mGluR5.	Reduction in mGluR5 mRNA in SZ and MD. Reduction in mGluR5 (monomer and dimer) protein in SZ and BP, and a reduction in mGluR5 protein (monomer only) in MD when standardised to NSE, but not ß-actin.
Matosin et al., 2013	37 SZ 37 CT	DLPFC (BA46)	Receptor autoradiography ([^{3}H]MPEP) labelling of mGluR5. Immunoblot analysis of mGluR5.	No change in mGluR5 binding or protein (monomer) expression in SZ.
Corti et al., 2011	21 SZ 35 CT	PFC (BA10)	Immunoblot analysis of mGluR5.	No change in mGluR5 protein expression in SZ.
Volk et al., 2010	28 SZ 14 SZA 42 CT	PFC (BA9)	qRT-PCR of *GRM5*.	mGluR5 mRNA levels were not altered overall in SZ. However, SZA subjects displayed reduced mGluR5 mRNA expression compared to SZ subjects.
Gupta et al., 2005	16 SZ 9 CT	PFC (BA9,11,32,46) caudate, putamen, nucleus accumbens	Immunoblot for mGluR5.	No change in mGluR5.
Ohnuma et al., 2000	5 SZ 6 CT	Dentate gyrus, cornu ammonis	In situ hybridisation of transcripts	No change in mGluR5 mRNA in SZ

		and parahippocampal gyrus	encoding for *GRM5*.	subjects.
Richardson-Burns et al., 2000	12 SZ 8 CT	Thalamus	In situ hybridisation of transcripts encoding for *GRM5*.	No change in mGluR5 mRNA in SZ subjects.
Ohnuma et al., 1998	7 SZ 10 CT	PFC (BA 9, 10, 11)	In situ hybridisation of transcripts encoding for *GRM5*.	mGluR5 mRNA increased in layer III of BA 11 in SZ.

Abbreviations: BA, Brodmann's Area; BP, bipolar disorder; CT, control; MD, major depression; MDP, major depression with psychosis; MDNP, major depression without psychosis; mGluR5, metabotropic glutamate receptor 5; [^{3}H]MPEP, tritiated 2-Methyl-6-(phenylethynyl)pyridine; PFC, prefrontal cortex; qRT-PCR, quantitative real time polymerase chain reaction; SZ, schizophrenia; SZA, schizoaffective disorder.

1.1.6 The effects of current APDs on mGluR5

APDs are currently the primary form of treatment for schizophrenia patients. Whilst both first, second and third generation APDs competitively antagonise dopamine D_2 receptors, second and third generation APDs have a much more complex mode of action with various affinities for dopaminergic, serotonergic, muscarinic, adrenergic and histamatergic receptors (Coward, 1992; Seeman, 2002). Despite being widely efficacious for positive schizophrenia symptoms, APDs display minimal benefits for negative and cognitive symptom profiles for many patients (Heresco-Levy, 2003). In addition, APDs cause a large array of adverse effects, including obesity, metabolic syndrome, sedation and motor dysfunction among more (Kane, 2011).

There are few studies investigating the involvement of mGluR5 in the mechanisms of current APDs. Chronic administration of clozapine in mGluR5 knockout mice improved sensorimotor gating and NMDAR-antagonist induced hyperactivity, although it did not improve memory deficits, suggesting modulation of mGluR5 is necessary for these therapeutic effects (Gray et al., 2009). For this reason, it is important to understand whether current medications can influence the expression and/or activity of mGluR5, and also to allow for the interpretation of postmortem human data where there was premortem exposure to APDs. The three previous studies investigating the effects of APDs on mGluR5 levels (notably, this is irrespective of modelling disease) are summarised in Table 1.3. We have recently shown that acute, subchronic and chronic treatments with typical (haloperidol) and atypical (olanzapine) APDs do not influence mGluR5 binding density in the rat PFC, hippocampus or striatum (Matosin et al., 2013). Another study however has shown that mGluR5 mRNA expression is increased by typical (haloperidol) and atypical (sertinadole) APD treatment in the anterior cingulate cortex (ACC) and striatum in Sprague Dawley rats (Iasevoli et al., 2010). However, it should be noted that Iasevoli and colleagues used almost double the dose of these APDs, and thus the ability to compare these results is impeded. Additionally, it must be noted that mRNA levels do not denote correspond-

ing changes in protein expression (Greenbaum et al., 2003). Furthermore, previous studies have not considered whether mGluR5 regulation may be affected by APD treatment.

Table 1.3 Summary of mGluR5 investigations in animal models of APD treatment. (Adapted from Newell et al., 2014)

Study	Animals	Treatment	Analysis	Outcome
Matosin et al., 2013	Male adult Sprague-Dawley rat (n=6/group).	Haloperidol, 0.1mg/kg 3 times per day, OR olanzapine, 1mg/kg, 3 times per day, p.o. for 8,16 or 36 days. Sacrificed 48 hours after last treatment.	Receptor autoradiography ([^{3}H]MPEP) labelling of mGluR5.	No effect of haloperidol or olanzapine treatment on mGluR5 binding.
Iasevoli et al., 2010	Male adult Sprague-Dawley rats (n=7).	Haloperidol, 0.8mg/kg, OR Sertindole, 2mg/kg, s.c. injection for 21 days, sacrificed 90 mins after the last treatment.	In situ hybridisation of mGluR5 mRNA.	Increased mGluR5 mRNA in medial (DM and VM) striatum, ACC and motor cortices in response to both treatments. No difference in hippocampus. Increased mGluR5 mRNA in DL striatum after sertindole treatment only.
Tascedda et al., 2001	Male Sprague-Dawley rats (n=6-8/group).	Haloperidol (1mg/kg), clozapine (10mg/kg), or olanzapine (2mg/kg) s.c., daily for 1 (acute) or 21 (chronic) days. Sacrificed 6 hours after last treatment.	Probe Preparation and RNase Protection Assay	No change in mGluR5 mRNA expression after any treatment.

Abbreviations: mGluR5, metabotropic glutamate receptor 5; [^{3}H]MPEP, tritiated 2-Methyl-6-(phenylethynyl)pyridine; p.o., per oral; s.c. subcutaneous.

It remains unclear whether mGluR5 is affected by current APDs, however, it has been suggested that interactions occur between mGluR5 and D_2 receptors, a major target of most APDs. mGluR5 protein

expression was reported to be decreased in the rat PFC following chronic adolescent treatment with the potent dopamine agonist, apomorphine (Shao et al., 2010). This mechanism of action may be attributable to "receptor mosaics", a term coined by the Agnati/Fuxe group, whereby receptors may be coupled physically resulting in more complex signalling pathways (Fuxe et al., 2008). This group reported that D_2 and mGluR5 form heterodimers in the striatum, particularly at the corticostriatal glutamate synapse, raising the possibility that mGluR5 was altered by apomorphine via this pathway. This linkage (mGluR5-D_2) provides an avenue of interaction between glutamatergic and dopaminergic transmission. Dysfunctions within these neurochemical systems are reflective of positive, negative and cognitive symptomatology. Thus, it is possible that current APDs may exert therapeutic effects indirectly via mGluR5, although this has not been experimentally confirmed.

1.1.7 mGluR5 as a future treatment target for schizophrenia

As aforementioned, currently available APDs have some efficacy for positive schizophrenia symptoms, but have minimal benefits for the negative and cognitive symptoms in many patients, and are often accompanied by severe side effects (Heresco-Levy, 2003). Due to the strong evidence that glutamate is involved in all aspects of schizophrenia symptomatology, including cognition, many groups have aimed to develop drugs aimed at the glutamatergic system which are well-tolerated with reduced adverse effects (Rubio et al., 2012). However, as the NMDAR cannot be directly agonised as a therapy for schizophrenia due to the high risk of excitotoxicity, receptor desensitisation, and adverse side effects, modulation of glutamatergic transmission via mGluR5 is promising. For example, the functional and physical link to the NMDAR provides a way to indirectly modulate dysfunctional NMDAR activity. In addition, mGluR5 is selectively distributed in brain regions relevant to schizophrenia, reducing the chances of excitotoxicity. mGluR5 agonists have been shown to ameliorate cognitive impairments induced by NMDAR antagonists in animals (Lecourtier et al., 2007; Stefani and Moghaddam, 2010; Vales et al., 2010). Additionally, Kinney et al. (2006) reported that mGluR5 agonism attenuated NMDAR antagonist induced deficits in parvalbumin, a calcium-binding protein which is well documented to be altered in schizophrenia. However, due to cytotoxic effects associated with direct agonism, positive allosteric modulators (PAMs) are the preferred candidates for drug development. PAMs do not activate receptors directly, rather they act on an allosteric site to potentiate activation by glutamate (Conn et al., 2009).

PAMs of mGluR5, the majority acting at the MPEP allosteric binding site, reverse a wide-range of positive, negative and cognitive schizophrenia-like behaviours induced in both dopaminergic (i.e. amphetamine) and glutamatergic (i.e. MK-801/PCP) animal models of schizophrenia. mGluR5 PAMs have also been reported not to cause sedation (Gilmour et al., 2013; Parmentier-Batteur et al., 2013), or other unwanted behavioural side effects (Balschun et al., 2006), although "very mild" catalepsy has been reported for some of these agents at high doses (Liu et al., 2008; Schlumberger et al., 2010).

Considering the role of mGluR5 in cognitive function, mGluR5-targeting drugs may be particularly useful for the treatment of cognitive deficits associated with the disorder. Nonetheless, there is limited knowledge regarding the status of mGluR5 in the pathological state (i.e. alterations in protein levels, binding ability, function, regulation or signalling), despite that pathological alterations to mGluR5 could impact on the therapeutic efficacy of these agents. It is therefore critical to understand the status of mGluR5 in the pathological state to guide the development of mGluR5-based treatments.

1.2 Literature summary

mGluR5 is an important modulator of glutamatergic neurotransmission, particularly via its interaction with the NMDAR, which is widely reported to be altered in schizophrenia pathology, and has therefore come to light as a protein that might also be involved in the pathophysiology of the disorder. In support, mGluR5 is critically involved in cognitive function, which is impaired in patients with schizophrenia. mGluR5 is thus a compelling therapeutic target for the treatment of schizophrenia, particularly the cognitive deficits.

Accumulating evidence suggests there could be aberrations of mGluR5 translation, expression or function in individuals with schizophrenia, including studies that indicate that genetic variation within *GRM5* is associated with schizophrenia, and evidence from animal studies indicating that altered mGluR5 activity induces schizophrenia-like behaviours. Specifically, pharmacological blockade of mGluR5 in animals produces a range of schizophrenia-related behaviours, especially deficits in working memory, instrumental learning, spatial memory and social interaction. Additionally, mGluR5 knockout mice display strong schizophrenia-like behaviours, including reduced sensorimotor gating and spatial memory. Finally, mGluR5 upregulation has promising therapeutic potential in preclinical animal models of schizophrenia. Studies aimed to confirm alterations to mGluR5 protein in postmortem human brain studies have however reported conflicting results, most likely due to small patient cohorts and the polygenic nature of schizophrenia. In addition, studies examining mGluR5 have not advanced beyond the surface to examine whether mGluR5 regulation is affected. This is important to explore, not only to increase the current understanding of the underlying molecular biology of schizophrenia, but also to determine whether mGluR5 - as a potential novel therapeutic target - will be affected in the patient.

Lastly, although preliminary data regarding APD effects on mGluR5 is conflicting, the effects of commonly used pharmacotherapies on mGluR5 regulation have not been explored. The effects of current APD medication on the mGluR5 system will contribute to the current understanding of (i) whether postmortem measures in the schizophrenia brain are confounded by the patients' premortem medication history, (ii) how mGluR5-targeted therapeutics might work in patients already exposed to current APD treatment, and (iii) whether mGluR5 might be used as an adjunct treatment (particularly to

treat cognitive dysfunctions) in patients already successfully treated with current APDs. Collectively, uncovering the molecular alterations (particularly protein expression and function) occurring in schizophrenia, and how these molecules are effected by current therapeutics, will help to build a solid foundation for the tailoring, development and employment of novel treatments for schizophrenia.

1.2.1 Aims and hypotheses

1.2.1.1 General aim

To investigate whether *GRM5,* mGluR5, and mGluR5 regulation is affected in the pathology of schizophrenia, to better understand the underlying neurobiology of schizophrenia and the treatment potential of mGluR5 in patients with schizophrenia.

1.2.1.2 Specific aims

The specific aims of this PhD were:

1. To characterise the binding density of mGluR5 in the ACC (which is highly involved in emotion and cognition) of patients with schizophrenia, bipolar disorder or major depression (with and without psychosis). This study was designed to deconstruct the overlapping nature of psychiatric disorders, to determine which changes are specific to mGluR5 and schizophrenia. (Presynaptic mGluR2/3 were also included in the original study design to provide additional context.)
2. To determine whether mGluR5 and mGluR5 regulators are altered in postmortem samples from the DLPFC, which is a region associated with working memory, cognitive flexibility, planning, inhibition, and abstract reasoning, of subjects with schizophrenia.
3. To determine whether alterations in mGluR5 and mGluR5 regulators extend to the hippocampal CA1 region (highly implicated in cognitive processes, including several types of learning and memory formation and storage) of subjects with schizophrenia.
4. To determine whether commonly used APD treatments for schizophrenia affect mGluR5 expression or regulation in a pharmacological animal model.
5. To determine whether novel SNPs within *GRM5* are associated with schizophrenia, and whether genetic variation within *GRM5* could contribute to cognitive dysfunction in schizophrenia subjects using a case-control cohort for this disorder.

1.2.1.3 Hypotheses

1. Despite the preliminary findings in the DLPFC that mGluR5 is not altered in schizophrenia subjects but is reduced in schizoaffective subjects of depressive subtype (Matosin et al., 2013), it was hypothesised that mGluR binding density would be reduced in the ACC of patients with schizophrenia, major depression and bipolar disorder, due to the role of the ACC in emotion and mood which are processes disrupted in these three disorders. However based on the previous findings in the DLPFC, it was further hypothesised that mGluR5 reduction would be pronounced in subjects with major depression.
2. Considering evidence of NMDAR hypofunction in this same postmortem cohort (Weickert et al., 2012), the results from animal studies that mGluR5 knockout or antagonism produces schizophrenia-like behaviours (Matosin and Newell, 2013), and reports of the importance of the DLPFC

in schizophrenia pathology (Callicott et al., 2000), it was hypothesised that mGluR5 protein and endogenous regulation would be reduced in the postmortem DLPFC in schizophrenia.

3. In view of the extensive evidence of (i) hippocampal-dependent cognitive impairment in schizophrenia (Harrison, 2004), (ii) the specific role of mGluR5 in modulating synaptic plasticity in the CA1 region in schizophrenia (Mukherjee and Manahan-Vaughan, 2013), and (iii) evidence that mGluR5 knockout or antagonism induces deficits in hippocampal-dependent cognitive functions in rodents, it was hypothesised that protein levels of mGluR5 and mGluR5 signalling partners would be reduced in the CA1 region of postmortem schizophrenia brains.
4. mGluR5 is highly involved in cognitive function, and current APD have minimal effects on cognitive deficits in many schizophrenia patients (Harvey, 2013). Accordingly, clozapine was unable to reverse cognitive deficits in mGluR5 knockout mice (Gray et al., 2009), suggesting mGluR5 plays a fundamental role in mediating cognitive behaviours. It was thus hypothesised that current APD treatment in rats would not affect protein expression of mGluR5 or mGluR5 signalling partners in the cortex or hippocampus.
5. As (i) Devon et al. (2001) and Timms et al. (2013) have both reported differential disruptions to *GRM5* in families with schizophrenia, (ii) an intronic microsatellite within *GRM5* was previously associated with schizophrenia in a case-control population (Devon et al., 2001), (iii) *GRM5* was recently reported to be a top candidate in psychiatric pathology (Ayalew et al., 2012), and (iv) the hypothesis that mGluR5 is dysregulated in schizophrenia, it was hypothesised that the tested polymorphisms within the 3' UTR of *GRM5* would be significantly associated with schizophrenia in the studied population. Furthermore, due to the extensive evidence that mGluR5 is involved in the mechanisms underlying synaptic plasticity, it was further hypothesised that allelic variation within *GRM5* would affect cognition in patients with schizophrenia.

1.2.1.4 Significance

Schizophrenia affects approximately 24 million people worldwide, but current therapeutic treatments for these patients are inadequate for many sufferers. Understanding the causes and neurobiology of schizophrenia is critical to developing more successful treatments with minimal side effects. For the first time, the studies within this thesis addressed whether mGluR5 and mGluR5 regulation is affected in schizophrenia or by current APD medication, and whether SNPs within *GRM5* are associated with schizophrenia and cognition. The studies presented in this thesis add to the limited literature regarding *GRM5*/mGluR5, and its role in the emergence and neurobiology of schizophrenia. Further, these studies provide critical information regarding the status of mGluR5 in the pathology of schizophrenia, which is necessary to guide the development of future therapeutics aimed at mGluR5.

Chapter Two

2.1 Brief introduction

The content of Chapter Two includes Study 1: Is mGluR5 binding density altered in the ACC in the pathophysiology of psychotic and mood disorders?

2.1.1 Relevant aims

This study was designed to address Aim 1:

1. To characterise the binding density of mGluR5 in the ACC (which is highly involved in emotion and cognition) of patients with schizophrenia, bipolar disorder or major depression (with and without psychosis). This study was designed to deconstruct the overlapping nature of psychiatric disorders, to determine which changes are specific to mGluR5 and schizophrenia. (Presynaptic mGluR2/3 were also included in the original study design to provide additional context, such as to determine if alterations are specific to mGluR5.)

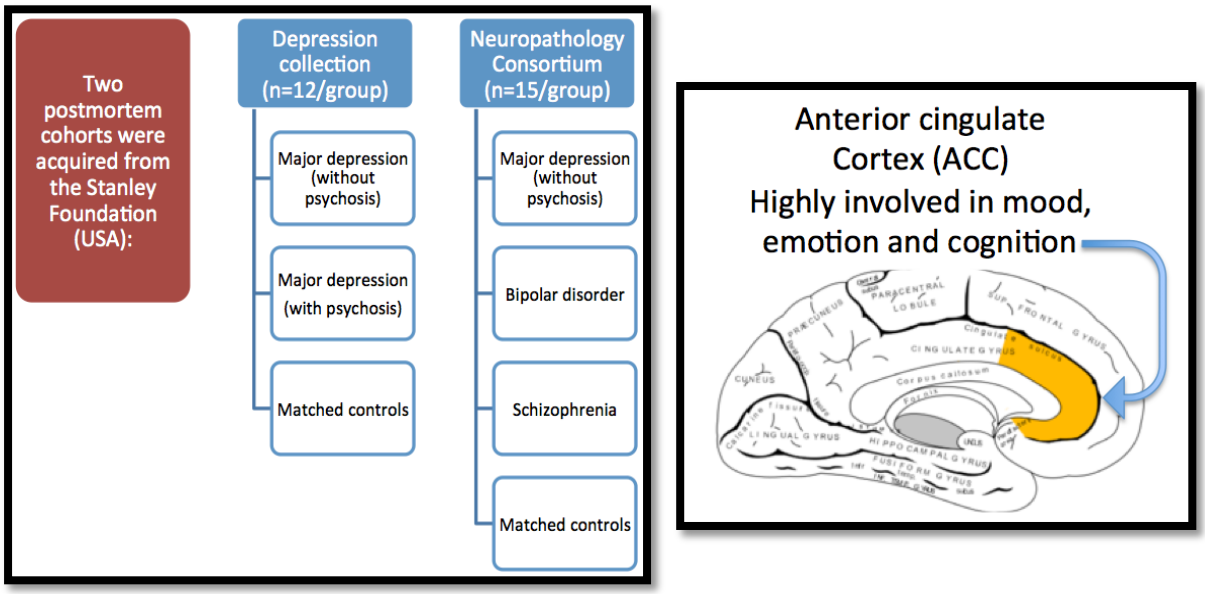
2.1.2 Overview and general methods

2.1.2.1 Study rationale

Previous studies that examined mGluR5 in the postmortem schizophrenia brain have largely reported little significant difference in mGluR5 mRNA or protein expression in schizophrenia compared to control subjects, although this might be brain-region dependent (see Chapter 1, section 1.1.5.3 and Table 1.2). However, we previously found preliminary evidence of a decrease in mGluR5 in schizoaffective subjects of depressive subtype (unpublished observations), suggesting alterations in mGluR5 may be associated with different symptom profiles in patients, and specifically, depressive-like symptoms. Reductions in mGluR5 binding have also been previously reported in the PFC of subjects with major depression (Deschwanden et al., 2011). This study therefore aimed to analyse whether alterations to mGluR5 are specific to depressive or psychotic symptoms using two well-established brain cohorts.

2.1.2.2 Methods overview

(a) SUBJECTS, STRUCTURE AND DESIGN:



(b) RECEPTOR AUTORADIOGRAPHY TECHNIQUE:

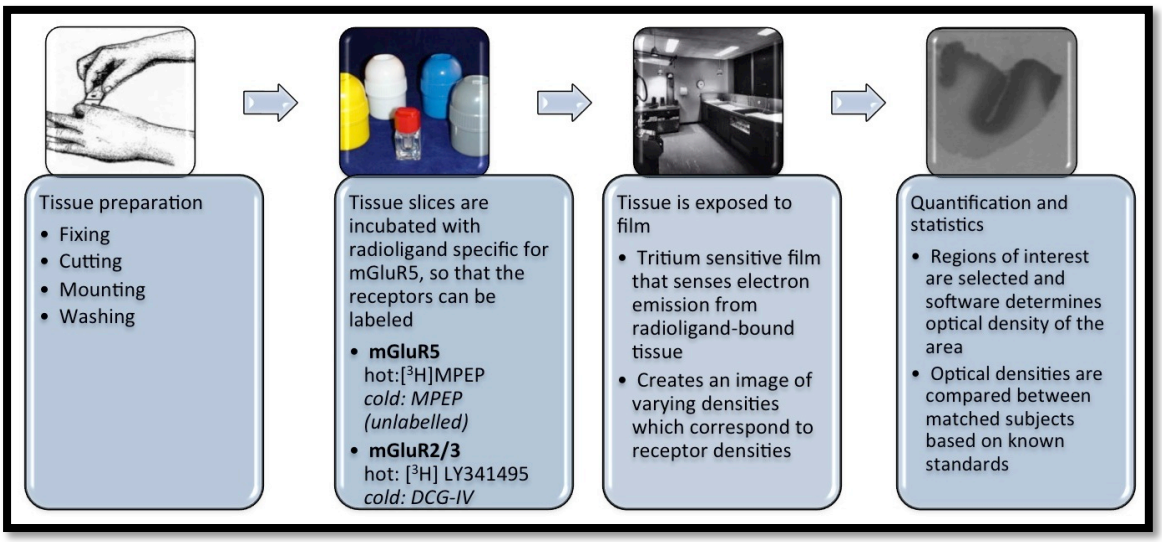


Figure 2.1 Methodological overview of Study 1. (a) Subjects in this study were derived from two well-characterised postmortem cohorts, consisting of subjects with schizophrenia, bipolar disorder or major depression (with or without psychosis). The anterior cingulate cortex of these subjects was examined using the receptor autoradiography technique as seen in (b). Brain slices were incubated with a tritiated specific ligand (hot) for either mGluR5 (MPEP) or mGluR2/3 (LY341495), and non specific binding (cold) determined by incubation in non-labeled specific ligands for these receptors (MPEP and DCG-IV). **Abbreviations:** DCG-IV, (1R,2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropane-1,2-dicarboxylic acid; LY341495, Eli Lilly and Company compound 341495, also known as Eglumegad; MPEP, 2-Methyl-6-(phenylethynyl)pyridine.

2.1.3 Result summary

The results from Study 1 (Chapter 2) indicate that mGluR5 (and mGluR2/3) binding is not altered in the ACC or in schizophrenia, bipolar disorder or major depression with or without psychosis, compared to control subjects. Although these results suggest that novel therapeutics that target mGluR5 will be unhindered, at least in this brain region, alterations to receptor regulation or function cannot be discounted. In addition, the finding of differential age-related differences in mGluR2/3 binding across the studied disorders suggest there may be differential treatment responses to these novel therapeutics depending on the pathology and age of the patient.

2.1.4 Author contributions

N. Matosin performed the experiments, acquired and analysed the data, wrote the first draft of the manuscript, which all authors reviewed and approved for publication.

2.1.5 Collaborator's statements

We hereby declare that the statement in section 2.1.4 pertaining to the contributions of N. Matosin are correct.

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Research Paper

Metabotropic glutamate receptor mGluR2/3 and mGluR5 binding in the anterior cingulate cortex in psychotic and nonpsychotic depression, bipolar disorder and schizophrenia: implications for novel mGluR-based therapeutics

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Background: Metabotropic glutamate receptors 2/3 (mGluR2/3) and 5 (mGluR5) are novel therapeutic targets for major depression (MD), bipolar disorder (BD) and schizophrenia. We aimed to determine whether mGluR2/3 and mGluR5 binding in the anterior cingulate cortex (ACC), a brain region essential for the regulation of mood, cognition and emotion, were differentially altered in these pathologies. **Methods:** Using postmortem human brains derived from 2 cohorts, [^{3}H]LY341495 binding to mGluR2/3 and [^{3}H]MPEP binding to mGluR5 were measured by receptor autoradiography in the ACC. The first cohort comprised samples from individuals who had MD with psychosis (MDP), MD without psychosis (MDNP) and matched controls (n = 11–12 per group). The second cohort comprised samples from individuals who had MDNP, BD, schizophrenia and matched controls (n = 15 per group). **Results:** No differences in mGluR2/3 or mGluR5 binding were observed in the MDP, MDNP, BD or schizophrenia groups compared with the control group (all $p > 0.05$). Importantly, there were also no differences in binding densities between the psychiatric disorders ($p > 0.05$). We did, however, observe age-related effects, with consistent negative associations between mGluR2/3 and age in the control group ($r < –0.575$, $p < 0.025$) and the psychotic disorder groups (MDP and schizophrenia: $r = –0.765$ to $–0.515$, $p < 0.05$), but not in the mood disorder groups (MDNP, BD). **Limitations:** Replication in larger independent cohorts and medication-naive individuals would strengthen these findings. **Conclusion:** Our findings suggest that mGluRs are unaltered in the ACC; however, the presence of altered receptor function cannot be discounted and requires further investigation. Taken together with previous studies, which report differential changes in mGluR2, 3 and 5 across these disorders, we suggest mGluRs may be affected in a brain region–specific manner.

Introduction

Major depression (MD) is an important cause of disability as well as financial and emotional burden worldwide. While traditionally the monoaminergic system has been implicated in the pathophysiology and treatment of MD, evidence from clinical, preclinical and postmortem studies are rapidly implicating glutamatergic dysregulation in MD.[1] Accordingly, metabotropic glutamate receptors (mGluRs), which slowly mediate glutamatergic neurotransmission, are promising therapeutic targets for the treatment of MD. The challenges, however, in elucidating the pathophysiology of MD, and therefore appropriate

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treatment strategies, are the heterogeneity of the disorder and its association with other psychiatric and mood disorders, such as bipolar disorder (BD) and schizophrenia.

Major depression with psychotic features (psychotic depression) is classified as a subtype of major depression in DSM-IV. As recently discussed by Rothschild,[2] psychotic depression is associated with BD. Studies have shown that patients with psychotic depression had an increased risk for BD compared with patients with nonpsychotic depression. In contrast, evidence suggests psychotic depression is distinct from schizophrenia.[2] It is necessary to characterize the pathophysiology of depression, including its psychotic and nonpsychotic types, and determine the differences and similarities between BD and schizophrenia, as this has crucial implications for advising the use of certain pharmacotherapeutics.

Several lines of evidence suggest the involvement of the anterior cingulate cortex (ACC) in the pathophysiology of depression. It has critical control over mood, emotion and cognition, as proven by bilateral cingulotomy psychosurgery, which can relieve depression and some psychiatric symptoms.[3] Concurrently, reduced glutamate levels have been reported in the ACC of patients with depression,[4–6] and this is reflected at the protein level for various markers expressed at the glutamatergic synapse.[7–9] The involvement of glutamatergic dysfunction in depression may be specific to this brain region, as 1 group found alterations of various postsynaptic glutamatergic proteins (NR1 subunit of the *N*-methyl-D-aspartate receptor [NMDAR], postsynaptic density protein 95 [PSD95] and postsynaptic density protein 93 [PSD93]) in the ACC, but not in the dorsolateral prefrontal cortex (DLPFC), in individuals with depression.[7]

Metabotropic glutamate receptors, particularly mGluR2/3 and mGluR5, have been identified as novel therapeutic targets for mood and psychiatric disorders because of their ability to modulate glutamatergic neurotransmission.[10–12] Whereas mGluR2/3 are predominately presynaptic receptors that inhibit glutamate and γ-aminobutyric acid (GABA) release into the synapse,[13] mGluR5 are primarily expressed postsynaptically, where they modulate the glutamate response, especially in association with the NMDAR.[11] In addition to their therapeutic potential, mGluRs are also implicated in the pathophysiology of these disorders. For example, numerous animal studies demonstrate that selected mGluRs are affected in animal models of depression,[14,15] and knockout animals display psychotic phenotypic behaviours.[11,13] From these studies, it is becoming increasingly clear that there may be opposing actions of the glutamatergic system in these disorders, particularly as mGluR2/3 and mGluR5 blockade appear to be beneficial for the treatment of depression, while potentiation of these receptors has preclinical efficacy for the treatment of psychosis.[1,11,13] Hence, mGluR2/3 and mGluR5 may be differentially involved in the pathophysiology of mood and psychotic disorders.

We sought to determine whether mGluR2/3 and mGluR5 were altered in the ACC of individuals with MD (in the presence and absence of psychosis) and the associated pathologies BD and schizophrenia. We studied 2 independent postmortem cohorts from the Stanley Medical Research Institute: the Stanley Depression Collection, consisting of samples from individuals who had MD without psychosis (MDNP), MD with psychosis (MDP) and controls, and the Stanley Neuropathology Consortium, consisting of samples from individuals who had diagnoses of MDNP, BD and schizophrenia and controls. We measured mGluR2/3 and mGluR5 binding in the ACC (Brodmann area [BA] 24) of these samples by in situ receptor autoradiography. As the ACC is implicated in the emotional and cognitive deficits associated with these disorders, the findings from this study have translational implications for novel drugs aimed to treat mood and cognitive deficits in patients with these disorders.

Methods

Human postmortem brain samples

Frozen postmortem brain tissue was acquired through the Stanley Medical Research Institute. From the Depression Collection, we obtained samples from 24 individuals who had MD (MDNP: $n = 12$; MDP: $n = 11$) and controls ($n = 12$) matched according to age, postmortem interval (PMI), refrigeration interval and brain pH (Table 1). Psychosis could not be confirmed in 1 of the MD samples, who was therefore excluded from psychosis-specific analyses. From the Neuropathology Consortium, we obtained samples from 60 individuals who had MDNP, BD and schizophrenia and controls ($n = 15$ per group), matched for age, sex, race and PMI (Table 2). Detailed clinical and demographic information regarding these cohorts have been published previously.[16,17] The work described in this study was approved by the Human Research Ethics Committee at the University of Wollongong and conducted according to their guidelines (HE13/069).

Receptor autoradiography

Frozen coronal sections at the level of the ACC (BA24; 14 μm) were mounted onto slides and stored at –80°C until the day of the assay. Receptor autoradiography studies were based on protocols described previously (mGluR2/3;[18,19] mGluR5[20,21]). Autoradiography exposures were optimized for mGluR2/3 and mGluR5 to ensure that each cortical layer could be quantified on a linear scale and none of the cortical layers with high expression were overexposed. The signal to noise ratio of mGluR2/3 and mGluR5 binding in all groups assayed were well above the nonspecific control. Two sections for each individual were analyzed for total binding, and adjacent sections were used to determine nonspecific binding. All experiments were performed blind to diagnoses.

mGluR2/3

Tissue sections were preincubated in a buffer solution containing 50 mM Tris buffer, 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (pH 7) at room temperature for 2 × 10 minutes. Subsequently, slides were incubated for 60 minutes at room temperature in buffer solution containing 50 nM [^{3}H]LY341495 (specific mGluR2/3 agonist; specific activity 40 Ci/mmol; American Radiolabelled

Chemicals). To determine nonspecific binding, adjacent sections were additionally incubated with 50 nM [^{3}H]LY341495 and 0.01 mM DCG-IV (specific mGluR2/3 agonist; Sigma). After incubation, the slides were washed in 4°C washing buffer (50 mM Tris buffer, pH 7) for 2 × 30 seconds and 1 × 1 minute. This protocol favours mGluR2 over mGluR3 binding.[19]

mGluR5

Tissue sections were preincubated for 15 minutes at room temperature in 50 mM TrisNaCl (pH 7.5). Slides were then incubated in solution containing 10 nM [^{3}H]MPEP (specific mGluR5 antagonist; specific activity 60 Ci/mmol; American Radiolabelled Chemicals) and 50 mM TrisNaCl for 90 minutes at room temperature. Adjacent sections were incubated in the same solution with the addition of 10 µM unlabelled MPEP (Sigma) to determine nonspecific binding. Slides were washed in 4°C TrisNaCl (pH 7.5) for 2 × 3 minutes and briefly dipped in 4°C MilliQ-water.

