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NR2B or not 2B? Alterations of NR2B in schizophrenia and the effect of an NR2B blockade in an exploratory mouse model. Is there a link between NR2B and schizophrenia pathology?

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Alterations of NR2B in schizophrenia and the effect of an NR2B blockade in an exploratory mouse model. Is there a link between NR2B and schizophrenia pathology?

A thesis submitted in (partial) fulfilment of the requirements for the award of the degree of

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From

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By

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DECLARATION

I, Amy E Geddes, declare that this thesis, submitted in (partial) fulfillment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science Medicine and Health, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. This manuscript has not been submitted for qualification at any other academic institution.

Amy Geddes

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PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS

BACE	β-site of amyloid precursor protein cleaving enzyme
CB_1R	cannabinoid receptor 1
CNS	central nervous system
DLPFC	dorsolateral prefrontal cortex
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
EGF	epidermal growth factor
$GABA_AR$	gamma-amino-butyric-acid A receptor
GRIN2B	glutamate (NMDA) receptor subunit epsilon 2
HRP	horseradish peroxidase
LTP	long-term potentiation
MAGUK	membrane associated guanylate kinases
mGluR5	metabotropic glutamate receptor 5
MK-801	dizoclipine
NMDAR	N-methyl-D-aspartate receptor
NRG1	neuregulin 1
NSW TRC	New South Wales Tissue Resource Centre
PBS-T	phosphate-buffered saline containing Tween 20
PCP	phencyclidine
PMI	postmortem interval
PN	postnatal day
PPI	prepulse inhibition
PSD	postsynaptic density
PVDF	polyvinylidene fluoride
SAP	synapse associated protein
SNP	single nucleotide polymorphism
SRL	Schizophrenia Research Laboratory
TACE	tumor necrosis factor-α converting enzyme
THC	Δ^9 -tetrahydrocannabinol
TM	transmembrane

ABSTRACT

Schizophrenia is a devastating disorder thought to evolve from a combination of environmental and genetic factors. Evidence suggests that the glutamatergic N-methyl-D-aspartate receptor (NMDAR) may play a central role in the development of the disorder. The NMDAR, including the NR2B subunit of this receptor, is critical for normal brain development and has been implicated in the pathophysiology of schizophrenia, particularly the cognitive symptoms. It is hypothesised that developmental disruption to the NMDAR can cause a cascade of events resulting in altered developmental trajectories of other systems known to be involved in schizophrenia, such as the neuregulin (NRG), gamma-aminobutyric acid (GABA), and cannabinoid systems. The overall aim of this PhD thesis was to assess the potential role of the NR2B subunit in the pathophysiology and development of schizophrenia, with a particular focus on the examination of brain regions that mediate cognitive functions.

Part A of the thesis used two postmortem human brain cohorts to determine whether NR2B alterations play a role in the pathophysiology of schizophrenia. NR2B protein and binding density (using the NR2B specific radioligand [^{3}H]Ifenprodil) were first examined in the hippocampus (specifically the dentate gyrus sub-region) of a cohort of 20 schizophrenia subjects and 20 matched controls (Chapter 2). There was a 43% reduction in NR2B protein levels, specifically in the left hemisphere, in schizophrenia subjects, but this was accompanied by no change in [^{3}H]Ifenprodil binding to NR2B, suggesting the reduction in protein may be specific to certain cellular compartments. These findings provide the first evidence of NR2 protein abnormalities in the hippocampus in schizophrenia, highlighting the hippocampal lateralisation in this disorder. Considering the importance of

hippocampal NR2B to cognitive function, it may be that these observed alterations in NR2B are related to cognitive dysfunction in schizophrenia subjects. Building on these findings, chapter three examined NR2B protein and binding density in another brain region highly implicated in cognitive symptoms, the dorsolateral prefrontal cortex (DLPFC). Using a larger cohort (n=37/group), there was no evidence of hemisphere specific alterations in schizophrenia, however a 37% decrease in NR2B protein density (but not binding) was observed in the female schizophrenia subjects (n=13) compared to the male schizophrenia subjects (n=24) suggesting a gender difference in NR2B protein in schizophrenia subjects.

Experimental part B of the thesis (Chapters 3-5) utilised a novel rodent model to assess whether developmental disruption to the NR2B subunit of the NMDAR could alter the developmental trajectory of key systems implicated in schizophrenia. In this model, mice were treated with the highly specific NR2B antagonist, Ro 63-1908 (30 mg/kg s.c.) from postnatal days (PN) 7-14. Brains from both male and female mice were then collected at PN14 (8 hours after treatment), PN35 (adolescence) or PN70 (adulthood). Immunoblot and receptor autoradiography were used to examine NMDA, NR2A, NR2B, NRG1, ErbB4, $GABA_A$ and CB_1 receptors, with a particular focus on the prefrontal cortex and hippocampus brain regions. It was shown that perinatal NR2B antagonism caused a 70% reduction in NR2B binding at PN14 in the prefrontal cortex and hippocampus ($p<0.001$), validating the NR2B blockade. It was also shown that this treatment caused a 23% increase in NR2B protein in the prefrontal cortex at PN35 ($p=0.020$) and a 13% decrease in NRG1 protein in the hippocampus at PN35 ($p=0.012$), effects that were independent of gender. Perinatal NR2B antagonism did not however alter the other receptors/proteins examined.

In chapters three, four and five, the normal developmental trajectory of the NMDA, NRG1/ErbB4, $GABA_AR$ and CB_1R systems was also examined. Gender specific developmental trajectories were observed with differences most evident at the adolescent time point. Female mice showed a 166% increase in NR2A binding compared to males in the prefrontal cortex and hippocampus (144-166%; $p<0.001$). This result was positively correlated to that of $GABA_AR$ binding in which there was a similar increase in binding in the female mice at adolescence (81-181%; $p<0.01$). In contrast, males showed greater expression of the CB_1R compared to females at adolescence (25-38%; $p<0.02$).

Collectively, this thesis has provided novel insights into the changes that occur in the NR2B subunit in schizophrenia, revealing for the first time that expression of NR2B is altered in the DLPFC and hippocampus of schizophrenia subjects, in a gender and hemisphere specific manner. These alterations may be related to cognitive symptoms experienced by patients, however further studies are required to determine this. This thesis also showed that perinatal NR2B antagonism causes some alterations to schizophrenia relevant systems including NR2B and NRG1 at adolescence, which could potentially make this animal model more vulnerable to further impacts at adolescence. It should be noted however, that this model did not cause widespread changes in systems (at least in terms of protein expression) relevant to the pathophysiology of schizophrenia. Finally, this thesis has identified that the developmental profiles of schizophrenia relevant systems, differs between the two genders (at least in a mouse model), highlighting the importance of considering this in the design of future studies.

1 THE ROLE OF THE NMDA RECEPTOR IN SCHIZOPHRENIA

Schizophrenia is a debilitating mental disorder characterised by a range of positive, negative and cognitive symptoms, which include delusions, hallucinations, anhedonia, social isolation and learning and memory impairments (Koenig, 2006). These symptoms commonly emerge during adolescence to young adulthood. Pathophysiological studies have revealed alterations in the brain structure of patients with schizophrenia, such as ventricular enlargement, reductions in cortical grey matter, and altered cytoarchitechture (Keshavan and Hogarty, 2000, Mueser and McGurk, 2004, Jarskog et al., 2005). These atypical physical traits do not deteriorate as the patient ages and are a sign of defective formation as opposed to damage and degeneration (Weinberger, 1995). Together with data showing an increased risk of developing schizophrenia following perinatal complications (Mueser and McGurk, 2004), these findings have lead researchers to consider schizophrenia as a neurodevelopmental disorder with origins in early life, even though the symptoms are not apparent until later in life.

Epidemiological evidence suggests that there is an earlier onset in males compared to females in many populations (Hafner et al., 1993, Shimizu, 1988, Szymanski et al., 1995). Gender differences have also been observed in the symptom profiles of schizophrenia patients (Goldstein and Link, 1988, Gur et al., 1996), for example in clinical cognitive studies, male schizophrenia patients show greater cognitive deficits than females (Aylward et al., 1984), with females displaying a reduced level of negative symptoms (Gur et al., 1996). Brain imaging studies have since been conducted to try to elucidate these gender differences, with studies showing differences in cortical size, for example in the inferior parietal lobule, between male and female patients (Frederikse et al., 2000). These gender differences

in schizophrenia may extend to brain neurochemistry with some studies reporting gender specific alterations (Schroder et al., 1997). Furthermore, Frederikse and colleagues revealed a gender difference in the lateralisation of the brain, with healthy male subjects displaying a greater asymmetry (greater volume on left than right side) than females (Frederikse et al., 1999). This asymmetry is reversed (more prominent on right than left side) in male schizophrenia patients, whereas female schizophrenia patients show no difference to healthy subjects (Frederikse et al., 2000). Findings of reduced cortical volumes or lateralisation effects specific to one gender could have an effect on proteins at a cellular level and therefore it is important to examine both gender and hemispheric effects in the brain in relation to schizophrenia studies.

Various family, twin and adoption studies have provided strong support for genetic involvement in the pathogenesis of schizophrenia (Pulver, 2000, Maynard et al., 2001), however the inheritance appears to be in a complex polygenetic and non-Mendelian manner (Risch and Baron, 1984). Genetic linkage analyses have identified several loci that are associated with the disorder and there are a number of candidate genes that are implicated in schizophrenia, including *Glutamate (NMDA) receptor epsilon-2* (*GRIN2B)* and *neuregulin 1 (NRG1*) (Brzustowicz et al., 2000, Martucci et al., 2006, Miyatake et al., 2002). This suggests that no single genetic mechanism can be responsible for the aetiology of schizophrenia and although there is a genetic component to schizophrenia, it is likely that schizophrenia develops from an interaction of genetic and environmental factors. Some of the environmental factors that have been found to increase the risk for developing schizophrenia include insults during early brain development, such as drug abuse during pregnancy, maternal stress or malnutrition, obstetric complications and neonatal anaesthetic agents (Ikonomidou et al., 2001, Abel et al., 2008, Shen et al., 2008), which all affect

the brain development of the foetus, as well as other insults during the periadolescent brain developmental period, such as cannabis use (Henquet et al., 2005). The N-methyl-D-aspartate receptor (NMDAR), a protein which is vitally important in brain development, appears to be a convergence point for many genetic and environmental factors implicated in schizophrenia (Snyder and Gao, 2013) and is the focus of one of the leading hypotheses of schizophrenia aetiology, the glutamate hypothesis.

1.1 The Glutamate Hypothesis: Excitatory Neurotransmission in Schizophrenia

It is widely acknowledged that an imbalance of neurotransmission is involved in the pathogenesis of schizophrenia, particularly the imbalance of excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission. A considerable amount of research has focused on the ionotropic NMDAR in the pathogenesis of schizophrenia since the discovery several decades ago that antagonists of this receptor, such as phencyclidine (PCP) and ketamine, mimic symptoms of the disorder in humans and exacerbate symptoms in schizophrenia patients (Olney et al., 1999). The NMDAR is critically involved in many central nervous system (CNS) functions, especially during early brain development. It is vital for neuronal proliferation, differentiation, migration and establishment in the developing brain (Komuro and Rakic, 1993, Contestabile, 2000). Altered or diminished function of the NMDAR during this time could therefore interfere with essential neuronal processes resulting in various long-term effects. As schizophrenia is considered to have origins in early neurodevelopment, with alterations to such processes as neuronal migration, the developmental NMDAR hypofunction hypothesis is certainly feasible. In the mature brain, the NMDAR plays a key role in synaptic plasticity processes such as

long-term potentiation, which underlie functions such as learning and memory, and therefore may be involved in the cognitive symptoms experienced by schizophrenia patients.

Various studies have been conducted using animal models in an effort to uncover the effects of developmental NMDAR hypofunction and its relationship to schizophrenia-like symptoms. NR1 hypomorphic mice which display 5-10% levels of functional NMDARs show deficits in prepulse inhibition, reduced social interaction, hyperactivity, cognitive deficits, which model the positive, negative and cognitive aspects of schizophrenia (Mohn et al., 1999). These behaviours were influenced by antipsychotic drug treatment, providing this model with face and predictive validity (Mohn et al, 1999). However it has been suggested that the global effects produced by this model are too general to model a specific disorder, suggesting that more targeted insults to the NMDAR, for example, at a specific developmental period, may result in a more relevant model (Barkus et al., 2012).

Pharmacological models provide the opportunity to target specific molecules at specific points in time. The administration of NMDAR antagonists such as dizoclipine (MK-801), PCP, nitrous oxide and ketamine, to rodents during the first two weeks of life [when NMDAR expression and brain developmental synaptogenesis are at their peak (du Bois and Huang, 2007)] produces, long-term behavioural effects such as enhanced locomotion (Facchinetti et al., 1993, Harris et al., 2003, du Bois et al., 2008), impairments in cognitive function as examined by spatial working memory, cognitive set-shifting and other learning tests (Andersen and Pouzet, 2004, Stefani and Moghaddam, 2005) and deficits in prepulse inhibition (Harris et al., 2003). These behavioural changes mimic certain aspects of schizophrenia and provide support for the involvement of the glutamatergic system

in schizophrenia pathophysiology. These behavioural changes are accompanied by neurotransmitter alterations (Wang et al., 2001, Sircar and Soliman, 2003, Wang et al., 2008, du Bois et al., 2009), which have to some extent paralleled that observed in the human schizophrenia brain. For example perinatal NMDAR antagonism has been shown to increase gamma-amino-butyric-acid A receptors ($GABA_ARs$) in various brain regions of female rats (du Bois et al., 2009). As well as this, a loss of GABAergic interneurons has been observed following perinatal NMDAR blockade (Nakatani-Pawlak et al., 2009), which is representative of the GABAergic dysfunction seen in schizophrenia pathology (Benes et al., 1996b, Dean et al., 1999, Deng and Huang, 2006, Newell et al., 2007b). Furthermore, these neurodevelopmental models promote an enhanced apoptotic response in several regions of the developing rodent brain (Ikonomidou et al., 1999, Ikonomidou et al., 2000, Wang et al., 2001, Harris et al., 2003, Jevtovic-Todorovic et al., 2003). It has been proposed that an alteration in apoptotic activity occurring early in development may contribute to human schizophrenia pathology (Jarskog, 2006), with excessive neuronal depletion contributing to the array of cognitive and behavioural effects observed later in life (Jevtovic-Todorovic et al., 2003).

1.2 NMDAR: Structure, Function and Developmental Distribution

NMDARs are composed of a heterometric assembly of subunits where at least one obligatory NR1 subunit combines with distinct arrangements of NR2 and/or NR3 subunits. To add to the complexity of these receptors, there are eight different splice variants of the gene encoding for the NR1 subunit and six other genes that encode for different forms of the NR2 (NR2A, NR2B, NR2C, NR2D) and NR3 (NR3A, NR3B) subunits, producing a multitude of different NMDARs (Paoletti and

Neyton, 2007). This formation of NMDARs gives rise to a diverse range of functional and pharmacological properties (Sheng et al., 1994, Flint et al., 1997, Kristiansen et al., 2007).

The extracellular N-terminal domain of the NR2 subunits contains the binding site for glutamate, the primary activator of the NMDAR, while the NR1 subunit (and NR3 subunits) binds glycine, a co-activator. Inside the cell, the C-terminal region of the NR2 subunits is anchored to PDZ (Post-synaptic density 95, Drosophila disc large tumor suppressor, Zonula occludens-1)-containing scaffold proteins in the postsynaptic density (PSD) including PSD-95, synapse associated protein (SAP)102, PSD-93 and SAP97, with PSD-95 being the most studied (Kornau et al., 1995, Muller et al., 1996, Niethammer et al., 1996, Bassand et al., 1999, Sans et al., 2000, Chen et al., 2006, Sato et al., 2008, Cousins et al., 2009). These scaffolding proteins form part of the membrane associated guanylate kinases (MAGUKs), which due to their close positioning to the postsynaptic membrane and their signalling capacity, are also involved in critical processes during synaptogenesis and synapse maturation including localisation, trafficking and organisation of many membranous and cellular proteins (Sheng and Hoogenraad, 2007, Elias et al., 2008, Howard et al., 2010).

The NR2 subunits of the NMDAR are spatially and developmentally regulated, providing an important level of receptor regulation (Herin and Aizenman, 2004, Mueller et al., 2004). Whereas NR2C subunits are primarily restricted to the cerebellum and NR2D subunits to the thalamus and brainstem, NR2A and NR2B are the predominant subunits in the cortex, striatum and hippocampus (Watanabe et al., 1992, Portera-Cailliau et al., 1996, Wenzel et al., 1997). Widespread NR2B expression during development becomes confined to forebrain areas in adulthood,

whereas the NR2A subunit is increasingly expressed in all brain regions throughout development (Watanabe et al., 1992, Portera-Cailliau et al., 1996, Wenzel et al., 1997). These forebrain areas in particular are closely associated with schizophrenia pathology as the neural circuits contained within and between these regions play a central role in eliciting the positive, negative and cognitive symptoms that are experienced by patients with the disorder (Heimer, 2000). Therefore this regional expression of NR2 subunits may play an important role in schizophrenia symptoms.

In terms of the developmental regulation, animal studies have revealed that the NR2B and NR2D subunits emerge early in development with NR2B and NR2D mRNA present prenatally in the rodent brain, as early as embryonic day 14, while the NR2A and NR2C mRNAs are not detectable until at or after birth (Watanabe et al., 1992, Monyer et al., 1994, Sheng et al., 1994). Similarly NR2B protein is already widely and strongly expressed by birth with very little expression of NR2A and NR2C protein at postnatal day (PN) zero (Portera-Cailliau et al., 1996, Wenzel et al., 1997, Sans et al., 2000, Liu et al., 2004). The expression pattern of the PSD and SAP proteins mirrors that of the NR2 subunits with the SAP97 and SAP102 proteins present early in development and involved in synaptogenesis while PSD-93 and PSD-95 emerge later during synapse maturation (Sans et al., 2000, Elias et al., 2008, Howard et al., 2010). There has been evidence to suggest that certain NR2 subunits preferentially associate with one or another of the postsynaptic density proteins; one study has shown that NR2A has a slight but not complete preference for PSD-95 and NR2B a preference for SAP102 (Sans et al., 2000). On the other hand it has been found that the number of binding domains for PSD-95 on the NR2B subunit is greater than on the NR2A subunit and thus NR2B may be more closely associated to

PSD-95 (Cousins et al., 2009), or the NR2 subunits may interact comparably with the postsynaptic density proteins (Al-Hallaq et al., 2007).

Throughout postnatal development, expression of NR2A mRNA and protein increases to adult levels in all areas at around the third postnatal week in the rodent brain (Monyer et al., 1994, Sheng et al., 1994, Zhong et al., 1995, Wenzel et al., 1997, Guilarte and McGlothan, 1998, Ritter et al., 2002). Peak NR2B expression in the rodent occurs during the first week in most brain regions (Monyer et al., 1994, Wenzel et al., 1997, Ritter et al., 2002). However there appears to be conflicting results of NR2B expression from this point until adulthood with some studies showing cortical NR2B expression remains constant from early development to adulthood (Sheng et al., 1994, Zhong et al., 1995, Portera-Cailliau et al., 1996) while others have found a decrease in NR2B levels to adulthood (Monyer et al., 1994, Wenzel et al., 1997). The expression of NR2C mRNA and protein in the rodent brain is fairly weak until the third postnatal week when there is a dramatic increase in the cerebellum and thalamus (Monyer et al., 1994, Zhong et al., 1995, Wenzel et al., 1997). This increase in NR2C levels correlates with a decrease in NR2B levels in the cerebellum at this time (Monyer et al., 1994). Compared to the other subunits NR2D expression peaks earlier in development, around PN7, but is restricted to regions such as the midbrain and thalamus (Monyer et al., 1994).

A similar pattern of NR2 mRNA and protein expression has been observed in a study of the human foetal brain, where NR2B is the predominant subunit expressed in all of the cortical layers as early as gestational week 8 with transient increases at week 11, 13 and 19 (Ritter et al., 2001). There is a decline in NR2B mRNA in the human hippocampus from its peak expression during the neonatal stage of life to adulthood, whereas NR2A mRNA expression appears to remain consistent

throughout the life stages (Law et al., 2003). Interestingly, NR2B mRNA levels remain significantly higher than NR2A in the dentate gyrus of the hippocampus during development and at adulthood (Law et al., 2003).

NR2A and NR2B containing NMDARs often have functionally different roles via their activation of opposing cellular pathways. For example, activation of synaptic NR2A containing receptors activates cell survival pathways e.g. CREB, whereas activation of extrasynaptic NR2B containing NMDARs deactivates these same pathways, leading to neuronal death (Hardingham et al 2002). Similarly, Hu et al (2008) reported that NR2A containing NMDARs in the hippocampus regulate neurogenesis positively, while NR2B containing NMDARs regulate it negatively (Hu et al., 2008). Further, it has been reported that blockade of NR2B subunits with ifenprodil, in rat hippocampal slices, results in an enhanced depolarisation response of NMDARs, mediated via NR2A subunits. This suggests that an inhibitory relationship exists between NR2A and NR2B subunits, whereby NR2B containing NMDARs normally maintain inhibitory control over NR2A containing NMDARs (Mallon et al., 2005). In vivo, Mao et al (2006) found that blocking NR2B containing NMDARs with ifenprodil during the mouse neurodevelopmental critical period reduced synaptic plasticity in the adult mouse auditory cortex, suggesting that it is the NR2B subunit that is critical to synaptic plasticity/modulation. This data highlights that the balance between NR2A and NR2B containing NMDARs is key in determining neuronal function, synaptic plasticity and normal brain development. Therefore abnormalities in the balance between NR2A and NR2B during development and maturation may play a role in the pathophysiology of schizophrenia. Evidence suggests that the NR2B subunit in the hippocampus is particularly important for NMDAR channel function (Akashi et al., 2009), long-term

potentiation (LTP) and associated cognitive functions such as spatial learning (Clayton et al., 2002). Accordingly, specifically targeting the NR2B subunit has been suggested as a novel mechanism to treat cognitive dysfunction in schizophrenia patients (Mony et al., 2009, Menniti et al., 2013).

There has been little investigation on whether this developmental regulation of NR2 subunits differs between the two genders. However, there are gender differences in brain maturation (De Bellis et al., 2001) and there is evidence to suggest that males and females are at different susceptibility to stress-induced cognitive disruptions mediated by the prefrontal cortex (Markham et al., 2013). Therefore it is likely that the developmental profile of underlying systems such as the NMDA system are differentially regulated between the two genders, especially at adolescence. The hormone oestrogen has also been shown to depress NMDAR function and reduce phosphorylation of the NR2B subunit in the prefrontal cortex (Liu et al., 2012), while in the hippocampus it enhances LTP via a functional increase in NR2B-containing NMDARs (Vedder et al., 2013). The higher density of NMDARs at the synapse however are possibly due to the recruitment of NR2B-containing receptors from extrasynaptic locations (Vedder et al., 2013) rather than increased expression of NR2B protein, as oestrogen does not appear to affect NMDAR, NR2A or NR2B protein levels (Snyder et al., 2011).). These findings reveal potential differences in NMDAR-associated biochemistry in the male versus female brain due to differing hormone levels.

1.3 NMDAR Subunits and Schizophrenia

1.3.1 Evidence from Genetic Studies

While genetic vulnerability is clearly associated with schizophrenia, schizophrenia has a heterogeneous aetiology involving multiple genes. Several of the genes associated with schizophrenia include sites on the NMDAR itself, including the receptor subunits NR1, NR2A and NR2B coded for by the *GRIN1* (Galehdari et al., 2009), *GRIN2A* (Tang et al., 2006) and *GRIN2B* (Qin et al., 2005) genes, or genes that regulate NMDAR function (Snyder and Gao, 2013). The *GRIN2B* gene appears particularly associated with schizophrenia with the frequency of a genetic variation in the promoter region found to be higher in schizophrenia patients and this has been shown to considerably affect the transcription of the gene (Miyatake et al., 2002). This genetic polymorphism was also found to be selective to schizophrenia in a similar study that examined overall haplotype frequency in schizophrenia, bipolar disorder and control subjects (Martucci et al., 2006). In a recent study investigating the association of *GRIN1* and *GRIN2A-D* with schizophrenia, a genetic variation in the 3' region of *GRIN2B*, but not the other GRINs, was found to be significantly associated with schizophrenia (Demontis et al., 2011).

1.3.2 Evidence from Environmental Factors

Environmental factors, such as early life stress, have shown aetiological relevance to schizophrenia in animal models, as early life stress has been found to reduce hippocampal NR2B levels and alter the synaptic NR2A/NR2B balance leading to potential learning and memory deficits in later life (Zhao et al., 2013). A similar study, adding weight to the above results, found that prenatal stress reduces NR1 and NR2A protein in the hippocampus and reduces NR1 protein in the

prefrontal cortex and striatum in male and female rats (NR2B protein was not examined) (Sun et al., 2013). Another study has shown a decrease in NMDAR binding and NR2A mRNA expression in the hippocampus of rats exposed to repeated postnatal hypoxia (a factor of obstetric complications) and an increase in NR1 mRNA that remained in the adult rat brain (Schmitt et al., 2007).

1.3.3 Evidence from Postmortem Human Brain Studies

Studies in postmortem human brain support the hypothesis of NMDAR dysfunction in schizophrenia. For example altered NMDAR binding density has been reported in several cortical brain regions in schizophrenia (Kornhuber et al., 1989, Zavitsanou et al., 2002, Newell et al., 2005, Pilowsky et al., 2005) although not all studies and/or brain regions have shown changes (Weissman et al., 1991, Scarr et al., 2005). Many studies have examined the status of the specific NMDAR subunits in schizophrenia, due to their different functional properties, with results showing alterations in the level of NR1 and NR2 mRNA and protein, as well as the proteins in the PSD, in schizophrenia subjects in comparison to control subjects (see Table 1.1). Most recently, protein and mRNA expression of the obligatory NR1 subunit was reported to be decreased in the dorsolateral prefrontal cortex in schizophrenia subjects, using one of the largest cohorts to date (n=37/group) (Weickert et al, 2012). Interestingly, genetic variation in the *GRIN2B* gene was associated with the reduced expression and was also predictive of cognitive dysfunction. While this study reported no change in NR2B mRNA, they did not explore NR2B protein levels; it is known that mRNA levels do not always denote corresponding changes in protein levels (Greenbaum et al, 2003), so assessing NR2B protein levels in this region in schizophrenia subjects may be important. They did however report that NR2B mRNA expression was 39% higher in the left versus right DLPFC and 33% greater

in males relative to females, independent of diagnosis (Weickert et al., 2013), but this highlights the importance of considering gender and hemisphere in studies of this nature.

NR1 mRNA has been reported to be decreased in the hippocampus and thalamus and increased in the occipital and anterior cingulate cortices in schizophrenia (Gao et al., 2000, Ibrahim et al., 2000, Dracheva et al., 2001, Kristiansen et al., 2006). However these results vary in terms of the specificity of the probe used to detect the mRNA with some studies using pan probes while others are specific to certain splice variants of NR1. There are alterations reported in NR2 subunit mRNA and protein in the prefrontal cortex, including an increase in NR2D mRNA, and a reduction in NR2A and NR2C mRNA as well as NR2B protein (Akbarian et al., 1996, Beneyto and Meador-Woodruff, 2008, Kristiansen et al., 2010b). NR2B subunit mRNA has been found to be increased in the hippocampus (Gao et al., 2000) while NR2B subunit protein has been found to be selectively upregulated in the superior temporal cortex (Grimwood et al., 1999). Furthermore alterations in associated transporter and regulatory proteins of the NMDAR subunits has been reported in schizophrenia, particularly associated with the NR2B subunit (Kristiansen et al., 2010a). These findings show significant changes however there are also studies showing no changes in the same brain regions (see Table 1.1). Clinton and colleagues have found that the use of an elderly versus a younger cohort of samples can influence direction of change in the subunits, particularly in the thalamus. The PSD and SAP proteins appear to be particularly vulnerable to age with opposite changes in both the prefrontal cortex and thalamus depending on whether the cohort is elderly or younger (Clinton et al., 2003, Clinton and Meador-Woodruff, 2004, Clinton et al., 2006). Furthermore differences in the specificity of the brain

region and cellular compartments, as well as examination of hemisphere and gender effects are also likely contributing factors to the various results found. For example, while Kristiansen (2010b) found no change in NR2B protein levels in the DLPFC in total cell homogenates, when examined in endoplasmic reticulum enriched fractions they revealed a significant reduction in schizophrenia subjects. Therefore due to the inconsistent findings, which could be attributed to differences in tissue cohorts and methodologies, these results must be interpreted with some caution.

1.3.4 Evidence from Rodent Studies

While human postmortem data supports that NMDARs and their subunits are altered in schizophrenia, at least in some cohorts, animal studies have and will continue to assist in unravelling the functional and developmental significance of those alterations in schizophrenia patients. There are a number of animal studies that have examined the role of a non-specific NMDAR blockade on behaviour and neurochemical alterations in the rodent (see du Bois and Huang, 2007), however there is currently limited animal data available with regard to the importance of the specific subunits. Blockade of the NR2A subunit with NVP-AAM077 (PEAQX) has been reported to reduce locomotor activity (5-10 mg/kg) and not alter prepulse inhibition (PPI) (5-20 mg/kg) (Chaperon et al., 2003). On the other hand, the NR2B antagonist Ro 25-6981, produced hyperlocomotor activity (20 mg/kg), disrupted PPI (5-20 mg/kg), an increase in anxiety-like behaviour (10mg/kg) and an impairment in learning and memory (5-10 mg/kg) in adult rats, which may be recognised as schizophrenia-like behaviours in the rodent (Chaperon et al., 2003, Duffy et al., 2007, Mathur et al., 2009). However, a lack of locomotor changes in rodents treated with 10 mg/kg Ro 25-6981 has also been reported (Kosowski and Liljequist, 2004). Adult rats treated with a more potent and selective NR2B antagonist, Ro 63-1908,

displayed increased locomotor activity (10-30 mg/kg) and impaired response inhibition (1-10 mg/kg) but no disruption to PPI (1-10 mg/kg) (Higgins et al., 2003).

Table 1.1. Changes in NMDAR subunits in human postmortem brain tissue in schizophrenia.

NMDAR subunit		Brain region	Cohort
NR1		**Prefrontal cortex**	
	mRNA	Increase in DLPFC by 100%[a]	n=13C/26SZ
		Decrease in DLPFC by 22%[b]	n=37C/37SZ
		Lamina specific reductions in DLPFC[c]	n=15C/15SZ
		No change in BA10[d]	n=15C/15SZ
	protein	No change in DLPFC[e] or ER fraction of DLPFC[f]	n=8C/13SZ; n=8C/13SZ
		Hippocampus	
	mRNA	Decrease in dentate gyrus by 15%[g]	n=35C/31SZ
		No change in any subdivision of hippocampus[h,i,j]	n=8C/8SZ; n=15C/15SZ; n=8C/13SZ
		Decrease in NR1-4 of hippocampus (subdivisions combined) in LH by 33%[j]	n=13C/16SZ
		Decrease in NR1-2 of hippocampus (subdivisions combined) in RH by 41%[j]	n=13C/16SZ
	protein	Decrease in male SZ subjects by 55% in LH[j]	n=7C/8SZ
		No change in dentate gyrus[k]	n=15C/15SZ
		Other regions	
	mRNA	Decrease in DM and CM thalamus by 55%[l]	n=8C/12SZ
		No change in any subdivision of thalamus[m,n]	n=16C/14SZ; 15C/15SZ
		Increase in occipital cortex by 230%[a]	n=13C/26SZ
		Increase in NR1 (pan) by 15% and NR1-1 (isoform) by 22% in STG[o]	n=6C/6SZ
		No change in parietotemporal cortex (BA39)[d]	n=15C/15SZ
		No change in middle frontal and visual[o], entorhinal and perirhinal cortex[i]	n=6C/6SZ; n=15C/15SZ
		No change in cerebellum[d]	n=15C/15SZ
	protein	No change in ventral or DM thalamus[p]	n=8C/15SZ
		Increase in ACC by 35%[e]	n=8C/13SZ
		Increase in NR1 by 30% of STG[s]	n=14C/14SZ
		No change in STG[k], orbitofrontal cortex[q] or ER fraction of ACC[f]	n=15C/15SZ; n=15C/15SZ; n=8C/13SZ
NR2A		**Prefrontal cortex**	
	mRNA	Lamina specific reductions in DLPFC[c]	n=15C/15SZ

		No change in DLPFC[a] or BA10[d]	n=13C/26SZ; n=15C/15SZ
	protein	No change in DLPFC[c,e]	n=15C/15SZ; n=8C/13SZ
		Hippocampus	
	mRNA	No change in any subdivision of hippocampus[g,h,i]	n=35C/31SZ; n=8C/8SZ; n=15C/15SZ
	protein	No change in any subdivision of hippocampus[i]	n=15C/15SZ
		Other regions	
	mRNA	No change in any subdivision of thalamus[l,m,n]	n=8C/12SZ; n=16C/14SZ; 15C/15SZ
		Increase in occipital cortex by 230%[a]	n=13C/26SZ
		No change in parietotemporal[d], entorhinal and perirhinal cortex[i]	n=15C/15SZ; n=15C/15SZ
		No change in cerebellum[d,r]	n=15C/15SZ; n=9C/10SZ
	protein	No change in any subdivision of the thalamus[l,p]	n=8C/12SZ; n=8C/15SZ
		No change in ACC[e,s]	n=8C/13SZ; n=14C/14SZ
NR2B		**Prefrontal cortex**	
	mRNA	No change in DLPFC or BA10[a,c,d]	n=13C/26SZ; n=15C/15SZ; n=15C/15SZ
	protein	Decrease in ER fraction of DLPFC by 55%[f]	n=8C/13SZ
		No change in DLPFC[c,e]	n=15C/15SZ; n=8C/13SZ
		Hippocampus	
	mRNA	Increase in CA2 by 40%[g]	n=35C/31SZ
		No change in any subdivision of the hippocampus[h,i]	n=8C/8SZ; n=15C/15SZ
	protein	No change in any subdivision of the hippocampus[i]	n=15C/15SZ
		Other regions	
	mRNA	Decrease in CM thalamus by 60%[l]	n=8C/12SZ
		Increase in combined subdivisions of thalamus by 30%[n]	n=15C/15SZ
		No change in any/other subdivision of thalamus[l,m]	n=8C/12SZ; n=16C/14SZ
		No change in occipital and parietotemporal[a,d], entorhinal and perirhinal cortex[i]	n=13C/26SZ; n=15C/15SZ; n=15C/15SZ

		No change in cerebellum[d,r]	n=15C/15SZ; n=9C/10SZ
	protein	Decrease in anterior, DM and CM thalamus by 20-29%[l]	n=8C/12SZ
		Increase in DM thalamus by 375% but no change in ventral thalamus[p]	n=8C/15SZ
		No change in LD, reticular or ventral thalamus[l]	n=8C/12SZ
		Increase in STG by 100%[s]	n=14C/14SZ
		No change in ACC[e], entorhinal and perirhinal cortex[i]	n=8C/13SZ; n=15C/15SZ
NR2C		**Prefrontal cortex**	
	mRNA	Decrease in BA10 by 22-41%[d]	n=15C/15SZ
		Lamina specific reductions in DLPFC[c]	n=15C/15SZ
	protein	No change in DLPFC[e]	n=8C/13SZ
		Hippocampus	
	mRNA	No change in any subdivision of hippocampus[h,i]	n=8C/8SZ; n=15C/15SZ
		Other regions	
	mRNA	Decrease in anterior, DM, LD and CM thalamus by 54-73%[l]	n=8C/12SZ
		No change in any subdivision of thalamus [m,n]	n=16C/14SZ; 15C/15SZ
		No change in parietotemporal[d], entorhinal and perirhinal cortex[i]	n=15C/15SZ; n=15C/15SZ
		No change in cerebellum[d,r]	n=15C/15SZ; n=9C/10SZ
	protein	No change in ACC[e]	n=8C/13SZ
NR2D		**Prefrontal cortex**	
	mRNA	No change DLPFC or BA10[e,d]	n=15C/15SZ; n=15C/15SZ
	protein	No change in DLPFC[e]	n=8C/13SZ
		Hippocampus	
	mRNA	No change in any subdivision of hippocampus[h,i]	n=8C/8SZ; n=15C/15SZ
		Other regions	
	mRNA	No change in any subdivision of thalamus[l,m,n]	n=8C/12SZ; n=16C/14SZ; 15C/15SZ
		No change in parietotemporal[d], entorhinal and perirhinal cortex[i]	n=15C/15SZ; n=15C/15SZ
		Increase in vermis and RH of cerebellum by 100-110%[r]	n=9C/10SZ

protein	No change in ACC[e]	n=8C/13SZ

Abbreviations: [a]Dracheva et al., 2001, [b]Weichert et al., 2012, [c]Beneyto and Meador-Woodruff, 2008, [d]Akbarian et al., 1996, [e]Kristiansen et al., 2006, [f]Kristiansen et al., 2010a, [g]Gao et al., 2000, [h]McCullumsmith et al., 2007, [i]Beneyto et al., 2007, [j]Vrajova et al., 2010, [k]Toro and Deakin, 2005, [l]Ibrahim et al., 2000, [m]Dracheva et al., 2008, [n]Clinton and Meador-Woodruff, 2004, [o]Le Corre et al., 2000, [p]Clinton et al., 2006, [q]Nudmamud-Thanoi and Reynolds, 2004, [r]Scmitt et al., 2010, [s]Grimwood et al., 1999. ACC=anterior cingulate cortex, BA=Brodmann's area, C=control, CA2=cornu ammonis 2 of hippocampus, CM=central medial thalamus, DLPFC=dorsolateral prefrontal cortex, DM=dorsomedial thalamus, ER=endoplasmic reticulum, LD=lateral dorsal thalamus, LH=left hemisphere, NMDAR=N-methy-D-aspartate receptor, NR1/2A/2B/2C/2D=NMDAR subunits, RH=right hemisphere, STG=superior temporal cortex, SZ=schizophrenia.

