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Neuregulin 1-Erbb4 in the rodent prefrontal cortex: Investigations of schizophrenia-related behaviours and signalling pathways

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The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other degree.

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Abstract

Schizophrenia is a severe, chronic and debilitating psychiatric disorder. Current therapies have no efficacy in treating the cognitive impairments which are largely responsible for the poor quality of life of schizophrenia patients and contribute to the massive economic burden that is associated with the disorder.

Although it is known that schizophrenia is highly heritable, the underlying genetic basis is still poorly understood due to the complex polygenetic nature of the disorder. Several candidate genes which are thought to increase risk for the incidence of schizophrenia have been identified. Two such schizophrenia candidate genes are *neuregulin 1* (*NRG1*) and *v-erb-a erythroblastic leukaemia viral oncogene homolog 4 (ERBB4)*. As well as the genetic evidence from genetic association studies, studies of animal models and the endogenous biological functions of NRG1 and ERBB4 in the CNS suggest that these genes may play an important role in the pathophysiology of schizophrenia. However, very little is known about the functions of these genes in specific brain regions in adulthood with respect to cognition.

To address this, I have utilised recombinant adeno-associated viral particles (rAAVs) as a vehicle to mediate knockdown of the expression of Erbb4 specifically within the medial prefrontal (mPFC) cortex of adult rats. This allows for a spatially and temporally controlled investigation of the role that Erbb4 signalling may play in prefrontal cortex-dependent behaviours in adulthood.

Following initial i*n vitro* and *in vivo* validation of the functionality of the rAAVs, further *in vivo* studies confirmed that, five weeks after stereotaxic injection of rAAVs encoding a short hairpin sequence corresponding to *Erbb4* (sh*Erbb4*.rAAV), into the mPFC of rats, there was significant Erbb4 protein knockdown, as analysed by ELISA. Subsequent western blot analysis revealed that Erbb4 knockdown consequently increased the level of Nrg1 expression and decreased the activity of Akt signalling, but had no effect on Erk signalling.

Erbb4 knockdown specifically within the mPFC increased performance accuracy in the 5-choice serial reaction time task at 5 weeks post-surgery. Furthermore, viral mediated Erbb4 knockdown specifically within the mPFC heightened the sensitivity to the locomotor inducing effects of amphetamine. There were, however, no effects of Erbb4 knockdown on pre-pulse inhibition at any time points assessed. These results indicate that Nrg1-Erbb4 signalling in the PFC modulates cognitive performance but not sensorimotor gating, and that dopaminergic transmission may be regulated by Nrg1-Erbb4 signalling.

In conclusion, this study highlights the ability of viral mediated gene manipulation to investigate regionally specific roles of schizophrenia candidate genes in adulthood in terms of cognition and downstream signalling pathways. This may translate to a better understanding of how these genes may exert potentially pathophysiological effects in patients and ultimately lead to improved treatments.

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List of abbreviations

5-CSRTT	5-choice serial reaction time task
5-HT	Serotonin
AChR	Acetylcholine receptor
ADHD	Attentional deficit hyperactivity disorder
Akt	v-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of variance
AP	Anterior-posterior
ARIA	Acetylcholine receptor inducing activity
BACE	β-amyloid precursor protein cleaving enzyme
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CATIE	Clinical Antipsychotic Trials of Intervention effectiveness
cDNA	Complementary DNA
CNS	Central nervous system
CNV	Copy number variant
COMT	*Catechol-O-methyl transferase*
CPT	Continuous performance task
CRD	Cysteine rich domain
CUtLASS	Cost Utility of the Latest Antipsychotic drugs in Schizophrenia Study
DA	Dopamine
DAO	D-amino acid oxidase
dB	Decibel
DEPC	Diethylpyrocarbonate
DISC1	Disrupted in schizophrenia 1
DLPFC	Dorsolateral prefrontal cortex
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
Drd2	Dopamine receptor D2
DSM IV	Diagnostic and Statistical Manual of Mental Disorders IV
dsRNA	Double stranded RNA
DV	Dorso-ventral
ECACC	European Collection of Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent assay
EPS	Extra-pyramidal symptoms
ERBB	*v-erb-a erythroblastic leukaemia viral oncogene homolog*
Erk	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
GABA	Gamma-Aminobutyric acid
GAD	Glutamic acid decarboxylase
gc	Genome copies
GFAP	Glial fibrillary acidic protein
GSK3-β	Glycogen synthase kinase 3β
GWAS	Genome wide association study

HRP	Horse radish peroxidase
i.p.	Intraperitoneal
ICD-10	International Classification of Diseases 10th revision
Ig	Immunoglobulin
ITI	Inter-trial interval
ITR	Inverted terminal repeat
Kb	Kilobase
kDa	Kilo Dalton
LH	Limited hold
LSD	lysergic acid diethylamide
LTP	Long term potentiation
MAM	Mitotoxin methylazoxymethanol
MAPK	Mitogen-activated protein kinase
MAPK	mitogen-activated protein kinase
MATRICS	Measurement and Treatment Research to Improve Cognition in Schizophrenia
Mb	Megabase
MEM	Minimum Essential Medium
mGluR	Metabotropic glutamate receptor
ML	Medio-lateral
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
N200	Neurofilament 200kDa
NMDA	N-Methyl-D-asparate
NRG	Neuregulin
NSE	Neuron-specific enolase
OCD	Obsessive compulsive disorder
PBS	Phosphate buffered saline
PCP	Phencyclidine
pERBB4	Phosphorylated ERBB4
PFA	Paraformaldehyde
PI3-K	Phosphoinositide-3 kinase
PPI	Prepulse inhibition
prL	Prelimbic
PRODH	*Proline dehydrogenase*
PSD	Post synaptic density
PTGS	Posttranscriptional gene silencing
Pvalb	Parvalbumin
qRT-PCR	Quantitative real time reverse transcriptase polymerase chain reaction
rAAV	Recombinant adeno-associated virus
RISC	RNAi silencing complex
RNA	Ribonucleic acid
RNA$_i$	RNA interference
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SCA1	spinocerebellar ataxia type 1
scr	Scrambled
SD	Stimulus duration
SDS	Sodium dodecyl sulphate
SE	Standard error
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
ssDNA	Single stranded DNA
TACE	tumour necrosis factor-α converting enzyme
tERBB4	Total ERBB4
THC	Δ-9-tetrahydrocannabinol
TMD	Transmembrane domain
VCSF	Velo-cardio-facial-syndrome
veh	Vehicle
vITI	Variable inter-trial interval
vSD	Variable stimulus duration
WCST	Wisconsin card sort test
WPRE	Woodchuck posttranscriptional regulatory element

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The hard work and commitment this thesis represents is dedicated in memory of my beloved godmother and aunt, Edith Paterson, who always told me I would be the first "Doctor" of the family.

1 Introduction

1.1 Schizophrenia

Schizophrenia is a chronic and debilitating psychiatric disorder in which, unlike many other neurological diseases, the onset of symptoms generally manifest in late adolescence/early adulthood. The causes of the disorder are unclear but mostly likely result from genetic and environmental factors which impact on neurotransmitter systems in particular brain circuits. Emil Kraepelin, a German psychiatrist, first described schizophrenia in 1893 and termed the disorder, *Dementia Praecox,* to differentiate it from other forms of dementia. However *Dementia Praecox*-meaning early dementia- was somewhat misleading as not all people with schizophrenia experience dementia. Later, the psychiatrist Eugene Bleuler renamed *Dementia Praecox* and coined the term “schizophrenia” in 1908, and made the observation that schizophrenia appeared to be a group of diseases as it had multiple effects on personality, thought, cognition and motivation. A recent systematic review of the epidemiology of schizophrenia indicates that approximately 7 per 1000 individuals will develop schizophrenia during their lifetime (McGrath *et al.,* 2008). Furthermore, men are at a slightly higher risk than women for developing schizophrenia, with a male:female risk ratio of 1.4:1 being identified in multiple studies (Aleman *et al.,* 2003; McGrath *et al.*, 2008). With the lifelong duration of schizophrenia, the economic burden of the disorder is a huge cost to UK society, with an estimated cost of £6.7 billion in 2004/5 in England alone (Mangalore & Knapp, 2007).

1.1.1 Symptoms of schizophrenia

Diagnosis of schizophrenia is carried out according to guidelines set by the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV) in the USA and the International Classification of Diseases 10th revision (ICD-10) in Europe. Both guidelines state that in order to diagnose a patient with schizophrenia they must have a specific number of symptoms, which have been presented for a specific duration of time. For example DSM IV states that the patient must have two or more of the “characteristic” symptoms of schizophrenia, present for a significant portion of time during a one month period. Schizophrenia is thought of as a spectrum disorder with many subtypes of schizophrenia being classified based on severity and presence of symptoms, including paranoid schizophrenia, catatonic schizophrenia, residual schizophrenia and undifferentiated schizophrenia (ICD-10).

The symptoms of schizophrenia have been broadly categorised into three groups including positive symptoms, negative symptoms and cognitive deficits. Positive symptoms of schizophrenia are clinical features that are normally absent in the general population, for example hallucinations most commonly of an auditory nature, delusions, thought disorder and disorganised speech (ICD-10). Unlike positive symptoms, which reflect an excess of normal functions, negative symptoms of schizophrenia are observed as a loss of thoughts, feelings or emotions normally present in the general population. For example negative symptoms include the loss of motivation, withdrawal from society, inability to experience pleasure (anhedonia), blunted emotions and apathy. It is these symptoms that are associated with the staggeringly high co-morbidity seen in schizophrenia with ~10% schizophrenics committing suicide (Meltzer *et al.*, 2002).

The third classification of symptoms of schizophrenia is cognitive deficits, which are now widely recognised as a core feature of the disorder. Seven cognitive domains have been identified to be deficient in patients with schizophrenia including; working memory, processing speed, visual and verbal learning and memory, social cognition and attention and vigilance (Nuechterlein *et al.*, 2004). Moreover, the importance of cognitive deficits in schizophrenia has been emphasised by studies showing cognitive performance correlates with functional outcome in schizophrenia patients (Green, 1996, Green, 2006). Poor cognitive performance, likely in combination with the effects of negative symptoms may explain why it is estimated that almost 80% of schizophrenia patients are unemployed (Mangalore & Knapp, 2007).

1.1.2 Neuropathology of schizophrenia

Unlike other disorders of the brain where distinct neuropathologies are evident with disease progression, such as amyloid plaques and neurofibrillary tangles in Alzheimer's disease, schizophrenia is not associated with gross neuroanatomical changes. Instead, subtle morphological and cytoarchitectural changes have been identified in the *post-mortem* studies of brains of patients that had schizophrenia.

Lateral ventricular enlargement has been reported in schizophrenia since 1976 (Johnstone *et al.,* 1976). A review of MRI studies revealed that the median increase in ventricular size in schizophrenia is 40% (Lawrie & Abukmeil, 1998). This review also revealed this enlargement was accompanied by reduction in brain volume of an average of 3%, with medial temporal structures being affected. The morphology of other brain regions has also been implicated in being affected by schizophrenia. The temporal lobe has been implicated in particular as a loss in size of this region, particularly the superior temporal gyrus, has been correlated to the extent of auditory hallucinations (Shenton *et al.,* 1992). Similarly, Wible *et al.*, (1995) found a correlation between the reduction in frontal lobe volume and the extent of negative symptoms. However, studies have been confounded due to the use of *post-mortem* tissue from patients who have undergone treatment with antipsychotics for long periods of time and also the variables between donors of the tissues e.g. age, period of antipsychotic treatment, and extent of clinical symptoms. However, magnetic resonance imaging (MRI) studies in first episode schizophrenia patients have also shown global brain volume reductions (Steen *et al.,* 2006; Vita *et al.*, 2006).

There have also been cytoarchitectural abnormalities found in schizophrenia; for example there is a decrease in cellular size of neurones in the dorsolateral prefrontal cortex and hippocampus (Harrison, 1999). This study also suggested that there is accompanying increase in neurone density in these brain regions. Dendritic spine and synapse densities have also been shown to be affected in cell subtypes in schizophrenia (Selemon & Goldman, 1999; Chen *et al.,* 2008). These cytoarchitectural changes may lead to a lack of connectivity between brain regions, possibly leading to the manifestation of positive, negative or cognitive symptoms of schizophrenia. Further evidence for dysconnectivity of brain regions in schizophrenia stems from reports showing deficits in myelination, resulting from myelin sheath or oligodendrocyte abnormalities (Konrad & Winterer, 2008).

Many of these findings have led to the development of the neurodevelopmental theory of schizophrenia. This theory posits that an as yet unidentified pre- or neonatal insult to the brain, disrupts the function of late maturing brain regions such as the PFC that ultimately manifests as behavioural abnormalities later in life (Weinberger, 1987).

1.2 Hypotheses of schizophrenia

The underlying causes of the symptoms of schizophrenia are thought to be due to an imbalance of neurotransmitter levels in distinct brain regions. However, it is common opinion that due to the complex nature of schizophrenia not a sole neurotransmitter pathway is affected. Instead it is thought that alterations in multiple neurotransmitter systems converge and have feedback effects on each other.

1.2.1 The dopamine hypothesis

The dopamine (DA) hypothesis of schizophrenia was originally based upon the observations of Carlsson and Lindquist (1963), who proposed that hyperactivity of DA transmission was linked to the positive symptoms of schizophrenia. Furthermore, all typical antipsychotic drugs, which are somewhat effective in managing positive symptoms, have high affinity for the DA D2 receptor which is predominantly expressed in subcortical regions of the brain (Seeman, 2004). In addition amphetamine, which enhances DA release, worsens positive symptoms of schizophrenia patients (Angrist *et al.,* 1974) and induces schizophrenia-like symptoms in unaffected individuals (Janowsky & Risch, 1979). From these studies it is thought that over-activity of subcortical dopamine DA systems is responsible for the positive symptoms of schizophrenia. Moreover a series of studies have shown increased D2 receptor binding in mesolimbic regions of the brains of schizophrenia patients (MacKay *et al.,* 1982; Seeman *et al.,* 1984; Crawley *et al.,* 1986)

In contrast, D1 receptors are predominantly expressed in cortical regions (Davis *et al.,* 1991). It is thought that a hypodopaminergic state in this prefrontal cortical region may be responsible for the cognitive deficits of schizophrenia, as

D1-mediated transmission is required for optimal PFC activity and thus performance in cognitive tests (Goldman-Rakic, 2000). This is supported by the fact that current antipsychotic treatments, which predominantly act by antagonising D2 receptors have little efficacy in the treatment of cognitive deficits. The imbalance between mesocortical dopamine deficiency and mesolimbic dopamine over-activity may then explain all three symptom domains of schizophrenia.

1.2.2 Glutamate hypothesis

There is growing support for the hypothesis of glutamatergic dysfunction in schizophrenia. This was primarily proposed due to non-competitive N-Methyl-D-asparate (NMDA) receptor antagonists, for example ketamine and phencyclidine (PCP), producing effects similar to positive, negative and cognitive symptoms of schizophrenia in healthy humans (Krystal *et al.,* 1994; Ardler *et al.,* 1999; Lahti *et al.,* 2001). Moreover, administration of these antagonists can exacerbate symptoms in schizophrenia patients (Luby *et al.,* 1959; Allen *et al.,* 1978; Lahti *et al.,* 2001). In line with these findings, it is thought that schizophrenia may involve NMDA receptor hypofunction. This is supported by evidence for reduced NMDA receptor subunit *GRIN1 (NR1)* mRNA levels in *post-mortem* brains of schizophrenia patients (Law & Deakin, 2001) and reductions in markers for glutamate transport for example vesicular glutamate transporter-1 (*SLC32A1/VGAT1)* (Reynolds and Harte, 2007).

In agreement with NMDA receptor hypofunction, GABAergic neurotransmission is thought to be affected in schizophrenia. NMDA receptors are particularly concentrated on parvalbumin-containing GABA interneurones, loss of function of these NMDA receptors impacts GABAergic dysregulation (Morris *et al.,* 2005). Reductions in parvalbumin-positive GABA-ergic interneurones in frontal and hippocampal regions have been observed in schizophrenia (Benes *et al.*, 2000; Reynolds *et al.,* 2004). Glutamic acid decarboxylase (GAD)-67 (GAD1) an enzyme important in the conversion of glutamate to GABA is also thought to be down-regulated in schizophrenia (Lipska *et al.,* 2003).

1.2.3 Other hypotheses

Other neurotransmitters have also been implicated in the pathology of schizophrenia, for example serotonin (5HT). 5-HT involvement in schizophrenia was first prompted by the observation that lysergic acid diethylamide (LSD), which binds with high affinity to 5HT receptors, induces psychosis-like symptoms (Hemmings & Hemming, 1978). The serotonin hypothesis was also strengthened by the observation that the antipsychotic clozapine, which was found to have higher efficacy in treating symptoms, with fewer extra-pyramidal side effects (EPS), had an ability to block $5HT_{2A}$ receptors (Baldessarini & Frankenburg, 1991). This implies that schizophrenia may be partly due to an over-activation of 5HT receptors.

1.3 Treatments of schizophrenia

Although there is, as of yet, no cure for schizophrenia, there are drugs available for the life-long management of positive symptoms, and to a lesser extent negative symptoms. However, there is to date no significantly effective treatment for the cognitive deficits. Some of the earlier treatments for schizophrenia resulted in unwanted side effects; nevertheless, with better pharmacological knowledge, more effective, safer antipsychotic drugs are being produced.

1.3.1 Typical antipsychotics

Typical antipsychotics, also referred to as first generation antipsychotics, include haloperidol, chlorpromazine, thioridazine and flupenthixol. These drugs have a high affinity for D2 receptors and are linked to a number of serious side effects. These side effects include dry mouth, muscle stiffness, tremors and extra-pyramidal symptoms (EPS). EPS are a group of side effects caused by hypodopaminergic transmission in the nigrostriatal pathway and resemble symptoms of Parkinson’s disease. These include dystonia (muscle spasms), tardive dyskinesia (involuntary movement of muscles, usually in the face) and akathisia (restlessness). Moreover, although there is high D2 receptor blockade, some patients are resistant to typical antipsychotic treatment. Importantly these drugs also have no effect on negative symptoms or cognitive deficits (Abi-Dargham & Laruelle, 2005).

1.3.2 Atypical antipsychotics

Schizophrenia treatment was revolutionised with the introduction of clozapine in the early 1990s. This drug was found to be better in treating positive symptoms in treatment-resistant schizophrenia patients and also produced fewer EPS. This reduction in EPS may be due to the drug having a different pharmacological profile from typical antipsychotics, with a lower blockade of DA receptors. Clozapine however does cause alternative side-effects such as weight gain, and more seriously, agranulocytosis. Therefore it is mainly prescribed to treatment-resistant schizophrenia patients. Other atypical antipsychotics, such as olanzapine, risperidone and amisulpride, like clozapine, do not act solely by antagonising D2 receptors, but have a wide range of affinities including 5HT receptors, other DA receptor subtypes and histamine receptors (Abi-Dargham & Laruelle, 2005). Despite this relatively broad tropism, Clinical Antipsychotic Trials of Intervention effectiveness (CATIE) studies and Cost Utility of the Latest Antipsychotic drugs in Schizophrenia Study (CUtLASS) failed to find atypical antipsychotics significantly more effective than their predecessors (Lieberman *et al.*, 2005; Jones *et al.*, 2006). Furthermore, cognitive deficits are poorly treated by atypical antipsychotics.

1.3.3 Developing therapies

With multiple hypotheses of the potential underlying neurotransmission deficits involved in schizophrenia now generally accepted, there has been a rapid development of drugs which target these alternative neurotransmitter systems. These drugs include metabotropic glutamate receptor 5 (mGluR5/GRM5) activators which enhance NMDA receptor function. However, there are difficulties in producing drugs which act specifically at subtypes of mGluRs, as their binding sites are very similar. Due to the complex genetic nature of schizophrenia, which most likely leads to the heterogeneity observed in symptom severity, it is probable that patients with symptom clusters or specific polymorphisms will respond better to more targeted treatments. However, the causal genetics and neurotransmitter impairments in schizophrenia are too poorly understood for the development of such drugs at this time.

1.4 Risk factors implicated in schizophrenia

Schizophrenia is a very complex disorder and it is thought that there are several causative factors that can increase the risk of subsequent development of the condition. However, the complexity of schizophrenia suggests that there is not one individual underlying causative factor. Instead it is feasible to say that additive or interactive effects between several candidate genes and several environmental factors are responsible.

1.4.1 Illegal substances

One such risk factor associated with schizophrenia is the use of illegal substances, in particular cannabis. Cannabis use is known to produce effects on the CNS that reflect some of the symptoms of schizophrenia such as impairments in memory and attention, anxiety and hallucinations. Moreover, long term heavy use of cannabis has been associated with long term cognitive deficits (Hall & Solowij, 1998); however, this has been the subject of much debate. Furthermore, there is evidence for increased cannabinoid receptor, CB1 (CNR1), expression in multiple brain regions of schizophrenia patients including the dorsolateral prefrontal cortex (Dean *et al.,* 2001). There have been many studies investigating the link between cannabis use and increased risk to schizophrenia development; however the results of these studies have been variable. In 2005 a meta-analysis of these studies showed that cannabis consumption is related to doubling the risk of schizophrenia (Henquet *et al.*, 2005). It has also been reported that the age of onset of cannabis use affects the risk factor. Arseneault *et al.,* (2002), demonstrated this by reporting that people who regularly used cannabis by early adolescent years (15 years old) were four times more likely to be diagnosed with schizophrenia or a schizophrenia-like-disorder by the age of 26 than controls (people who had never used cannabis or had used it once or twice as an adolescent).