Imaging and quantification

Together with [^{3}H] microscale autoradiographic standards (Amersham Biosciences), sections were exposed to tritium sensitive Kodak Biomax MR film (Kodak) for 10 weeks. Films were developed using the AGFA CP1000 film developer (Agfa-Gavaert N.V.), scanned using a GS800 densitometer (BioRad) and analyzed using Multi-Analyst software (Bio-Rad). Quantification was performed blind to diagnoses. Radioligand binding signals were expressed in counts per minute (cpm)/mm^2 and, with the use of standards and the specific activities of the respective radioligands, were converted to fmol/mg tissue equivalents.

Statistical analysis

As data were normally distributed across both cohorts, we used parametric tests. No data points were identified as outliers (± 2

Table 1: Cohort demographic and clinical descriptives of tissue sample donors within the Depression Collection

	Group; mean (range)*			
Descriptive	Control	MD (total)	MDP	MDNP
No. of patient donors	12	24	11	12
Age at death, yr	46.8 (24–62)	42.2 (24–63)	39.6 (28–63)	42.8 (24–56)
Illness duration, yr	—	11.7 (0.1–31)	11 (3–21)	12.3 (0.1–31)
Age at illness onset, yr	—	29.7 (13–45)	28.6 (13–39)	30.5 (14–45)
Sex, male:female	8:4	13:11	5:6	7:5
PMI, h	25.3 (9–65)	29.6 (13–65)	33.1 (19–52)	23.6 (13–38)
Refrigeration, h†	7.42 (3–18)	7.96 (1–31)	7.18 (1–14)	8 (2–31)
pH	6.64 (6.31–6.57)	6.6 (6.3–6.9)	6.6 (6.3–6.74)	6.7 (6.52–6.9)
Hemisphere, right:left	6:6	15:9	8:3	7:5
Antipsychotics‡	0	12	9	3
Fluphenazine equivalents	—	2 242 (100–6 500)	2 533 (100–6 500)	1 367 (100–3 000)

MD = major depression; MDNP = major depression without psychosis; MDP = major depression with psychosis; PMI = postmortem interval.
*Unless otherwise indicated.
†Values refer to the mean interval between patient death and refrigeration of the body at the medical examiner's office.
‡Number of patients with a prescription for antipsychotics at the time of death.

Table 2: Cohort demographic and clinical descriptives of tissue sample donors within the Neuropathology Consortium

	Group; mean (range)*			
Descriptive	Control	MD	Bipolar disorder	Schizophrenia
No. of patient donors	15	15	15	15
Age at death, yr	48.1 (29–68)	46.5 (30–65)	42.3 (25–61)	44.2 (25–62)
Illness duration, yr	—	12.6 (1–42)	20.13 (6–43)	21.3 (5–45)
Age at illness onset, yr	—	33.9 (11–54)	21.5 (7–39)	23.2 (13–42)
Sex, male:female	9:6	9:6	9:6	9:6
PMI, h	23.7 (8–42)	27.5 (7–47)	32.5 (13–62)	33.7 (12–61)
FST, d	338.27 (31–774)	434 (86–931)	620 (224–836)	621.13 (68–938)
pH	6.3 (5.8–6.6)	6.2 (5.6–6.5)	6.2 (5.8–6.5)	6.1 (5.8–6.6)
Hemisphere, right:left	7:8	6:9	8:7	6:9
Antipsychotics†	0	0	12	14
Fluphenazine equivalents	—	—	26 033 (200–60 000)	56 000 (1 000–2 000)

FST = freezer storage time; MD = major depression; PMI = postmortem interval.
*Unless otherwise indicated.
†Number of patients with a prescription for antipsychotics at the time of death.

standard deviations). The depression collection and the neuropathology consortium were analyzed separately. We used analysis of variance (ANOVA) followed by a Tukey post hoc test to compare differences in mGluR2/3 and mGluR5 binding between the diagnoses in the 2 cohorts (i.e., MDNP, MDP v. control and MDNP, BD, schizophrenia and control). Subsequently, we completed Pearson correlations to determine any influence of continuous variables on the data. We then carried out analyses of covariance (ANCOVAs) in which we compared binding values between the diagnostic groups in the 2 cohorts, controlling for variables that significantly correlated with the data. Statistical analyses were performed using SPSS software version 19.0. We considered results to be significant at $p < 0.05$, and data are presented as means ± standard errors of the mean (SEM).

The Stanley Neuropathology Consortium Integrative Database

The Stanley Neuropathology Consortium Integrative Database (SNCID; http://sncid.stanleyresearch.org)[22] includes 1762 pathological markers measured in 12 different brain regions by the Neuropathology Consortium. In line with the hypotheses of the present study, we sought to determine if there were associations between our measures of mGluR2/3 and mGluR5 and measures of other glutamate-related proteins, specifically within the cingulate cortex. Exploratory correlation analyses of glutamatergic markers (various measures of NMDAR, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl propionate receptor [AMPAR] and kainate receptors) and signalling partners (PSD95, brain-derived neurotrophic factor [BDNF] and protein kinase C [PKC]) that have previously been measured in the cingulate cortex (BA not specified) was performed using the SNCID as previously described.[22] We used a nonparametric method (Spearman correlation) to minimize effects caused by differences in units and/or distribution patterns.[23] All 60 individuals were included, and significance was set at $p < 0.05$. Detailed information regarding the measurement and experimental protocol for each marker is available on the SNCID database (http://sncid.stanleyresearch.org).

Results

Distribution

We found that mGluR2/3 ([^{3}H]LY341495) and mGluR5 ([^{3}H]MPEP) binding were highly expressed in the cortical areas of brain slices across the 2 cohorts (Fig. 1). Whereas mGluR5 binding was homogeneously distributed across all cortical layers, mGluR2/3 binding showed pronounced layer differences; the superficial one-third of the cortex had significantly higher binding than the lower layers ($p < 0.001$) and was analyzed accordingly.

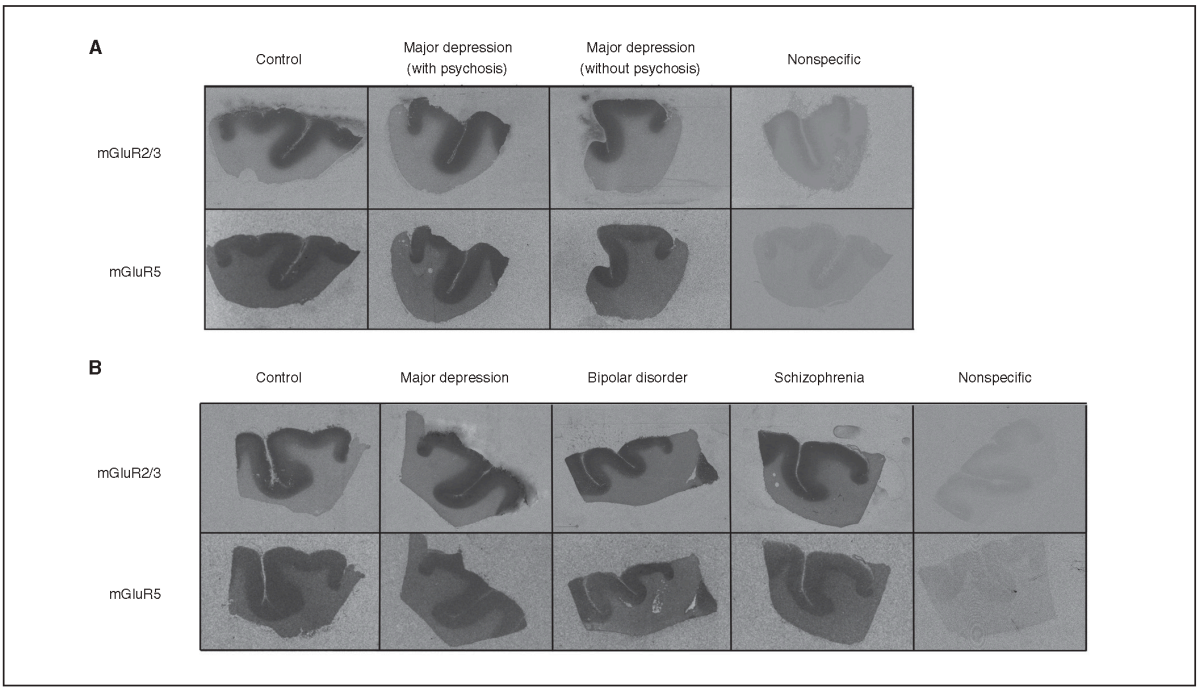

Fig. 1: Representative receptor autoradiographs of mGluR2/3 [^{3}H]LY341495 and mGluR5 [^{3}H]MPEP binding from the anterior cingulate cortex of samples from the **(A)** Stanley Depression Collection (control, major depression with psychosis, major depression without psychosis) and the **(B)** Stanley Neuropathology Consortium (control, major depression, bipolar disorder and schizophrenia). A representative nonspecific binding has been included for each cohort. mGluR2/3 [^{3}H]LY341495 and mGluR5 [^{3}H]MPEP binding densities were not different between pathological and control groups.

Depression Collection

Analysis of demographic and clinical data

Mean pH, age at death, PMI and brain weight did not differ among the MDNP, MDP and control groups (F = 0.054–3.171, all $p > 0.05$). Excluding controls, further analyses revealed no significant differences between diagnoses (MDNP v. MDP) for age of onset (t_{21} = –0.379, p = 0.71) or illness duration (t_{21} = –0.417, p = 0.68). Individuals in the MDNP and MDP groups were exposed to antipsychotic medication (measured as fluphenazine equivalent units), although average estimated lifetime exposure did not differ significantly between the groups (3 in the MDNP group v. 9 in the MDP group, t_{10} = 0.940, p = 0.37). Quantitative antidepressant and mood stabilizer medication histories were not available.

Diagnosis-related effects

One-way ANOVA revealed no significant diagnostic alterations in mGluR2/3 (total: $F_{2,32}$ = 0.306, p = 0.74; upper: $F_{2,32}$ = 1.502, p = 0.24; lower: $F_{2,32}$ = 0.338, p = 0.72) or mGluR5 ($F_{2,32}$ = 0.074, p = 0.93) comparing the MDP, MDNP and control groups (Fig. 2). Subsequently, we performed ANCOVAs to control for age at death, brain pH, refrigeration interval, brain weight and PMI; however, significance was unchanged for mGluR2/3 (total: $F_{2,27}$ = 0.934, p = 0.43; upper: $F_{2,27}$ = 0.761, p = 0.72; lower: $F_{2,27}$ = 0.025, p = 0.98) or mGluR5 ($F_{2,27}$ = 1.010, p = 0.46). In addition, we performed an independent t test to compare all individuals with depression (n = 24) and controls (n = 12). No significance was seen for either mGluR2/3 (total: t_{34} = –0.009, p = 0.99; upper: t_{34} = –0.576, p = 0.57; lower: t_{34} = 0.693, p = 0.49) or mGluR5 (t_{34} = –0.106, p = 0.92).

Effects of demographic and clinical variables

Pearson correlations for continuous variables (pH, age at death, PMI, refrigeration interval, brain weight, age at disease onset, duration of illness and lifetime fluphenazine equivalents) are presented in Table 3 and in the Appendix, Table S1, available at jpn.ca. Age at death was consistently associated with mGluR2/3 binding in the control group (total: r = –0.551, p = 0.06; upper: r = –0.747, p = 0.005; lower: r = –0.134; p = 0.94) and in the MDP group (total: r = –0.765, p = 0.006; upper: r = –0.748, p = 0.008; lower: r = –0.692, p = 0.018) but not in the MDNP group (total: r = –0.371, p = –0.24; upper: r = –0.383, p = 0.22; lower: r = –0.335, p = 0.29). Age at death correlated positively with mGluR5 in the MDNP group (r = 0.587, p = 0.045) but not in the MDP group (r = 0.551, p = 0.08) or the control group (r = 0.023, p = 0.94; Table 3).

Overall, there was no effect of lifetime antipsychotic exposure on total or lower mGluR2/3 (total: r = –0.289, p = 0.19; lower: r = –0.047, p = 0.84) or mGluR5 (r = 0.005, p = 0.98) in all individuals who had MD (MDNP and MDP), but we observed a negative association with mGluR2/3, specifically in the upper layer (r = –0.460, p = 0.031). Power for the subgroups was notably low (MDNP: n = 3; MDP: n = 9) and was therefore not reported (Table 3).

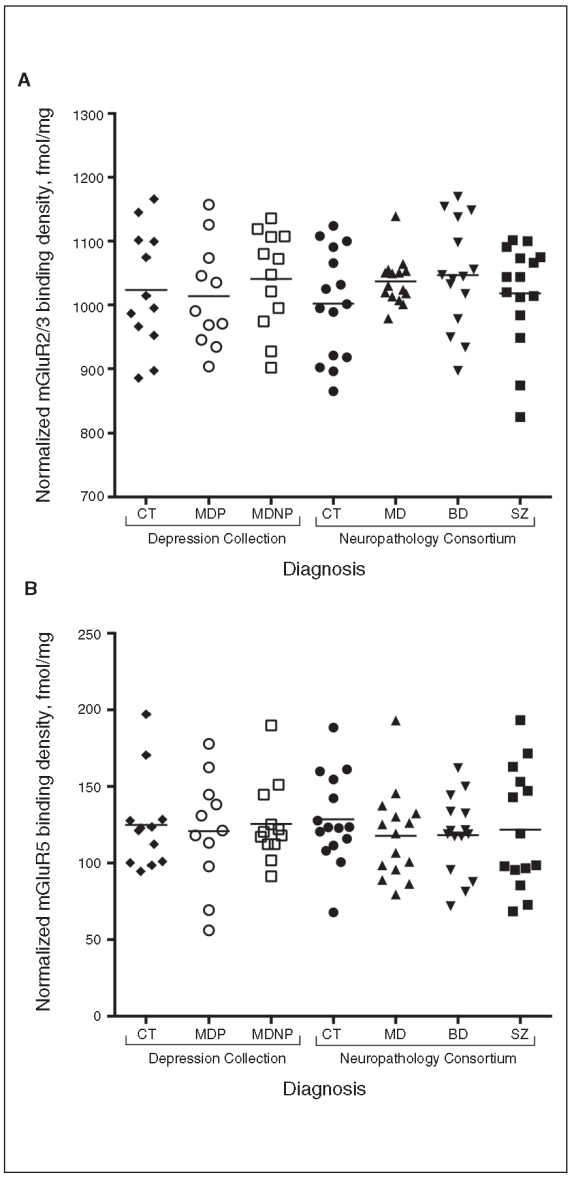

Fig. 2: Normalized **(A)** mGluR2/3 [^{3}H]LY341495 and **(B)** mGluR5 [^{3}H]MPEP binding density (as measured in fmol/mg tissue) in the anterior cingulate cortex of control (CT; diamonds = Depression Collection; solid circles = Neuropathology Consortium), major depression with psychosis (MDP; hollow circles), major depression without psychosis (MDNP; Depression Collection = hollow squares; Neuropathology Consortium = upward arrowheads), bipolar disorder (BD; downward arrowheads), and schizophrenia (SZ; solid squares) groups. mGluR2/3 and mGluR5 binding densities were not altered or differentially expressed in the anterior cingulate cortex in these neuropathologies.

Neuropathology Consortium

Analysis of demographic and clinical data

Mean pH, age at death, PMI and brain weight did not differ among the MDNP, BD, schizophrenia and control groups (F = 0.420–1.857, all $p > 0.147$). However, freezer storage time was significantly greater in the BD group (281.73 d longer, $p = 0.010$) and the schizophrenia group (282.86 d longer, $p = 0.009$) than the control group. Excluding controls, further ANOVAs and post hoc tests revealed significant differences between diagnoses, with the MDNP group having a mean age at onset that was 10.73 years older than the schizophrenia group (p = 0.016) and 12.46 years older than the BD group ($p = 0.005$). Illness duration also approached significance, being 8.63 years longer in the schizophrenia group than in the MDNP group (p = 0.08). Of the patients who had been exposed to antipsychotic medication (12 in the BD group and 14 in the schizophrenia group), lifetime antipsychotic exposure did not differ between the groups ($t_{24} = -1.557$, $p = 0.13$). Quantitative antidepressant and mood stabilizer medication histories were not available.

Diagnosis-related effects

One-way ANOVA revealed no significant diagnostic alterations in mGluR2/3 (overall: $F_{3,56} = 1.387$, $p = 0.26$; upper: $F_{3,56}$ = 1.586, $p = 0.20$; lower: $F_{3,56} = 0.847$, $p = 0.47$) or mGluR5 ($F_{3,56}$ = 0.541, $p = 0.66$) binding densities in the MDNP, BD and schizophrenia groups compared with the control group (Fig. 2). The ANCOVA controlling for age at death, brain pH, brain weight, freezer storage time and PMI confirmed this finding for both mGluR2/3 (total: $F_{3,51} = 0.934$, $p = 0.43$; upper: $F_{3,51} = 0.761$, $p = 0.52$; lower: $F_{3,51} = 0.818$, $p = 0.49$ and mGluR5 ($F_{3,51} = 1.010$, $p = 0.40$). As age at disease onset, illness duration and lifetime fluphenazine equivalents were observed to correlate with binding (Table 4 and Appendix, Table S1), we performed additional ANCOVAs excluding control samples. The data remained nonsignificant for both mGluR2/3 (total: $F_{2,35} = 0.998$; $p = 0.38$; upper: $F_{2,35} = 1.203$; $p = 0.31$; lower: $F_{2,35}$ = 0.497, $p = 0.61$) and mGluR5 ($F_{2,35} = 0.382$, $p = 0.69$), suggesting no significant difference in mGluR2/3 and mGluR5 binding among the MDNP, BD and schizophrenia groups.

Effects of demographic and clinical variables

Pearson correlations for mGluR2/3 and mGluR5 with continuous variables (pH, age at death, PMI, freezer storage time, brain weight, age at disease onset, duration of illness, and lifetime fluphenazine equivalents) are presented in Table 4 and in the Appendix, Table S1. Various correlations for pH, brain weight, PMI and freezer storage time were observed across the diagnostic groups; these were accounted for by ANCOVA and did not affect the diagnostic findings.

Consistent with our results in the Depression Collection, age at death had a significant negative effect on mGluR2/3

Table 3: Pearson correlations for variables influencing mGluR2/3 and mGluR5 binding densities in the anterior cingulate cortex within diagnostic groups of the Depression Collection

Diagnosis; variable	Age at death		Fluphenazine equivalent	
All samples, $n = 36$				
mGluR2/3 total	$r = -0.551$	$p < 0.001$	—	—
mGluR2/3 upper	$r = -0.569$	$p < 0.001$	—	—
mGluR2/3 lower	$r = -0.403$	$p = 0.015$	—	—
mGluR5 total	$r = 0.359$	$p = 0.032$	—	—
Controls, $n = 12$				
mGluR2/3 total	$r = -0.551$	$p = 0.06$	—	—
mGluR2/3 upper	$r = -0.747$	$p = 0.005$	—	—
mGluR2/3 lower	$r = -0.134$	$p = 0.68$	—	—
mGluR5 total	$r = 0.023$	$p = 0.94$	—	—
Major Depression, all samples; $n = 24$				
mGluR2/3 total	$r = -0.570$	$p = 0.004$	$r = -0.289$*	$p = 0.19$*
mGluR2/3 upper	$r = -0.507$	$p = 0.011$	$r = -0.460$*	$p = 0.031$*
mGluR2/3 lower	$r = -0.529$	$p = 0.008$	$r = -0.047$*	$p = 0.84$*
mGluR5 total	$r = 0.545$	$p = 0.006$	$r = -0.005$*	$p = 0.98$*
Major Depression, with psychosis; $n = 11$				
mGluR2/3 total	$r = -0.765$	$p = 0.006$	—	—
mGluR2/3 upper	$r = -0.748$	$p = 0.008$	—	—
mGluR2/3 lower	$r = -0.692$	$p = 0.018$	—	—
mGluR5 total	$r = 0.551$	$p = 0.08$	—	—
Major Depression, without psychosis; $n = 12$				
mGluR2/3 total	$r = -0.371$	$p = 0.24$	—	—
mGluR2/3 upper	$r = -0.383$	$p = 0.22$	—	—
mGluR2/3 lower	$r = -0.335$	$p = 0.29$	—	—
mGluR5 total	$r = 0.587$	$p = 0.045$	—	—

*$n = 12$.

binding in the control group (total: $r = –0.695$, $p = 0.004$; upper: $r = –0.666$, $p = 0.007$; lower: $r = –0.575$, $p = 0.025$) and in the schizophrenia group (total: $r = –0.528$, $p = 0.043$; upper: $r = –0.458$, $p = 0.09$; lower: $r = –0.515$, $p = 0.049$) groups, but not in the MDNP or BD groups. Age at death displayed a borderline significant correlation with mGluR5 in the schizophrenia group ($r = 0.505$, $p = 0.06$) but not in the MDNP, BD or control groups. Illness duration also significantly and negatively correlated in the schizophrenia group for mGluR2/3 (total: $r = –0.697$, $p = 0.004$; upper: $r = –0.734$, $p = 0.002$; lower $r = –0.539$, $p = 0.038$), although this correlation was not seen with mGluR5. Although lifetime antipsychotic medication did not differ between the schizophrenia and BD diagnostic groups, a significant negative correlation with mGluR2/3 was observed, specifically in the BD group (Table 4).

Correlations with glutamatergic markers and signalling partners in the SNCID

Explorative Spearman correlations with the data from the present study and those from previous studies (available in the SNCID) are presented in the Appendix, Table S2. Various significant associations were observed, both overall and in individual pathologies, between mGluR2/3 and mGluR5 with measures of NMDAR, AMPAR and kainate receptors as well as signalling partners PSD95, PKC and BDNF.

Discussion

The mGluR2/3 and mGluR5 receptors are targets of new therapeutic approaches for psychiatric conditions, including MD, BD and schizophrenia. Furthermore, animal studies have suggested the involvement of these receptors in the pathophysiologies of these disorders.[1,11] In light of this, we have examined binding to mGluR2/3 and mGluR5 in postmortem brains, specifically the ACC, of individuals with MD (MDP, MDNP), BD and schizophrenia and compared them to matched controls. In particular, we chose the receptor binding technique and radioligands that target the same binding sites as novel drugs currently under development to determine how drugs targeting these receptor sites may be affected in the patient. We report no significant diagnostic alterations of mGluR2/3 binding or mGluR5 binding in the ACC in individuals with these pathologies.

Unaltered levels of mGluR2/3 and mGluR5 in depression (with or without psychosis)

mGluR2/3 and mGluR5 are prominent prospective targets in the treatment of mood disorders, as both clinical and preclinical studies have shown antidepressant properties of antagonists or negative allosteric modulators targeted at

Table 4: Pearson correlations for variables influencing mGluR2/3 and mGluR5 binding densities in the anterior cingulate cortex within diagnostic groups of the Neuropathology Consortium.

Diagnosis; variable	Age at death		Fluphenazine equivalent	
All samples, $n = 60$				
mGluR2/3 total	$r = –0.410$	$p = 0.001$	—	—
mGluR2/3 upper	$r = –0.464$	$p < 0.001$	—	—
mGluR2/3 lower	$r = –0.273$	$p = 0.035$	—	—
mGluR5 total	$r = 0.087$	$p = 0.51$	—	—
Controls, $n = 15$				
mGluR2/3 total	$r = –0.695$	$p = 0.004$	—	—
mGluR2/3 upper	$r = –0.666$	$p = 0.007$	—	—
mGluR2/3 lower	$r = –0.575$	$p = 0.025$	—	—
mGluR5 total	$r = –0.305$	$p = 0.27$	—	—
Major depression, $n = 15$				
mGluR2/3 total	$r = –0.258$	$p = 0.35$	—	—
mGluR2/3 upper	$r = –0.355$	$p = 0.19$	—	—
mGluR2/3 lower	$r < 0.001$	$p = 0.10$	—	—
mGluR5 total	$r = –0.453$	$p = 0.09$	—	—
Bipolar disorder, $n = 15$				
mGluR2/3 total	$r = –0.045$	$p = 0.87$	$r = –0.554$	$p = 0.032$
mGluR2/3 upper	$r = –0.286$	$p = 0.30$	$r = –0.525$	$p = 0.045$
mGluR2/3 lower	$r = 0.170$	$p = 0.55$	$r = –0.482$	$p = 0.07$
mGluR5 total	$r = 0.023$	$p = 0.93$	$r = –0.131$	$p = 0.64$
Schizophrenia, $n = 15$				
mGluR2/3 total	$r = –0.528$	$p = 0.043$	$r = –0.109$	$p = 0.70$
mGluR2/3 upper	$r = –0.458$	$p = 0.09$	$r = 0.016$	$p = 0.96$
mGluR2/3 lower	$r = –0.515$	$p = 0.049$	$r = –0.225$	$p = 0.42$
mGluR5 total	$r = 0.505$	$p = 0.06$	$r = 0.477$	$p = 0.07$

mGluR2/3 and mGluR5.[1,12] To our knowledge, we are the first to report that neither mGluR2/3 nor mGluR5 binding levels are altered in the ACC of individuals with MD, in the presence (MDP) or absence (MDNP) of psychosis. Our results are in line with a previous study reporting unaltered mGluR2 and mGluR3 gene expression in the postmortem ACC of individuals with MD.[9] We found no other studies that have investigated mGluRs in the ACC in individuals with depression. In other brain regions of depressed patients, an increase in mGluR2/3[24] and reduction of mGluR5 protein in the prefrontal cortex (BA10) have been reported, together with reduced (in vivo) prefrontal cortical mGluR5 binding[25] and increased mGluR5 gene expression in the locus coeruleus.[26] Although it is difficult to draw conclusions from only a small number of studies, the inconsistencies between these findings highlight the inherent regional differences in mGluRs in the pathophysiology of MD as well as the importance of considering psychiatric subclass. These prior studies have an important limitation in that they do not distinguish between MDP and MDNP, particularly since differential proteome profiles have been recently reported, specifically between MDNP and MDP.[17]

mGluR2/3 and mGluR5 binding does not differ in BD and schizophrenia compared with depression

In line with depression with and without psychosis we report that mGluR2/3 and mGluR5 binding density are unaltered in the ACC in individuals with schizophrenia and BD. While mGluR binding, to our knowledge, has not previously been measured in individuals with BD, studies in the prefrontal cortex, thalamus and striatum of individuals with schizophrenia have likewise reported no change in mGluR2/3[19,27–30] or mGluR5[11,21] (binding, protein or mRNA). Our findings highlight that in the ACC there are no distinct differences between the overlapping diagnoses of MDNP, MDP, schizophrenia and BD with regards to binding to mGluR2/3 and mGluR5. This suggests that novel pharmacological modulators of mGluR2/3 and mGluR5 may have unhindered binding sites in all these disorders.

It should, however, be noted that there may be wider dysregulations in mGluR2/3 and mGluR5 function that were not measured within the scope of our study or that there may be alterations in other mGluR subtypes dependent on specific brain regions.[12] For example, Volk and colleagues[31] found reductions in mGluR1a mRNA, but not mGluR5, in the prefrontal cortex (BA9 and BA42) of patients with schizophrenia. Conversely another more recent study found mGluR5 mRNA and protein to be reduced specifically in BA9 and the lateral cerebellum in individuals with schizophrenia.[32] These examples highlight the heterogeneity and intricacies of spectrum disorders such as schizophrenia.[33]

Current and novel pharmacotherapy implications

In the present study we found evidence of a negative association between mGluR2/3 binding and antipsychotic dose, specifically in individuals with BD and MD, but not in those with schizophrenia. Previous postmortem studies have reported no association between antipsychotic dose and mGluR2/3[19,27,28] or mGluR5,[21] specifically in patients with schizophrenia. Animal studies have reported no effect of antipsychotics on mGluR2/3[34] or mGluR5 binding.[21] The effects of antidepressants have not been as well studied, and while reliable quantitative lifetime antidepressant drug history was not available in the present study, our previous work found no association of antidepressant history and mGluR5 binding and protein in the DLPFC in individuals with schizophrenia.[21] Deschwanden and colleagues,[25] however, found that antidepressant treatment may reduce mGluR5 binding specifically in the precentral gyrus of antemortem patients with MD. Animal studies have shown that tricyclic antidepressant treatment significantly increases mGluR2/3[35] and mGluR5a[36] protein in the rat hippocampus, although another study found that total mGluR5 protein levels were unaltered.[35] To our knowledge, there are no prior studies on antidepressant effects specifically in the ACC to shed light on these findings; therefore, further investigation is required.

The mGluR-targeted modulators have shown promising antipsychotic and antidepressant therapeutic potential in preclinical and clinical studies. Orthosteric and allosteric modulators of mGluRs target binding sites on either the N-terminus (mGluR2/3) or transmembrane (mGluR5) domain, respectively.[1,11,13,37] These orthosteric and allosteric binding sites were the same targets of the radioligands used in the present study. As we report that these binding sites are not altered in the ACC in psychopathology, mGluR2/3 and mGluR5 may be unimpeded targets for novel antidepressant and antipsychotic intervention. As results from recent clinical trials with mGluR modulators indicated that specific single nucleotide polymorphisms (SNPs) were associated with treatment response,[38] further study into these same binding sites in individuals with these SNPs would be of value. In addition, we consistently found a negative correlation between mGluR2/3 binding and age in the control, schizophrenia and MDP groups, with no association in the MDNP and BD groups. Age-related differences in treatment approaches remain an issue,[39] and our findings suggest there may be differential treatment responses to these novel therapeutics depending on the pathology and age of the patient.

Association of mGluRs with markers in the wider glutamatergic system

In addition to the metabotropic group of glutamate receptors, glutamate signalling is also transduced through the ionotropic glutamate receptors NMDAR, AMPAR and kainate receptors. The NMDAR and AMPAR receptors are of particular interest as they signal closely with mGluR5[11] and mGluR2/3,[12] respectively, and novel mGluR2/3- and 5- based drugs are proposed to exert their therapeutic effects, at least partially, through their influence on NMDAR and AMPAR.[11,12] In addition, PSD-95 is a key signalling partner of mGluR5 as it physically and functionally links it to NMDAR in the postsynapse, while PKC and BDNF are downstream signalling effectors of mGluR5/NMDAR signalling.[11] We explored the association of mGluR2/3

and mGluR5 measured in the present study in relation to measures of these glutamatergic markers as available on the SNCID. We chose a total of 29 markers representing glutamatergic markers and signalling partners within the cingulate cortex.

As expected, we observed a number of differential associations between mGluR2/3, mGluR5 and these glutamatergic markers and signalling partners (see the Appendix, Table S2). Many of these associations were specific to 1 of the 3 psychiatric disorders examined, indicating that there may be differential associations in the pathophysiologies of these disorders. However, although correlation analyses are useful to identify associations between markers, they do not indicate causality and must be treated as exploratory. These associations may provide direction for future studies aiming to distinguish pathological differences between these psychiatric disorders.