Although these studies show behavioural abnormalities that mirror schizophrenia symptoms following NR2B but not NR2A antagonist treatments in adult animals, these abnormalities are not as extensive as those observed with non-selective NMDAR antagonists. Due to the expression pattern of the NR2 subunits in early development and the relevance of the developmental neurobiology of schizophrenia, NR2 antagonist treatment may need to be further examined in a perinatal model in order to verify its relevance to the aetiology of schizophrenia. Gene knockout studies have shown that targeting the NR2 subunits specifically causes different outcomes. For example, NR2A knockout mice exhibit reduced anxiety and depression-like behaviour (Boyce-Rustay and Holmes, 2006) and normal PPI (Spooren et al, 2004) but do also show evidence of cognitive deficits with reduced LTP and spatial memory impairments (Bannerman et al., 2008). Mice with a complete knockout of NR2B die shortly after birth due to an impaired suckling response and cannot undergo behavioural analysis without hand feeding (Kutsuwada et al., 1996), highlighting the importance of this subunit to development. A recent study has shown that there are spatial and non-spatial memory deficits following a gene-targeted forebrain-specific deletion of NR2B in adult mice, whereas hippocampal-restricted deletion of NR2B induces a selective working memory deficit (von Engelhardt et al., 2008).

Anastasio and colleagues (2009) have investigated the behavioural and cellular effects of perinatal NR2A and NR2B antagonism. They reported enhanced apoptosis following perinatal (PN7, 9 and 11) NR2A antagonist treatment (10 and 20 mg/kg NVP-AAM077) in addition to a locomotor sensitisation effect to a PCP challenge, while perinatal NR2B antagonist treatment (1 or 5 mg/kg ifenprodil) did not produce elevated apoptosis or a locomotor sensitisation to PCP (Anastasio et al.,

2009). The NR2B antagonist (ifenprodil) used, is not as potent or specific to the NR2B subunit as Ro 63-1908 (Zhou et al., 1999) in which doses of up to 30 mg/kg have been used in adult rodents. Therefore, this dose of ifenprodil may not be high enough to see any behavioural effects and hence there remains the possibility that NR2B antagonist-induced apoptosis does occur. Furthermore NVP-AAM077 is not completely specific to NR2A, it also has NR2B binding properties (Frizelle et al, 2006). While it is possible that the apoptotic effect may be related to the NR2A-containing NMDAR's, this may leave an imbalance of NR2 subunits (possibly via a responsive upregulation of NR2A subunits and hence a relative decrease in NR2B), which may cause behavioural and neurochemical abnormalities reminiscent of schizophrenia. To our knowledge no-one has investigated the effect of a perinatal NR2B blockade on neurotransmitter receptors or proteins in the brain.

Changes in NMDAR subunit expression have been observed following NMDAR antagonist treatment. Acute treatment with PCP on PN7, increased the expression of membrane NR2B protein and decreased the level of NR2B protein eight hours after administration in the endoplasmic reticulum in the frontal cortex of the rat brain (Anastasio and Johnson, 2008). This was suggested to be due to an increase in the trafficking of the NR2B subunit to the membrane. This short-term upregulation of NR2B was accompanied by an increase in the level of PSD-95 but not the NR2A subunit (Anastasio and Johnson, 2008). On the other hand sub-chronic treatment with PCP resulted in an elevated membrane and endoplasmic reticulum level of NR2A but not NR2B or PSD-95 which has been attributed to an increase in synthesis of NR2A protein (Anastasio and Johnson, 2008). Eight hours following NMDAR blockade an increase in the insertion of new NR2A subunit containing NMDARs has also been found in hippocampal cell cultures, however no change in

the NR2B subunit (von Engelhardt et al., 2009). Similarly Wilson et al (1998) found that NR2A mRNA was significantly increased in the striatum, cortex and hippocampus four hours following MK-801 treatment to rats on PN7 (Wilson et al., 1998). It has been hypothesised that this imbalance of the NMDAR subunits, particularly the disproportionate NR2A/NR2B ratio, after treatment with NMDAR antagonists causes an acceleration of the normal NMDAR development processes (Wilson et al., 1998). This disproportionate development of the NMDAR subunits and the consequent alteration in NMDAR function could trigger a cascade of modifications to other signalling pathways and hence, render the brain more susceptible to schizophrenia onset later in life. Long-term analysis of NMDAR subunits in adult rats following perinatal PCP treatment has shown that the NR2A and NR2B subunit protein are increased in the prefrontal cortex but not the hippocampus (Owczarek et al., 2011). A similar study from our group found increases to NR2A in the hippocampus and NR2B in the prefrontal cortex at five weeks of age after perinatal PCP treatment but decreases to NR2A and NR2B in the hippocampus, but not prefrontal cortex, at adulthood highlighting the region-specific natures of these models (du Bois et al., 2012).

1.4 The NRG1 Signalling Pathway and Schizophrenia:

As previously mentioned, genetic linkage studies in several populations have provided evidence of *NRG1* as a susceptibility gene for schizophrenia (Stefansson et al., 2002, Williams et al., 2003, Bakker et al., 2004, Fukui et al., 2006, Munafo et al., 2006, Walss-Bass et al., 2006). *NRG1* belongs to a family of genes encoding for growth/differentiation factors that exert their effects on various organs, including the brain, by activating the ErbB tyrosine kinase receptors (Garcia et al., 2000). The

NRG1 gene transcripts can be classed as six types and comprise over 31 different isoforms of *NRG1* due to the presence of multiple promoter regions and alternative splicing of the gene sequence (Falls, 2003, Harrison and Law, 2006, Mei and Xiong, 2008).

NRG1 is synthesised in a membrane-bound form, often referred to as pro-NRG1, a precursor to the mature NRG1 signalling protein that is generated from the proteolytic cleavage at the extracellular transmembrane (TM) region. The release of the soluble extracellular portion (inclusive of the epidermal growth factor (EGF) domain) of NRG1 occurs in most isoforms, with the exception of type III which remains in contact with the cell as both the N-terminal and C-terminal of the protein are located intracellularly (For review see Mei and Xiong, 2008). The cleavage is catalysed by the proteases tumor necrosis factor-α converting enzyme (TACE), β-site of amyloid precursor protein cleaving enzyme (BACE) and meltrin β, and leads to the diffusion of mature NRG1 into the extracellular space for functional purposes (Hu et al., 2006, Yokozeki et al., 2007, Mei and Xiong, 2008).

The pattern of expression of NRG1 mRNA and protein in the rodent central nervous system during gestational development varies between the different isoforms. Type I mRNA is predominant during early embryogenesis whereas type II and III mRNA are not detected until midgestation but display a broader expression pattern in the brain (Meyer et al., 1997). Similarly NRG1 type I protein is expressed early in embryonic development with a strong distribution already present in the cortical plate, piriform cortex, septum, putamen and basal telencephalon by embryonic day 17 (Pinkas-Kramarski et al., 1994). This differential expression indicates that each isoform may have a distinct functional role during development (Meyer et al., 1997). At adulthood, the regional distribution of NRG1 transcripts and

protein is widespread throughout the rodent brain, mirroring that seen in the adult human brain (Law et al., 2004), however the expression remains in a distinct nuclei and cortical layer specific manner (Meyer and Birchmeier, 1994, Pinkas-Kramarski et al., 1994). To our knowledge the normal developmental expression of NRG1 protein has not been studied from postnatal to adult levels but is essential to our understanding of the NRG1 signalling system, its location, function and interactions with other proteins.

ErbB4 is the predominant receptor for NRG1 in neurons (Bjarnadottir et al., 2007) with emerging evidence showing a preferential location on parvalbumin-containing GABAergic interneurons (Fazzari et al., 2010). Alternative splicing of ErbB4 transcripts is known to generate at least four isoforms made up of different combinations of juxtamembrane (JMa and JMb) and intracellular regions (CYT1 and CYT2), which consequently are coupled to distinct signalling pathways inside the cell (For review see Mei and Xiong, 2008). The activation of ErbB receptors by NRG1 results in the formation of homodimers or heterodimers of the various receptor subtypes (ErbB1-4) and the phosphorylation of the intracellular kinase domain. These phosphorylated tyrosine residues are protein binding sites for downstream signalling molecules and enzymes that can have varying effects on the regulation of transcriptional and translational factors in the cell (Mei and Xiong, 2008).

Researchers have found altered levels of NRG1 subtypes in postmortem brain tissue of schizophrenia patients. An upregulation of NRG1 type I mRNA has been observed in the dorsolateral prefrontal cortex and hippocampus of schizophrenia subjects compared to control (Hashimoto et al., 2004, Law et al., 2006), however results are inconsistent for the protein levels and depend on the

fragment or isoform of NRG1 examined (Chong et al., 2008, Boer et al., 2009, Barakat et al., 2010, Stuart et al., 2011, Marballi et al., 2012). Furthermore, an association between a single nucleotide polymorphism (SNP) in the at-risk haplotype of NRG1 and altered NRG1 transcript expression in the hippocampus has been reported (Law et al., 2006).

An association between the ErbB4 gene and schizophrenia has also been reported. The identification of single SNP's in the non-coding, intronic region of the candidate gene and the increased disease risk for individuals who carry these SNP's has lead researchers to investigate the possible functional consequences of these genetic variations (Norton et al., 2006, Silberberg et al., 2006, Law et al., 2007). Indeed it has been shown that certain splice-variants of ErbB4 mRNAs are altered in the brain of schizophrenia subjects (Law et al., 2007). CYT-1 and JM-a isoforms of ErbB4 are increased in the prefrontal cortex but not the hippocampus compared to controls and interestingly a positive correlation has been found between the abnormal expression of these isoforms and the various risk SNPs for schizophrenia (Silberberg et al., 2006, Law et al., 2007). A similar increase in ErbB4 protein in the prefrontal cortex but not the hippocampus has also been observed in schizophrenia subjects (Chong et al., 2008, Stuart et al., 2011).

Several genetic knockout animal studies have also highlighted the potential role of NRG1 in brain and behavioural abnormalities that are related to schizophrenia. Results have revealed that TM-domain *Nrg1* heterozygous mutant mice display hyperlocomotion, increased aggressive and exploratory behaviours, social interaction deficits and have impaired PPI compared to wild type mice when tested at adulthood (Stefansson et al., 2002, Karl et al., 2007, O'Tuathaigh et al., 2007, O'Tuathaigh et al., 2008). Chen and colleagues (2008) recently reported that

adult mutant mice heterozygous for type III *Nrg1* also show disrupted PPI as well as impairments in working memory tasks, but no change in locomotor activities (Chen et al., 2008). These behavioural alterations are similar to those observed with NMDAR hypofunction in rodents and are also representative of schizophrenia symptomology. While these animal models support the possibility that genetic mutations in *NRG1* could contribute to schizophrenia symptomology, as stated by Li and colleagues (2007), mutations in *NRG1* genes are only likely to account for a small fraction of schizophrenia cases. In other cases, primary dysfunction in other genes/signalling pathways (eg glutamate/NMDAR) may lead to disrupted development of the NRG1 system and subsequently schizophrenia symptomology.

1.5 NMDAR and NRG1 Interactions:

The NR2 subunits of the NMDAR and the ErbB4 receptor share a common anchoring region on the PDZ2 domain of PSD-95 and other molecules in the PSD and through this commonality it is thought that interactions occur (Garcia et al., 2000) (Figure 1.1).

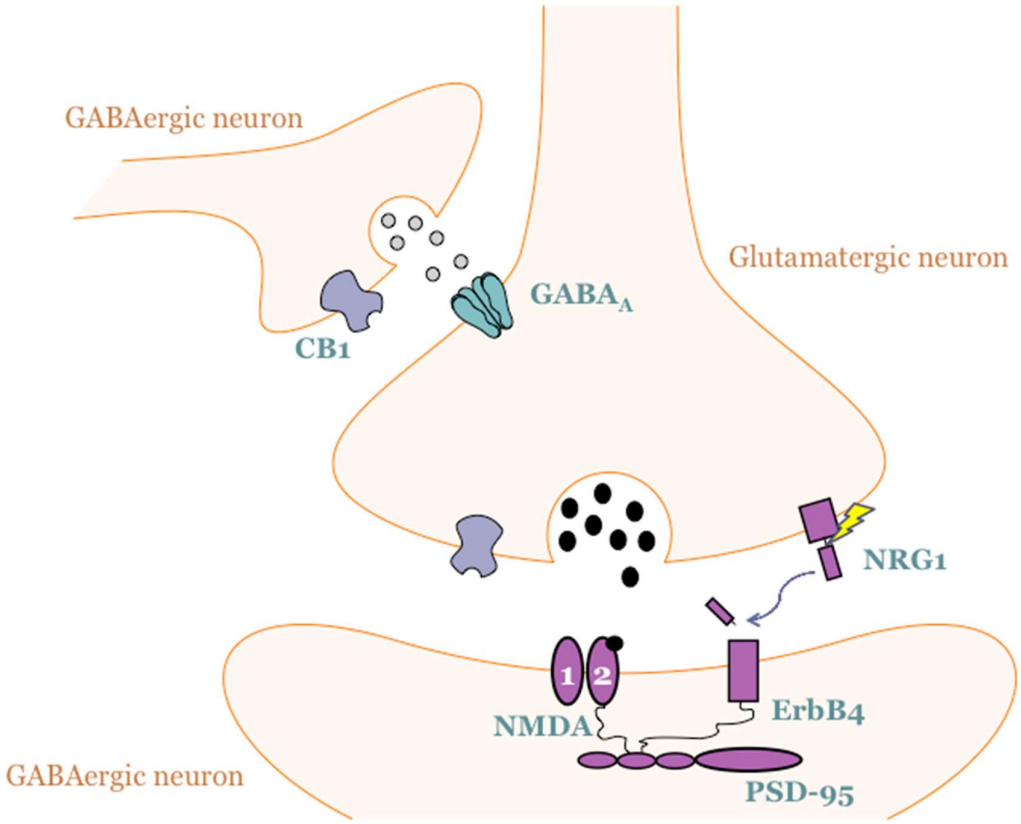

Figure 1.1. Interactions and locations of NMDAR, NRG1, ErbB4, $GABA_AR$ and CB_1R molecules on glutamatergic and GABAergic neurons.

There have been various reports of NMDAR modulation by NRG1 signalling. Firstly, it has been shown that *Nrg1* EGF-domain heterozygous mutant mice display a reduction in MK-801 binding in the prefrontal cortex, suggestive of reduced NMDAR expression (Stefansson et al., 2002). Other studies have found that NRG1 stimulation in the prefrontal cortex enhances the association of ErbB4 with both PSD-95 and the NR1 subunit in schizophrenia patients and causes a reduction in NR2A phosphorylation and NMDAR activation and an increase in NMDAR internalisation (Gu et al., 2005, Hahn et al., 2006). Highlighting a regional difference in signalling pathways, the disruption of NRG1-mediated ErbB4 stimulation in the hippocampus, also results in the loss of NMDARs (Li et al., 2007). Application of

NRG1 to cultured hippocampal neurons does not affect the mRNA expression levels of the NR2 subunits (Okada and Corfas, 2004) however, it has been found that NRG1 signalling in the hippocampus enhances phosphorylation of the NR2B subunit, concurrent with ErbB4 phosphorylation and the activation of Fyn and Pyk2 (proline-rich tyrosine kinase 2), two non-receptor tyrosine kinases thought to be involved in the downstream amplification of NRG1 signalling (Bjarnadottir et al., 2007). NR2B was also found to be hypophosphorylated in the hippocampus of TM-domain NRG1$^{+/-}$ mutant mice, as well as in ErbB4$^{+/-}$ mutant mice, which can lead to reduced channel opening and functioning of the NMDAR, but the hypophosphorylation was reversed with clozapine treatment (Moghaddam, 2003, Bjarnadottir et al., 2007). Interestingly, clozapine has also been shown to increase NR2B-containing NMDAR currents in the nucleus accumbens (Wittmann et al., 2005). From these findings it has been speculated that attenuated NRG1 signalling in the hippocampus may cause abnormal modulation of NMDAR function through the altered regulation of NR2B phosphorylation and hence, contribute to the pathophysiology of schizophrenia (Bjarnadottir et al., 2007). Adding further complications to the regional specificity of NRG1 signalling, NR2C mRNA expression has been found to be decreased in the cerebellar molecular layer of the right hemisphere and vermis in a population of schizophrenia patients with a NRG1 polymorphism (Schmitt et al., 2010). Supporting this finding, the NRG1-β isoform has been shown to upregulate the expression of NR2C mRNA in cultured cerebellar slices (Ozaki et al., 1997). Together these findings reveal the differential effects that NRG1 signalling has on the NMDAR subunits in the different brain regions, however there is still work to be done in order to further understand this complex signalling system.

While it has been shown that the NRG1-ErbB4 signalling pathway can affect glutamatergic function, the reverse may also be true and the activity of NRG1 could be influenced by the NMDAR. The link between the NR2 subunits and the PSD provides support for a disruption to the NRG1/ErbB4 system following an NMDAR insult, as ErbB4 is also physically linked to PSD proteins (Figure 1.1). Results have shown that PSD-95 and ErbB4 are capable of forming a ternary complex in neurons, which considerably increases tyrosine phosphorylation of the ErbB4 receptor and enhances NRG1-induced ErbB4 signalling (Huang et al., 2000). It is therefore likely that this regulation of the NRG1/ErbB4 pathway by PSD-95 could be affected by disruptions to the NMDAR, or more specifically the NR2 subunits, and play a major role in schizophrenia pathophysiology. Recent data from our laboratory has shown that perinatal PCP treatment produces alterations in NRG1 and ErbB4 protein expression throughout development in the prefrontal cortex but not the hippocampus (du Bois et al., 2012). In addition, an article by Feng and colleagues (2010) found that chronic treatment with the specific NMDAR antagonist MK-801 in adult rats produced an increase in NRG1 and ErbB4 protein expression in the prefrontal cortex and hippocampus (Feng et al., 2010). Therefore regulation of NRG1/ErbB4 signalling via the NMDAR is possible, however the exact mechanism of how this occurs remains unknown and whether there is region or subunit specificity also remains elusive. Furthermore, a blockade of the NMDAR has the ability to produce functional deficits in GABAergic interneurons in certain cortical areas and the hippocampus (Li et al., 2002, Braun et al., 2007, Homayoun and Moghaddam, 2007) which similarly has been noted in schizophrenia pathology (Wassef et al., 2003). More specifically it has been shown that the loss of parvalbumin and GAD67 in cortical interneurons following NMDA antagonism can be attributed to the NR2A

subunit (Kinney et al., 2006). As mentioned previously these interneurons are the preferential location of ErbB4 receptors and so it is highly probable that alterations to NMDAR functioning will affect ErbB4.

1.6 Inhibitory Neurotransmission and Schizophrenia

The GABAergic and cannabinoid systems are key regulators of inhibition within the brain. Both systems play an important role in regulating glutamatergic transmission (Figure 1.1). GABA signals through the ionpotropic $GABA_AR$ as well as the metabotropic $GABA_AR$. The endogenous cannabinoids (anadamide, 2-arachidonylglycerol) signal via the g-protein coupled CB_1 and CB_2 receptors. Cannabinoid CB_1 receptors (CB_1R) are more abundant in the CNS than CB_2, with the latter playing a leading role in immune related functions. CB_1Rs are highly abundant on glutamatergic and GABAergic neurons in the CNS where it plays a role in the inhibition of glutamate and GABA release. In humans and rodents, CB_1Rs are involved in the regulation of a variety of behaviours including, but not limited to, motor activity, pain sensitivity, learning, memory and cognition (Rodriguez de Fonseca et al., 1998; Strangman et al., 1998; Varvel et al., 2009). CB_1Rs are functionally active in early gestation (Berrendero et al., 1998; Berrendero et al., 1999; Mato et al., 2003) and throughout development there is a general increase in CB_1R levels in the human, monkey and rat brain, although expression remains region specific (Belue et al., 1995; Berrendero et al., 1999; Eggan et al., 2010a; Mato et al., 2003). The $GABA_AR$ also displays a distinct developmental and regional expression in the rat brain (Xia and Haddad, 1992), particularly in terms of the individual subunits, with expression emerging in embryonic development, highlighting the

major role of the GABAergic system in neuronal migration, differentiation and survival (for review see Galanopoulou, 2008).

Both the GABAergic and cannabinoid systems have been implicated in neurodevelopmental disorders of psychosis, such as schizophrenia. A deficit of GABAergic signalling has been implicated in schizophrenia as reductions in GABAergic interneurons and increased levels of $GABA_ARs$ have been observed in postmortem human schizophrenia brain tissue compared to controls (Benes et al., 1992; Benes et al., 1996a; Benes et al., 1996b; Dean et al., 1999; Deng and Huang, 2006; Newell et al., 2007b). Increased levels of CB_1R binding in the prefrontal and cingulate cortices have also been found in schizophrenia brain tissue (Dalton et al., 2011; Giuffrida et al., 2004; Newell et al., 2006; Zavitsanou et al., 2004), however a decrease in CB_1Rs has been found in two areas of the prefrontal cortex (Egan et al., 2008; Egan et al., 2010b). Furthermore it has been shown that susceptible individuals who use cannabis (which activates the CB_1R), especially at the developmental stage of adolescence, display a range of schizophrenia-like symptoms and have an increased risk for the development of schizophrenia (Caspari, 1999; Linszen et al., 1994; van der Stelt and Di Marzo, 2003). Considering not every individual who uses cannabis develops schizophrenia symptoms it is thought that there may be an underlying predisposition that renders that brain more vulnerable to the effects of cannabis (Boucher et al., 2007b).

Young adult rats treated subchronically with PCP show schizophrenia-like cognitive deficits, which are worsened when subjected to Δ^9-tetrahydrocannabinol (THC; the active ingredient in cannabis) (Viagno et al, 2009). These rats also display a reduction in functional CB_1Rs in the prefrontal cortex, hippocampus and substantia nigra, which may contribute to the greater sensitivity (Vigano et al, 2009). Similarly,

a genetic knockout animal study has highlighted the potential role of a disruption in the NRG1 signalling system in inducing a vulnerability to the effects of cannabis (Boucher et al., 2007b). As previously mentioned, TM-domain *Nrg1* heterozygous mutant mice already display schizophrenia-like behaviours (Stefansson et al., 2002, Karl et al., 2007, O'Tuathaigh et al., 2007, O'Tuathaigh et al., 2008). Interestingly, when treated with THC these mice show a greater sensitivity to the behavioural effects of this main psychoactive ingredient of cannabis (Boucher et al., 2007a). These mice also show a borderline significant alteration in CB_1Rs, which may contribute in part, to this increased susceptibility (Newell et al, 2013).

1.7 The Hippocampus and Prefrontal Cortex in Schizophrenia

The hippocampus and prefrontal cortex are important brain regions that display structural abnormalities and functional disruptions in the schizophrenia brain (Breier et al., 1992). The hippocampus plays an important role in learning and memory. Several lines of research have shown that there are various structural and functional abnormalities in the hippocampus of the schizophrenia brain, including a reduction in hippocampal volume, altered synaptic connections and changes in neurotransmitter receptors (for review see Harrison 2004), as well as impairments in declarative and recognition memory that are associated with reduced hippocampal activation (for review see Preston et al., 2005). Furthermore animal models involving a neonatal hippocampal lesion have shown schizophrenia relevant behaviours and neurochemical changes at the periadolescent period such as deficits in social interactions, deficits in learning and memory, increased locomotor activity and disruptions to dopaminergic and glutamatergic signalling (for review see Tseng et al., 2009). This animal model has also shown gender differences in terms of age of

onset and symptom severity that closely model that seen in the human disease (Tseng et al., 2009). Therefore study into the levels of proteins implicated in the disorder in the hippocampus and associated connections appears critical to understanding the disorder and providing better treatment options.

The prefrontal cortex plays a crucial role in cognitive planning and working memory and has been heavily implicated in schizophrenia (for review see Glahn et al., 2005). Many neuroimaging studies have found a decrease in activation of the prefrontal cortex and a reduction in blood flow to the dorsolateral prefrontal cortex specifically in schizophrenia patients when performing tasks related to working memory, a cognitive function performed by the prefrontal cortex (Glahn et al., 2005; Grace, 2000, Weinberger et al., 1986). While the frontal cortex as a whole appears to show no differences in cortical volume between schizophrenia and control subjects or gender or hemispheric differences (Highley et al., 2001), structural anomalies have been found in the prefrontal cortex, including reduced grey matter volume in the inferior prefrontal cortex of schizophrenia patients (Buchanan et al., 1998, Harms et al., 2010). Animal studies with a genetic knockout targeted to the NR1 subunit of the NMDAR in the excitatory neurons of the medial prefrontal cortex have shown impairments of certain aspects of schizophrenia-like behaviours such as PPI and short-term memory (Rompala et al., 2013). Although there were no impairments in other schizophrenia related behaviours this suggests that the prefrontal cortex or glutamatergic connections to and/or from the prefrontal cortex play a role in a subset of cognitive symptoms integral to schizophrenia (Rompala et al., 2013). Therefore with the hippocampus and prefrontal cortex consistently reported to be involved in schizophrenia we chose to focus on these brain regions in this study.

1.8 Summary

Schizophrenia is a complex, neurodevelopmental disorder that is thought to involve NMDAR dysfunction in the hippocampus and prefrontal cortex. The NR2B subunit of the NMDAR is the most abundantly expressed in early development and is the only NR2 subunit to show protein changes in the postmortem schizophrenia brain to date. The NR2 subunits and NRG1 are functionally connected, with alterations to NRG1 stimulation of ErbB4 affecting NMDAR channel properties and subunit specific phosphorylation. More research is required to determine whether this interaction is reciprocal, developmentally regulated, brain region specific and particularly whether it is altered in disorders such as schizophrenia. Furthermore, cannabis use, particularly among adolescents, is also implicated as being a risk factor for developing schizophrenia symptoms however it is unknown why some individuals are more vulnerable than others. An early developmental disruption, such as a blockade of the NR2B subunit and consequent imbalance of NR2 subunits, may render the brain more susceptible to the effects of cannabis via altering the endogenous cannabinoid system. Alterations to NR2B functioning during this time may consequently affect NRG1 signalling which has already been shown to increase sensitivity to the effects of cannabis. Ultimately the determination of the relationship between NR2B and NRG1 during development and the role these systems may play in modulating other aspects of brain chemistry will further our understanding of schizophrenia pathophysiology.

1.9 Aims and Hypotheses

The overall aim of this PhD thesis was to assess the role of the NR2B subunit in the pathophysiology and development of schizophrenia. To assess this, the thesis

was divided into two parts. Part A utilised postmortem human brain tissue to examine whether there are alterations in NR2B protein in schizophrenia. Part B utilised an animal model to examine whether disruptions to NR2B during early brain development could produce changes in brain systems that are involved in schizophrenia.

Aims - Part A:

1. To examine protein and binding levels of the NR2B subunit in the hippocampus of schizophrenia subjects compared to controls, and determine any gender or hemisphere specific alterations. To investigate the relationship between NR2B and NRG1 in the hippocampus of the schizophrenia brain.

2. To examine protein and binding levels of the NR2B subunit in the dorsolateral prefrontal cortex of schizophrenia subjects compared to controls, and determine any gender or hemisphere specific alterations.

Aims – Part B:

1. To determine the normal developmental profile of NMDAR, NR2A, NR2B, NRG1, ErbB4, $GABA_AR$ and CB_1Rs in the mouse brain.

2. To determine the effect of a perinatal disruption to brain development (NR2B antagonist treatment) on the developmental profile of the NMDAR, NRG1, GABAergic and cannabinoid signalling systems.

3. To investigate the effect of gender on the developmental profiles of the NMDAR, NRG1, GABAergic and cannabinoid signalling systems.

Hypotheses

Disruptions to the NMDAR are widely believed to contribute to the cognitive deficits associated with schizophrenia. It was hypothesised that this thesis will show evidence of altered expression of the NR2B subunit of the NMDAR in cognitive related brain regions (prefrontal cortex and hippocampus) in schizophrenia. As there is evidence to suggest that a) schizophrenia shows gender-specific phenotypes and b) glutamatergic alterations in the schizophrenia brain may be lateralised, it was hypothesised that changes in NR2B in these regions in schizophrenia will be gender and hemisphere specific.

Evidence from both genetic and environment studies suggest that the NR2B subunit of the NMDAR may not only be involved in the pathophysiology and symptomology of schizophrenia, but may also be causal to the development of schizophrenia. It is hypothesised that impacts that target this receptor subunit at a critical point in development will lead to long-term changes, not only in the NMDAR, but also in other related systems (such as neuregulin, GABA, cannabinoid) that are implicated in the pathophysiology of schizophrenia.

1.10 Methodologies:

The techniques of Western blot and *In situ* Radioligand Binding Autoradiography were used throughout this thesis to measure protein and binding densities, respectively, of the proteins of interest. Western blot utilises specific antibodies to determine the levels of particular proteins in homogenised tissue

samples. This technique can be applied to crude tissue homogenates (which include membrane, cytosol, nucleus, organelles etc) or fractionated samples to measure levels in specific cell compartments. Throughout this study, Western blot was performed on crude homogenates to allow the measure of total protein expression in the brain region of interest.

In situ radioligand binding autoradiography uses radiolabelled ligands that bind to specific sites on protein receptors or receptor subunits in tissue slices and is widely used to characterise receptors and their anatomical distribution. In addition, these binding sites are often the sites for binding by endogenous and exogenous ligands, which compete for that site with the radiolabelled ligand. As a result, radioligand binding is often used in pharmacological studies to measure receptor occupancy following administration of various pharmacological compounds to assess drug targets and general ability of pharmacological compounds to bind to sites of interest.

Western blot and radioligand binding are often used in parallel to measure protein levels in tissue samples (Dean et al., 2002). It should not be expected however that these two methods always yield the same result. Often the antibodies and radioligands will bind to different sites on the protein and due to the homogenised versus sliced tissue approach, they have different access to cellular compartments to label the relevant proteins. For example, due to the major cell lysis that occurs following tissue homogenisation it is likely that Western blot in crude homogenates will label the majority of available proteins. In contrast, *in situ* radioligand binding on tissue slices may not have access to label all sites within the cell. Previous studies have shown differences between findings obtained from radiologand binding and crude Western blot, for example reported changes in binding to the Mu opioid receptor in schizophrenia that were not observed when the

protein levels were measured via Western blot. The authors interpreted this as representing a change in availability of receptor binding sites, independent of changes in total protein levels (Scarr et al., 2012).

1.11 Significance:

Schizophrenia is a severely debilitating mental disorder that affects approximately 1% of the Australian population. Currently, the treatments available for the disorder are unable to provide relief of all symptoms, as the underlying neuronal mechanisms of schizophrenia pathophysiology are not known. Furthermore, there are various side effects associated with these treatments and both the treatment and support costs place a major burden on many Australian families. Unravelling the neurochemical disturbances that contribute to the cause of schizophrenia and their interactions with other neurotransmitter systems would benefit not only those suffering from the disorder, but their families and friends and those that surround them.

This study was the first to examine the gender and hemisphere specific alterations of NR2B in the prefrontal cortex and hippocampus in the postmortem schizophrenia brain, which hopefully will encourage others to investigate these variables in the future in pursuit of a more specific pathophysiological discovery of schizophrenia or a gender specific treatment. This study was also the first to examine the neurochemical effects of perinatal NR2B antagonist treatment in a mouse model and provides further understanding of the relationship between the NMDAR and NRG1 signalling pathways, both of which have been implicated in schizophrenia, as well as the role they may play in increasing sensitivity to the psychosis-inducing effects of cannabis.

EXPERIMENTAL PART A: POSTMORTEM

HUMAN BRAIN ANALYSES

2 HEMISPHERE SPECIFIC ALTERATIONS IN NR2B PROTEIN IN THE HUMAN HIPPOCAMPUS

2.1 Introduction

The hippocampus plays an important role in learning and memory, cognitive functions that are widely believed to be disrupted in schizophrenia (Boyer et al., 2007). Several lines of research have shown that there are various structural and functional abnormalities in the hippocampus of the schizophrenia brain, including a reduction in hippocampal volume, altered synaptic connections (evidenced through reductions in synaptosomal proteins such as synaptophysin, SNAP-25 and synapsin) and changes in neurotransmitter receptors (for review see Harrison, 2004). Furthermore, with schizophrenia being a disorder of neurodevelopment, animal models involving a neonatal hippocampal lesion have shown promising results in terms of schizophrenia relevant behaviours and neurochemical changes at the periadolescent to adult period (Tseng et al., 2009). Collectively, studies point to the hippocampus as central to the pathophysiology of schizophrenia.