1.4.2 Environmental factors

Environmental risk factors have also been associated with increased risk of schizophrenia. It has been reported that risk of schizophrenia is altered by whether birth and childhood upbringing took place in an urban or rural environment. There is an increased risk of schizophrenia in people who are born in urban areas, and this risk is increased relative to the length of time spent in this environment (Pedersen and Mortensen, 2001). The reason for this change in risk level is unknown; however, differences in diet, exposure to toxins and infections as well as social class have all been suggested as an explanation. In addition to area of birth, time of birth has been proposed as a risk factor for developing schizophrenia. It has been shown in both Northern and Southern hemisphere countries that people born in winter and spring months compared to other seasons are more likely to develop schizophrenia (Torrey *et al.,* 1997).

1.4.3 Intrauterine environmental factors

The environment during prenatal life is also an important causative risk factor for schizophrenia. There is an increased risk of schizophrenia in people whose mother was malnourished during pregnancy. A well documented case of this is the Dutch Hunger Winter during 1944-45, where people conceived during the famine were later shown to have a two fold higher risk of schizophrenia than people conceived in the country in years previous to the famine (Hoek *et al.,* 1998). This increase in risk was replicated in another study investigating the Chinese famine of 1959-61 (St Clair *et al.*, 2005). The most accepted reasoning behind this increase in risk is that intrauterine nutrients are necessary for normal foetal development including development of the brain. Other complications during pregnancy can also lead to the delivered child having an increase in the risk of developing schizophrenia, for example obstetric complications during labour (Cannon *et al.,* 2001) and viral infections during pregnancy such as Influenza (Limosin *et al.,* 2003), Rubella (Brown *et al.,* 2001) and Herpes Simplex (Buka *et al.,* 2001).

1.4.4 Genetic factors

Schizophrenia is known to be a highly heritable disorder and there is a high incidence running through families. The concordance rate of schizophrenia is 80% (Cannon *et al.,* 1998; Cardno *et al.,* 1999; Sullivan *et al.,* 2003). In monozygotic twins, who share 100% genetic material, if one twin has schizophrenia the other twin has a 40-50% risk of also developing schizophrenia (Gottesman *et al.* 1987; Sullivan *et al.,* 2003). This risk drops to approximately 15% in dizygotic twins or siblings who share 50% genetic material (Gottesman *et al.* 1987; Sullivan *et al.,* 2003). Although the concordance rate of schizophrenia is very high, it is not 100%, which suggests schizophrenia is not purely a genetic disorder.

1.4.5 Gene x environment interaction

It is now widely accepted that other factors other than genes (as described in sections 1.4.1, 1.4.2, 1.4.3) are capable of subtly increasing risk for schizophrenia, and in recent years attention has turned to investigating potential interactions between genetic and environmental risk factors. However, these studies are difficult to carry out due to their longitudinal requirements.

To date the most successful gene x environment studies have been those investigating the interaction between genes and cannabis use. One such example of this additive effect is that adolescents who smoke cannabis and have a specific single nucleotide polymorphism (SNP) in the gene *Catechol-O-methyl transferase (COMT)*, which encodes an enzyme which degrades catecholamines (involved in dopamine catabolism), have a greater risk for schizophrenia than an adolescent individual that solely smokes cannabis or solely has the *COMT* gene polymorphism (Caspi *et al.,* 2005). This result however, was not confirmed in a similar subsequent study (Zammit *et al.,* 2007). Another gene, *Neuregulin 1 (NRG1)*, has also been recently linked to having a potential interaction with cannabis, as mice with a mutation in the *Nrg1* gene show a heightened response to the psychoactive component of cannabis, Δ-9-tetrahydrocannabinol (THC) (Boucher *et al.,* 2007).

1.5 Identification of schizophrenia candidate genes

Although it is known that schizophrenia runs in families, it is also known that schizophrenia is not inherited in a Mendelian inheritance pattern, and the precise mechanism of inheritance in schizophrenia remains unknown. Over the past few decades, as advances in molecular biology have significantly progressed, many genes have been identified that may be associated with increased liability to schizophrenia. Advances in technology have facilitated the identification of these schizophrenia candidate genes.

1.5.1 Structural chromosomal studies

Several schizophrenia candidate genes have been identified via studies investigating changes in chromosome structure. Deletion of the chromosomal region 22q11 has been found to lead to velo-cardio-facial-syndrome (VCFS). This microdeletion syndrome is estimated to affect 1:2500-1:4000 live births, and is associated with a 30-fold increase in the risk of affected individuals with VCFS to develop schizophrenia (Murphy *et al.,* 2002; Gothelf *et al.,* 2007). Deletion of this chromosomal region will affect the expression of multiple genes, however. Two of these are of particular interest in terms of schizophrenia. *COMT* which encodes an enzyme initiating the degradation of catecholamines such as the neurotransmitter dopamine by catalysing their methylation, and *Proline dehydrogenase (PRODH)* which encodes an enzyme which initiates the degradation of proline: an amino acid important in the synthesis of glutamate (Harrison & Weinberger, 2005). Since several aspects of schizophrenia have been linked to the manifestation of altered dopaminergic and glutamatergic states in specific brain regions, *COMT* and *PRODH* are likely candidate genes for schizophrenia.

An autosomal balanced translocation involving chromosomes 1q42.1 and 11q14.3 was identified in a large Scottish family that were found to have a very high incidence of schizophrenia, bipolar disorder and major depression (St Clair *et al.*, 1990). Subsequent sequencing of the 1q42 and 11q14.3 breakpoints led to the finding of two novel genes disrupted by the previous mutation, *DISC1* and *DISC2,* with only *DISC1* having the ability to encode protein (Millar *et al.*, 2000).

DISC1 is a promising schizophrenia candidate gene as it has many functions which relate to the pathophysiology of the disorder, with Morris *et al.,* (2003) concluding that "DISC1 is a multifunctional protein whose truncation may contribute to schizophrenia susceptibility by disrupting intracellular transport, neurite architecture, and/or neuronal migration".

More recent studies have shifted from looking at gross chromosomal structural changes to more focussed studies of sub-microscopic copy number variants (CNVs) - microdeletions or microduplications of small stretches of DNA. Several studies utilising genome wide searches for CNVs have found CNVs are more abundant in schizophrenia patients (Kirov *et al.,* 2008; Walsh *et al.,* 2008; Xu *et al.,* 2008). Genes which appear to be affected by these deletions or duplications include those previously implicated in schizophrenia including *Neurexin* (*NRXN1*) and *ERBB4* (Kirov *et al.,* 2008; Walsh *et al.,* 2008; St Clair, 2009). These chromosomal aberrations are highly penetrant variants, which are thought to exist in combination with other genetic variants to increase the genetic risk for schizophrenia.

1.5.2 Linkage studies

The aim of linkage analysis studies, which are carried out using samples from affected families, is to identify regions of the genome which are shared between affected family members but not among unaffected family members (I.e. inherited genetic variation). Many linkage studies have implicated "risk regions" of the genome that appear to be linked with schizophrenia. Meta-analyses of over 30 schizophrenia linkage studies found that chromosomal regions 8p21-22 and 22q11-12 are likely to contain potential schizophrenia candidate genes (Badner & Gershon, 2002; Lewis *et al.,* 2003). Although linkage studies do not identify individual candidate genes they do narrow down regions in which these genes may be positioned; further studies within these regions can then identify individual genes. Several plausible schizophrenia candidate genes have been identified from these aforementioned "risk regions" such as *NRG1* which is located on 8p21-22 (see section 1.8).

1.5.3 Association studies

Unlike linkage studies, association studies can be carried out on unrelated individuals, and aim to investigate if SNPs within a specific gene are associated with disease. SNPs represent common genetic variations which, individually, have low penetrance. Support for many schizophrenia candidate genes has been strengthened by positive findings of association studies, including *dystrobrevin binding protein* (also known as *dysbindin*) *(DTNBP1)* (Straub *et al.,* 2002). A useful resource for the overview of association studies of schizophrenia candidate genes is SZGene (www.schizophreniaforum.org/res/sczgene) which lists both positive and negative findings for many genes. From these findings SZGene composes a list of the current top schizophrenia genetic risk factors (based on genetic evidence only), the top schizophrenia candidate gene as of 1/12/2010 is *PRSS16* which encodes a serine protease. Often negative findings of previously positively associated genes are found, this may be due to limitations in sample size, ethnicities tested and which SNPs were investigated.

Genome wide association studies (GWAS) utilise a similar approach to the aforementioned association studies. However, instead of investigating SNPs in an individual gene, genotypes of cases and controls for half a million to a million SNPs are simultaneously tested on a chip. Because of the large number of statistical comparisons required in such studies, the risk of false positive findings is increased by using this method. Therefore the threshold for a significant finding of a difference in genotype frequency between cases and controls has to be set much higher than that of regular association studies. Several schizophrenia GWAS scans have been carried out since 2007 with many interesting genes being identified, such as the zinc finger gene, *ZNF804A* (O'Donovan *et al.,* 2008). However, three meta-analyses of GWAS scans only found converging results for the major histocompatibility region on chromosome 6 being associated with schizophrenia (Purcell *et al.,* 2009; Shi *et al.,* 2009; Stefansson *et al.,* 2009).

Although converging evidence is emerging for genetic risk factors for schizophrenia, the polygenic nature of the disorder makes the identification of the underlying genetic risks very complex. It has been suggested that the complex genetic risk of schizophrenia is due to low penetrance variations in 1000s of individual genes (Purcell *et al.,* 2009). However, it is also likely that it is a combination of multiple common polymorphisms in schizophrenia candidate genes and rare *de novo* mutations that contributes to the risk of schizophrenia. With the ongoing development of new technologies, such as next generation sequencing, the genetic burden of schizophrenia will be better understood.

1.6 Measuring cognition in rodents

Cognitive deficits are now considered to be a core symptom domain of schizophrenia, with cognitive performance being closely correlated with functional outcome in patients (Green, 1996; Green, 2006). Unfortunately current antipsychotic therapies are inefficient at treating these cognitive deficits (Harvery & Keefe, 2001; Keefe *et al.,* 2007). Therefore, an initiative was developed to identify the cognitive symptoms that have unmet therapeutic need, called the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative. This initiative identified seven domains of cognition that are affected in schizophrenia 1) attention/vigilance 2) working memory 3) reasoning and problem solving 4) processing speed 5) visual learning and memory 6) verbal learning and memory 7) social cognition (Marder *et al.,* 2004).

Preclinical assessment that maps onto these symptom domains in rodents may allow the discovery and selection of potentially useful therapies for treating the cognitive deficits of schizophrenia. For example attention/vigilance and processing speed can be measured in the 5-choice serial reaction time task (5-CSRTT), a preclinical equivalent to the continuous performance task (CPT) in humans (Robbins, 2002). Problem solving and reasoning can be assessed by the attentional set-shifting task which is analogous to the Wisconsin Card Sorting Task (WCST) used in humans (Birell & Brown, 2000). Other tasks such as social recognition and social interaction allow assessment of social cognition, whereas working memory can be assessed by the radial arm maze and odour span tests (Young *et al.,* 2009).

1.7 Animal models

With the complexity of schizophrenia come limitations to the creation of animal models of the disorder. Although it is unlikely that animal models are capable of encapsulating all aspects of the disorder, it is possible to model components of schizophrenia. To date animal models have allowed a better understanding of the underlying neurotransmitter systems that are likely to be dysfunctional in schizophrenia. They also allow preclinical testing of potential new therapies for schizophrenia. There are several different mechanisms by which animal models of schizophrenia have been generated, each mechanism generating models reflecting slightly different aspects of schizophrenia, with their own distinct advantages and disadvantages.

1.7.1 Pharmacological models

Pharmacological animal models of schizophrenia are based upon the current knowledge of the alterations in neurotransmitter systems that are thought to lead to the symptoms of schizophrenia. For example, based on the glutamate hypothesis of schizophrenia (section 1.2.2), administration of PCP to rats has been shown to result in not only biochemical changes but also behavioral deficits that reflect those observed in schizophrenia patients. Previous studies from our laboratory have shown that repeated PCP administration to rats led to reduced *parvalbumin* (*Pvalb*) mRNA and *Kv3.1* (*Kcnc1*) potassium channel mRNA, which are both expressed in GABAergic interneurones (Cochran *et al.,* 2002; Cochran *et al.,* 2003). Additionally, PCP causes reduced metabolic activity in PFC and reduces network connectivity (Cochran *et al.*, 2003; Dawson *et al.,* 2010). Furthermore, transcriptome analysis of PFC tissue, from rats chronically treated with PCP, showed that PCP treatment altered the expression of many genes with functions related to the glutamate and GABA systems (Catherine Winchester, personal communication). These alterations included genes located on chromosomal loci that have been previously implicated in schizophrenia including 8p22-21 (Winchester *et al.,* 2007). In addition PCP treatment in rats has resulted in deficits in behaviours that are dysfunctional in schizophrenia, including sensorimotor gating (Egerton *et al.,* 2005), executive function as assessed by set-shifting (Egerton *et al.*, 2003; Egerton *et al.*, 2005; Dawson *et al.*, 2010) and attention, vigilance and processing speed (Thomson *et al.*, 2010).

Other pharmacological substances, including toxins, have also been utilized to generate animals which resemble some of the aspects of schizophrenia. For example prenatal exposure of rats to the mitotoxin methylazoxymethanol (MAM) decreases cortical mass, causes deficits in PPI, heightened locomotor response to amphetamine and deficits in working memory (Moore *et al.*, 2006).

1.7.2 Environmental models

As described in section 1.4.2, environmental factors have also been implicated in increasing the risk to schizophrenia. Rodents with environmental insults show biochemical and behavioral changes that resemble those of schizophrenia. Rats or mice that are moved into social isolation post-weaning show deficits in prepulse inhibition, locomotor activity and object recognition (Jones *et al.*, 2010) along with decreased cortical and hippocampal synaptic plasticity (Comery *et al.*, 1995; Silva-Gomez *et al.*, 2003).

1.7.3 Genetic models

Genetic association studies have identified polymorphisms within many genes that increase risk for schizophrenia; in addition numerous genes have been identified/hypothesised as functional candidate genes based on their endogenous roles within the CNS which are known to be dysfunctional in schizophrenia. However, to date no causative genes have yet been identified. Therefore, in order to understand further the functions of these genes and to gain a better insight into how they may be potentially pathophysiological in schizophrenia, animal models have been generated with altered forms of schizophrenia candidate genes.

For example animal models with deletion of chromosome 22q11.2 show sensorimotor gating and working memory deficits that reflect aspects of schizophrenia (Paylor *et al.*, 2001). Targeted mutation of selected schizophrenia genes in rodents has permitted a greater understanding of how the gene of interest may play a role in structural and biochemical changes of schizophrenia. Animal studies of *Disc1* show a strong link between the gene and schizophrenia. For instance Kamiya *et al.*, (2005) demonstrated that loss of expression of *Disc1* by RNAi (quantified by western blot) resulted in impaired cerebral cortex development, whereas another study demonstrated that Disc1 dysfunction in schizophrenia may lead to altered glycogen synthase kinase 3β (GSK3β) signalling (Mao *et al.,* 2009). In addition functions of Nrg1 and Erbb4 have been better understood by animal models of these genes (see section 1.9.3).

1.8 Neuregulin-Erbb signalling

1.8.1 Neuregulin 1 (NRG1) nomenclature and structure

Neuregulins are a family of four growth factor genes (*NRG1-4*) that mediate important roles in a number of different organs by activating receptor tyrosine kinases. Human *NRG1* (also known as heregulin) was first identified in the early 1990's (Holmes *et al.,* 1992), with the identification of *NRG2*, *NRG3* and *NRG4* occurring in subsequent years (Carraway *et al.,* 1997; Zhang *et al.,* 1997; Harari *et al.,* 1999). Of the neuregulins, *NRG1* has been most widely studied due to its important roles in CNS and cardiac development. However, recently there has been a surge of interest in *NRG3* as it is the only *neuregulin* with expression restricted to the brain and its binding ability restricted to specific receptor tyrosine kinase sub-types (Zhang *et al.,* 1997), making *NRG3* a potentially attractive molecule to study in terms of neurological disorders.

The structure of the human *NRG1* gene has been widely investigated since its discovery. *NRG1* is a large gene (1.4 megabases (Mb)) located on chromosome 8p12.21, and due to multiple promoter transcription start sites and alternative splicing there are approximately 31 transcript isoforms (Mei & Xiong, 2008). The nomenclature of these isoforms is based upon four structural characteristics: 1) The N-terminal encoded sequence determines the "type" of NRG1, with six distinct types of NRG1 (NRG1 type I-VI) having been identified to date. Types I, II, IV and V are similar in that they possess an immunoglobulin (Ig) domain whereas type III possesses a cysteine rich domain (CRD). Type VI *NRG1* lacks the exons that encode for Ig and CRD domain and so little is known about its processing and signalling capabilities. 2) All NRG1s contain an epidermal growth factor (EGF)-like domain; however there are three types of EGF-like domains termed α, β and γ. 3) The linker region between the EGF-like encoding domain and the C-terminal transmembrane encoding domain (TMD) is also subject to alternative splicing, resulting in four variants (1-4) of the linker region. 4) The C-terminal domain can consist of a cytoplasmic tail of type a, b or c (Falls, 2003; Mei & Xiong, 2008). These isoform defining characteristics determine the function and regional expression of NRG1, for example NRG1 isoforms with a β EGF-like domain and a type "a" cytoplasmic tail are the most prominently expressed in the brain (Falls, 2003). A simplified summary of the NRG1 nomenclature is shown in Figure 1-1.

The *NRG1* gene encodes a membrane bound pro-protein which is susceptible to cleavage by proteases. Tumour necrosis factor-α converting enzyme (ADAM17, TACE), β-amyloid precursor protein cleaving enzyme (BACE) and meltrin β (ADAM19) act at a juxtamembrane site close to the EGF-like domain (Loeb & Fischbach, 1995; Sunnarborg *et al.*, 2002; Yokozeki *et al.*, 2007). This cleavage results in the release of the bioactive extracellular fragment of NRG1 that can then initiate signalling cascades via binding to receptor tyrosine kinases expressed on the same cell type (autocrine signalling) or other cell types (paracrine signalling). Type III NRG1 is unique in that not only is it the only NRG1 type to consist of a CRD but also because its transmembrane domain is located within the N-terminal domain. Thus cleavage of the type III pro-protein does not result in release of a bioactive extracellular fragment, but instead exposes the bioactive EGF-domain as a tethered ligand still membrane bound. Type III NRG1 isoforms are therefore thought only to be capable of juxtacrine signalling.

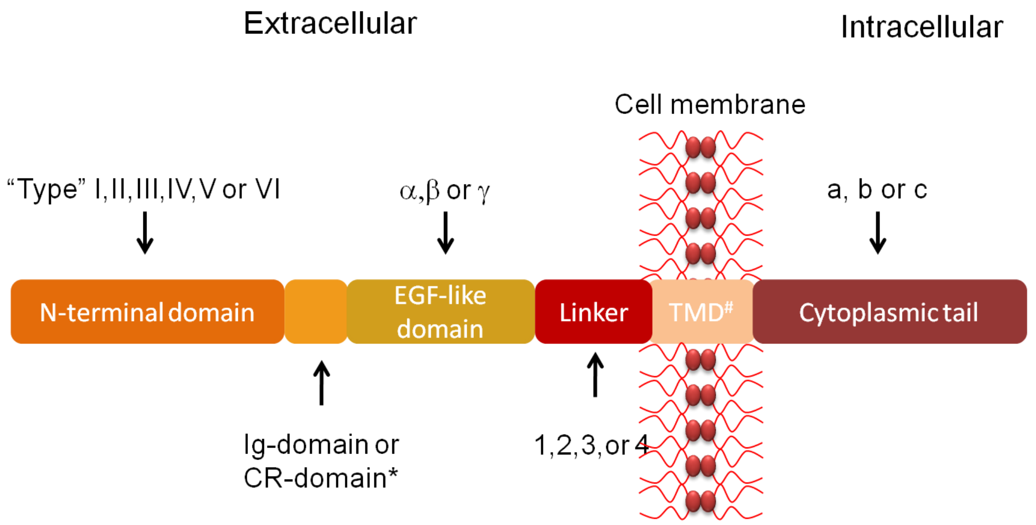


Figure 1.1 Neuregulin-1 isoform nomenclature. NRG1 isoforms arise from combinations of type (I-VI), presence of Ig or CR domain, α,β or γ EGF domain, linker region (1-4), cytoplasmic tail a, b or c.* Type VI Nrg1 does not contain either Ig or CR domain. # Type III Nrg1 has an additional TMD located within the N-terminal domain. Ig: Immunoglobulin, CRD: cysteine rich domain, EGF: epidermal growth factor, TMD: transmembrane domain.