Limitations

While our findings suggest possible age-related differences between the psychotic and mood components, further analysis in a larger postmortem cohort is required to support this notion. In addition, further studies are required to determine if mGluR2/3 or mGluR5 function or downstream signalling are altered in these pathologies. It should be noted that despite no pathological alteration of mGluR2/3 and mGluR5 binding sites in the present study, the ACC remains highly involved in these pathologies as alterations of various neurotransmitters and their associated receptors have been reported in this brain region.[3,7,40–42] Although our study provides valuable information on these binding sites in psychiatric pathology, additional studies examining binding specifically to membranous mGluR2/3 and mGluR5 and the affinity of novel mGluR2/3- and mGluR5-targeting drugs for these receptors in psychiatric disorders will provide valuable insight into the effectiveness of these drugs in the patient.[33]

One confound of postmortem studies in neuropsychiatric disorders, is the presence of comorbid substance abuse, especially alcohol and drug abuse, or at least substantially higher alcohol[43] and illicit drug[44] intake in patients with psychiatric disorders. While reliable quantitative patient histories were not available in the present study, mGluR1/5 densities in the prefrontal cortex may be affected by alcohol,[45] and mGluR2 gene expression was reported to be reduced in the ACC of patients with alcohol addiction.[46] Furthermore, there is a growing body of evidence suggesting that mGluRs may be involved in drug and alcohol addiction disorders.[47] Therefore, the molecular effects of alcohol and drug use should be considered when interpreting the present and prior/future studies.

Conclusion

To our knowledge, we have assessed for the first time mGluR2/3 and mGluR5 binding capacity in the ACC in individuals with MD with and without psychosis as well as those with BD and schizophrenia. While we provide evidence that age is differentially associated with mGluR2/3 binding in individuals with psychotic and mood disorders, we found no alterations in mGluR2/3 or mGluR5 binding compared with controls or between any studied pathological state. This suggests that mGluR2/3- and mGluR5-based therapeutics used to treat emotional and cognitive deficits may have unhindered binding capacity in these pathological states. Future studies may extend on this work by investigating mGluR protein/mRNA expression and their association with other mGluRs, specifically in the ACC and other brain limbic structures; this would increase our understanding of the role of mGluRs in the neuropathologies of psychiatric disease and elucidate their treatment potential.

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Competing interests: None declared.

Contributors: K.A. Newell designed the study. N. Matosin and K.A. Newell acquired the data, which all authors analyzed. N. Matosin and K.A. Newell wrote the article, which all authors reviewed and approved for publication.

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Chapter Three

3.1 Brief introduction

The content of Chapter Three includes Study 2: Is mGluR5 or mGluR5 regulation affected in the DLPFC of schizophrenia subjects, or by APD treatment?

3.1.1 Relevant aims

This study was designed to address Aims 2 and 4:

2. To determine whether mGluR5 and mGluR5 regulators are altered in postmortem samples from the DLPFC, which is a region associated with working memory, cognitive flexibility, planning, inhibition, and abstract reasoning, of subjects with schizophrenia.

4. To determine whether commonly used APD treatments for schizophrenia affect mGluR5 or mGluR5 endogenous regulators in the PFC of a pharmacological animal model.

3.1.2 Overview and general methods

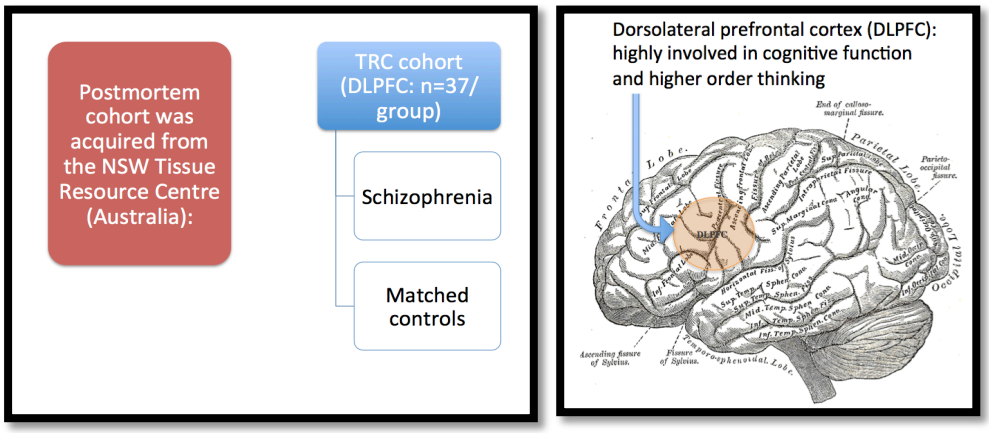
3.1.2.1 Study rationale

Although the levels of cortical mGluR5 protein and mRNA are largely reported to be unaltered in schizophrenia, the regulation of mGluR5 in schizophrenia, or response of mGluR5 to commonly used therapeutics, has never before been considered. Studies solely examining mGluR5 are not sufficient to conclude that mGluR5 is unaffected in schizophrenia. Therefore, this study was designed to assess whether mGluR5, as well as important novel mGluR5 endogenous regulators (Norbin, Preso1 and Tamalin) were altered in the postmortem schizophrenia brain or affected by current and commonly prescribed APDs.

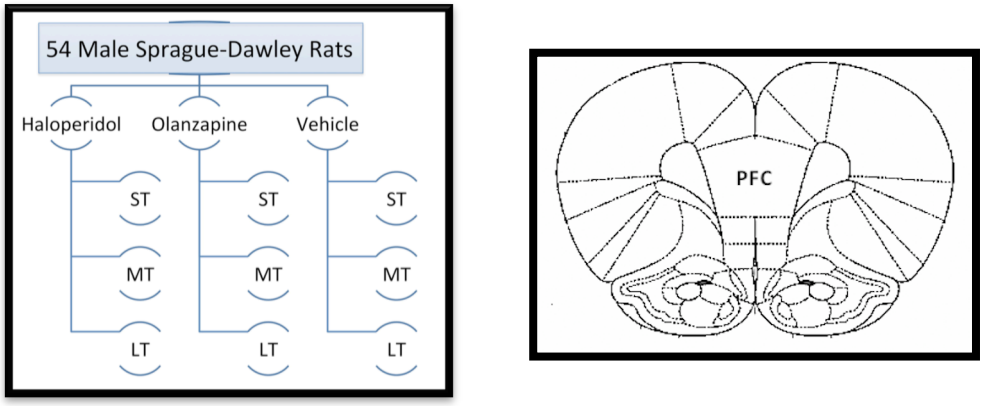
3.1.2.2 Methods overview

(a) SUBJECTS, STRUCTURES AND DESIGN:

Human:



Animal:



(b) IMMUNOBLOT PROTEIN DETECTION TECHNIQUE:

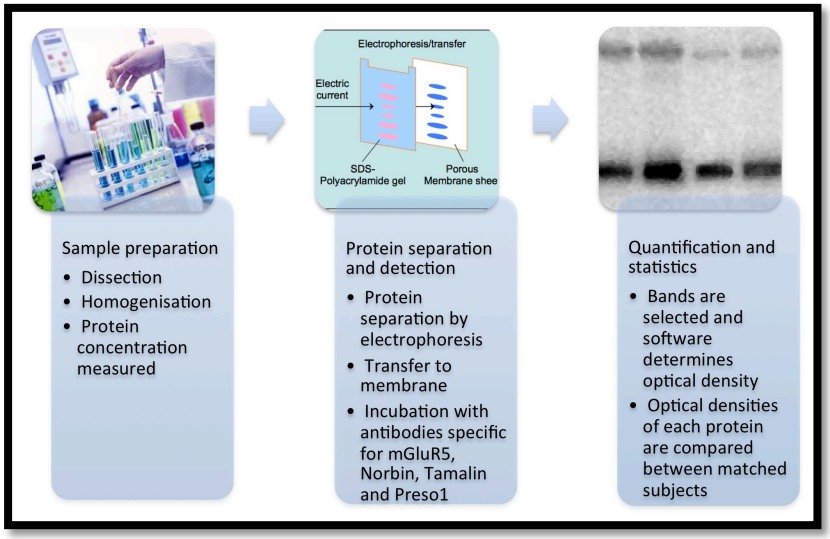


Figure 3.1 Methodological overview of Study 3. (a) A large cohort of 37 schizophrenia subjects compared to 37 matched controls were used for this study. The DLPFC was examined. Subsequently, 54 adult male rats were divided into six groups of 3 treatments (haloperidol, olanzapine and vehicle/control) and 3 treatment-durations (8, 15 and 35 days). The PFC area of the rat brain was analysed. (b) mGluR5, Norbin, Tamalin and Preso1 were analysed by immunoblot analysis; these tissues are derived from the same animals as previously published in Matosin et al., 2013 (*Schizophrenia Research*) **Abbreviations:** DLPFC, dorsolateral prefrontal cortex; ST, short term; MT, medium term; LT, long term; PFC, prefrontal cortex; TRC, tissue resource centre; SDS polyacrylamide, sodium dodecyl sulphate-polyacrylamide.

3.1.3 Results summary

Using a large postmortem cohort, the results from Study 2 showed that mGluR5 protein is increased and its regulatory proteins are decreased in the DLPFC region of schizophrenia patients. In addition, Study 4 showed that current commonly used APDs do not influence the levels of mGluR5 and its regulatory proteins. Due to the prominent role of cortical mGluR5 in the emergence of learning and memory deficits, dysregulation of mGluR5 in this region might contribute to the cognitive dysfunctions that are observed in schizophrenia patients. Further, as mGluR5 and mGluR5 modulators are not influenced by current APDs, this provides support for the use of mGluR5-targeting drugs for the treatment of the observed alterations. This study provides strong support of mGluR5 dysregulation in schizophrenia, at least in the DLPFC.

3.1.4 Author contributions

N. Matosin was a designer of this study, performed the experiments, acquired protein measures, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication. mGluR5 mRNA data was contributed by S. Fung and C. S. Weickert. Antipsychotic drug treated rat brain tissue was provided by X.F. Huang, which N. Matosin analysed.

3.1.5 Collaborator's statements

We hereby declare that the statement in section 3.1.4 pertaining to the contributions of N. Matosin are correct.

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ORIGINAL PAPER

Alterations of mGluR5 and its endogenous regulators Norbin, Tamalin and Preso1 in schizophrenia: towards a model of mGluR5 dysregulation

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Abstract Knockout of genes encoding metabotropic glutamate receptor 5 (mGluR5) or its endogenous regulators, such as Norbin, induce a schizophrenia-like phenotype in rodents, suggesting dysregulation of mGluR5 in schizophrenia. Human genetic and pharmacological animal studies support this hypothesis, but no studies have explored mGluR5 dysfunction at the molecular level in the postmortem schizophrenia brain. We assessed mGluR5 mRNA and protein levels in the dorsolateral prefrontal cortex (DLPFC) using a large cohort of schizophrenia and control subjects ($n = 37$/group), and additionally measured protein levels of recently discovered mGluR5 endogenous regulators, Norbin (neurochondrin), Tamalin (GRASP-1), and Preso1 (FRMPD4), which regulate mGluR5 localization, internalization and signaling. While mGluR5 mRNA expression was unchanged, mGluR5 protein levels were significantly higher in schizophrenia subjects compared to controls (total: +22 %; dimer: +54 %; $p < 0.001$). Conversely, mGluR5 regulatory proteins were expressed at lower levels in schizophrenia subjects compared to controls (Norbin −37 %, $p < 0.001$; Tamalin −30 %, $p = 0.084$; Preso1 −29 %, $p = 0.001$). mGluR5 protein was significantly associated with mGluR5 mRNA and mGluR5 endogenous regulators in control subjects, but these associations were lost in schizophrenia subjects. Lastly, there were no associations between protein measures and lifetime antipsychotic history in schizophrenia subjects. To confirm no antipsychotic influence, all proteins were measured in the prefrontal cortex of rats exposed to haloperidol or olanzapine; there were no effects of antipsychotic drug treatment on mGluR5, Norbin, Tamalin or Preso1. The results from our study provide compelling evidence that mGluR5 regulation is altered in schizophrenia, likely contributing to the altered glutamatergic signaling that is associated with the disorder.

Chapter Four

4.1 Brief introduction

The content of Chapter Four includes Study 3: Is mGluR5 or mGluR5 regulation affected in the CA1 region of schizophrenia subjects, or by APD drug treatment?

4.1.1 Relevant aims

These studies were designed to address Aims 3 and 4:

3. To determine whether alterations in mGluR5 and mGluR5 regulators extend to the hippocampal CA1 region (highly implicated in cognitive processes, including several types of learning and memory formation and storage) of subjects with schizophrenia.

4. To determine whether commonly used APD treatments for schizophrenia affect mGluR5 or mGluR5 endogenous regulators in the PFC of a pharmacological animal model.

4.1.2 Overview and general methods

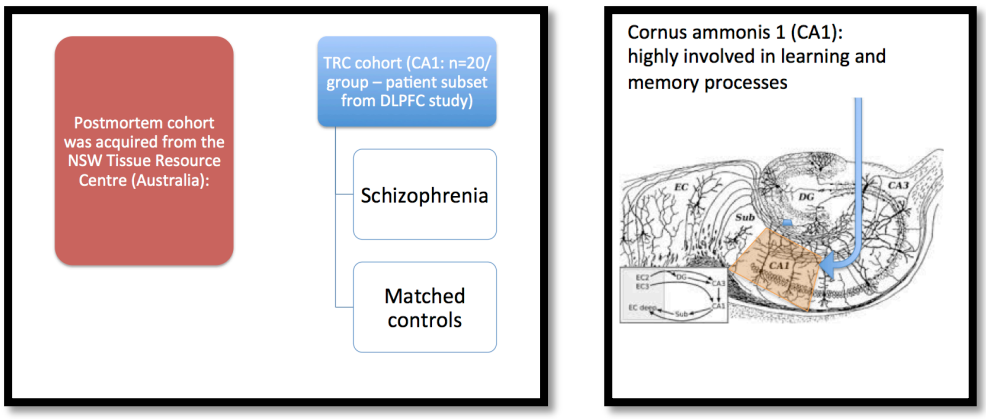
4.1.2.1 Study rationale

Extensive evidence indicates that mGluR5 activity in CA1 is critically involved in LTP/LTD, the mechanisms underlying proper synaptic plasticity and thus cognitive function. In accordance, mGluR5 signalling has been reported to modulate working memory, instrumental learning, spatial memory and proper sensorimotor gating in rodents, which are processes also disrupted in patients with schizophrenia. However, the status of mGluR5 in the hippocampus is largely unknown. This study therefore aimed to determine whether mGluR5 is altered in the hippocampal CA1 region in schizophrenia subjects.

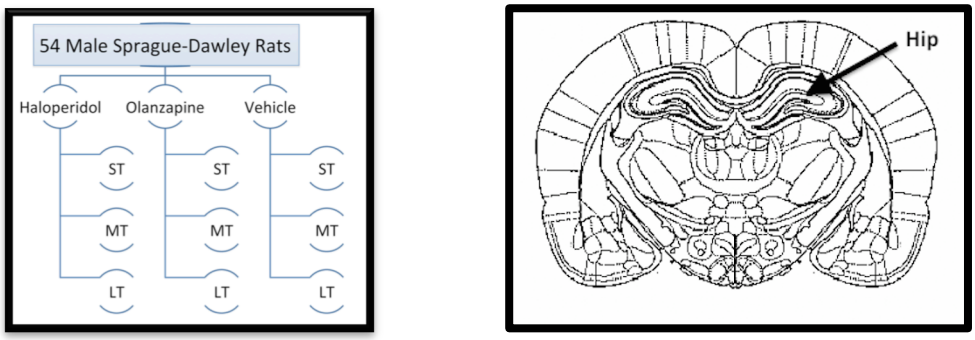
4.1.2.2 Methods overview

(a) SUBJECTS, STRUCTURES AND DESIGN:

Human:



Animal:



(b) IMMUNOBLOT PROTEIN DETECTION TECHNIQUE:

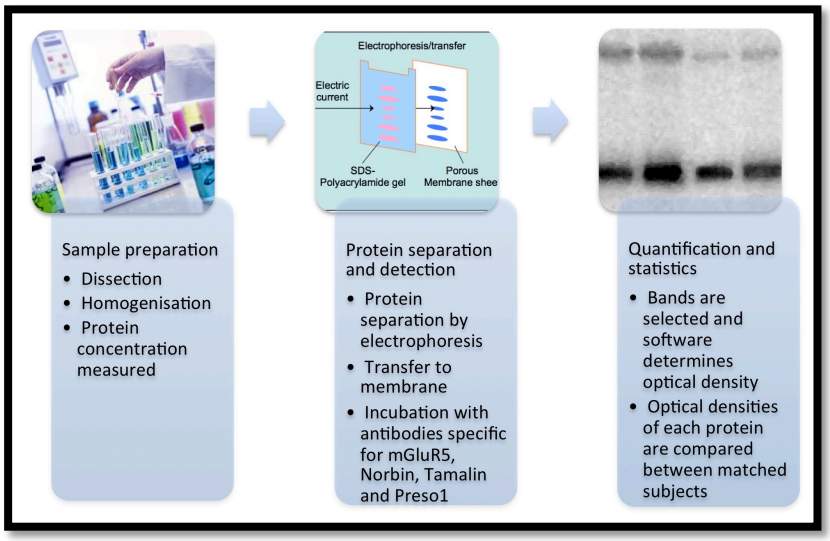


Figure 4.1 Methodological overview of Study 4. (a) 20 schizophrenia subjects were compared to 20 matched controls in this study. The examined hippocampal CA1 region was obtained from the level of the lateral geniculate nucleus, and visually verified by researchers at the NSW Tissue Resource Centre. Subsequently, 54 adult male rats were divided into six groups of 3 treatments (haloperidol, olanzapine and vehicle/control) and 3 treatment-durations (8, 15 and 35 days). The hippocampus area of the rat brain was analysed; these tissues are derived from the same study described in Chapter 3 and Matosin et al., 2013 (*Schizophrenia Research*). (b) mGluR5, Norbin, Tamalin and Preso1 were analysed by immunoblot analysis. **Abbreviations:** CA1, cornu ammonis 1; Hip, hippocampus; ST, short term; MT, medium term; LT, long term; TRC, Tissue Resource Centre; SDS polyacrylamide, sodium dodecyl sulphate-polyacrylamide.

4.1.3 Results Summary

For the first time, the study from Chapter 4 examined whether mGluR5 protein levels are affected in the CA1 hippocampal region in postmortem samples from schizophrenia subjects. Although a significant increase in mGluR5 was detected in CA1, the results from this study were surprising considering a decrease/abolition of mGluR5 induces cognitive-like deficits in rodent models. Thus, it is hypothesised that this increase in mGluR5 does not represent a functional increase, but rather a core deficit is present affecting mGluR5 activity in CA1, resulting in increased levels of mGluR5 and mGluR5 endogenous regulators to compensate for this signalling deficit. Further, considering mGluR5 protein levels are increased in both the CA1 and DLPFC regions, whilst mGluR5 endogenous regulators are increased in CA1 but deceased in DLPFC, this suggests mGluR5 may be regulated in a brain-region dependent manner.

4.1.4 Author contributions

N. Matosin was a designer of this study, performed the experiments, acquired protein measures, analysed all the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication. Antipsychotic drug treated rat brain tissue was provided by X.F. Huang, which N. Matosin analysed.

4.1.5 Collaborator's statements

We hereby declare that the statement in section 4.1.4 pertaining to the contributions of N. Matosin are correct.

Francesca Fernandez-Enright

Jeremy Lum

Jessica Andrews

Martin Engel

Xu-Feng Huang

Kelly Newell

A newer version with a subset of the data in this original transcript is now accepted at *Schizophrenia Research*, 2015: **Matosin N**, Fernandez-Enright F, Lum JS, Andrews JL, Engel M, Huang XF, Newell KA. Metabotropic glutamate recepto 5 and its trafficking molecules, Norbin and Tamalin, are increased in the CA1 hippocampal region of subjects with schizophrenia. (see Appendix)

Full-length paper

mGluR5 and mGluR5 endogenous regulators are increased in the CA1 hippocampal region of subjects with schizophrenia

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Abstract

Metabotropic glutamate receptor 5 (mGluR5) is involved in hippocampal-dependent learning and memory, which are processes disrupted in schizophrenia. Recent evidence from human genetic and animal studies suggests that the regulation of mGluR5, including its interaction with endogenous regulators, may be altered in the disorder. However there have been no investigations of hippocampal mGluR5 or mGluR5 regulatory molecules in the postmortem schizophrenia brain to confirm this notion. In the present study, we investigated whether protein expression of mGluR5 and its endogenous regulators Norbin, Tamalin and Preso1 in postmortem samples from the hippocampal CA1 region of schizophrenia subjects and matched controls (n=20/group). Protein levels of mGluR5 (total: 42%, $p<0.001$; monomer: 25%, $p=0.011$; dimer: 52%, $p< 0.001$) and mGluR5 endogenous regulators (Norbin: 47%, $p<0.001$; Tamalin: 34%, $p=0.009$; Preso1: 83%, $p<0.001$) were significantly higher in schizophrenia subjects compared to controls. To determine any influence of antipsychotic drug treatment, all proteins were also correlated with lifetime chlorpromazine equivalents in patients, and separately measured in the hippocampus of rats exposed to haloperidol or olanzapine treatment. mGluR5 negatively correlated with lifetime antipsychotic drug exposure in schizophrenia patients, suggesting antipsychotic drugs could reduce mGluR5 protein in schizophrenia subjects. In contrast, mGluR5 and mGluR5 endogenous regulators were not altered in the hippocampus of antipsychotic drug treated rats. This investigation provides strong support for the hypothesis that mGluR5 is involved in the pathology of schizophrenia, and that mGluR5 alterations might contribute to the hippocampal-dependent cognitive dysfunction associated with this disorder.

Keywords: mGluR5, Hippocampus, Norbin, Tamalin, Preso1, Schizophrenia

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APD, antipsychotic drug; DLPFC, Dorsolateral prefrontal cortex; mGluR5, metabotropic glutamate receptor 5; NMDAR, N-methyl-D-aspartate receptor; PAM, positive allosteric modulator; PFC, prefrontal cortex; PMI, postmortem interval.

1. Introduction

Metabotropic glutamate receptor subtype 5 (mGluR5) is a postsynaptic G-protein coupled receptor (GPCR), best known for its ability to modulate postsynaptic currents induced by the ionotropic N-methyl-D-aspartate glutamate receptor (NMDAR; Matosin and Newell, 2013). A substantial body of evidence indicates that mGluR5 is critically involved in hippocampal-dependent learning and memory. Notably, several studies have reported that hippocampal mGluR5 activity is important for long-term potentiation (LTP) and long-term depression (LTD), molecular mechanisms underpinning synaptic plasticity (see Mukherjee and Manahan-Vaughan, 2013). *mGluR5* knockout mice display reduced LTP, specifically in the hippocampal cornu ammonis 1 (CA1) region, as well as deficits in spatial learning and memory (Lu et al., 1997). Chronic mGluR5 antagonism in rats similarly induces deficits in LTP in the CA1 region, which correlates with reduced hippocampal mGluR5 protein levels and poorer working and reference memory (Manahan-Vaughan and Braunewell, 2005). Accordingly, mGluR5 positive allosteric modulators (PAMs) enhance the balance of hippocampal LTP and LTD at CA1 synapses, and consequently improve spatial learning in mice (Ayala et al., 2009).

Despite extensive examination in animal models, the status of hippocampal mGluR5 protein in patients with schizophrenia is largely unknown. Only one study has previously investigated mGluR5 mRNA expression in the hippocampus of schizophrenia subjects (in a small postmortem cohort of 5 schizophrenia subjects and 6 controls), reporting decreased mGluR5 expression in the parahippocampal gyrus, and no alterations in the dentate gyrus, CA1, CA3 or CA4 (Ohnuma et al., 2000). Based on studies indicating the importance of mGluR5 in synaptic plasticity, as well reports that suggest mGluR5 deficits lead to schizophrenia-like cognitive dysfunctions, it is important to extend and thoroughly investigate whether hippocampal mGluR5 is altered in schizophrenia, specifically at the protein level, as this might contribute to the manifestation of learning and memory deficits observed in patients with schizophrenia.

Accumulating evidence indicates the importance of mGluR5 protein-protein interactions in the regulation of mGluR5 trafficking, internalization and signalling (Enz, 2007). For instance, the neuron-specific protein Norbin (neurochondrin) plays a critical role in mGluR5 cell-surface targeting, and positively regulates mGluR5 signalling. Interestingly, *Norbin* knockout was found to reduce mGluR5-dependent LTP and abolished LTD in CA1 synapses (Wang et al., 2009). In cultured mouse hippocampal CA1/CA3 neurons, the multiscaffold protein Tamalin proved critical to mGluR5 neuritic localization processes (Kitano et al., 2002) and deletion of the Tamalin binding site on mGluR5 induced mGluR5 internalisation (Timms et al., 2013). Lastly, phosphorylation of mGluR5 by the novel scaffold protein Preso1 (also known as FRMPD4) was shown to down-regulate mGluR5

signalling in hippocampal neurons (Hu et al., 2012). However, the status of these endogenous mGluR5 regulators in the schizophrenia postmortem brain has not been assessed.

In the present study, we therefore determined whether mGluR5 protein levels are altered in postmortem samples from the CA1 hippocampal region of schizophrenia subjects compared to healthy controls (n=20/group). We further examined protein levels of the mGluR5 regulators Norbin, Tamalin and Preso1, which collectively play an important role in mGluR5 trafficking, internalisation and signalling (Hu et al., 2012; Kitano et al., 2003; Wang et al., 2009). The CA1 region was chosen for examination due to evidence that mGluR5-mediated LTP might be specific to CA1 synapses (Lu et al., 1997). Subsequently, we ascertained whether any observed changes in protein levels of mGluR5 or its endogenous regulators were influenced by antipsychotic drug (APD) treatment. For this purpose, we treated rats with first- and second-generation APDs (haloperidol and olanzapine respectively) and subsequently measured hippocampal protein levels of mGluR5, Norbin, Tamalin and Preso1.

2. Materials and methods

2.1 Human postmortem brain samples and tissue preparation

Postmortem human CA1 samples from 20 control (no history of psychiatric diagnosis) and 20 schizophrenia subjects, diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), were obtained from the New South Wales Brain Bank Network (Sydney, Australia). Subjects were matched according to tissue pH, postmortem interval, RNA integrity number (RIN) and age at death (Table 1). APD treatment premortem was standardised to a lifetime chlorpromazine equivalent for each patient, and antidepressant drug treatment history was specified on a qualitative scale (i.e. yes/no). The samples analysed in this study were derived from a cohort we previously used to study mGluR5 (DLPFC, n=37/group; Matosin et al., 2013). This study was approved by the Human Research Ethics Committees at the University of Wollongong (HE99/222) and the University of New South Wales (HREC 07261). Anatomical identification and preparation of the tissue has been previously described in detail (Fernandez-Enright et al., 2014).

2.2 Animal housing, treatment and tissue preparation

To assess the influence of haloperidol and olanzapine on mGluR5 and mGluR5 endogenous regulator expression, rats were treated with APD medication as previously described (Matosin et al., 2013). Briefly, adult (10 weeks of age) male Sprague-Dawley rats were purchased from the Animal Resource Centre (Perth, Australia). Rats were separated into groups according to the 3 treatments and 3

treatment-durations (9 groups; n=6/group). Animals were fed prepared food pellets (sweet cookie dough containing 62% carbohydrate, 22% protein, 10% vitamins, 6% fibre and minerals) 3 times/day at 8 hour intervals, containing either typical antipsychotic, haloperidol (0.1mg/kg body weight; daily total 0.3mg/kg), or atypical antipsychotic, olanzapine (1mg/kg body weight; daily total 3mg/kg), or vehicle control (no drug), for short term (8 days), medium term (15 days) and long term (35 days) durations. Food and water was available ad libitum, and consumption of food pellets was visually validated. APD doses were chosen to model a clinical setting (Han et al., 2008; Weston-Green et al., 2012).

48 hours after final treatment, rats were euthanized using carbon dioxide asphyxiation. The hippocampus (Bregma −2.30 to −5.20; total of dorsal and ventral regions) was dissected on ice, snap frozen in liquid nitrogen and then stored at -80°C until use. Tissue was homogenized in NP-40 lysis buffer (Invitrogen, Australia), containing ß-glycerophosphate and pheylmethanesulfonylfluoride and protease inhibitor cocktail (Sigma). Protein concentration was determined by DC assay (Bio-Rad), according to the manufacturer's instructions. All animal experiments in this study were approved by the University of Wollongong Animal Ethics Committee (AE10/18) and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3 Immunoblot analyses

Immunoblotting was performed as described previously (Matosin et al., 2013), with minor modifications optimized for the measurement of each protein of interest. 5μg (human) or 10μg (animal) of total protein was loaded per lane. Relative protein densities were determined by immunoblot analyses using polyclonal antibodies as previously reported: mGluR5 [ABCAM ab27190; human 1:250, rat 1:500 (Matosin et al., 2013)], Norbin [ABCAM ab130507; human and rat 1:500), Tamalin [ABCAM ab30576; human 1:100, rat 1:1000 (Tai et al., 2010)], Preso1 [Santa Cruz sc-242862; human 1:100, rat 1:1000 (Piton et al., 2010)]. mGluR5 monomer was detected at 135kDa whilst the mGluR5 dimer was detected at 270kDa. Individual bands were totalled before normalization to respective ß-actin and pooled samples to gain a measure of total mGluR5. Norbin, Tamalin, and Preso1 proteins were identified at the expected molecular weights (Figure 1a). Samples were visualised using an enhanced chemiluminescent detection kit (Bio-Rad). Band density was detected by the Gel Doc 2200 Pro (Carestream Molecular Imaging, USA) and quantified with Carestream MI software (v 5.0.4.44, Carestream Molecular Imaging). All bands were within the limits of saturation. Protein measures were subsequently normalized to their respective ß-actin density. Experiments and quantification were performed blind to diagnosis (human) or treatment group (rat).

2.4 Statistical Analyses

Statistical analyses were performed with SPSS software (v19.0). The level of significance was set to $p<0.05$ and data are presented as mean±SEM.

2.4.1 Human brain analyses

Distributions of mGluR5 total measures (but not individual monomer or dimer measures) as well as Norbin, Tamalin and Preso1 were skewed to the right (Kolmogorov-Smirnov; d=0.152-0.246, $p<0.001$). Normalized distribution for these proteins was therefore achieved by transforming to the natural logarithm of the relative protein values. Outliers were screened as mean ±2 standard deviations and removed. Analyses of Variance (ANOVA) were used to detect differences in protein expression between diagnostic groups (schizophrenia/control) as well as gender (male/female), hemisphere (left/right) and antidepressant history (yes/no) for each target. Spearman's correlations were used to determine whether sample characteristics (Table 2) were associated with protein measures, and to examine if additional measures of disease characteristics were correlated specifically with the schizophrenia group (lifetime APD history, age of disease onset and duration of illness). Analyses of covariance (ANCOVA) for diagnostic effects on protein expression were subsequently performed, accounting for sample characteristics that were associated with protein measures. Further Spearman's correlations were performed to determine the strength of associations between mGluR5 measures and its endogenous regulators in schizophrenia compared to control subjects.

2.4.2 Animal brain analyses

Values for mGluR5 (total) and Norbin were skewed to the right (Kolmogorov-Smirnov; d=0.103-0.169, $p<0.001$), and thus were normalized by converting to the natural logarithm. Protein differences between treatment (haloperidol/olanzapine/vehicle) and duration (short term/medium term/long term) were analysed by two-way ANOVA.

3. Results

3.1 Protein levels of mGluR5 in schizophrenia subjects compared to controls

We detected a robust increase in mGluR5 protein levels in the CA1 region of schizophrenia subjects compared to controls (total: +42%, $F_{1,37}$=138.579, p<0.001; monomer: +25%, $F_{1,37}$=7.194, p=0.011; dimer: +52%, $F_{1,37}$= 51.705, p<0.001; Figure 1a-b). Demographic and clinical measures that correlated significantly with mGluR5 protein levels (Table 2a) were assessed for their influence on the data. After co-varying for freezer storage time and brain pH, which significantly correlated with mGluR5 (total and dimer), significant differences between schizophrenia and control were maintained (mGluR5 total: $F_{1,35}$=172.974, p<0.001; mGluR5 dimer: $F_{1,35}$=59.433, p<0.001). An effect of gender was observed, whereby mGluR5 total protein was decreased in male subjects independent of diagnosis ($F_{1,37}$=5.357, p=0.026; -35.5%); however, there was no gender-specific difference in mGluR5 protein levels within the schizophrenia or control groups, and no hemispheric differences in mGluR5 protein expression.