Dysregulation of glutamatergic neurotransmission is a widely accepted occurrence in the schizophrenia brain (Konradi and Heckers, 2003). The NMDAR is an ionotropic glutamate receptor that appears to play a central role in schizophrenia pathophysiology. The NMDAR is assembled in various subunit combinations, with two obligatory NR1 subunits and two other NR2 A, B, C, D or rarely NR3 A, B subunits (Paoletti, 2011). Various studies have found alterations in NMDAR binding and NR1 subunit mRNA and protein in the hippocampus of schizophrenia subjects (for review see Geddes et al, 2011). Some replicated findings include a decrease in NR1 mRNA in the dentate gyrus of schizophrenia patients (Gao et al., 2000, Law and Deakin, 2001), a decrease or no change in NMDAR binding density (Gao et al.,

2000, Pilowsky et al., 2005, Beneyto et al., 2007) and most interestingly a more pronounced decrease in NMDAR binding and NR1 mRNA and protein measurements in the left hippocampal hemisphere of schizophrenia subjects compared to the right hemisphere (Law and Deakin, 2001, Pilowsky et al., 2005, Vrajova et al., 2010). These studies suggest a lateralised dysregulation of NR1 mRNA and protein in the hippocampus of schizophrenia subjects. With regards to the NR2 subunits, one study has reported an increase in NR2B subunit mRNA in the CA2 region of the hippocampus in schizophrenia, with two further studies reporting no change, and no changes being reported for the NR2A mRNA, or the less expressed 2C and 2D subunits (Gao et al., 2000, Beneyto et al., 2007, McCullumsmith et al., 2007). One study did measure NR2B binding density in the hippocampus, and while they found no change in schizophrenia subjects (Beneyto et al., 2007), it is important to highlight that they did not consider hemisphere effects, which appear to be important to schizophrenia pathology in the hippocampus (Harrison et al, 2003).

The NR2 subunit composition defines the functional properties of the NMDAR (Hardingham et al., 2002, Hu et al., 2008), therefore to understand the NMDAR dysregulation in schizophrenia it is critical to investigate alterations to the NR2 subunits. NR2A and NR2B subunits are the principle NR2 subunits in the hippocampus (Law et al., 2003); evidence suggests that the NR2B subunit in the hippocampus is particularly important for NMDAR channel function (Akashi et al., 2009), LTP and associated cognitive functions such as spatial learning (Clayton et al., 2002). Accordingly, specifically targeting the NR2B subunit has been suggested as a novel mechanism to treat cognitive dysfunction in schizophrenia patients (Mony et al., 2009, Menniti et al., 2013). Of the studies that have examined NR2A and

NR2B proteins in various brain regions in schizophrenia, NR2B is predominantly implicated with four studies showing altered NR2B protein (Grimwood et al., 1999, Ibrahim et al., 2000, Clinton et al., 2006, Kristiansen et al., 2010a) and none reporting altered NR2A (see Geddes et al, 2011), however there are also a number of studies that have found no change in NR2B protein levels (Grimwood et al., 1999, Ibrahim et al., 2000, Clinton et al., 2006, Kristiansen et al., 2006, Beneyto et al., 2007, Beneyto and Meador-Woodruff 2008). Furthermore, a number of studies have suggested an association or reciprocal signalling between the NMDAR and NRG1 protein, NRG1 being one of the leading candidate genes for schizophrenia, (Stefansson et al., 2002, Gu et al., 2005, Hahn et al., 2006, Walss-Bass et al., 2006, Li et al., 2007, Feng et al., 2010, Geddes et al., 2011, du Bois et al., 2012), with NR2B and NRG1 particularly associated in the hippocampus (Bjarnadottir et al., 2007). The NR2 subunits share a structural link with NRG1 via its receptor, ErbB4, with both anchoring to the PSD95 family of scaffolding proteins, providing a basis for this association. NRG1 mRNA is reported to be altered in the hippocampus of the schizophrenia brain (Law et al., 2006) and the 50 kDa fragment of NRG1 protein is reduced in the hippocampus of the schizophrenia brain (Marballi et al., 2012). Our group has shown that NRG1 protein is decreased, specifically in the left hippocampal hemisphere of schizophrenia subjects compared to controls (Stuart et al., 2011), further supporting a hemisphere specific deficit in the hippocampus.

The aim of this study was to determine whether there were alterations in NR2B subunit protein and [^{3}H]Ifenprodil binding density in the hippocampus of schizophrenia subjects compared to control subjects and to determine whether any effects are hemisphere or gender specific. In addition, we aimed to determine whether there was a relationship between the observed NR2B protein and binding

measurements and NRG1 and ErbB4 protein measured in the same tissue cohort (Stuart et al., 2011).

2.2 Materials and Methods

2.2.1 Brain Tissue

Postmortem human brain tissue was acquired from the New South Wales Tissue Resource Centre (NSW TRC) at the University of Sydney. The cohort consisted of schizophrenia samples, with the diagnosis of schizophrenia confirmed according to the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV), and healthy controls, with no known history of psychiatric illness. For the protein analysis, 20 schizophrenia subjects versus 20 control subjects, and for the binding analysis, 20 schizophrenia subjects and 21 control subjects were included from the same cohort (Table 2.1). There were no significant differences in age, brain pH, postmortem interval (PMI) or brain weight between the schizophrenia and control subjects included in the protein (Age: $t_{38} = -0.036$, $p = 0.971$; Brain pH: $t_{26.801} = 0.069$, $p = 0.945$; PMI: $t_{38} = -0.307$, $p = 0.761$; Brain Weight: $t_{38} = 1.085$, $p = 0.285$) or binding (Age: $t_{39} = 0.132$, $p = 0.895$; Brain pH: $t_{28.384} = -0.381$, $p = 0.706$; PMI: $t_{39} = 0.388$, $p = 0.700$; Brain Weight: $t_{39} = 0.792$, $p = 0.433$) studies. All clinical assessments, selection of cases and brain region dissections were performed by the NSW TRC. The study was approved by the University of Wollongong Human Research Ethics Committee (HE99/222).

Table 2.1. Subject demographic and clinical characteristics.

Control Subjects								Schizophrenia Subjects									
Gender	Hemi		Age	PMI	Brain pH	BW	Analysis	Gender	Hemi		Age	PMI	Brain pH	BW	Onset Age	DOI	Chlor equiv	Analysis
M/F	L/R		(years)	(hours)		(grams)		M/F	L/R		(years)	(hours)		(grams)	(years)	(years)	(mg)	
F	L		52	9.5	5.75	1320	WB/RA	M	L		27	10	6.5	1420	14	13	649.5	WB/RA
F	L		71	16	6.2	1260	WB/RA	M	L		67	5	6.36	1400	26	41	1300	WB/RA
F	L		52	11	6.21	1200	WB/RA	M	L		57	38	6.35	1590	31	26	250	WB
M	L		34	20.5	6.73	1580	WB	M	L		44	29.5	6.55	1420	27	17	575	WB/RA
M	L		37	21	6.64	1610	WB/RA	F	L		67	27	6.23	1130	21	46	625	WB/RA
M	L		51	20	5.88	1530	WB/RA	M	L		32	26	6.24	1540	19	13	780	WB/RA
M	L		74	10	6.22	1528	WB/RA	M	L		75	36	6.36	1440	31	44	700	WB/RA
M	L		44	50	6.6	1220	WB/RA	F	L		56	34	6.56	1360	16	40	1000	WB/RA
M	L		57	18	6.6	1360	WB/RA	M	L		33	48	6.65	1480	21	12	222	WB/RA
M	L		68	45.5	6.12	1408	WB/RA	F	L		55	33.5	6.69	1120	17	38	2362.5	WB/RA
F	R		56	23	6.65	1360	WB	M	L		51	18	6.62	1560	21	30	800	WB/RA
F	R		72	25	7	1500	WB/RA	F	R		56	39	6.59	1020	24	32	350	WB/RA
F	R		51	37.5	6.92	1294	WB/RA	F	R		58	19	6.14	1120	19	39	350	WB/RA
F	R		63	42	7.02	1346	WB/RA	F	R		66	12.5	6.52	1220	19	47	1850	WB/RA
F	R		52	43	6.33	1304	WB/RA	M	R		57	48	6.66	1420	40	17	600	WB/RA
M	R		73	48	6.8	1380	WB/RA	F	R		61	42	6.65	1140	19	42	1150	WB/RA
M	R		53	27	6.64	1450	WB/RA	M	R		52	46	6.43	1548	19	33	590.8	WB/RA
M	R		24	43	6.27	1490	WB/RA	M	R		53	27.5	6.27	1398	35	18	502.5	WB/RA
M	R		50	30	6.37	1426	WB/RA	M	R		72	21	6.49	1344	44	28	227.5	WB/RA
M	R		56	24	6.53	1635	WB/RA	F	R		54	29	6.5	1490	19	35	762.5	WB/RA
F	R		63	50	6.46	1100	RA	M	R		59	26.5	6.48	1586	21	29	784	RA
F	R		62	35	6.06	1424	RA											
M	L		36	34	6.67	1540	RA											
SUMMARY																		
12M/9F	10L/11R	Av:	54.50	28.20	6.47	1410.1	WB	12M/8F	10L/10R	Av:	54.65	29.45	6.47	1358.00	24.10	30.55	782.37	WB
		SEM:	3.07	3.05	0.08	29.29				SEM:	2.93	2.86	0.04	39.53	1.88	2.73	124.63	
12M/8F	10L/10R	Av:	55.29	30.45	6.44	1396.4	RA	12M/8F	11L/9R	Av:	54.75	28.88	6.47	1357.80	23.60	30.70	809.07	RA
		SEM:	2.86	2.97	0.08	30.38				SEM:	2.86	2.76	0.04	38.46	1.80	2.65	118.21	

Abbreviations: AV = average, BW = brain weight, Chlor equiv = Chlorpromazine equivalent, DOI = duration of illness, F = female, L = left, M = male, PMI = postmortem interval, R = right, RA = receptor autoradiography, SEM = standard error of the mean, WB = Western blot.

2.2.2 Western Blot

Tissue from the dentate gyrus of the hippocampus was weighed and manually homogenised in nine volumes of buffer containing 0.1 M Tris-HCl, 10 µl/ml protease inhibitor cocktail (Sigma), 0.625 µl/ml apoprotinin and 0.5 µl/ml glycerol. The amount of protein in each sample was determined using the Bradford Reagent Assay (BioRad, Hercules, CA, USA) following manufacturer's instructions. Samples were diluted to 4 µg/µl and stored at -80°C until use. The following procedure was performed in triplicate. Samples were diluted in Laemmli's sample buffer (Bio-Rad) and loaded into precast 4-12% bis-tris gels (Bio-Rad) with 10 µg of total protein per well, along with a molecular weight ladder (Precision Plus Protein™ Standards; Bio-Rad) and run in XT-MOPS buffer (Bio-Rad) at 200 V for 55 minutes. The protein was transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) at 100 V for 1 hour using Tris-glycine buffer (Bio-Rad) containing 20% methanol and blocked in phosphate-buffered saline containing Tween-20 (PBS-T) containing 5% skim milk for 1 hour at room temperature. Blots were then incubated overnight (4°C) with an anti-NR2B antibody (#MAB5778, 1/625; Millipore, Billerica, MA, USA) diluted in PBS-T containing 1% skim milk. A mouse anti-actin antibody was used as an internal standard (#MAB1501, 1/100 000; Millipore). Following primary antibody exposure, blots were washed for 3 x 5 minutes in PBS-T and incubated for 1 hour (room temperature) with a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit: #AP307P, anti-mouse: #AP308P, 1/3 000; Millipore). Blots were washed for 3 x 5 minutes in PBS-T and the proteins of interest detected on Kodak BioMax MR Film (Sigma-Aldrich, Castle Hill, NSW, Australia) using the ECL Plus Western Blotting Detection Reagents kit (GE Healthcare, Piscataway, NJ, USA). The films were developed using the AGFA CP1000 film developer (AGFA

Healthcare, Rydalmere, NSW, Australia) and analysed using Quantity One software version 4.6.7 (BioRad) connected to a GS-800 Calibrated Densitometer (BioRad). Quantification was performed blind to diagnosis. The optical density of each band was normalised to the respective β-actin band and averaged over the three runs. Western blot results for NR2B were correlated with NRG1 and ErbB4 protein levels from a previous study conducted in the laboratory (Stuart et al., 2011).

2.2.3 *In situ* Radioligand Binding Autoradiography

Hippocampus tissue sections for radioligand binding were cut on a cryostat at -18°C, at a thickness of 14 µm, mounted onto SuperFrost Plus slides (Menzel, Brunswick, Germany) and stored at -80°C until use. [^{3}H]Ifenprodil binding was performed based on the methods previously used by Ibrahim et al., (2000) and Basham et al., (1999). Three slides from each subject were incubated in 0.05 M TrisHCl buffer (pH 7.4) with 20 nM [^{3}H]Ifenprodil (NR2B antagonist: specific activity 40 Ci/mmol; PerkinElmer, Boston, MA, USA). Due to previous findings that [^{3}H]ifenprodil has some affinity for sigma receptors, piperazine acceptor sites, and non-GluN2B polyamine sites (Basham et al., 1999, Hashimoto et al., 1993, Hashimoto et al., 1994), the following pharmacological inhibitors were included in the incubation assay: 3 µM R(+)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride (+3PPP; to block non-specific binding to the sigma receptor), 30 µM GBR-12909 (to block piperazine sites) and 1 mM trifluoperazine (to block low affinity polyamine sensitive sites on adrenergic, dopaminergic and cholinergic receptors) for 3 hours at 4^{o}C to determine total binding. Adjacent sections were incubated in the same buffer, with the addition of 20 µM Ifenprodil (Sigma-Aldrich) to determine non-specific binding. All sections were then washed in 0.05 M Tris-HCl (pH 7.4) for 3 x 5 minutes at 4^{o}C, rinsed in distilled water and air-dried overnight.

All slides were placed in cassettes and exposed to tritium-sensitive Kodak BioMax MR Film (Sigma-Aldrich) for 10 weeks. The films were developed using the AGFA CP1000 film developer (AGFA) and analysed using Quantity One software version 4.6.7 (BioRad) connected to a GS-800 Calibrated Densitometer (BioRad). Quantification was performed blind to diagnosis by obtaining the optical density (background corrected) in the dentate gyrus and averaging it from the three slides.

2.2.4 Statistics

The statistical package used for data analysis was SPSS version 17 (IBM, Armonk, NY, USA). The data were checked for triplicate (standard deviation>30% mean) and population outliers (two standard deviations above or below the mean), as is widely used in this field (Weickert et al., 2013) and a Kolmogrov-Smirnov test was used to confirm the normality of the data. An independent samples t-test was used to determine differences between the schizophrenia and control subjects. Pearson's correlations were then used to determine correlations between NR2B protein or binding density and diagnostic variables such as age, PMI and pH with all the data and antipsychotic medication, age of illness onset, and duration of illness in the schizophrenia group. An ANCOVA was then performed if there were any significant correlations with the demographic variables. Two-Way ANCOVAs, adjusted for demographic variables, were performed to examine the effect of hemisphere and gender on the diagnosis and if a significant effect was found, a one-way ANOVA with Tukeys post-hoc analysis was conducted. Pearson's correlations were then performed between NR2B binding and protein measurements and between NR2B protein and NRG1 and ErbB4 protein measurements from the same cohort (Stuart et al., 2011).

2.3 Results

2.3.1 Left hippocampal deficits in NR2B subunit protein in schizophrenia subjects.

We observed one band for NR2B protein at the expected molecular weight of approximately 175 kDa (see Figure 2.1a) as previously reported (Laurie et al., 1997). Two outliers were removed due to technical error, one from each diagnostic group. The schizophrenia and control groups remained matched for age (t_{36} = -0.402, p = 0.690), PMI (t_{36} = -0.551, p = 0.585), brain pH ($t_{25.626}$ = -0.076, p = 0.939) and brain weight (t_{36} = 1.129, p = 0.266).

An Independent t-test showed that there was no difference between the NR2B protein density in the dentate gyrus of schizophrenia and control subjects (t_{36} = 1.452, p = 0.155). NR2B protein density correlated significantly with brain pH (r = -0.386, p = 0.017) and there was a trend for a correlation with the PMI (r = -0.318, p = 0.051) in the whole cohort (see Table 2.2). Similar correlation results were observed in the control subjects only (pH: r = -0.662, p = 0.002; PMI: r = -0.483, p = 0.036) but were not observed in the schizophrenia subjects only (pH: r = 0.162, p = 0.506; PMI: r = -0.116, p = 0.636). An ANCOVA correcting for brain pH and PMI confirmed that there was no difference in NR2B protein density between schizophrenia and control subjects ($F_{1,34}$ = 2.004, p = 0.166). There was no correlation between protein density in the schizophrenia subjects and duration of illness, age of onset or medication history (Table 2.2).

Table 2.2. Pearson's correlations for continuous variables with NR2B binding and protein.

Variable		All subjects		Control		Schizophrenia	
		NR2B binding	NR2B protein	NR2B binding	NR2B protein	NR2B binding	NR2B protein
Age	r	**-0.309**	0.160	**-0.408**	0.207	-0.212	0.152
	p	**0.049***	0.338	**0.067^**	0.395	0.369	0.534
PMI	r	0.054	**-0.318**	0.061	**-0.483**	0.030	-0.116
	p	0.738	**0.051^**	0.794	**0.036***	0.901	0.636
Brain pH	r	0.068	**-0.386**	0.226	**-0.662**	-0.255	0.162
	p	0.671	**0.017***	0.324	**0.002***	0.278	0.506
BW	r	-0.029	-0.067	0.152	-0.028	-0.218	-0.023
	p	0.885	0.688	0.512	0.908	0.356	0.926
Age Onset	r					-0.007	0.313
	p					0.977	0.193
DOI	r					-0.192	-0.056
	p					0.430	0.819
APD use	r					-0.368	-0.156
	p					0.111	0.525

Abbreviations: AP = antipsychotic drug, BW = body weight, DOI = duration of illness, pH = brain acidity, PMI = post mortem interval. *: $p<0.05$; ^: $0.05<p<0.07$.

A two-way ANCOVA with the main effects of DIAGNOSIS and HEMISPHERE revealed a significant effect of hemisphere ($F_{1,31}=5.222$, $p=0.011$) but no significant effect of diagnosis ($F_{1,31}=3.225$, $p=0.082$) and no significant interaction ($F_{1,31}=0.673$, $p=0.418$). A one-way ANOVA with Tukey's post-hoc analysis revealed a significant difference between groups ($F_{3,34}=2.882$, $p=0.050$) and a trend for a 43% decrease in the left hemisphere of the schizophrenia group compared to the left hemisphere of the control group ($p=0.066$) (see Figure 2.1b) (see Table 1 in Appendix for comparison of demographic variables by hemisphere). There was no effect of gender on NR2B protein density in the hippocampus (see Table 2 in Appendix for comparison of demographic variables by gender).

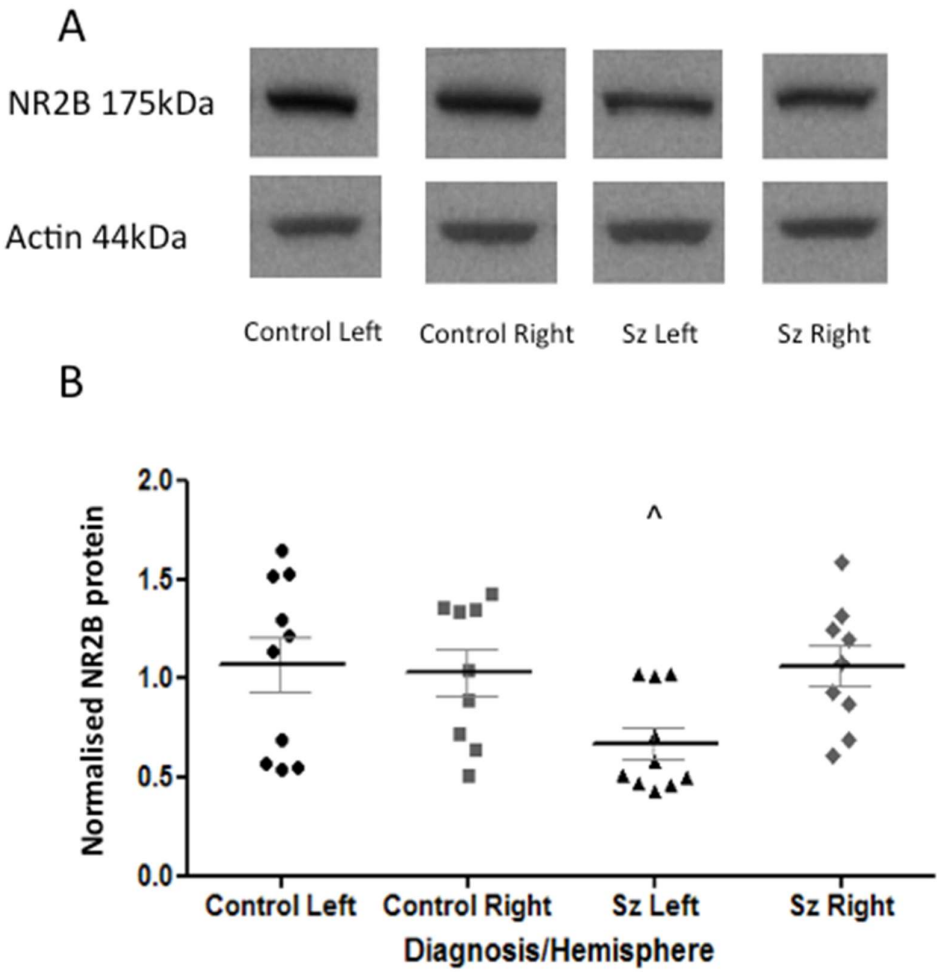

Figure 2.1. A: Representative Western blot bands of NR2B and actin protein. B: Hippocampal NR2B protein density in the left and right hemisphere of the schizophrenia and control brain. There was a 43% decrease in the left hemisphere of the schizophrenia subjects compared to the left hemisphere of the control subjects. ^: p = 0.066 compared to Control Left. Horizontal and vertical bars represent mean±SEM for each group. Abbreviations: Sz = schizophrenia.

2.3.2 No change in NR2B binding density in schizophrenia subjects compared to control.

An Independent t-test showed that there was no difference between [^{3}H]Ifenprodil binding in the schizophrenia and control subjects (t_{39} = 0.808, p = 0.424).

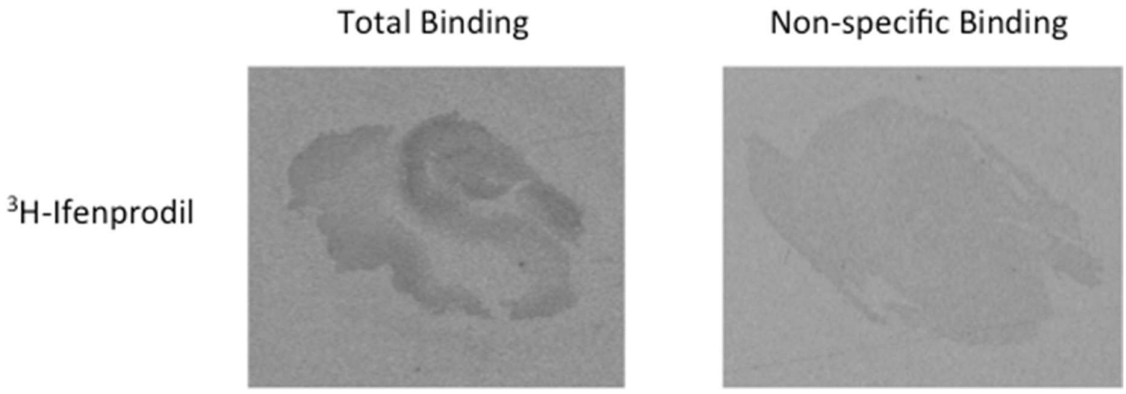

Figure 2.2. Representative total and non-specific [^{3}H]Ifenprodil binding in the hippocampus of this human tissue cohort.

[^{3}H]Ifenprodil binding density correlated with age at death of the subjects in the whole cohort ($r = -0.309$, $p = 0.049$); there was a trend for a correlation in the control subjects only ($r = -0.408$, $p = 0.067$) but no correlation between the schizophrenia subjects and age at death ($r = -0.212$, $p = 0.369$) (Table 2.2). An ANCOVA to correct for age at death confirmed no difference in [^{3}H]Ifenprodil binding density between schizophrenia and control subjects ($F_{1,38} = 0.780$, $p = 0.383$). There was no correlation between [^{3}H]Ifenprodil binding density in the schizophrenia subjects and length of illness, age of onset or medication history (Table 2.2). There were no effects of [^{3}H]Ifenprodil binding on gender or hemisphere (Figure 2.3) (see Table 1 and 2 in Appendix for comparison of demographic variables in gender and hemisphere subgroups).

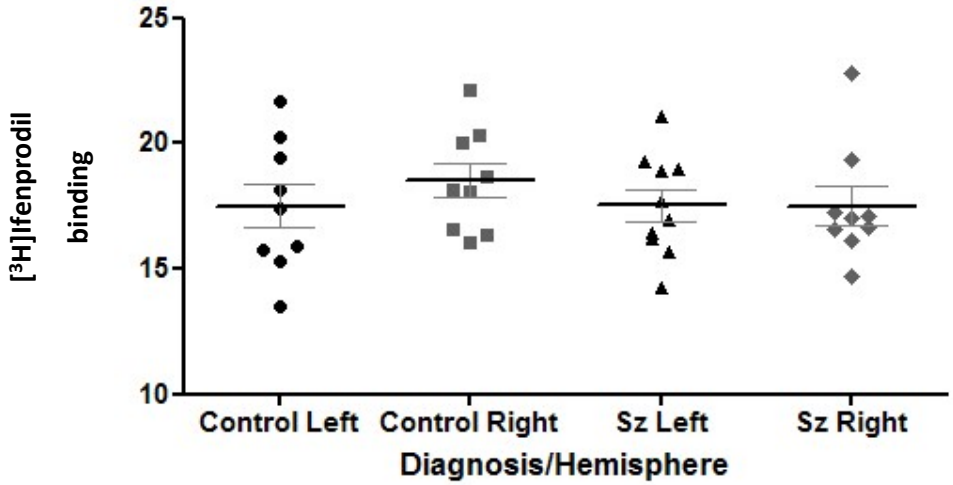

Figure 2.3. Hippocampal [^{3}H]Ifenprodil binding density in the left and right hemisphere of the schizophrenia and control brain. Horizontal and vertical bars represent mean±SEM for each group. Abbreviations: Sz = schizophrenia.

2.3.3 Positive relationship between NR2B and NRG1 protein.

There was no correlation between NR2B binding and protein measurements (Table 2.3). The NR2B protein measurements and NRG1 (130-140 kDa) protein levels (from a previous study in our laboratory using the same cohort (Stuart et al., 2011)) were found to have a significant positive correlation (r=0.343, p=0.043) (Table 2.3). Interestingly, this positive correlation was significant in the left hemisphere (r=0.462, p=0.047) but not right hemisphere (r=0.211, p=0.433) of the hippocampus (Table 2.3). There was no correlation between NR2B and ErbB4 (180 kDa) protein levels (from a previous study using the same cohort (Stuart et al., 2011)) (Table 2.3).

Table 2.3. Pearson's correlations between NR2B binding and protein, and NR2B, NRG1 and ErbB4 protein levels.

		NR2B binding vs NR2B protein	NR2B protein vs NRG1 protein	NR2B protein vs ErbB4 protein	NRG1 protein vs ErbB4 protein
All subjects	r	-0.050	**0.343**	0.210	0.157
	p	0.776	**0.043***	0.213	0.354
Control	r	0.003	0.296	0.141	0.141
	p	0.990	0.218	0.564	0.554
Schizophrenia	r	-0.187	0.281	0.167	0.070
	p	0.457	0.293	0.509	0.789
Left hemi	r	0.134	**0.462**	0.269	0.117
	p	0.596	**0.047***	0.251	0.624
Right hemi	r	-0.344	0.211	0.133	0.210
	p	0.192	0.433	0.667	0.418

Abbreviations: hemi = brain hemisphere, vs = versus. *: $p<0.05$.

2.4 Discussion

2.4.1 Left hippocampal deficits in NR2B subunit protein in schizophrenia

In the present study we identified a 43% reduction in NR2B protein levels in the hippocampal dentate gyrus in schizophrenia. Interestingly, this reduction was present in the left hemisphere and not the right hemisphere, highlighting the hippocampal lateralisation in the pathology of schizophrenia. Further, we identified for the first time, a positive correlation between NR2B protein and NRG1 protein, which was also specific to the left hippocampal hemisphere. In addition, we have confirmed previous results, this time in a larger cohort (n=15 cf. n=20), indicating no change in [^{3}H]-Ifenprodil binding to NR2B subunits in the hippocampus of schizophrenia subjects in comparison to control subjects (Beneyto et al., 2007) and extended these findings to report no hemisphere specific alteration in NR2B binding density.

It is well documented that the hippocampus is altered in schizophrenia and there is evidence to suggest that the left hemisphere shows more pronounced deficits in NMDAR expression than the right hemisphere. Studies investigating the effect of hemisphere have found a more pronounced decrease in NR1 mRNA and protein in the left hemisphere of schizophrenia subjects (Law and Deakin, 2001, Vrajova et al., 2010) as well as a reduction in [^{123}I]-CNS-1261 binding to the NMDAR channel in the left hippocampus of medication free schizophrenia subjects (Pilowsky et al., 2005). Ours is the first study in the hippocampus to report such a lateralisation effect with any of the NR2 subunits, however, to our knowledge, the protein density of the other NR2 subunits has not yet been investigated in the hippocampus. It has previously been discussed (Beneyto and Meador-Woodruff, 2008) that deletion of the NR1 subunit in the CA1 region of the hippocampus results in reduced expression of the NR2B subunit in the dendrites of pyramidal neurons in mice (Fukaya et al., 2003), suggesting our current findings could be associated with the reductions in NR1 previously reported in the left hippocampus in schizophrenia.

While the present study found a reduction in NR2B protein levels, this was accompanied by no change in NR2B binding density. [^{3}H]Ifenprodil binds to the polyamine site on NR2B subunits. While ifenprodil does also have some affinity for sigma, piperazine, adrenergic receptors and other polyamine sites (Basham et al., 1999, Hashimoto et al., 1993, Hashimoto et al., 1994), pharmacological inhibitors were used to block these sites in the binding assay. Therefore, it is unlikely that the discrepancy between binding and protein is a result of [^{3}H]Ifenprodil binding to sites other than NR2B. It is possible however that that these two techniques label different populations of NR2B subunits. Under the conditions used in the present study (i.e. the use of tissue sections rather than cell lysed homogenates), [^{3}H]ifenprodil would

bind primarily to cell surface receptors. This suggests that the reduction observed in NR2B protein in the present study, might represent a reduction in intracellular levels. The implications of such localised reductions remain unclear (McCullumsmith et al., 2014). Similar to our study, in cortical brain areas it has been shown that despite no change in NR2B binding (Beneyto and Meador-Woodruff, 2008), there was a reduction in NR2B protein in schizophrenia compared to control subjects, which was specific to the endoplasmic reticulum fraction of the cells (Kristiansen et al., 2010b). While we did not examine NR2B mRNA in the present study, two previous studies reported no change in NR2B mRNA levels in the dentate gyrus in schizophrenia (Beneyto et al., 2007, McCullumsmith et al., 2007), therefore it is unlikely the findings in the present study represent changes in NR2B production. It is possible however that our findings reflect disturbances to NR2B trafficking, degradation, or other aspects of NR2B regulation. Further studies, specifically examining isolated cellular compartments and proteins that regulate trafficking/degradation of NR2B, as well as assessments of NR2B phosphorylation and activation would be important to further understand the NR2B alterations in the hippocampus in schizophrenia.

It is possible that our results and others reporting left hippocampal deficits in NMDAR density could be due to asymmetries in hippocampal volume reduction in schizophrenia subjects, their first degree relatives or high risk individuals as described in studies utilising MRI technology to analyse various brain parameters (Breier et al., 1992, Shenton et al., 1992, Hirayasu et al., 1998, Narr et al., 2002, Seidman et al., 2002, Prestia et al., 2011). Although, not all of these studies have shown consistent results (Nelson et al., 1998, Wright et al., 2000) and it has been shown that even though hippocampal volume is reduced in schizophrenia the neuronal density has been found unchanged in the dentate gyrus (Zaidel et al., 1997,

Walker et al., 2002), the specific hippocampal region examined in the present study. Therefore these NMDAR deficits observed in the left hippocampus in the present study and others may represent actual alterations in protein amount, as opposed to reductions in protein due to associated reductions in neuronal density.

NR2B-containing NMDARs play a critical role in cognitive functions, especially in the hippocampus (For review see Nakazawa et al., 2004). Selective reduction of NR2B-containing NMDARs in the hippocampus has been reported to produce cognitive deficits in rodents (Brigman et al., 2010; Clayton et al., 2002) as well as disrupting underlying molecular processes such as LTP (Clayton et al., 2002). Specifically, the left hippocampus has previously been associated with verbal and visual memory performance in schizophrenia subjects (DeLisi et al., 1991; Seidman et al., 2002). Therefore, a reduction in NMDARs or altered composition of the NMDAR subunits in hippocampal subregions, such as that found in the present study, may be a contributing factor to the cognitive deficits observed in these patients. Therapies aiming to enhance NR2B function or activity have been shown to improve cognitive function in animal models of ageing-induced cognitive impairment (Brim et al., 2013). Similarly, over-expression of NR2B in transgenic mice results in increased cognitive ability (Tang et al., 1999). Along these lines, positive allosteric modulation, selective for NR2B-containing NMDARs has been proposed as a novel therapeutic strategy for the treatment of schizophrenia and cognitive dysfunction more generally (Menniti et al., 2013; Mony et al., 2009). Our findings therefore of reduced NR2B protein in the hippocampus, where it plays a critical role in cognitive function, and is possibly related to cognitive dysfunction in schizophrenia patients, supports the idea that NR2B positive allosteric modulators could potentially be therapeutic for cognitive dysfunctions in schizophrenia.

2.4.2 Effects of confounding variables on NR2B binding and protein in the hippocampus

As with all postmortem human brain studies of this nature, we cannot rule out an influence of ante-mortem medication on our data. However, we observed no correlation between antipsychotic dose (measured as chlorpromazine equivalents) and NR2B binding or protein levels. Furthermore, there is evidence from animal studies that antipsychotic drug treatment does not alter NR2B mRNA or protein levels in the brain (Fumagalli et al., 2008; O'Connor et al., 2006; Schmitt et al., 2003), suggesting that our findings are unlikely to be due to the effects of pre-mortem antipsychotic drug treatment. While the confounding variables, brain pH and PMI, correlated significantly with NR2B protein levels, these variables were factored into ANCOVA analyses, in which the finding of a decrease in the left hippocampal hemisphere of schizophrenia subjects remained.