1.8.2 NRG1 expression in the CNS

NRG1 is widely expressed in the adult human brain, including the prefrontal cortex, hippocampus, cerebellum and substantia nigra- areas which have been linked to being important in schizophrenia (Law *et al.,* 2004; Bernstein *et al.,* 2006). Similarly, in the adult rat brain *Nrg1* expression has been shown in these structures (Chen *et al.,* 1994; Kerber *et al.,* 2003). *NRG1* isoform expression in the brain appears to be spatially and temporally controlled. Where types I, II and III *NRG1* are expressed in the cortex of adult human and rat brain (Kerber *et al.,* 2003; Hashimoto *et al.,* 2004), type IV NRG1 appears to be brain specific and is restricted to human expression (Steinhorsdottir *et al.,*2004; Tan *et al.,* 2007). Moreover *NRG1* isoform expression changes during development. Type IV *NRG1* is expressed 3.5 fold times higher in foetal human brain than that of the adult (Tan *et al.,* 2007). Moreover, in rodents Nrg1 protein expression in the hippocampus, cerebellum and cortex is lower in adulthood than in foetal tissues (Chen *et al.,* 1994). In mice the first embryonic expression of Nrg1 is type III NRG1 expression, which is followed by the expression of type I then type III, suggesting the isoforms have distinct roles during different stages of development.

NRG1 mRNA has been shown to be clearly expressed in cortical pyramidal neurons and interneurones of the adult human cortex and hippocampus, and in Golgi and Purkinje cells of the cerebellum (Law *et al.,* 2004; Bernstein *et al.,* 2006). In agreement, Kerber *et al.,* (2003) found Nrg1 protein expression in granular and Purkinje cells of cerebellum in adult rat (Kerber *et al.,* 2003). Type II NRG1 is expressed in oligodendrocytes, microglia and astrocytes of the adult human brain (Cannella *et al.,* 1999). Subcellular location of NRG1 has been shown to be predominantly in the neuronal cell body and synapse rich regions, with clustering at cell membranes (Chaudhury *et al.,* 2003; Law *et al.,* 2004).

1.8.3 ERBB4 nomenclature and structure

ERBB4 is one of four members of the ERBB receptor tyrosine kinase (RTK) family. *ERBB1-4* are located on human chromosomes 7, 17, 12 and 2, respectively, and encode type 1 receptor tyrosine kinases ERBB1 (epidermal growth factor receptor; EGFR), ERBB2, ERBB3 and ERBB4 (Ullrich *et al.,* 1984; Yamamoto *et al.,* 1986; Kraus *et al.,* 1989; Plowman *et al.,* 1993). Human *ERBB4*, like *NRG1*, is a large gene of approximately 1.15Mb, consisting of 28 exons (Mei & Xiong, 2008). The *ERBB4* gene is also subject to alternative splicing, and as a result four isoforms of *ERBB4* have been identified. *JMa ERBB4* isoforms contain exon 16 but not exon 15, while *JMb* isoforms contain exon 15 but not 16, consequently these isoforms differ structurally in their juxtamembrane sequence affecting their susceptibility to proteolytic cleavage (Elenius *et al.,* 1997a). *CYT* isoforms differ in their cytoplasmic tail sequence, with *CYT1* isoforms containing exon 26 and thus a 16 amino-acid sequence that is the binding site for PI3-K. *CYT2* isoforms lack exon 26 and therefore activation of CYT2 containing ERBB4 receptors by NRG1 does not activate the PI3-K signal transduction pathway (Elenius *et al.,* 1999). A very recent study has identified novel *ERBB4* transcripts with alternative splicing of exon 3. These isoforms have been termed *del.3* and appear to exist only in *CYT2* transcripts (i.e. not containing exon 26) (Tan *et al.,* 2010). It is unlikely that these are the only naturally occurring *ERBB4* isoforms, and indeed further novel isoforms have been identified in rare forms of cancers termed *JMc* and *JMd* (Gilbertson *et al.,* 2001). The nomenclature for *ERBB4* isoforms is diagrammatically shown in Figure 1-2.

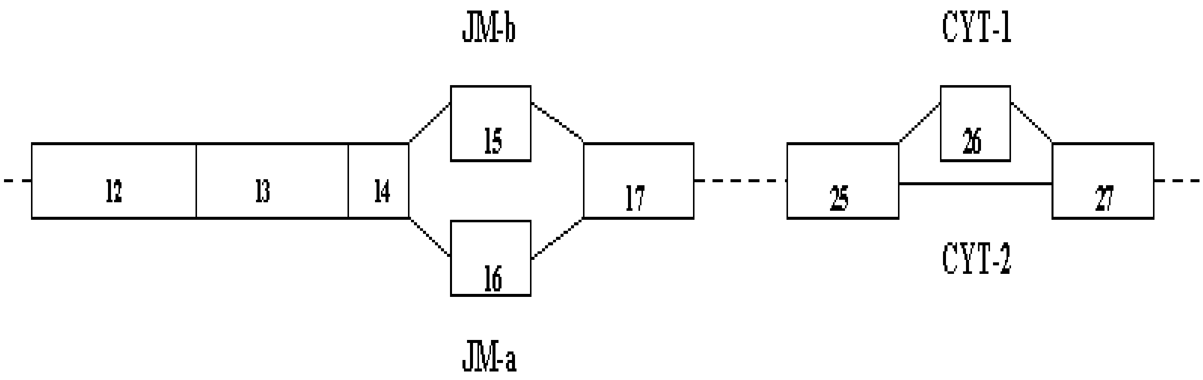


Figure 1.2 Partial exon structure of *ERBB4* isoforms *JMa, JMb, CYT1* and *CYT2* produced by alternative splicing. *JMa* and *JMb* isoforms resulting from the inclusion of exon 16 or 15, which in turn renders them sensitive or insensitive to proteolytic cleavage by presenilin dependent γsecretase respectively. *CYT1* and *CYT2* isoforms result from the inclusion or exclusion of exon 26, respectively, resulting in the ability or inability to activate the PI3-K transduction pathway. Therefore four combinations of receptor are possible, JMa/CYT1, JMa/CYT2, JMb/CYT1 or JMb/CYT2. Dashed line indicates continuation of exons not shown in diagram. Exons are numbered and represented as boxes, with intronic DNA represented by dashed lines (size is not to scale).

1.8.4 ERBB4 expression in the CNS

Human *ERBB4* is highly expressed in the heart, kidney and neuronal tissues (Plowman *et al.,* 1993). The levels of *ERBB4* isoform expression appears to be tissue dependant, with *JMa* isoforms being solely expressed in the kidney, whereas the heart only expresses *JMb ERBB4* isoforms (Elenius *et al.,* 1997a). In addition *ERBB4 CYT2* isoforms are predominantly expressed in neural and renal tissues while *CYT1* is predominantly expressed in the breast and heart (Elenius *et al.,* 1999).

ERBB4 is expressed in most regions of the adult human brain, and in all cortical areas with intense expression specifically in cortical layers II and V (Bernstein *et al.,* 2006). This general expression pattern is also seen in the brains of non-human primates (Thompson *et al.,* 2007). To date the regional expression of *ERBB4* isoforms is unknown, however it has been shown that all four isoforms (JMa, JMb. CYT1 and CYT2) are expressed to some extent in cortical and hippocampal tissues of human brain (Law *et al.,* 2006). In the adult rat brain *Erbb4* is widely expressed in the cortex, amygdala, hippocampus, reticular thalamic nucleus and cerebellum (Steiner *et al.,* 1999; Gerecke *et al.,* 2001).

All isoforms of *ERBB4* including the newly identified *del.3* transcript are expressed in the foetal brain (Tan e*t al.,* 2010). *Erbb4* expression in rats has been shown to increase with development (Mechawar *et al.,* 2007). The subcellular location of Erbb4 has been identified. In general Erbb4 expression has been identified as somatodendritic, with Erbb4 immunoreactivity or mRNA expression localising to the cytoplasm and/or plasma membrane (Gerecke *et al.,* 2001; Mechawar *et al.,* 2007). However, the expression has also been observed within the nucleus of cortical and hippocampal cells (Mechawar *et al.,* 2007). The complex proteolytic processing of ERBB4 may explain this diffuse cellular expression.

Moreover, ERBB4 is expressed in many cell types. ERBB4 has been identified in interneurones in human brain (Bernstein *et al.,* 2006), and rat brain (Steiner *et al.,* 1999; Mechawar *et al.,* 2007). More recently Erbb4 protein expression has been shown to be in GABAergic interneurones (Yau *et al.,* 2003; Fishan *et al.,* 2009; Vullhorst *et al.,* 2009; Neddens & Buonanno, 2010). ERBB4 has also been identified in pyramidal neurons (Berstein *et al.,* 2006; Mechawar *et al.,* 2007; Thompson *et al.,* 2007). However, this pyramidal cell expression has been subject to debate recently as studies utilising newly synthesised antibodies generated against Erbb4 have found no expression of Erbb4 in pyramidal cells of the mouse hippocampus (Vullhorst *et al.,* 2009; Neddens & Buonanno, 2010). Expression of ERBB4 in glia is also somewhat unclear with studies showing presence (Ma *et al.,* 1999; Bernstein *et al.,* 2006) and absence (Mechawar *et al.,* 2007) of co-localisation of ERBB4 with glial markers. Erbb4 has also been shown to be expressed in dopaminergic neurons of the substantia nigra and ventral tegmental area (Steiner *et al.,* 1999).

1.8.5 NRG-mediated ERBB4 activation

All ERBB receptors are alike in that they contain four domains; an ectodomain containing two cysteine rich domains, a single transmembrane domain, intracellular juxtamambrane domain and a carboxy-terminal tail (Mei & Xiong, 2008). Ligand binding to the ectodomain triggers receptor dimerisation and subsequent auto and trans-phosphorylation of the intracellular tyrosine kinase residues on the receptor, which serve as docking sites for various adaptor proteins. These proteins then activate numerous intracellular signalling cascades.

Each type of ERBB receptor has a distinct ligand binding profile. There are seven known ligands that bind with high affinity to ERBB4, including three known ERBB1 ligands; betacellulin (Riese *et al.,* 1996), heparin binding epidermal growth factor (Elenius *et* al., 1997b) and epiregulin (Komurasaki *et al.*, 1997) as well as neuregulins 1-4 (NRG1-4) the most potent being NRG1-1β (Tzahar *et al.,* 1994). Upon activation, receptor dimerisation can be homodimerisation, where two ERBB receptors of the same type come together, or heterodimerisation, where two differing ERBB receptors form a complex. ERBB4 receptors are capable of homodimerisation (i.e. ERBB4/ERBB4), whereas, ERBB3 lacks an enzymatically active tyrosine kinase domain and so requires heterodimerisation with ERBB2 as a co-receptor for signalling (Guy *et al.,* 1994). ERBB2 itself is thought to be an orphan receptor as no ligands have been identified; instead ERBB2 forms heterodimers with the other ERBB receptors, activating signalling as

ERBB2 does have an active tyrosine kinase domain. ERBB2/ERBB4 heterodimers are thought to potentiate ERBB4's signalling capacity by increasing the affinity of the ligand binding site, thus accentuating the signalling response to ligand binding (Graus-Porta *et al.,* 1997; Wang *et al.,* 1998; Jones *et al.,* 1999).

Following ligand binding and ERBB4 dimerisation, phosphorylation of the intracellular domains generates docking sites for adapter proteins such as SHC (Culouscou *et al.,* 1995) and GRB7 (Fiddes *et al.,* 1998). This initiates mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3-K) signalling cascades (Puricelli *et al.,* 2002).

Like NRG1, the ERBB4 receptor is susceptible to proteolytic cleavage. Cleavage of the juxtamembrane domain of JMa containing ERBB4 isoforms by TACE releases an extracellular fragment containing the ligand binding site (Elenius *et al.,* 1997a; Cheng *et al.,* 2003). This extracellular fragment can then bind to pro-protein membrane bound NRG's or type III NRG1, to initiate intracellular signalling. Furthermore, the remaining 80kDa membrane bound fragment of ERBB4 can be further cleaved by γ-secretase to release an intracellular fragment (Ni *et al.,* 2001; Vidal *et al.,* 2005). This intracellular fragment can translocate to the nucleus where it has been shown to interact with transcriptional factors which may regulate gene expression (Omerovic *et al*., 2004; Linggi & Carpenter, 2006). This backwards signalling has also been proposed for NRG1, which can also be cleaved by γ-secretase. This intracellular fragment can also translocate to the nucleus and has been shown to regulate the promoter of post synaptic density 95 (PSD95) (Bao *et al.,* 2004).

1.9 Evidence for aberrant NRG1-ERBB4 signalling in schizophrenia

NRG1 and *ERBB4* have both been implicated as strong candidate genes for increasing the risk to schizophrenia due to evidence from studies of their endogenous functions in the CNS, genetic studies and animal models. Based on the evidence from genetic studies, *NRG1* is currently at position 26 in the SZGene database.

1.9.1 Biological functions related to schizophrenia

NRG1 signalling has been shown to have central functions in the regulation and development of several organs including those of the cardiovascular system (Erickson *et al.,* 1997; Rentschler *et al.,* 2002; Kuramochi *et al*., 2006) and breast tissue (Yang *et al.,* 1995; Jones *et al.,* 1996; Li *et al.,* 2002), however, it is the important roles that NRG1 plays in the CNS that have linked it to schizophrenia.

NRG1 plays central roles in systems that are affected in schizophrenia (for example the regulation of oligodendrocytes and myelination, which appears to be abnormal in schizophrenia) (Hakak *et al.,* 2001; Hof *et al.,* 2002; Flynn *et al.,* 2003; Tkachev *et al.,* 2003). In addition, studies of ERBB4 have identified many important functions including mediating axonal navigation between the thalamus and neocortex (Lopez-Bendito *et al.,* 2006), regulation of neuroblast chain migration, orientation and organisation (Anton *et al.,* 2004), promotion of proliferation and organisation of neuronal precursors in the sub ventricular zone (Ghashghaei *et al.,* 2006), and regulation of neuronal migration along radial glial fibres (Rio *et al.,* 1997). These actions appear to be dysfunctional in schizophrenia and are a potential explanation for the loss of synaptic connectivity observed in the disease, which could implicate ERBB4's role in the cognitive deficits seen in schizophrenia.

NRG1-ERBB4 signalling has also been implicated in regulating the expression of neurotransmitter receptor subunits, indicating NRG1-ERBB4 signalling dysfunction may affect neurotransmitter systems that have previously been proposed to be altered in schizophrenia. For example, ERBB4 appears to have distinct interactions with GABA receptors: ERBB4 signalling through activation by immunoglobulin-domain containing isoforms of Nrg1 increases the expression of the $GABA_A$ receptor $\beta 2$ subunit as well as increasing the number of functional $GABA_A$ receptors in cerebellar granule cells *(*Rieff *et al.,* 1999*)*. However, Okada *et al.,* (2004), found that Ig-NRG, which was in close proximity to ERBB4 receptors, down-regulated the expression of $GABA_A$ receptor α subunits in hippocampal slices. It is probable to assume that this effect of NRG1, hypothetically through ERBB4 receptors, is brain region and/or $GABA_A$ subunit dependent. Flames *et al., (*2004), showed that NRG1/ERBB4 signalling has an important role in the development and migration of GABAergic interneurons, as loss of the functionality of either NRG1 or ERBB4 resulted in a decrease in the number of GABAergic interneuron's migrating to the cortex. Therefore this creates a direct link between dysfunction of NRG1/ERBB4 signalling and defective GABA neurotransmission as seen in schizophrenia (Lewis *et al.,* 2000; Benes & Berretta 2001). In addition NRG1 signalling, particularly through type III NRG1, regulated neuronal acetylcholine receptor expression (Yang *et al.,* 1998; Liu *et al.,* 2001). NMDA subunit expression is also regulated by NRG1 (Ozaki *et al.,* 1997).

NRG1-ERBB4 signalling has been further linked to regulating NMDA neurotransmission. Huang *et al.,* (2000) reported that ERBB4 is co-localised with PSD95, and more recently it has been shown both NRG1 and ERBB4 bind to PSD95 through the PDZ domain of their C termini (Fujikawa *et al.,* 2007). Altering the level of PSD95, by over-expression or inhibition, enhanced or attenuated activation of ERK by means of NRG1 (Huang *et al.,* 2000). Furthermore, PSD95, ERBB4 and NMDA receptors co-localise at excitatory synapses, offering potential that there is an interaction between these proteins (Garcia *et al.,* 2000). Following these studies Gu *et al.,* (2005) found that Nrg1 decreased NMDA receptor currents in both cultured rat PFC neurons and rat PFC tissue slices, and that this decrease was attenuated by inhibiting Erbb receptors as well as blockers of downstream molecules. The authors suggest that the decrease in NMDA receptor currents is due to an increase in receptor internalisation. However, as the inhibitors were non-specific for all Erbb receptors, it is unclear as to which were responsible. A direct link between NRG1/ERBB4/PSD95 and schizophrenia was made by Hahn *et al.,* (2006), who showed that in human schizophrenic *post-mortem* prefrontal cortex tissue slices there was an increase in PSD95-ERBB4 interaction versus tissue from unaffected individuals. This study also showed that although there were no significant changes in the levels of NRG1 or ERBB4 between groups, there was enhanced NRG1-ERBB4 signalling in the tissue from schizophrenic patients and that this enhanced signaling led to a greater reduction of NMDA receptor function in schizophrenia. This observation directly links with the hypothesis that NMDA receptor function is reduced in schizophrenia, and thus alterations in NRG1-ERBB4 signalling caused by a genetic deficit in schizophrenia may contribute to this hypofunction.

Because of the findings that ERBB4 and NRG1 are enriched at the post synaptic density, in recent years the role NRG1-ERBB4 signalling may play in regulating synaptic plasticity has been investigated. NRG1-ERBB4 has been shown constitutively to regulate long term potentiation (LTP) (Kwon *et al.,* 2005; Pitcher *et al.,* 2008) through a dopamine dependent mechanism (Kwon *et al.,* 2008).

Another connecting factor linking NRG1's biological function to schizophrenia is that NRG1 is important in the onset of puberty and sexual development (Toyoda-Ohno *et al.,* 1999). Mice that have dysfunctional NRG1 signalling have delayed onset of puberty and reproductive development due to levels of reproductive hormones being reduced (Prevot *et al.*, 2003). As these hormones are of utmost importance at the time of the onset age of schizophrenia, this indicates that if indeed there was a link between schizophrenia and NRG1 signalling dysfunction, it could be viable that the neurodevelopmental abnormality is due to a change in levels of these critical hormones.

1.9.2 Genetic evidence for NRG1-ERBB4 dysfunction in schizophrenia

NRG1 and *ERBB4* are located at 8p21-22 and 2q33.3-34, respectively, regions highlighted as risk regions in one or both of the aforementioned meta-analyses of linkage studies (Badner & Gershon, 2002; Lewis *et al.* 2003). There have been several lines of research strengthening the association of both *NRG1* and *ERBB4* with schizophrenia. A major breakthrough came from an Icelandic familial study when Stefansson *et al.* (2002) reported that there was significant linkage between a core haplotype of *NRG1*, consisting of five single nucleotide polymorphisms (SNPs) and two microsatelites, and schizophrenia. This association between *NRG1* and schizophrenia has been subsequently replicated in 20 case control studies and a further 9 family based studies in multiple populations including those of European, American and Asian origins. However, there have also been several studies finding no association in these populations.

A summary of all of the schizophrenia association studies carried out on *NRG1* to date can be found on SZGene (http://www.szgene.org/geneoverview.asp?geneid=311).

Several association studies have strengthened the link of *ERBB4* as a risk gene for schizophrenia. In a large case/control study from the UK and Ireland, association was found between variants in *ERBB4* with an increased risk of schizophrenia, and this association may be through the interaction with the Icelandic *NRG1* haplotype (Norton *et al.*, 2006). This association was replicated by two further studies (Silberberg *et al.*, 2006; Law *et al.*, 2007), where both found association of three intronic SNPs; rs7598440, rs839523 and rs707284, which form a core risk haplotype in exon 3 of *ERBB4,* in linkage disequilibrium in three separate populations. Further association studies have positively associated *ERBB4* with schizophrenia; however, as with *NRG1,* negative findings have also been documented. A summary of all of the schizophrenia association studies carried out on *ERBB4* to date can be found on SZGene (http://www.szgene.org/geneoverview.asp?geneid=273). In addition, in a study of rare structural variants in schizophrenia a novel deletion in *ERBB4* was found (Walsh *et al.*, 2008).

1.9.3 Biochemical evidence for NRG1-ERBB4 dysfunction in schizophrenia

A number of studies in human *post-mortem* brain tissue have shown an alteration in the expression of *NRG1* in schizophrenia. Hashimoto *et al.*, (2004) found that in the human dorsolateral prefrontal cortex (DLPFC) there was a 23% increase in *NRG1* type I mRNA in tissue from schizophrenia patients compared to samples from unaffected individuals, with no alterations in expression of the other *NRG1* subtypes. These findings were also replicated in the hippocampus (Law *et al.*, 2006); however this study did not find small decreases in subtype ratios that were observed by Hashimoto *et al.*, (2004). More recently NRG1 protein levels were found to be increased selectively within the PFC of schizophrenia patients (Chong *et al.*, 2008). Conversely, a study by Hahn and colleagues (2006), found no alteration in the expression of NRG1 in the DLPFC of *post-mortem* human schizophrenic samples in comparison to unaffected control samples.

Furthermore, there is also evidence of altered expression of *ERBB4* in schizophrenia. Two studies have found that *ERBB4* expression in the DLPFC is increased in schizophrenia, and that these increases are due to significant increases in *JMa* and *CYT1* splice isoforms (Silberberg *et al.,* 2006; Law *et al.,* 2007). Additionally Law *et al.*, (2007) observed that this increase was not present in the hippocampus but appeared to be specific to the PFC, and was associated with specific SNP allelic expression of *ERBB4.* Although no changes in ERBB4 protein expression have been identified in one study (Hahn *et al.,* 2006), the previous findings of increased ERBB4 in schizophrenia have been supported by two recent studies (Chong *et al.,* 2008; Law *et al.,* in press).