3.2 Protein levels of mGluR5 endogenous regulators in schizophrenia subjects compared to controls

Protein levels of mGluR5 endogenous regulators were also significantly increased in schizophrenia (Figure 1c). Specifically, Norbin was increased by 46.7% ($F_{1,37}$=19.600, p<0.001), Tamalin by 34.0% ($F_{1,37}$=7.630, p=0.009) and Preso1 by 83.3% ($F_{1,37}$=199.983, p<0.001). There were no effects of gender or hemisphere on Norbin, Tamalin or Preso1. Considering the nature of our results whereby all measured proteins of interest were increased, we further analysed protein levels of α-tubulin as a negative control. Protein levels of α-tubulin (05-829, Millipore) were not significantly different between schizophrenia and control subjects ($F_{1,37}$=1.991, p=0.167; Supplementary Figure 1), validating our findings of increased mGluR5 and mGluR5 endogenous regulators.

3.3 Effects of clinical and demographic variables, and premortem medication estimates on protein measures

Spearman's correlations were performed to determine any relationships between demographic/clinical variables and protein levels of mGluR5 (total, monomer and dimer; Table 2, A), Norbin, Tamalin and Preso1 (Table 2, B). There was a significant correlation between mGluR5 total protein and brain pH in all subjects, but this was not maintained in the individual diagnostic groups. There was an effect of freezer storage time on mGluR5 total and dimer levels. A significant negative correlation was observed between lifetime antipsychotic drug dose and mGluR5 monomer levels in schizophrenia

subjects; this association was also trending for mGluR5 dimer levels. For mGluR5 regulatory proteins, age of disease onset was positively associated with Preso1, but there were no effects of illness duration on the protein levels. APD measures did not correlate with Norbin, Tamalin or Preso1 protein levels. Lastly, there were no effects of antidepressant medication (yes/no; $F_{17\text{-}18} \geq 0.015$, $p \geq 0.189$) on mGluR5, Norbin, Tamalin or Preso1 protein levels.

3.4 Relationships of mGluR5 protein (total, monomer and dimer) with Norbin, Tamalin and Preso1 in schizophrenia subjects compared to controls

Spearman's correlations were performed to determine the presence or loss of associations between mGluR5 and its endogenous regulators in control and schizophrenia subjects (Table 3, A and B). Although mGluR5 monomer and dimer levels were strongly and positively correlated in both control and schizophrenia subjects ($r \geq 0.790$, $p \leq 0.001$), there were no correlations between measures of mGluR5 (total, monomer or dimer) and its endogenous regulators in control or schizophrenia groups.

3.5 Protein levels of mGluR5 and its endogenous regulators in the hippocampus of APD treated rats

To assess the effects of current antipsychotic medications on the mGluR5 system, two-way ANOVA were used to compare the effects of haloperidol and olanzapine treatment (8, 16 or 36 days) on protein levels of mGluR5 (total, monomer or dimer), Norbin, Tamalin and Preso1 proteins in the hippocampus. There were no significant effects of olanzapine or haloperidol treatment overall or following the specific treatment periods ($F \geq 0.229$, $p \geq 0.296$) (Figure 2).

4. Discussion

In the present study, we provide the first evidence that protein expression of mGluR5 is significantly higher (total: 42%; monomer: 25%; dimer: 52%) in the hippocampal CA1 region of schizophrenia subjects relative to healthy controls. Schizophrenia subjects also show a marked increase in Norbin, Tamalin and Preso1 proteins (34-83%), which are endogenous regulators of mGluR5 signalling, trafficking and internalisation. We found no influence of APD treatment on mGluR5 regulatory proteins in human subjects, however there was a negative association between lifetime APD dose and mGluR5 protein expression levels in schizophrenia subjects. There were no alterations in mGluR5 or its regulatory proteins in the hippocampus in response to APD treatment in our animal model.

mGluR5 activation in CA1 is critically involved in cognitive functions via mGluR5-mediated modulation of synaptic plasticity (Mukherjee and Manahan-Vaughan, 2013). mGluR5 knockout mice display deficits in CA1-dependent LTP, coupled with deficits in spatial learning and memory (Lu et al., 1997). mGluR5 PAMs, which upregulate mGluR5 activity, have been reported to enhance hippocampal-dependent LTP and LTD in the CA1 region, congruent with improved performance in the Morris water maze, a measure of hippocampal-dependent spatial learning (Ayala et al., 2009). mGluR5-dependent differences in spatial learning in rats correlate with measures of synaptic plasticity in CA1 and with mGluR5 protein levels (Manahan-Vaughan and Braunewell, 2005). As cognitive dysfunction is a common symptom among schizophrenia patients (Harvey, 2013), the observed increase in mGluR5 in the present study likely represents a compensatory upregulation for mGluR5 dysfunction in this region or in response to wider deficits in glutamatergic signalling. As reviewed by Rubio and colleagues, reductions in the glutamatergic receptors, NMDA (Vrajová et al., 2010), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; Eastwood et al., 1997, 1995) and kainate (Benes et al., 2001), have been reported in the hippocampus. Although one study also reported no changes in these receptors (Breese et al., 1995), it is notable that there has been no increase detected.

In support of a core deficit in mGluR5, Timms and colleagues recently reported the presence of a missense mutation in the *mGluR5* gene in a schizophrenia pedigree, transmitting to the extracellular domain of the mGluR5 protein and likely altering mGluR5 sensitivity to glutamate (Timms et al., 2013). Additionally, the authors reported a frameshift mutation (P1148fs) that caused deletion of the Tamalin-binding site on the carboxyl tail of mGluR5, disrupting Tamalin/mGluR5 interactions and increasing mGluR5 internalisation in primary hippocampal cultures (Timms et al., 2013). We have also recently discovered a significant association of a single nucleotide polymorphism located in the regulatory 3' untranslated region (highly involved in regulation of protein-protein interactions) within

the *mGluR5* gene and schizophrenia in a Caucasian case-control population (unpublished results). Considering this evidence, Tamalin (but also Norbin and Preso1) proteins might be upregulated in schizophrenia to compensate for reduced protein-protein interactions. Norbin, Tamalin and Preso1 are also critical regulators of mGluR5 signalling; whilst Preso1 limits the amplitude and duration of mGluR5 activation (Hu et al., 2012), Norbin positively regulates mGluR5 signalling (Wang et al., 2009). Therefore our finding of increased levels of these proteins further support that mGluR5 signalling might be altered in the CA1 region of schizophrenia subjects, and these endogenous regulators are upregulated to increase mGluR5 signalling.

Although it is accepted that mGluR5 is expressed on the postsynaptic neuronal membrane, increasing evidence supports that the majority (60-90%) of mGluR5 is expressed at intracellular locations, including the endoplasmic reticulum membrane and nuclear membrane (Jong et al., 2014). Depending on its cellular location, mGluR5 reportedly activates different signalling cascades leading to unique cellular responses (Jong et al, 2009). In accordance, intracellular and extracellular mGluR5 differentially mediate synaptic plasticity in the CA1: cell surface receptors contribute to both LTD and LTP, whereas activation of intracellular mGluR5 are involved in LTD only (Purgert et al., 2014). Considering our finding of alterations to proteins that regulate mGluR5 movement to/from neuronal membranes, it will be important to determine whether the increase in mGluR5 observed in this study represents increases in intracellular or cell-surface expression of mGluR5, or both.

Premortem antipsychotic drug exposure can influence molecular measures in postmortem tissue analyses. In the present study, estimated lifetime APD exposure was negatively correlated with mGluR5 monomer levels and a correlation approaching significance was observed with mGluR5 dimers. This finding suggests an influence of APD on mGluR5 protein levels in the CA1 whereby APDs might act to reduce mGluR5 protein levels in the CA1. However considering we conversely detected increased mGluR5 protein levels in this region, and we found no influence of APD treatment on mGluR5 in the hippocampus of antipsychotic drug treated rats, it is unlikely that APD exposure is confounding the results of our study. Furthermore, there was no association between Norbin, Tamalin and Preso1 and APD in the present study, and we have previously reported that mGluR5 binding was not associated with APD exposure in the hippocampus (Matosin et al., 2013).

mGluR5 is a promising therapeutic target for the treatment of cognitive deficits in schizophrenia (Matosin and Newell, 2013). The present study reports that mGluR5 and mGluR5 endogenous regulators are altered in the CA1 region in subjects with schizophrenia, suggesting that mGluR5 signalling is altered. As mGluR5 has been shown to play an important role in hippocampal-dependent cognitive dysfunctions seen in schizophrenia patients, future studies should aim to investigate the

correlation between hippocampal mGluR5 expression and cognition in humans. Additional studies are also required to elucidate the nature of possible functional changes in hippocampal mGluR5 in schizophrenia subjects, to determine how to best target mGluR5 in a manner that will be therapeutic in the context of pathological alterations. For example, altered cell surface expression might limit available targets for drugs aimed at mGluR5, whilst insensitivity to glutamate may (positively or negatively) affect positive allosteric modulators that rely on simultaneous glutamate activation. It is important for future studies to elucidate the exact underlying mechanisms of these alterations and their implications for novel mGluR5-targeting therapeutics.

To conclude, we report in the present study that hippocampal CA1 protein levels of mGluR5 and mGluR5 endogenous regulators are increased in the pathology of schizophrenia. We hypothesise this may be related to a functional deficit within mGluR5, whereby glutamate sensitivity, protein-protein interactions or mGluR5 localisation are affected. Due to the prominent role of hippocampal mGluR5 in the emergence of learning and memory deficits, we further suggest that alterations to mGluR5 in CA1 may contribute to an imbalance of LTD:LTP, thus contributing to cognitive dysfunctions that are observed in schizophrenia patients. Nonetheless, these findings strongly support a role for mGluR5 in schizophrenia, and consequently its potential as a novel therapeutic target.

Role of funding source

The funding sources had no role in this study, including study design, data collection and publication decisions.

Contributors

All authors contributed to the study design and have contributed to and approved the final manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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Figures and Tables

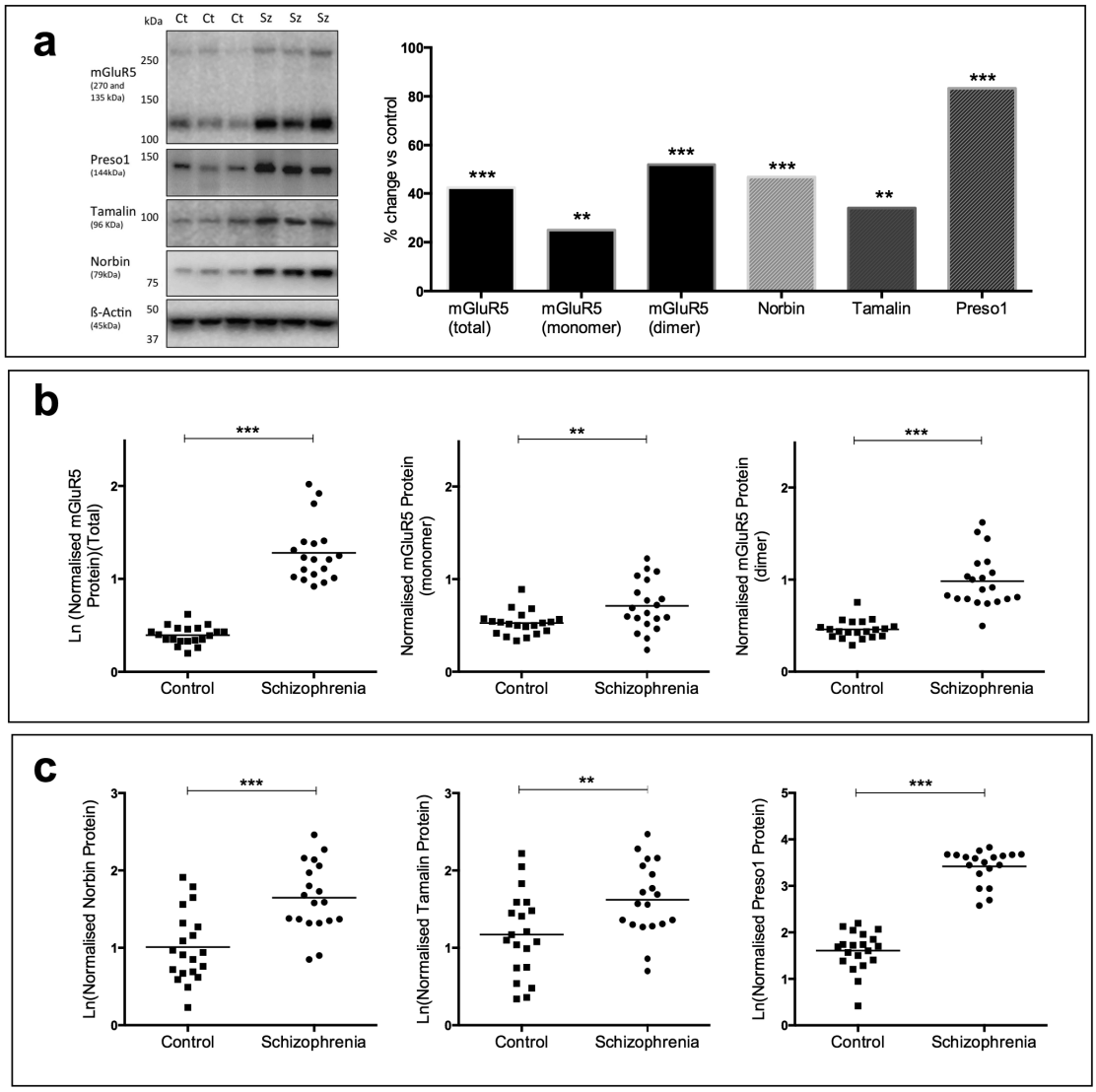


Figure 1. Protein levels of mGluR5 and mGluR5 signalling partners Norbin, Tamalin and Preso1 in the CA1 region of subjects with schizophrenia and matched controls. (**a**) Representative immunoblots and graphical depiction of percentage differences between protein measures in patients with schizophrenia compared to control subjects. (**b**) Normalised protein levels of mGluR5 (total, monomer and dimer). (**c**) Normalised protein levels of mGluR5 endogenous regulators, Norbin, Tamalin and Preso1. mGluR5, Norbin, Tamalin and Preso1 were significantly increased in schizophrenia subjects compared to controls. **Abbreviations:** Ct: control; DLPFC: dorsolateral prefrontal cortex; Ln: natural logarithm; kDa: kilodaltons; mGluR5: metabotropic glutamate receptor 5; Sz: schizophrenia; Tot: total. ***P<0.05, **P<0.01, ***P<0.001.**

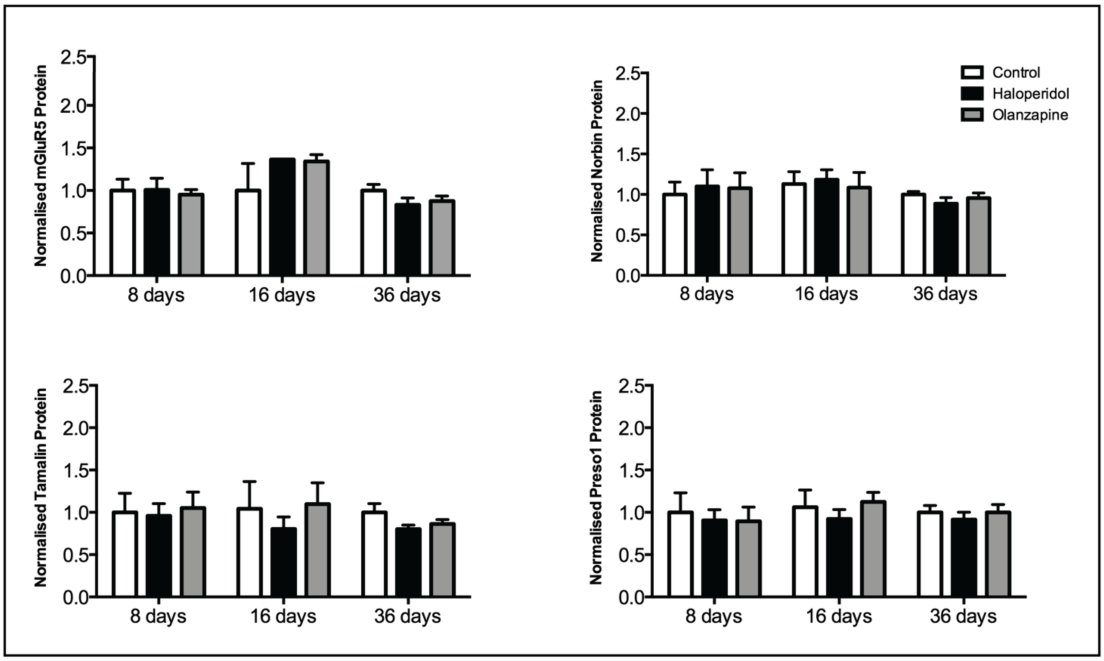
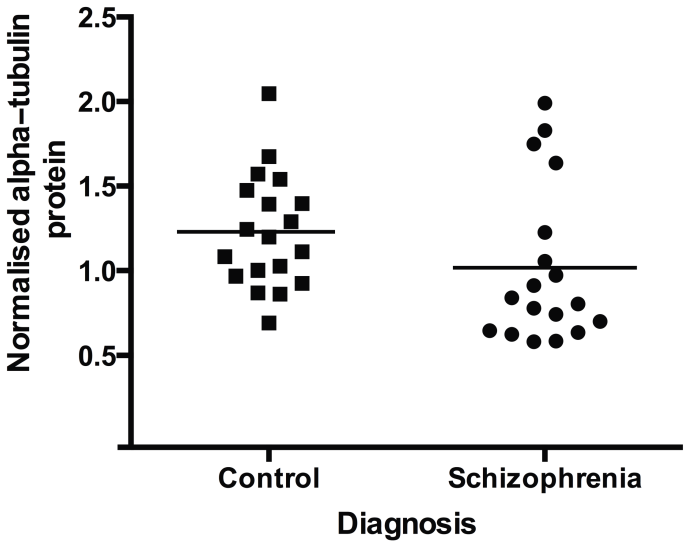


Figure 2. Effects of current antipsychotic drug treatment on mGluR5, Norbin, Tamalin and Preso1. Protein levels in the hippocampus of rats treated for 8, 16 or 36 days with haloperidol (0.3mg/kg/day) or olanzapine (3mg/kg/day). There were no significant effects of antipsychotic drug treatment on levels of mGluR5 or its endogenous regulators.



Supplementary Figure 1. Normalised protein levels of α-tubulin protein were measured as a positive control. Protein levels of α-tubulin were not significantly different in schizophrenia subjects compared to control subjects.

Table 1. Demographic and clinical characteristics of the postmortem cohort.

	CA1 (n=20/group)	
	Control	Schizophrenia
Brain pH	6.6 ± 0.03	6.6 ± 0.3
Postmortem interval (hours)	26.1 ± 12.8	28.8 ± 14.1
RNA integrity number	7.2 ± 0.7	7.2 ± 0.5
Age at Death (years)	58.2 ± 12.6	55.5 ± 13.5
Gender	2 F, 18 M	9 F, 11 M
Hemisphere	13 R, 7L	10R, 10L
Age of disease onset (years)	-	23.5 ± 6.8
Duration of illness (years)	-	32.05 ± 13.7
Lifetime antipsychotic drug medication (Standardised chlorpromazine equivalent, mg)	-	668 ± 421
Antidepressant history (yes/no)	-	12

Abbreviations: DLPFC: dorsolateral prefrontal cortex; F: female; L: left; M: male; R: right. Data are expressed as mean ± standard deviation

Table 2. Spearman's Correlations for continuous clinical and demographic variables and protein levels of (A) mGluR5 total, monomer and dimer, and (B) mGluR5 endogenous regulators, Norbin, Tamalin and Preso1, in the hippocampal CA1 region. Significant values ($p<0.05$) are highlighted in bold.

A. mGluR5 (total, monomer and dimer).

Variable	All subjects			Controls			Schizophrenia		
	mGluR5 (total)	**mGluR5 (monomer)**	**mGluR5 (dimer)**	**mGluR5 (total)**	**mGluR5 (monomer)**	**mGluR5 (dimer)**	**mGluR5 (total)**	**mGluR5 (monomer)**	**mGluR5 (dimer)**
Brain pH	**r = -0.355** **p = 0.026**	r = -0.001 p = 0.993	r =-0.088 p =0.593	r = -0.304 p = 0.192	r = -0.040 p = 0.867	r = -0.134 p = 0.573	r = -0.448 p = 0.055	r = -0.084 p = 0.732	r =-0.116 p = 0.497
Age at Death	r = -0.028 p = 0.868	r = -0.154 p = 0.349	r =-0.164 p = 0.320	r = -0.188 p = 0.427	r = -0.087 p = 0.716	r = 0.136 p = 0.569	r = 0.287 p = 0.233	r = -0.184 p =0.452	r = -0.211 p = 0.386
Postmortem interval	r = -0.167 p = 0.309	r = 0.100 p = 0.546	r = 0.170 p = 0.302	r = -0.199 p = 0.400	r = -0.199 p = 0.401	r = -0.118 p = 0.620	r = -0.328 p = 0.170	r = 0.250 p = 0.302	r = 0.359 p = 0.131
Freezer storage time	**r = 0.356** **p = 0.026**	r =0.243 p =0.136	**r = 0.347** **p =0.030**	r = 0.274 p = 0.243	r = 0.335 p = 0.149	r = 0.332 p = 0.152	**r = 0.531** **p = 0.019**	r = 0.207 p =0.395	r = 0.354 p = 0.137
Brain weight	r = -0.144 p = 0.383	r = -0.039 p = 0.815	r = -0.232 p = 0.156	r = -0.267 p = 0.255	r = -0.083 p = 0.729	r = -0.170 p =0.473	r = 0.030 p = 0.903	r = -0.069 p = 0.780	r = -0.194 p = 0.425
Age of disease onset	- -	- -	- -	- -	- -	- -	r = 0.259 p = 0.285	r = 0.241 p = 0.321	r = 0.156 p = 0.523
Duration of illness	- -	- -	- -	- -	- -	- -	r = 0.199 p = 0.415	r = -0.234 p = 0.336	r = -0.232 p = 0.339
Standardised chlorpromazine equivalent (mg; lifetime APD)	- -	- -	- -	- -	- -	- -	r = -0.160 p = 0.514	**r = -0.502** **p = 0.029**	r = -0.426 p = 0.069

B. mGluR5 endogenous regulators Norbin, Tamalin and Preso1.

Variable	All subjects			Controls			Schizophrenia		
	Norbin	**Tamalin**	**Preso1**	**Norbin**	**Tamalin**	**Preso1**	**Norbin**	**Tamalin**	**Preso1**
Brain pH	r = 0.015 p = 0.929	r = 0.026 p = 0.874	r = 0.020 p = 0.960	r = 0.142 p = 0.551	r = 0.127 p = 0.593	r = 0.084 p = 0.726	r = -0.170 p = 0.486	r = -0.147 p = 0.549	r = 0.197 p = 0.419
Age at Death	r = -0.096 p = 0.561	r = -0.096 p = 0.560	r = -0.148 p = 0.368	r = -0.068 p = 0.777	r = -0.061 p = 0.798	r = -0.324 p = 0.163	r = -0.199 p = 0.414	r = -0.187 p = 0.444	r = -0.211 p = 0.387
Postmortem interval	r = -0.051 p = 0.758	r = -0.085 p = 0.606	r = -0.097 p = 0.555	r = 0.113 p = 0.635	r = 0.049 p = 0.838	r = 0.212 p = 0.610	r = -0.158 p = 0.517	r = -0.170 p = 0.487	r = -0.391 p = 0.098
Freezer storage time	r = 0.173 p = 0.292	r = 0.159 p = 0.334	r = 0.180 p = 0.273	r = 0.219 p = 0.354	r = 0.162 p = 0.496	r = 0.035 p = 0.885	r = 0.220 p = 0.365	r = 0.210 p = 0.389	r = 0.155 p = 0.528
Brain weight	r = -0.258 p = 0.113	r = -0.176 p = 0.283	r = -0.153 p = 0.352	r = -0.123 p = 0.606	r = -0.042 p = 0.860	r = -0.373 p = 0.106	r = -0.145 p = 0.553	r = -0.142 p = 0.563	r = 0.397 p = 0.093
Age of disease onset	- -	- -	- -	- -	- -	- -	r = 0.051 p = 0.837	r = 0.078 p = 0.750	**r = 0.498** **p = 0.030**
Duration of illness	- -	- -	- -	- -	- -	- -	r = -0.231 p = 0.341	r = -0.224 p = 0.357	r = -0.035 p = 0.886
Standardised chlorpromazine equivalent (mg; lifetime APD)	- -	- -	- -	- -	- -	- -	r = -0.251 p = 0.300	r = -0.256 p = 0.290	r = -0.247 p = 0.307

Table 3. Spearman's correlations for associations between protein levels of mGluR5 (total, monomer and dimer) and novel mGluR5 signalling partners Norbin, Tamalin and Preso1, within (A) control and (B) schizophrenia subjects. Significant values ($p<0.05$) are highlighted in bold.

A. Control

	mGluR5 total	**mGluR5 monomer**	**mGluR5 dimer**
mGluR5 (total)	-	r = 0.066	r = 0.102
	-	p = 0.782	p = 0.668
mGluR5 (monomer)	r = 0.066	-	**r = 0.853**
	p = 0.782	-	**p < 0.001**
mGluR5 (dimer)	r = 0.102	**r = 0.853**	-
	p = 0.668	**p < 0.001**	-
Norbin	r = -0.036	r = 0.274	r = 0.266
	p = 0.980	p = 0.243	p = 0.257
Tamalin	r = 0.017	r = 0.334	r = 0.292
	p = 0.945	p = 0.150	p = 0.212
Preso1	r = 0.161	r = 0.365	r = 0.275
	p = 0.498	p = 0.113	p = 0.240

B. Schizophrenia

	mGluR5 total	**mGluR5 monomer**	**mGluR5 dimer**
mGluR5(total)	-	r = 0.140	r = 0.137
	-	p = 0.557	p = 0.566
mGluR5(monomer)	r = 0.140	-	**r = 0.790**
	p = 0.557	-	**p < 0.001**
mGluR5 (dimer)	r = 0.137	**r = 0.790**	-
	p = 0.566	**p < 0.001**	-
Norbin	r = 0.037	r = 0.001	r = 0.064
	p = 0.876	p = 0.996	p = 0.788
Tamalin	r = 0.027	r = 0.038	r = 0.029
	p = 0.910	p = 0.875	p = 0.902
Preso1	r = 0.217	r = 0.367	r = 0.095
	p = 0.357	p = 0.112	p = 0.690

Chapter Five

5.1 Brief introduction

The content of Chapter Five includes Study 4: Are SNPs within the regulatory 3' untranslated region of *GRM5* significantly associated with schizophrenia diagnosis, and associated with or predictive of cognitive function in these patients?

5.1.1 Relevant aims

This study was designed to address Aim 5:

5. To determine whether novel SNPs within *GRM5* are associated with schizophrenia, and whether genetic variation within *GRM5* could contribute to cognitive dysfunction in schizophrenia subjects using a case-control cohort for this disorder.

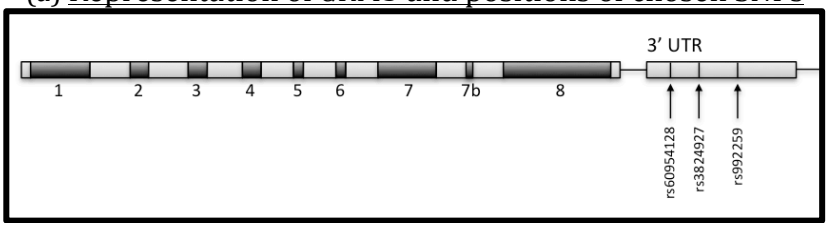
5.1.2 Overview and general methods

5.1.2.1 Study rationale

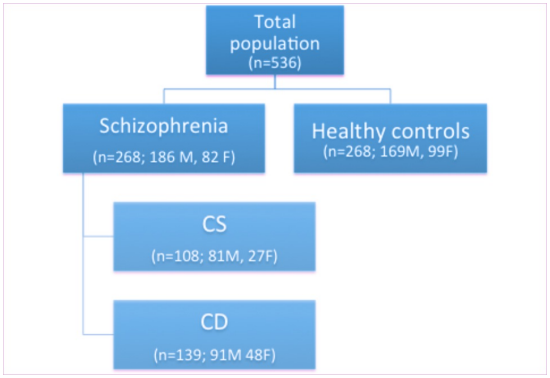
Despite that *GRM5* was recently identified as one of the top candidate genes for schizophrenia aetiology (Ayalew et al., 2012), and growing evidence from animal models for a role of *GRM5* in cognition, no studies have investigated the potential association between SNPs within *GRM5* and cognitive features of schizophrenia. As our recent postmortem studies suggested dysregulation of mGluR5, we aimed to investigate whether dysregulation might originate with genetic variation within *GRM5.* This study thus attempted to investigate possible associations between three SNPs located within the regulatory 3' untranslated region (a non-coding region highly involved in protein conformation and regulation of sites responsible for protein-protein interactions) of *GRM5,* with schizophrenia and cognitive features in a Caucasian case-control cohort.

5.2.2.2 Methods overview

(a) Representation of *GRM5* and positions of chosen SNPs



(b) Patient population:



(c) Cognitive measures:

Abbreviated Test name	Feature assessed
WTAR	IQ estimate
WASI	Intelligence scale
LNS	Working memory
COWAT	Executive functioning
RBANS scores for:	Delayed memory
	Attention
	Immediate memory
	Construction
	Language

(d) Statistical methods:

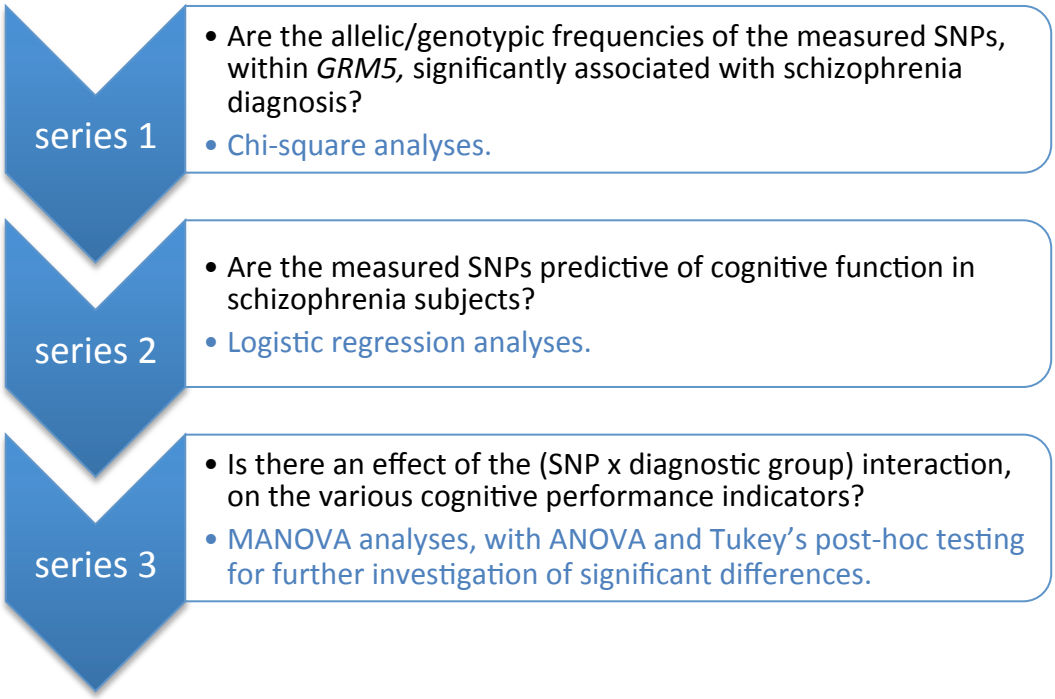


Figure 5.1 Methodological overview of Study 5. **Abbreviations:** 3' UTR, 3' untranslated region; ANOVA, analyses of variance; CD, schizophrenia patients characterised by severe cognitive deficits; COWAT, Controlled Oral Word Association Test; CS, schizophrenia subjects characterised as cognitively spared relative to CD; F, female; LNS, Letter Number Sequencing; M, male; MANOVA, multiple analyses of variance; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; SNP, single nucleotide polymorphism; WASI, Wechsler Abbreviated Scale for Intelligence; WTAR, Wechsler Test of Adult Reading; rs, reference single nucleotide polymorphism; SNP, single nucleotide polymorphism.