2.4.3 Relationship between NR2B and the NRG1 signalling system in the hippocampus in schizophrenia

This study found a significant positive correlation between NR2B and NRG1 protein, particularly in the left hemisphere of the hippocampus. This is the first study to correlate NR2B and NRG1 protein levels in a human brain tissue cohort. Both NR2B and NRG1 protein were decreased in the left hippocampal hemisphere in schizophrenia subjects and since the correlation was found in the left but not the right hemisphere, this suggests that the positive relationship between these two proteins may be related to schizophrenia pathology. Unfortunately the cohort was not powered to enable diagnosis/hemisphere specific correlations and therefore we cannot confirm this suggestion. It is possible that an abnormal relationship between NR2B and NRG1 protein in the left hemisphere of the hippocampus contributes to

the pathology of schizophrenia. A functional correlation between NRG1 and NR2B has been previously reported in the hippocampus of a human cell line, with NRG1 stimulation increasing the tyrosine phosphorylation of the NR2B subunit (Bjarnadottir et al., 2007). The phosphorylation of NR2B in the hippocampus of schizophrenia subjects has not yet been studied, but it is possible that the relationship between NRG1 and NR2B protein expression in the left hippocampal hemisphere of schizophrenia subjects may contribute to associated functional alterations/deficits in this region.

ErbB4 protein did not correlate with NR2B protein and has also shown no correlation with NRG1 in the hippocampus (Stuart et al., 2011). The NRG1 signalling system, via an interaction with ErbB proteins, is both physically and functionally associated with the NR2 subunits of the NMDAR and this connection appears to occur via proteins in the postsynaptic density (Geddes et al., 2011). However ErbB4 has been found to be exclusively located on GABAergic interneurons in the hippocampus (Vullhorst et al., 2009) and with NMDAR location not confined to a certain cell type, this could explain the lack of correlation with NR2B. Furthermore ErbB4 mRNA has been found predominantly in the CA1 region of the hippocampus (Okada and Corfas, 2004) and it has been suggested that ErbB2 is the predominant ErbB protein in the dentate gyrus of the hippocampus (Thompson et al., 2007) which adds further weight to the lack of correlation seen with NR2B and ErbB4 protein.

2.4.4 Conclusion

In conclusion, the present study for the first time, revealed that NR2B protein levels are decreased in the left hippocampal (dentate gyrus) hemisphere of schizophrenia subjects compared to control subjects and that there is a left

hemisphere specific positive relationship between the NR2B and NRG1 proteins. This reduction may be associated with cognitive symptoms arising in schizophrenia subjects, although caution is warranted in interpreting this result as it did not reach significance (p-=0.066). While further studies are warranted to confirm this finding and determine if this reduction is common to other NR2 subunits or hippocampal subregions, our findings provide support for the development of NR2B selective positive modulators as a novel therapy for schizophrenia, especially the cognitive dysfunctions.

3 GENDER SPECIFIC ALTERATIONS IN NR2B PROTEIN IN THE HUMAN PREFRONTAL CORTEX

3.1 Introduction

Glutamatergic dysregulation in the cortex and hippocampus plays an intrinsic role in schizophrenia pathology (Falkenberg et al., 2014, Konradi and Heckers, 2003). We previously showed that the NR2B subunit of the NMDAR is reduced in the hippocampus in schizophrenia, specifically in the left hemisphere, which we suggest may contribute to the cognitive deficits in schizophrenia. The prefrontal cortex plays a crucial role in cognitive planning and working memory as well as personality, judgement and thought differentiation and has been heavily implicated in schizophrenia (Glahn et al., 2005). Many neuroimaging studies have found abnormalities in prefrontal cortical function in schizophrenia patients when performing tasks related to working memory, especially in the right hemisphere (Callicott et al., 2000, Perlstein et al., 2001, Barch et al., 2003, Lee et al., 2008). Structural anomalies have also been found, including reduced grey matter volume in the inferior prefrontal cortex of schizophrenia patients (Buchanan et al., 1998, Harms et al., 2010).

In the prefrontal cortex, it has been found that NMDAR binding levels remain largely unchanged (Kornhuber et al., 1989, Weissman et al., 1991, Scarr et al., 2005), however alterations to specific subunit mRNA has been observed in schizophrenia subjects compared to controls (Akbarian et al., 1996, Dracheva et al., 2001, Beneyto and Meador-Woodruff, 2008). This suggests that there may be alterations to specific NMDAR subunit protein in the prefrontal cortex of schizophrenia subjects. NR2B is the only NR2 subunit to show protein alterations in any brain region of the schizophrenia brain to date (Grimwood et al., 1999, Ibrahim

et al., 2000, Clinton et al., 2006), including a reduction in NR2B protein in the endoplasmic reticulum cellular fraction in the prefrontal cortex (Kristiansen et al., 2010b), however none of these studies have examined the effect of hemisphere or gender. Considering the hemisphere dependent alteration in NR2B protein we found in the hippocampus of schizophrenia subjects compared to control (see Chapter 2) and the deficits in prefrontal cortical function seen in the right hemisphere (as mentioned above), it is important to examine protein changes in the brain in relation to hemisphere. Furthermore, there are gender differences in the onset, symptom profile and pathology of schizophrenia (Frederikse et al., 2000, Gur et al., 1996) and we may be missing valuable information on gender specific neurochemical changes in the brain by not examining gender effects in postmortem brain tissue cohorts.

Therefore the aim of this study was to determine whether there is an alteration in density of NR2B subunit protein and binding in the DLPFC of schizophrenia subjects compared to controls in a brain tissue cohort with enough power to examine the effects of hemisphere and gender.

3.2 Materials and Methods

3.2.1 Brain Tissue

Postmortem human brain tissue was acquired from the NSW TRC at the University of Sydney. The cohort consisted of 37 schizophrenia samples, with the diagnosis of schizophrenia confirmed according to the DSM-IV, and 37 healthy controls, with no known history of psychiatric illness. Samples were matched for age, brain pH, PMI and RNA integrity (Table 3.1). All clinical assessments, selection of cases, matched controls, assessment of tissue quality, identification of the DLPFC (Figure 3.2) and preparation of the tissue were performed by the NSW TRC and/or Schizophrenia Research Laboratory (SRL) (see Weickert et al., 2010). Clinical and

demographic characteristics of the subjects were obtained including gender, brain hemisphere, antipsychotic drug history, subclass of schizophrenia and duration of illness (see Table 3.1) The study was approved by the Human Research Ethics Committees of the University of Wollongong (HE99/222) and University of New South Wales (HREC 07261).

Table 3.1. Subject demographic and clinical characteristics.

Control subjects									Schizophrenia subjects										
Gender	Hemi		Age	PMI	Brain pH	RIN	BW	FST	Gender	Hemi		Age	PMI	Brain pH	RIN	BW	FST	Onset Age	DOI	Chlor equiv
M/F	R/L		(years)	(hours)			(grams)	(months)	M/F	R/L		(years)	(hours)			(grams)	(months)	(years)	(years)	(mg)
F	L		60	13	6.52	7.4	1200	82	F	L		67	27	6.44	6.3	1130	109	21	46	625
F	L		33	24	6.91	8	1390	66	F	L		73	20	6.9	7.2	1400	58	36	37	300
F	R		56	23	6.47	7.1	1360	75	F	L		56	34	6.81	7.7	1360	56	17	39	1000
F	R		21	39.5	6.83	8.1	1570	21	F	L		55	33.5	6.3	7.6	1120	29	17	38	2362.5
F	R		49	15	6.64	8.3	1330	27	F	L		68	32	6.24	7.5	1426	11	23	45	224
F	R		78	11	6.37	6.1	1320	39	F	R		51	12	5.69	6.2	1160	139	35	16	556.25
F	R		51	37.5	7.15	7.7	1294	5	F	R		56	39	7.09	7.6	1020	114	24	32	350
M	L		60	21	6.55	7.8	1440	146	F	R		61	17	6.43	6.8	1290	105	31	30	1866.5
M	L		37	21	6.8	7.6	1610	128	F	R		58	19	6.34	6.8	1120	96	19	39	350
M	L		18	33	6.79	7	1740	121	F	R		66	12.5	6.33	6.6	1220	72	19	47	1850
M	L		58	12	6.56	7.6	1350	92	F	R		61	42	6.91	6.8	1140	54	19	42	1150
M	L		50	19	6.61	6.9	1500	80	F	R		54	29	6.41	7.2	1490	53	19	35	762.5
M	L		74	10	6.31	7.4	1528	80	F	R		33	50	6.93	8.1	1420	16	14	19	162.5
M	L		44	50	6.72	7.2	1220	63	M	L		67	5	6.82	8.4	1400	125	26	41	1300
M	L		46	29	5.84	6.7	1320	28	M	L		27	10	6.56	7	1420	137	18	9	649.5
M	L		78	6.5	6.29	7.5	1320	54	M	L		51	21	6.53	6.9	1490	133	27	24	400
M	L		57	18	6.86	8.4	1360	57	M	L		57	38	6.98	7.5	1590	125	30	27	250
M	L		55	20	7.19	7.3	1400	152	M	L		27	38.5	6.82	8	1700	121	23	4	287.5
M	R		50	29	6.68	8.1	1320	112	M	L		44	29.5	6.52	7.3	1420	110	27	17	575
M	R		56	24	6.64	7.1	1635	107	M	L		30	24	6.77	7.3	1390	108	26	4	552.5
M	R		59	20	6.96	7.4	1360	106	M	L		56	15	6.16	6.7	1310	102	21	35	425
M	R		43	13	6.71	7.4	1500	102	M	L		32	26	6.99	7.9	1540	99	19	13	780
M	R		37	24	6.94	7.5	1520	100	M	L		51	18	6.68	7.9	1560	93	21	30	800
M	R		46	25	6.65	7.3	1490	97	M	L		40	21.5	6.5	7.6	1360	71	17	23	1012.5
M	R		34	20.5	6.51	7.1	1580	76	M	L		75	36	6.56	6.9	1440	65	32	43	700
M	R		73	48	6.57	8.1	1380	28	M	L		73	14	6.79	7	1590	60	21	52	650
M	R		53	27	6.7	7.1	1450	26	M	L		30	26	7.01	7.2	1550	53	27	3	187.5
M	R		24	43	6.95	7.1	1490	22	M	L		33	48	6.75	8.3	1480	49	22	11	222
M	R		64	39.5	7.01	7	1470	17	M	R		27	33	6.84	8.2	1500	130	19	8	315
M	R		62	37.5	6.57	7.5	1480	16	M	R		52	8.4	6.7	7.7	1140	114	21	31	430
M	R		60	25	6.02	7.1	1610	53	M	R		55	72	6.37	6.6	1500	67	30	25	900
M	R		60	21.5	6.95	6.9	1620	19	M	R		54	27.5	6.19	6.3	1460	64	19	35	325
M	R		54	29	6.8	6	1510	45	M	R		57	48	6.72	6.5	1420	62	40	17	600
M	R		56	37	6.98	6.2	1510	62	M	R		57	28	6.41	7.4	1400	62	30	27	1100
M	R		61	27.5	6.31	6.6	1438	8	M	R		34	26	6.95	7.3	1500	48	27	7	195
M	R		37	11	6.23	6.9	1280	113	M	R		52	46	6.35	7.1	1458	38	19	33	590.8
M	L		38	13.5	6.73	7.7	1620	151	M	R		59	26.5	6.87	7.6	1586	8	21	38	784
SUMMARY																				
30M/7F	14L/23R	Av:	51.14	24.80	6.66	7.30	1446.35	69.62	24M/13F	20L/17R	Av:	51.32	28.46	6.61	7.27	1391.89	79.89	23.70	27.62	691.64
		SEM:	2.40	1.80	0.05	0.09	20.90	7.02			SEM:	2.32	2.26	0.05	0.10	26.96	6.12	1.00	2.27	82.56

Abbreviations: Av = average, BW = brain weight, Chlor equiv = chlorpromazine equivalent, DOI = duration of illness, F = female, FST = freezer storage time, L = left, M = male, PMI = postmortem interval, R = right, RIN = RNA integrity. All subjects were used for both Receptor Autoradiography and Western Blot experiments.

3.2.2 Western Blot

Homogenised tissue from the DLPFC was obtained at a concentration of 2 µg/µL from the SRL. The following procedure was performed in triplicate. Samples were diluted in Laemmli's sample buffer (Bio-Rad, Hercules, CA, USA) and loaded into precast 4-12% bis-tris gels (Bio-Rad) with 10 µg of total protein per well, along with a molecular weight ladder (Precision Plus Protein™ Standards; Bio-Rad) and run in XT-MOPS buffer (Bio-Rad) at 200 V for 55 minutes. The protein was transferred to PVDF membrane (Bio-Rad) at 100 V for 1 hour using Tris-glycine buffer (Bio-Rad) containing 20% methanol and blocked in PBS-T containing 5% skim milk for 1 hour at room temperature. Blots were then incubated overnight (4°C) with an anti-NR2B antibody (#MAB5778, 1/500; Millipore) diluted in PBS-T containing 1% skim milk. A mouse anti-actin antibody was used as an internal standard (#MAB1501, 1/100000; Millipore). Following primary antibody exposure, blots were washed for 3 x 5 minutes in PBS-T and incubated for 1 hour (room temperature) with a HRP-conjugated secondary antibody (anti-rabbit: #AP307P, anti-mouse: #AP308P, 1/3000; Millipore). Blots were washed for 3 x 5 minutes in PBS-T and the proteins of interest detected on Kodak BioMax MR Film (Sigma-Aldrich, Castle Hill, NSW, Australia) using the ECL Plus Western Blotting Detection Reagents kit (GE Healthcare, Piscataway, NJ, USA). The films were developed using the AGFA CP1000 film developer (AGFA Healthcare, Rydalmere, NSW, Australia) and analysed using Quantity One software version 4.6.7 (BioRad) connected to a GS-800 Calibrated Densitometer (BioRad). Quantification was performed blind to diagnosis. The optical density of each band was normalised to the respective β-actin band and averaged over the three runs.

3.2.3 *In situ* Radioligand Binding Autoradiography

DLPFC tissue for radioligand binding was coronally sectioned on a cryostat at -18°C, at a thickness of 14 µm, mounted onto gelatinised slides and stored at -80°C until use. [^{3}H]Ifenprodil binding was performed based on the methods previously used by Ibrahim et al., (2000) and Basham et al., (1999). Three slides from each subject were incubated in 0.05 M TrisHCl buffer (pH 7.4) with 5 nM [^{3}H]Ifenprodil (NR2B antagonist: specific activity 40 Ci/mmol; PerkinElmer, Boston, MA, USA), 3 µM R(+)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride (+3PPP; to block non-specific binding to the sigma receptor), 30 µM GBR-12909 (to block piperazine sites) and 1 mM trifluoperazine (to block low affinity polyamine sensitive sites on adrenergic, dopaminergic and cholinergic receptors) for 3 hours at 4°C to determine total binding. Adjacent sections were incubated in the same buffer, with the addition of 10 µM Ifenprodil (Sigma-Aldrich) to determine non-specific binding. All sections were then washed in 0.05 M Tris-HCl (pH 7.4) for 3 x 5 minutes at 4°C, rinsed in distilled water and air-dried overnight.

Autoradiographic images were taken using a Beta-Imager camera (BioSpace, Paris, France). Quantification was performed blind to diagnosis using the B vision+ program (BioSpace), with the aid of histological standards obtained from Weickert et al (2010). The relative density from three slides per case was averaged to give the final analysis value.

3.2.4 Statistics

The statistical package used for data analysis was SPSS version 17 (IBM). The data was checked for triplicate (standard deviation>30% mean) and population outliers (two standard deviations above or below the mean) and a Kolmogrov-Smirnov test was used to confirm the normality of the data. An Independent samples

t-test was used to determine differences between the schizophrenia and control subjects. Pearson's correlations were then used to determine relationships to diagnostic variables such as age, PMI, brain pH and RNA integrity with all the data and antipsychotic medication and duration of illness in the schizophrenia group. An ANCOVA was then performed if there were any significant correlations with the diagnostic variables. Two way ANCOVAs, adjusted for demographic variables, were performed to test the effect of hemisphere and gender on the diagnosis and if a significant difference was found, a one way ANOVA with Tukey's post-hoc analysis was conducted. Pearson's correlations were then performed between NR2B binding and protein measurements.

3.3 Results

3.3.1 Gender difference in NR2B protein in schizophrenia subjects.

One band was observed and quantified at approximately 175 kDa as previously reported (Laurie et al., 1997). An Independent t-test showed that there was no difference in NR2B protein density between schizophrenia and control subjects ($t_{72} = 0.671$, $p = 0.504$). NR2B protein density correlated significantly with PMI ($r = -0.332$, $p = 0.004$) and there was a trend for a correlation with age at death of the subjects ($r = 0.212$, $p = 0.069$) when assessed in the whole cohort (See Table 3.2). A similar correlation of NR2B protein density and PMI was observed in the control subjects ($r = -0.408$, $p = 0.012$) but not in the schizophrenia subjects ($r = -0.277$, $p = 0.096$). An ANCOVA correcting for these variables showed no difference in NR2B protein density between schizophrenia and control subjects ($F_{1,71} = 0.073$, $p = 0.787$). There was no correlation between NR2B protein density in the schizophrenia subjects and duration of illness, age of onset or medication history (Table 3.2). A two way ANCOVA with the main effects of DIAGNOSIS and

GENDER showed a significant diagnosis x gender interaction ($F_{1,69}$=4.073, p=0.047) but no significant main effect of diagnosis (p=0.377) or gender (p=0.099). Post hoc analyses revealed a 37% decrease in the female schizophrenia subjects compared to the male schizophrenia subjects (p=0.040) (see Figure 3.1) with a one way ANOVA showing that the male/female control/schizophrenia groups were still matched for age ($F_{3,70}$=1.711, p=0.173), PMI ($F_{3,70}$=0.563, p=0.641), pH ($F_{3,70}$=0.766, p=0.517), RNA integrity ($F_{3,70}$=1.014, p=0.392) and freezer storage time ($F_{3,70}$=1.954, p=0.129). Brain weight was different between the groups ($F_{3,70}$=11.240, p=0.000) with the schizophrenia female group having a significantly smaller brain weight than the control male (p=0.000) and the schizophrenia male (p=0.000) (see Table 4 in Appendix for comparison of demographic variables).

There was no difference in NR2B levels between the female schizophrenia and female control subjects (p=0.244) however a power analysis, as performed by Scarr and colleagues 2012, (http://www.biomath.info/power/ttest.htm) (Scarr et al., 2012) revealed that our cohort was underpowered and needed 17 per/group in order to detect a difference between the female control and female schizophrenia groups. There was no effect of hemisphere on NR2B protein density (see Table 3 in Appendix for comparison of demographic variables).

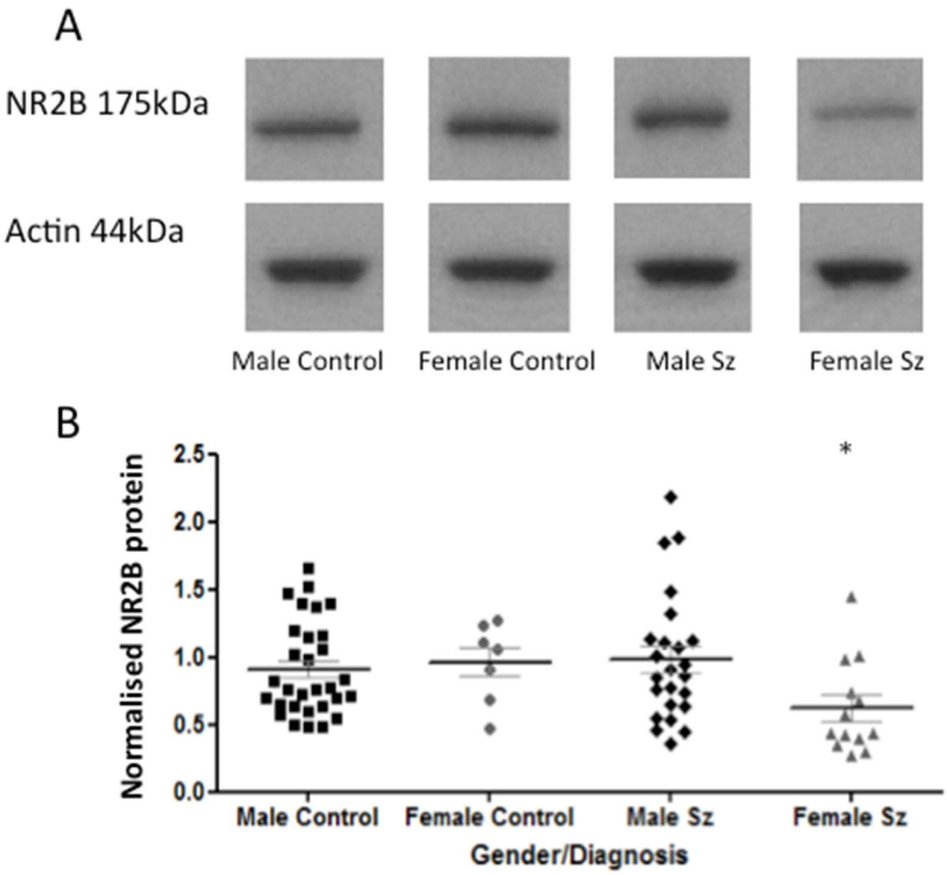

Figure 3.1. A: Representative Western blot bands of NR2B and actin protein. B: NR2B protein density in the dorsolateral prefrontal cortex of the male and female, schizophrenia and control brain. There was a 37% decrease in the female schizophrenia subjects compared to the male schizophrenia subjects. *: p = 0.040 compared to Female Sz. Horizontal and vertical bars represent mean±SEM for each group. Abbreviations: Sz = schizophrenia.

3.3.2 No change in NR2B binding density in schizophrenia subjects compared to control.

An Independent t-test showed that there was no difference between [^{3}H]Ifenprodil binding in the schizophrenia and control subjects (t_{72} = -0.263, p = 0.793).

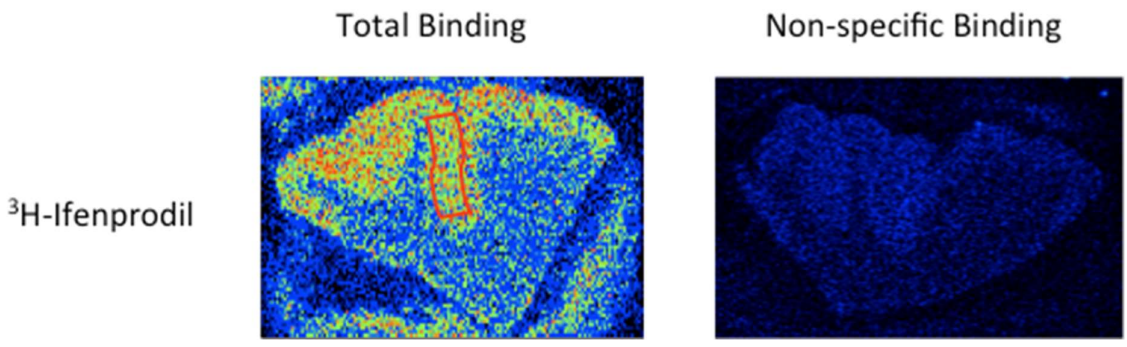

Figure 3.2. Representative total and non-specific [^{3}H]Ifenprodil binding in the dorsolateral prefrontal cortex of this human tissue cohort. Box highlights area of the dorsolateral prefrontal cortex as identified by the SRL.

[^{3}H]Ifenprodil binding density correlated with freezer storage time ($r = 0.359$, $p = 0.002$) and there was a trend for a correlation with age at death of the subjects ($r = -0.222$, $p = 0.057$) in the whole cohort. There was a correlation with freezer storage time in the control subjects only ($r = 0.324$, $p = 0.051$) and the schizophrenia subjects only ($r = 0.405$, $p = 0.013$) (Table 3.2). An ANCOVA to correct for freezer storage time and age at death showed no difference in [^{3}H]Ifenprodil binding sites between schizophrenia and control subjects ($F_{1,70} = 0.009$, $p = 0.927$). There was no correlation between [^{3}H]Ifenprodil binding density in the schizophrenia subjects and length of illness, age of onset or medication history (see Table 3.2). There were no effects of hemisphere or gender on [^{3}H]Ifenprodil binding in the prefrontal cortex (see Table 3 and 4 in Appendix for comparison of demographic variables).

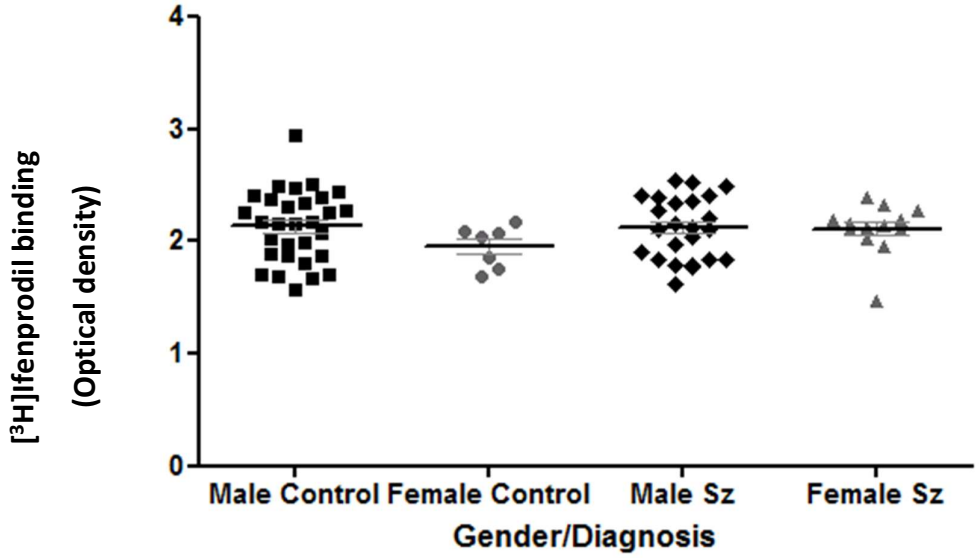

Figure 3.3. [^{3}H]Ifenprodil binding density in the dorsolateral prefrontal cortex of the male and female, schizophrenia and control brain. Horizontal and vertical bars represent mean±SEM for each group. Abbreviations: Sz = schizophrenia.

Table 3.2. Pearson's correlations for continuous variables with NR2B binding and protein.

Variable		All subjects		Control		Schizophrenia	
		NR2B binding	NR2B protein	NR2B binding	NR2B protein	NR2B binding	NR2B protein
Age	r	**-0.222**	**0.212**	-0.221	0.248	-0.226	0.194
	p	**0.057^**	**0.069^**	0.188	0.139	0.179	0.250
PMI	r	-0.058	**-0.332**	0.120	**-0.408**	-0.240	-0.277
	p	0.622	**0.004***	0.481	**0.012***	0.153	0.096
Brain pH	r	0.066	-0.082	0.091	-0.113	0.044	-0.074
	p	0.574	0.486	0.592	0.506	0.795	0.665
BW	r	-0.112	0.080	-0.005	-0.114	-0.209	0.169
	p	0.341	0.497	0.978	0.500	0.214	0.317
RIN	r	0.147	-0.045	0.251	0.131	0.029	-0.173
	p	0.212	0.706	0.134	0.440	0.863	0.305
FST	r	**0.359**	0.014	**0.324**	-0.072	**0.405**	0.105
	p	**0.002***	0.906	**0.051^**	0.673	**0.013***	0.537
Age Onset	r					-0.062	0.147
	p					0.716	0.385
DOI	r					-0.204	0.134
	p					0.226	0.429
APD use	r					-0.082	-0.036
	p					0.627	0.833

Abbreviations: AP = antipsychotic drug, BW = body weight, DOI = duration of illness, FST = freezer storage time, pH = brain acidity, PMI = post mortem interval, RIN = RNA integrity. *: $p<0.05$; ^: $0.05<p<0.07$.

3.3.3 Correlations

Overall there was no significant correlation between NR2B binding density and NR2B protein density ($r = -0.161$, $p = 0.171$). This was similar for the control subjects only ($r = -0.262$, $p = 0.117$), however in the schizophrenia subjects there was a significant negative correlation ($r = -0.346$, $p = 0.036$) between NR2B binding and protein density.

3.4 Discussion

This study has shown a significant reduction in NR2B protein but not binding density in the DLPFC of female schizophrenia subjects compared to male schizophrenia subjects. There were however no significant differences in NR2B protein or binding density between schizophrenia and control subjects in this tissue cohort.

3.4.1 Gender difference in NR2B protein in the prefrontal cortex in schizophrenia

This study is the first to observe a gender difference in NR2 subunit protein in the schizophrenia brain. Despite the uneven grouping/decreased sample number when analysing gender effects and the lack of effect of oestrogen on NMDAR, NR2A or NR2B protein levels (Snyder et al., 2011), this suggests that the influence of gender in the schizophrenia brain may be important. While oestrogen levels and oestrous cycle status were not controlled for in this study, previous reports suggest that oestrogen does not affect NMDAR, NR2A or NR2B protein levels (Snyder et al., 2011), albeit it may effect NR2B phosphorylation and synaptic localisation (Liu et al., 2012; Vedder et al., 2013) The present study did find a decrease in brain weight in the schizophrenia female group compared to the control male and schizophrenia male groups, which may contribute to the difference in NR2B levels,

although no correlation between NR2B levels and brain weight were observed and there was no difference in NR2B levels between the schizophrenia female and control male groups, so this is unlikely. Known gender differences in schizophrenia that may contribute to the observed change in NR2B in female schizophrenia subjects include a later age of onset in females (although it should be noted that age of onset did not correlate with NR2B binding or protein in this study), higher prevalence of affective symptoms in females and differences in cognitive impairments, although results suggest worse cognitive deficits in males (for review see Ochoa et al., 2012). It is therefore surprising to find a reduction in NR2B protein levels in female schizophrenia subjects compared to males given the importance of the NR2B subunit in cognitive functions in the prefrontal cortex (Zhao et al., 2005, Wang et al., 2008). Female schizophrenia subjects in the present study also had a longer duration of illness than the male schizophrenia group ($p=0.006$) (see Table 4 in the Appendix). However, there was no correlation of NR2B with duration of illness, so this is unlikely to account for the gender specific findings observed. Similar to our findings in the hippocampus, this reduction in NR2B protein levels was accompanied by no change in NR2B binding density suggesting a change in intracellular protein levels but not cell surface receptors.

Previous studies on the same postmortem brain cohort have reported a 33% increase in NR2B mRNA in males relative to females, however this was independent of diagnosis (Weickert et al., 2013), and a gender x diagnosis interaction was found with regards to metabotropic glutamate receptor 5 (mGluR5) binding density (Matosin et al, 2013). This provides support to our finding in another aspect of the glutamatergic system, suggesting that changes in the glutamatergic system in schizophrenia are likely to be gender specific. The use of NMDAR antagonists, such

as PCP, to induce a schizophrenia-like state are known to have gender specific effects, with females generally being more susceptible to its effects (Kokras and Dalla 2014). Gender differences in brain chemistry in healthy subjects, including the dopaminergic, serotonergic and GABAergic systems, have been reported showing differences in neurotransmitter and receptor levels, mainly an increase in the female brain (for review see Cosgrove et al., 2007). Although our study found no difference in NR2B expression between male and female control subjects, future studies exploring neurochemical changes in the schizophrenia brain should examine gender effects with the aim of linking any changes to schizophrenia symptomology or clinical characteristics.

Gender is often overlooked in postmortem studies despite the gender specific differences observed in the onset and symptom profile of schizophrenia (Ochoa et al., 2012) and it may be important to consider gender specific treatments. Animal models are widely used to study the aetiology and treatments of psychiatric disorders such as schizophrenia, however, most commonly only males are used in these models (Kokras and Dalla 2014). Our findings show clear gender differences with regards to the NR2B subunit in the prefrontal cortex in schizophrenia and therefore a gender analysis should be included in future studies of both postmortem and animal models of schizophrenia.

This study found no hemisphere effects of NR2B protein levels in the prefrontal cortex of schizophrenia subjects compared to controls despite the observed deficits in the right hemisphere in neuroimaging studies (Callicott et al., 2000, Perlstein et al., 2001, Barch et al., 2003, Lee et al., 2008). This is in contrast to our finding of a decrease in NR2B protein specifically in the left hippocampal hemisphere in schizophrenia subjects. This suggests that hemisphere effects of

NR2B protein may be brain region specific. In a previous study of the DLPFC using this same postmortem cohort, NR2B mRNA expression was reported to be 39% higher in the left versus the right prefrontal cortex however this effect was independent of diagnosis (Weickert et al, 2013).

A previous study examining NMDAR subunit protein levels in the prefrontal cortex of the schizophrenia brain found no effect of diagnosis (Kristiansen et al., 2006) however they did not examine gender differences probably due to low sample size. No effect of diagnosis has also been observed in a previous study examining [^{3}H]Ifenprodil binding in the prefrontal cortex of the schizophrenia brain (Beneyto and Meador-Woodruff, 2008). Furthermore, no difference in NR2B mRNA in the prefrontal cortex has been reported in the schizophrenia compared to the control brain (Arkbarian et al., 1996, Beneyto and Meador-Woodruff, 2008, Dracheva et al., 2001).

There is one study which shows that NR2B protein is decreased in the endoplasmic reticulum cellular fraction in the prefrontal cortex of schizophrenia compared to control subjects (Kristiansen et al., 2010b) suggesting an alteration in intracellular production or trafficking of the NR2B subunit protein. We cannot rule out changes in the endoplasmic reticulum in our cohort as we did not separate the protein into different cellular compartments.

3.4.2 Effects of confounding variables

Due to the fact that there was no correlation between the NR2B binding or protein in the schizophrenia subjects and antipsychotic drug use, it appears that medication history does not affect NR2B levels. This is further supported by animal studies showing no change in NR2B levels in the brain following antipsychotic drug treatment (Schmitt et al., 2003, O'Connor et al., 2006, Fumagalli et al., 2008).

3.4.3 Conclusion

In conclusion, this study reports a significant reduction in NR2B protein expression in the DLPFC of female compared to male schizophrenia subjects. This finding highlights the gender specific nature of the biology of schizophrenia. Sex differences are often overlooked in the biological examination of schizophrenia, despite the clear gender differences that are observed in the clinic including later age of disease onset, higher incidence of affective symptoms. The present findings suggest, where possible, both male and female subjects should be included in future schizophrenia studies. Furthermore, given this study was underpowered to detect differences between female schizophrenia subjects and controls (due to the nature of the cohort), future studies in a larger cohort are necessary to confirm these findings.