It should be noted however, that several studies have suggested that *Nrg1* and *Erbb4* expression is altered in rats chronically treated with anti-psychotic drugs (Wang *et al.,* 2008; Chana *et al.,* 2009), although these results were not replicated in a recent study (Law *et al.,* in press). Therefore, changes in expression of these genes in schizophrenia may be partly attributable to long-term exposure to antipsychotic treatments.

1.9.4 Animal models of aberrant Nrg1-Erbb4 signalling

In addition to the association and expression studies, animal models have also provided positive evidence for *NRG1* and *ERBB4* as functional candidate genes for schizophrenia. Heterozygous *Nrg1* knockout mice have shown open field hyperactivity (Gerlai *et al.,* 2000; Stefansson *et al.,* 2002; O'Tuathaigh *et al.,* 2005; Karl e*t al.,* 2007). However, with the complexity of the *NRG1* gene it is not surprising that this behavioural phenotype was not observed in other mouse models with mutated *Nrg1* (Rimer *et al.,* 2005; Erlichman *et al.,* 2009). Sensorimotor gating has also been shown as dysfunctional in some *Nrg1* mouse models (Stefansson *et al.,* 2002; Chen *et al.,* 2008; Deakin *et al.,* 2009) but not in others (Erlichman *et al.,* 2009). Other behaviours more representative of the cognitive deficits have been observed in *Nrg1* mouse models, such as disrupted latent inhibition (Rimer *et al.,* 2005), fear conditioning and novel object recognition (Duffy *et al.,* 2010).

In agreement that NRG1 signalling is dysfunctional in schizophrenia, mutations in the *Erbb4* gene in mice result in similar behavioural deficits reflective of schizophrenia. These behaviours include hyperactivity (Stefansson *et al.,* 2002; Golub *et al.,* 2004; Wen *et al.,* 2010), dysfunctional pre-pulse inhibition (Stefansson *et al.,* 2002; Wen *et al.,* 2010), social interaction (Roy *et al.,* 2007) and working memory deficits (Golub *et al.,* 2004; Wen *et al.,* 2010). Furthermore, Gerlai *et al.,* (2000) showed that the schizophrenia-related behavioural phenotype seen in heterozygous *Erbb4* rodents was specific and not observed in heterozygous mutants of *Erbb2* or *Erbb3*. Although there has been a recent advance in animal modelling of these two genes, to date these studies have been limited to mice, and investigate the effect of gene dysfunction throughout the entire brain.

As well as behavioural changes, animal models of *Nrg1* and *Erbb4* have allowed the elucidation of other neurobiological roles of these genes within the CNS. For example *Nrg1* mutant mice have impaired short and long term potentiation (Bjarnadottir *et al.,* 2007) and alterations in the expression of serotonin-related genes (Dean *et al.,* 2008). Moreover mice with alterations in *Erbb4* show reduced dendritic spine density (Barros *et al.,* 2009) and altered GABAergic neurotransmission (Wen *et al.,* 2010).

1.10 Regulating gene expression

For several decades the genetic composition of rodents has been manipulated to allow the understanding of the underlying function of the targeted genes. Knockout animals have been successfully bred and are an invaluable tool in scientific research; however, as in the case of *Nrg1* and *Erbb4* homozygous mice, knockout can be lethal (Gassmann *et al.,* 1995; Seshadri *et al.,* 2010). Heterozygous “knockouts” of schizophrenia candidate genes are commonly bred where only one copy of the endogenous gene is deleted or manipulated, permitting the gene expression to be significantly reduced but still allowing viability of the animal. One of the major problems with knockout animals is that the effect of the mutation is not regional but is widespread throughout the entire body. In the past 10 years it has come to light that there are alternative approaches to regulating the expression levels of specific genes. One such approach is RNA interference (RNA_i).

1.10.1 RNAi

This method of endogenous gene silencing allows for double stranded RNA (dsRNA) with a sequence complementary to the gene of interest to be introduced into cell cultures or organisms, where it recognises and causes initiation of the RNAi pathway, knocking down transcription of the target gene. The RNAi pathway can be effectively induced *in vitro and in vivo* by the application of short interfering RNAs (siRNA) or by the expression of short hairpin RNAs (shRNA) via viral or non-viral vectors.

The term RNAi was coined in 1998 by Fire and colleagues who discovered that injecting dsRNA into the body or gonad of nematode *Caenorhabditis elegans* resulted in silencing of a gene with sequence complementary to that of the dsRNA (Fire *et al.,* 1998). This phenomenon had already been observed in plants (Napoli *et al.,* 1990) and fungi (Romano and Macino, 1992), where the phenomenon observed was termed posttranscriptional gene silencing (PTGS) and quelling, respectively.

1.10.2 RNAi pathway

When long dsRNA enters the cell, it is recognised by a dsRNA-specific endonuclease called *Dicer*. This protein cleaves the dsRNA into short dsRNAs of around 21 to 23 nucleotides with 2 nucleotide long 3 prime (3') overhangs. These siRNAs then form a complex with several proteins including Argonaut, this complex is termed the RNAi silencing complex (RISC). RISC causes unwinding of the ds siRNA sequence leaving one single antisense strand of complementary RNA bound to the RISC, which binds to the target mRNA in a sequence specific manner.

Binding of the siRNA-RISC to the target mRNA results in the target mRNA being cleaved in the middle of the siRNA complementary sequence. The cleaved mRNA is recognised by the cell as foreign material and is digested by nucleases. mRNA cleavage prevents translation into the target protein thus silencing gene function (Hoyer, 2007).

1.10.3 RNAi applications

RNAi has been used as an effective tool for investigating the function of several schizophrenia candidate genes including *DISC1*, *DTNBP1* and *CAMK2* (Numakawa *et al*., 2004; Babcock *et al*., 2005; Duan *et al*., 2007). Several studies have observed the behavioral outcome following specific knockdown of the gene of interest in rodents, for example behavioral testing of: nociception (Tan *et al*., 2005), auditory processing and spatial learning (Threlkeld *et al*., 2007), Alzheimer’s related behavioural deficits (Singer *et al*., 2005), exploratory behaviour (Babcock *et al*., 2005) and motor performance deficits (Hommel *et al*., 2003).

1.11 Hypotheses and aims

It is well established from genetic associations, expression studies, and animal models that *NRG1* and *ERBB4* are promising candidate genes for schizophrenia, yet the understanding of how these genes function in distinct regions of the brain in adulthood, and the relation of these functions to cognition and schizophrenia-related behaviors, is poor. Therefore we sought to investigate the function of Nrg1-Erbb4 signalling within a distinct brain region of the adult rat, the mPFC, by utilising recombinant adeno-associated viral particles designed to knockdown the expression of *Erbb4*.

We hypothesised that by knocking down expression of Erbb4 within the mPFC of rats, a brain region highly associated with the cognitive deficits of schizophrenia, we would initiate changes in schizophrenia-related behaviours and in particular measures of cognition. In addition, it was also hypothesised that as ERBB4 signals as part of a regulated signal transduction pathway, dysregulation of Erbb4 expression would have a knock-on effect on the levels of other components of this pathway-which may be reflective of biochemical changes that are pathophysiological in schizophrenia.

By validating *in vivo* viral-mediated knockdown of Erbb4 and measuring the downstream effects of this knockdown on behavioural and biochemical function in rats, we aimed to gain a greater knowledge of the function of Erbb4 within the mPFC in adulthood, and overcome the spatial and temporal restrictions of previous animal models of Nrg1-Erbb4 dysfunction. We propose that investigating Nrg1-Erbb4 signalling in this way will potentially lead to a greater understanding of how Nrg1-Erbb4 may be pathophysiological in schizophrenia, and could give a greater insight in to prospective therapeutic targets for treating the cognitive deficits of schizophrenia.

In addition we also aimed to validate a neuronal cell line NG108-15 as a potential *in vitro* model for investigating Nrg1-Erbb4 signalling. Thus as an alternative to *in vivo* studies this model system would allow a reduction in the numbers of animals required, and also enable validation of recombinant adeno-associated viral particles to be tested on a cell line before being utilised in more physiologically relevant tissues.

2 Materials and Methods

2.1 Materials

Recombinant adeno-associated viral particles (rAAVs) were produced by the University of Pennsylvania, Penn Vector Core facility, USA. Three types of rAAVs were supplied expressing either: enhanced green fluorescent protein (eGFP) only (rAAV.eGFP), eGFP and a hairpin sequence corresponding to rat *Erbb4* (rAAV.shErbb4.eGFP) or eGFP and a scrambled version of the hairpin sequence corresponding to rat *Erbb4* which does not correspond to any known gene (rAAV.scr.eGFP). The shErbb4 sequence specifically targets a 19 nucleotide sequence of exon 28 of the rat *Erbb4* gene: TGG AGA CCT TCA AGC TTT A whereas the scrambled hairpin sequence: AGT TCA TGA GTG TCA CAT C is not known to target any gene. In brief, the recombinant replication deficient AAV vectors were produced by the co-transfection of HEK293 cells with plasmids that encoded 1) shRNA driven by the U6 promoter and/or eGFP driven by the CMV promoter 2) AAV *ori* proteins of serotype 2 *and rep* and *cap* proteins of serotype 9 and 3) adenoviral “helper” proteins VA, E2a and E4. The constructs encoded additional elements to aid viral packaging and transduction properties including the SV40 intron, woodchuck posttranscriptional regulatory element (WPRE) and bovine growth hormone polyadenylation signal. The viral particles were supplied with titres quantified as genome copies (gc)/ml.

Phencyclidine hydrochloride (PCP) and d-amphetamine sulphate were purchased from Sigma-Aldrich, Poole, UK. Human recombinant NRG1β was purchased from R&D Systems, Abingdon, UK and reconstituted in 0.1% BSA (w/v)/1xPBS. 0.5M ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$) solution pH8 and all cell culture media (Neurobasal, Dulbecco’s Modified Eagle Medium (DMEM, 4500mg/L D-Glucose), Minimum Essential Medium (MEM) and Opti-MEM® I Reduced Serum Medium) were purchased from GIBCO®, Invitrogen, Paisley, UK.

Several chemicals were routinely used in the preparation of buffers described within this section. Table 1 lists these routinely used chemicals and the source from which they were purchased. The source of all other materials and equipment used in this study is stated in brackets in the appropriate section of the description of methods.

Ringers solution was composed by diluting one quarter-strength Ringers Solution tablet (Oxoid, Cambridge, UK) in 500ml distilled H_2O. 10x Phosphate Buffered Saline (PBS) consisted of a final concentration of 1.3M NaCl, 70mM Na_2HPO_4, 30mM $NaH_2PO_4.2H_2O$ in diethylpyrocarbonate (DEPC) treated-H_2O, and was diluted to a 1x solution in distilled H_2O before use.

Table 2-1 List of chemicals routinely used and their source

Chemical	Chemical formula	Source
Potassium phosphate	KH_2PO_4	Fisher Scientific, Leicestershire, UK
Sulphuric acid	H_2SO_4	
Tris Base	$C_4H_{11}NO_3$	
Glycerol	$C_3H_8O_3$	BDH, Poole, UK
Potassium chloride	KCl	
Sodium chloride	NaCl	
Sodium hydrogen phosphate	Na_2HPO_4	
Calcium chloride dihydrate	$CaCl_2.2H_2O$	Sigma-Aldrich, Poole, UK
D-Glucose	$C_6H_{12}O_6$	
Magnesium sulphate heptahydrate	$MgSO_4.7H_20$	
Methanol	CH_3OH	
Sodium azide	NaN_3	
Sodium bicarbonate	$NaHCO_3$	
Sodium deoxycholate	$C_{24}H_{39}NaO_4$	
Sodium dodecyl sulphate (SDS)	$CH_3(CH_2)_{11}OSO_3Na$	
Sodium orthovanadate	Na_3VO_4	
Sodium phosphate dihydrate	$NaH_2PO_4.2H_2O$	
Triton® X-100	$(C_4H_4O)_nC_{14}H_{22}O$	
Tween®-20	$C_{58}H_{114}O_{26}$	

2.2 Cell culture

2.2.1 NG108-15 cell culture

Mouse neuroblastoma x rat glioma NG108-15 cells (European Collection of Cell Cultures, ECACC) were maintained in DMEM (4500mg/L D-Glucose) supplemented with 10% (v/v) Foetal Bovine serum (FBS, Sigma-Aldrich), 100 units/ml penicillin and 0.1mg/ml streptomycin (Invitrogen). Cells were grown in 25cm^2/75cm^2 flat bottomed plates (Corning® CoStar®, Sigma-Aldrich) and incubated at a constant temperature of 37°C in air containing 5% $C0_2$. Once cells were at approximately 75-80% confluency they were detached from the plate surface with 0.05% (v/v) trypsin/ 1xEDTA (Sigma-Aldrich) and resuspended in fresh supplemented DMEM. The cells were then replated into 75cm^2 plates or counted using a haemocytometer and plated at a specific density into 6/12 well flat bottomed plates (Corning® CoStar®, Sigma-Aldrich).

2.2.2 Primary dissociated cortical cultures

Dissociated cortical cultures were prepared from postnatal day 1/2 rat pups using a dissection surface and surgical equipment sterilised with 70% (v/v) ethanol. The pups were euthanised by a 100μl intraperitoneal (i.p.) injection of Euthatal (Merial, Harlow, UK), followed by decapitation. The brains were removed and placed in MEM that had been chilled to 4°C, the hemispheres were separated and the cortical tissue dissected out. The cortical tissue was then cut with a size 10 scalpel blade into small pieces and incubated in 1.5mg/ml papain (Sigma-Aldrich)/preparation buffer (116mM NaCl, 5.4mM KCl, 26mM $NaHCO_3$, 1.3mM $NaH_2PO_4.2H_2O$, 1mM $MgSO_4.7H_2O$, 1mM $CaCl_2.2H_2O$, 0.5mM EDTA pH8, 25mM D-Glucose) for 30 minutes at 37°C to disrupt the cortical tissue enzymatically.

The cortical tissue was then removed from the papain and transferred into 10mg/ml BSA (Sigma-Aldrich)/ preparation buffer pre-warmed to 37°C. The tissue was then gently triturated using a plastic Pasteur pipette. Next, the dissociated tissue was centrifuged in a Sigma 2K15 refrigerated centrifuge (Sigma-Aldrich) at 4°C, 2000rpm for 3 minutes, the supernatant removed and the tissue resuspended in Neurobasal® media supplemented with 2% (v/v) B27 (Invitrogen), 100 units/ml penicillin and 0.1mg/ml streptomycin (Invitrogen) and 2mM L-Glutamine (Invitrogen). The cells were then plated out into 8-well chamber slides or 6/12 well flat bottom plates pre-coated with 4mg/ml poly-D-lysine (Sigma-Aldrich)/DEPC treated H_2O. Two days after plating half the medium was removed from the wells to remove any dead cells or debris and replaced with fresh supplemented Neurobasal® medium.

The cells were then left to culture for a further 4 days before any treatments were carried out.

2.2.3 NRG1β stimulation

NG108-15 cells were grown in 6-well flat bottom plates as described in section 2.2.1 for 24 hours before their cell culture media was removed and replaced with serum free DMEM. The cells were left to culture for a further 24 hours before being treated with vehicle (0.1% BSA (w/v)/1xPBS) or NRG1β at final concentrations ranging from 10ng/ml-200ng/ml. The cells were then incubated for 10, 30 or 60 minutes at a constant temperature of 37˚C in air containing 5% $C0_2$ before protein was isolated (section 2.12) from the cells. Phosphorylated ERBB4 levels or levels of other proteins of interest were assessed by ELISA (section 2.14) or western blotting (section 2.13) respectively, following NRG1β application.

2.3 RNAi *in vitro*

2.3.1 siRNA transfection

NG108-15 cells at a density of $1.5x10^5$ cells/ml were plated into 6 or 12 well flat bottomed plates (Corning® CoStar®, Sigma-Aldrich) and incubated at a constant temperature of 37˚C in air containing 5% $C0_2$ for 24 hours before transfection with siRNAs. Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) was allowed to come to room temperature and then diluted in Opti-MEM® I reduced serum medium according to the manufacturer's protocol. Custom *Silencer*® select siRNA for mouse *Erbb4* and *Silencer*® siRNA for *Gapdh* and a non-targeting sequence (Ambion, Austin, TX, USA) were diluted to required concentrations in Opti-MEM® I and incubated at room temperature for 5 minutes. Equal volumes of diluted Lipofectamine™ RNAiMAX and diluted siRNA were incubated together at room temperature for 20 minutes to allow complexes to form. Following incubation, the siRNA/transfection reagent complex was added to the plated NG108-15 cells. For each experiment control wells were included which contained untreated NG108-15 cells (no transfection reagent or siRNA) and cells treated with transfection reagent only (mock transfection, no siRNA). Cells were incubated at a temperature of 37˚C in air containing 5% $C0_2$ until further processing.

The target sequence of the *Erbb4* siRNA was the mouse equivalent of the hairpin sequence used in the shErbb4 recombinant adeno-associated viral particles.

2.3.2 rAAV transfection

24 hours (NG108-15 cells) or 4 days (dissociated cortical cultures) after plating, cells were treated with recombinant adeno-associated viral particles. Viral particles were allowed to thaw on ice before being diluted in the appropriate cell culture media (DMEM or Neurobasal®). The correct volumes of diluted particles were applied directly into the well to give the desired amount of genome copies (gc). For each experiment wells were included that were treated with 3% (w/v) sucrose/PBS to control for the effect of viral particles alone. Following rAAV treatment, cells were incubated at a temperature of 37˚C in air containing 5% $C0_2$ until further processing.

2.4 *In vivo* studies

2.4.1 Animals

All experimental procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986. Male Hooded Lister rats (Harlan, UK) weighing 260-280g upon arrival were dual housed at a maintained temperature and humidity of 20-22˚C and 55%, respectively. Following a one week quarantine period the rats were ready to begin training or undergo surgery as described in the following sections. Three individual *in vivo* studies were performed.

1) 9 rats were used to study the location of injection sites and at which titre to use rAAV particles. There were two treatment groups in this study: the first group of 3 rats received a 2μl injection into the medial prefrontal cortex (mPFC) of vehicle (3% (w/v) sucrose/PBS) in one hemisphere and a 2μl injection of “high titre” $1x10^9$gc/μl rAAV.eGFP into the contralateral hemisphere. The second group of 6 rats received a 2μl injection into the mPFC of “low titre” $1x10^8$gc/μl rAAV.eGFP in one hemisphere and a 2μl injection of “high titre” $1x10^9$gc/μl rAAV.eGFP into the contralateral hemisphere (surgeries were performed as described in section 2.4.2). Rats then underwent transcardial perfusion (see section 2.4.3) at 1, 3 or 8 weeks following surgery (1 veh/high titre rat and 2 low/high titre rats at each time point). The prefrontal cortex (PFC) was sectioned and then stained to detect the presence of multiple proteins (see section 2.15.2)

2) 24 rats were used to study the effect of viral particles on schizophrenia-related behaviours and to quantify mRNA and protein levels of members of the Nrg1-Erbb4 signalling pathway. This study included 3 treatment groups of 8 rats receiving 2μl bilateral injections into the mPFC of vehicle (3% (w/v) sucrose/PBS), $1x10^9$gc/μl rAAV.scr.eGFP or $1x10^9$gc/μl rAAV.shErbb4.eGFP (surgeries were performed as described in section 2.4.2). Rats were then allowed to recover for one week before being tested in the open field and prepulse inhibition (PPI) behavioural tasks (as described in sections 2.5 and 2.6, respectively). The tasks were repeated at 3 weeks and 5 weeks post-surgery, after which the rats were euthanised by cervical dislocation and the PFC tissue collected (see section 2.4.4). The mRNA expression of genes of interest was analysed in this tissue by qRT-PCR (see section 2.11), while protein expression of members of the Nrg1-Erbb4 signalling pathway was analysed by western blot and ELISA (see sections 2.13 and 2.14, respectively).

3) 36 rats were used to study the effect of viral particles on performance in the 5-choice serial reaction time task. First rats were trained in the task

(see section 2.7.3) then split into three treatment groups based on performance at baseline testing (see section 2.7.4). This study included three treatment groups of 12 rats receiving 2μl bilateral injections into the mPFC of vehicle (3% (w/v) sucrose/PBS), 1x10^9gc/μl rAAV.scr.eGFP or 1x10^9gc/μl rAAV.shErbb4.eGFP (surgeries were performed as described in section 2.4.2). At one, three and five weeks post-surgery the rats were tested in the task (see section 2.7.5).

2.4.2 Stereotaxic surgery

Rats were initially anaesthetised in a clear acrylic chamber via the inhalation of isoflurane (Isoflo®, Abbot Animal Health, Maidenhead, UK) set at level 5. Oxygen and nitrous oxide were continuously supplied at 0.3L/min and 0.7L/min, respectively, throughout the duration of the surgical procedure. Once no pedal or corneal reflexes were present the fur from the level of the eyes to muscular band of the neck was shaved using a small set of clippers. Next, the rat was transferred to a heat mat (Fine Science Tools, Inc, Cambridge, UK) and positioned in front of the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The level of anaesthesia was reduced to 3 and the rat was then secured into the frame using atraumatic ear bars (David Kopf Instruments), the incisor bar was set to 3.3mm below the ear bars to achieve flat skull position (Paxinos & Watson, 2007).