5.1.3 Result summary

In Chapter 5, it is reported that (i) rs60954128 minor allele (T) and homozygous genotype (TT) were significantly associated with schizophrenia in males; (ii) rs3824927 genotypes significantly predicted membership of a subtype of schizophrenia cases characterised by severe cognitive deficits in male cases; and, (iii) both rs60954128 and rs3824927 exerted differential effects on particular cognitive domains in men and women. These findings are in line with current evidence indicating a role for mGluR5 in schizophrenia and cognition. The findings from this study suggest there is a complex association between genetic variation within the *GRM5* gene and cognitive function in schizophrenia. However, the effects of *GRM5* polymorphisms on cognition may be sex-specific, differentially affecting cognitive performance in men and women with and without schizophrenia.

5.1.4 Author contributions

N. Matosin was a designer of this study, prepared samples for analyses, analysed the data, and wrote the first draft of the manuscript, which all authors reviewed and approved for publication. MassARRAY genotyping was performed by researchers at the Garvan Institute, Sydney. Cognitive data and relevant analyses were contributed by M. Green.

5.1.5 Collaborator's statements

We hereby declare that the statement in section 5.1.4 pertaining to the contributions of N. Matosin are correct.

Melissa Green

Kelly Newell

Jessica Andrews

Francesca Fernandez-Enright

The American Journal of Psychiatry

Sex-specific associations of novel gene variants in metabotropic glutamate receptor 5 with schizophrenia and cognitive dysfunction

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Sex-specific associations of novel gene variants in metabotropic glutamate receptor 5 with schizophrenia and cognitive dysfunction

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ABSTRACT

Objective: *GRM5,* a gene encoding for metabotropic glutamate receptor 5, is now recognized among the top candidate genes for schizophrenia, with glutamate signaling implicated in the cognitive deficits characterizing this disorder. Yet, there have been no investigations of association between single nucleotide polymorphisms (SNP) within *GRM5* and cognitive features of schizophrenia.

Method: We set out to identify associations between three SNPs within *GRM5* (rs60954128, rs3824927, and rs992259, located within the regulatory 3'untranslated region), with schizophrenia and cognitive features in a case-control cohort (n=268/group, from the Australian Schizophrenia Research Bank). We further explored the effects of these SNPs within Grade of Membership-derived cognitive subtypes of schizophrenia. Cognition was assessed using estimates of premorbid and current intelligence quotients and subscales from the Controlled Oral Word Association test, the Repeatable Battery for the Assessment of Neurophysiological Status and the Letter Number Sequencing Test.

Results: We report significantly greater frequency of T-carriers of rs60954128 in men suffering from schizophrenia compared to controls. Genotype variants for this SNP also had significant effects on working memory in schizophrenia patients, but driven by effects in females. Whilst neither allelic nor genotypic frequencies of rs3824927 were associated with schizophrenia, men with schizophrenia carrying the minor T allele for this SNP showed greater cognitive dysfunction compared to schizophrenia patients carrying the major G allele.

Conclusions: These findings strongly support a role for *GRM5* in cognitive dysfunction associated with genetic vulnerability to schizophrenia, which may operate in a sex-specific manner.

INTRODUCTION

Cognitive dysfunction is a core feature of schizophrenia that is strongly predictive of patients' long-term functional outcomes (1). Glutamatergic neurotransmission is critically linked with cognitive function, and several genetic variants involved in glutamate signaling have reached genome-wide significance in schizophrenia case-control populations (2). These studies have highlighted both the biological importance of glutamatergic molecules and their potential as novel therapeutic targets. Among them, *GRM5* was reported as one of the top candidate genes in schizophrenia etiology in a study integrating not only gene-level data from genome wide association studies (GWAS), but also gene expression data from available human, animal and cell-based studies (3).

GRM5 is a large gene located at the chromosomal region 11q14.3. It encodes for metabotropic glutamate receptor 5 (mGluR5), a compelling novel target for the treatment of schizophrenia owing to its ability to mediate post-synaptic *N*-methyl-D-aspartate receptor (NMDAR) currents (4; 5). Only few studies have examined genetic variation within *GRM5* in the context of schizophrenia. Over a decade ago, one group reported that a long-range restriction map covering the *GRM5* gene was associated with schizophrenia within a large Scottish pedigree (6). A novel intragenic microsatellite was subsequently associated with schizophrenia in a case-control population (7). Although *GRM5* has not reached significance in recent GWAS (8; 9), knockout of *GRM5* or pharmacological blockade of mGluR5 in rodent models induces a wide range of schizophrenia-like behaviors, such as deficits in sensorimotor gating, spatial learning and memory, working memory and instrumental learning (10–13). Accordingly, mGluR5 is critically involved in synaptic plasticity, the mechanism underlying cognitive functions such as learning and memory (14). However, no previous studies have aimed to identify genetic polymorphisms within *GRM5* and their potential association with cognition in schizophrenia.

Notably, cognitive deficits in schizophrenia are not uniformly severe. Accumulating evidence supports the existence of putative cognitive subtypes of schizophrenia (15). The use of a clustering method known as Grade of Membership (GoM) has been particularly useful to delineate homogenous classes of schizophrenia cases on the basis of performance across a range of cognitive domains (16); the existence of 'cognitive deficit' (CD) and (relatively) 'cognitively spared' (CS) subtypes of

schizophrenia have been replicated in independent samples (17; 18), and assisted in the detection of previous genetic linkage and single nucleotide polymorphism (SNP) associations that would otherwise have been obscured by the heterogeneity of cognitive function among schizophrenia cases (18–20). Recent evidence for differential brain structure among cognitive subtypes of schizophrenia supports the idea that these subtypes reflect biologically distinct classes (21). Additionally, it is notable that the ability to distinguish CD from CS cases on the basis of brain structure is stronger in female cases (21). It is therefore possible that biological associations with CD/CS subtypes may operate in a sex-specific manner.

The present study thus aimed to determine if variation in SNPs within *GRM5* are associated with the diagnosis of schizophrenia, and cognitive features of the disorder, in a strictly selected Caucasian case-control population drawn from a national bio-banking facility. As our group has recently discovered evidence that mGluR5 regulation is altered in cortical and hippocampal postmortem schizophrenia samples (22), we chose to examine three novel SNPs in the regulatory 3' untranslated region (3' UTR) of *GRM5*; this region is strongly implicated in protein conformation and regulation of sites critical for protein-protein interactions (23). Following case-control allelic association analyses, each SNP was subsequently analyzed for its ability to predict membership of schizophrenia cases classified previously as relatively cognitively spared (CS) or showing severe cognitive deficits (CD) compared to healthy controls (18). We also examined the association of *GRM5* SNPs with each of nine cognitive performance domains in both schizophrenia and healthy control participants. As extensive evidence from schizophrenia rodent models and human postmortem studies implicates mGluR5 in cognitive impairment (24; 25), we hypothesized that the selected *GRM5* SNPs would be significantly associated with schizophrenia and cognition, and predictive of cognitive performance. Considering potential sex-specific effects (26), all statistical analyses were further conducted in males and females separately.

METHOD

Subjects

Samples were acquired from the Australian Schizophrenia Research Bank (ASRB), a national repository of patient samples and associated data (27). For recruitment, participants were required to be fluent in English, have no history of an organic brain disorder, post-traumatic amnesia, mental retardation, movement disorder, substance dependence or received electroconvulsive therapy in the past six months. The Diagnostic Interview for Psychosis (DIP) was performed by trained researchers to obtain clinical and diagnostic data from patients (28). Further details describing clinical and demographic characterization, sampling frameworks and consent procedures has been previously described in detail (27).

For the present study, subjects were selected from the ASRB using strict criteria to prevent population stratification. Selected cases were of Caucasian ethnicity and diagnosed with schizophrenia according to the DSM-IV. Cases were subsequently matched with controls (no prior personal or family history of mental disorders) according to sex and age. The final sample consisted of 268 schizophrenia cases (186 males and 82 females; average age 38.94±10.99 years) and 268 matched controls (169 males and 99 females; average age 38.60±12.57 years). The majority of schizophrenia subjects were medicated (58 typical antipsychotics, 211 atypical antipsychotics, 54 mood stabilizer, 92 antidepressants). This study was approved by and conducted according to the guidelines of the University of Wollongong Human Research Ethics Committee (HE10/161) and the University of New South Wales Human Ethics Committee (HC12658).

Neuropsychological measures

Standardized estimates of premorbid and current intelligence quotient (IQ) were obtained using Wechsler Tests for Adult Reading (WTAR; 29) and Abbreviated Scale of Intelligence (WASI; 30). Executive function was assessed using the Controlled Oral Word Association Test (COWAT; 31) and the Letter Number Sequencing Test (LNS; 30). The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) was used to derive indices of attention and memory function, specifically delayed memory, immediate memory, construction, language and attention (32).

Cognitive subtyping

A large subset of the schizophrenia patients (247 cases) were classified as either cognitively spared (CS) or displaying a generalized cognitive deficit (CD) on the basis of previous GoM analyses of the ASRB sample (18). The final GoM sample consisted of 108 CS cases (81 males and 27 females; average age, 38.00±10.17 years) and 139 CD cases (91 males and 48 females; average age 39.64±11.50 years).

Genotyping

Three SNPs within the 3' UTR region of the *GRM5* gene (rs60954128, rs3824927 and rs992259; Figure 1) were selected based on their Minor Allele Frequencies (MAF) reported in a Caucasian population (MAF>10%) and/or previous associations with schizophrenia (3; 33). High-throughput SNP genotyping was performed using the MassARRAY® genotyping assay (Sequenom, Inc., San Diego, CA, USA). Analysis was subsequently performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). PCR and extension primer design, selection and multiplexing were performed using MassARRAY® Designer Software (Sequenom, Inc.).

Statistical Method

Analyses were performed using SPSS (version 21) with significance set to $P<0.05$. Bonferroni correction was utilized to adjust for multiple comparisons and to control for type 1 error, resulting in a corrected significance level of $P<0.016$ to give a 95% probability of correctly concluding not to reject the null hypothesis in the chi-square (χ^2) test. The genotypic distributions of SNPs rs60954128, rs3824927 and rs992259 were initially assessed for deviation from Hardy Weinberg Equilibrium (HWE; $P>0.05$), and SNP(s) which deviated from HWE were removed from further analyses. All analyses were firstly performed in both sexes combined (referred to as "all subjects") and then in male and female subjects individually.

Three series of statistical analyses were performed:

(i) Case-control genotypic/allelic frequencies and differences among SNPs: χ^2 analyses were performed for each SNP to test for significant differences in allelic and genotypic frequencies between the case and control groups. A linkage disequilibrium (LD) plot based on absolute D' values (pairwise comparison of tested SNPs) was also generated using SAS statistical software (v8.2 SAS Institute, Cary, NC, USA; SNP & Variation Suite).

(ii) Prediction of CD/CS group membership within schizophrenia cases: Investigation of the capacity of risk-allele carriers to predict membership in GoM-derived cognitive phenotypes was conducted using a series of multinomial logistic regressions, and categorical GoM classes as dependent variables. The first series of multinomial logistic regressions used healthy control participants as the reference category, to determine whether risk-allele carriers were more highly represented among members of the CD or CS subtype. The second series of analyses examined whether risk-allele carriers were more highly represented among members of the CD subtype, relative to CS subtypes.

(iii) Effects of genotype on specific cognitive domains: For each of nine cognitive performance measures, the main effects of genotype, group (schizophrenia:control), and the interaction of SNP genotype and group status was examined using a series of mixed design multiple analyses of variance (MANOVA), with two levels of the within-subjects factor of genotype (minor allele carrier/non-minor allele carrier) and two levels of the between-subjects factor of group (case/control). Significant interaction effects were followed up with analysis of variance (ANOVA) and subsequently verified with Tukey's post-hoc tests to identify genotype effects on cognitive outcomes across diagnosis.

RESULTS

(i) Genotypic/allelic frequencies and differences among SNPs

rs992259 deviated from HWE and was thus excluded from further analyses. Allelic frequencies of the remaining SNPs (rs60954128 and rs3824927) did not deviate from HWE, and the control group showed a similar pattern as those reported for Caucasian populations in genetic database results (Minor Allelic Frequency [MAF]=14.2 % and 51.5%, for rs60954128 and rs3824927 respectively; Table 1).

For rs60954128, significant associations with schizophrenia compared with healthy controls were found for allelic (χ^2=5.32, df=1, P=0.021) and genotypic (χ^2=7.41, df=2, P=0.025) frequencies, whereby T allele carriers and TT homozygote genotypes were overrepresented in schizophrenia cases; however, these analyses did not withstand Bonferroni correction (Table 1). Examination of sex-specific effects revealed a greater number of TT homozygote genotypes on rs60954128 in male cases compared to male controls, with significant associations between both genotypic (χ^2=9.09, df=2, P=0.011) and allelic (χ^2=6.66, df=1, P=0.009) frequencies withstanding Bonferroni correction for male participants only. There were no significant associations of genotypic/allelic frequency distributions with schizophrenia for rs3824927 in all subjects or when examined according to sex (P>0.05). Pairwise LD statistics revealed evidence of low to moderate LD between rs60954128 and rs3824927 (D'=0.371: R^2=0.233; Supplementary Figure 1).

(ii) Do **GRM5** *genotypes predict membership of CD/CS subtypes within SZ cases?*

Multinomial logistic regressions were implemented to investigate the ability of *GRM5* SNP polymorphisms to predict membership of GoM-derived cognitive phenotypes. Regression parameters and models are summarized in Table 2. In Model 1, GoM-derived subtypes were entered as dependent variables, with healthy controls dummy coded as the reference variable and reference SNPs entered as fixed factors. In this model, neither rs60954128 nor rs3824927 were able to significantly predict membership of either CS or CD cognitive subtypes in reference to healthy control status; however, specifically in males, T allele carriers of rs60954128 were able to predict membership to the CS group (B=-0.649, df=2, P=0.037), although this did not reach model significance (χ^2=4.382, df=2, P=0.112).

In Model 2, we examined the ability of rs60954128 and rs3824927 risk allele carriers to predict membership to the CD group, using the CS subtype as the reference category. Risk allele carriers for rs3824927 were able to significantly predict membership to the CD subtype relative to the CS subtype (χ^2=4.702, df=1, P=0.030), whereby T allele carriers were more highly represented in the CD group (B=-0.704, df=1, P=0.034). Further sex-specific analyses revealed that this was likely driven by an effect of T allele carriers specifically in male cases compared to male controls (χ^2=4.216, df=1, P=0.040), whereby male T allele carriers of rs3824927 were more highly represented in the CD group compared to male controls (T allele carriers: B=-0.826, df=1, P=0.045).

(iii) Effect of genotypes on cognitive function

Given the low frequency of minor homozygote genotypes in the tested cohort, cases were grouped into all minor allele carriers (rs60954128: CT/TT; rs3824927: GT/TT), and compared to major allele homozygotes (rs60954128: CC; rs3824927: GG). MANOVA results for the effects of group-status (i.e. schizophrenia or control) and genotype interactions on cognitive performance measures are presented in Table 3. Main effects of group-status confirmed that schizophrenia subjects scored significantly lower test scores in all cognitive assessments, compared to healthy controls (F≤210.077, P≤0.007; results of all main effects and interactions are provided in Supplementary Table 1).

For the rs60954128 genotype (CC:CT/TT) there were main effects on RBANS subscales of attention, independent of diagnosis, in female subjects only ($F_{3,133}$=6.453, P=0.012; Supplementary Table 1). Post-hoc analyses detected significantly worse performance on these cognitive tests among T allele carriers (CT/TT; P=0.011) relative to major allele homozygotes (CC). In addition, there were significant effects of group-status by genotype interactions on RBANS delayed memory for the entire group ($F_{3,408}$=4.971, P=0.026) and in males only ($F_{3,271}$=4.988, P=0.026). In females, the interaction of group-status and genotype affected LNS scores ($F_{3,133}$=9.104, P=0.003), with female schizophrenia T allele carriers (CT/TT) having significantly lower LNS scores compared to both CC major allele homozygotes in schizophrenia and all allele carrier groups (CC, CT/TT) in healthy controls (P<0.031).

These allelic differences failed to reach significance in post-hoc tests for the entire group (and male subset) of schizophrenia and healthy control cases.

For the rs3824927 genotype (GG:GT/TT), a main effect of genotype on COWAT scores was observed for males only ($F_{3,296}$=7.444, P=0.007); post-hoc analyses revealed significantly worse performance on these cognitive measures among T allele carriers (GT/TT: P=0.018) relative to major allele homozygotes (GG). In addition, significant group-status (both genders) by genotype interaction effects were detected on the WASI ($F_{3,451}$=10.866, P=0.001), LNS ($F_{3,451}$=4.407, P=0.036), RBANS total score ($F_{3,451}$=4.193, P=0.027) and RBANS delayed memory domain ($F_{3,451}$=6.267, P=0.013). Sex-specific investigation revealed that these significant main effects were specific to male schizophrenia subjects (WASI: $F_{3,296}$=11.073, P<0.001; LNS: $F_{3,296}$=0.509, P=0.025; RBANS total: $F_{3,296}$=6.890, P=0.009; RBANS delayed memory: $F_{3,296}$=7.795, P=0.006). WASI test scores were significantly lower in male schizophrenia T allele carriers (GT/TT) compared to male schizophrenia major homozygotes (GG), and compared to both allele carrier groups (GG, GT/TT) in healthy males (P=0.010). However, post-hoc testing did not detect significant differences between allele group carriers (GG:GT/TT) within schizophrenia or control subjects for LNS, RBANS total or RBANS delayed memory measures (Supplementary Table 1).

DISCUSSION

Here, we report the first evidence for genetic variation amongst two SNPs located in the regulatory 3' UTR of the *GRM5* gene in association with schizophrenia, and in relation to cognitive features of the disorder, with the occurrence of several sex-specific effects. Specifically, we found that (i) rs60954128 minor allele (T) and homozygous genotype (TT) were significantly associated with schizophrenia in males; (ii) rs3824927 genotypes significantly predicted membership of a subtype of schizophrenia cases characterized by severe cognitive deficits in male cases; and, (iii) both rs60954128 and rs3824927 exerted differential effects on particular cognitive domains in men and women. In line with emerging evidence indicating a role for mGluR5 in schizophrenia and cognition (14; 24), we provide strong support for a complex association between genetic variation within the *GRM5* gene and cognitive function in schizophrenia. Since all significant associations reported are largely dependent on sex, we further provide the first evidence that the effects of *GRM5* polymorphisms on cognition may be sex-specific, differentially affecting cognitive performance in men and women with and without schizophrenia.

The first main finding in our study was that men with schizophrenia had significantly higher minor allelic (T; P=0.009) and genotypic (TT; P=0.011) frequencies of *GRM5* SNP rs60954128 compared to their matched controls. This finding is in line with previous studies implicating *GRM5* in the genetic susceptibility for schizophrenia, which have demonstrated the association of different genomic variants than those examined here. For example, schizophrenia has been associated with a microsatellite (CA) within exon 7 in *GRM5,* reported in a Scottish case-control population (7), and a *de novo* non-synonymous SNP (G369V) and frameshift substitution within *GRM5* was detected in a schizophrenia pedigree, hypothesized to affect glutamate sensitivity and demonstrated to interrupt mGluR5 protein-protein interactions at the mGluR5 C-terminal respectively (34). However, these previous studies did not address the potential specificity of findings for males or females.

The sex-specific differences in our case-control study mirror the apparently inherent sex differences in the etiology of the disease, with a slightly higher incidence of schizophrenia reported in men (26). Our recent postmortem brain studies also showed sex-specific effects on mGluR5. We reported a significant sex x diagnosis interaction in the dorsolateral prefrontal cortex (35), and despite

post-hoc analyses not reaching significance, we observed reduced mGluR5 binding in men with schizophrenia compared to male controls (t_{35}=1.773, p=0.085). In addition, we have also observed reduced protein levels in the CA1 region (22) of men compared to women, independent of diagnosis (unpublished observations). Notably, previous animal studies analyzing *GRM5*/mGluR5 are almost exclusively conducted on one sex (24), owing to known sex-specific effects on molecular measures. It is additionally interesting that *PDZ-GEF 2*, a gene coding for a PDZ ligand which binds to the C-terminal end of mGluR5, was also found to be sex-specifically associated with schizophrenia in an Irish case-control population (36). Considering rs60954128 is located at the beginning of the 3' UTR DNA region proximal to the region coding for the mGluR5 C-terminus, the site of endogenous protein-protein interactions, it would be of considerable interest to investigate the effects of rs60954128 genetic variation on genomic regions responsible for the interaction of modulators/cofactors, and whether these effects are sex-specific.

Despite the lack of association between rs3824927 genetic variation and schizophrenia in our case-control population, this polymorphism was able to significantly predict membership of an arguably more severe type of schizophrenia, characterized by widespread cognitive deficits (18). Once again, this significant effect was sex-specific, with a higher frequency of cognitive deficits observed in male minor allele carriers compared to male major allele homozygotes, and female subjects with schizophrenia. This finding supports the potential utility of mGluR5 as a novel target for cognitive enhancing drugs that might be useful to treat a variety of neurocognitive disorders including schizophrenia (37). Although it should be considered that male dominant findings in the present study might be representative of the higher number of men compared to women in this cohort, sex-specific biological distinctions between brain structures of CD and CS types are evident, providing support for sex-specific differences between the GoM-derived cognitive subtype classes (21). Replication in a larger cohort with power sufficient to detect robust sex differences is now required.

Our additional characterization of the effects of variation in *GRM5* SNPs on specific cognitive domains suggest widespread effects of rs60954128 on general neuropsychological performance, working memory and IQ, with only a specific effect on delayed memory evident for rs3824927 in the entire schizophrenia group. Sex specific effects included that of minor T allele carriers (CT/TT) of

rs60954128 on reduced processing speed in females (30), consistent with previous findings (10–12; 38–40). Furthermore, in men only, rs60954128 scores had a significant effect on IQ and delayed memory. Secondly, rs3824927 strongly affected IQ, with lower IQ scores evident in male schizophrenia minor T allele carriers compared to their matched controls. However, post-hoc tests in these latter measures did not detect a significant difference in scores between genotypes, although this was likely due to reduced power within these groups. Considering the extensive evidence of *GRM5* knockout causing deficits in synaptic plasticity, and affecting various measures of learning and memory in male animal models (24), these findings should certainly be further investigated in independent cohorts with robust power following sex-wise analyses.

GRM5 encodes mGluR5, best known for its ability to modulate NMDAR-mediated post-synaptic currents (4; 24). Although little is known about genetic markers within *GRM5* in schizophrenia, *GRM5* gene expression and mGluR5 protein is differentially affected in multiple regions of the postmortem schizophrenia brain (24). Of note, we recently found that mGluR5 mRNA and protein levels were significantly correlated in control but not schizophrenia subjects in the dorsolateral prefrontal cortex (a region highly involved in executive function), indicating dysregulation or dysfunction of mGluR5 in schizophrenia (unpublished data). SNPs located within the 3' UTR are reported to have regulatory functions, such as control of RNA splicing, stability, translation, microRNA interactions, and transcription (23). As the SNPs studied in the present study are located within the regulatory 3' UTR of *GRM5*, they have potential regulatory roles and may contribute to the dysregulation of mGluR5 mRNA synthesis and stability that we have previously observed. It is likely that genetic variation in *GRM5* regulatory regions may affect mGluR5 regulation, and subsequent investigation to uncover the potential functionality of the tested SNPs is warranted but not presented here.

In summary, this study provides novel evidence of potential sex-specific contributions of *GRM5* to schizophrenia etiology. Although we have not explored the exact mechanisms of action in the present study, we hypothesize that SNPs within the 3' UTR region of *GRM5* translate to downstream signaling changes causing effects to the regulation of the mGluR5 protein. Disruption to this protein is widely reported to cause a range of learning and memory deficits (14; 24). Although the cohort examined in the present study demonstrated extensive matching and cognitive phenotyping, further

studies in independent and larger cohorts with high power for sex-wise analyses are warranted. Nonetheless, in combination with the previous pathological evidence of a role for *GRM5* in schizophrenia, the results from the present study elaborate on these findings and support the involvement of *GRM5* in schizophrenia and cognition.

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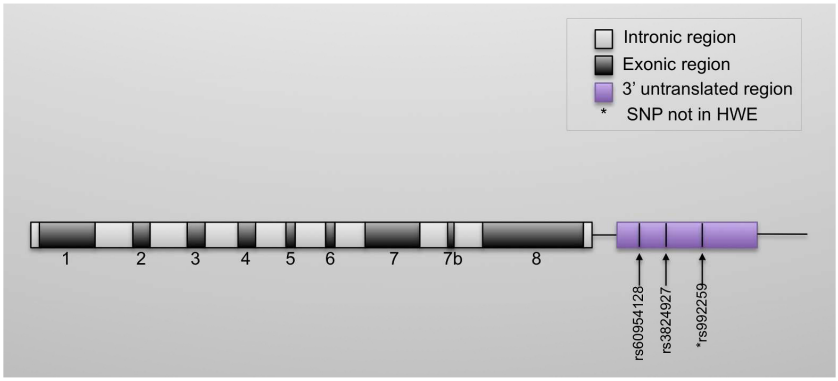


FIGURE 1. Schematization of tested SNPs within the 3' untranslated region of the GRM5 gene.
Abbreviations: HWE, Hardy Weinberg Equilibrium; SNP, single nucleotide polymorphism; rs, reference SNP.
913x412mm (72 x 72 DPI)

TABLES

Table 1. Positions of chosen novel SNPs within the *GRM5* 3' UTR, minor genotypic and allelic frequencies (HC, SZ; CS, CD), and tests of association (SZ:HC only). Significant associations withstanding Bonferroni correction are in bold.

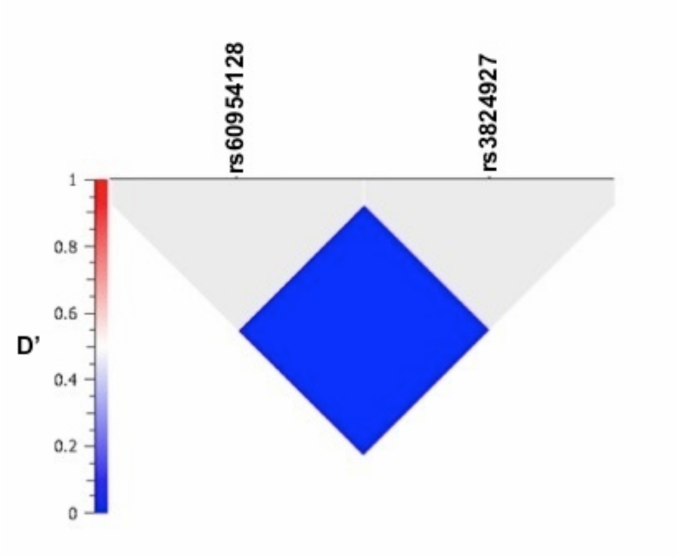
SNP	Location	Minor allele	HWE		MGF (%)				Genotypic Association (HC:SZ only)		MAF (%)				Allelic Association (HC:SZ only)	
			P (HC/SZ)		*HC*	*SZ*	*CS*	*CD*	χ^2	*P*	*HC*	*SZ*	*CS*	*CD*	χ^2	*P*
rs60954128	88506288	T	0.224/0.289	*all*	1.0	5.0	5.2	5.1	7.41	0.025	13.8	19.7	20.3	18.4	5.13	0.023
				male	0.0	5.7	6.7	3.4	**9.09**	**0.011**	11.6	19.6	22.0	14.7	**6.11**	**0.013**
				female	2.5	3.4	2.4	9.5	0.47	0.791	17.5	20.2	17.1	28.6	0.45	0.504
rs3824927	88508028	T	0.273/0.437	*all*	23.7	28.6	25.4	30.0	2.77	0.251	49.6	49.1	47.1	57.0	0.13	0.721
				male	23.4	30.0	27.3	22.0	2.72	0.256	48.7	47.6	48.7	57.9	0.25	0.616
				female	24.1	25.7	22.2	8.0	0.43	0.806	48.9	47.8	44.4	54.3	0.03	0.861
rs992259	88644873	G	1.906×10^{-8}/0.026													

Abbreviations: CD, schizophrenia subjects displaying cognitive deficits; CS, schizophrenia subjects cognitively spared relative to the CD group; HC, healthy controls; SZ, schizophrenia patients; MAF, minor allelic frequency; MGF, minor genotypic frequency; HWE, Hardy-Weinberg equilibrium; OR, odds ratio. Significant values are in bold.

Supplementary Table 1. Complete results of MANOVAs performed to determine Group, SNP, and Group x SNP effects on cognitive performance measures, in all subjects (both sexes) and male and female subjects individually. Note that Tukey's post-hoc tests are for the results of significant GRP x SNP interactions only (highlighted in bold).