EXPERIMENTAL PART B: THE EFFECT OF A PERINATAL NR2B BLOCKADE ON THE DEVELOPMENTAL PROFILE OF RELATED NEUROTRANSMITTER SYSTEMS. AN ANIMAL MODEL

4 THE EFFECT OF PERINATAL NR2B ANTAGONISM ON THE NMDA RECEPTOR AND ITS PRIMARY SUBUNITS 2A AND 2B: A MOUSE MODEL

4.1 Introduction

As detailed in Chapter 1, the perinatal NMDAR hypofunction hypothesis of schizophrenia is widely studied in animal models. Models of developmental NMDAR hypofunction (eg NR1 hypomorphic mice, perinatal NMDAR antagonist administration) demonstrate an array of behavioural and neurochemical effects in adult animals that mimic schizophrenia-like symptoms and neurochemical alterations that are observed in the schizophrenia brain (Facchinetti et al., 1993, Wang et al., 2001, Harris et al., 2003, Sircar and Soliman, 2003, Andersen and Pouzet, 2004, Stefani and Moghaddam, 2005, du Bois et al., 2008, Wang et al., 2008, du Bois et al., 2009). NMDARs are composed of different combinations of NR1, NR2 (A, B, C, D) and/or NR3 (A, B) subunits, with the NR2 subunits displaying distinct developmental and regional patterns of expression (For review see Geddes et al., 2011). The NMDAR is vital for early brain development and the NR2B subunit shows a stronger and earlier expression in the brain during the perinatal period than the other NR2 subunits (Watanabe et al., 1992, Monyer et al., 1994, Wenzel et al., 1997, Ritter et al., 2001). Furthermore the distribution of NR2B subunits is quite broad during early development and becomes more restricted to schizophrenia relevant forebrain structures, such as the prefrontal cortex and hippocampus, later in development (Wenzel et al., 1997, Heimer, 2000). Therefore an alteration to NR2B levels during this early, critical point in development could cause a disruption to the normal brain growth pattern and change the developmental profile of associated proteins. Indeed it has been suggested that an imbalance of NR2 subunits, particularly NR2A and NR2B, during early brain development could cause an

alteration of the normal NMDAR developmental processes, including synaptogenesis and synapse maturation (Gambrill and Barria, 2011). This is based on the ability of the C-terminus of the NR2B subunit to recruit signalling and scaffolding proteins necessary for synapse formation (Wilson et al., 1998, Akashi et al., 2009, Gambrill and Barria, 2011).

NR2B blockade (using Ro 25-6981) in adult rodents causes hyperlocomotor activity, disrupted PPI, increases in anxiety-like behaviour and impairments in learning and memory, which are recognised as schizophrenia-like behaviours in the rodent (Chaperon et al., 2003, Duffy et al., 2007, Mathur et al., 2009). However, a lack of locomotor changes in rodents treated with Ro 25-6981 has also been reported (Kosowski and Liljequist, 2004). Adult rats treated with the more selective NR2B antagonist, Ro 63-1908, displayed increased locomotor activity and impaired response inhibition (but no disruption to PPI) (Higgins et al., 2003). These behavioural alterations are similar, although not to the same extent, to those seen with total antagonism of the NMDAR (Higgins et al., 2003) and are not observed with NR2A blockade (Chaperon et al., 2003).

Perinatal NR2B blockade (using ifenprodil) in a single study has shown that rats are not susceptible to hyperlocomotion following a PCP challenge at adolescence, while rats treated with the NR2A antagonist PEAQX display hyperlocommotive behaviour following the PCP challenge (Anastasio et al., 2009). This study suggests that it may be perinatal NR2A blockade that mirrors the behaviours seen with PCP administration, however the NR2B antagonist (ifenprodil) used, is not as potent or specific to the NR2B subunit as Ro 63-1908 (Zhou et al., 1999) in which doses of up to 30 mg/kg have been used in adult rodents. Therefore further investigation is needed into the effects of perinatal NR2 antagonism,

particularly as the NR2B subunit is critical for early synaptogenesis (Gambrill and Barria, 2011) and any impact that hinders synaptic formation and connection could have adverse consequences throughout brain development.

While perinatal PCP treatment produces alterations in NMDAR proteins in the short (Anastasio and Johnson, 2008) and longer terms (du Bois et al., 2012), the effect of a perinatal NR2 blockade on related proteins has not been investigated. Considering the role that NR2B plays in early synaptic connections, a perinatal blockade of NR2B could have substantial effects, not only on other proteins at this perinatal period but also on protein levels later in development, particularly at the adolescent period when synaptic pruning is abundant. Furthermore, it is possible there is a gender difference in the normal developmental profile of NMDAR associated proteins or a difference in protein response to the perinatal NR2B antagonist treatment between genders, considering our findings of a gender difference in NR2B protein in the prefrontal cortex of schizophrenia patients. Therefore the aims of this study were to 1. Examine the effect of perinatal NR2B antagonist treatment on the NMDAR and its primary subunits, NR2A and NR2B at juvenile, adolescent and adult time points, 2. Study the normal developmental profile of the NMDAR, NR2A and NR2B subunits, and 3. Examine the developmental and treatment effects in male and female mice.

4.2 Materials and Methods

4.2.1 Animals and Drug Treatment Procedure

Thirty-eight female and nineteen male adult C57BL/6 mice were obtained from the Animal Resources Centre (Perth, Australia). They were housed at the University of Wollongong Animal House in a temperature controlled environment (20^{o}C ± 2) under 12:12 hour light-dark cycle (lights off at 1900h). The mice were

provided with food and water ad libitum. The mice were acclimatised to these housing conditions for one week before mating. Upon successful mating, female mice were housed individually for the 21 days of gestation. The day of birth of the pups is considered PN0. On the morning of PN7 the cage of pups was assigned to either a treatment or control group. The treatment group was injected subcutaneously with 30 mg/kg of the NR2B antagonist Ro 63-1908 (Tocris, Bristol, UK) at a volume of 5 ul/g daily for eight days from PN7 to PN14. This dose is based on a previous experiment showing psycho-mimetic behavioural changes similar to MK-801 in adult rodents (Higgins et al., 2003). The duration of treatment was chosen based on the critical period for NMDAR antagonist-induced apoptosis in the rodent brain (Ikonomidou et al., 1999). The control group were similarly injected with the same volume of saline, containing 0.9% NaCl. One third of the litters were euthanased eight hours after the last treatment day on PN14 (n = 6 per gender per group). The remaining litters were weaned on PN28 with the mice housed 2/cage of the same gender and then cages were randomly allocated to either an adolescent or adult group, and euthanased on PN35 (adolescence) or PN70 (adult) (5-6 per gender per group). All procedures involving animals were approved by the University of Wollongong Animal Ethics Committee (AE08/12) under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) guidelines. All efforts were made to minimise numbers of animals used.

4.2.2 Autoradiography Procedures and Quantification

Immediately following euthanasia mouse brains from 6 mice/group were removed and frozen whole in liquid nitrogen and stored at -80°C until sectioning. Brains were sectioned coronally (14 μm thick) with a Shandon Cryotome E (Thermo Fischer Scientific, Waltham, MA, USA) under -18°C conditions and then mounted

on Polysine™ microscope slides (Menzel-Glaser, Braunschweig, Germany). Sections were collected at levels corresponding to Bregma 2.34 and -1.94 mm (Paxinos and Franklin, 2001), which includes the structures of the prefrontal cortex and hippocampus and kept in -20°C conditions.

4.2.3 NMDAR Binding

[^{3}H]MK-801 binding was performed based on the methods used previously (Newell et al., 2007a). Sections were incubated in 30 mM HEPES buffer (pH 7.5) containing 100 µM glycine, 100 µM glutamate and 1 mM EDTA, with the addition of 20 nM [^{3}H]MK-801 (NMDA receptor antagonist: specific activity 27.5 Ci/mmol; PerkinElmer, Waltham, MA, USA) for 2½ hours at room temperature to determine total binding. Adjacent sections were incubated with 20 nm [^{3}H]MK-801 in the presence of 20 µM MK-801 (Sigma-Aldrich, Castle Hill, NSW, Australia) to determine non-specific binding. All sections were then washed in 30 mM HEPES buffer (pH 7.5) containing 1 mM EDTA for 2 x 20 minutes at 4°C. Finally the sections were rinsed in distilled water and air-dried overnight.

4.2.4 NR2A Binding

[^{3}H]CGP39653 binding was performed based on the methods previously used by Scarr et al (2005). All sections were preincubated in 0.05 M TrisHCl buffer (pH 7.6) with the addition of 0.0025 M $CaCl_2$ for 45 minutes at room temperature. Sections were then incubated in the same buffer containing 20 nM [^{3}H]CGP39653 (NR2A antagonist: specific activity 47.25 Ci/mmol; PerkinElmer) for 45 minutes at room temperature to determine total binding, with adjacent sections incubated in the presence of 100 µM PEAQX (NR2A antagonist; Sigma-Aldrich) to determine non-specific binding. All sections were then washed in 0.05 M TrisHCl buffer (pH 7.6)

with 0.0025 M $CaCl_2$ for 2 x 30 seconds at 4°C, rinsed in distilled water and air-dried overnight.

4.2.5 NR2B Binding

[^{3}H]Ifenprodil binding was performed based on the methods used previously (Basham et al., 1999). All sections were incubated in 0.05 M Tris HCl buffer (pH 7.4) with the addition of 20 nM [^{3}H]Ifenprodil (NR2B antagonist: specific activity 40 Ci/mmol; PerkinElmer), 3 μM R(+)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride (+3PPP) (to block non-specific binding to the sigma receptor), 30 μM GBR-12909 (to block piperazine sites) and 1 mM trifluoperazine (to block low affinity polyamine sensitive sites on adrenergic, dopaminergic and cholinergic receptors) for 3 hours at 4°C to determine total binding, with adjacent sections incubated in the presence of 20 μM Ifenprodil (Sigma-Aldrich) to determine non-specific binding. All sections were then washed in 0.05 M Tris-HCl (pH 7.4) for 3 x 5 minutes at 4°C, rinsed in distilled water and air-dried overnight.

4.2.6 Quantification

All slides were placed in cassettes and exposed to tritium-sensitive Kodak BioMax MR Film (Sigma-Aldrich) for 6-12 weeks. The films were developed using the AGFA CP1000 film developer (Agfa-Gevaert N.V.) and analysed using Quantity One software version 4.6.7 (BioRad) connected to a GS-800 Calibrated Densitometer (BioRad). Quantification was performed blind to grouping by measuring the average optical density in each brain region in three adjacent sections and then averaging across six mice per group.

4.2.7 Western Blot

Brains from 5 mice/group were removed immediately upon euthanasia and dissected into prefrontal cortex and hippocampus regions, frozen in liquid nitrogen and stored at -80ºC until use. The prefrontal cortex and hippocampus tissue was weighed and manually homogenised in 9 volumes of buffer containing 0.1 M Tris-HCl, 10 µl/ml protease inhibitor cocktail (Sigma), 0.625 µl/ml apoprotinin and 0.5 µl/ml glycerol. Samples were quantified using the DC protein assay (BioRad, Hercules, CA, USA) following manufacturer's instructions. The standard curve was determined using 2 mg/mL BSA serial dilution as a control. Samples were then diluted in Laemmli's sample buffer (Bio-Rad) and loaded in triplicate into precast 4-12% bis-tris gels (Bio-Rad) with 3 µg of total protein per well, along with a molecular weight ladder (Precision Plus Protein™ Standards, Bio-Rad) and run with XT-MOPS buffer (Bio-Rad) at 200 V for 55 minutes. The protein was transferred to PVDF membrane (Bio-Rad) at 100 V for 1 hour using Tris-glycine buffer (Bio-Rad) containing 20% methanol and blocked in PBS-T and 5% skim milk for 1 hour at room temperature. Blots were then incubated overnight (4°C) with one of the following antibodies diluted in PBS-T containing 1% skim milk: anti-NR2B (#ab28373, 1/1250; Abcam, Cambridge, UK) or anti-NR2A (#ab65799, 1/250; Abcam). A mouse anti-actin antibody was used as an internal standard (#MAB1501, 1/50000; Millipore, Billerica, MA, USA). Following primary antibody exposure, blots were washed for 3 x 5 minutes in PBS-T and incubated for 1 hour (room temperature) with a HRP-conjugated secondary antibody (anti-rabbit: #AP307P, anti-mouse: #AP308P, 1/3000; Millipore). Blots were washed for 3 x 5 minutes in PBS-T and the proteins of interest detected using the ECL Plus Western Blotting Detection Reagents kit (GE Healthcare, Piscataway, NJ, USA). Images were

captured using the Gel Logic 2200 PRO (Carestream Health Inc, New Haven, CT, USA) with exposure times of 5-10 minutes and quantified blind to grouping with the Carestream molecular imaging software, version 5.0 (Carestream Health Inc) and the average density of each band normalised to actin.

4.2.8 Statistics

The statistical package used for data analysis was SPSS version 17 (IBM). The data was checked for triplicate (SD>30% mean) and population outliers (two standard deviations above or below the mean) and a Kolmogrov-Smirnov test was used to determine the normality of the data. A three-way ANOVA with AGE, TREATMENT and GENDER as the main factors was performed for each brain region. With a significant effect or interaction effect, either a one-way ANOVA with tukey's or dunnett's T3 post hoc depending on levene's test of homogeneity of variances (for AGE) or an Independent samples t-test with Bonferroni adjustment (for TREATMENT and GENDER) was performed (with adjustments to the degrees of freedom using the Welch-Satterthwaite method if Levene's test was significant). Significance was set at $p<0.05$, with bonferroni adjustments set at $p<0.017$ when data was split for age, gender and treatment. All data is presented as mean±SEM.

4.3 Results

[^{3}H]MK-801 displayed strong binding to the NMDAR in the prefrontal cortex and hippocampus. In comparison, [^{3}H]CGP39653 and [^{3}H]Ifenprodil showed moderate binding to NR2A and NR2B subunits respectively in the prefrontal cortex and hippocampus. For all radioligands, non-specific binding represented less than 5% of total binding (see Figure 4.1).

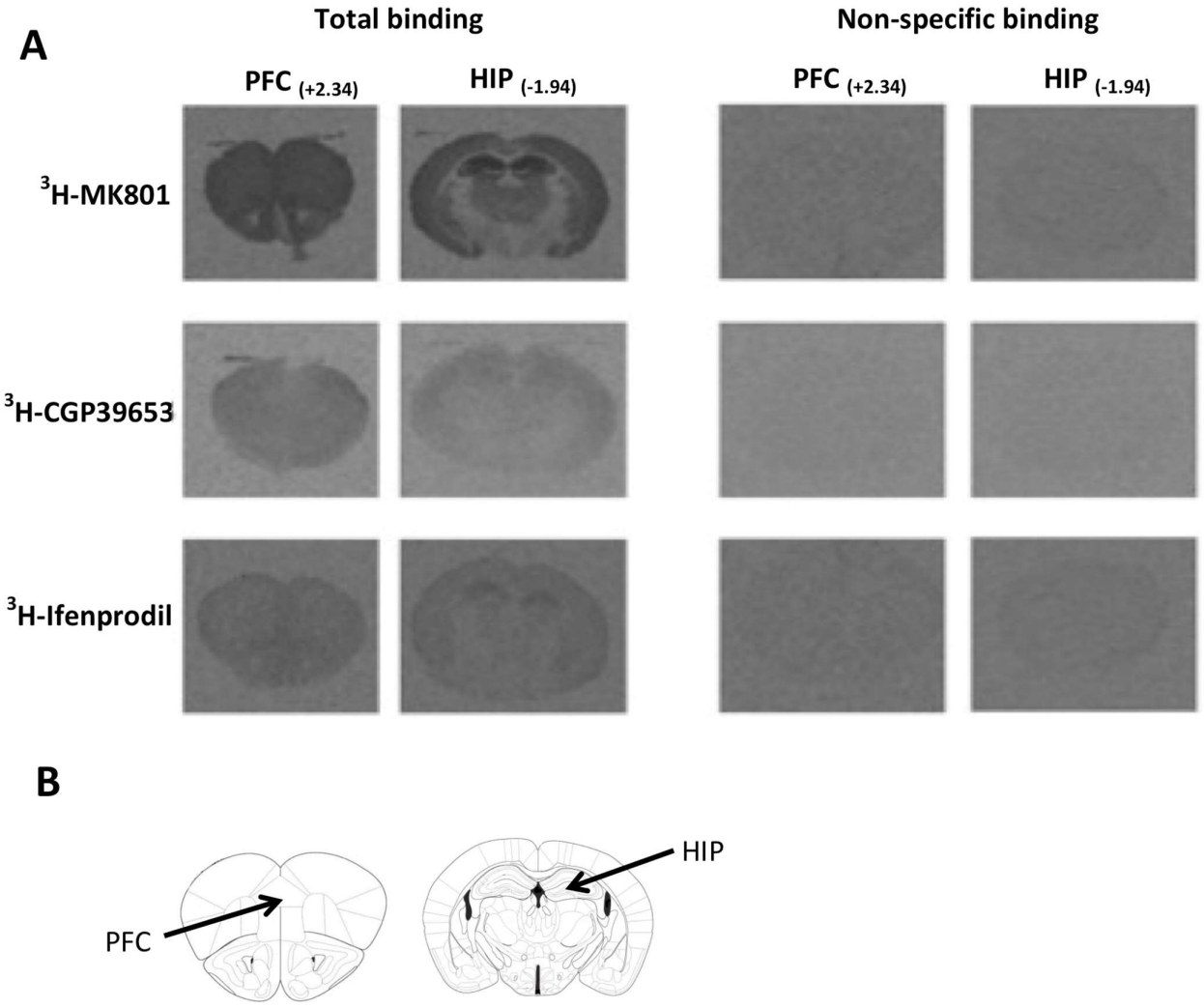

Figure 4.1. A: Representative autoradiograms of total and non-specific NMDAR ([^{3}H]MK801), NR2A ([^{3}H]CGP39653) and NR2B ([^{3}H]Ifenprodil) binding in the prefrontal cortex (Bregma +2.34) and hippocampus (Bregma -1.94) of adult mice. B: Mouse brain atlas pictures showing bregma levels and locations for prefrontal cortex (+2.34) and hippocampus (-1.94). Abbreviations: HIP = hippocampus, PFC = prefrontal cortex.

4.3.1 NMDAR Binding

A three way ANOVA revealed a significant main effect of AGE in the prefrontal cortex ($F_{2,54}$=19.4, p<0.001) and hippocampus ($F_{2,52}$=45.4, p<0.001) on [^{3}H]MK-801 binding. There was no significant main effect of TREATMENT or GENDER in the prefrontal cortex or hippocampus or any interactions observed.

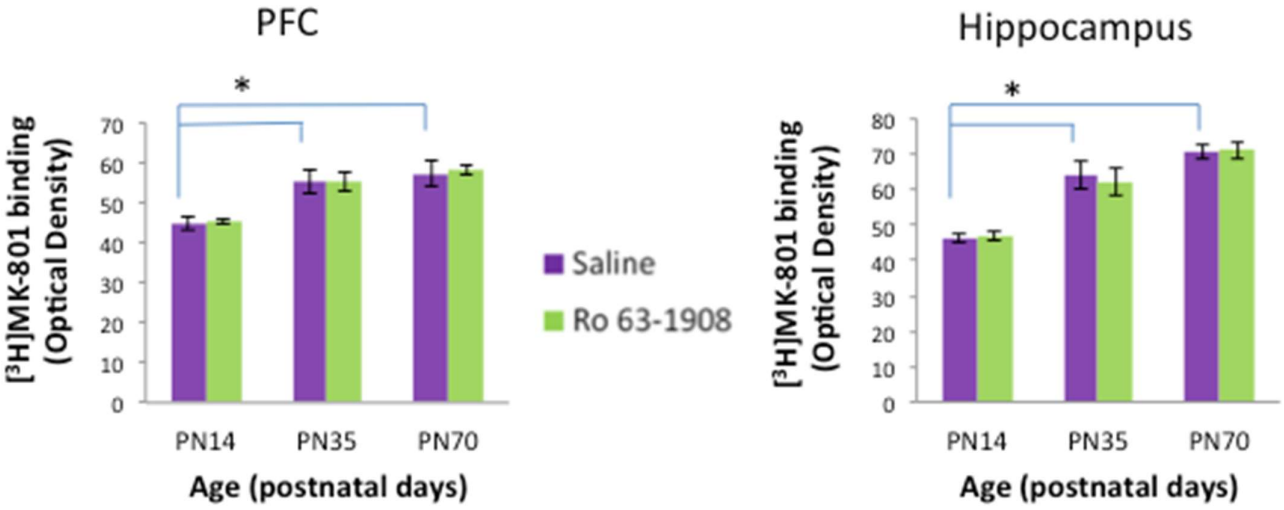

Figure 4.2. [^{3}H]MK-801 binding to NMDARs in the prefrontal cortex and hippocampus of mice at postnatal days 14, 35 and 70, following perinatal treatment with saline or the NR2B antagonist Ro 63-1908. Data represents males and females combined, n=10-12/group. Data presented as mean optical density ± SEM. *: 0.000<p<0.05. Abbreviations: PFC = prefrontal cortex; PN = postnatal day.

Using the control data of both genders combined, [^{3}H]MK-801 binding was significantly different between time points in the prefrontal cortex ($F_{2,30}$=6.7, p=0.004) and hippocampus ($F_{2,29}$=28.8, p<0.001) (see Figure 4.2). Post-hoc tests revealed an increase in binding from PN14 to PN35 in the prefrontal cortex (+23%, p=0.021) and hippocampus (+39%, p=0.005) but no change from PN35 to PN70 in the prefrontal cortex (p=0.846) and hippocampus (p=0.432). Overall there was an

increase from PN14 to PN70 in the prefrontal cortex (+28%, p=0.006) and hippocampus (+53%, p<0.001) (see Figure 4.2).

4.3.2 NR2A Binding

A three way ANOVA revealed a significant main effect of AGE (prefrontal cortex: $F_{2,54}$=28.6, p<0.001; hippocampus: $F_{2,54}$=13.1, p<0.001) and GENDER (prefrontal cortex: $F_{1,54}$=43.8, p<0.001; hippocampus: $F_{1,54}$=44.3, p<0.001) and an AGE x GENDER interaction (prefrontal cortex: $F_{1,54}$=25.0, p<0.001; hippocampus: $F_{1,54}$=13.7, p<0.001) on [^{3}H]CGP39653 binding. There was also a trend for an AGE x TREATMENT interaction in the prefrontal cortex ($F_{1,54}$=3.055, p=0.055). There was no significant main effect of TREATMENT in the prefrontal cortex or hippocampus or any other interactions observed.

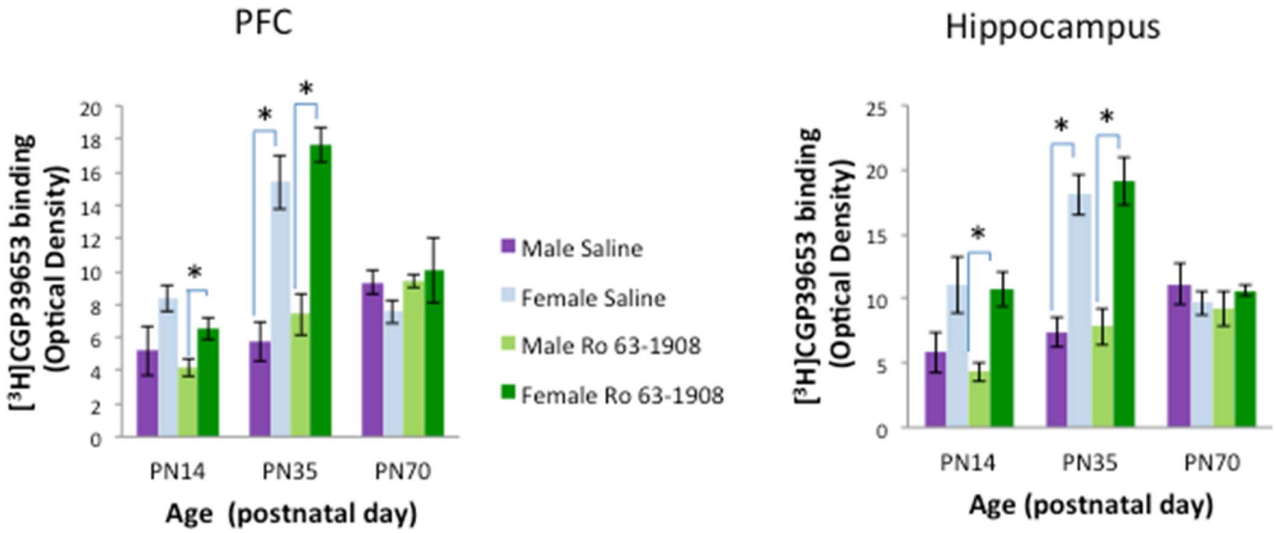

Figure 4.3. [^{3}H]CGP39653 binding to the NR2A subunit in the prefrontal cortex and hippocampus of male and female mice at postnatal days 14, 35 and 70, following perinatal treatment with saline or the NR2B antagonist Ro 63-1908. n=5-6/group. Data presented as mean optical density ± SEM. *: p<0.001. Abbreviations: PFC = prefrontal cortex; PN = postnatal day. Columns at each age group correspond to the descending order of the groups in the graph key.

In the saline animals there was an increase in [^{3}H]CGP39653 binding in the female compared to the male mice at PN35 in the prefrontal cortex (+166%, t_9=-4.9, p=0.001) and hippocampus (+144%, t_9=-5.7, p<0.001) but not at PN14 (prefrontal cortex: p=0.078; hippocampus: p=0.096) or PN70 (prefrontal cortex: p=0.130; hippocampus: p=0.486) (see Figure 4.3). In the Ro 63-1908 treated animals there was an increase in [^{3}H]CGP39653 binding in the female compared to the male mice at PN14 (prefrontal cortex: +56%, t_{11}=-2.8, p=0.017; hippocampus: +146%, t_{11}=-4.3, p=0.001) and PN35 (prefrontal cortex: 138%, t_9=-6.1, p<0.001; hippocampus: +145%, t_9=-5.0, p=0.001) but not PN70 (prefrontal cortex: p=0.762; hippocampus: p=0.387) (see Figure 4.3).

Using the control data, [^{3}H]CGP39653 binding density was significantly different between time points in the prefrontal cortex (male: $F_{2,14}$=3.9, p=0.044; female: $F_{2,13}$=15.1, p<0.001) and hippocampus (female: $F_{2,13}$=6.5, p=0.011; male (trend): $F_{2,14}$=3.5, p=0.060). Post-hoc tests revealed a trend for an increase in binding from PN14 to PN70 in the prefrontal cortex (+80%, p=0.059) and hippocampus (+90%, p=0.059) of the male mice. In the female mice, there was a significant increase in binding from PN14 to PN35 in the prefrontal cortex (+84%, p=0.001) and hippocampus (+63%, p=0.031) and then a decrease from PN35 to PN70 in the prefrontal cortex (-51%, p=0.001) and hippocampus (-46%, p=0.014). Overall there was no difference between PN14 to PN70 in the prefrontal cortex (p=0.864) and hippocampus (p=0.841) in the female mice.

4.3.3 NR2A Protein

There was a single band at 165 kDa as expected. A three way ANOVA revealed a significant main effect of AGE in the hippocampus ($F_{2,47}$=8.1, p=0.001). Despite this, when using the control data, NR2A protein expression was not

significantly different between time points in the hippocampus ($F_{2,26}$=2.3, p=0.120) (see Figure 4). There were no significant main effects of GENDER or TREATMENT or significant interactions in the hippocampus and no significant effects in the prefrontal cortex.

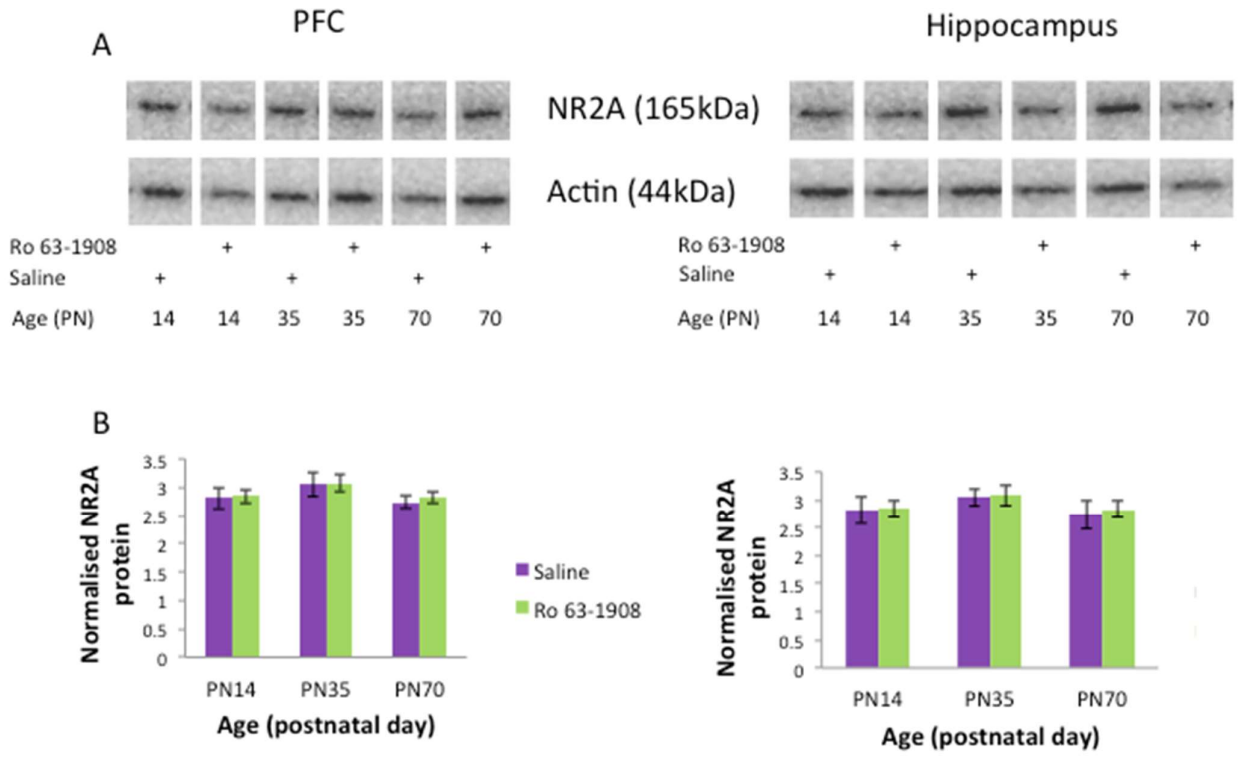

Figure 4.4. A: Representative Western blot bands of NR2A and actin protein. B: Graphs showing no difference in NR2A protein in the prefrontal cortex and hippocampus of mice at postnatal days 14, 35 and 70, following perinatal treatment with saline or the NR2B antagonist Ro 63-1908. Data represents males and females combined, n=10-12/group. Data presented as mean optical density ± SEM. Abbreviations: PFC = prefrontal cortex; PN = postnatal day.

4.3.4 NR2B Binding

A three way ANOVA revealed a significant main effect of AGE (prefrontal cortex: $F_{2,49}$=115.6, p<0.001; hippocampus: $F_{2,49}$=69.2, p<0.001) and TREATMENT (prefrontal cortex: $F_{1,49}$=30.4, p<0.001; hippocampus: $F_{1,49}$=55.2, p<0.001) and an

AGE x TREATMENT interaction (prefrontal cortex: $F_{1,49}$=37.1, p<0.001; hippocampus: $F_{1,49}$=37.6, p<0.001) on [^{3}H]Ifenprodil binding. There was no significant main effect of GENDER in the prefrontal cortex or hippocampus or any other interactions observed.

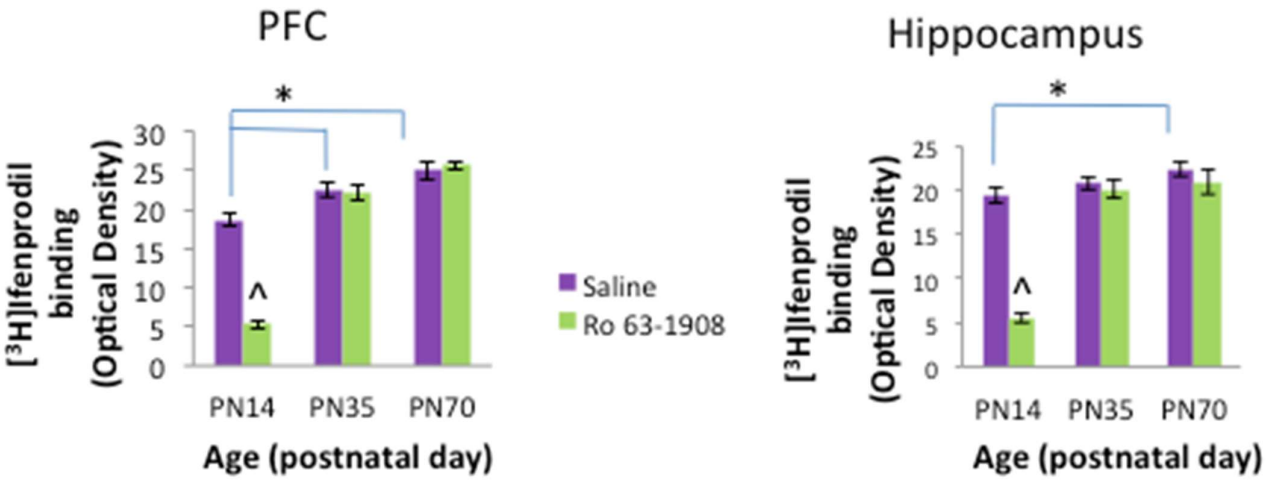

Figure 4.5. [^{3}H]Ifenprodil binding to the NR2B subunit in the prefrontal cortex and hippocampus of mice at postnatal days 14, 35 and 70, following perinatal treatment with saline or the NR2B antagonist Ro 63-1908. Data represents males and females combined, n=10-12/group). Data presented as mean density ± SEM. *: 0.000<p<0.05, ^: p<0.001 compared to saline at PN14. Abbreviations: PFC = prefrontal cortex; PN = postnatal day.

An Independent samples t-test revealed a decrease in [^{3}H]Ifenprodil binding in the Ro 63-1908 treated mice compared to the controls at PN14 in the prefrontal cortex (-72%, t_{21}=12.2, p<0.001) and hippocampus (-71%, t_{21}=14.4, p<0.001) but not at PN35 (prefrontal cortex: p=0.883; hippocampus: p=0.560) or PN70 (prefrontal cortex: p=0.640; hippocampus: p=0.371).

Using the control data with genders combined, [^{3}H]Ifenprodil binding expression was significantly different between time points in the prefrontal cortex ($F_{2,27}$=10.8, p<0.001) and hippocampus ($F_{2,28}$=4.0, p=0.029). Post-hoc tests revealed

an increase in binding from PN14 to PN35 in the prefrontal cortex (+20%, p=0.033) but not the hippocampus (p=0.413) and no change from PN35 to PN70 in the prefrontal cortex (p=0.197) or hippocampus (p=0.299). Overall there was an increase from PN14 to PN70 in the prefrontal cortex (+34%, p<0.001) and hippocampus (+16%, p=0.022).

4.3.5 NR2B Protein

There was a single band at 165 kDa as expected. A three way ANOVA revealed a significant main effect of TREATMENT in the hippocampus ($F_{1,47}$=4.5, p=0.040), with an overall 8% increase in NR2B protein expression following Ro 63-1908 treatment. A significant AGE x TREATMENT interaction was observed in the prefrontal cortex ($F_{1,48}$=4.0, p=0.024). There was no significant main effect of GENDER in the prefrontal cortex or hippocampus or any other interactions observed.