The shaved area was swabbed with 70% (v/v) ethanol and a single incision was made with a sterile scalpel from between the eyes to between the ears. Next, the skull was exposed by blunt dissection and Bregma located. The flat skull position was confirmed by checking the dorso-ventral (DV) co-ordinates of Bregma and Lambda were the same, if not the incisor bar was adjusted. From Bregma the location of the prelimbic area of the mPFC (prL) in each hemisphere was identified and marked on the skull using the co-ordinates +3.2mm anterior-posterior (AP), +/- 2mm medio-lateral (ML) (Paxinos & Watson, 2007). Using a dental drill with a small 1mm dental burr attached (Royem Scientific Ltd, Bedfordshire, UK) small burr holes were made at these co-ordinates. Next the 5μl Hamilton syringes fitted with Hamilton needles (Hamilton Company, Reno, NV, USA) were secured to both arms of the stereotaxic frame at an angle of 20°, and lowered through the burr holes to 4mm below the surface of the skull (injections were performed at an angle to reduce the chance of disturbing blood vessels running along the midline of the brain). 2µl bilateral injections of vehicle (PBS/3% (w/v) sucrose), rAAV.eGFP, rAAV.scr.eGFP or rAAV.shErbb4.eGFP were then performed over a 10 minute time period; following completion of the injection the needle was left in place for a further 5 minutes to reduce the chance of back-flow of injected material. The needle was slowly retracted and the incision site closed using Vicryl™ sutures (Ethicon® coated Vicryl™ 4.0, Dunlop Veterinary Supplies, Dumfries, UK).

Following surgery the rats were removed from the stereotaxic frame and placed single housed in one warmed paper-lined cage per rat. The following day the cage bedding was changed back to sawdust and the rats were kept separate for a further 48 hours to prevent damage to the incision wound. The rats' were then re-housed with their cage mates from before surgery and allowed to recover for another 4 days before any further experimental procedures were performed. Rats' weights were closely monitored to check for post-surgical weight changes.

2.4.3 Transcardial perfusion

Rats were removed from their home cage and given a lethal i.p. injection of Euthatal (Merial). Once no pedal or corneal reflexes were present the chest cavity was exposed and a needle was inserted into the left ventricle of the heart. The right atrium was then cut and 1x Ringer's/PBS solution was then pumped into the heart until the liver had paled. 250ml freshly made 4% (w/v) paraformaldehyde (PFA, prilled form, Sigma-Adrich)/PBS was then pumped through the heart, at a slow rate until complete fixation had taken place (approximately 30 minutes). The whole brain was then dissected out and placed in 20% (w/v) sucrose/PBS and stored at 4°C for cryoprotection purposes.

2.4.4 Cervical dislocation and PFC dissection

Rats that were involved in studies 2 and 3 (see section 2.4.1) were euthanised by cervical dislocation. The whole brain was immediately removed and brains from rats in study 3 were snap frozen in isopentane (VWR International Ltd, Poole, UK) cooled to -42°C on dry ice and stored at -80°C until further processing (data not shown in thesis). Brains from rats in study 2 were transferred to an ice cold tile and the whole PFC dissected out. The PFC tissue was exposed by the initial removal of the olfactory bulb tissue; a coronal slice of approximately 1.5mm was then collected. Diagonal cuts were then made with a size 10 scalpel blade through the cingulum of this coronal slice, isolating the prefrontal cortical tissue. All dissections were carried out by the same individual to minimise variation in sample collection. Each hemisphere was individually stored in ribolyser tubes (Lysing Matrix D, Qbiogene, Cambride, UK) at -80°C until being used for mRNA or protein expression analysis.

2.5 Open Field

2.5.1 Open field apparatus

The open field test apparatus consisted of two 100cmx100cmx40cm infra-red translucent acrylic plastic arenas situated on top of infra-red light boxes and positioned directly under a ceiling mounted infra-red sensitive camera. The camera was linked to a computer in an adjacent room where distance travelled was analysed using EthoVision Pro, Version 3.0 video tracking software (Noldus Information Technology, The Netherlands). All locomotor experiments were carried out in the same room with lighting level fixed at 190 Lux.

2.5.2 Habituation to open field

Rats were removed from their home cages and individually housed in the room where the locomotor activity was to be assessed. The rats were habituated to the open field arenas for 30 minutes prior to locomotor activity assessment.

2.5.3 Amphetamine challenge

The effect of an amphetamine challenge on locomotor activity was assessed in the rats that had previously undergone stereotaxic surgery. In a cross-over design rats received subcutaneous injections in the flank of vehicle (0.9% (w/v) saline) or d-amphetamine sulphate (1mg/kg) on the first day of locomotor testing. The following day the alternative treatment was administered to the rats (e.g. vehicle day 1, amphetamine day 2). In brief, the rats were removed from the arena following the 30 minute habituation period and administered the appropriate drug treatment. They were then immediately placed back into the same arena as where the habituation had been monitored to assess locomotor activity over a 60 minute period. Arenas were cleaned with disinfectant between rats.

2.6 Prepulse inhibition

2.6.1 PPI apparatus

The effect of stereotaxic surgery on PPI was carried out using three identical sound-attenuated chambers (MED Associates, St. Albans, VT, USA) and the magnitude of startle responses recorded by MED Associates Startle Software (SOF-825).

2.6.2 Habituation to PPI

The day prior to the PPI testing (and at least 2 hours after locomotor activity assessment) rats were habituated to the PPI procedure. PPI experimental procedure was as follows, rats were placed into restrainers connected to a platform measuring the extent of movement. The restrained rats were firstly acclimatised for 5 minutes to a background noise of 65 decibels (dB). They were then tested in three blocks of trials, in all of which the interval between trials was on average 15 seconds. Blocks 1 and 3 consisted of 6 stimulus-alone trials of 120 dB pulses of white noise lasting 40 milliseconds (ms). Block 2 consisted of 52 trials 12 of which were stimulus alone trials, 10 of which were 40ms trials of background noise (no-stimulus), and 30 of which were 40ms 120 dB pulses preceded by a 20ms lower amplitude "pre-pulse" of 4, 8 or 16dB above background noise 100ms before the stimulus. Each pre-pulse intensity was presented 10 times in a pseudorandom order.

2.6.3 PPI testing

The next day (day 2) the rats were again exposed to this PPI procedure, this time the test data were acquired and PPI calculated from the trials in Block 2 for each pre-pulse amplitude using the following equation: % PPI= 100x ((stimulus alone response)-(pre-pulse + stimulus response))/ (stimulus alone response). Startle *per se* was taken from the startle response to stimulus alone.

2.7 5-choice serial reaction time task

2.7.1 Animals

36 Male Hooded Lister rats (Harlan) weighing 260-280g upon arrival were dual housed at a maintained temperature and humidity of 20-22°C and 55%, respectively. Following a one week quarantine period the rats were maintained at 85% of their free feeding weight by a strict daily food restriction regime of 25-30g standard rat chow per cage. All animals were allowed water *ad libitum,* except during the 30 minute training/testing periods. The rats' weights were monitored three times a week throughout the duration of the study (except the period following surgery, where the rats' weights were monitored daily for a week). This study was carried out in accordance with the UK animals (scientific procedures) Act 1986.

2.7.2 Habituation phase

For three days prior to the habituation to the apparatus, each rat was handled daily for approximately 5 minutes to allow familiarisation to being handled. The day prior to the rats being introduced to the apparatus for the first time reward pellets (45mg, Sandown Scientific, Middlesex, UK) were placed in each cage to familiarise the rats to the rewards and thus prevent neophobia.

On the first day of habituation to the apparatus, the rats were placed within the operant box with reward pellets in the food hopper. This purpose of this was two-fold 1) To accustom the rat to the operant box and 2) To build the association between the food hopper and reward collection. This association was strengthened on the second habituation day where reward pellets were again available in the food hopper, in addition each aperture was baited with a reward pellet to build the association between nose poking and food reward.

2.7.3 Training of the 5-choice serial reaction time task

Once the rats were familiarised with the apparatus and food rewards, training of the task began. The rats were placed into the operant box and a nose-poke into the food hopper initiated the beginning of the task. After a 5 second inter-trial interval (ITI) one of the five open apertures illuminated for a fixed period of time (stimulus duration, SD). Responses into this illuminated aperture during the SD or within a specified duration of time afterwards (limited hold, LH) the rat would be rewarded with a food reward and a correct response was recorded. Responses into an incorrect aperture during SD or LH periods would be recorded as an incorrect response and the rat would be punished by a time out period of darkness of 5 seconds. Time out punishments were also initiated if the rat made an omission - no response into any aperture during SD or LH, or a premature response – a response in an aperture within the ITI period. The next trial was again initiated by a nose-poke in the food hopper. This was repeated for 100 trials or 30 minutes, whichever was completed first.

Training of the five choice serial reaction time task was performed on weekdays. Training of the task initially involved the stimulus duration and limited hold being set at 60 seconds; these durations were gradually reduced once the rat had performed the task to criteria at that training level with an accuracy of 80% or more with less than 20% omissions. Each rat was trained to the level of performing the task with an accuracy of 80% or more with less than 20% omissions with a stimulus duration of 0.6 seconds and limited hold duration of 5 seconds. As not all rats met criteria at level 12 at the same time, the rats that had met criteria at level 12 were reduced to 3 training sessions a week to prevent over-training. The training levels are summarised in Table 2.

2.7.4 Baseline testing

Once all rats had reached criteria at training level 12 their performance in 3 variations of the 5-choice serial reaction time task was assessed. Parameters of these tasks are summarised in Table 3. Performance in the basic task was assessed on Monday, Tuesday and Thursday and an average performance over the three days was calculated. Performance in the variable inter-trial interval task and variable stimulus duration task were assessed on Wednesday and Friday, respectively. The rats were split into 3 groups of 12 with equal performance in all three task types based on the baseline testing period to ensure any differences following surgery was due to the surgical treatment carried out.

2.7.5 Post-surgical testing

Rats were re-tested in all three task types 1, 3 and 5 weeks post-surgery as described in Table 4. Again, an average performance in the basic task was calculated from three basic task sessions at each time point. In the weeks between testing (weeks 2 and 4), the rats were exposed to the basic task three times a week to maintain their performance.

Table 2-2 Summary of 5-choice serial reaction time task training levels. s, seconds

Training level	Stimulus duration (s)	Inter-trial interval (s)	Limited hold (s)	Time out (s)
1	60	5	60	5
2	30	5	30	5
3	20	5	20	5
4	10	5	10	5
5	5	5	5	5
6	2.5	5	5	5
7	1.5	5	5	5
8	1	5	5	5
9	0.9	5	5	5
10	0.8	5	5	5
11	0.7	5	5	5
12	0.6	5	5	5

Table 2-3 5-choice serial reaction time task types. s, seconds

Test type	Stimulus duration (s)	Inter-trial interval (s)	Limited hold (s)	Time out (s)
Basic	0.5	5	5	5
vITI	0.5	5 / 7 / 10 / 12	5	5
vSD	0.5 / 0.4 / 0.2 / 0.1	5	5	5

Table 2-4 Summary of 5-choice serial reaction time task testing post-surgery.

Day		5-choice test type	Treatment
0		-	Stereotaxic injections into mPFC veh/scr.rAAV/shErbb4.rAAV
WEEK 1	7	Basic	-
	8	Basic	-
	9	vITI	-
	10	Basic	-
	11	vSD	-
WEEK 3	21	Basic	-
	22	Basic	-
	23	vITI	-
	24	Basic	-
	25	vSD	-
WEEK 5	35	Basic	-
	36	Basic	-
	37	vITI	-
	38	Basic	-
	39	vSD	-

2.8 Total RNA isolation

2.8.1 RNA isolation from cultured cells

RNA was isolated from NG108-15 cells or dissociated cortical cultures 24 hours and 4 days, respectively after transfection using RNeasy mini kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. First the medium was removed from the wells and cells were subsequently washed with ice-cold Dulbecco's phosphate-buffered saline (D-PBS, Invitrogen). NG108-15 cells were then detached from the well surface using 0.05% (w/v) trypsin/ 1xEDTA pH8 (Sigma-Aldrich) which was inactivated with DMEM. The detached cells were transferred to an Eppendorf tube and pelleted by centrifugation in a Boeco M-24 desktop centrifuge (Boeco, Germany) at 10,000rpm for 5 minutes at room temperature. The cell pellet was washed with ice cold D-PBS and centrifuged again at 10,000rpm for 5 minutes at room temperature.

The cells were lysed (either directly in the plate for dissociated cortical cultures or as a cell pellet for NG108-15 cells) with 350µl buffer RDD+β-mercaptoethanol (Sigma-Aldrich). Lysed cells were then transferred to a ribolyser tube (Lysing Matrix D, Qbiogene) and disrupted mechanically for 20 seconds at 6.5g (Hybaid Ribolyser, Hybaid Ltd, Middlesex, UK). An equal volume of 70% (v/v) ethanol was then added to the lysed cells which were transferred to an RNeasy column and centrifuged at 10,000rpm for 15 seconds at room temperature. The total RNA, bound to the column matrix was washed with the kit wash buffers and treated with DNase to remove any genomic DNA from the sample. Total RNA was eluted from the column with 30µl RNase free water. Table 5 summarises the steps involved in total RNA isolation.

2.8.2 RNA isolation from PFC tissue

Total RNA was isolated from the PFC tissue (one hemisphere) using RNeasy mini kit (Qiagen) as described above with the following modifications. Tissue was lysed with 350µl buffer RDD+β-mercaptoethanol (Sigma-Aldrich) directly in the ribolyser tube and total RNA eluted with 50µl RNase free H_2O (Ambion).

Samples were stored at -80°C until further processing. Table 5 summarises the steps involved in total RNA isolation.

Table 2-5 Steps involved in total RNA isolation from cultured cells and PFC tissue.

Step	Buffer	Volume	Spin cycle
1	RW1	350µl	15s, 10,000rpm
2	RDD+DNase I*	80µl	-----
3	RW1	350µl	15s, 10,000rpm
4	RPE	500µl	15s, 10,000rpm
5	RPE	500µl	2min, 10,000rpm
6	-	-	1min, 13,000rpm
7	RNase free H_2O	30/50µl	1min, 10,000rpm

2.8.3 Qualification and quantification of total RNA

1.5µl isolated total RNA was used to quantify the concentration of total RNA in each sample by NanoDrop™ 1000 (Thermo Scientific, Leicestershire, UK) and quality of RNA in selected samples was checked using the RNA6000 Nano Chip Kit on the Bioanalyser 2100 (Agilent Technologies, Berkshire, UK).

2.9 First strand cDNA synthesis

First strand cDNA was synthesised from up to 2µg total RNA isolated from NG108-15 cells, dissociated cortical culture cells, rat PFC tissue or rat and mouse brain total RNA (Ambion) using SuperScript®VILO™ cDNA synthesis kit (Invitrogen) according to the manufacturers instructions. In a final volume of 20µl first strand cDNA was synthesised using up to 2µg total RNA 1xVILO™ reaction mix, 1xSuperScript® III reverse transcriptase enzyme and nuclease free water (Ambion). First strand cDNA was then generated by incubating the tube contents at 25˚C for 25 minutes, 42˚C for 60 minutes then terminating the reaction at 85˚C for 5 minutes (Veriti™ 96-well Thermal Cycler, Applied

Biosystems, Foster City, CA, USA). Samples were stored at -20°C until they were further processed.

2.10 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.10.1 *Primer design*

RT-PCR primers specific for *Erbb4* were purchased from Sigma-Genosys (Poole, UK). Primers were designed so that the forward and reverse primers were located in different exons and therefore any product amplified from genomic DNA would have a much bigger amplicon length than that of a product amplified from intronless mRNA. Mouse and rat *Erbb4* exon sequences were taken from ENSEMBL (www.ensembl.org). PCR primers were designed so that the resulting product amplified would include differences in sequence between rat and mouse *Erbb4* or differences between *Erbb4* isoforms. Specificity of primers to *Erbb4* was confirmed by ensuring the primer sequences had no sequence identity to any other known genes using the Basic Local Alignment Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequences of PCR primers used in this study are shown in table 6. It also shows the species and isoform specificity of the primers.

2.10.2 *Polymerase chain reaction*

KOD Hot Start DNA polymerase enzyme mixture (Novagen, Merck Chemical Ltd, Nottingham, UK) was used to amplify up to 2μg 1st strand cDNA synthesised from NG108-15 cell RNA or total RNA from rat or mouse brain (Ambion). A reaction mix was prepared containing a final concentration of: 1x KOD Hot Start PCR Buffer, 0.2mM dNTP mixture, 1mM $MgSO_4$, 0.3μM forward and reverse primer mix, 1-2μl cDNA, 1U KOD Hot Start DNA polymerase, RNase free H_2O up to 50μl). The reaction mix was incubated in a thermocycler (Veriti™ 96-well Thermal Cycler, Applied Biosystems) 95°C for 2 minutes, followed by 35-40 cycles of 95°C for 20 seconds, 62-67°C for 10 seconds, 70°C for 10 seconds.

5-20μl of PCR products were loaded with BlueJuice™ gel loading buffer (Invitrogen) into 2.5% (w/v) agarose gels stained with 0.01% (v/v) GelStar® Nucleic Acid Gel Stain (Lonza, Slough, UK) and electrophoresed at a constant supply of 80volts. Products were then viewed by ultraviolet light and size confirmed by comparison to 1kb Plus DNA ladder (Invitrogen).

2.10.3 Extraction and purification of PCR products

The PCR product was then carefully cut out of the gel and the DNA fragments were extracted and purified using QIAquick Gel Extraction Kit (Qiagen) as recommended by the manufacturer's protocol. In brief, the product was weighed and added to an eppendorf tube with 6x v/w of buffer QG, the sample was heated at 50˚C for 10 minutes with brief vortexing every three minutes. Next an equal volume to product weight of isopropanol was added and the sample applied to spin column in 800µl intervals and centrifuged in a Boeco M-24 desktop centrifuge (Boeco, Germany) at 13,000rpm/1min until the entire sample was used. Next 500µl buffer QG was applied to the column and centrifuged again at 13,000rpm/1min. 750µl buffer PE was pipetted directly onto the column and incubated at room temperature for 1 minute before being centrifuged at 13,000rpm/1min. The purified product was then eluted from the spin column with 30µl RNase free H_20. A small amount of the purified product was then electrophoresed on a 2.5% (w/v) agarose gel to ensure correct size was maintained.

2.10.4 PCR product sequencing and sequence analysis

Purified PCR products were sequenced using 3.2µM forward and reverse PCR primers (GeneService, Nottingham).

The resulting sequence of the PCR product was analysed by aligning against human, mouse and rat genomes and transcripts (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2-6 Summary of RT-PCR primers used

Primer ID	Primer sequence (5'-3')	Species specificity	Isoform specificity	Exon location
FwdM1	AGGCTACATGACTCCAATGC	Mouse	All	27
FwdR1	AGGCTACATGACTCCCATGC	Rat	All	27
RevRM1	TTCCAGTAGTCGGGGTTGTC	Rat/Mouse	All	28
M/R *JMa/b* Fwd	CCACCCTTGCCATCCAAA	Rat/Mouse	JMa/JMb	14
M/R *JMa/b* Rev	CCAATGACTCCGGCTGCAATCA	Rat/Mouse	JMa/JMb	17
M *CYT1/2* Fwd	CAACATACCTCCTCCCATCTACAC	Mouse	CYT1/2	25
M *CYT1/2* Rev	GCATTCCTTGTTGTGTAGCAAA	Mouse	CYT1/2	27

2.11 Quantitative real time RT-PCR (qRT-PCR)

2.11.1 Plate set-up

qRT-PCR was carried out using TaqMan® based chemistry (Applied Biosystems) in MicroAmp™ optical 96-well reaction plates (Applied Biosystems). Each experimental well contained up to 125ng 1st strand cDNA, 1x TaqMan®Gene Expression Master Mix (Applied Biosystems), 1x Gene expression assay (Applied Biosystems) and RNase free H_2O in a final volume of 25μl. In each experiment, wells containing RNase free H_2O instead of template were included to monitor for contamination. Each sample or non-template control was performed in triplicate. The reaction plate was then sealed with a piece of MicroAmp™ optical adhesion film (Applied Biosystems) and briefly centrifuged. cDNA was amplified for 40 cycles of 95˚C/2min, 95˚/15sec, 60˚/1min using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Simultaneously, levels of gene expression were quantified in real time from the levels of excited fluorescent probe incorporated into the gene expression assay. The gene expression assays used in this study are listed in table 7.

Table 2-7 TaqMan® gene expression assays used in this study

Gene expression assay	Target gene	Species
Rn00665492_m1	*Erbb4*	Rat
Mm01256803_m1	*Erbb4*	Mouse
Mm00437762_m1	*β-2-m*	Mouse
Mm99999915_g1	*Gapdh*	Mouse
Hs99999901_s1	*r18s*	Human/rat/mouse
Rn01482168_m1	*Nrg1*	Rat
Rn00583646_m1	*Akt1*	Rat
Mm00433800_m1	*Grin1*	Mouse

2.11.2 Gene expression quantification

The Comparative C_T Method ($\Delta\Delta C_T$ Method - where the amount of target is normalised to an endogenous reference then expressed relative to a calibrator sample) was used to quantify changes gene expression. The cycle at which amplification begins was set as the end cycle of the baseline; this was determined for each gene expression assay in each experiment performed. The threshold level of fluorescence was set to 0.3 in all experiments. The cycle at which the amount of fluorescence emitted from a given sample crosses the threshold value is recorded as that sample's C_T value. The C_T value of the non-template wells was checked to ensure any amplification in experimental samples was not due to contamination, the C_T values of each triplicate of each experimental sample were averaged (outliers were removed if the C_T value was notably different from the other two replicates).