		(a) GRM5 rs60954128 (CC:CT/TT)			(b) GRM5 rs3824927 (GG: GT/TT)		
		all	male	female	all	male	female
WTAR	GRP	F=33.378, P<0.001	F=21.905, P<0.001	F=8.590, P=0.004	F=26.449, P<0.001	F=17.521, P<0.001	F=7.972, P=0.005
	SNP	F=0.015, P=0.903	F=0.001, P=0.972	F=0.007, P=0.933	F=0.876, P=0.350	F=0.078, P=0.780	F=2.266, P=0.134
	GRP x SNP	F=0.042, P=0.838	F=0.118, P=0.731	F=0.868, P=0.353	F=2.321, P=0.128	F=1.562, P=0.212	F=0.457, P=0.500
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-
WASI	GRP	F=113.643, P<0.001	F=83.588, P<0.001	F=25.210, P<0.001	F=93.520 P<0.001	F=67.630, P<0.001	F=23.651,P <0.001
	SNP	F=1.775, P=0.183	F=1.047, P=0.307	F=0.239, P=0.626	F=0.450, P=0.502	F=0.524, P=0.470	F=0.008, P=0.929
	GRP x SNP	F=1.888, P=0.170	**F=4.156, P=0.042**	F=0.673, P=0.414	**F=10.866, P=0.001**	**F=11.073, P<0.001**	F=0.547, P=0.461
	Tukey's	-	SZ: CT/TT>CC NS	-	**SZ: GG>GT/TT P=0.015**	**SZ: GG>GT/TT P=0.010**	-
		-	HC: CC>CT/TT NS	-	HC: GT/TT>GG NS	HC: GT/TT>GG NS	-
LNS	GRP	F=107.653, P<0.001	F=74.481, P<0.001	F=30.864, P<0.001	F=78.183, P<0.001	F=50.560, P<0.001	F=25.955, P <0.001
	SNP	F=0.251, P=0.617	F=1.169, P=0.281	F=1.616, P=0.206	F=0.022, P=0.883	F=0.276, P=0.600	F=1.229, P=0.269
	GRP x SNP	F=0.257, P=0.612	F=0.814, P=0.368	**F=9.104, P=0.003**	**F=4.407, P=0.036**	**F=0.509, P=0.025**	F=0.022, P=0.881
	Tukey's	-	-	**SZ: CC>CT/TT, P=0.031**	SZ: GT/TT>GG NS	SZ: GG>GT/TT NS	-
		-	-	HC: CT/TT>CC NS	HC: GT/TT>GG NS	HC: GT/TT>GG NS	-
COWAT	GRP	F=35.198, P<0.001	F=23.106, P<0.001	F=8.044, P=0.005	F=42.860, P<0.001	F=36.284, P<0.001	F=7.357, P=0.007
	SNP	F=0.123, P=0.726	F=0.115, P=0.735	F=0.477, P=0.491	F=2.496, P=0.115	**F=7.444, P=0.007**	F=1.571, P=0.212
	GRP x SNP	F=1.063, P=0.303	F=3.547, P=0.061	F=0.904, P=0.334	F=0.153, P=0.696	F=0.057, P=0.812	F=0.015, P=0.904
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-
RBANS TOTAL	GRP	F=210.077, P<0.001	F=159.169, P<0.001	F=47.964, P<0.001	F=188.397, P<0.001	F=128.653, P<0.001	F=56.374, P<0.001
	SNP	F=0.005, P=0.945	F=0.335, P=0.563	F=1.529, P=0.218	F=0.990, P=0.320	F=0.032, P=0.858	F=0.882, P=0.349
	GRP x SNP	F=2.706, P=0.101	F=3.750, P=0.054	F=0.101, P=0.751	**F=4.913, P=0.027**	**F=6.890, P=0.009**	F=0.003, P=0.956
	Tukey's	-	-	-	SZ: GG>GT/TT NS	SZ: GG>GT/TT NS	-
		-	-	-	HC: GT/TT>GG NS	HC: GT/TT>GG NS	-
RBANS delayed memory	GRP	F=90.448, P<0.001	F=69.667, P<0.001	F=16.557, P<0.001	F=75.612, P<0.001	F=51.451, P<0.001	F=22.007, P <0.001
	SNP	F=0.294, P=0.588	F=0.237, P=0.627	F=0.092, P=0.762	F=0.140, P=0.709	F=0.913, P=0.340	F=2.998, P=0.085
	GRP x SNP	**F=4.971, P=0.026**	**F=4.988, P=0.026**	F=0.049, P=0.825	**F=6.267, P=0.013**	**F=7.795, P=0.006**	F=0.003, P=0.958
	Tukey's	SZ: CT/TT>CC NS	SZ: CT/TT>CC NS	-	SZ: GT/TT>GG NS	SZ: GG>GT/TT NS	-
		HC: CC>CT/TT NS	HC: CC>CT/TT NS	-	HC: GT/TT>GG NS	HC: GT/TT>GG NS	-
RBANS attention	GRP	F=128.275, P<0.001	F=94.350, P<0.001	F=30.844, P<0.001	F=125.991, P<0.001	F=86.272, P<0.001	F=37.329, P <0.001
	SNP	F=0.567, P=0.452	F=0.109, P=0.741	**F=6.453, P=0.012**	F=1.762, P=0.185	F=3.188, P=0.075	F=0.468, P=0.495
	GRP x SNP	F=0.687, P=0.408	F=2.665, P=0.104	F=2.094, P=0.150	F=0.806, P=0.370	F=1.640, P=0.201	F=0.458, P=0.500
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-
RBANS immedia-te memory	GRP	F=137.081, P<0.001	F=98.369, P<0.001	F=31.511, P<0.001	F=119.652, P<0.001	F=77.893, P<0.001	F=37.809, P <0.001
	SNP	F=0.159, P=0.690	F=0.381, P=0.538	F=0.105, P=0.746	F=0.067, P=0.796	F=0.428, P=0.513	F=0.686, P=0.409
	GRP x SNP	F=0.030, P=0.864	F=0.184, P=0.668	F=0.878, P=0.350	F=1.287, P=0.257	F=2.967, P=0.086	F=0.274, P=0.601
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-
RBANS construct-ion	GRP	F=51.062, P<0.001	F=49.979, P<0.001	F=8.824, P=0.004	F=46.860, P<0.001	F=36.116, P<0.001	F=12.510, P=0.001
	SNP	F=0.212, P=0.646	F=1.763, P=0.185	F=0.365, P=0.547	F=0.884, P=0.348	F=0.203, P=0.653	F=1.629, P=0.204
	GRP x SNP	F=2.961, P=0.086	F=1.552, P=0.214	F=0.311, P=0.578	F=2.941, P=0.087	F=2.601, P=0.108	F=0.427, P=0.514
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-
RBANS language	GRP	F=69.497, P<0.001	F=44.405, P<0.001	F=20.857, P<0.001	F=66.001, P<0.001	F=43.984, P<0.001	F=22.712, P <0.001
	SNP	F=1.598, P=0.207	F=1.034, P=0.310	F=0.047, P=0.829	F=1.360, P=0.244	F=0.390, P=0.533	F=0.202, P=0.654
	GRP x SNP	F=1.176, P=0.279	F=0.743, P=0.390	F=0.861, P=0.355	F=1.730, P=0.189	F=0.846, P=0.358	F=0.906, P=0.343
	Tukey's	-	-	-	-	-	-
		-	-	-	-	-	-

Abbreviations: COWAT, Controlled Oral Word Association Test; GRP, Group (schizophrenia:control); HC, healthy control; LNS, Letter Number Sequencing; MANOVA, Multiple Analyses of Variance; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; SNP, single nucleotide polymorphism; SZ, schizophrenia; Tukey's, Tukey's post-hoc testing; WASI, Wechsler Abbreviated Scale for Intelligence; WTAR, Wechsler Test of Adult Reading.



Supplementary Figure 1. Linkage disequilibrium between SNPs rs60954128 and rs3824927, located within the 3' untranslated region of *GRM5*.

Chapter Six

6.1 Overall discussion and conclusion

The series of studies presented in this thesis collectively support that mGluR5 is dysregulated in the pathophysiology of schizophrenia. The main findings from these studies are:

(i) Binding to mGluR5 (and related presynaptic receptors, mGluR2/3) is not altered in the ACC of schizophrenia subjects or subjects with depression (with or without psychosis), bipolar disorder compared to controls (Chapter 2; Matosin et al., 2014).

(ii) In the DLPFC, mGluR5 mRNA is unaltered, whilst protein levels of mGluR5 are increased, and mGluR5 endogenous regulators (Norbin, Tamalin and Preso1) are decreased in schizophrenia subjects compared to controls. Also in this region, mGluR5 protein levels are significantly correlated with mGluR5 mRNA, and protein levels of mGluR5 endogenous regulators, in control subjects but not schizophrenia subjects; this indicates dysregulation of mGluR5 in schizophrenia (Chapter 3; Matosin et al. 2015, *Acta Neuropathologica*).

(iii) mGluR5 is increased in the hippocampal CA1 region in individuals with schizophrenia. However, as mGluR5 knockout induces cognitive deficits in animal models, it is hypothesised that this increase (in CA1 and also the DLPFC) does not represent a functional increase, but rather a compensatory increase for a core deficit in mGluR5. Furthermore, considering mGluR5 endogenous regulators are increased in CA1, compared to the DLPFC where they are significantly decreased, this suggests mGluR5 may be regulated in a brain-region specific manner (Chapter 4; Matosin et al, accepted *Schizophrenia Research* [see Appendix]).

(iv) Evidence from the animal studies in Chapters 3 and 4 (and the majority of evidence from our human studies) suggest commonly used APDs do not influence the mGluR5 system (Matosin et al., 2013, Chapter 3 and Chapter 4), further suggesting that our postmortem findings are not confounded by premortem APD history. Additionally, these findings provide evidence that novel drugs that aim to correct potential changes in mGluR5 (levels, signalling or regulation) might be useful as an adjunct treatment with current APDs, as it is unlikely current APDs target the observed alterations in mGluR5.

(v) Lastly, SNPs located within the regulatory 3' UTR region (a key region involved in protein conformation and regulation of protein-protein interactions) of *GRM5,* were significantly

associated with schizophrenia in men, and had gender-specific effects on several cognitive tests in both men and women, in an Australian Caucasian case-control population. This suggests that *GRM5*/mGluR5 may operate in a sex-specific manner (Chapter 5; Matosin et al, submitted *American Journal of Psychiatry*).

Detailed discussions for each study are included within Chapters 2-5. The remainder of Chapter 6 will provide a general discussion of the main findings of this thesis. I will further discuss necessary considerations, the limitations of these past and present works, and recommended avenues for future investigations.

6.1.1 mGluR5 in schizophrenia: a summary of what we knew

As discussed in Chapter 1, converging lines of evidence from genetic and animal studies over the last two decades have consistently indicated the involvement of mGluR5 in the pathology of schizophrenia. *GRM5* knockout or pharmacological blockade of mGluR5 induces a wide range of schizophrenia-like behaviours, including social interaction deficits, impaired working memory, reduced instrumental learning and potentiation of deficits induced by NMDAR antagonists. An additional body of evidence suggests the involvement of mGluR5 in LTD and LTP, which are the mechanisms underlying learning and memory processes. Of these studies, it is interesting to note that differences in spatial learning of mice significantly correlated with protein levels of mGluR5, suggesting mGluR5 protein expression may be a marker of deficits in synaptic plasticity and therefore cognitive processes (Manahan-Vaughan and Braunewell, 2005).

Despite the results from animal studies supporting an important role of mGluR5 in the manifestation of schizophrenia-like behaviours, the evidence from human studies has been largely inconsistent with these findings. Postmortem studies inarguably impart an important understanding of the molecular processes that underpin neuropsychiatric disorders. However, these investigations are commonly inconsistent with animal and cellular-based mechanistic studies, highlighting the complexity of the processes involved. Several groups have previously examined mGluR5 measures of expression in postmortem samples from brain regions that are highly involved in learning, memory, executive processing and emotion, including multiple regions of the prefrontal cortex, hippocampus, cingulate cortices and deeper structures including the thalamus, caudate and putamen. However, the vast majority of evidence from these prior investigations (summarised in Chapter 1) have largely shown mGluR5 binding, mRNA and protein levels are unaltered in various regions of the schizophrenia postmortem brain.

It is however important to consider that there are significant limitations of many of these earlier studies. These include the use of cohorts with limited power (n<20/group; Gupta et al., 2005; Matosin et al., 2014; Ohnuma et al., 1998; Ohnuma et al., 2000b; Richardson-Burns et al., 2000), samples from elderly subjects (Gupta et al., 2005), and differences in experimental techniques used, which cannot be compared across studies (for example, receptor autoradiography compared with immunoblot in total homogenates, as previously demonstrated: Matosin et al., 2013). Additionally, there is also the possibility of cohort-dependent differences, which can only be overcome by repetitive experimentation within independent cohorts of significant power. These limitations, coupled with the findings from animal studies that mGluR5 is involved in the manifestation of schizophrenia-related behaviours, strongly suggested the need to further investigate mGluR5 in schizophrenia before strong conclusions can be drawn.

More recently, one study for the first time investigated both mGluR5 mRNA and protein measures within the same tissue samples, which has provided additional information regarding the regulation of mGluR5 in schizophrenia. Fatemi and colleagues last year reported that although there was no change in mGluR5 mRNA levels in BA9, there was decreased levels of mGluR5 (monomer) coupled with both decreased (pan)mRNA, as well as reduced protein levels of mGluR5 (monomer) in the lateral cerebellum in individuals with schizophrenia (Fatemi et al., 2013). This investigation highlighted two important points. Firstly, mGluR5 is likely altered in a brain region-specific manner. Secondly, due to the lack of change in mGluR5 mRNA expression but alteration of mGluR5 protein levels in BA9, this suggests that the rate of mGluR5 protein synthesis or degradation might be affected in this region. This potential dysregulation has not been detected in previous studies due to measurements of only mRNA or protein levels independently. However, Fatemi and colleagues (2013) did not present specific correlations between mGluR5 mRNA and protein levels in schizophrenia and control patients, despite that this would be further indicative of mGluR5 synthesis and stability in this region.

In addition to the possibility of altered synthesis and stability of mGluR5, there are additional processes that endogenously occur to regulate mGluR5 that may subsequently affect mGluR5 function. Amongst the known modulatory molecules, there are well-reported roles for Homer, PSD-95, GKAP, Shank, calmodulin and others (Table 1.1; Enz, 2007). These molecules modulate mGluR5 signalling and localisation, and are reportedly affected in schizophrenia (Broadbelt and Jones, 2008; Ting et al., 2012). Some other compelling examples include Norbin, Tamalin and Preso1 (discussed in Chapters 1, 3 and 4). Despite that alterations in these molecules could impair mGluR5 activity and regulation (and thus contribute to the pathophysiology of schizophrenia) Norbin, Tamalin and Preso1 have not been previously examined in any region of the postmortem schizophrenia brain.

A very recent study demonstrated that genetic variation within the *GRM5* gene can effect these protein-protein interactions (Timms et al., 2013). A frameshift within *GRM5* in one schizophrenia pedigree was demonstrated to delete the Tamalin binding site on the C-terminus of mGluR5, disrupting Tamalin binding and increasing endocytosis of mGluR5. Further, a missense translating to the Venus flytrap domain of mGluR5 was hypothesised to alter glutamate sensitivity to mGluR5. Only one study previously investigated whether markers within mGluR5 are associated with schizophrenia diagnosis. Devon and colleagues microsatellite-typed a Scottish case-control population, and reported the novel microsatellite was significantly associated with schizophrenia cases (Devon et al., 2001). More recently mGluR5 has been named as one of the top candidate genes in schizophrenia aetiology, although SNPs within *GRM5* have never before been investigated for their association with the disorder. It is possible that dysregulation of mGluR5 protein might originate within *GRM5*, but this is yet to be explored.

There are therefore several avenues by which alterations of mGluR5 signalling/functioning may occur, which are beyond the direct approaches that have been implemented in the past. Although postmortem studies are disadvantaged as manipulation of the studied systems is not feasible, it is still possible to study some aspects of protein regulation using postmortem tissue. By measuring mRNA and protein levels, as well as the levels of regulatory proteins, alterations and correlational analyses between measures in schizophrenia subjects compared to controls provide substantial information. Thus following the completion of Chapter 2 of this thesis, where no change in mGluR5 binding was again detected in the postmortem schizophrenia brain, I set out to advance the current knowledge regarding the status of mGluR5 in schizophrenia pathology by exploring whether mGluR5 is dysregulated in the disorder.

6.1.2 mGluR5 dysregulation in schizophrenia: what we know now

In Chapter 2 of this thesis (Matosin et al., 2014), mGluR5 binding was for the first time investigated in the ACC of individuals with schizophrenia compared to controls, but was not significantly different in the disorder. As mGluR5 levels had not been assessed before in the ACC, there are no studies with which these results can be compared, although the results from this study were consistent with my previous work, where no change in mGluR5 binding was reported in the closely related DLPFC region of a large cohort of schizophrenia patients (Matosin et al., 2013). In the 2013 study, a significant reduction of mGluR5 was observed in schizoaffective subjects of depressive subtype; this observation was not published due to insufficient power (n=4), although the finding was interesting considering Deschwanden and colleagues (2011) previously reported reductions of in vivo binding to the MPEP site of mGluR5 in several PFC areas in individuals with depression, and binding levels correlated with depressive symptom severity.

Considering these results, it was necessary to further and extensively investigate whether alterations of mGluR5 were specific to depressive/negative symptoms, both within schizophrenia but also related disorders such as major depression and bipolar disorder, in which psychosis and depressive symptoms overlap (McCullumsmith et al., 2014). The Stanley cohorts (Neuropathology Consortium and Depression Collection) were chosen for this purpose, as it allowed some degree of dissection of the overlapping symptoms across schizophrenia and related disorders. The results indicated that there was no alteration in mGluR5 binding or presynaptic mGluR2/3 binding (which was added to the original study design to provide additional context) specific to schizophrenia, depression (psychotic or non-psychotic), or bipolar disorder. As binding was not significantly different in these neuropathological states compared to controls, these results also suggest that mGluR5 and mGluR2/3 binding sites are unaltered and therapeutics aimed at these receptors will be unhindered in the pathological states of schizophrenia and the related neuropsychiatric disorders studied.

Despite the fact that my binding studies provide a valuable understanding of the therapeutic utility of mGluR5 and mGluR2/3, this method is inadequate to assess wider dysregulations of these receptors that may occur within these pathologies, especially considering the accumulating evidence that emerged during the time of these studies. This included studies from: (i) Fatemi and colleagues, indicating brain-region specific dysregulation of mGluR5 mRNA and protein levels in schizophrenia (Fatemi et al., 2013); (ii) Hu and colleagues, who highlighted the importance of signalling molecules such as Preso1, that endogenously regulate mGluR5 signalling and function (Hu et al., 2012); (iii) and lastly, Timms and colleagues, who demonstrated that mGluR5 dysregulations might originate within the *GRM5* gene to disrupt downstream protein-protein interactions with molecules that endogenously regulate mGluR5, as well as the presence of genetic variations within *GRM5* in some patients that could potentially alter glutamate sensitivity (Timms et al., 2013). Considering the newly available evidence, the subsequent studies in this thesis aimed to specifically determine whether mGluR5 was dysregulated in two critical brain regions involved in schizophrenia pathology (the DLPFC and CA1), and whether dysregulation of mGluR5 might originate within the *GRM5* gene.

Building on the results from Matosin et al. (2013) and Chapter 2 (Matosin et al., 2014) that indicated unaltered mGluR5 binding in the DLPFC and ACC regions in schizophrenia, the findings in Chapters 3 and 4 demonstrate that there are wider dysregulations occurring within the mGluR5 system not detectable by prior binding studies. In the DLPFC (Chapter 3), although there was no change in mGluR5 mRNA expression, a significant increase in mGluR5 protein (total and dimer measures) was found. These measures were significantly correlated in control subjects, but not in schizophrenia subjects. Further, marked reductions in Norbin, Tamalin and Preso1 were also detected in the DLPFC, and the-

se were significantly associated with mGluR5 protein expression in control subjects but not in schizophrenia subjects. In Chapter 4, a consistent increase in mGluR5 (total, monomer and dimer measures) was found in the hippocampal CA1 region in schizophrenia subjects. Contrasingly in this region, protein levels of all measured mGluR5 endogenous regulators were significantly *increased*, and displayed differential correlations with mGluR5 protein levels, compared to the results in the DLPFC. Additionally, although mGluR5 mRNA measures were not included in the scope of this study, mGluR5 expression was previously reported to be unaltered in the CA1 region, albeit in a small cohort (n=5; Ohnuma et al., 2000b). These findings suggest that alterations to mGluR5 synthesis and stability within the DLPFC likely extend to the CA1 region.

With regards to the exact mechanism underlying the dysregulation of mGluR5, hypotheses have been suggested throughout the end-of-chapter discussions in Chapters 2-5. In addition to the proposed alteration of mGluR5 synthesis and stability, the observed alterations of the endogenous regulators Norbin, Tamalin and Preso1, lend to the possibility of disruption to mGluR5 signalling, trafficking and internalisation, which are processes critically controlled by these proteins (Hu et al., 2012; Kitano et al., 2003; Wang et al., 2009). In this regard, the lack of change in mGluR5 binding (Matosin et al., 2013; Matosin et al., 2014), in addition to evidence that mGluR5 trafficking might be impaired (due to alterations in trafficking molecules Norbin and Tamalin, Chapters 3 and 4), might be indicative of intracellular retention of mGluR5; this is considering binding studies likely measure cell-surface receptors only. As G-protein coupled receptor dimerisation occurs soon after biosynthesis, it is likely that the captive mGluR5 are in the dimer form (see Chapter 3). A growing body of evidence indicates that mGluR5 proteins are only functional in the form of disulfide-linked dimers (El Moustaine et al., 2012; Romano et al., 1996), suggesting an increase in mGluR5 dimers represents an increase in functional mGluR5 units in the present study. However, considering *reduced* mGluR5 signalling (by mGluR5 knockout or pharmacological antagonism) produces schizophrenia-like behaviors, and mGluR5 PAMs have therapeutic efficacy in preclinical rodent models (see Matosin and Newell, 2013), I hypothesise a model of mGluR5 dysregulation in which higher protein expression and dimer units does not represent increased activity and/or function in the schizophrenia brain, but rather, alterations to mGluR5 localisation (Chapter 3, Figure 4a and b) and also signalling (Chapter 3, Figure 4a and c); however, further studies investigating the expression of mGluR5 mRNA/protein within specific cellular compartments will be necessary.

It should also be considered that regulation (and subsequent dysregulation) of mGluR5 is likely brain-region specific. Unlike the DLPFC where protein levels of mGluR5 signalling partners were decreased (Chapter 3), increased levels of expression of these same proteins were observed in CA1 (Chapter 4). Additionally in CA1, there was no correlation between mGluR5 and mGluR5 signaling

partners in control subjects in this region whereas strong correlations were present in the DLPFC in control subjects. This suggests the existence of fundamental differences in mGluR5 regulation in the cortex compared to hippocampal regions irrespective of diseased state. Although both the prefrontal cortex and hippocampus are involved in memory and learning tasks, the differing cell populations, network structures and functions of these regions support that the regulation of mGluR5 in these networks may also differ (Otani, 2004; Schobel et al., 2009; Yoon et al., 2008).

The observed changes to mGluR5 could also represent a compensatory response due to wider dysregulation of the glutamate system (Chapter 3). For example, considering the NMDAR hypofunction hypothesis of schizophrenia, and evidence that expression of the NMDAR is decreased in the DLPFC of these same schizophrenia subjects (Weickert et al., 2012), mGluR5 could be maintained at a high levels on cortical neurons to account for potential NMDAR signalling deficits, at least in these regions. Furthermore in schizophrenia, it has been suggested that region-dependent alterations in glutamate are present, with reduced glutamatergic function in the cortex coupled with hyperglutamatergic function in the hippocampus (Ohnuma et al., 2000b). Reflectively, ionotropic glutamate receptors in these regions are reported to be differentially regulated in subjects with schizophrenia; for example, the majority of evidence indicates alterations to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) are specific to the hippocampus, whilst NMDAR alterations are primarily observed in cortical regions (Meador-Woodruff and Healy, 2000). As mGluR5 modulates both these receptor subtypes, the reasons for regional differences in the relationships of mGluR5 with its signalling partners might be due to differential regulation of glutamate and ionotropic glutamate receptors in cortical compared to hippocampal brain regions. Additionally, mGluR5 exists as three differently sized isoforms with varying length of the intracellular C-terminus and functions (Newell and Matosin, 2014). These isoforms with differing functions are reportedly expressed differentially across brain regions, such as the hippocampus and cerebellum (Malherbe et al., 2002). As previous studies have not individually measured mGluR5 isoforms, it is possible that whilst no change was detected in mRNA levels overall, there may be isoform-dependent differences, and future studies are required to explore isoform-specific differences. Thus, it will be critical for future studies to uncover how mGluR5 is regulated differently in different brain regions.

Additionally, the results from Matosin et al. (2013), Chapter 3 and Chapter 4 provide substantial evidence that short-term (8 days), medium-term (15 days) or long-term exposure (35 days) to commonly used APD agents (haloperidol and olanzapine) do not influence the mGluR5 system, suggesting that the findings from these postmortem studies are unlikely to be confounded by antemortem medication history. However, the 'long-term' treatment period used in the context of these studies is comparable to subchronic treatment, considering more recent studies examining the effects of APDs on mo-

lecular systems have employed significantly longer (chronic) treatment periods used by others in the field (e.g. 12 weeks treatment period was employed by Deng et al., 2015); thus the effects of APD on mGluR5 should be confirmed by future studies in a model of chronic exposure.

In Chapter 5, analyses of SNPs within the 3' UTR region of *GRM5* support that mGluR5 is dysregulated in schizophrenia, and provides additional information suggesting this dysregulation might arise from variation at the gene-level. These findings provide strong support that *GRM5* is associated with schizophrenia, and particularly the cognitive deficits associated with the disorder. This study is the first to bridge pathological findings in mGluR5 with clinical measures in humans, providing invaluable insight into the potential mechanisms involved in mGluR5 dysregulation and its consequences. However, it is important to note that all findings in Chapter 5 were gender-specific, suggesting *GRM5* may operate in a sex-specific manner. Thus, whilst the association of *GRM5*, cognition and schizophrenia is not surprising based on the extensive evidence from animal models of a role for mGluR5 in this context (Matosin and Newell, 2013), the gender-specific findings from this study provide the first evidence that mGluR5 may be affected differently in men and women.

6.2 Further considerations, limitations and recommendations for future research

Collectively, the results from the studies presented in this thesis provide evidence from the gene, mRNA and protein levels that strongly support a model of mGluR5 dysregulation in the pathophysiology of schizophrenia. However, there are numerous issues that must be further deliberated, and caution taken with the interpretation of these results as the present studies do not uncover all the details necessary to draw absolute conclusions.

As postmortem human brain tissue cohorts are a limited resource, it is a significant strength of the current body of work that three brain regions of interest were examined across three independent postmortem cohorts covering four major neuropsychiatric disorders (schizophrenia, bipolar disorder, major depression without psychosis, major depression with psychosis). However these strengths also engender several caveats; specifically, the postmortem studies within this thesis are inconsistent with regards to the experiments performed, brain regions studied and cohorts employed with these factors varying across Chapters 2 to 4. This can lead to holes in the current understanding. For example, it is unclear whether the conflicting results from Chapter 2, in which mGluR5 binding is unaltered, and Chapters 3 and 4, where mGluR5 total protein levels are significantly increased, is driven by cohort-specific differences or brain region heterogeneity of expression. However considering my previous binding study also detected no change in mGluR5 binding in the closely related DLPFC region (Matosin et al., 2013), it is more likely that these differences are a result of different experi-

mental methods (binding versus immunoblot), although replication of these experimental methods in well-powered independent cohorts of identical brain-regions is necessary. Notably, the subjects studied in Chapter 4 (CA1) are a subset of the same subjects examined in Chapter 3 (DLPFC), and mGluR5 was uniformly increased in both these regions, reducing the chance of cohort-specific differences.

One of the limitations of previous work, as previously noted, has been the small sample sizes within earlier studies. This issue also encroaches on my works within Chapters 2 (n=12-15/group), 4 (n=20) and 5 (n=236/group), particularly when sex-wise statistics were performed. These issues are inherent in the field of postmortem human brain analyses, considering their increasingly limited availability due to the field's improved understanding of developing methodically rigorous cohorts that are strictly matched for pH, postmortem interval, age of death, and other demographic and clinical variables; it thus requires many resources and a lot of time to develop high quality cohorts, reducing their availability and size. For the postmortem brain analyses in this thesis, power calculations based on Matosin et al. (2013) suggested that a sample size of 20 subjects per group is required to reliably detect molecular changes as small as 5% with 95% confidence. When investigating differences between mRNA/protein measurements in all schizophrenia subjects compared to controls in the DLPFC and CA1 (Chapter 3 and 4), there was sufficient power to reliably detect alterations (n>20). However, in instances when subjects were reclassified, such as in the case of gender within diagnostic groups (schizophrenia/control), power was reduced. For example, in Chapter 4, although a gender effect was observed overall whereby mGluR5 protein levels were reduced in male (n=28) compared to female (n=11) subjects, the study was slightly underpowered for the mean differences observed, requiring 12 extra female subjects (power level 0.8; power analysis via: http://www.biomath.info/power/ttest.htm) to detect a significant change within schizophrenia/control groups. Similarly in Chapter 5, a power calculation indicates that for the smaller of the two cohorts, a sample size of 268 cases (536 alleles) with a MAF of 0.14 has >90% has a priori power to detect a significant allelic association conferring an odds ratio of 1.5 or greater. Although the case-control cohort possessed sufficient power overall, when separated for gender and/or GoM-subtype analyses, the power was reduced. Thus, it is imperative that future studies specifically investigating mGluR5/*GRM5* should consider that alterations might be gender-dependent, and account for this series of analyses when choosing the sample size in the study design.

Considering that the evidence from this collection of studies suggests that mGluR5 is dysregulated in the pathophysiology of schizophrenia, these findings have numerous implications for future novel mGluR5-targeting therapies. Traditionally, the identification of pathological alterations in mGluR5 is considered supportive of the use of therapeutics directed at mGluR5 to correct these dysfunctions.

However, evidence suggests there is a possibility that currently developed mGluR5-targeted drugs may not be of therapeutic value:

- Many drugs targeting mGluR5 are not membrane permeable, and depend on cell-surface expression of mGluR5; thus, it must be confirmed whether there are available surface targets for mGluR5 in schizophrenia regions involved in the pathology. Whilst the binding studies previously reported suggest the availability of binding sites at least in the DLPFC and ACC (Matosin et al., 2013; Matosin et al., 2014), this should be confirmed in cell-membrane fractions from postmortem samples.
- There is extensive evidence that mGluR5 is subjected to "location bias", whereby 60-90% of mGluR5 are reportedly located within intracellular membranes. Purgert and colleagues published an interesting study earlier this year, demonstrating that mGluR5 is able to signal from these intracellular locations, where they play a particularly important role in modulation of hippocampal synaptic plasticity (Purgert et al., 2014). Considering this evidence, it is critical for future studies to determine whether novel mGluR5-targeting drugs should in fact be designed to target intracellular mGluR5.
- The previously discussed alterations to mGluR5 localisation, dimerisation, glutamate insensitivity (as hypothesised by Timms et al., 2013), or disrupted protein-protein interactions with endogenous regulators known to modulate mGluR5 function (such as Norbin and Preso1 that modulate mGluR5 signalling, and evidence to support this hypothesis in Chapters 3-5) might render mGluR5 units present, but not functional. Even considering this possibility, there is still potential for the use of pharmacological agents targeted at the mGluR5 system. However, first, future pathological studies are required to determine the nature of these alterations so that the appropriate therapeutics may be developed; for example, if trafficking deficits in mGluR5 are confirmed, these deficits may be more successfully treated with pharmacoperones rather than allosteric modulators (including PAMs). It is recommended that future studies uncover the precise nature of mGluR5 dysfunction, so that the appropriate types of therapeutics may be developed.

6.3 Conclusion

In addition to the widely reported role of glutamate in the emergence of cognitive deficits in schizophrenia, and animal studies demonstrating that mGluR5 critically controls the mechanisms underlying cognitive function, the findings from this thesis provide the first evidence of mGluR5 dysregulation in schizophrenia. These findings significantly contribute to the existing body of evidence that mGluR5 is involved in the pathophysiology of the disorder. Furthermore, these findings support the development of agents that modulate mGluR5 regulation and/or signalling to treat the cognitive dys-

functions in patients with schizophrenia, although additional studies are required to first uncover the consequences of these alterations.

The present study offers a substantial contribution to the existing knowledge of the role of mGluR5 in the pathophysiology of schizophrenia. Furthermore, in line with the existing evidence from animal studies that mGluR5 alterations induce deficits in synaptic plasticity, the results form Chapter 5 show, for the first time, that *GRM5* has a modulatory effect on cognitive function in humans. As mGluR5-targeting drugs have cognitive-enhancing properties in preclinical paradigms, and current APDs minimally treat the cognitive deficits associated with schizophrenia, these studies collectively suggest that mGluR5 may offer value as an adjunct cognitive-enhancing drug in those patients suffering from the cognitive aspects of the disorder. However, due to the heterogeneity of schizophrenia, an individualised treatment approach will likely be necessary. Therefore future studies will be required to identify the patient subset that will benefit from mGluR5-targeted treatments.

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Appendix

1. Supplementary Materials, Chapter 2

Supplementary Table 1. Pearson's Correlations for continuous clinical and demographic variables against mGluR2/3 and mGluR5 binding densities in the anterior cingulate cortex within diagnostic groups of the (a) Depression Collection and the (b) Neuropathology Consortium.