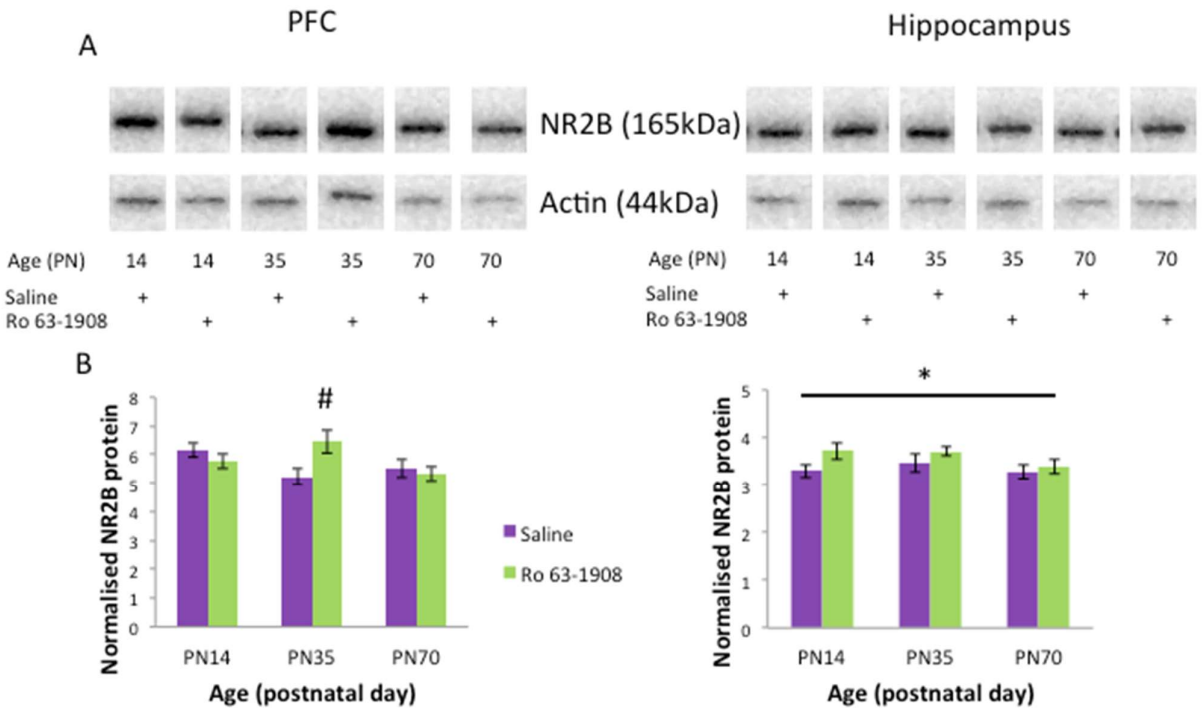

Figure 4.6. A: Representative Western blot bands of NR2B and actin protein. B: Graphs showing a trend for an increase in NR2B protein in mice following perinatal NR2B antagonist (Ro 63-1908) compared to those treated with saline at postnatal day 35 in the prefrontal cortex. Data represents males and females combined, n=10-12/group). Data presented as mean relative density ± SEM. #: p=0.020 (bonferroni corrected). 8% increase (p=0.040) in Ro 63-1908 treated mice, independent of age, compared to saline in the hippocampus. Abbreviations: PFC = prefrontal cortex; PN = postnatal day.

An Independent samples t-test showed a trend (using Bonferroni correction) for an increase in the Ro-631908 treated group compared to the control at PN35 in the prefrontal cortex (+23%, t_{18}=-2.5, p=0.020) but not at PN14 (p=0.309) or PN70 (p=0.684). Despite the main effect of treatment in the hippocampus there was no difference between the Ro 63-1908 treated group and control at any individual age (PN14: p=0.087; PN35: p=0.283; PN70: p=0.610) (see Figure 4.6).

Using the control data, there was a trend for a difference in NR2B protein expression between time points in the prefrontal cortex ($F_{2,27}$=2.8, p=0.077), with a

post hoc test revealing a trend for a decrease between PN14 and PN35 (-15%, p=0.070) and no other differences.

4.4 Discussion

The main findings from this study are: 1. Perinatal NR2B antagonism produced a reduction in NR2B binding at PN14 in the prefrontal cortex and hippocampus, but did not affect NMDAR or NR2A binding. 2. Perinatal NR2B antagonism produced small but significant increases in NR2B (but not NR2A) protein in the hippocampus and prefrontal cortex. 3. The adolescent brain showed evidence of gender specific NMDAR stoichiometry; NR2A binding was substantially increased in the prefrontal cortex and hippocampus of female mice, compared to males, at PN35.

4.4.1 Perinatal NR2B antagonism produces alterations specific to the NR2B subunit

Perinatal NR2B antagonism resulted in a substantial 70% decrease in [^{3}H]Ifenprodil binding at PN14 in both the prefrontal cortex and hippocampus. This reduction was observed eight hours after the final injection of the NR2B antagonist, Ro 63-1908, and was not apparent in the long-term. Ro 63-1908 and [^{3}H]ifenprodil are NR2B antagonists that both bind to the polyamine site on the NR2B subunit (Coughenour and Barr 2001, Zhou et al., 1999). Considering we did not observe a concomitant reduction in NR2B protein following Ro 63-1908 treatment, this finding strongly suggests that Ro 63-1908 displaced the [^{3}H]Ifenprodil binding in the brain, providing evidence of its ability to cross the blood brain barrier in this model. It has previously been shown that Ro 63-1908 is present in the cerebrospinal fluid following intravenous administration to adult animals (Gill et al., 2002). However,

there are no published studies showing evidence of direct binding of this compound to NR2B binding sites in the brain. The present study has therefore provided evidence that 8 hours following a subcutaneous administration, that Ro 63-1908 does displace [^{3}H]Ifenprodil from NR2B binding sites in the juvenile rat brain. Ro 63-1908 has previously been characterised to have an apparent half-life of only 0.30-0.39 hours following a 10 mg/kg intravenous bolus administration (Gill et al., 2002), however the half-life after subcutaneous administration has not been characterised. Based on our findings, it appears that the half-life following this administration, at least in juvenile mice, may in fact be much longer.

While NR2B protein levels were unchanged at PN14 following Ro 63-1908 treatment, there was a small, but significant, increase in NR2B protein levels in the hippocampus, independent of age and gender, as well as an increase in NR2B protein levels specifically at PN35 in the prefrontal cortex. These changes were not accompanied by changes in NR2B binding. As we have seen from the previous chapters examining NR2B binding and protein levels in the human prefrontal cortex and hippocampus, this disparity between binding and protein levels is not uncommon. This increase in NR2B protein levels observed following Ro 63-1908 treatment might represent a compensational increase in endogenous NR2B protein stores due to the perinatal blockade of NR2B. An investigation of the NR2B levels of subcellular compartments would be an important avenue to pursue to further characterise the cellular alterations in this model, especially considering cell compartment specific alterations in NR2B have been found in schizophrenia (Kristiansen et al., 2010b).

This is the first study to examine the effects of perinatal NR2B antagonism on the NMDAR and subunit levels. Our group has previously examined the effects of

the non-specific NMDAR antagonist PCP, on the developmental expression of NMDAR, including the NR2A and NR2B subunits. Perinatal PCP treatment showed an increase in NR2B protein and NMDAR binding at adolescence and similar to the results from the present study, these findings from du Bois and colleagues were observed in the prefrontal cortex but not the hippocampus (du Bois et al., 2009, du Bois et al., 2012). It is possible that the prefrontal cortex is not as efficient at returning a normal protein balance in the cell or it may be that the perinatal antagonism caused an imbalance in the developmental pattern of NR2B protein at adolescence that was specific to the prefrontal cortex. Du Bois and colleagues (2012) also showed that the NR2A subunit was increased in the hippocampus at adolescence, which contrarily did not occur in the present study. This suggests that non-specific antagonism of the NMDAR can affect both the NR2A and NR2B subunits in different parts of the brain whereas specific NR2B antagonism appears to only alter the NR2B subunit protein.

At the adult time point of PN70, the mouse model used in this study showed no changes in NR2A or NR2B subunit binding or protein, which is similar to some studies in adult human schizophrenia tissue in the prefrontal cortex and hippocampus (Beneyto et al., 2007, Beneyto and Meador-Woodruff, 2008, Kristiansen et al., 2006). In Chapter 2 of this thesis, it was reported that NR2B protein was reduced in the hippocampus of schizophrenia subjects, specifically in the left hemisphere. Unfortunately hemisphere specific effects were not examined as part of our animal study. Functional lateralisation is reported to occur in rodents in both the hippocampus and cortical areas (Cowell et al., 1999, Kim et al., 2012, Shipton et al., 2014). Furthermore, in the hippocampal CA1 region, the larger synapses found on the right correspond with a higher density of NR1 subunit, but not the NR2B subunit

(Shinohara et al., 2008) and this suggests hemisphere specific differences in some subunits that should be followed up in future studies. In Chapter 3 of this thesis, it was reported that NR2B protein was reduced in female schizophrenia subjects compared to males, but no gender effects were observed in this study in regards to NR2B. This animal model produced selective subtle changes, primarily at adolescence. It is likely that a secondary insult, perhaps at the adolescent period, is required to develop this model into one that more closely models the pathological alterations observed in schizophrenia.

4.4.2 Developmental profile of the NMDAR and NR2A and NR2B subunits in the mouse brain

In this study, NMDAR binding showed an overall increase from PN14 to PN70 in both the prefrontal cortex and the hippocampus of the mouse brain, which confirms previous findings in rats (du Bois et al., 2009).

This was the first study to investigate [^{3}H]Ifenprodil binding during development of the rodent brain. There was an increase from PN14 to PN70 in both the prefrontal cortex and the hippocampus. This pattern was unlike that of the NR2B protein in which there was a small decrease observed between PN14 and PN35 in the prefrontal cortex, and no other differences in protein levels between the time points. In support of our protein findings, previous studies have shown that NR2B protein levels decrease or remain constant from peak expression at around PN21 (Sheng et al., 1994, Zhong et al., 1995, Portera-Cailliau et al., 1996, Wenzel et al., 1997). A similar pattern of tapering expression of NR2B protein during the postnatal period has been found in the human brain (Jantzie et al., 2013). An increase in binding throughout development, as observed in the present study, suggests that the availability or affinity of the binding sites in the membrane may change over time

but the amount of total protein remains fairly constant from early adolescence to adulthood.

In contrast to NR2B, NR2A binding showed a gender specific developmental profile. There was no change in NR2A binding during development for the male mice but there was an increase from PN14 to PN35 and then a decrease from PN35 to PN70 in the female mice, showing a peak in expression at adolescence. No gender difference was observed in NR2A protein and there was no difference in NR2A protein between the developmental time points. This disparity between binding and protein, similar to that found with NR2B suggests that the binding sites in the membrane may display different developmental patterns to the total protein amount. The NR2A ligand, [^{3}H]CGP39653, binds to the glutamate binding site on the NR2 subunit with a higher affinity for NR2A but can also bind to NR2B (Christie et al., 2000). Therefore it is possible that the discrepancy between binding and protein may be related to the lack of specificity of the ligand. However, considering the NR2B binding in this study did not show these gender specific developmental changes, this is unlikely. While ours is the first to examine the developmental profile in mice, one previous study in rats has shown an increase in NR2A protein from the early post-natal period to PN21 and then a slight decline or plateau to adult (Wenzel et al., 1997).

4.4.3 Gender specific NMDAR stoichiometry at adolescence

The gender specific developmental profile in NR2A binding results in substantially higher NR2A binding in the prefrontal cortex and hippocampus of female mice, compared to males, at PN35. This effect was specific to NR2A binding, with no gender effects observed for total NMDAR binding or NR2B binding/protein. To our knowledge a gender specific expression of NR2A binding has not been

previously reported. While it is possible that the gender differences observed in NR2A binding are associated with the oestrous cycle and hormone levels in the female mice, it has been reported that NR2A mRNA levels do not fluctuate with the oestrous cycle in adult rodents (Adams et al., 2001). Oestrogen does not appear to affect the protein levels of NMDAR, NR2A or NR2B either (Snyder et al., 2011). The disparity between male and female NR2A binding may underlie gender specific susceptibilities apparent in schizophrenia patients at this time point, although further research into the association between NR2A levels, particularly in the female, and schizophrenia symptom profiles is needed. Furthermore, gender differences in NR2A and other receptor or subunit levels elucidated in future human post mortem tissue studies and animal models of schizophrenia may assist in the development of gender specific treatments for the disorder.

4.4.4 Conclusion

In conclusion, perinatal NR2B antagonism had minimal effects on the NMDAR complex, other than a subtle increase in NR2B protein the hippocampus (irrespective of gender and age) and in the prefrontal cortex specifically at adolescence. Despite this there may indeed be altered functional capacity or activity of these receptors and/or the downstream signalling capacity, which was not measured in this study. While the significance of this subtle change in NR2B protein at adolescence remains to be determined, it provides the first evidence of the effects of perinatal NR2B antagonism on brain neurochemistry. Furthermore we provide the first evidence of gender specific alterations in the development of the glutamatergic system in mice, specifically at adolescence, although our findings are limited as we did not measure oestrogen levels in females or determine stage in oestrous cycle.

Further research is needed to confirm these gender differences in the human brain and whether they occur in schizophrenia pathology.

5 THE EFFECT OF PERINATAL NR2B ANTAGONISM ON NRG1 AND ERBB4 PROTEIN: A MOUSE MODEL

5.1 Introduction

NRG1 is a principal candidate gene implicated in schizophrenia. Although present in over 30 isoforms, the NRG1 protein contains a common EGF domain with which it signals primarily through ErbB4. Alterations in NRG1 and ErbB4 have been noted in the schizophrenia brain, particularly in the areas of the prefrontal cortex and hippocampus (Hashimoto et al., 2004, Law et al., 2006, Law et al., 2007, Chong et al., 2008, Barakat et al., 2010, Marballi et al., 2012).

NRG1 plays a vital role in brain development, including synapse formation and plasticity, glial cell development and myelination and neuronal survival (Mei and Xiong, 2008). The pattern of expression of NRG1 mRNA and protein in the rodent central nervous system during gestational development varies between the different isoforms. Type I mRNA is predominant during early embryogenesis whereas type II and III mRNA are not detected until midgestation but display a broader expression pattern in the brain (Meyer et al., 1997). Similarly NRG1 type I protein is expressed early in embryonic development (Pinkas-Kramarski et al., 1994). It has also been shown that the amount and composition of NRG1 mRNA subtypes in the cerebral cortex differs between embryonic, perinatal and adult rat brain, with levels of NRG1 mRNA increasing from juvenile to adult ages in both the rat and human cerebral cortex (Liu et al., 2001). At adulthood, the regional distribution of NRG1 transcripts and protein is widespread throughout the rodent brain, mirroring that seen in the adult human brain (Law et al., 2004). The mRNA expression does not necessarily translate to protein expression (Greenbaum et al.,

2003) and to date, the developmental expression of NRG1 protein has not been reported in the literature.

Both the NR2 subunits of the NMDAR and the ErbB4 protein are linked to the PSD-95 family of proteins (Figure 1.1) and therefore it is possible that a functional association is present between these proteins. NRG1 signalling has been found to alter the expression and function of the NMDAR, as well as influence NR2B subunit phosphorylation in a brain region specific manner (Hahn et al., 2006, Bjarnadottir et al., 2007). More recently it has been shown in animal models that a blockade of the NMDAR can affect the NRG1 signalling pathway. Chronic MK-801 treatment in adult rats results in an upregulation of NRG1 and ErbB4 proteins in the prefrontal cortex (Feng et al., 2010), while perinatal PCP treatment produces age-specific alterations in NRG1 and ErbB4 protein, that are more prominent in the prefrontal cortex than the hippocampus (du Bois et al., 2012). These models suggest NRG1 and ErbB4 alterations could play a role in the development of schizophrenia pathology either primarily, through NRG1 mutations, or as a consequence of NMDAR hypofunction.

The aims of this study were to 1. Examine NRG1 and ErbB4 protein expression at the juvenile, adolescent and adult time points following perinatal NR2B antagonist treatment, 2. Study the normal developmental profile of NRG1 and ErbB4, 3. Examine the developmental and treatment effects in both male and female mice and 4. Assess the relationship between the NRG1/ErbB4 and NMDAR subunits.

5.2 Materials and Methods

The animal and drug treatment procedure, Western blot protocol and statistics were the same as detailed in Chapter 4. The antibodies used for Western blot were anti-NRG1 (#ab2994, 1/500; Abcam) targeting the EGF domain of the NRG1 protein and anti-ErbB4 (#ab32375, 1/2500; Abcam) targeting the C-terminal of the ErbB4 protein. Pearson's correlations were performed for each age group to determine if there was a relationship between the NRG1 and ErbB4 proteins, as well as with the NR2A and NR2B protein data (from Chapter 4).

5.3 Results

NRG1 was presented as a single 60 kDa band, which has been suggested to be the EGF domain common to all isoforms and therefore represent total NRG1 levels (Fung et al., 2012). ErbB4 protein was also presented as a single 147 kDa band and quantified as previously reported (Wakuda et al., 2015).

5.3.1 Reduction in NRG1 protein in the hippocampus at adolescence after perinatal NR2B antagonism

A three way ANOVA revealed a significant main effect of AGE in the prefrontal cortex ($F_{2,48}$=168.5, $p<0.001$) and hippocampus ($F_{2,47}$=277.2, $p<0.001$) and a significant AGE x TREATMENT interaction in the hippocampus ($F_{2,47}$=4.6, $p=0.014$). There was no significant main effect of GENDER or TREATMENT or any other significant interactions.

A one way ANOVA using the control data with genders combined showed that NRG1 protein expression was significantly different between time points in the hippocampus ($F_{2,26}$=188.9, $p<0.001$) and prefrontal cortex ($F_{2,27}$=88.0, $p<0.001$). Post-hoc tests revealed a substantial increase from PN14 to PN35 in the prefrontal

cortex (+330%, p<0.001) and hippocampus (+326%, p<0.001) and a further increase from PN35 to PN70 in the prefrontal cortex (+34%, p=0.012) but not the hippocampus (p=0.745). Overall there was an increase from PN14 to PN70 in the prefrontal cortex (+479%, p<0.001) and hippocampus (+305%, p<0.001) (see Figure 5.1).

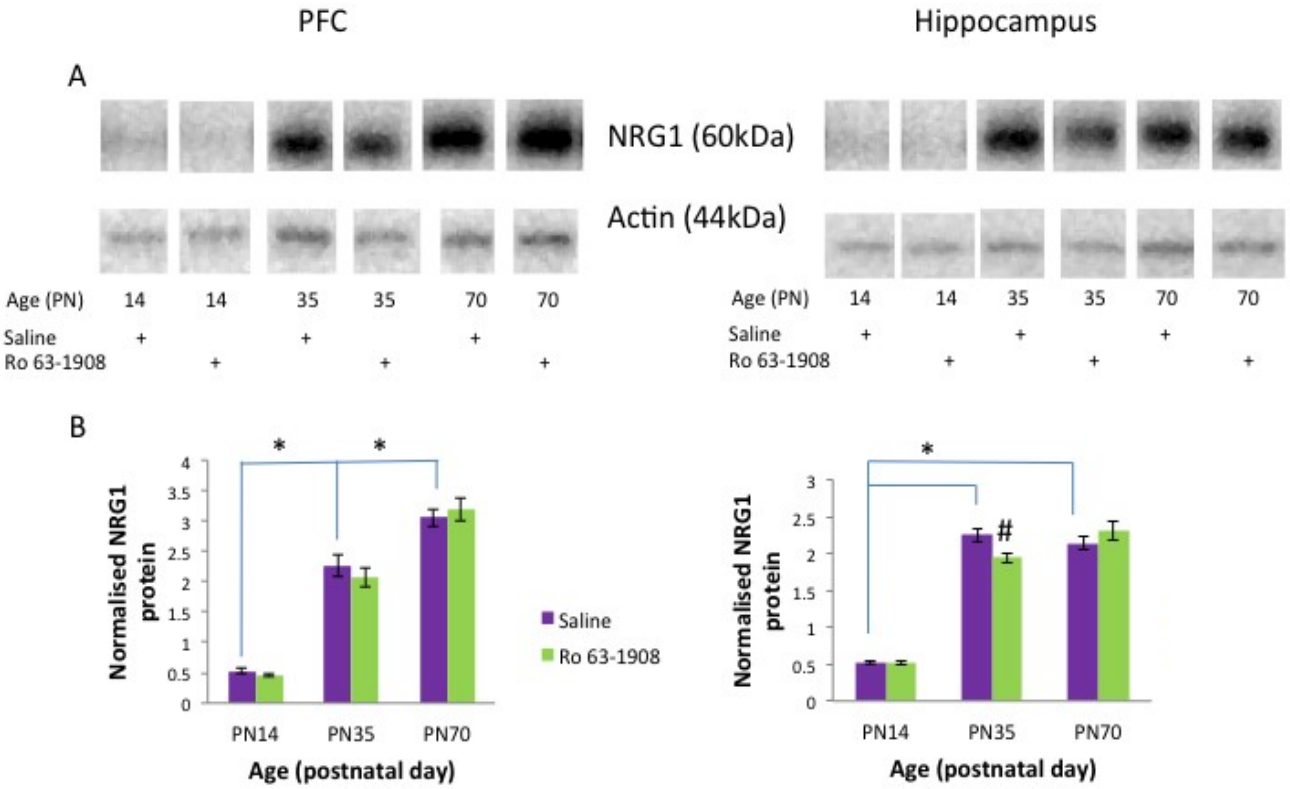

Figure 5.1. A: Representative Western blot bands of NRG1 and actin protein. B: Graphs showing an increase in NRG1 protein throughout development and a decrease in NRG1 protein in mice following perinatal NR2B antagonist (Ro 63-1908) treatment compared to those treated with saline at postnatal day 35 in the hippocampus. Data represents males and females combined, n=10-12/group. Data presented as mean relative density ± SEM. *: 0.000<p<0.05, #: p=0.012, 13% decrease in NRG1 protein levels of Ro 63-1908 treated mice compared to saline at postnatal day 35. Abbreviations: PFC = prefrontal cortex, PN = postnatal day.

An Independent samples t-test revealed a decrease in the Ro-631908 treated group compared to the control at PN35 in the hippocampus (-13%, t_{18}=2.8, p=0.012) but no difference at PN14 (p=0.859) or PN70 (p=0.316) (see Figure 5.1).

5.3.2 No change in ErbB4 protein after perinatal NR2B antagonism

A three way ANOVA revealed a significant GENDER x TREATMENT interaction in the hippocampus ($F_{2,47}$=6.5, p=0.014) but there were no significant differences in post hoc analyses. There were no significant main effects and no other significant interactions in the hippocampus and no significant effects in the prefrontal cortex (see Figure 5.2).

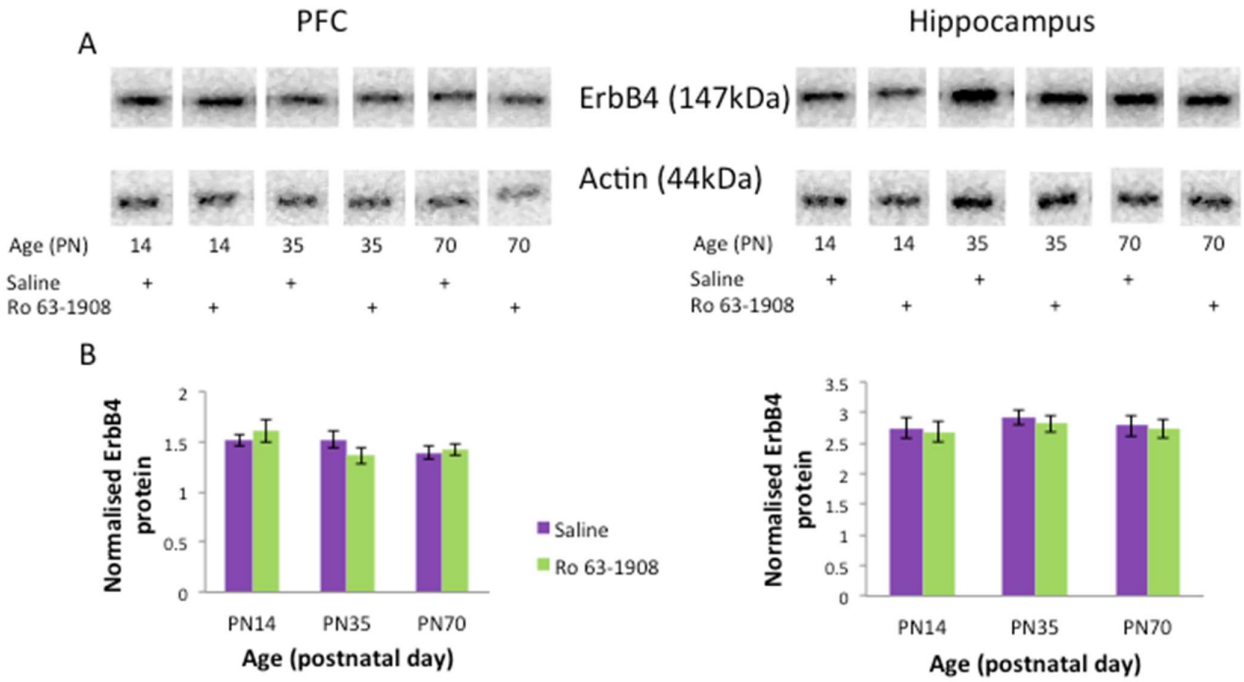

Figure 5.2. A: Representative Western blot bands of ErbB4 and actin protein. B: Graphs showing no difference in ErbB4 protein in the prefrontal cortex and hippocampus of mice at postnatal days 14, 35 and 70 following perinatal treatment with saline or the NR2B antagonist Ro 63-1908. Data represents males and females combined, n=10-12/group. Data presented as mean relative density ± SEM. Abbreviations: PFC = prefrontal cortex, PN = postnatal day.

5.3.3 Relationship between NRG1 signalling system and the NR2A and NR2B subunits

NRG1 and ErbB4 protein showed significant positive correlations in the hippocampus specifically at PN14 (r=0.506, p=0.023, n=20) and PN70 (r=0.476,

p=0.039, n=20), but not at PN35. In contrast NRG1 and ErbB4 proteins were positively correlated in the prefrontal cortex only at PN35 (r=0.605, p=0.005, n=20) (see Table 5.1).

NR2B and NRG1 protein showed significant positive correlations at PN14 (r=0.445, p=0.049, n=20) and PN70 (r=0.445, p=0.049, n=20) in the prefrontal cortex as well as at PN70 in the hippocampus (r=0.466, p=0.044, n=20). NRG1 and NR2A protein was significantly correlated only at PN70 in the hippocampus (r=0.512, p=0.025, n=20) (see Table 5.1). The NR2 subunits did not correlate with ErbB4 at any age.

Table 5.1. Pearson's correlations between NRG1, ErbB4, NR2A and NR2B proteins.

		PN14	PN35	PN70
PFC				
NRG1/ErbB4	r	-0.008	**0.605**	0.011
	p	0.975	**0.005***	0.965
NRG1/NR2A	r	0.362	-0.132	-0.264
	p	0.117	0.579	0.260
NRG1/NR2B	r	**0.445**	0.193	**0.445**
	p	**0.049***	0.415	**0.049***
ErbB4/NR2A	r	**0.431**	-0.213	0.044
	p	**0.058^**	0.367	0.855
ErbB4/NR2B	r	**-0.495**	0.108	-0.291
	p	**0.028***	0.650	0.213
HIP				
NRG1/ErbB4	r	**0.506**	0.381	**0.476**
	p	**0.023***	0.098	**0.039***
NRG1/NR2A	r	-0.375	-0.363	**0.512**
	p	0.103	0.116	**0.025***
NRG1/NR2B	r	0.040	-0.301	**0.466**
	p	0.868	0.197	**0.044***
ErbB4/NR2A	r	-0.182	0.054	**0.449**
	p	0.442	0.822	**0.054^**
ErbB4/NR2B	r	0.096	0.026	0.187
	p	0.688	0.912	0.443

Abbreviations: HIP = hippocampus, PFC = prefrontal cortex, PN = postnatal day. *: $p<0.05$, ^: $0.05<p<0.07$.

5.4 Discussion

The main findings from this study were: 1. There was a selective decrease in NRG1 at adolescence after perinatal NR2B antagonism. 2. NRG1 protein expression significantly increased from the juvenile to the adult ages in the prefrontal cortex and hippocampus. 3. There were age dependent positive correlations between NRG1/ErbB4 and NRG1/NR2B.

5.4.1 Perinatal NR2B antagonism produces alterations in NRG1 protein at adolescence

This study revealed a decrease in hippocampal NRG1 protein expression, specifically at adolescence, following perinatal NR2B antagonism. Alterations of proteins in the brain at adolescence like we see in NR2B and NRG1 in this study could have functional outcomes that affect behaviour, memory and cognition. Studies conducted on NRG1 mutant mice, for example, that have reduced expression of NRG1 show that this results in susceptibility to behavioural abnormalities and further impacts to the brain, particularly at adolescence (Long et al., 2013). Therefore while the alterations observed in the present study are subtle, collectively they may increase the susceptibility of Ro 63-1908 treated animals to secondary impacts. NRG1 and NR2B proteins were positively correlated in the prefrontal cortex and hippocampus at PN70 and in the prefrontal cortex at PN14 but there was no correlation at PN35. Due to the opposing changes seen in NR2B and NRG1 protein at adolescence in our mouse model, it is likely that the perinatal NR2B antagonism disrupts the positive relationship at this age and this could alter other signalling systems and pathways during the adolescent brain growth period, possibly rendering the brain vulnerable to impacts during this time. Behavioural analyses were not

conducted in the present study as the stress of behavioural tests themselves could influence brain measures (Rosenzweig and Bennett, 1996).

One study that examined NRG1 protein following perinatal PCP treatment in rats found no alteration in NRG1 protein in the hippocampus at juvenile, adolescent or adult ages but found a decrease in the prefrontal cortex at the adolescence (du Bois et al., 2012). The discrepancy between our results and those of du Bois et al. may be due to the non-specific nature of PCP versus the specific NR2B antagonist used as the perinatal treatment or the differing antibodies used in the Western blot protocol, which could have identified different isoforms of the NRG1 protein. Du Bois and colleagues (2012) measured the 85 kDa band (Santa Cruz Biotechnology antibody sc-348) antibody of NRG1 that is thought to represent NRG1 type III, while we measured the 60 kDa band of NRG1 that is thought to be the EGF domain that is common to all isoforms of NRG1.

Perinatal NB2B antagonist treatment had no effect on levels of ErbB4 in the prefrontal cortex or hippocampus at any age in our study. This is in contrast to the study by du Bois and colleagues, which showed alterations in ErbB4 protein throughout development in the prefrontal cortex (du Bois et al., 2012). Again this discrepancy could be due to the non-specific nature of PCP or the differing antibodies used for detection (the present study using Abcam antibody ab32375 to detect a single band at 147 kDa and du Bois et al. 2012 using Santa Cruz Biotechnology antibody sc-283 and quantified the 185 kDa band).

Our study found no alteration in either NRG1 or ErbB4 protein at PN70. In the schizophrenia brain, studies using adult post mortem tissue have found various changes depending on the fraction of NRG1 and ErbB4 examined. In the prefrontal cortex, there has been no change in NRG1(1α) protein (Boer et al., 2009), an

increase in the 53 kDa cytoplasmic fragment of NRG1 protein (Chong et al., 2008), a decrease in the C-terminal fragment of NRG1 protein (Barakat et al., 2010), a decrease in the 50 kDa NRG1 protein fragment and an increase in the N-terminal fragment of NRG1 protein (Marballi et al., 2012) in schizophrenia. For ErbB4 protein, an increase in the 180 kDa cytoplasmic fraction has been found in the prefrontal cortex (Chong et al., 2008). In the hippocampus, NRG1 protein is decreased in the left hemisphere (Stuart et al., 2011) and the 50 kDa fragment of NRG1 protein is decreased (Marballi et al., 2012) in schizophrenia subjects compared to controls. Considering these mixed results, it is hard to determine where the results of the present study lie in terms of matching the occurrence in the schizophrenia brain. Miraballi and colleagues suggest that an alteration in NRG1 cleavage/cleavage enzymes rather than NRG1 protein levels may be an underlying factor in the schizophrenia brain (Miraballi et al., 2012). More research is required in both human and animal models to come to any conclusions.

5.4.2 Increasing NRG1 protein in the mouse brain throughout development

This is the first study in any species to our knowledge to examine NRG1 protein levels during brain development. We found a dramatic increase from PN14 to PN35 in the prefrontal cortex and hippocampus and then a further increase to PN70 in the prefrontal cortex. NRG1 mRNA has been examined throughout development with a decrease in the total amount from perinatal to adulthood in the monkey and human (Liu et al., 2011). NRG1 protein plays a vital role in brain development and so it is interesting that protein levels increase throughout development, however it also has an importance in the mature brain and can modulate synaptic transmission, dendrite growth, receptor expression and many other aspects of neuronal function (Harrison and Law, 2006). Our study used an antibody that recognises the EGF

domain of NRG1 protein that is present in each isoform, this suggests that total NRG1 protein levels increase with age however it is possible that different isoforms have different developmental profiles. Isoforms of NRG1 mRNA have different developmental profiles and are regulated by neuronal activity (Liu et al., 2011). Therefore it is likely that particular NRG1 isoforms and isoform ratios are more important during early development and not the total levels of NRG1 protein.

In terms of ErbB4 protein, there was no change in protein levels throughout development or with gender and this is consistent with that shown previously in the monkey prefrontal cortex (Thompson et al., 2007). Considering that ErbB4 is one of the primary receptors for NRG1, it is surprising that it did not reflect the developmental profile of NRG1. It may be that the different isoforms of ErbB4 show distinct developmental expressions, that we were unable to detect with our use of a non-specific ErbB4 antibody. In addition, the full-length form of ErbB4 may remain unaltered over time but the ErbB4 fragments for signalling or the ErbB4 signalling efficiency may change. We must also consider that NRG1 does signal through other ErbB receptors and that ErbB4 can also interact with NRG2 and NRG3 (Hobbs et al., 2002). While we provide novel evidence of the developmental expression of these proteins, clearly there is a need for more extensive investigations of the developmental profile of this system.

5.4.3 Conclusion

In conclusion, perinatal NR2B antagonism produced a selective alteration in NRG1 levels at the adolescent time point and this appeared to alter the dynamics of the relationship with the NR2B subunit protein. In addition, we have for the first time found evidence of a dramatic increase of NRG1 protein levels throughout

development which provides a strong basis to further investigate the developmental expression and function of NRG1 signalling and its role of NRG1 schizophrenia.

6 THE EFFECTS OF PERINATAL NR2B ANTAGONISM ON THE GABAERGIC AND CANNABINERGIC SYSTEMS: A MOUSE MODEL

6.1 Introduction

The cannabinoid and GABAergic systems are key regulators of inhibition within the brain. The $GABA_AR$ functions to inhibit excitatory output while the CB_1R is highly abundant on GABAergic and glutamatergic neurons where it plays a role in the inhibition of GABA and glutamate release as well as the modulation of other neurotransmitter systems.