The ΔC_T was calculated by subtracting the mean C_T value of the endogenous control gene from the mean C_T value of target gene. For each sample, the ΔC_T value of a calibrator sample (e.g. untreated cells or vehicle treated rats) was then subtracted from the ΔC_T value of the test sample to give the $\Delta\Delta C_T$ value. The antilog ($2^{-\Delta\Delta C_T}$) of this value was then calculated to give a fold change in gene expression of the target gene.

2.11.3 Gene expression assay validation

To ensure the validity of using the Comparative C_T Method ($\Delta\Delta C_T$ method), the amplification efficiencies of the TaqMan® Gene expression Assays used for endogenous control genes and target genes in this study were tested. To test if the amplification efficiencies were relatively equivalent (and therefore valid for $\Delta\Delta C_T$ analysis) the amplification of endogenous control genes and target genes was quantified over a range of dilutions of template cDNA.

cDNA synthesised from 200ng, 100ng, 40ng, 20ng, 10ng, 4ng and 2ng of rat brain or mouse brain total RNA (Ambion) using the protocol described in section 2.9 was diluted 1/2.5 in RNase free H_2O (Ambion). The experiment was set-up as described in the previous section with the appropriate gene expression assay being used. The ΔC_T was calculated for the target gene as described in the previous section. An XY scatter plot was then created plotting ΔC_T for each sample against template amount, a linear regression was applied to this data to calculate the value of the slope. If the value of the slope is less than 0.1 then it is evident that the efficiency of the two assays tested were of equal efficiency and therefore the $\Delta\Delta C_T$ Method can be used to quantify changes in gene expression.

2.12 Protein isolation

2.12.1 Protein lysate isolation from cultured cells

48 hours to 8 days post-treatment protein lysates were isolated from NG108-15 cells and dissociated cortical cultures. On ice the cell culture medium was removed from the wells and cells were washed with ice cold D-PBS. 125μl-250μl ice cold RIPA buffer (10mM Tris, pH7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton® X-100, 10% (v/v) glycerol) supplemented with 10µl/ml protease inhibitor cocktail (Sigma-Aldrich), 1mM NaF and 2mM Na_3VO_4 was added to each well and incubated on ice for 5 minutes. Lysed cells were scraped from the wells and transferred to an ice cold eppendorf tube and centrifuged in a Sigma 2K15 refrigerated centrifuge (Sigma-Aldrich) at 4˚C for 10 minutes at 10,000rpm. The supernatant was collected and stored at -80°C until further processing.

2.12.2 Protein lysate isolation from PFC tissue

The tissue from one hemisphere of the PFC, weighing 25-60mg, was allowed to thaw on ice. Once thawed, 500μl ice cold RIPA buffer supplemented with 10µl/ml protease inhibitor cocktail (Sigma-Aldrich), 1mM NaF and 2mM Na_3VO_4 was added to each sample. The tissue was then mechanically disrupted and cells lysed three times at 3.5g for 20 seconds (Hybaid Ribolyser). The lysates were then centrifuged in a Sigma 2K15 refrigerated centrifuge (Sigma-Aldrich) at 4°C for 10 minutes at 10,000rpm, before the supernatant was collected and stored at -80°C until further processing.

2.12.3 Protein quantification

The concentration of protein in each sample was determined using the Bradford’s colorimetric assay (Bradford, 1976). In brief, BSA (Sigma-Aldrich) was diluted in H_2O to known concentrations of 250µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25µg/ml. Each protein lysate sample (from cells or tissue) was diluted in H_2O so that its concentration would be within the range of that of the BSA standards used (generally 1/10 or 1/20 dilution). As a standard for 0µg/ml protein RIPA buffer was diluted to the same factor as the samples. 10µl of each BSA standard, lysis buffer and sample were pipetted, in triplicate, into a flat bottom 96 well plate (Corning® CoStar®, Sigma-Aldrich). 200µl 5x Bradford's reagent (BioRad, Hemel Hemstead, UK) diluted 1:4 in H_2O was then added to each well and incubated at room temperature for 10 minutes to allow the dye to bind to protein and to convert colour.

The plate was then inserted into a MRX microplate reader (Dynex Technologies, Ashford, UK) and the optical density of each well was measured at 630nm. Using Revelation software v3.01 a linear regression of optical density vs. protein concentration was calculated using the optical density value from lysis buffer to represent 0µg/ml protein and the serial dilution of BSA as known protein concentration standards. The concentration of each sample was then calculated from the linear regression curve. The concentration of each sample was multiplied by the dilution factor (10 or 20) to give the final concentration.

2.13 Western blotting

2.13.1 Sample preparation

Protein lysates from cells or tissue were prepared for western blotting by the addition of 4x NuPAGE® LDS sample buffer (Invitrogen) and 10x NuPAGE® reducing agent (Invitrogen) to the lysate sample. This was then denatured at 70°C for 10 minutes. As the samples were further diluted by the addition of the sample buffer and reducing agent, the protein concentration quantified by Bradford's assay was adjusted by multiplying by 0.65. This final concentration was then used to determine the volume of sample required to give a desired amount of protein, so that the same amount of protein was used for each sample.

2.13.2 Electrophoresis

Separation of proteins by molecular weight was achieved using 10% NuPAGE® Novex® Bis-Tris gels (Invitrogen). In brief, the NuPAGE® gels were secured into the XCell *SureLock*® Mini-Cell system (Invitrogen) and the inner and outer chambers filled with 1x NuPAGE® SDS MES Running Buffer (Invitrogen). The inner chamber was supplemented with NuPAGE® Antioxidant (Invitrogen) to maintain the state of the protein samples during electrophoresis. Next, the correct volumes of the protein samples were pipetted into the wells of the NuPAGE® gel, in each experiment at least one well of each gel contained 5μl Precision Plus Kailedescope™ ladder (BioRad) as a colorimetric marker of protein molecular weights.

Samples were then electrophoresed at 200V for 35 minutes or until the loading dye had visibly reached the bottom of the gel.

2.13.3 Protein transfer

Following electrophoresis, the protein samples were then transferred to PVDF membrane (0.2μm pore size, Invitrogen). First, sponges, PVDF membranes (pre-wet in 100% Methanol) and filter papers were pre-soaked in 1x NuPAGE® Transfer Buffer/ 10% (v/v) methanol/ NuPAGE® Antioxidant. The gels were carefully removed from their cassettes and assembled on top of a piece of pre-wet filter paper, and the pre-wet PVDF membrane was then placed on top of the gel and this was topped by another piece of pre-wet filter paper. This gel-membrane “sandwich” was then secured into the XCell II™ Blot Module (Invitrogen) with enough pre-wet sponges under the “sandwich” (on the cathode core) and on top of the “sandwich” (the anode core) until there was a tight fit and any trapped air bubbles were removed.

The blot module was then filled with 1x NuPAGE® Transfer Buffer/ 10% (v/v) methanol/ NuPAGE® Antioxidant and the outer chamber filled with H_2O to dissipate any heat during the transfer process. A supply of 30V was then passed through the blot module (from the cathode core to the anode core) for 1 hour to transfer proteins from the gel onto the PVDF membrane.

2.13.4 Antibody incubations

Detection of proteins of interest was performed using several blocking, washes and antibody incubation steps. Initially, the PVDF membrane were incubated in TBS (20mM Tris, 137mM NaCl, pH 7.6)/ 0.1% (v/v) Tween-20/ 5% (w/v) dried milk powder (Marvel) for 2 hours at room temperature with agitation to block non-specific binding of antibodies. Next, the membrane was washed 5 times in TBS/0.1% (v/v) Tween®-20 for 5 minutes per wash at room temperature with agitation before being incubated in the appropriate primary antibody diluted to the correct concentration in TBS/0.1% (v/v) Tween®-20/ 5% (w/v) dried milk overnight at 4°C with agitation. The list of antibodies and dilutions used are shown in table 8.

The following day the membrane was washed as before, with an additional 10 minute final wash. The membrane was then incubated for 1-2 hours at room temperature with agitation in the appropriate HRP-conjugated secondary antibody diluted to the correct concentration in TBS/0.1% (v/v) Tween®-20/ 5% (w/v) dried milk. When multiple proteins of different sizes were being detected from the same sample two primary and, if necessary, secondary antibodies were used in conjunction. When the primary antibody was already conjugated to HRP and no secondary antibody necessary (e.g. Actin-HRP antibody), overnight incubation was not necessary, the membrane was simply incubated for 1-2 hours at room temperature in diluted HRP antibody then processed as if after secondary antibody incubation had taken place. Next, the membranes were washed as before, with an additional 10 minute final wash.

2.13.5 Visualisation and quantification of proteins

The proteins were then detected using enhanced chemiluminescence (ECL) Plus reagents (Amersham, GE Healthcare, Buckinghamshire, UK). The reagents were allowed to come to room temperature and then diluted as described in the manufacturer's instructions (1ml Solution A + 50μl Solution B per membrane) this was then pipetted onto the PVDF membrane and allowed to incubate for 5 minutes at room temperature. Next, the PVDF membrane was wrapped in Clingfilm, ensuring no air bubbles were present and taped inside an x-ray cassette. In complete darkness x-ray film (ECL Plus Hyperfilm, Amersham, GE Healthcare or Kodak) were placed against the membrane and exposed for 5 seconds to 1 hour. The film was then developed (X-O MAT) and protein levels in each sample were quantified by measuring the optical density of each sample (ImageJ, NIH, Bethesda, MD, USA).

Each sample's optical density was expressed as normalised to the optical density of actin, a protein for which the expression should not change with the treatments used in this study, and therefore can serve as a control for variations in the amount of sample initially loaded. The same membrane was used several times to identify the levels of multiple proteins in the same sample. To do this the antibodies were stripped from the membranes by incubating the membrane in pre-warmed stripping buffer (62.5mM Tris HCL (pH 6.7), 2% (w/v), 100mM β-mercaptoethanol) for 30 minutes at 50°C. Membranes were then washed twice in TBS/0.1% (v/v) Tween-20 for 10 minutes before undergoing blocking, washes and antibody incubations as described above.

2.14 Enzyme linked immunosorbant assay (ELISA)

2.14.1 Plate preparation

Protein levels of total ERBB4 (tERBB4) and phosphorylated ERBB4 (pERBB4) were quantified using DuoSet® IC Human total-ERBB4 ELISA and DuoSet® IC Human Phospho-ERBB4 ELISA (R&D Systems). Firstly, wells of high bind polystyrene flat bottom 96-well microplates (R&D Systems) were coated with 100μl 1μg/ml (pERBB4) or 4μg/ml (tERBB4) capture antibody diluted in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2) overnight at 4°C.

2.14.2 Sample and Standard application

The following day wells were washed five times with wash buffer (PBS, 0.05% (v/v) Tween®-20) then non-specific antibody binding was blocked with 300μl blocking buffer (PBS, 1% (w/v) BSA, 0.05% (w/v) NaN_3 pH 7.2) for 2 hours at room temperature after which the wells were washed again (as before). Next, Human tERBB4 standards of known concentration (31.25pg/ml-4000pg/ml) and

Human pERBB4 standards of known concentrations (46.875pg/ml-6000pg/ml) were diluted in IC Diluent#12 (R&D Systems) and pipetted in triplicate into the 96-well plate. NG108-15 cell, dissociated cortical cell or rat PFC tissue lysates were diluted 1/2 (for tERBB4 ELISA) or 1/4 (for pERBB4 ELISA) in IC Diluent#12 (R&D Systems) and pipetted in duplicate or triplicate into the 96-well plate. Standards and samples were incubated at room temperature for 2 hours. For each experiment triplicates of RIPA buffer diluted to the same factor as samples were included to serve as a 0pg/ml standard. Wells including capture antibody only or detection antibody only were included to ensure specificity of antibodies (no optical densities were observed with these treatments).

2.14.3 Antibody incubations

The wells were again washed (as before) and then incubated with detection antibody (1x phosphotyrosine-HRP for pERBB4 ELISA or 1μg/ml tERBB4 detection antibody) diluted in IC Diluent#14 (R&D Systems) for 2 hours at room temperature. For the tERBB4 ELISA an additional antibody incubation step of 20 minutes in 1x streptavidin-HRP diluted in IC Diluent#14 was required as the detection antibody used in this ELISA was not HRP-conjugated.

2.14.4 Detection and quantification of proteins

Following antibody incubations the wells underwent a final wash step (as before) then were incubated in Substrate solution (1:1 Solution A : Solution B, R&D Systems) for 30 minutes at room temperature. The enzymatic reaction was stopped by the addition of 50% (v/v) H_2SO4 to each well. The optical density of each well was measured on a MRX microplate reader (Dynex Technologies) at 450nM. A standard curve of the standards was generated plotting mean optical of each standard against standard concentration (pg/ml). The levels of tERBB4 or pERBB4 in the samples were then determined from this using linear regression analysis.

2.15 Immuno-fluorescent staining

2.15.1 Immuno-fluorescent staining in cultured cells

Cell culture media was removed from the wells of an 8-well chamber slide and cells were fixed in ice-cold 4% (w/v) PFA (prilled form, Sigma-Aldrich)/PBS for 20 minutes at 4°C. Following fixation, cells were washed twice in PBS for 3 minutes at room temperature before being permeablised in PBS/0.5% (v/v) Triton® X-100. Non-specific antibody binding was then prevented by incubating the cells in blocking buffer (PBS/15% (v/v) donkey serum) for 1 hour at room temperature. The cells were then incubated in the correct primary antibody diluted in PBS/3% (v/v) donkey serum/0.5% (v/v) Triton-X-100 overnight at 4°C.

The following day, the cells were washed three times in PBS before being incubated with the appropriate Alexa-Fluor conjugated secondary antibody diluted in 1xPBS/5% (v/v) donkey serum for 1 hour at room temperature. The cells were again washed three times in PBS, the chamber removed from the slide and a coverslip being applied with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

Immunofluorescent staining was then observed using the correct filter on a Nikon Eclipse E600 epifluorescent microscope and images were taken using a Nikon CoolPix 4500 camera mounted to the microscope.

2.15.2 Immuno-fluorescent staining in PFC brain sections

Once the brains from the transcardially perfused rats had sunk to the bottom of the 20% (w/v) sucrose/PBS solution they were believed to be cryoprotected. The brains were then snap frozen in isopentane (VWR International Ltd) cooled to -42°C on dry ice, then placed in the cryostat (Leica CM1850) to equilibrate to -20°C. The brains were then cut into 20μm sections and mounted onto poly-L-lysine (0.01%, Sigma-Aldrich) coated slides. Using a hydrophobic barrier pen (Vector Laboratories), boundaries were drawn around each brain section.

Firstly the sections were dehydrated in 50% (v/v) ethanol for 30 minutes at room temperature. Next sections underwent three 10 minute washes in wash buffer

(2.5mM $NaH_2PO_4.2H_2O$, 7mM Na_2HPO_4 , 0.3M NaCl) before being incubated in the correct primary antibody diluted in wash buffer/0.3% (v/v) Triton®- X-100 overnight at 4°C. The next day the sections were washed again 3 times for 10 minutes in wash buffer before being incubated in the appropriate Alexa Fluor conjugated secondary antibody diluted in buffer/0.3% (v/v) Triton®- X-100 for two hours at room temperature. For co-localisation studies combinations of primary and secondary antibodies were used together in the incubation stages, ensuring that the antibodies were raised in different species. The list of antibodies used is shown in table 8. After another series of washes (as before) the sections were allowed to air-dry then coverslips were mounted onto the slides with VECTASHIELD® Mounting Medium with DAPI.

Immunofluorescent staining was then observed using the correct filter on a Nikon Eclipse E600 epifluorescent microscope and images were taken using a Nikon CoolPix 4500 camera mounted to the microscope. For co-localisation studies further images were taken using a Leica TCS S-P5 laser scanning confocal microscope. Images were merged using ImageJ (NIH, Bethesda, MD, USA).

2.16 Statistical analysis

All statistical analyses were carried out using Minitab version 13.1. For data to be assessed by parametric statistical tests (ANOVA, followed by Tukey's *post hoc* pair-wise comparisons tests or Student's t-test) all data sets were tested for normal distribution using the Anderson Darling normality test. Where data were not normally distributed they were transformed by Box-Cox transformation. Non-parametric tests were utilised where transformed data did not result in normal distribution (Mann-Whitney or Wilcoxon-rank tests). Statistical significance was set at $p<0.05$. Data were expressed as mean ± standard error of the mean (SEM).

Table 2-8 Summary of antibodies and dilutions used for immunofluorescence and western blotting experiments

Antibody	Supplier	Cat No.	Species	Dilution
Primary antibodies				
α-Erbb4	Santa Cruz	sc-283	Rabbit	1:1000
α-Nrg1	Santa Cruz	sc-348	Rabbit	1:1000
α-Actin-HRP	Santa Cruz	sc-1616	Rabbit	1:2000-1:7000
α-GFP	Serotec	4745-1051	Sheep	1:1000-1:3000
α-GFAP	Autogen Bioclear	AB-J158	Mouse	1:400-1:500
α-Akt	Cell Signalling	#9272	Rabbit	1:1000
α-phospho-Akt	Cell Signalling	#4058	Rabbit	1:1000
α-Erk	Cell Signalling	#4696	Mouse	1:2000
α-phospho-Erk	Cell Signalling	#4377	Rabbit	1:1000
α-N200	Sigma-Aldrich	N 0142	Mouse	1:400
α-Parvalbumin	Swant	PV 235	Mouse	1:5000
Secondary antibodies				
α-sheep-HRP	Santa Cruz	sc-2473	Donkey	1:1000-1:2000
α-mouse-HRP	Santa Cruz	sc-2306	Donkey	1:1000-1:2000
α- rabbit-HRP	Santa Cruz	sc-2305	Donkey	1:1000-1:2000
Alexa Fluor® 488 α-sheep IgG	Invitrogen Molecular Probes®	A-11015	Donkey	1:1000
Alexa Fluor® 594 α-mouse IgG	Invitrogen Molecular Probes®	A-11005	Goat	1:200

3 Validation of the NG108-15 cell line as a tool for researching Erbb4

3.1 Introduction

3.1.1 Neuronal cell lines as a tool for investigating CNS systems

Cell lines can serve as effective models for investigating several aspects of the CNS, such as researching pharmacological responses to new therapeutics before *in vivo* testing and exploring mechanisms of receptor mediated signalling. Although it is unlikely cell lines encapsulate the entire complex neuronal network system of the CNS, they are useful in determining roles of specific proteins in different cell types by utilising cell lines of different cellular origins; for example C6 and JS1 cells for investigating glial cells or Schwann cells, respectively. Moreover, the use of cell lines can help reduce the amount of animals required for neuroscience research.

In addition, neuronal-like cell lines are generally easy to maintain in culture, and although optimisation is required, they can be manipulated more simply than primary cultured cells. Much research has been performed in neuronal and non-neuronal cell lines to investigate proteins potentially involved in neuronal disorders. However, many studies over-express genes of interest in these cell lines without prior determination of whether the gene is endogenously expressed. It is important to characterise which genes/proteins are endogenously expressed in neuronal cell lines, as this will allow the study of processes that may be involved in disorders of the CNS in an environment where the cell has complete control over the expression of all components involved. Manipulation of gene expression i.e. by over-expression systems, may thus take over the endogenous control the cell has over these components of the process being investigated, and may lead to potentially unnatural interactions.

3.1.2 NG108-15 cell line

The NG108-15 cell line is a hybrid clonal cell line formed by Sendai virus-induced fusion of the mouse neuroblastoma cell clone N18TG-2 and the rat glioma cell clone C6 BV-1 (Klee & Nirenberg, 1974). This cell line has been widely used to investigate many aspects of the CNS and is known to express genes/proteins of multiple neuronal signalling systems endogenously. A large proportion of studies utilising NG108-15 cells have been to research the renin-angiotensin system (Gendron *et al.,* 2003; Kilian *et al.,* 2008) and δ-opioid receptor signalling (Eisinger & Ammer, 2011).

NG108-15 cells express multiple important neurotransmitter receptors including $5HT_3$ receptors (Emerit *et al.,* 1995; Matsushima *et al.,* 2010), NMDA receptors (Ohkuma *et al.*, 1994), glutamate receptors (Tanaka *et al.*, 2010) and acetylcholine receptors (both muscarinic and nicotinic) (Hamprecht, 1985). NG108-15 cells also express the inhibitory neurotransmitter GABA and its synthesising enzyme GAD (Searles & Singer, 1988). Since NG108-15 cells express aspects of these neurotransmitter systems, which have also been linked with Nrg1-Erbb4 function, it is likely that this signalling pathway is expressed in NG108-15 cells but this hypothesis requires confirmation.

3.2 Aims

The aims of this chapter were to validate the neuronal cell line NG108-15, as a potential tool for exploring the Nrg1-Erbb4 signalling pathway by investigating the mRNA and protein expression of Erbb4 in this cell line, and to determine if Erbb4 levels could be altered by siRNA mediated RNA interference and to study the consequences of such knockdown. In addition, the functionality of the Erbb4 receptors in NG108-15 cells was studied to explore if Nrg1-Erbb4 signalling in this cell line was a relevant model of Nrg1-Erbb4 signalling within the CNS.