(a)

	All subjects (n=36)				Controls (n=12)				Major Depression (all subjects) (n=24)				Major Depression (with psychosis) (n=11)				Major Depression (without psychosis) (n=12)			
Variable	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**
Brain pH	r = 0.297	r = 0.270	r = 0.263	**r = -0.486**	r = -0.103	r = -0.188	r = 0.044	**r = -0.734**	**r = 0.568**	**r = 0.630**	r = 0.389	r = -0.343	**r = 0.611**	**r = 0.634**	r = 0.528	r = -0.515	r = 0.477	r = 0.536	r = 0.374	r = -0.257
	p = 0.079	p = 0.112	p = 0.121	**p = 0.003**	p = 0.749	p = 0.559	p = 0.892	**p = 0.007**	**p = 0.004**	**p = 0.001**	p = 0.060	p = 0.101	**p = 0.046**	**p = 0.036**	p = 0.095	p = 0.105	p = 0.117	p = 0.072	p = 0.232	p = 0.420
Brain weight	r = 0.207	r = 0.153	r = 0.226	r = 0.025	r = 0.361	r = 0.361	r = 0.274	r = 0.242	r = 0.147	r = 0.074	r = 0.200	r = -0.053	r = 0.179	r = 0.114	r = 0.204	r = 0.030	r = 0.148	r = 0.099	r = 0.206	r = -0.183
	p = 0.226	p = 0.373	p = 0.186	p = 0.885	p = 0.248	p = 0.249	p = 0.388	p = 0.449	p = 0.493	p = 0.731	p = 0.350	p = 0.804	p = 0.598	p = 0.738	p = 0.547	p = 0.930	p = 0.645	p = 0.759	p = 0.521	p = 0.570
Post-mortem interval	**r = -0.415**	**r = -0.516**	r = -0.197	r = -0.022	**r = -0.824**	**r = -0.870**	r = -0.558	r = -0.008	r = -0.235	r = -0.330	r = -0.084	r = -0.024	r = 0.114	r = 0.285	r = -0.016	r = 0.079	r = -0.203	r = -0.129	r = -0.291	r = -0.326
	p = 0.012	**p = 0.001**	p = 0.250	p = 0.899	**p = 0.001**	**p < 0.001**	p = 0.059	p = 0.980	p = 0.269	p = 0.116	p = 0.698	p = 0.913	p = 0.738	p = 0.395	p = 0.963	p = 0.817	p = 0.527	p = 0.690	p = 0.359	p = 0.301
Refrigerator Interval	**r = -0.341**	**r = -0.337**	**r = 0.609**	r = 0.033	r = -0.194	r = -0.205	r = -0.132	r = -0.117	**r = -0.418**	r = -0.330	**r = -0.437**	r = 0.097	r = -0.020	r = 0.281	r = -0.224	r = 0.274	**r = -0.604**	r = -0.520	**r = -0.685**	r = -0.031
	p = 0.045	**p = 0.048**	**p < 0.001**	p = 0.851	p =0.545	p = 0.523	p = 0.683	p = 0.717	**p = 0.047**	p = 0.124	**p = 0.037**	p = 0.659	p = 0.954	p = 0.402	p = 0.507	p = 0.416	**p = 0.049**	p = 0.101	**p = 0.020**	p = 0.927
Age of Disease Onset	-	-	-	-	-	-	-	-	**r = -0.656**	**r = -0.507**	**r = -0.529**	r = 0.343	**r = -0.715**	**r = -0.779**	**r = -0.591**	r = 0.461	**r = -0.658**	**r = -0.635**	**r = -0.655**	r = 0.177
	-	-	-	-	-	-	-	-	**p < 0.001**	**p = 0.011**	**p = 0.008**	p = 0.101	**p = 0.013**	**p = 0.005**	**p = 0.055**	p = 0.153	**p = 0.020**	**p = 0.026**	**p = 0.021**	p = 0.583
Duration of illness	-	-	-	-	-	-	-	-	r = 0.207	r = 0.181	r = 0.196	r = 0.246	r = 0.142	r = 0.321	r = 0.003	r = 0.018	r = 0.500	r = 0.452	r = 0.537	r = 0.447
	-	-	-	-	-	-	-	-	p = 0.333	p = 0.398	p = 0.360	p = 0.246	p = 0.677	p = 0.336	p = 0.993	p = 0.958	p = 0.098	p = 0.140	p = 0.072	p = 0.145

(b)

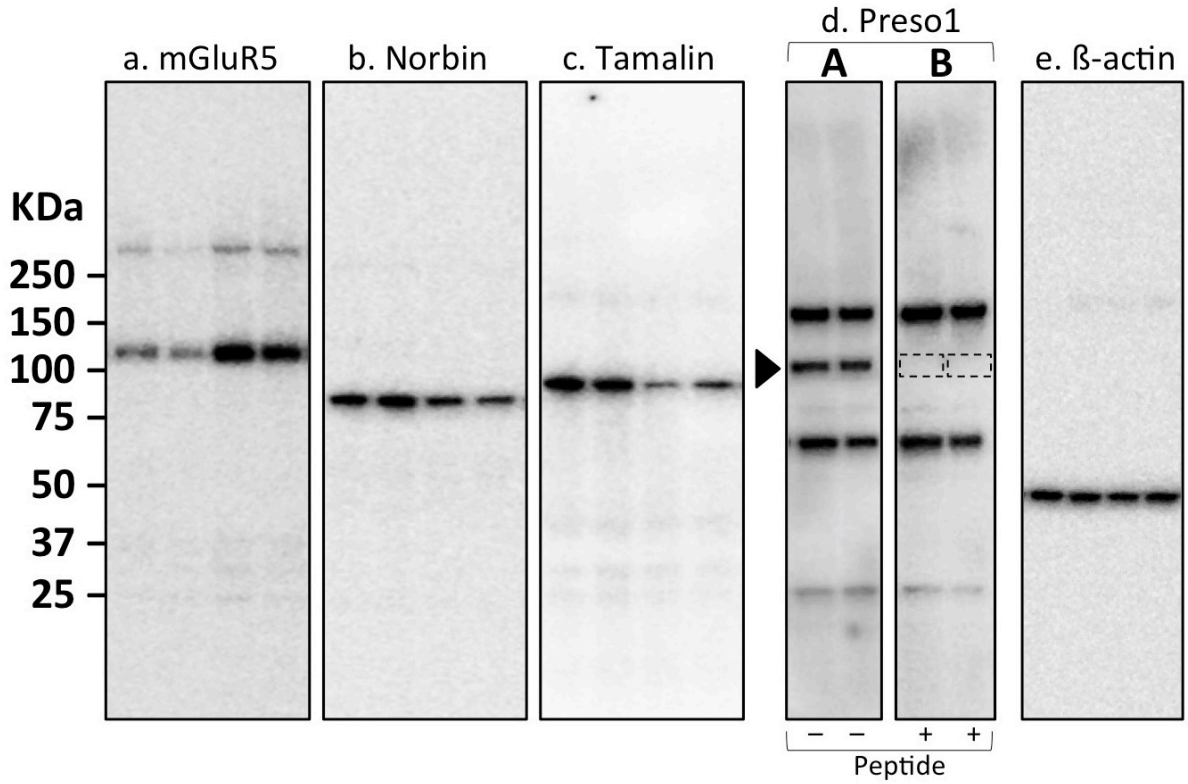
	All subjects (n=60)				Controls (n=15)				Major Depression (n=15)				Bipolar Disorder (n=15)				Schizophrenia (n=15)			
Variable	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**	**mGluR2/3 total**	**mGluR2/3 upper**	**mGluR2/3 lower**	**mGluR5 total**
Brain pH	**r = -0.263**	r = -0.127	r = -0.246	**r = -0.263**	r = -0.279	r = -0.123	r = -0.349	r = -0.181	r = 0.365	r = 0.325	r = 0.200	r = -0.441	**r = -0.554**	r = -0.436	**r = -0.558**	r = -0.058	r = 0.007	r = -0.011	r = 0.025	r = -0.381
	p = 0.042	p = 0.334	p = 0.058	**p = 0.042**	p = 0.313	p = 0.663	p = 0.203	p = 0.519	p = 0.181	p = 0.238	p = 0.476	p = 0.100	**p = 0.032**	p = 0.104	**p = 0.031**	p = 0.837	p = 0.981	p = 0.969	p = 0.931	p = 0.162
Brain weight	r = -0.032	r = 0.252	r = -0.003	r = -0.032	r = -0.210	r = -0.011	r = -0.328	r = 0.163	r = 0.234	r = 0.453	r = -0.417	r = 0.100	r = 0.494	**r = 0.579**	r = 0.334	r = -0.047	r = 0.265	r = 0.214	r = 0.276	r = -0.376
	p = 0.809	p = 0.052	p = 0.981	p = 0.809	p = 0.453	p = 0.969	p = 0.233	p = 0.562	p = 0.401	p = 0.090	p = 0.601	p = 0.724	p = 0.061	**p = 0.024**	p = 0.224	p = 0.868	p = 0.339	p = 0.444	p = 0.319	p = 0.167
Post-mortem interval	r = -0.129	r = -0.123	r = -0.108	r = 0.023	r = 0.281	r = 0.437	r = 0.096	r = 0.145	r = -0.398	r = -0.262	r = -0.323	r = 0.113	r = -0.129	r = -0.174	r = -0.067	r = 0.004	r = -0.512	**r = -0.558**	r = -0.376	r = -0.040
	p = 0.325	p = 0.350	p = 0.413	p = 0.861	p = 0.310	p = 0.103	p = 0.733	p = 0.606	p = 0.142	p = 0.346	p = 0.241	p = 0.689	p = 0.647	p = 0.534	p = 0.812	p = 0.989	p = 0.051	**p = 0.031**	p = 0.167	p = 0.888
Freezer storage time	**r = -0.327**	r = 0.097	r = 0.003	**r = -0.327**	r = -0.219	r = -0.142	r = -0.236	r = -0.294	r = 0.144	r = 0.001	r = 0.222	**r = -0.559**	r = 0.294	**r = 0.569**	r = 0.006	r = -0.286	r = -0.105	r = -0.164	r = -0.023	r = -0.358
	p = 0.011	p = 0.461	p = 0.981	**p = 0.011**	p = 0.433	p = 0.613	p = 0.398	p = 0.288	p = 0.608	p = 0.997	p = 0.426	**p = 0.030**	p = 0.287	**p = 0.027**	p = 0.983	p = 0.302	p = 0.710	p = 0.559	p = 0.936	p = 0.190
Age of Disease Onset	-	-	-	-	-	-	-	-	r = -0.184	r = -0.312	r = 0.066	r = -0.378	r = 0.209	r = 0.093	r = 0.273	r = 0.463	r = 0.131	r = 0.300	r = -0.074	r = 0.390
	-	-	-	-	-	-	-	-	p = 0.511	p = 0.257	p = 0.816	p = 0.165	p = 0.455	p = 0.742	p = 0.326	p = 0.082	p = 0.642	p = 0.278	p = 0.792	p = 0.151
Duration of illness	-	-	-	-	-	-	-	-	r = 0.006	r = 0.077	r = -0.077	r = 0.065	r = -0.184	r = -0.325	r = -0.030	r = -0.378	**r = -0.697**	**r = -0.734**	**r = -0.539**	r = 0.308
	-	-	-	-	-	-	-	-	p = 0.982	p = 0.786	p = 0.786	p = 0.817	p = 0.511	p = 0.237	p = 0.915	p = 0.164	**p = 0.004**	**p = 0.002**	**p = 0.038**	p = 0.263

Supplementary Table 2: Correlation (r) and signifance (p) values from Spearman's correlations between mGluR2/3 and mGluR5 measured in the Neuropathology Consortium in the present study, with (a) glutamatergic receptor markers and (b) related signalling partners measured in the same cohort from previous studies.

	Marker	Ref	Method		All subjects		Control		Major Depression		Bipolar		Schizophrenia	
					mGluR2/3	mGluR5	mGluR2/3	mGluR5	mGluR2/3	mGluR5	mGluR2/3	mGluR5	mGluR2/3	mGluR5
(a)	**NMDA receptor**	a	[3H]MK801 Binding	r	0.253	-0.169	0.418	0.400	0.154	-0.382	0.411	-0.189	0.436	**-0.650**
	(ion channel)			p	0.051	0.198	0.121	0.140	0.585	0.160	0.128	0.499	0.104	**0.009**
	NMDA receptor		[3H]ifenprodil Binding	r	-0.095	-0.083	**-0.582**	-0.221	0.014	**-0.567**	0.350	-0.061	-0.311	0.214
	(NR1/NR2B-containing NMDARs)			p	0.469	0.529	**0.023**	0.428	0.960	**0.028**	0.201	0.830	0.260	0.443
	NMDA receptor		[3H]MDL105,519 Binding	r	-0.028	**-0.311**	-0.129	-0.361	-0.018	0.107	-0.089	**-0.611**	0.104	-0.307
	(glycine site)			p	0.831	**0.016**	0.648	0.187	0.950	0.704	0.752	**0.016**	0.713	0.265
	NMDA receptor		[3H]CGP39653 Binding	r	0.022	**-0.257**	0.226	-0.174	-0.175	-0.304	0.014	-0.289	0.079	-0.264
	(NMDA agonist site)			p	0.868	**0.049**	0.436	0.553	0.533	0.271	0.960	0.296	0.781	0.341
	NMDA receptor		In situ hybridisation	r	-0.019	-0.189	-0.068	**-0.593**	0.121	-0.325	-0.186	-0.004	0.393	-0.146
	(NR1 subunit)			p	0.884	0.149	0.810	**0.020**	0.666	0.237	0.508	0.990	0.147	0.603
	NMDA receptor			r	0.172	-0.232	0.293	0.021	0.214	-0.275	0.075	-0.204	**0.600**	-0.464
	(NR2A subunit)			p	0.193	0.076	0.289	0.940	0.443	0.321	0.791	0.467	**0.023**	0.095
	NMDA receptor			r	0.210	**-0.272**	0.304	0.396	0.050	**-0.689**	0.086	-0.021	0.225	**-0.604**
	(NR2B subunit)			p	0.107	**0.035**	0.271	0.143	0.860	**0.004**	0.761	0.940	0.420	**0.017**
	NMDA receptor			r	0.079	0.003	0.325	0.318	0.050	0.207	0.108	0.134	0.404	0.211
	(NR2C subunit)			p	0.553	0.982	0.237	0.248	0.860	0.459	0.714	0.648	0.136	0.451
	NMDA receptor			r	-0.026	**-0.368**	-0.364	-0.457	-0.061	-0.350	0.186	**-0.543**	0.039	-0.025
	(NR2D subunit)			p	0.845	**0.004**	0.182	0.087	0.830	0.201	0.508	**0.037**	0.889	0.930
	NMDA receptor	b	Immunoprecipitation	r	-0.131	-0.163	0.175	-0.504	0.116	-0.416	**-0.695**	0.218	-0.493	0.068
	(NR1 subunit)			p	0.320	0.213	0.533	0.056	0.680	0.123	**0.004**	0.435	0.062	0.810
	NMDA receptor			r	-0.012	-0.014	-0.100	0.050	-0.234	0.361	0.002	0.020	0.391	-0.452
	(NR2A subunit)			p	0.925	0.914	0.723	0.860	0.401	0.186	0.995	0.945	0.150	0.091
	NMDA receptor			r	-0.018	-0.069	-0.261	0.132	-0.029	**-0.718**	-0.002	0.472	0.322	-0.222
	(NR2B subunit)			p	0.893	0.600	0.348	0.639	0.919	**0.003**	0.995	0.076	0.242	0.427
	NMDA receptor			r	-0.035	-0.019	-0.132	0.207	0.025	0.202	-0.306	-0.225	0.415	-0.282
	(NR3A subunit)			p	0.790	0.885	0.639	0.459	0.929	0.470	0.268	0.419	0.124	0.308
	AMPA receptor	a	In situ hybridisation	r	0.226	0.038	-0.021	0.175	**0.596**	-0.311	0.118	0.236	0.389	0.059
	(GluR1 subunit)			p	0.086	0.776	0.940	0.533	**0.019**	0.260	0.676	0.398	0.169	0.840
	AMPA receptor			r	-0.037	0.031	-0.275	-0.284	-0.077	0.288	-0.059	0.305	0.393	-0.367
	(GluR2 subunit)			p	0.787	0.819	0.342	0.326	0.794	0.318	0.840	0.288	0.164	0.197
	AMPA receptor			r	-0.042	0.181	-0.504	0.282	0.011	0.266	0.182	**0.547**	0.446	-0.024
	(GluR3 subunit)			p	0.759	0.178	0.056	0.308	0.970	0.358	0.533	**0.043**	0.110	0.935
	AMPA receptor			r	0.055	0.007	0.146	**-0.575**	0.032	0.221	-0.086	-0.111	-0.046	0.451
	(GluR4 subunit)			p	0.681	0.956	0.603	**0.025**	0.909	0.428	0.761	0.694	0.876	0.106
	Kainate receptor		[3H]kainate Binding	r	-0.008	-0.076	-0.468	0.179	0.143	-0.305	0.292	0.020	0.114	-0.421
	(Kainate agonist site)			p	0.953	0.572	0.079	0.524	0.626	0.288	0.311	0.946	0.685	0.118
	Kainate receptor		In situ hybridisation	r	0.162	-0.035	0.175	-0.100	0.475	-0.004	-0.007	0.307	0.189	-0.075
	(GluR5 subunit)			p	0.217	0.790	0.533	0.723	0.074	0.990	0.980	0.265	0.499	0.791
	Kainate receptor			r	0.067	-0.163	-0.021	-0.371	0.409	-0.064	-0.156	-0.064	**0.673**	-0.212
	(GluR6 subunit)			p	0.653	0.275	0.948	0.236	0.212	0.853	0.594	0.829	**0.033**	0.556
	Kainate receptor			r	**0.273**	-0.252	0.329	-0.250	0.161	-0.254	0.154	0.150	**0.534**	**-0.618**
	(GluR7 subunit)			p	**0.036**	0.054	0.232	0.369	0.567	0.362	0.585	0.594	**0.049**	**0.019**
	Kainate receptor			r	0.173	-0.151	0.464	0.004	0.157	-0.343	-0.104	0.107	0.240	-0.336
	(KA1 subunit)			p	0.189	0.255	0.081	0.990	0.576	0.211	0.713	0.704	0.409	0.240
	Kainate receptor			r	-0.049	-0.055	-0.089	0.064	0.064	-0.275	**-0.650**	0.482	0.490	-0.138
	(KA2 subunit)			p	0.712	0.677	0.752	0.820	0.820	0.321	**0.009**	0.069	0.075	0.637
(b)	**PSD95**	b	Immunoprecipitation	r	**0.280**	**-0.271**	0.021	0.261	0.461	0.068	0.374	-0.440	0.342	**-0.767**
				p	**0.030**	**0.036**	0.940	0.348	0.084	0.810	0.170	0.101	0.213	**0.001**
	BDNF	c	In situ hybridization	r	-0.010	0.086	-0.211	-0.186	0.254	-0.089	-0.025	**0.550**	-0.046	-0.214
				p	0.943	0.511	0.451	0.508	0.362	0.752	0.930	**0.034**	0.869	0.443
	PKC alpha	d	Western blot	r	0.016	-0.229	-0.400	-0.321	-0.209	0.242	0.181	0.071	0.525	**-0.727**
	total, cytostolic			p	0.910	0.093	0.140	0.243	0.494	0.426	0.553	0.817	0.054	**0.003**
	PKC alpha			r	-0.036	-0.109	-0.104	-0.239	0.077	0.137	-0.349	-0.033	0.499	-0.380
	phosphorylation, cytostolic			p	0.793	0.425	0.713	0.390	0.803	0.655	0.221	0.911	0.069	0.180
	PKC epsilon			r	-0.093	-0.158	0.385	-0.357	-0.373	-0.009	-0.119	-0.619	-0.048	0.071
	total, cytostolic			p	0.569	0.330	0.194	0.231	0.259	0.979	0.779	0.102	0.911	0.867
	PKC epsilon			r	-0.164	-0.186	0.196	-0.035	-0.442	-0.055	-0.167	-0.667	-0.600	0.300
	phosphorylation, cytostolic			p	0.347	0.285	0.542	0.914	0.200	0.881	0.693	0.071	0.285	0.624

P<0.05 are bolded/highlighted. **Abbreviations:** NMDA: n-methyl-D-aspartate; NMDAR: NMDA receptor; 3H: tritiated; PKC: protein kinase C; PSD95: post-synaptic density protein 95. References: all data referred to are available on the Stanley Neuropathology Consortium Integrative Database (http://sncid.stanleyresearch.org/), and were produced by the following researchers: **a.** Meador Woodruff et al; **b.** Hahn et al; **c.** Webster et al, ; **d.** Manji et al.

2. Supplementary Materials, Chapter 3



Supplementary Fig. 1 Full-length immunoblots for (a) mGluR5, (b) Norbin, (c) Tamalin, and (d) Preso1 (A. with no peptide, B. with blocking peptide). mGluR5 (a) appeared as 2 distinct bands at 135kDa (monomer) and 270kDa (dimer) and these bands have previously been shown to be specific for mGluR5 in HEK cells transfected with mGluR5 (www.abcam.com/Metabtropic-Glutamate-Receptor-5-antibody-ab27190). Norbin (b) and Tamalin (c) were reliably detected as single bands at the expected molecular weights (79kDa and 96kDa respectively) with no non-specific binding. The Preso1 antibody (Santa Cruz sc-242862) produced several bands at various molecular weights (d); A single specific band was verified at 144kDa by peptide pre-absorption/neutralization,and this band was quantified.The representation of ß-actin (e); is taken from the mGluR5 immunoblot which was reprobed with ß-actin as a loading control. ß-actin was measured in the same way for all immunoblots in this study.

Supplementary Methods: Determination of Preso1 specificity by peptide pre-absorption (FRMPD4 Antibody, Santa Cruz: sc-242862 and sc-242862 P)

Peptide pre-absorption/neutralization was performed according to the protocol described by Santa Cruz (http://www.scbt.com/protocols.html?protocol=peptide_neutralization). The highest concentration of antibody dilution for which a consistently positive result was achieved,was determined to be 1:1000. For blocking/competition, 10µL of antibody, 50µL of peptide and 500µL of PBS, was combined and incubated for 2 hours at room temperature. The antibody/peptide solution was subsequently diluted up to 10mL with 0.5% skim milk in PBST, and applied to membranes containing 15µg of electrophoresed protein from the rat prefrontal cortex. These immunoblots were prepared and electrophoresed as described in the main methods.

Supplementary Table 1. Immunoblot conditions: all immunoblots were run on bis-trispolyacrymalide gels (4-12% Bis-Tris, BioRad), at a loading concentration of 5µg (human) or 10µg (rat) of total protein per lane. Samples were reduced with laemmli buffer containing ß-mercaptoethanol.

				1º antibody			2º antibody			
Protein	Buffer	Block	Denaturing	Dilution (human, rat)	Temp.	Time	Type	Dilution	Temp	Time
mGluR5	PBST	Skim milk	95˚C for 5min	1:250, 1:500	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Tamalin	PBST	BSA	95˚C for 5min	1:100, 1:1000	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Norbin	PBST	BSA	95˚C for 5min	1:500, 1:500	4ºC	16 hr	Rabbit	1:3000	RT	1 hr
Preso1	PBST	BSA	95˚C for 5min	1:100, 1:1000	4ºC	16 hr	Goat	1:3000	RT	1 hr
ß-actin	As for primary antibody			1:5000, 1:5000	RT	1 hr	Mouse	1:3000	RT	1 hr

Abbreviations: BSA: Bovine serum albumin; mGluR5: metabotropic glutamate receptor 5; PBST: phosphate-buffered saline containing Tween-20; RT: room temperature.

Supplementary Table 2. Spearman's Correlations for continuous clinical and demographic variables and mGluR5 (mRNA, total and dimer), Norbin, Tamalin and Preso1 protein levels in the postmortem dorsolateral prefrontal cortex. Significant values ($p<0.05$) are highlighted in bold.

Variable	All subjects						Controls						Schizophrenia					
	mGluR5 mRNA	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**	**mGluR5 mRNA**	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**	**mGluR5 mRNA**	**mGluR5 (total)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**Preso1**
Brain pH	**r = 0.267**	r = 0.001	r = 0.006	r = 0.123	r = -0.021	r = -0.062	r = 0.137	r = 0.002	r = -0.133	r = -0.028	r = -0.150	r = 0.110	r = 0.351	r = 0.063	r = 0.176	r = 0.175	r = 0.096	r = -0.078
	p = 0.021	p = 0.994	p = 0.960	p = 0.297	p = 0.860	p = 0.598	p = 0.420	p = 0.992	p = 0.431	p = 0.870	p = 0.375	p = 0.517	p = 0.033	p = 0.714	p = 0.303	p = 0.301	p = 0.574	p = 0.645
Age at Death	**r = -0.242**	r = -0.037	r = 0.012	r = -0.009	r = 0.175	r = 0.108	**r = -0.417**	r = -0.110	r = 0.103	r = 0.171	**r = 0.353**	r = 0.218	r = -0.106	r = 0.192	r = 0.095	r = -0.193	r = 0.012	r = 0.009
	p = 0.037	p = 0.758	p = 0.920	p = 0.939	p = 0.136	p = 0.358	**p = 0.010**	p = 0.524	p = 0.543	p = 0.311	**p = 0.032**	p = 0.194	p = 0.531	p = 0.261	p = 0.580	p = 0.253	p = 0.946	p = 0.959
RNA integrity number	**r = 0.229**	r = 0.038	r = 0.134	r = 0.122	r = 0.069	r = 0.098	r = 0.050	r = -0.061	r = 0.047	r = 0.046	r = -0.102	r = -0.020	**r = 0.437**	r = 0.176	**r = 0.355**	r = 0.312	r = 0.305	r = 0.265
	p = 0.050	p = 0.748	p = 0.258	p = 0.301	p = 0.560	p = 0.408	p = 0.767	p = 0.724	p = 0.784	p = 0.788	p = 0.547	p = 0.905	**p = 0.007**	p = 0.306	**p = 0.034**	p = 0.060	p = 0.066	p = 0.113
Post-mortem interval	r = 0.124	r = -0.055	r = 0.174	r = 0.132	r = 0.102	r = 0.002	r = -0.017	r = -0.179	r = -0.081	r = 0.155	r = 0.184	r = 0.119	r = 0.213	r = -0.066	r = 0.273	r = -0.259	r = 0.083	r = -0.027
	p = 0.292	p = 0.649	p = 0.140	p = 0.262	p = 0.385	p = 0.987	p = 0.921	p =0.297	p = 0.636	p = 0.358	p = 0.275	p = 0.483	p = 0.205	p =0.703	p = 0.107	p = 0.122	p = 0.623	p = 0.874
Freezer storage time	r = 0.043	r =0.221	r = 0.146	**r = -0.132**	**r = -0.446**	**r = -0.369**	r = 0.301	**r = 0.501**	r = 0.275	**r = -0.348**	**r = -0.533**	**r = -0.391**	r = -0.254	**r = -0.373**	**r = -0.446**	**r = -0.375**	r = -0.242	r = -0.245
	p = 0.715	p = 0.062	p = 0.217	**p = 0.001**	**p< 0.001**	**p = 0.001**	p = 0.070	**p =0.002**	p = 0.099	**p = 0.035**	**p< 0.001**	**p = 0.017**	p = 0.129	**p =0.025**	**p = 0.006**	**p = 0.022**	p = 0.149	p = 0.145
Brain weight	**r = 0.270**	r = -0.009	r = -0.114	r = 0.120	r = 0.065	r = 0.039	r = 0.181	r = -0.022	r = -0.189	r = 0.055	r = -0.001	r = 0.110	**r = 0.398**	r = 0.057	r = -0.061	r = -0.023	r = 0.084	r = -0.103
	p = 0.020	p = 0.940	p = 0.339	p = 0.309	p = 0.581	p = 0.740	p = 0.282	p =0.899	p = 0.263	p = 0.747	p = 0.993	p = 0.515	**p = 0.015**	p =0.470	p = 0.724	p = 0.895	p = 0.620	p = 0.543
Age of disease onset	-	-	-	-	-	-	-	-	-	-	-	-	r = -0.213	r = 0.130	r = -0.072	**r = -0.362**	r = -0.108	**r = -0.364**
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.206	p =0.450	p = 0.674	**p = 0.028**	p = 0.524	**p = 0.027**
Duration of illness	-	-	-	-	-	-	-	-	-	-	-	-	r = -0.009	r = 0.120	r = 0.082	r = -0.076	r = -0.079	r = 0.172
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.959	p =0.486	p = 0.634	p = 0.654	p = 0.641	p = 0.308
Lifetime antipsychotic drug medication	-	-	-	-	-	-	-	-	-	-	-	-	r = 0.038	r = 0.015	p = 0.051	r = 0.043	r = 0.037	r = -0.030
	-	-	-	-	-	-	-	-	-	-	-	-	p = 0.823	p =0.932	p = 0.768	p = 0.802	p = 0.826	p = 0.861

Supplementary Table 3 Spearman's correlations for associations between mGluR5 (total and dimer protein measures, and mRNA) and novel mGluR5 signaling partners, Norbin, Tamalin and Preso1 protein levels in the dorsolateral prefrontal cortex of the postmortem schizophrenia brain. Significant values ($p<0.05$) are highlighted in bold.

CONTROL	mGluR5 total (protein)	mGluR5 dimer (protein)	mGluR5 expression (mRNA)
mGluR5(tot) (protein)	- -	**r = 0.512 p = 0.001**	**r = 0.361 p = 0.030**
mGluR5(dim) (protein)	**r = 0.512 p = 0.001**	- -	r = 0.050 p = 0.770
mGluR5 (mRNA)	**r = 0.361 p = 0.030**	r = 0.050 p = 0.770	- -
Norbin (protein)	**r = -0.657 p< 0.001**	**r = -0.227 p = 0.018**	**r = -0.500 p = 0.002**
Tamalin (protein)	**r = -0.560 p< 0.001**	r = -0.196 p = 0.254	**r = -0.470 p = 0.003**
Preso1 (protein)	**r = -0.428 p = 0.009**	**r = -0.367 p = 0.026**	**r = -0.372 p = 0.023**

SCHIZOPHRENIA	mGluR5 total (protein)	mGluR5 dimer (protein)	mGluR5 expression (mRNA)
mGluR5(tot) (protein)	- -	**r = 0.608 p< 0.001**	r = 0.059 p = 0.732
mGluR5(dim) (protein)	**r = 0.608 p< 0.001**	- -	r = 0.125 p = 0.468
mGluR5 (mRNA)	r = 0.059 p = 0.732	r = 0.125 p = 0.468	- -
Norbin (protein)	r = 0.096 p = 0.578	**r = 0.388 p = 0.019**	**r = 0.466 p = 0.004**
Tamalin (protein)	r = 0.202 p = 0.238	r = 0.175 p = 0.307	r = 0.051 p = 0.764
Preso1 (protein)	r = 0.080 p = 0.644	r = -0.069 p = 0.689	r = -0.009 p = 0.960

3. Accepted manuscript, with a subset of data from Chapter 4

Full-length paper

Metabotropic glutamate receptor 5, and its trafficking molecules Norbin and Tamalin, are increased in the CA1 hippocampal region of subjects with schizophrenia

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Abstract

Metabotropic glutamate receptor 5 (mGluR5) is involved in hippocampal-dependent learning and memory, which are processes disrupted in schizophrenia. Recent evidence from human genetic and animal studies suggests that the regulation of mGluR5, including its interaction with trafficking molecules, may be altered in the disorder. However there have been no investigations of hippocampal mGluR5 or mGluR5 trafficking molecules in the postmortem schizophrenia brain to confirm this. In the present study, we investigated whether protein expression of mGluR5, as well as Norbin and Tamalin (modulators of mGluR5 signalling and trafficking), might be altered in the schizophrenia brain, using postmortem samples from the hippocampal CA1 region of schizophrenia subjects and matched controls (n=20/group). Protein levels of mGluR5 (total: 42%, $p<0.001$; monomer: 25%, $p=0.011$; dimer: 52%, $p< 0.001$) and mGluR5 trafficking molecules (Norbin: 47%, $p<0.001$; Tamalin: 34%, $p=0.009$) were significantly higher in schizophrenia subjects compared to controls. To determine any influence of antipsychotic drug treatment, all proteins were also correlated with lifetime chlorpromazine equivalents in patients, and separately measured in the hippocampus of rats exposed to haloperidol or olanzapine treatment. mGluR5 was negatively correlated with lifetime antipsychotic drug exposure in schizophrenia patients, suggesting antipsychotic drugs could reduce mGluR5 protein in schizophrenia subjects. In contrast, mGluR5 and mGluR5 trafficking molecules were not altered in the hippocampus of antipsychotic drug treated rats. This investigation provides strong support for the hypothesis that mGluR5 is involved in the pathology of schizophrenia, and that alterations to mGluR5 trafficking might contribute to the hippocampal-dependent cognitive dysfunctions associated with this disorder.

Keywords: mGluR5, Hippocampus, Norbin, Tamalin, Trafficking, Schizophrenia

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APD, antipsychotic drug; DLPFC, Dorsolateral prefrontal cortex; mGluR5, metabotropic glutamate receptor 5; NMDAR, N-methyl-D-aspartate receptor; PAM, positive allosteric modulator; PFC, prefrontal cortex; PMI, postmortem interval.

1. Introduction

Metabotropic glutamate receptor subtype 5 (mGluR5) is a postsynaptic G-protein coupled receptor (GPCR), best known for its ability to modulate postsynaptic currents induced by the ionotropic N-methyl-D-aspartate glutamate receptor (NMDAR; Matosin and Newell, 2013). A substantial body of evidence indicates that mGluR5 is critically involved in hippocampal-dependent learning and memory. Specifically, several studies have reported that hippocampal mGluR5 activity is important for long-term potentiation (LTP) and long-term depression (LTD), molecular mechanisms underpinning synaptic plasticity (see Mukherjee and Manahan-Vaughan, 2013). *mGluR5* knockout mice display reduced LTP, specifically in the hippocampal cornu ammonis 1 (CA1) region, as well as deficits in spatial learning and memory (Lu et al., 1997). Chronic mGluR5 antagonism in rats similarly induces deficits in LTP in the CA1 region, which correlates with reduced hippocampal mGluR5 protein levels and poorer working and reference memory (Manahan-Vaughan and Braunewell, 2005). Accordingly, mGluR5 positive allosteric modulators (PAMs) enhance the balance of hippocampal LTP and LTD at CA1 synapses, and consequently improve spatial learning in mice (Ayala et al., 2009). Notably, very recent evidence suggests localisation of mGluR5 in this region might modulate the direction or balance of synaptic plasticity (e.g. LTP versus LTD; Purgert et al., 2014).