Both the cannabinoid and GABAergic systems have been implicated in schizophrenia. A deficit of GABAergic signalling has been implicated in schizophrenia as reductions in GABAergic interneurons and increased levels of $GABA_ARs$ have been observed in postmortem human schizophrenia brain compared to controls (Benes et al., 1992; Benes et al., 1996a; Benes et al., 1996b; Dean et al., 1999; Deng and Huang, 2006; Newell et al., 2007b, Verdurand et al., 2013). Increased levels of CB_1R binding in the prefrontal and cingulate cortices have also been found in schizophrenia brain tissue (Dalton et al., 2011; Giuffrida et al., 2004; Newell et al., 2006; Zavitsanou et al., 2004), however a decrease in CB_1Rs has been found in two areas of the prefrontal cortex (Eggan et al., 2008; Eggan et al., 2010b).

As a model of schizophrenia, perinatal NMDAR antagonism has been shown to increase $GABA_ARs$, particularly at adolescence, in various regions of the female rat brain (du Bois et al., 2009). As well as this, a loss of GABAergic interneurons has been observed following perinatal NMDAR blockade (Nakatani-Pawlak et al., 2009), which is representative of the GABAergic dysfunction seen in schizophrenia pathology. There are no published studies to date that have examined CB_1R density following perinatal NMDAR antagonist treatment however an alteration in CB_1R

mRNA has been observed (Mailleux and Vanderhaeghen, 1992). The CB_1R has been found to co-localise with the NMDAR in the postsynaptic membrane and this association along with excessive cannabinoid activity can destabilise NMDARs in the membrane and disrupt their function (for review see Sanchez-Blazquez et al., 2013). This restriction of NMDAR function by cannabinoid activity allows the reversal of NMDAR excitotoxicity but could also cause NMDAR hypofunction and contribute to schizophrenia pathology. Furthermore both $GABA_AR$ subunit and NR1 and NR2B mRNA have been found altered in the hippocampus and striatum of CB_1 mutant mice (Warnault et al, 2007). Whether or not the reverse is true and CB_1R levels are affected by NMDAR or NR2B antagonism has not been studied.

The NR2B subunit of the NMDAR has been found on GABAergic interneurons and plays an important role in NMDAR mediated signalling and synaptic plasticity at these synapses (Szinyei et al., 2003) and NR2A and NR2B are thought to differentially regulate GABA release (Fantin et al, 2007). So while it is known that non-specific NMDAR antagonism can affect GABAergic interneuron and $GABA_AR$ levels it is not known if specific NR2B antagonism can affect $GABA_ARs$ and if so are the alterations similar to that seen in schizophrenia.

To further our understanding of the interactions between the glutamatergic, GABAergic and cannabinoid signalling systems, particularly with reference to NMDAR hypofunction, the aims of this study were to 1. Examine the effects of perinatal NR2B antagonism on $GABA_AR$ and CB_1R density at the juvenile, adolescent and adult time points, 2. Investigate the normal developmental profile of $GABA_A$ and CB_1Rs in the mouse brain and 3. Examine the developmental and treatment effects in both male and female mice.

6.2 Materials and Methods

The animal and drug treatment procedure is the same as that from Chapter 4.

6.2.1 Autoradiography Procedures and Quantification

6.2.2 $GABA_AR$ Binding

Sections for $GABA_AR$ binding were collected at levels corresponding to Bregma 2.34 and -1.94 mm (Paxinos and Franklin, 2001) including the structures of the prefrontal cortex, hippocampus and thalamus (showed a high density of $GABA_ARs$). [^{3}H]Muscimol binding was performed based on the methods previously used by du Bois et al (2009). All sections were preincubated in 50 mM TrisCitrate (pH 7) for 3 x 5 minutes at 4°C. The sections were then incubated in the same buffer with the addition of 12 nM [^{3}H]Muscimol (selective $GABA_AR$ agonist: specific activity 25.5 Ci/mmol; PerkinElmer) for 45 minutes at 4°C to determine total binding, with adjacent sections incubated in the presence of 100 µM GABA (Sigma-Aldrich) to determine nonspecific binding. All sections were then washed in 50 mM TrisCitrate (pH 7) for 4 x 2 seconds at 4°C, rinsed in distilled water and air dried overnight.

6.2.3 CB_1R Binding

Sections for CB_1R binding were collected at levels corresponding to Bregma 2.34, 1.32 and -1.94 mm (Paxinos and Franklin, 2001) including the structures of the prefrontal cortex, striatum, hippocampus and globus pallidus (showed a high density of CB_1Rs). [^{3}H]CP55940 binding was performed based on the methods previously used by Newell et al (2006). All sections were preincubated in 50 mM TrisHCl (pH 7.4) with 5% bovine serum albumin (BSA) for 30 minutes at room temperature. The sections were then incubated in the same buffer with the addition of 10 nM

[^{3}H]CP55940 (specific cannabinoid receptor agonist: specific activity 174.6 Ci/mmol, PerkinElmer, Waltham, MA, USA) for 2 hours at room temperature to determine total binding, with adjacent sections incubated in the presence of 10μM CP55940 (Sigma Aldrich, Castle Hill, NSW, Australia) to determine nonspecific binding. All sections were then washed, firstly in 50mM TrisHCl (pH 7.4) containing 1% BSA for 1 hour at 4°C, then in 50mM TrisHCl (pH 7.4) for 2 hours at 4°C and lastly in 50 mM TrisHCl (pH 7.4) for 5 minutes at 4°C. Finally the sections were rinsed in distilled water and air-dried overnight.

6.2.4 Quantification

All slides were then placed in cassettes and exposed to tritium-sensitive Kodak BioMax MR Film (Sigma-Aldrich) for 6-12 weeks. The films were developed using the AGFA CP1000 film developer (Agfa-Gevaert N.V.) and analysed using Quantity One software version 4.6.7 (BioRad) connected to a GS-800 Calibrated Densitometer (BioRad). Quantification was performed by measuring the average optical density in each brain region in three adjacent sections and then averaging across six mice per group.

6.2.5 Statistics

A Pearson's correlation was conducted to determine if there was any correlation between the binding levels of $GABA_AR$, CB_1R, NMDAR, NR2A subunits and NR2B subunits in the prefrontal cortex or hippocampus. All data is presented as mean±SEM.

6.3 Results

6.3.1 No effect of perinatal NR2B antagonism on $GABA_AR$ Binding

$GABA_AR$ binding density was observed in the prefrontal cortex, hippocampus and thalamus regions (see Figure 6.1).

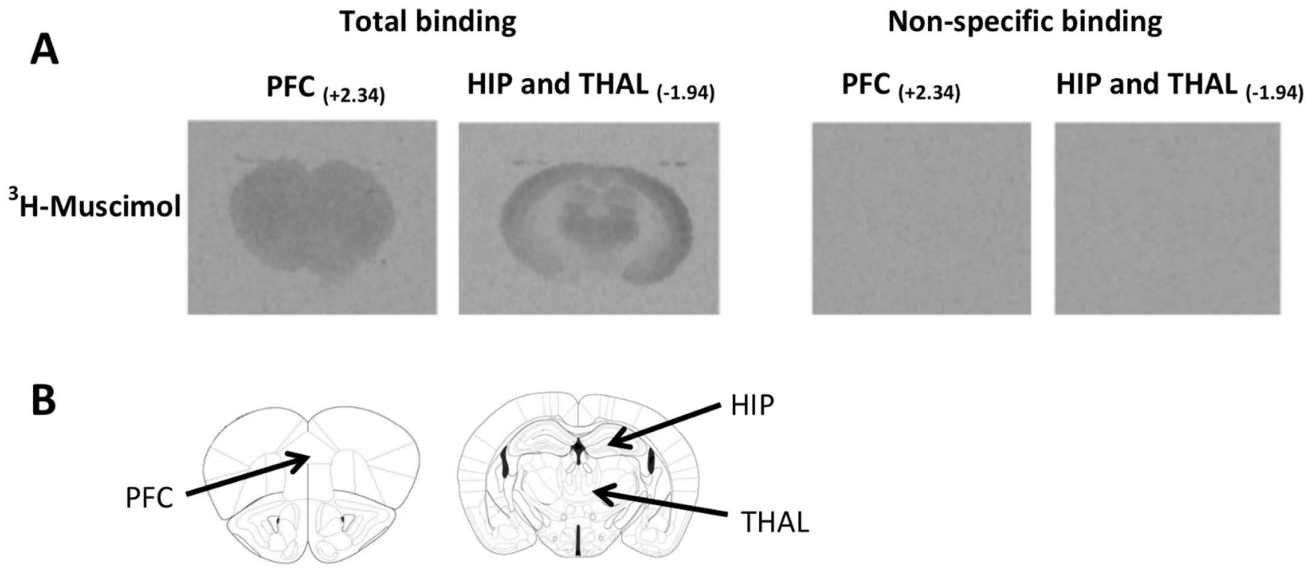

Figure 6.1. A: Representative autoradiograms of total and non-specific $GABA_AR$ (^{3}H-Muscimol) binding in the prefrontal cortex (Bregma +2.34), thalamus and hippocampus (Bregma -1.94) of adult mice. B: Mouse brain atlas pictures showing bregma levels and locations of prefrontal cortex (+2.34), thalamus and hippocampus (-1.94). Abbreviations: HIP = hippocampus, PFC = prefrontal cortex, THAL = thalamus.

A three way ANOVA revealed a significant main effect of AGE in the prefrontal cortex ($F_{2,55}$=15.6, $p<0.001$), hippocampus ($F_{2,54}$=22.1, $p<0.001$) and thalamus ($F_{2,55}$=11.9, $p<0.001$) and of GENDER in the prefrontal cortex ($F_{1,55}$=38.7, $p<0.001$), hippocampus ($F_{1,54}$=29.9, $p<0.001$) and thalamus ($F_{2,55}$=18.2, $p<0.001$) on [^{3}H]Muscimol binding. There was an AGE x GENDER interaction in the hippocampus ($F_{2,54}$=6.1, $p=0.004$) and thalamus ($F_{2,55}$=4.6, $p=0.014$) and a trend for

one in the prefrontal cortex ($F_{2,55}$=3.0, p=0.059). There was no significant effect of TREATMENT in any brain region or any other interactions observed.

Using the control data, [^{3}H]Muscimol binding expression was significantly different between time points in the prefrontal cortex (male: $F_{2,15}$=8.7, p=0.003; female: $F_{2,15}$=19.2, p<0.001), hippocampus (male: $F_{2,15}$=15.2, p<0.001; female: $F_{2,15}$=3.8, p=0.050) of both genders and in the thalamus of the male mice ($F_{2,15}$=4.2, p=0.036) but not the female mice ($F_{2,15}$=3.1, p=0.081). Post hoc tests revealed a significant decrease in binding from PN14 to PN35 in the prefrontal cortex (-45%, p=0.003) and hippocampus (-64%, p<0.001) of male mice, but no change in the thalamus between these two ages (p=0.258). This was followed by an increase from PN35 to PN70 in all the brain regions (prefrontal cortex: +53%, p=0.042; hippocampus: +119%, p=0.012; thalamus: +130%, p=0.029), resulting in no overall difference between PN14 and PN70 (prefrontal cortex: p=0.360; hippocampus: p=0.114; thalamus: p=0.452). In the female mice there was a decrease from PN14 to PN35 in the prefrontal cortex (-26%, p=0.001), similar to the male mice, but no change in the hippocampus (p=0.195) or thalamus (p=0.098). There was no change from PN35 to PN70 in any brain region in the female mice (prefrontal cortex: p=0.539; hippocampus: p=0.713; thalamus: p=0.947), resulting in an overall decrease from PN14 to PN70 in the prefrontal cortex (-33%, p<0.001) and hippocampus (-27%, p=0.047) and no change in the thalamus (p=0.169).

Independent samples t-tests with Bonferroni adjustment showed that in the saline animals there was a significant increase in [^{3}H]Muscimol binding in female compared to male mice at PN14 in the prefrontal cortex (females: +35%, t_{10}=-5.6, p<0.001) but not the hippocampus (p=0.061) and thalamus (p=0.107) and at PN35 in the prefrontal cortex (females: +81%, t_9=-4.2, p=0.002), hippocampus (females:

+181%, t_9=-5.4, p<0.001) and thalamus (females: +190%, t_9=-5.2, p=0.001) (see Figure 6.2). There was no significant difference between the male and female mice at PN70 in the prefrontal cortex (p=0.613), hippocampus (p=0.185) or thalamus (p=0.169). In the Ro 63-1908 treated animals there was an increase in [^{3}H]Muscimol binding in the female compared to the male mice at PN14 in the thalamus (+40%, t_{11}=-3.0, p=0.012) but not the prefrontal cortex (p=0.035) or hippocampus (p=0.039) and at PN35 in the prefrontal cortex (females: +54%, t_9=-4.4, p=0.002) and hippocampus (females: +107%, t_9=-3.3, p=0.009) but not the thalamus (p=0.051). There were no differences in gender at PN70 (prefrontal cortex: p=0.578; hippocampus: p=0.997; thalamus: p=0.924) (see Figure 6.2).

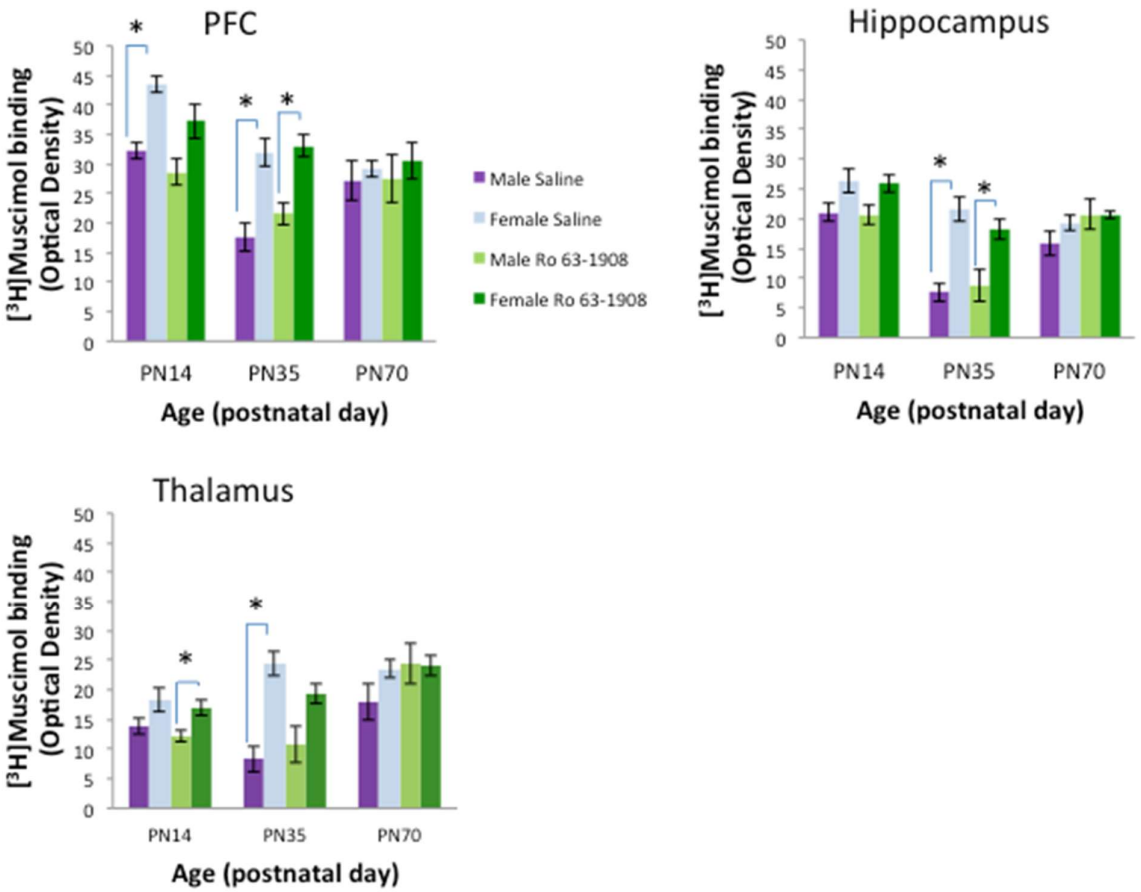

Figure 6.2. [^{3}H]Muscimol binding to the $GABA_A$ receptor in the prefrontal cortex, hippocampus and thalamus of male and female mice following perinatal treatment with saline or the NR2B antagonist Ro 63-1908 at postnatal days 14, 35 and 70. n=5-6/group. Data presented as mean relative density ± SEM. *: $0.000<p<0.0167$. Abbreviations: PFC = prefrontal cortex, PN = postnatal day. Columns at each age group correspond to the descending order of the groups in the graph key.

6.3.2 No effect of perinatal NR2B antagonism on CB_1R Binding

CB_1R binding was examined in the prefrontal cortex, striatum, hippocampus and globus pallidus (see Figure 6.3).

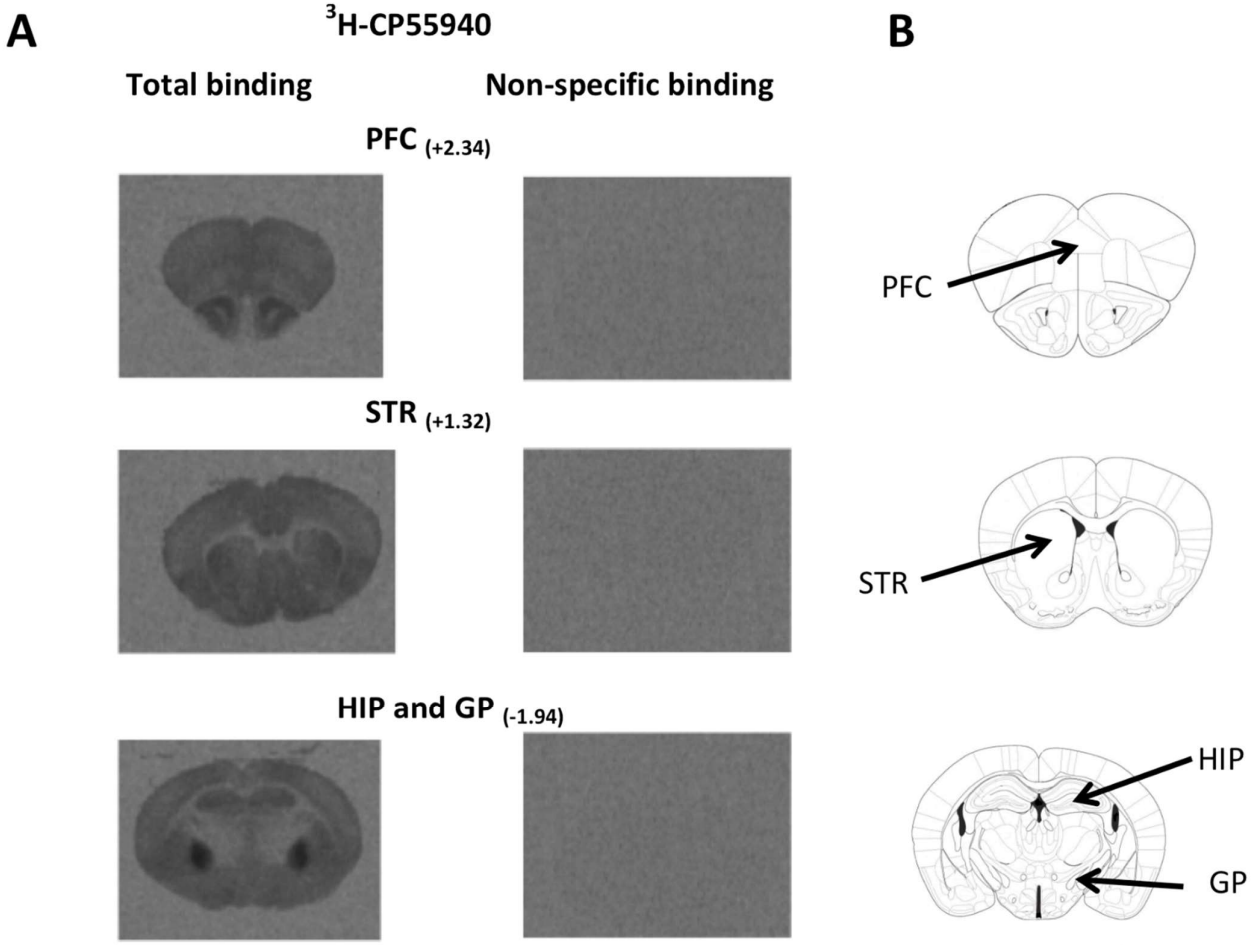

Figure 6.3. A: Representative autoradiograms of total and non-specific CB_1 receptor (^{3}H-CP55940) binding in the prefrontal cortex (Bregma +2.34), striatum (Bregma +1.32), hippocampus and globus pallidus (Bregma -1.94) of adult mice. B: Mouse brain atlas pictures showing bregma levels and locations of prefrontal cortex (+2.34), striatum (+1.32), hippocampus and globus pallidus (-1.94). Abbreviations: GP = globus pallidus, HIP = hippocampus, PFC = prefrontal cortex, STR = striatum.

A three way ANOVA revealed a significant main effect of AGE in the prefrontal cortex ($F_{2,54}$=198.2, P<0.001), hippocampus ($F_{2,55}$=132.6, P<0.001), striatum ($F_{2,54}$=281.3, P<0.001) and globus pallidus ($F_{2,55}$=112.7, P<0.001) and a significant interaction of AGE x GENDER in the prefrontal cortex ($F_{2,54}$=19.7, p<0.001), hippocampus ($F_{2,55}$=8.2, p=0.001) and striatum ($F_{2,54}$=43.9, p<0.001) on [^{3}H]CP55940 binding. There was a significant main effect of GENDER in the prefrontal cortex ($F_{1,54}$=6.3, p=0.015) but not in the hippocampus, striatum or globus pallidus. There was no significant main effect of TREATMENT in any brain region or any other interactions observed.

A one way ANOVA using the control data showed that [^{3}H]CP55940 binding was significantly different between time points in the prefrontal cortex (male: $F_{2,15}$=100.4, p<0.001; female: $F_{2,13}$=90.4, p<0.001), hippocampus (male: $F_{2,15}$=36.1, p<0.001; female: $F_{2,13}$=23.2, p<0.001), striatum (male: $F_{2,15}$=86.6, p<0.001; female: $F_{2,12}$=177.6, p<0.001) and globus pallidus (male: $F_{2,15}$=58.4, p<0.001; female: $F_{2,12}$=68.2, p<0.001). A post hoc test revealed a large increase in binding from PN14 to PN35 in the male mice in all brain regions (prefrontal cortex: +174%, p<0.001; hippocampus: +109%, p<0.001; striatum: +157%, p<0.001; globus pallidus: +133%, p<0.001) and then a decrease from PN35 to PN70 in the prefrontal cortex (-32%, p<0.001) and striatum (-21%, p=0.001) but not the hippocampus (p=0.278) or globus pallidus (p=0.894). Overall there was an increase from PN14 to PN70 in all brain regions (prefrontal cortex: +86%, p<0.001; hippocampus: +87%, p<0.001; striatum: +103%, p<0.001; globus pallidus: +126%, p<0.001).

Similarly, in the female mice there was an increase from PN14 to PN35 in all the brain regions (prefrontal cortex: +134%, p<0.001; hippocampus: +56%, p=0.001; striatum: +74%, p<0.001; globus pallidus: +63%, p<0.001), although these increases

were of a smaller magnitude than the male mice. In contrast to the male mice there was a further increase from PN35 to PN70 in the prefrontal cortex (+19%, $p=0.025$) and striatum (+29%, $p<0.001$) but not the hippocampus ($p=0.184$) or globus pallidus ($p=0.342$) resulting in an overall increase from PN14 to PN70 in all brain regions (prefrontal cortex: +178%, $p<0.001$; hippocampus: +79%, $p<0.001$; striatum: +125%, $p<0.001$; globus pallidus: +78%, $p<0.001$).

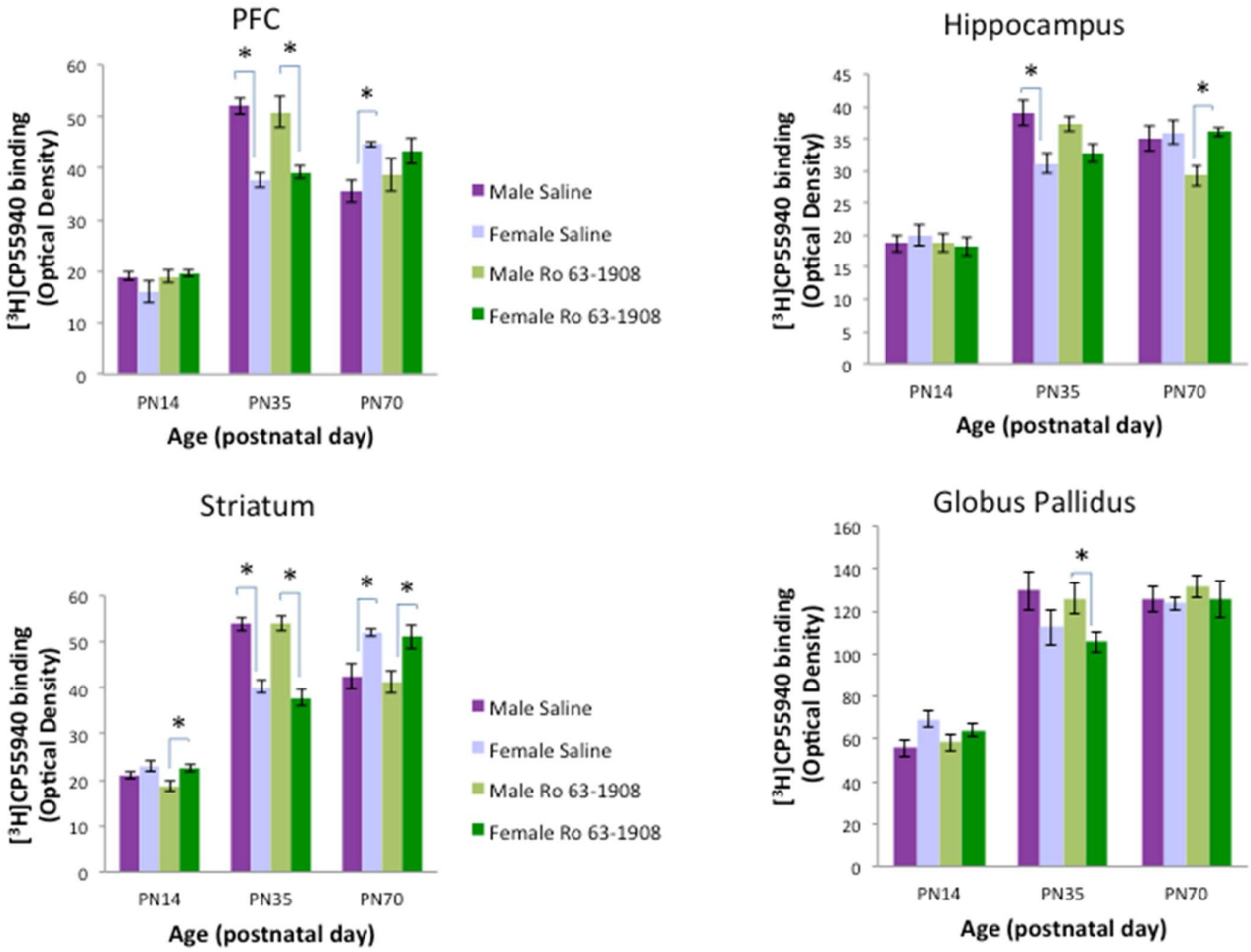

Figure 6.4. [^{3}H]CP55940 binding to the CB_1 receptor in the prefrontal cortex, hippocampus, striatum and globus pallidus of male and female mice following perinatal treatment with saline or the NR2B antaonist Ro 63-1908 at postnatal days 14, 35 and 70. n=5-6/group. Data presented as mean relative density ± SEM. *: 0.000<p<0.0167. Abbreviations: PFC = prefrontal cortex, PN = postnatal day. Columns at each age group correspond to the descending order of the groups in the graph key.

Independent samples t tests with Bonferroni adjustment showed that in the saline animals there was no significant difference in [^{3}H]CP55940 binding in the female compared to the male mice at PN14 the prefrontal cortex (p=0.236), hippocampus (p=0.549), striatum (p=0.126) or globus pallidus (p=0.032). In contrast, at PN35 there was a significant increase in binding in the male compared to the female mice in the prefrontal cortex (males: +38%, t_9=6.7, p<0.001), hippocampus (males: +25%, t_9=2.9, p=0.016) and striatum (males: +34%, t_9=6.5, p<0.001) but no

difference in the globus pallidus (p=0.208). There was also a difference between the genders at PN70 in the prefrontal cortex (females: +26%, t_9=-3.7, p=0.005) and striatum (females: +22%, t_9=-3.2, p=0.011) but not in the hippocampus (p=0.759) or globus pallidus (p=0.763) (see Figure 6.4). In the Ro 63-1908 treated animals there was an increase in [^{3}H]CP55940 binding in the female compared to the male mice at PN14 in the striatum (females: +20%, t_{11}=-2.8, p=0.017) but not the prefrontal cortex (p=0.626), hippocampus (p=0.837) or globus pallidus (p=0.270). At PN35 there was an increase in binding in the male compared to the female mice in the prefrontal cortex (+30%, $t_{6.4}$=3.5, p=0.012), striatum (+43%, t_9=6.8, p<0.001) and globus pallidus (+19%, t_9=2.3, p=0.048) but not the hippocampus (p=0.031) and at PN70 there was an increase in binding in the female compared to male mice in the hippocampus (females: +24%, t_7=-3.5, p=0.010) and striatum (females (trend): +24%, t_7=-2.9, p=0.024) but not the prefrontal cortex (p=0.294) or globus pallidus (p=0.547) (see Figure 6.4).

6.3.3 Correlations between the GABAergic, cannabinoid and glutamatergic systems

Overall there was a significant negative correlation between CB_1R and $GABA_AR$ binding in the prefrontal cortex (r=-0.544, p<0.001) and hippocampus (r=-0.535, p<0.001). The correlation remained significant in the male (prefrontal cortex: r=-0.560, p<0.001; hippocampus: r=-0.638, p<0.001) and female (prefrontal cortex: r=-0.680, p<0.001; hippocampus: r=-0.599, p<0.001) groups when analysed separately (see Table 6.1). Correlations were also run by gender due to the amount of gender differences found in $GABA_AR$ and CB_1R binding.

Table 6.1. Pearson's correlations between NMDAR, NR2A, NR2B, $GABA_AR$ and CB_1R binding in male and female mice.

		Male		Female	
PFC					
$GABA_AR$		r	p	r	p
	CB_1R	**-0.560**	**0.000***	**-0.680**	**0.000***
	NMDAR	0.196	0.251	**-0.468**	**0.009***
	NR2A	0.007	0.969	-0.104	0.557
	NR2B	-0.152	0.376	**-0.373**	**0.042***
CB_1R					
	NMDAR	**0.441**	**0.007***	**0.672**	**0.000***
	NR2A	0.278	0.106	**0.360**	**0.046***
	NR2B	**0.563**	**0.000***	**0.712**	**0.000***
HIP					
$GABA_AR$		r	p	r	p
	CB_1R	**-0.638**	**0.000***	**-0.599**	**0.000***
	NMDAR	-0.216	0.227	**-0.447**	**0.013***
	NR2A	-0.046	0.796	-0.331	0.069
	NR2B	-0.312	0.068	**-0.361**	**0.046***
CB_1R					
	NMDAR	**0.716**	**0.000***	**0.664**	**0.000***
	NR2A	0.326	0.056^	0.257	0.164
	NR2B	**0.580**	**0.000***	**0.647**	**0.000***

Abbreviations: CB_1R = cannabinoid receptor 1, $GABA_AR$ = $GABA_A$ receptor, HIP = hippocampus, NMDAR = N-methyl-D-aspartate receptor, NR2A/B = NMDAR subunits A and B, PFC = prefrontal cortex, PN = postnatal day.
* = $p<0.05$, ^ = $0.05<p<0.06$.

NR2A binding showed a significant positive correlation with $GABA_AR$ binding in the prefrontal cortex (r=0.656, p=0.001) and the hippocampus (r=0.604, p=0.004) at PN35. This positive correlation was also seen in the hippocampus at PN70 (r=0.445, p=0.049) and in the prefrontal cortex at PN14 (r=0.700, p<0.001) (see Table 6.2). NR2A binding was also negatively correlated to CB_1R binding at PN35 (prefrontal cortex: r=-0.703, p=0.000; hippocampus: r=-0.433, p=0.044).

Table 6.2. Pearson's correlations between NMDAR, NR2A, NR2B, $GABA_AR$ and CB_1R binding at postnatal days 14, 35 and 70.

		PN14		PN35		PN70	
PFC							
$GABA_AR$		r	p	r	p	r	p
	CB_1R	-0.320	0.119	**-0.664**	**0.001***	0.108	0.651
	NMDAR	-0.048	0.821	0.245	0.285	**0.678**	**0.001***
	NR2A	**0.700**	**0.000***	**0.656**	**0.001***	0.387	0.092
	NR2B	0.254	0.231	0.254	0.255	-0.053	0.825
CB_1R							
	NMDAR	-0.103	0.624	-0.025	0.915	0.067	0.780
	NR2A	-0.330	0.116	**-0.703**	**0.000***	-0.283	0.226
	NR2B	-0.126	0.556	-0.125	0.579	0.284	0.226
HIP							
$GABA_AR$		r	p	r	p	r	p
	CB_1R	0.074	0.725	-0.376	0.093	**-0.570**	**0.009***
	NMDAR	0.346	0.090	0.082	0.740	**0.487**	**.034***
	NR2A	0.295	0.161	**0.604**	**0.004***	**0.445**	**0.049***
	NR2B	0.075	0.720	0.190	0.409	0.244	0.300
CB_1R							
	NMDAR	-0.022	0.916	0.167	0.482	-0.087	0.722
	NR2A	0.069	0.749	**-0.433**	**0.044***	-0.257	0.273
	NR2B	0.128	0.543	0.107	0.636	-0.035	0.882

Abbreviations: CB_1R = cannabinoid receptor 1, $GABA_AR$ = GABAA receptor, HIP = hippocampus, NMDAR = N-methyl-D-aspartate receptor, NR2A/B = NMDAR subunits A and B, PFC = prefrontal cortex, PN = postnatal day.
* = $p<0.05$, ^ = $0.05<p<0.06$.

6.4 Discussion

The main findings from this study were: 1. NR2B antagonist treatment during the perinatal period did not alter the developmental profile of $GABA_AR$ or CB_1Rs in either gender. 2. $GABA_AR$ binding shows significant gender differences in receptor development, which are positively correlated to NR2A binding results, particularly at adolescence. 3. CB_1R binding density was also significantly different between genders, particularly at adolescence. 4. There was a negative correlation between CB_1 and $GABA_AR$ binding density in the prefrontal cortex and hippocampus.