3.3 Results

3.3.1 Expression of Erbb4 mRNA in NG108-15 cells

RT-PCR carried out on total RNA isolated from NG108-15 cells (sections 2.8.1 and 2.9) confirmed that *Erbb4* mRNA is expressed in this cell line (Figure 3.1). Species-specific PCR primers were utilised (section 2.10.2) as the genome of this cell line contains rat and mouse chromosomes and therefore potentially rat and/or mouse *Erbb4* transcripts. An amplicon of the predicted molecular weight (approximately 300bp) was obtained using a mouse specific forward PCR primer (FwdM1, section 2.10.1) in combination with a universal reverse primer (RevRM1, section 2.10.1) (Figure 3.1, lane 3) indicating that mouse *Erbb4* transcripts are expressed in this cell line. However no amplicons were obtained using a rat specific forward PCR primer (FwdR1, section 2.10.1) in combination with a universal reverse primer (RevRM1, section 2.10.1) (Figure 3.1, lane 5). These data suggest that the *Erbb4* transcripts in NG108-15 cells are of mouse origin.

To confirm that the NG108-15 *Erbb4* PCR product obtained with the mouse specific primers (Figure 3.1, lane 3) was indeed derived from mouse chromosomes in the hybrid cell line, the DNA sequence of this amplicon was obtained (sections 2.10.3 and 2.10.4). Bioinformatic analyses (section 2.10.4) showed that there was 100% sequence identity with this amplicon and mouse *Erbb4* mRNA (Figure 3.2A) and that no matches were found with any other mouse or rat transcripts or genomic DNA or sequences from other species, suggesting that this *Erbb4* PCR product was indeed of mouse origin. Furthermore, when the sequence of the PCR product was aligned with the 288bp rat *Erbb4* mRNA sequence there were multiple differences between the two sequences, confirming that the sequenced amplicon was not of rat origin (Figure 3.2B).

These data showed that *Erbb4* mRNA was expressed in the NG108-15 cell line and that it was likely to be solely of mouse origin. However the lack of a rat PCR product could be a technical artefact and therefore further PCR analyses were carried out. RT-PCR was performed on total RNA isolated from rat and mouse brains (sections 2.10.2) using the rat specific primers (FwdR1 and RevRM1). An amplicon of the correct molecular weight (approximately 300bp) was obtained from the rat brain cDNA (Figure 3.3A, lane 4), confirming that the technical conditions employed could amplify a rat *Erbb4* transcript if it was present in the NG108-15 sample. However, no amplicon was obtained from the mouse brain cDNA using the rat primers suggesting that the rat primers were indeed specific for the rat *Erbb4* mRNA (Figure 3.3A, lane 3). The species specificity of the PCR primers was confirmed when the mouse specific primers (FwdM1 and RevRM1) were used for RT-PCR on total RNA isolated from rat and mouse brains (sections 2.10.2). The mouse specific primers used to amplify an *Erbb4* product in NG108-15 cells (Figure 3.1, lane 3) were able to generate an amplicon from mouse brain cDNA (Figure 3.3B, lane 3) but not from rat brain cDNA (Figure 3.3B, lane 4).

Moreover, analysis of the DNA sequence of the PCR product produced from amplifying rat brain cDNA with the rat specific primers (FwdR1 and RevRM1) showed 100% sequence identity with rat *Erbb4* mRNA (Figure 3.4A). In addition there were no 100% identity matches with transcripts or genomic DNA from other species with. Furthermore, when the sequence of the rat brain cDNA PCR product was aligned with mouse *Erbb4* mRNA sequence there were multiple differences in the nucleotide sequence (Figure 3.4B) whereas alignment of the PCR product with the expected rat *Erbb4* amplicon sequence showed 100% sequence identity.

In addition as the correct molecular weight of the expected amplicon was achieved in all reactions, along with the 100% sequence identity of mouse *Erbb4* in NG108-15 PCR products and rat *Erbb4* in rat brain PCR products and that the resultant sequences of these PCR products showed two exons of Erbb4 confirm that the PCR products are very unlikely to be due to a genomic artefact. In summary, these data show that *Erbb4* is expressed in the NG108-15 cell line and despite these cells being a hybridoma of rat and mouse chromosomes, *Erbb4* is expressed from mouse chromosomes only.

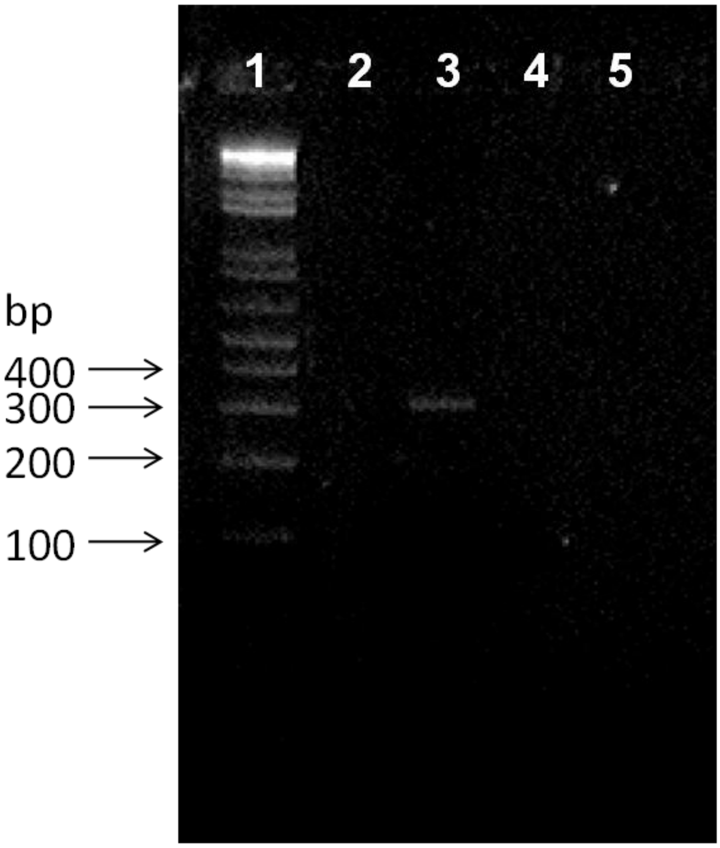
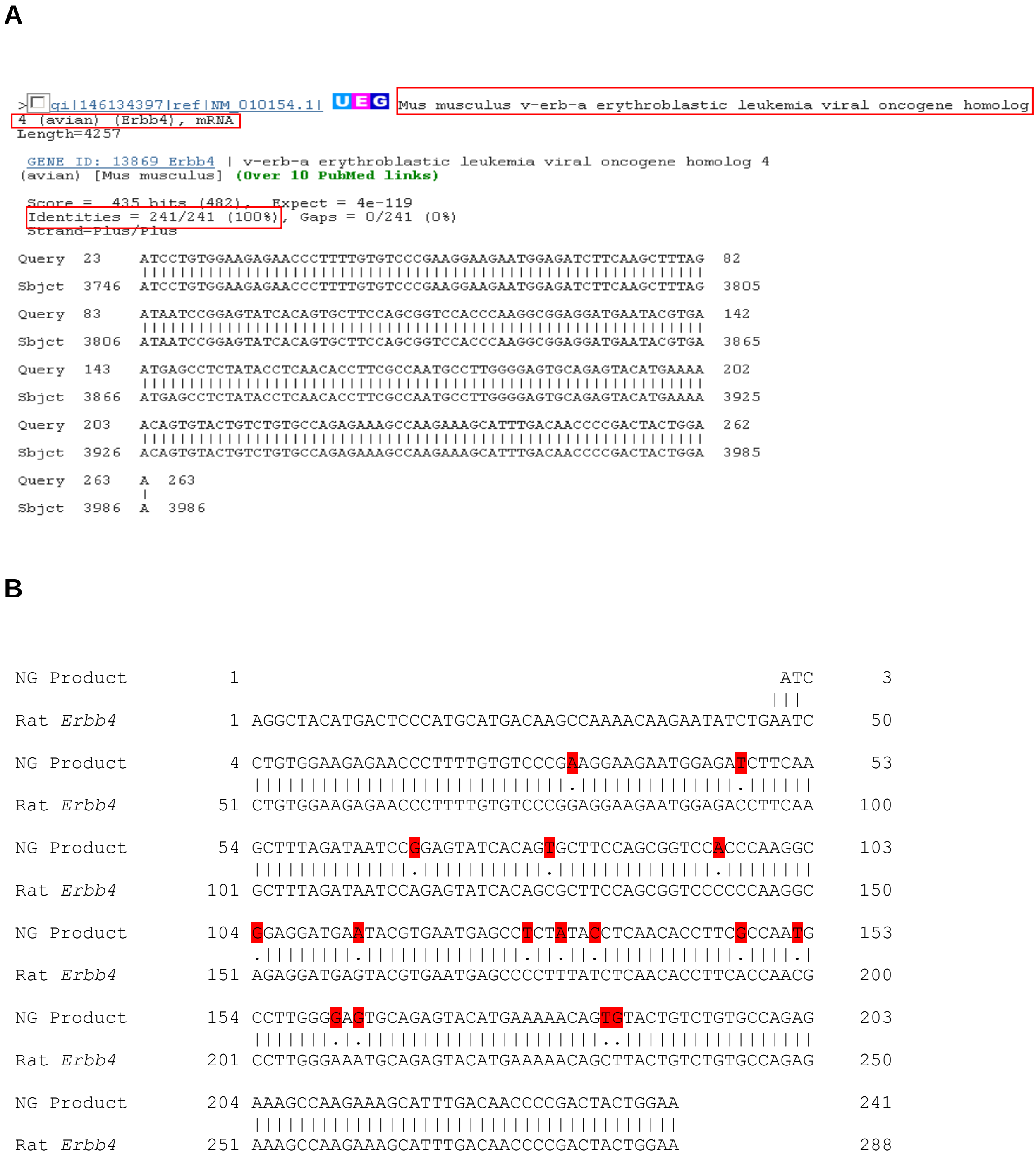


Figure 3.1 Expression of *Erbb4* in NG108-15 cells. Analysis of 30% (v/v) of RT-PCR products in a 2.5% (w/v) agarose gel visualised by ethidium bromide DNA stain under ultraviolet light. The molecular weight marker (lane 1) is 0.1μg 1Kb+ DNA ladder (Invitrogen). Lanes 2 and 3 contain products from using mouse specific primers (mouse exon 27 forward primer FwdM1 and exon 28 rat/mouse universal primer) in water (lane 2) and NG108-15 cell cDNA (lane 3). Lanes 4 and 5 contain products from using rat specific primers (rat exon 27 forward primer FwdR1 and exon 28 rat/mouse universal primer) in water (lane 4) and NG108-15 cell cDNA (lane 5). There are no amplicons from reactions using water as the template or using rat specific primers. A band of approximately 300bp, consistent with the predicted molecular weight, was obtained with mouse specific primers in cDNA from the NG108-15 cells.



A

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4 (avian) (Erbb4), mRNA
Length=4257

 GENE ID: 13869 Erbb4 | v-erb-a erythroblastic leukemia viral oncogene homolog 4
(avian) [Mus musculus] (Over 10 PubMed links)

 Score =  435 bits (482),  Expect = 4e-119
 Identities = 241/241 (100%), Gaps = 0/241 (0%)
 Strand=Plus/Plus

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Query  83    ATAATCCGGAGTATCACAGTGCTTCCAGCGGTCCACCCAAGGCGGAGGATGAATACGTGA  142
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Sbjct  3806  ATAATCCGGAGTATCACAGTGCTTCCAGCGGTCCACCCAAGGCGGAGGATGAATACGTGA  3865

Query  143   ATGAGCCTCTATACCTCAACACCTTCGCCAATGCCTTGGGGAGTGCAGAGTACATGAAAA  202
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Sbjct  3866  ATGAGCCTCTATACCTCAACACCTTCGCCAATGCCTTGGGGAGTGCAGAGTACATGAAAA  3925

Query  203   ACAGTGTACTGTCTGTGCCAGAGAAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGA  262
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3926  ACAGTGTACTGTCTGTGCCAGAGAAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGA  3985

Query  263   A  263
             |
Sbjct  3986  A  3986
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B

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NG Product            1                                                ATC     3
                                                                       |||
Rat Erbb4             1 AGGCTACATGACTCCCATGCATGACAAGCCAAAACAAGAATATCTGAATC    50

NG Product            4 CTGTGGAAGAGAACCCTTTTGTGTCCCGAAGGAAGAATGGAGATCTTCAA    53
                        ||||||||||||||||||||||||||||.||||||||||||||.||||||
Rat Erbb4            51 CTGTGGAAGAGAACCCTTTTGTGTCCCGGAGGAAGAATGGAGACCTTCAA   100

NG Product           54 GCTTTAGATAATCCGGAGTATCACAGTGCTTCCAGCGGTCCACCCAAGGC   103
                        ||||||||||||||.|||||||||||.||||||||||||||.||||||||
Rat Erbb4           101 GCTTTAGATAATCCAGAGTATCACAGCGCTTCCAGCGGTCCCCCCAAGGC   150

NG Product          104 GGAGGATGAATACGTGAATGAGCCTCTATACCTCAACACCTTCGCCAATG   153
                        .||||||||.||||||||||||||.||.||.||||||||||||.||||.|
Rat Erbb4           151 AGAGGATGAGTACGTGAATGAGCCCCTTTATCTCAACACCTTCACCAACG   200

NG Product          154 CCTTGGGGAGTGCAGAGTACATGAAAAACAGTGTACTGTCTGTGCCAGAG   203
                        |||||||.|.|||||||||||||||||||||..|||||||||||||||||
Rat Erbb4           201 CCTTGGGAAATGCAGAGTACATGAAAAACAGCTTACTGTCTGTGCCAGAG   250

NG Product          204 AAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGAA               241
                        ||||||||||||||||||||||||||||||||||||||
Rat Erbb4           251 AAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGAA               288
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Figure 3.2 Sequence analyses of the NG108-15 cell mouse specific *Erbb4* PCR product. (A) BLAST analysis (section 2.10.4) of the DNA sequence of the *Erbb4* amplicon generated with mouse specific PCR primers (section 2.10.1). Query = NG108-15 cell PCR product sequence, Sbjct = mouse *Erbb4* mRNA sequence. The alignment of the *Erbb4* PCR product sequence (Query) with mouse *Erbb4* mRNA (Sbjct) shows 100% sequence identity, confirming that this amplicon is of mouse origin. Gene name and sequence identity are highlighted by red boxes. (B) Alignment of the DNA sequence of the *Erbb4* amplicon (NG product) generated with mouse specific PCR primers with rat *Erbb4* mRNA sequence. Sequence identity was 93% indicating that this was not a rat transcript. The nucleotides that did not match between the two sequences are highlighted in red.

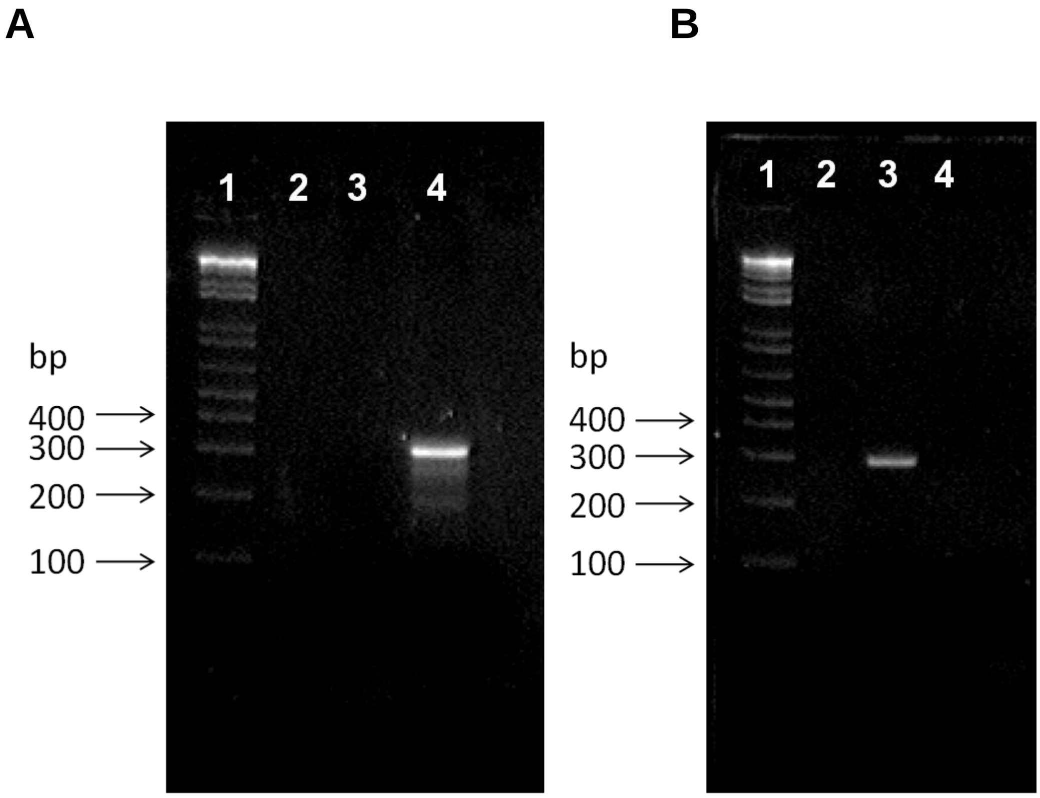
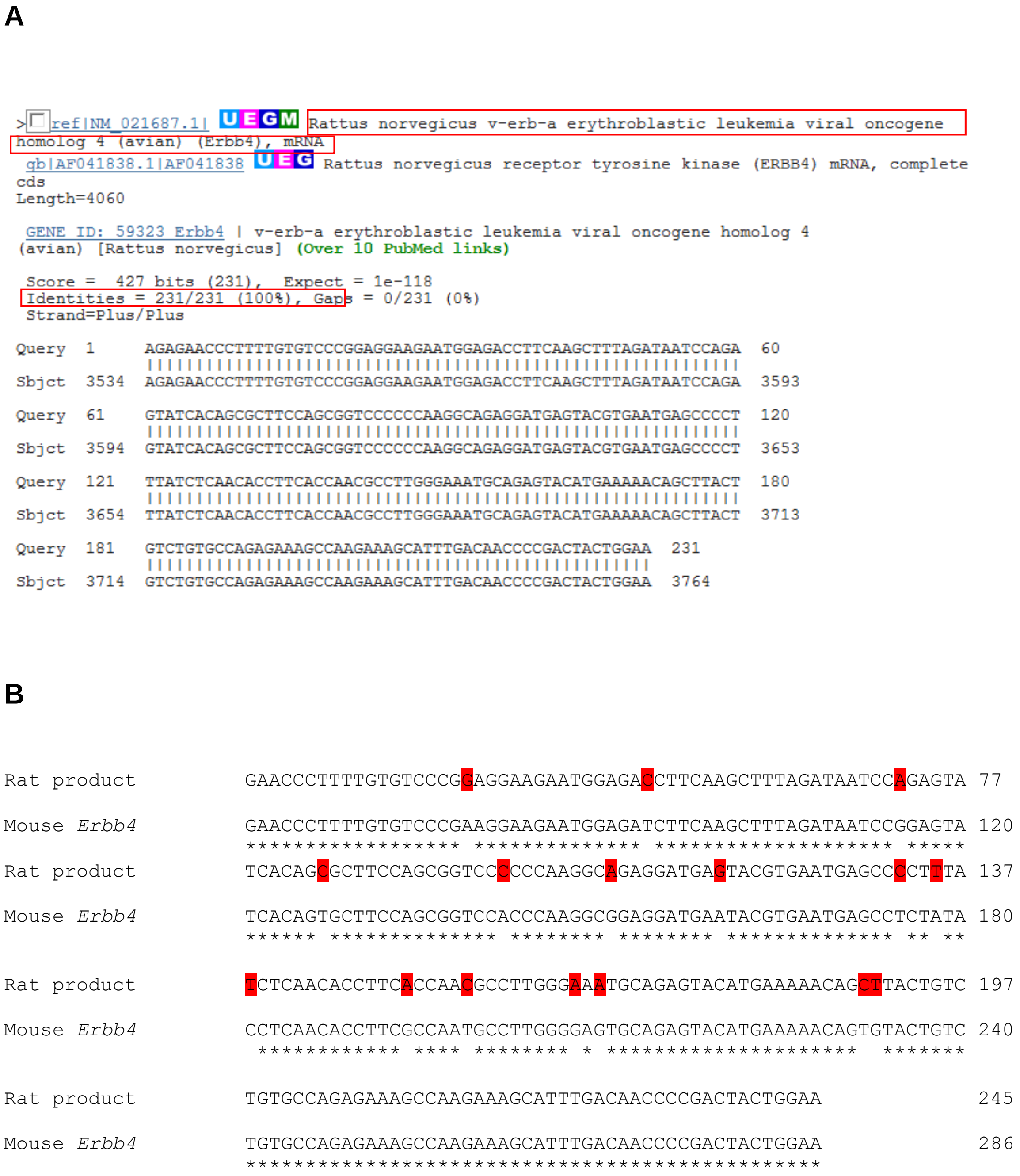


Figure 3.3 Validation of mouse and rat *Erbb4* PCR primers. Analysis of 10% (v/v) of RT-PCR products in a 2.5% (w/v) agarose gel visualised by ethidium bromide DNA stain under ultraviolet light. The molecular weight marker (lane 1) is 0.1μg 1Kb+ DNA ladder (Invitrogen). (A) Lanes 2, 3 and 4 contain products from using rat specific primers (rat exon 27 forward primer FwdR1 and exon 28 rat/mouse universal primer) in water (lane 2), mouse brain cDNA (lane 3) and rat brain cDNA (lane 4). (B) Lanes 2,3 and 4 contain products from using mouse specific primers (mouse exon 27 forward primer FwdM1 and exon 28 rat/mouse universal primer) in water (lane 2), mouse brain cDNA (lane 3) and rat brain cDNA (lane 4). There are no amplicons from reactions using water as the template or using rat specific primers with mouse brain cDNA or using mouse specific primers with rat specific primers. A band of approximately 300bp, consistent with the predicted molecular weight, was obtained with mouse specific primers in cDNA from mouse brain and with rat specific primers in cDNA from rat brain.