Despite extensive examination in animal models, the status of hippocampal mGluR5 protein in patients with schizophrenia is largely unknown. Only one study has previously investigated mGluR5 mRNA expression in the hippocampus of schizophrenia subjects (in a small postmortem cohort of 5 schizophrenia subjects and 6 controls), reporting decreased mGluR5 expression in the parahippocampal gyrus, and no alterations in the dentate gyrus, CA1, CA3 or CA4 (Ohnuma et al., 2000). Based on studies indicating the importance of mGluR5 in synaptic plasticity, including reports that suggest mGluR5 deficits lead to schizophrenia-like cognitive dysfunctions, it is important to extend on these works and thoroughly investigate whether hippocampal mGluR5 is altered in schizophrenia, specifically at the protein level, as this might contribute to the manifestation of learning and memory deficits observed in patients with schizophrenia.

Accumulating evidence indicates the importance of mGluR5 protein-protein interactions in the regulation of mGluR5 trafficking, internalisation and signalling (Enz, 2007). The first discovered modulator of mGluR5 was Homer1 (Brakeman et al., 1997), which has since been extensively studied in the context of schizophrenia (Szumlinski et al., 2006). Both Homer1 protein and gene are reported as altered in, or associated with, schizophrenia (Engmann et al., 2011; Spellmann et al., 2011). However, in recent years, other molecules that modulate mGluR5 localisation and trafficking have become apparent. The neuron-specific protein Norbin (neurochondrin) plays a critical role in mGluR5 localisation, and positively regulates mGluR5 signalling. Interestingly, *Norbin* knockout was found to reduce

mGluR5-dependent LTP and abolished LTD in CA1 synapses (Wang et al., 2009). In cultured mouse hippocampal CA1/CA3 neurons, the multiscaffold protein Tamalin also proved critical to mGluR5 neuritic localisation processes (Kitano et al., 2002) and deletion of the Tamalin binding site on mGluR5 induced mGluR5 internalisation in cellular assays (Timms et al., 2013). Furthermore, evidence from a schizophrenia pedigree suggests that mGluR5/Tamalin interactions might be disrupted in the disorder (Timms et al., 2013). As mGluR5 localisation appears to impact on mGluR5 functions (Purgert et al., 2014), it is important to consider the possibility of altered mGluR5 trafficking in the context of schizophrenia. However, the status of these mGluR5 trafficking molecules in any neuropsychiatric pathology, including schizophrenia, has not been assessed.

In the present study, we therefore determined whether mGluR5 protein levels are altered in postmortem samples from the CA1 hippocampal region of schizophrenia subjects compared to healthy controls (n=20/group). We further examined protein levels of the mGluR5 trafficking molecules Norbin and Tamalin, which collectively play an important role in mGluR5 trafficking, internalisation and signalling (Kitano et al., 2003; Wang et al., 2009). The CA1 region was chosen for examination due to evidence that mGluR5-mediated LTP might be specific to CA1 synapses (Lu et al., 1997). Subsequently, we ascertained whether any observed changes in protein levels of mGluR5 or its trafficking molecules were influenced by antipsychotic drug (APD) treatment. For this purpose, we treated rats with first- and second-generation APDs (haloperidol and olanzapine respectively) and measured hippocampal protein levels of mGluR5, Norbin and Tamalin.

2. Materials and methods

2.1 Human postmortem brain samples and tissue preparation

Postmortem human CA1 samples from 20 control (no history of psychiatric diagnosis) and 20 schizophrenia subjects, diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), were obtained from the New South Wales Brain Bank Network (Sydney, Australia). Subjects were matched according to tissue pH, postmortem interval, RNA integrity number (RIN) and age at death (Table 1). APD treatment premortem was standardised to a lifetime chlorpromazine equivalent for each patient, and antidepressant drug treatment history was specified on a qualitative scale (i.e. yes/no). The samples analysed in this study were derived from a cohort we previously used to examine mGluR5 (Brodmann's Area 46, n=37/group; Matosin et al., 2013). This work was approved by the Human Research Ethics Committees at the University of Wollongong (HE99/222) and the University of New South Wales (HREC 07261). Anatomical identification and preparation of this CA1 tissue has been previously described in detail (Fernandez-Enright et al., 2014).

2.2 Animal housing, treatment and tissue preparation

To assess the influence of haloperidol and olanzapine on mGluR5 and mGluR5 endogenous regulator expression, rats were treated with APD medication as previously described (Matosin et al., 2013). Briefly, adult (10 weeks of age) male Sprague-Dawley rats were purchased from the Animal Resource Centre (Perth, Australia). Rats were separated into groups according to the 3 treatments and 3 treatment-durations (9 groups; n=6/group). Animals were fed prepared food pellets (sweet cookie dough containing 62% carbohydrate, 22% protein, 10% vitamins, 6% fibre and minerals) 3 times/day at 8 hour intervals, containing either typical antipsychotic, haloperidol (0.1mg/kg body weight; daily total 0.3mg/kg), or atypical antipsychotic, olanzapine (1mg/kg body weight; daily total 3mg/kg), or vehicle control (no drug), for short term (8 days), medium term (15 days) and long term (35 days) durations. Food and water was available ad libitum, and consumption of food pellets was visually validated. APD doses were chosen to model a clinical setting (Han et al., 2008; Weston-Green et al., 2012).

Forty-eight hours after final treatment, rats were euthanised using carbon dioxide asphyxiation. The hippocampus (Bregma –2.30 to –5.20; total of dorsal and ventral regions) was dissected on ice, snap frozen in liquid nitrogen and then stored at -80°C until use. Tissue was homogenised in NP-40 lysis buffer (Invitrogen, Australia), containing ß-glycerophosphate and pheylmethanesulfonylfluoride and protease inhibitor cocktail (Sigma). Protein concentration was determined by DC assay (Bio-Rad), according to the manufacturer's instructions. All animal experiments in this study were approved by the University of Wollongong Animal Ethics Committee (AE10/18) and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3 Immunoblot analyses

Immunoblotting was performed on total homogenates as described previously (Matosin et al., 2013), with minor modifications optimised for the measurement of each protein of interest. Each lane was loaded with 5μg (human) or 10μg (animal) of total protein. These protein concentrations were determined to be in the linear range of detection (data not shown). Relative protein densities were determined by immunoblot analyses using polyclonal antibodies as previously reported: mGluR5 [ABCAM ab27190; human 1:250, rat 1:500 (Matosin et al., 2013)], Norbin [ABCAM ab130507; human and rat 1:500), and Tamalin [ABCAM ab30576; human 1:100, rat 1:1000 (Tai et al., 2010)]. mGluR5 monomer was detected at 135kDa whilst the mGluR5 dimer was detected at 270kDa. Individual bands were totalled before normalisation to respective ß-actin and pooled samples to gain a measure of total mGluR5. Samples were visualised using an enhanced chemiluminescent detection kit (Bio-Rad). Band density was detected by the Gel Doc 2200 Pro (Carestream Molecular Imaging,

USA) and quantified with Carestream MI software (v 5.0.4.44, Carestream Molecular Imaging). All bands were within the limits of saturation. Protein measures were subsequently normalised to their respective ß-actin density. Experiments and quantification were performed blind to diagnosis (human) or treatment group (rat).

2.4 Statistical Analyses

Statistical analyses were performed with SPSS software (v19.0). The level of significance was set to $p<0.05$ and data are presented as mean±SEM.

2.4.1 Human brain statistical analyses

Distributions of mGluR5 total measures (but not individual monomer or dimer measures) as well as Norbin and Tamalin were skewed to the right (Kolmogorov-Smirnov; d=0.152-0.246, $p<0.001$). Normalised distribution for these proteins was therefore achieved by transforming to the natural logarithm of the relative protein values. Outliers were screened as mean ±2 standard deviations and removed. Analyses of Variance (ANOVA) were used to detect differences in protein expression between diagnostic groups (schizophrenia/control) as well as gender (male/female), hemisphere (left/right) and antidepressant history (yes/no) for each target. Spearman's correlations were used to determine whether sample characteristics (Table 2) were associated with protein measures, and to examine if additional measures of disease characteristics were correlated specifically with the schizophrenia group (lifetime APD history, age of disease onset and duration of illness). Analyses of covariance (ANCOVA) for diagnostic effects on protein expression were subsequently performed, accounting for sample characteristics that were associated with protein measures. Further Spearman's correlations were performed to determine the strength of associations between mGluR5 measures and its trafficking molecules in schizophrenia compared to control subjects.

2.4.2 Animal brain statistical analyses

Values for mGluR5 (total) and Norbin were skewed to the right (Kolmogorov-Smirnov; d=0.103-0.169, $p<0.001$), and thus were normalised by converting to the natural logarithm. Protein differences between treatment (haloperidol/olanzapine/vehicle) and duration (short term/medium term/long term) were analysed by two-way ANOVA.

3. Results

3.1 Protein levels of mGluR5 in schizophrenia subjects compared to controls

We detected a robust increase in mGluR5 protein levels in the CA1 region of schizophrenia subjects compared to controls (total: +42%, $F_{1,37}$=138.579, p<0.001; monomer: +25%, $F_{1,37}$=7.194, p=0.011; dimer: +52%, $F_{1,37}$= 51.705, p<0.001; Figure 1). Demographic and clinical measures that correlated significantly with mGluR5 protein levels (Table 2) were assessed for their influence on the data. After co-varying for freezer storage time and brain pH, which significantly correlated with mGluR5 (total and dimer), significant differences between schizophrenia and control were maintained (mGluR5 total: $F_{1,35}$=172.974, p<0.001; mGluR5 dimer: $F_{1,35}$=59.433, p<0.001). An effect of gender was observed, whereby mGluR5 total protein was decreased in male subjects independent of diagnosis ($F_{1,37}$=5.357, p=0.026; -35.5%); however, there was no gender-specific difference in mGluR5 protein levels within the schizophrenia or control groups, and no hemispheric differences in mGluR5 protein expression.

3.2 Protein levels of mGluR5 trafficking molecules, Norbin and Tamalin, in schizophrenia subjects compared to controls

Norbin and Tamalin proteins were identified at the expected molecular weights (79kDa and 96 kDa respectively; Figure 2). Protein levels of these mGluR5 trafficking molecules were significantly increased in schizophrenia (Figure 2). Specifically, Norbin was increased by 46.7% ($F_{1,37}$=19.600, p<0.001) and Tamalin by 34.0% ($F_{1,37}$=7.630, p=0.009). There were no effects of gender or hemisphere on Norbin or Tamalin. Considering the nature of our results whereby all measured proteins of interest were increased, we further analysed protein levels of α-tubulin as a negative control to eliminate the possibility of a global protein increase within the schizophrenia samples. Protein levels of α-tubulin (05-829, Millipore) were not significantly different between schizophrenia and control subjects ($F_{1,37}$=1.991, p=0.167), validating our findings of increased mGluR5 and mGluR5 trafficking molecules.

3.3 Effects of clinical and demographic variables, and premortem medication estimates on protein measures

Spearman's correlations were performed to determine any relationships between demographic/clinical variables and protein levels of mGluR5 (total, monomer and dimer), Norbin and Tamalin (Table 2). There was a significant correlation between mGluR5 total protein and brain pH in all subjects, but this

was not maintained in the individual diagnostic groups. There was an effect of freezer storage time on mGluR5 total and dimer levels. A significant negative correlation was observed between lifetime antipsychotic drug dose and mGluR5 monomer levels in schizophrenia subjects; this association was also trending for mGluR5 dimer levels. APD measures did not correlate with Norbin or Tamalin protein levels. Lastly, there were no effects of antidepressant medication (yes/no; $F_{17\text{-}18} \geq 0.015$, $p \geq 0.189$) on mGluR5, Norbin or Tamalin protein levels.

3.4 Relationships of mGluR5 protein (total, monomer and dimer) with Norbin and Tamalin in schizophrenia subjects compared to controls

Spearman's correlations were performed to determine the presence or loss of associations between mGluR5 and its trafficking molecules in control and schizophrenia subjects (Table 3). Although mGluR5 monomer and dimer levels were strongly and positively correlated in both control and schizophrenia subjects ($r \geq 0.790$, $p \leq 0.001$), there were no correlations between measures of mGluR5 (total, monomer or dimer) and its trafficking molecules in control or schizophrenia groups.

3.5 Protein levels of mGluR5 and its trafficking molecules in the hippocampus of APD treated rats

To assess the effects of current antipsychotic medications on the mGluR5 system, two-way ANOVA were used to compare the effects of haloperidol and olanzapine treatment (8, 16 or 36 days) on protein levels of mGluR5 (total, monomer or dimer), Norbin and Tamalin proteins in the hippocampus. There were no significant effects of olanzapine or haloperidol treatment overall or following the specific treatment periods ($F \geq 0.229$, $p \geq 0.296$).

4. Discussion

In the present study, we provide the first evidence that protein expression of mGluR5 is significantly higher (total: 42%; monomer: 25%; dimer: 52%) in the hippocampal CA1 region of schizophrenia subjects relative to healthy controls. Schizophrenia subjects also show a marked increase in Norbin, and Tamalin proteins (47% and 34% respectively), which are endogenous regulators of mGluR5 signalling and trafficking not before analysed in neuropsychiatric pathology. In addition, we found no influence of APD treatment on mGluR5 regulatory proteins in human subjects, however a negative association between lifetime APD dose and mGluR5 protein expression levels was observed in schizophrenia subjects. There were no alterations in mGluR5 or its trafficking molecules in the hippocampus in response to APD treatment in our animal model.

mGluR5 activation in CA1 is critically involved in cognitive functions via mGluR5-mediated modulation of synaptic plasticity (Mukherjee and Manahan-Vaughan, 2013). *mGluR5* knockout mice display deficits in CA1-dependent LTP, coupled with deficits in spatial learning and memory (Lu et al., 1997). mGluR5 PAMs, which upregulate mGluR5 activity, have been reported to enhance hippocampal-dependent LTP and LTD in the CA1 region, congruent with improved performance in the Morris water maze, a measure of hippocampal-dependent spatial learning (Ayala et al., 2009). mGluR5-dependent differences in spatial learning in rats correlated with measures of synaptic plasticity in CA1 and with mGluR5 protein levels (Manahan-Vaughan and Braunewell, 2005). As cognitive dysfunction is a common symptom among schizophrenia patients (Harvey, 2013), the observed increase in mGluR5 in the present study likely represents a compensatory upregulation for mGluR5 dysfunction in this region or in response to wider deficits in glutamatergic signalling. As recently reviewed by Rubio Hu and colleagues (2014), reductions in the ionotropic glutamatergic receptors, NMDA (Vrajová et al., 2010), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; Eastwood et al., 1997, 1995) and kainate (Benes et al., 2001), have been reported in the hippocampus. Although one study also reported no changes in these receptors (Breese et al., 1995), it is notable that there has been no increase detected.

In support of a deficit in mGluR5, Timms and colleagues recently reported the presence of a missense mutation in the *mGluR5* gene in a schizophrenia pedigree, transmitting to the extracellular domain of the mGluR5 protein and likely altering mGluR5 sensitivity to glutamate (Timms et al., 2013). Additionally, the authors reported a frameshift mutation (P1148fs) that caused deletion of the Tamalin-binding site on the carboxyl tail of mGluR5, disrupting Tamalin/mGluR5 interactions and increasing mGluR5 internalisation in primary hippocampal cultures (Timms et al., 2013). We have also recently discovered the significant association of a single nucleotide polymorphism, located in the regulatory

3' untranslated region (highly involved in regulation of protein-protein interactions) within the *GRM5* gene, and schizophrenia in a Caucasian case-control population (unpublished results). Considering this evidence, Tamalin (but also Norbin) proteins might be upregulated in schizophrenia to compensate for reduced protein-protein interactions. Whilst Norbin has been previously demonstrated to positively regulate mGluR5 signalling (Wang et al., 2009), both Norbin and Tamalin are also critical regulators of mGluR5 trafficking (Kitano et al., 2002; Wang et al., 2009). Therefore our finding of increased levels of these proteins further support that mGluR5 signalling might be altered in the CA1 region of schizophrenia subjects, and these trafficking molecules are upregulated in an attempt to compensate for alterations to mGluR5 signalling and trafficking.

Although it is accepted that mGluR5 is expressed on the postsynaptic neuronal membrane, increasing evidence supports that the majority (60-90%) of mGluR5 is distributed at intracellular locations, including the endoplasmic reticulum membrane and nuclear membrane (Jong et al., 2014). Depending on its cellular location, mGluR5 reportedly activates different signalling cascades leading to unique cellular responses (Jong et al, 2009). In accordance, intracellular and extracellular mGluR5 differentially mediate synaptic plasticity in the CA1: cell surface receptors contribute to both LTD and LTP, whereas activation of intracellular mGluR5 are involved in LTD only (Purgert et al., 2014). Considering our finding of alterations to proteins that regulate mGluR5 movement to/from neuronal membranes, using total homogenate preparations, it will be important to determine whether the increase in mGluR5 observed in this study represents increases in intracellular or cell-surface distribution of mGluR5, or both.

Premortem APD exposure can influence molecular measures in postmortem tissue analyses. In the present study, estimated lifetime APD exposure was negatively correlated with mGluR5 monomer levels and a correlation approaching significance was observed with mGluR5 dimers. This finding suggests an influence of APD on mGluR5 protein levels in the CA1 whereby APDs might act to reduce mGluR5 protein levels in the CA1. However considering we conversely detected increased mGluR5 protein levels in this region, and we found no influence of APD treatment on mGluR5 in the hippocampus of APD treated rats, it is unlikely that APD exposure is confounding the results of our study. Furthermore, we additionally report no assocation between Norbin, Tamalin, and APDs in our present human or rat studies, and we have previously reported that mGluR5 binding was not associated with APD exposure in the hippocampus (Matosin et al., 2013). Thus although an interaction is unlikely, additional studies are required to explore the mechanism underlying a potential association. Further investigation of the consequences of APD treatment on the mGluR5 system in animal models of schizophrenia would be valuable.

mGluR5 is a promising therapeutic target for the treatment of cognitive deficits in schizophrenia (Matosin and Newell, 2013). The present study reports that mGluR5 and mGluR5 trafficking molecules are altered in the CA1 region in subjects with schizophrenia, suggesting that mGluR5 signalling and trafficking is altered. It will be important for future studies to elucidate the exact mechanisms underlying these alterations and their implications for novel mGluR5-targeting therapeutics, to determine how to best target mGluR5 (if at all) in a manner that will be therapeutic in the context of pathological alterations. For example, altered cell surface expression might limit available targets for drugs aimed at mGluR5 and the use of pharmacological chaperones to attenuate mGluR5 trafficking deficits may be beneficial (Ulloa-Aguirre and Conn, 2011), whilst insensitivity to glutamate (as proposed by Timms et al., 2013) may positively or negatively affect PAMs that rely on simultaneous glutamate activation.

We conclude that hippocampal CA1 protein levels of mGluR5 and mGluR5 trafficking molecules are increased in the pathology of schizophrenia. We hypothesise that this may be related to a functional deficit within mGluR5, whereby glutamate sensitivity, protein-protein interactions or mGluR5 localisation are affected. Due to the prominent role of hippocampal mGluR5 in the emergence of learning and memory deficits, we further suggest that alterations to mGluR5 in CA1 may be associated with an imbalance of LTD:LTP, thus contributing to cognitive dysfunctions that are observed in schizophrenia patients. However, future studies investigating the correlation between hippocampal mGluR5 expression and cognition in humans is required to confirm this hypothesis.

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Figures and Tables

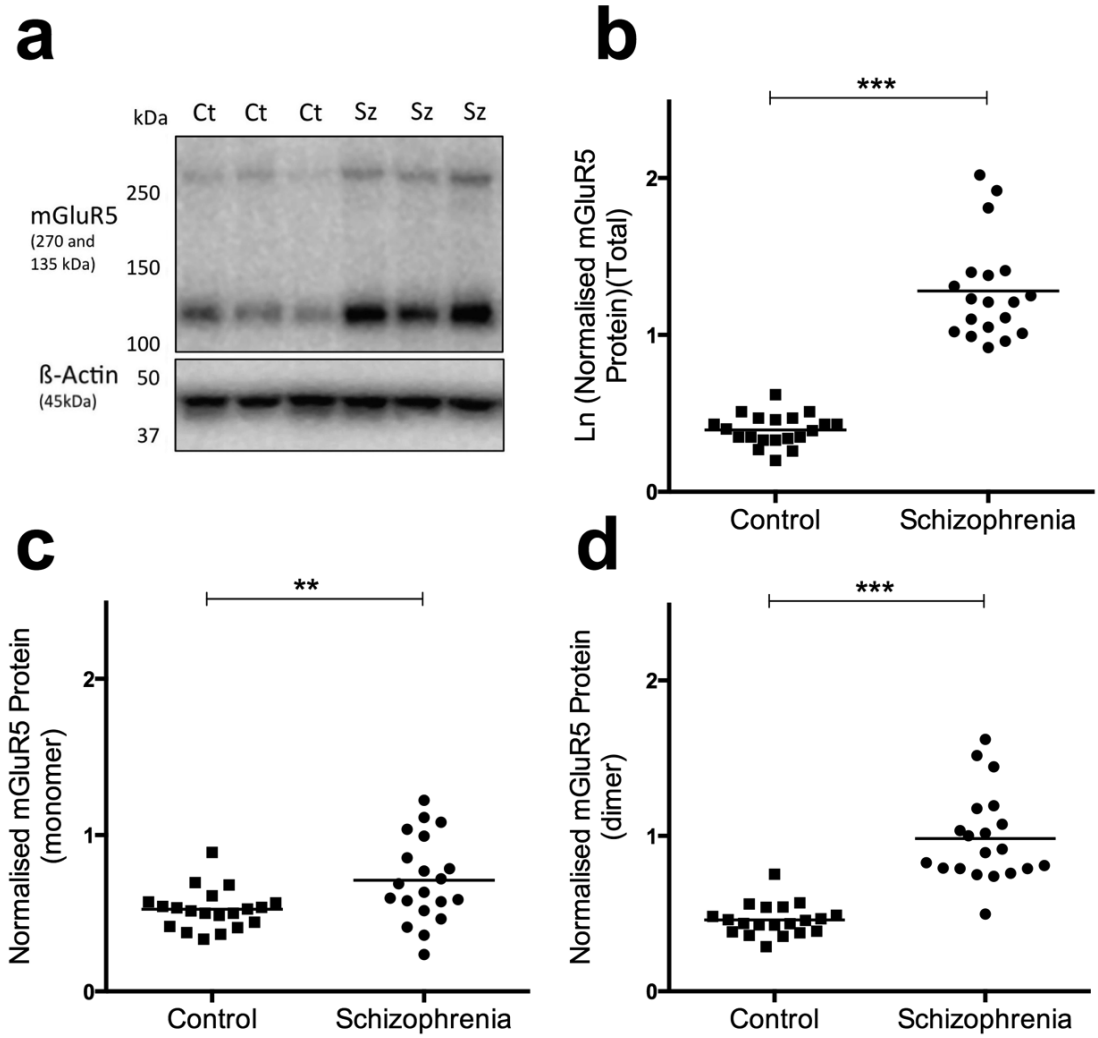

Figure 1. Protein levels of mGluR5 in the CA1 region of subjects with schizophrenia and matched controls. (a) Representative immunoblots, and normalised levels of mGluR5 **(b)** total, **(c)** monomer, and **(d)** dimer protein measures, in schizophrenia subjects compared to controls. **Abbreviations:** Ct: control; kDa: kilodaltons; Ln: natural logarithm; mGluR5: metabotropic glutamate receptor 5; Sz: schizophrenia. ***P<0.05, **P<0.01, ***P<0.001.**

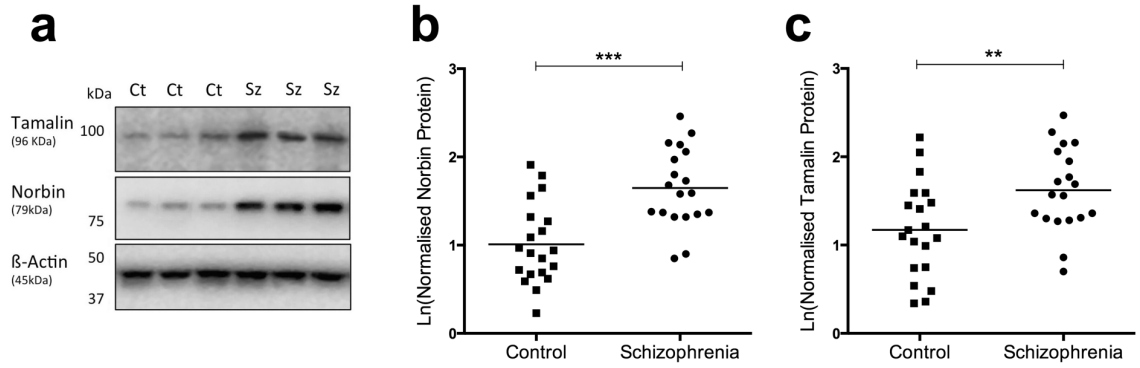

Figure 2. mGluR5 trafficking molecules Norbin and Tamalin in the CA1 region of subjects with schizophrenia. (a) Representative immunoblots, and normalised protein levels of mGluR5 trafficking molecules **(b)** Norbin and **(c)** Tamalin in the CA1 region of subjects with schizophrenia compared to matched controls. **Abbreviations:** Ct: control; kDa: kilodaltons; Ln: natural logarithm; Sz: schizophrenia. ***P<0.05, **P<0.01, ***P<0.001.**

Table 1. Demographic and clinical characteristics of the postmortem cohort.

	CA1 (n=20/group)	
	Control	Schizophrenia
Brain pH	6.6 ± 0.03	6.6 ± 0.3
Postmortem interval (hours)	26.1 ± 12.8	28.8 ± 14.1
RNA integrity number	7.2 ± 0.7	7.2 ± 0.5
Age at Death (years)	58.2 ± 12.6	55.5 ± 13.5
Gender	2 F, 18 M	9 F, 11 M
Hemisphere	13 R, 7L	10R, 10L
Age of disease onset (years)	-	23.5 ± 6.8
Duration of illness (years)	-	32.05 ± 13.7
Lifetime antipsychotic drug medication*	-	668 ± 421
Antidepressant history (yes/no)	-	12

*Standardised chlorpromazine equivalent, mg.

Abbreviations: DLPFC: dorsolateral prefrontal cortex; F: female; L: left; M: male; R: right. Data are expressed as mean ± standard deviation

Table 2. Spearman's Correlations for continuous clinical and demographic variables and protein levels of mGluR5 total, monomer and dimer, and mGluR5 trafficking molecules Norbin and Tamalin, in the hippocampal CA1 region. Significant values ($p<0.05$) are highlighted in bold.

Variable	All subjects					Controls					Schizophrenia				
	mGluR5 (total)	**mGluR5 (monomer)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**mGluR5 (total)**	**mGluR5 (monomer)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**	**mGluR5 (total)**	**mGluR5 (monomer)**	**mGluR5 (dimer)**	**Norbin**	**Tamalin**
Brain pH	**r = -0.355** **p = 0.026**	r = -0.001 p = 0.993	r =-0.088 p =0.593	r = 0.015 p = 0.929	r = 0.026 p = 0.874	r = -0.304 p = 0.192	r = -0.040 p = 0.867	r = -0.134 p = 0.573	r = 0.142 p = 0.551	r = 0.127 p = 0.593	r = -0.448 p = 0.055	r = -0.084 p = 0.732	r =-0.116 p = 0.497	r = -0.170 p = 0.486	r = -0.147 p = 0.549
Age at Death	r = -0.028 p = 0.868	r = -0.154 p = 0.349	r =-0.164 p = 0.320	r = -0.096 p = 0.561	r = -0.096 p = 0.560	r = -0.188 p = 0.427	r = -0.087 p = 0.716	r = 0.136 p = 0.569	r = -0.068 p = 0.777	r = -0.061 p = 0.798	r = 0.287 p = 0.233	r = -0.184 p =0.452	r = -0.211 p = 0.386	r = -0.199 p = 0.414	r = -0.187 p = 0.444
Postmortem interval	r = -0.167 p = 0.309	r = 0.100 p = 0.546	r = 0.170 p = 0.302	r = -0.051 p = 0.758	r = -0.085 p = 0.606	r = -0.199 p = 0.400	r = -0.199 p = 0.401	r = -0.118 p = 0.620	r = 0.113 p = 0.635	r = 0.049 p = 0.838	r = -0.328 p = 0.170	r = 0.250 p = 0.302	r = 0.359 p = 0.131	r = -0.158 p = 0.517	r = -0.170 p = 0.487
Freezer storage time	**r = 0.356** **p = 0.026**	r =0.243 p =0.136	**r = 0.347** **p =0.030**	r = 0.173 p = 0.292	r = 0.159 p = 0.334	r = 0.274 p = 0.243	r = 0.335 p = 0.149	r = 0.332 p = 0.152	r = 0.219 p = 0.354	r = 0.162 p = 0.496	**r = 0.531** **p = 0.019**	r = 0.207 p =0.395	r = 0.354 p = 0.137	r = 0.220 p = 0.365	r = 0.210 p = 0.389
Brain weight	r = -0.144 p = 0.383	r = -0.039 p = 0.815	r = -0.232 p = 0.156	r = -0.258 p = 0.113	r = -0.176 p = 0.283	r = -0.267 p = 0.255	r = -0.083 p = 0.729	r = -0.170 p =0.473	r = -0.123 p = 0.606	r = -0.042 p = 0.860	r = 0.030 p = 0.903	r = -0.069 p = 0.780	r = -0.194 p = 0.425	r = -0.145 p = 0.553	r = -0.142 p = 0.563
Age of disease onset	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	r = 0.259 p = 0.285	r = 0.241 p = 0.321	r = 0.156 p = 0.523	r = 0.051 p = 0.837	r = 0.078 p = 0.750
Duration of illness	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	r = 0.199 p = 0.415	r = -0.234 p = 0.336	r = -0.232 p = 0.339	r = -0.231 p = 0.341	r = -0.224 p = 0.357
Lifetime antipsychotic drug intake*	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	r = -0.160 p = 0.514	**r = -0.502** **p = 0.029**	r = -0.426 p = 0.069	r = -0.251 p = 0.300	r = -0.256 p = 0.290

* Standardised chlorpromazine equivalent (mg)

Table 3. Spearman's correlations for associations between protein levels of mGluR5 (total, monomer and dimer) and novel mGluR5 signalling partners Norbin and Tamalin, within (A) control and (B) schizophrenia subjects. Significant values ($p<0.05$) are highlighted in bold.

A. Control	*mGluR5 total*	*mGluR5 monomer*	*mGluR5 dimer*
mGluR5 (total)	-	r = 0.066	r = 0.102
	-	p = 0.782	p = 0.668
mGluR5 (monomer)	r = 0.066	-	**r = 0.853**
	p = 0.782	-	**p < 0.001**
mGluR5 (dimer)	r = 0.102	**r = 0.853**	-
	p = 0.668	**p < 0.001**	-
Norbin	r = -0.036	r = 0.274	r = 0.266
	p = 0.980	p = 0.243	p = 0.257
Tamalin	r = 0.017	r = 0.334	r = 0.292
	p = 0.945	p = 0.150	p = 0.212

B. Schizophrenia	*mGluR5 total*	*mGluR5 monomer*	*mGluR5 dimer*
mGluR5(total)	-	r = 0.140	r = 0.137
	-	p = 0.557	p = 0.566
mGluR5(monomer)	r = 0.140	-	**r = 0.790**
	p = 0.557	-	**p < 0.001**
mGluR5 (dimer)	r = 0.137	**r = 0.790**	-
	p = 0.566	**p < 0.001**	-
Norbin	r = 0.037	r = 0.001	r = 0.064
	p = 0.876	p = 0.996	p = 0.788
Tamalin	r = 0.027	r = 0.038	r = 0.029
	p = 0.910	p = 0.875	p = 0.902