6.4.1 Perinatal NR2B antagonist treatment does not alter the developmental profile of CB_1 and $GABA_A$ receptors

This study found that perinatal NR2B antagonist treatment did not affect CB_1R or $GABA_AR$ binding density in any brain region, age or gender. While this may appear that this treatment does not alter the developmental profile of CB_1 or $GABA_AR$ density, we cannot rule out that there may be alterations in receptor function, localization, or regulation, that have not been measured in this study. It is possible that receptor binding can be unaltered, but there can be deficits in functional activation of those receptors. Therefore, further studies would be required to determine whether this is the case for the present study.

This is the first study to examine perinatal NR2B antagonist treatment on $GABA_ARs$. The present study found no effect of NR2B antagonist treatment on $GABA_ARs$ while an increase in $GABA_ARs$ at adolescence following perinatal PCP treatment has been reported previously (du Bois et al., 2009). The non-specific nature of PCP blocking all NMDARs may account for the discrepancy between results.

While this is the first study to report on CB_1Rs following any form of perinatal NMDAR insult, our finding of no change in the brain regions examined is consistent with studies using adult NMDAR antagonist treatment. Phencyclidine (PCP) treatment has been reported to cause no change in CB_1R binding density in the adult rat brain 72 hours following treatment (Vigano et al., 2009). Similarly, there is no change in CB_1R density five or 10 days following PCP treatment (Seillier et al., 2010). These studies did however find changes in CB_1R stimulation following PCP treatment and a further study found changes in CB_1 mRNA following MK801 treatment (Mailleux and Vanderhaeghen, 1994). Therefore considering these

functional changes were associated with a lack of binding density change, we cannot rule out the possibility of CB_1R functional changes in our animal model.

6.4.2 Developmental profile of the $GABA_AR$ binding in the mouse brain

This study showed that $GABA_AR$ binding changes significantly during mouse brain development, with different developmental profiles between male and female mice. We found male $GABA_AR$ expression decreased from PN14 to PN35 and then increased to PN70, and female $GABA_AR$ expression decreased from PN14 to PN70 in both the prefrontal cortex and hippocampus. We also examined $GABA_AR$ binding in the thalamus, which increased from juvenile to adult levels, in contrast to that occurring in the prefrontal cortex and hippocampus. Similar to the present study, another study found a decrease in $GABA_ARs$ throughout development in the hippocampus in female rats (du Bois et al., 2009). Although, in contrast, an increase in $GABA_AR$ binding was found in the prefrontal cortex and a decrease was found in the thalamus (du Bois et al., 2009) however this was in a different species.

6.4.3 Gender differences in $GABA_AR$ binding

An increase in female compared to male $GABA_AR$ expression in the saline animals at PN14 (prefrontal cortex) and PN35 (prefrontal cortex, hippocampus and thalamus) was revealed in this study, with no gender differences observed in the adult mouse brain. Other studies which have examined gender differences in the adult mouse brain have similarly found no difference in $GABA_AR$ density between genders in the hippocampus and thalamus (Canonaco et al., 1996; Skilbeck et al., 2008) but have found an increase in $GABA_ARs$ in cortical regions in female compared to male mice (Skilbeck et al., 2008) (see Table 6.3). Studies in the developing (PN1 to 20) rat brain have found no differences in $GABA_AR$ expression

between genders in the medial preoptic area, ventrolateral thalamus, cingulate cortex, ventromedial nucleus of the hypothalamus, CA1 and CA2 regions of the hippocampus and amygdala (Davis and McCarthy 2000). From the present study and others, it appears important for future studies to examine both genders in studies focused on $GABA_AR$ expression and function as well as treatments that may affect the GABAergic system. It will also be important to consider the possible effects of oestrous cycle on these measures, especially considering the finding that some $GABA_AR$ subunits fluctuate with the oestrous cycle (Lovick et al., 2005, Maguire et al., 2005).

Table 6.3. Gender differences in CB_1 and $GABA_A$ receptors in the rodent brain.

Reference	Species	Method	Brain Region	Age	Differences
CB1					
Rodriguez de Fonseca et al., 1993	Wistar rat	Membrane binding	Limbic forebrain	PN2	↑ female 61%
				PN5	↑ male 42%
				PN10,15,20,30,40,70	No difference
			Striatum	PN10	↑ male 45%
				PN20	↑ female 36%
				PN15,30,40,70	No difference
			Mesencephalon	PN10,15,40,70	↑male 25,45,31,23%
				PN20,30	No difference
Mateos et al., 2011	Wistar rat	Autoradiography - [^{3}H]CP55940	Hippocampus (CA1,2,3)	PN81	↑ male 11%
			Dentate gyrus, Cingulate cortex, Amygdala		No difference
Castelli et al., 2014	Lister Hooded rat	Autoradiography – [^{3}H]CP55940	Prefrontal cortex		↑ male 28-30%
			Amygdala		↑ male 27%
			Nucleus accumbens, Striatum, Ventral tegmental area, Hippocampus		No difference
Riebe et al., 2010	Sprague-Dawley rat	Membrane binding	Hypothalamus	Adult (300-400g)	↑ male 54%
			Amygdala		↑ female 31%
			Hippocampus		No difference
Rodriguez de Fonseca et al., 1994	Wistar rat	Membrane binding	Hypothalamus, Limbic forebrain, Striatum	Adult (>8weeks)	No difference
			Mesencephalon		↑ male 31%

Riech et al., 2009	Sprague-Dawley rat	Western blot	Hippocampus	Adult (7-8 weeks)	↑ male 66%
Marco et al., 2007	Wistar rat	Western blot	Hippocampus	PN43	↑ male 10%
				PN75	No difference
			Striatum	PN43,75	No difference
Suarez et al., 2009	Wistar rat	Immuno-histochemistry	Hippocampus	PN13	No difference
Gonzalez et al., 2000	Sprague-Dawley rat	In situ hybridisation	Anterior pituitary	Adult	↑ male 40-610%
$GABA_A$					
Davis and McCarthy, 2000	Sprague-Dawley rat	Autoradiography - [^{3}H]Muscimol	Medial preoptic area, Ventrolateral thalamus, Cingulate cortex, Ventromedial hypothamalus, Hippocampus, Amygdala	PN1,5,10,20	No difference
Skilbeck et al., 2008	QS mice	Autoradiography - [^{3}H]GABA	Cingulate and Motor cortex	Adult	↑ female 15-20%
			Temporal cortex, Striatum, Lateral septum, Hippocampus, Amygdala		No difference
Canonaco et al., 1996	Wood mice	Autoradiography - [^{3}H]Muscimol	Striatum, Anterior hypothalamus	Adult	↑ male 36-50%
			Stria terminalis, Ventromedial hypothalamus, Ventrolateral thalamus, Central grey pontine, Substantia nigra		↑ female 81%,43%, 36%, 62%, 51%
			Medial preoptic area, Globus pallidus, Medial dorsal thalamus, Medial and dorsolateral geniculate		No difference

Juptner and Heimke, 1990	Sprague-Dawley rat	Membrane binding	Medial preoptic area, Hypothalamus	Adult	↑ male 305-430%
			Amygdala, Cortex		No difference

Abbreviations: PN = postnatal day.

6.4.4 Sexual dimorphism in the developmental profile of CB_1 receptors

To our knowledge, the present study is the first to report the developmental profile of CB_1R binding density in the mouse brain. Our findings are in general agreement with others who have reported increased levels from juvenile to adulthood in CB_1R levels in the rat, monkey and human brains (Belue et al., 1995; Berrendero et al., 1999; Eggan et al., 2010a; Mato et al., 2003, Van Laere et al., 2008), however our study furthered these previous studies by reporting clear gender differences in the developmental profile of CB_1R s in the mouse brain (peak expression of male CB_1Rs at adolescence and female CB_1Rs at adulthood). One previous developmental study in rats reported peak expression of CB_1R density at adolescence in the striatum and at adolescence to adulthood in the forebrain of both male and female rats (Rodriguez de Fonseca et al., 1993). It is hard to directly compare our findings to those in the rat because of the conflicting findings in the literature, but also it is clear from our study and others that there are species specific differences in CB_1R (and $GABA_ARs$) between rats and mice and even within species disparities due to strain differences (Haller et al., 2007; Hungund and Basavarajappa, 2000).

The differential profiles between the male and female mice in the present study resulted in gender differences in CB_1R expression at the adolescent (prefrontal cortex, striatum and hippocampus) and adult (prefrontal cortex and striatum) time periods in the mouse brain of the saline animals. While no other studies have examined gender differences in CB_1R binding density in the mouse brain, a previous study using the same receptor binding technique in adult rats found no difference in gender in the dentate gyrus and only a 10% reduction in the CA1, CA2 and CA3 regions of the hippocampus in females compared to males (Mateos et al., 2011). A human study using [^{18}F]MK-9470 PET to determine in vivo CB_1R distribution found

an increase CB_1Rs with age only in females (Van Laere et al., 2008), mirroring our finding of an incremental increase in CB_1R density in female but not male mice. Other studies using different techniques, such as Western blot and membrane binding, to examine CB_1Rs in the rat have found both similar and contrasting results to that of the present study (summarised in Table 6.3), indicating the presence of brain regional and species and/or strain variations when examining neurotransmitter receptors. It has been reported previously that there are gender-specific behavioural effects of CB_1R agonist treated rats at adolescence (Biscaia et al., 2003; Mateos et al., 2011), possibly due to differences in cannabinoid system development and regulation. We suggest that the gender specific findings observed in the present study would render the genders different susceptibilities to CB_1R impacts, which should be considered in the design of future studies, and that caution should be applied in the extrapolation of data from one gender to another. The literature, combined with the results from the present study, therefore points to the importance of examination of gender specific development of the cannabinoid system in the human brain in terms of both expression and function.

One obvious reason for gender differences in CB_1R and $GABA_A$R levels, particularly at adolescence, is sex steroids. One study has shown that although CB_1R density fluctuates throughout the oestrous cycle in adult female rodents in the hypothalamus, there were no changes in the striatum, limbic forebrain and mesencephalon (Rodriguez de Fonseca et al., 1994), which are similar brain regions to those examined in the present study. Another, more recent study has found that CB_1R density is reduced in cycling females compared to male and ovariectomised rats in the prefrontal cortex but not the hippocampus (Castelli et al., 2014). To our knowledge, studies of this nature have not been conducted with regards to whole

$GABA_AR$s, however it has been found that some $GABA_AR$ subunits fluctuate with the oestrous cycle in adult female rodents (Lovick et al., 2005; Maguire et al., 2005) as previously mentioned. This however does not explain the disparity between the gender differences seen at adolescence and the lack of or reversed gender differences seen at adulthood.

6.4.5 Correlations

As expected, there was a significant correlation between CB_1 and $GABA_AR$s. While the relationship between the cannabinoid and GABAergic systems is well established, i.e. CB_1Rs located on GABAergic interneurons act to inhibit GABA release, the relationship between the $GABA_A$ and CB_1R, especially during development is not well known. CB_1R density increased throughout development to adulthood, while there was a reduction in $GABA_AR$ density from juvenile to adult levels. The GABAergic system is vitally important in early neurodevelopment (Deidda et al., 2014) and therefore we would expect levels to be high. The cannabinoid system acts to inhibit the GABAergic system, so with low levels early in life this allows for less inhibition of the GABAergic system to allow GABA to perform its developmental functions. Later in life as CB_1R density increases, there is more inhibition of GABA, and CB_1Rs on GABAergic neurons in ageing rodents have been found to protect against cognitive decline by decreasing pyramidal cell degeneration and neuroinflammation (Albayram et al., 2011).

We found a positive correlation between NR2A and $GABA_AR$ binding (and a negative correlation between NR2A and CB_1R binding) in the prefrontal cortex and hippocampus at adolescence and this correlation was not observed with NMDAR or NR2B binding. This appears to be a unique finding and although the relevance of this correlation at adolescence is not clear, it has been found previously that the

NR2A subunit plays a more important role in the regulation of GABAergic function in parvalbumin interneurons than NR2B and the presence of NR2A in these neurons is much higher than NR2B (Kinney et al., 2005). Whether this then affects the levels of $GABA_AR$s is not known but may contribute to this association at adolescence, during a period of enhanced synaptic pruning and regulation. While our study did not examine the levels of proteins in specific neuron subtypes, it may be necessary to do so in future studies in order to further understand this relationship.

6.4.6 Conclusion

In conclusion, $GABA_A$ and CB_1Rs have opposing developmental profiles. $GABA_AR$ density differed between genders in the early to adolescent ages, whereas CB_1R density showed gender differences at the adolescence to adult ages. NR2B antagonist treatment in the perinatal period had no effect on CB_1 or $GABA_AR$ levels throughout development and therefore this model does not demonstrate neurochemical alterations in the GABAergic and cannabinoid systems similar to schizophrenia.

7 OVERALL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Overall Discussion

This study investigated the role of the NR2B subunit of the NMDAR in the pathophysiology and development of schizophrenia. Two approaches were used; First, NR2B subunit protein and binding density was examined in the postmortem human brain, specifically in the brain regions of the hippocampus and prefrontal cortex, including an analysis of gender and hemispheric effects. Postmortem human brain is a valuable tool to understand molecular processes occurring in the neuropsychiatric disorders such as schizophrenia. One of the limitations of this however is that there is an inability to assess molecular changes that occur prior to the onset of the disorder, to develop an understanding of the development of the disorder. This is where animal models provide value. Second, an animal model was employed to assess the effects of perinatal NR2B antagonism on the developmental profile of systems implicated in schizophrenia, including NMDA, NRG1/ErbB4, GABAergic and canabinnoid systems. In this animal model we also examined the effects of gender on the developmental profile of the proteins. The main findings from these studies are: 1. A decrease in NR2B protein in the left hippocampal hemisphere of the schizophrenia brain compared to control; 2. A decrease in NR2B protein in the prefrontal cortex of the female schizophrenia brain compared to males; 3. A reduction in NR2B binding following perinatal NR2B antagonism in the prefrontal cortex and hippocampus of the mouse brain; 4. Alterations in NR2B protein in the prefrontal cortex and NRG1 protein in the hippocampus at adolescence following perinatal NR2B antagonism; 5. Increases in NR2B binding and NRG1 protein during development; 6. Gender specific developmental profiles of NR2A, $GABA_AR$ and CB_1R binding.

7.1.1 Hemispheric alterations in the human hippocampus:

This is the first study to report a lateralisation effect of NR2B protein in the hippocampus in schizophrenia subjects and this could be associated with the reductions in NR1 previously reported in the left hippocampus in schizophrenia (Vrajova et al., 2010). Selective reduction of NR2B-containing NMDARs in the hippocampus has been reported to produce cognitive deficits in rodents (Brigman et al., 2010; Clayton et al., 2002) as well as disrupting underlying molecular processes such as LTP (Clayton et al., 2002). Our findings therefore of reduced NR2B protein in the hippocampus, where it plays a critical role in cognitive function, and is possibly related to cognitive dysfunction in schizophrenia patients, supports the idea that NR2B positive allosteric modulators could potentially be therapeutic for cognitive dysfunctions in schizophrenia.

7.1.2 Gender alterations in the human prefrontal cortex:

This is the first study to report a gender difference in NR2B protein in the prefrontal cortex of schizophrenia subjects. The decrease of NR2B protein in the female schizophrenia subjects compared to the male schizophrenia subjects shows it is important to consider gender when analysing post mortem cohorts. Although this result is only preliminary, as the sample size was not large enough to detect an observed difference between the female control and female schizophrenia groups, this is one of the largest schizophrenia post mortem cohorts to date and highlights the need for larger cohorts to investigate variables such as gender and hemisphere.

7.1.3 Treatment effects from the perinatal NR2B antagonist animal model:

In our animal model a decrease in NR2B binding at the juvenile time point in both the prefrontal cortex and hippocampus as well as a small increase in NR2B

protein at adolescence in the prefrontal cortex and a small reduction in NRG1 protein in the hippocampus following perinatal NR2B antagonist treatment. There was also an overall reduction in NR2B protein in the hippocampus, independent of age or gender. There were no treatment effects on NMDAR binding, NR2A subunit binding or protein, ErbB4 protein, $GABA_AR$ binding or CB_1R binding at any time point in any brain region.

Our study provides the first evidence of the effects of perinatal NR2B antagonism on brain neurochemistry. Perinatal NR2B antagonism had minimal effects on the NMDAR complex and NRG1 signalling system however despite this there may be altered functional activity of these receptors and/or the downstream signalling capacity, which is an avenue of research that would further the results of this study. Reduced expression of NRG1, as seen in the hippocampus at adolescence in our study, has been found to increase susceptibility to behavioural abnormalities and further impacts to the brain, particularly at adolescence (Long et al., 2013). Therefore while these alterations are subtle, collectively they may increase the susceptibility of Ro 63-1908 treated animals to secondary impacts. In addition to the alterations in NR2B and NRG1 at adolescence in our animal model we found NRG1 and NR2B proteins to be positively correlated in the prefrontal cortex and hippocampus at PN70 and in the prefrontal cortex at PN14 but there was no correlation at PN35. It is possible that perinatal NR2B antagonism disrupts the positive relationship between these proteins at this age and this could alter other signalling systems and pathways during the adolescent brain growth period, possibly rendering the brain vulnerable to impacts during this time. Furthermore while there was no effect of the perinatal NR2B antagonist treatment on $GABA_AR$ and CB_1R levels, previous studies have shown functional changes in the CB_1R despite a lack of

binding density change. Therefore we cannot rule out the possibility of functional changes in our animal model and again these experiments are recommended to further the results of our study.

7.1.4 Gender effects from the perinatal NR2B antagonist animal model:

Our study is the first to provide evidence in mice of gender differences in the glutamatergic system, particularly of NR2A binding at adolescence, which may underlie gender specific susceptibilities apparent in schizophrenia patients at this time point. Gender differences were also observed in the GABAergic and cannabinoid systems, similar to what has been reported previously however interestingly there was a significant correlation between the NR2A, $GABA_AR$ and CB_1R binding results. Again this highlights the need to examine gender differences, not only in post mortem cohorts, but also in animal models.

7.1.5 Developmental effects from the perinatal NR2B antagonist animal model:

This was the first study to investigate [^{3}H]Ifenprodil binding during development of the mouse brain. There was an increase from PN14 to PN70 in both the prefrontal cortex and the hippocampus unlike that of the NR2B protein, which confirmed previous findings of a small decrease between PN14 and PN35 in the prefrontal cortex, otherwise, no difference between the time points. An increase in binding throughout development suggests that the availability or affinity of the binding sites in the membrane may change over time but the amount of total protein remains fairly constant. No difference in NR2A protein was observed between the time points in either gender or with NR2A binding in the male mice however in the female mice there was a peak expression of NR2A binding at adolescence. This highlights the importance of gender separation in future NR2A related studies.

The developmental profile of the NRG1 protein was also an interesting finding in our study and the first to be presented in any species. Previous studies of NRG1 mRNA have found a decrease during development however in our study we found an increase in NRG1 protein during development. Given the importance of NRG1 in early development this was unexpected. Isoforms of NRG1 mRNA have different developmental profiles therefore it is likely that particular NRG1 isoforms and isoform ratios are more important during early development and not the total levels of NRG1 protein that were measured in this study. Examining NRG1 isoform expression during development would be a valuable experiment to perform to further our research.

7.2 Limitations

There are several limitations of this thesis that must be considered. In our animal study we did not measure the oestrous cycle status of the female mice and therefore it is possible that differing levels of oestrogen may have affected the proteins that were examined. Previous studies have shown that oestrogen does not appear to affect NMDAR, NR2A, NR2B levels (Snyder et al., 2011) (which were measured in this study) although it could affect the function of these proteins (Vedder et al., 2013). CB_1R and $GABA_AR$ proteins on the other hand could be affected by oestrous cycle status (Castelli et al., 2014, Lovick et al., 2005, Maguire et al., 2005).

This study did not examine lateralisation in the animal tissue. While laterality is present in the rodent brain (Cowell et al., 1999) and functional processing, such as long-term memory, does appear to be lateralised in mice (Shipton et al., 2014), we combined the left and right hemisphere brain structures for tissue

collection for our Western blot studies to assure we had sufficient protein for our studies. Considering our finding in the human tissue of a decrease in NR2B protein (using Western blot) in the left hippocampal hemisphere but not the right it appears that this may indeed be worthwhile to pursue in the future.

The protein expression levels in this study were examined in a specific brain regions, however they were not examined in different subpopulations of neurons (ie. excitatory vs inhibitory) or in separate compartments of the cell (ie. ER vs soma vs synaptic terminal). Despite no change in total binding or protein, it has previously been shown that NR2B is altered in schizophrenia subjects specifically in the endoplasmic reticulum fraction of cells in the prefrontal cortex (Kristiansen et al., 2010b). In an animal model changes were also observed in NR2A and NR2B protein in the endoplasmic reticulum after perinatal treatment with the NMDAR antagonist PCP (Anastasio and Johnson 2008). Furthermore, it has been found that the expression of NR2A is much higher than NR2B in a specific subtype of inhibitory interneurons (Kinney et al., 2005) and therefore the examination of tissue in different cellular populations and compartments of the cell would follow nicely from the experiments in this thesis.

7.3 Future Directions

In addition to addressing some of the limitations presented above, there are several avenues to be pursued in future investigations. Considering the close relationship between the NMDAR subunits (and NRG1 and ErbB4) and the MAGUK family of proteins in the postsynaptic density (PSD-95, PSD-93, SAP97 and SAP102) and the changes that have been found in these postsynaptic density proteins in the schizophrenia brain (for review see Geddes et al., 2011), it will be

worthwhile in the future to examine the effect an NMDAR antagonist (global or subunit specific) model has on these proteins. Conversely it may be that a disassociation with a particular postsynaptic density protein is what causes impairment in NMDAR stoichiometry or function. Therefore examination of the coupling or co-localisation between the NMDAR subunits and these MAGUKs in schizophrenia should be investigated.

As discussed throughout this thesis, there is evidence to suggest that despite no change in protein levels, disturbances to trafficking, degradation and regulation of the NR2B subunit may occur in schizophrenia (Kristiansen et al., 2010a). Further research to investigate whether any alterations in NR2B in schizophrenia are specific to the cell surface, the synapse or extrasynaptic region, and whether proteins that regulate the trafficking of NR2B are altered in schizophrenia. This may have implications for the effectiveness of NR2B positive allosteric modulators or similar drugs that are in preclinical development for the potential treatment of schizophrenia (Menniti et al., 2013).

Future studies on NR2B and schizophrenia, particularly animal models, may also want to consider the behavioural aspects of an impact to the NR2B subunit during early development and whether they correspond to functional changes in NR2B or other NMDAR associated proteins. Although there were limited alterations to NMDAR subunit expression in the present study, it remains possible that there are functional changes to these proteins as this has been shown in regards to the CB_1R (Vigano et al., 2009, Seillier et al., 2010).

Finally, while this study focused on the hippocampus and prefrontal cortex brain regions, others have found alterations in the NR2B subunit in schizophrenia in the superior temporal cortex and various parts of the thalamus (Grimwood et al.,

1999, Ibrahim et al., 2000, Clinton and Meador-Woodruff, 2004, Clinton et al., 2006). Therefore there are other parts of the cortex and brain that could be examined in relation to the animal model in this thesis, as well as numerous other parts of the brain that have not been studied in relation to the NMDAR subunit expression in schizophrenia.

7.4 Conclusion

In conclusion, this thesis has provided novel insights into the changes that occur in the NR2B subunit in schizophrenia, revealing for the first time that expression of NR2B is altered in the DLPFC and hippocampus of schizophrenia subjects, in a gender and hemisphere specific manner. These alterations may be related to cognitive symptoms experienced by patients, however further studies are required to determine this. There are numerous studies showing the functional importance of the NR2B subunit in both the prefrontal cortex and hippocampus, particularly in terms of cognitive performance; future investigations should therefore focus on the functional changes to NR2B including any alterations to NR2B trafficking or regulation. Importantly, this will align with research into the development of novel glutamatergic agents, including NR2B positive allosteric modulators, for the treatment of cognitive dysfunction in schizophrenia patients.

This thesis also showed that a pharmacological impact to the NR2B subunit during perinatal development causes some alterations to schizophrenia relevant systems, including NR2B and NRG1, at adolescence, which could potentially make this animal model more vulnerable to further impacts at adolescence. It should be noted however, that this model did not cause widespread changes in systems (at least in terms of protein expression) relevant to the pathophysiology of schizophrenia.

Finally, this thesis has identified that the developmental profiles of schizophrenia relevant systems, differs between the two genders (at least in a mouse model), highlighting the importance of considering this in the design of future studies.

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APPENDIX

Tables showing average demographic values split by hemisphere and gender in the human tissue hippocampus and prefrontal cortex.

Table 1. Average demographic values in the hippocampus by hemisphere.

Variable	Cohort	Control Left	Control Right	Sz Left	Sz Right	p
n	WB	10	10	11	9	
	RA	10	11	10	10	
Age (years)	WB	54±4.1 (34-74)	55±4.0 (24-73)	51.3±4.7 (27-75)	58.8±2.1 (52-72)	0.652
mean±SEM (range)	RA	54.2±4.0 (36-74)	56.3±3.9 (24-73)	50.7±5.0 (27-75)	58.8±2.0 (52-72)	0.556
PMI (hours)	WB	22.2±4.3 (9.5-50)	34.3±2.7 (23-48)	27.7±3.8 (5-48)	31.6±4.0 (12.5-48)	0.167
mean±SEM (range)	RA	23.5±4.4 (9.5-50)	36.8±2.7 (24-50)	26.7±3.9 (5-48)	31.1±3.8 (12.5-48)	0.093
Brain pH	WB	6.3±0.1 (5.75-6.73)	6.7±0.1 (6.27-7.02)	6.5±0.05 (6.23-6.69)	6.5±0.05 (6.14-6.66)	**0.026***
mean±SEM (range)	RA	6.3±0.1(5.75-6.67)	6.6±0.1 (6.06-7.02)	6.5±0.05 (6.23-6.69)	6.5±0.05 (6.14-6.66)	0.095
BW (grams)	WB	1401.6±46.0 (1200-1610)	1418.5±30.2 (1294-1635)	1405.5±47.0 (1120-1590)	1300±57.7 (1020-1548)	0.314
mean±SEM (range)	RA	1397.6±44.6 (1200-1610)	1395.4±39.9 (1100-1635)	1387±45.5 (1120-1560)	1328.6±61.5 (1020-1586)	0.734
Gender (M/F)	WB	7M/3F	5M/5F	8M/3F	4M/5F	
	RA	7M/3F	5M/6F	7M/3F	5M/5F	
Onset (age)	WB			22.2±1.8 (14-31)	26.4±3.3 (19-44)	0.258
mean±SEM (range)	RA			21.3±1.6 (14-31)	25.9±3.1 (19-44)	0.210
DOI (years)	WB			29.1±4.1 (12-46)	32.3±3.2 (17-47)	0.558
mean±SEM (range)	RA			29.4±4.3 (12-46)	32±3.0 (17-47)	0.637
Chlor equiv (mg)	WB			842.2±177.4 (222-2362.5)	709.3±160.4 (227.5-1850)	0.600
mean±SEM (range)	RA			901.4±176.3 (222-2362.5)	716.7±151.4 (227.5-1850)	0.450

Abbreviations: BW = brain weight,Chlor equiv = chlorpromazine equivalent, DOI = duration of illness, F = female, L = left, M = male, PMI = postmortem interval, R = right, RA =receptor autoradiography, SEM = standard error of the mean, Sz = schizophrenia, WB = Western blot. * = Control left brain pH is significantly less than Control right.

Table 2. Average demographic values in the hippocampus by gender.

Variable	Cohort	Control Male	Control Female	Sz Male	Sz Female	p
n	WB	12	8	12	8	
	RA	12	9	12	8	
Age (years)	WB	51.8±4.5 (24-74)	58.6±3.1 (51-72)	51.7±4.5 (27-75)	59.1±1.8 (54-67)	0.410
mean±SEM (range)	RA	51.9±4.4 (24-74)	59.8±2.8 (51-72)	51.8±4.5 (27-75)	59.1±1.8 (54-67)	0.327
PMI (hours)	WB	29.8±3.9 (10-50)	25.9±4.8 (9.5-43)	29.4±4.1 (5-48)	29.5±3.5 (12.5-42)	0.917
mean±SEM (range)	RA	30.9±3.8 (10-50)	29.9±5.0 (9.5-50)	28.5±4.1 (5-48)	29.5±3.5 (12.5-42)	0.977
Brain pH	WB	6.5±0.1 (5.88-6.8)	6.5±0.2 (5.75-7.02)	6.5±0.04 (6.24-6.66)	6.5±0.07 (6.14-6.69)	0.965
mean±SEM (range)	RA	6.4±0.08 (5.88-6.8)	6.4±0.2 (5.75-7.02)	6.5±0.04 (6.24-6.66)	6.5±0.07 (6.14-6.69)	0.984
BW (grams)	WB	1468.1±34.3 (1220-1635)	1323±30.9 (1200-1500)	1463.3±22.6 (1344-1590)	1200±54.1 (1020-1490)	**0.000***
mean±SEM (range)	RA	1464.8±33.4 (1220-1635)	1305.3±39.0 (1100-1500)	1463±22.4 (1344-1586)	1200±54.1 (1020-1490)	**0.000***
Hemisphere (L/R)	WB	7L/5R	3L/5R	8L/4R	3L/5R	
	RA	7L/5R	3L/6R	7L/5R	3L/5R	
Onset (age)	WB			27.3±2.6 (14-44)	19.3±0.9 (16-24)	**0.026**
mean±SEM (range)	RA			26.5±2.7 (14-44)	19.3±0.9 (16-24)	**0.045**
DOI (years)	WB			24.3±3.2 (12-44)	39.9±1.8 (32-47)	**0.002**
mean±SEM (range)	RA			24.6±3.2 (12-44)	39.9±1.8 (32-47)	**0.002**
Chlor equiv (mg)	WB			599.8±86.8 (222-1300)	1056.3±254.2 (350-2362.5)	0.064
mean±SEM (range)	RA			644.3±81.5 (227.5-1300)	1056.3±254.2 (350-2362.5)	0.088

Abbreviations: BW = brain weight,Chlor equiv = chlorpromazine equivalent, DOI = duration of illness, F = female, L = left, M = male, PMI = postmortem interval, R = right, RA =receptor autoradiography, SEM = standard error of the mean, Sz = schizophrenia, WB = Western blot. * = Control male brain weight significantly larger than both Control female and Sz female, Sz male brain weight significantly larger than Control female and Sz female.

Table 3. Average demographic values in the prefrontal cortex by hemisphere.

Variable	Cohort	Control Left	Control Right	Sz Left	Sz Right	p
n	WB/RA	14	23	20	17	
Age (years)	WB/RA	50.6±4.3 (18-78)	51.5±2.9 (21-78)	50.6±3.7 (27-75)	52.2±2.6 (27-66)	0.988
mean±SEM (range)						
PMI (hours)	WB/RA	20.7±3.0 (6.5-50)	27.3±2.2 (11-48)	25.9±2.4 (5-48)	31.5±4.0 (8.4-72)	0.166
mean±SEM (range)						
Brain pH	WB/RA	6.6±0.1 (5.87-7.19)	6.7±0.06 (6.02-7.15)	6.7±0.06 (6.16-7.01)	6.6±0.09 (5.69-7.09)	0.615
mean±SEM (range)						
RIN	WB/RA	7.5±0.1 (6.7-8.4)	7.2±0.1 (6-8.3)	7.4±0.1 (6.3-8.4)	7.1±0.1 (6.2-8.2)	0.272
mean±SEM (range)						
BW (grams)	WB/RA	1428.4±41.5 (1200-1740)	1457.3±22.7 (1280-1635)	1433.8±31.9 (1120-1700)	1342.6±42.2 (1020-1586)	0.068
mean±SEM (range)						
FST (months)	WB/RA	92.9±10.7 (28-152)	55.5±8.0 (5-113)	85.7±8.2 (11-137)	73.1±9.2 (8-139)	0.087
mean±SEM (range)						
Gender (M/F)	WB/RA	12M/2F	18M/5F	15M/5F	9M/8F	
Onset (age)	WB/RA			23.6±1.2 (17-36)	23.9±1.7 (14-40)	0.871
mean±SEM (range)						
DOI (years)	WB/RA			27.1±3.5 (3-52)	28.3±2.8 (7-47)	0.789
mean±SEM (range)						
Chlor equiv (mg)	WB/RA			665.2±112.0 (187.5-2362.5)	722.8±125.5 (162.5-1866.5)	0.733
mean±SEM (range)						

Abbreviations: BW = brain weight,Chlor equiv = chlorpromazine equivalent, DOI = duration of illness, F = female, FST = freezer storage time, L = left, M = male, PMI = postmortem interval, R = right, RA =receptor autoradiography, RIN = RNA integrity, SEM = standard error of the mean, Sz = schizophrenia, WB = Western blot.

Table 4. Average demographic values in the prefrontal cortex by gender.

Variable	Cohort	Control Male	Control Female	Sz Male	Sz Female	p
n	WB/RA	30	7	24	13	
Age (years)	WB/RA	51.5±2.5 (18-78)	49.7±7.0 (21-78)	47.5±3.0 (27-75)	58.4±2.8 (33-73)	0.173
mean±SEM (range)						
PMI (hours)	WB/RA	25.1±2.0 (6.5-50)	23.3±4.3 (11-39.5)	28.6±3.1 (5-72)	28.2±3.2 (12-50)	0.641
mean±SEM (range)						
Brain pH	WB/RA	6.6±0.06 (5.84-7.19)	6.7±0.1 (6.37-7.15)	6.7±0.05 (6.16-7.01)	6.5±0.1 (5.69-7.09)	0.517
mean±SEM (range)						
RIN	WB/RA	7.3±0.1 (6-8.4)	7.5±0.3 (6.1-8.3)	7.4±0.1 (6.3-8.4)	7.1±0.2 (6.2-8.1)	0.392
mean±SEM (range)						
BW (grams)	WB/RA	1468.4±22.2 (1220-1740)	1352±42.8 (1200-1570)	1466.8±22.9 (1140-1700)	1253.5±42.0 (1020-1490)	**0.000***
mean±SEM (range)						
FST (months)	WB/RA	75.4±8.0 (8-152)	45±11.2 (5-82)	85.2±7.2 (8-137)	70.2±11.1 (11-139)	0.129
mean±SEM (range)						
Hemisphere (L/R)	WB/RA	12L/18R	2L/5R	15L/9R	5L/8R	
Onset (age)	WB/RA			24.3±1.1 (17-40)	22.6±2.0 (14-36)	0.430
mean±SEM (range)						
DOI (years)	WB/RA			23.2±2.8 (3-52)	35.8±2.7 (16-47)	**0.006**
mean±SEM (range)						
Chlor equiv (mg)	WB/RA			584.6±61.3 (187.5-1300)	889.2±199.8 (162.5-2362.5)	0.078
mean±SEM (range)						

Abbreviations: BW = brain weight,Chlor equiv = chlorpromazine equivalent, DOI = duration of illness, F = female, FST = freezer storage time, L = left, M = male, PMI = postmortem interval, R = right, RA =receptor autoradiography, RIN = RNA integrity, SEM = standard error of the mean, Sz = schizophrenia, WB = Western blot. * = Sz female brain weight is significantly smaller than Control male and Sz male brain weight, but no difference to Control female brain weight.