A

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homolog 4 (avian) (Erbb4), mRNA
 gb|AF041838.1|AF041838 UEG Rattus norvegicus receptor tyrosine kinase (ERBB4) mRNA, complete
cds
Length=4060

 GENE ID: 59323 Erbb4 | v-erb-a erythroblastic leukemia viral oncogene homolog 4
(avian) [Rattus norvegicus] (Over 10 PubMed links)

 Score =  427 bits (231),  Expect = 1e-118
 Identities = 231/231 (100%), Gaps = 0/231 (0%)
 Strand=Plus/Plus

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Query  61    GTATCACAGCGCTTCCAGCGGTCCCCCCAAGGCAGAGGATGAGTACGTGAATGAGCCCCT  120
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Sbjct  3594  GTATCACAGCGCTTCCAGCGGTCCCCCCAAGGCAGAGGATGAGTACGTGAATGAGCCCCT  3653

Query  121   TTATCTCAACACCTTCACCAACGCCTTGGGAAATGCAGAGTACATGAAAAACAGCTTACT  180
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Sbjct  3654  TTATCTCAACACCTTCACCAACGCCTTGGGAAATGCAGAGTACATGAAAAACAGCTTACT  3713

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Sbjct  3714  GTCTGTGCCAGAGAAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGAA  3764
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B

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Rat product      GAACCCTTTTGTGTCCCGGAGGAAGAATGGAGACCTTCAAGCTTTAGATAATCCAGAGTA 77

Mouse Erbb4      GAACCCTTTTGTGTCCCGAAGGAAGAATGGAGATCTTCAAGCTTTAGATAATCCGGAGTA 120
                 ****************** ************** ******************** *****
Rat product      TCACAGCGCTTCCAGCGGTCCCCCCAAGGCAGAGGATGAGTACGTGAATGAGCCCCTTTA 137

Mouse Erbb4      TCACAGTGCTTCCAGCGGTCCACCCAAGGCGGAGGATGAATACGTGAATGAGCCTCTATA 180
                 ****** ************** ******** ******** ************** ** **

Rat product      TCTCAACACCTTCACCAACGCCTTGGGAAATGCAGAGTACATGAAAAACAGCTTACTGTC 197

Mouse Erbb4      CCTCAACACCTTCGCCAATGCCTTGGGGAGTGCAGAGTACATGAAAAACAGTGTACTGTC 240
                  ************ **** ******** * *********************  *******

Rat product      TGTGCCAGAGAAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGAA             245

Mouse Erbb4      TGTGCCAGAGAAAGCCAAGAAAGCATTTGACAACCCCGACTACTGGAA             286
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Figure 3.4 Sequence analyses of rat brain PCR product. (A) BLAST analysis (section 2.10.4) of the DNA sequence of the *Erbb4* amplicon generated with rat specific PCR primers (section 2.10.1). Query = rat brain PCR product sequence, Sbjct = rat *Erbb4* mRNA sequence. The alignment of the rat brain PCR product sequence (Query) with rat *Erbb4* mRNA (Sbjct) shows 100% sequence identity, confirming that this amplicon is of rat origin. Gene name and sequence identity are highlighted by red boxes. (B) Alignment of the DNA sequence of the *Erbb4* amplicon (rat brain product) generated with rat specific PCR primers with mouse *Erbb4* mRNA sequence. Sequence identity was 93% indicating that this was not a mouse transcript. The nucleotides that did not match between the two sequences are highlighted in red.

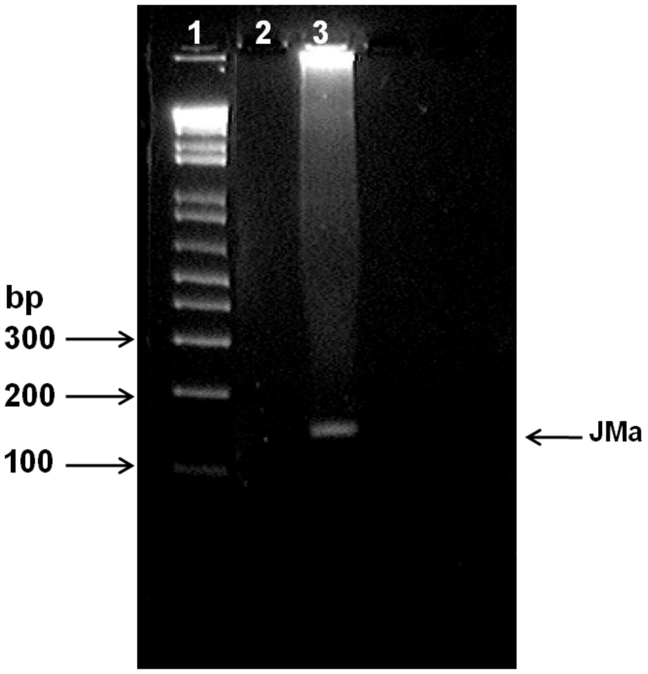
3.3.2 Expression of Erbb4 isoforms in NG108-15 cells

The splicing pattern of Erbb4 is complex (Elenius *et al.*, 1997; Elenius *et al.*, 1999; Tan *et al.*, 2010) and so having confirmed that *Erbb4* is expressed in the NG108-15 cells, it was necessary to determine which splice variants are expressed. Isoform specific primers were designed that would distinguish between the *JMa* and *JMb* isoforms and also between the *CYT1* and *CYT2* isoforms (*JMa/b* isoforms: exon 14, M/R JMa/b Fwd primer and exon 17, M/R JMa/b Rev primer and *CYT1/2* isoforms: exon 25, M CYT1/2 Fwd and exon 27, M CYT1/2 Rev).

An amplicon of the predicted molecular weight for the *Erbb4 JMa* isoform (approximately 140bp) was detected in the NG108-15 cells (Figure 3.5A, lane 3) but there was no amplicon for the predicted molecular weight of the *JMb* isoform. The presence of a product with an amplicon of 140bp but not 100bp in the NG108-15 cell sample indicates that NG108-15 cells express the mouse *JMa Erbb4* isoform only. The RT-PCR conditions were tested using total RNA isolated from mouse brain, in which *Erbb4* seems to be more highly expressed. Amplicons were obtained for *JMa* (140bp) and *JMb* (100bp) in the mouse brain cDNA (Figure 3.5B, lane 3), indicating that if the *JMb* Isoform is expressed in the NG108-15 cells it would have been detected.

Amplicons of the predicted molecular weight of the *Erbb4 CYT1* and *CYT2* isoforms (approximately 100 bp and 150bp, respectively) were detected in the NG108-15 cell cDNA (Figure 3.6A, lane 4). Again, the RT-PCR conditions were tested using total RNA isolated from mouse brain, in which *Erbb4* seems to be more highly expressed. Amplicons were obtained for both *Erbb4 CYT1* and *CYT2* isoforms (Figure 3.6B, lane 3). The presence of products with amplicons of 100bp and 150bp in the NG108-15 cell sample indicates that NG108-15 cells express the mouse *CYT1* and *CYT2 Erbb4* isoforms.

A



B

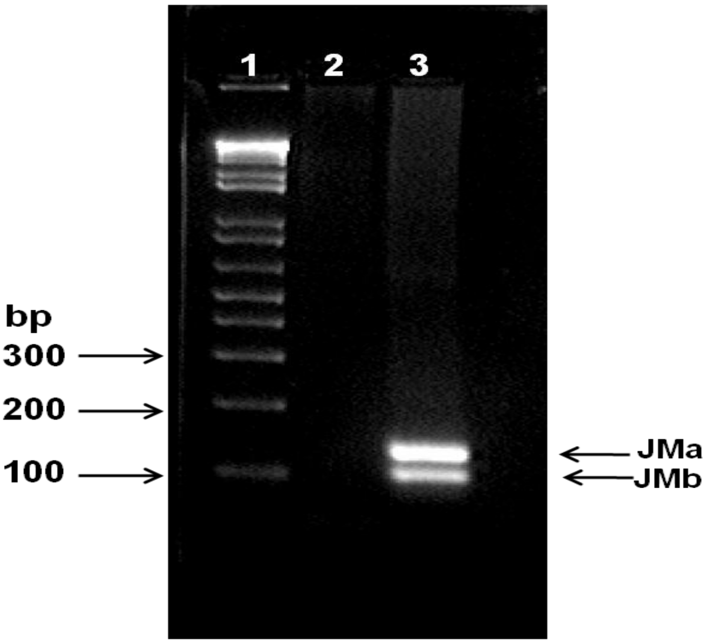
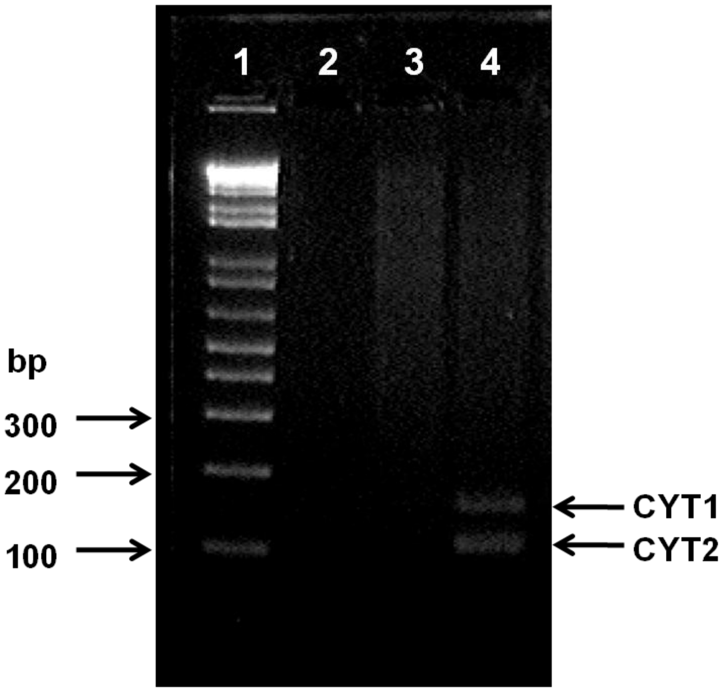


Figure 3.5 Investigation of *Erbb4 JMa/JMb* isoform expression in NG108-15 cells. Analysis of RT-PCR products in a 2.5% (w/v) agarose gel visualised by ethidium bromide DNA stain under ultraviolet light. The molecular weight marker (lane 1) is 0.1μg 1Kb+ DNA ladder (Invitrogen). (A) Lanes 2, and 3 contain 40% (v/v) PCR products from using primers designed to detect JMa/JMb isoforms with expected amplicon lengths of 135bp and 103bp, respectively (mouse/rat exon 14 forward primer M/R JMa/b Fwd and mouse/rat exon 17 reverse primer JMa/b Rev) in water (lane 2), and NG108-15 cell cDNA (lane 3). (B) Lanes 2, and 3 contain 20% (v/v) PCR products from using primers designed to detect JMa/JMb isoforms with expected amplicon lengths of 135bp and 103bp, respectively (mouse/rat exon 14 forward primer M/R JMa/b Fwd and mouse/rat exon 17 reverse primer JMa/b Rev) in water (lane 2), and mouse brain cDNA (lane 3). There are no amplicons from reactions using water as the template. A band of approximately 140bp, consistent with the predicted molecular weight of *JMa*, was obtained with JMa/b specific primers in cDNA from NG108-15 cell, two bands of approximately 100bp and 140bp, consistent with the predicted molecular weight of *JMa* and *JMb*, were obtained with JMa/b specific primers in cDNA from mouse brain.

A



B

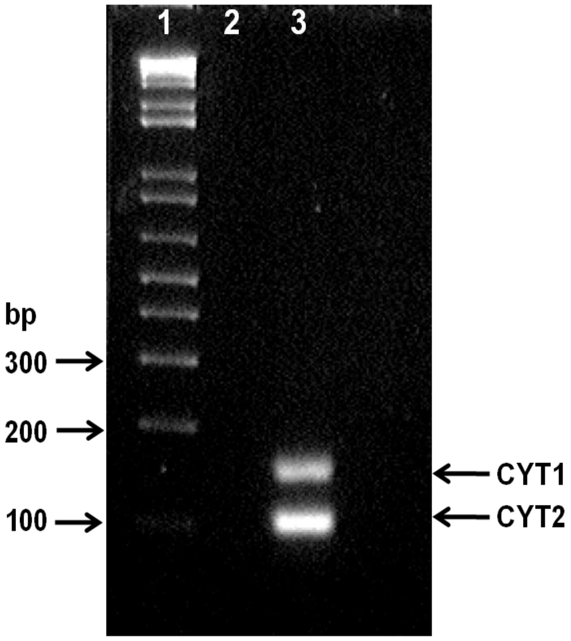


Figure 3.6 Investigation of *CYT1/2* isoform expression in NG108-15 cells. Analysis of RT-PCR products in a 2.5% (w/v) agarose gel visualised by ethidium bromide DNA stain under ultraviolet light. The molecular weight marker (lane 1) is 0.1μg 1Kb+ DNA ladder (Invitrogen). (A) Lanes 2, and 4 contain 40% (v/v) PCR products from using primers designed to detect *CYT1/CYT2* isoforms with expected amplicon lengths of 149bp and 101bp, respectively (mouse exon 25 forward primer M CYT1/2 Fwd and mouse exon 27 reverse primer M CYT1/2 Rev) in water (lane 2), and NG108-15 cell cDNA (lane 4). (B) Lanes 2, and 3 contain 20% (v/v) PCR products from using primers designed to detect *CYT1/CYT2* isoforms with expected amplicon lengths of 149bp and 101bp, respectively (mouse exon 25 forward primer M CYT1/2 Fwd and mouse exon 27 reverse primer M CYT1/2 Rev) in water (lane 2), and mouse brain cDNA (lane 3). There are no amplicons from reactions using water as the template. Bands of approximately 100bp and 150bp, consistent with the predicted molecular weight of CYT1 and CYT2, were obtained with *CYT1/2* specific primers in cDNA from NG108-15 cell and cDNA from mouse brain.

3.3.3 Expression of Erbb4 *and* Nrg1 *protein in NG108-15 cells*

Western blotting was carried out to determine whether the *Erbb4* mRNA is translated to protein and whether Nrg1 protein is expressed in NG108-15 cells (Section 2.13) and also to determine if expression of these proteins is similar to other neural tissues.

Erbb4 immunoreactive bands of approximately 180kDa, 150kDa, and 60kDa were observed in the NG108-15 cell sample. All of these immunoreactive bands were also present in rat and mouse brain samples with faint bands at 100kDa also present (Figure 3.7). The 180kDa band corresponds to the predicted full length molecular weight of Erbb4, whereas the lower molecular weight bands correspond to predicted molecular weights of cleaved and truncated forms of Erbb4. To compare the expression of Erbb4 in neuronal tissue (rat and mouse brain) and neuronal-like cells (NG108-15) with a peripheral tissue, immunoreactive bands of Erbb4 in rat heart tissue were also examined. Very similar immunoreactive bands were observed in the rat heart lysate than that of the NG108-15 cells, rat and mouse brain, but there was no 60kDa immunoreactive band in the rat heart sample. To achieve the 180kDa immunoreactive band, predicted to represent full length Erbb4, 45μg of NG108-15 cell lysate was required whereas 15μg of the brain and heart tissue was enough tissue to give prominent immunoreactive bands. This is suggestive that the expression of Erbb4 in NG108-15 cells is relatively low compared to cardiac or neuronal tissue.

Nrg1 immunoreactive bands of approximately 150kDa, 100kDa, 80kDa, 55kDa, 53kDa and 48kDa were observed in the NG108-15 cell sample (Figure 3.8). All these immunoreactive bands were also present in the rat and mouse brain samples with additional lower molecular weight bands of less than 48kDa also present (Figure 3.8). These bands represent both full length and cleaved forms of Nrg1. The intensities of the immunoreactive bands did differ between samples, with the NG108-15 cell sample having a much lower intensity of the 100kDa immunoreactive band than the brain samples despite the amount of NG108-15 cell total protein lysate analysed being much greater than that of the brain samples (45μg from the NG108-15 cells and 15μg from the brain samples).

The NG108-15 cells did however appear to have a greater abundance of the 48kDa immunoreactive band.

In summary, the western blot analysis of NG108-15 cell total protein lysates showed that multiple Erbb4 and Nrg1 protein isoforms, including the full length proteins, are expressed in NG108-15 cells.

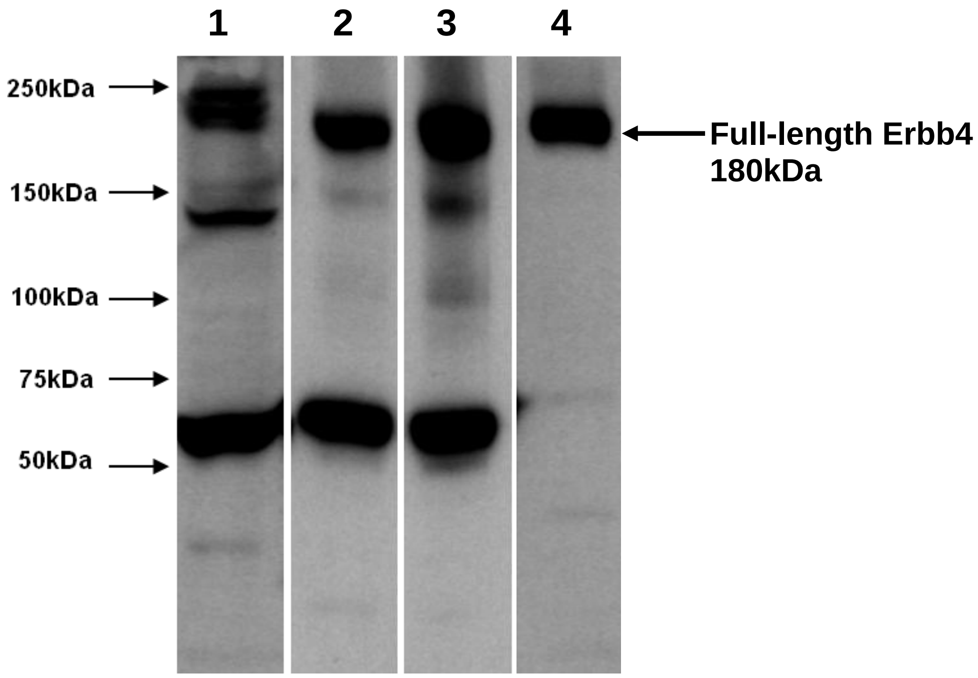


Figure 3.7 Comparison of the expression of Erbb4 protein in NG108-15 cells with neuronal and non-neuronal tissue. Representative western blots of 45μg NG108-15 cell (lane 1), 15μg rat brain (lane 2), 15μg mouse brain (lane 3) and 15μg rat heart (lane 4) total protein lysates depicting immunoreactive Erbb4 protein.

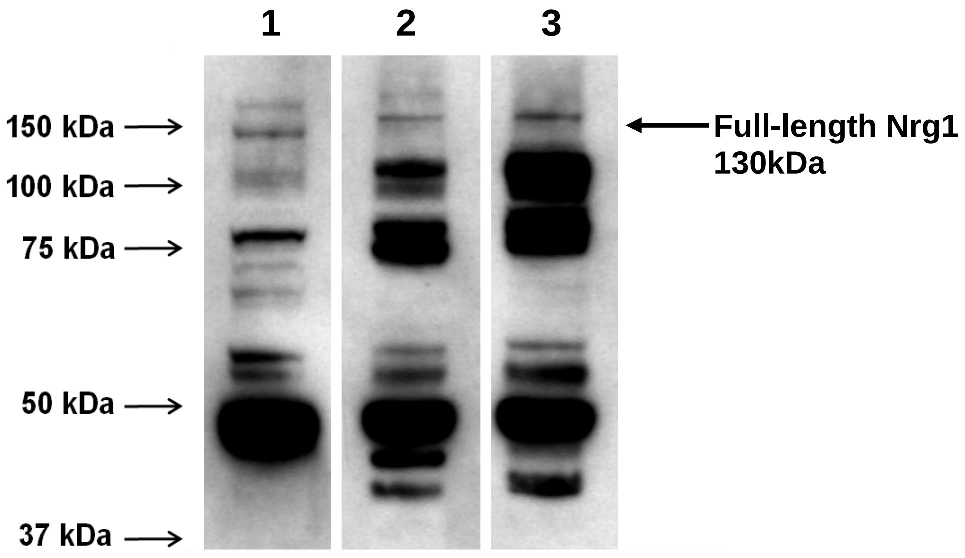
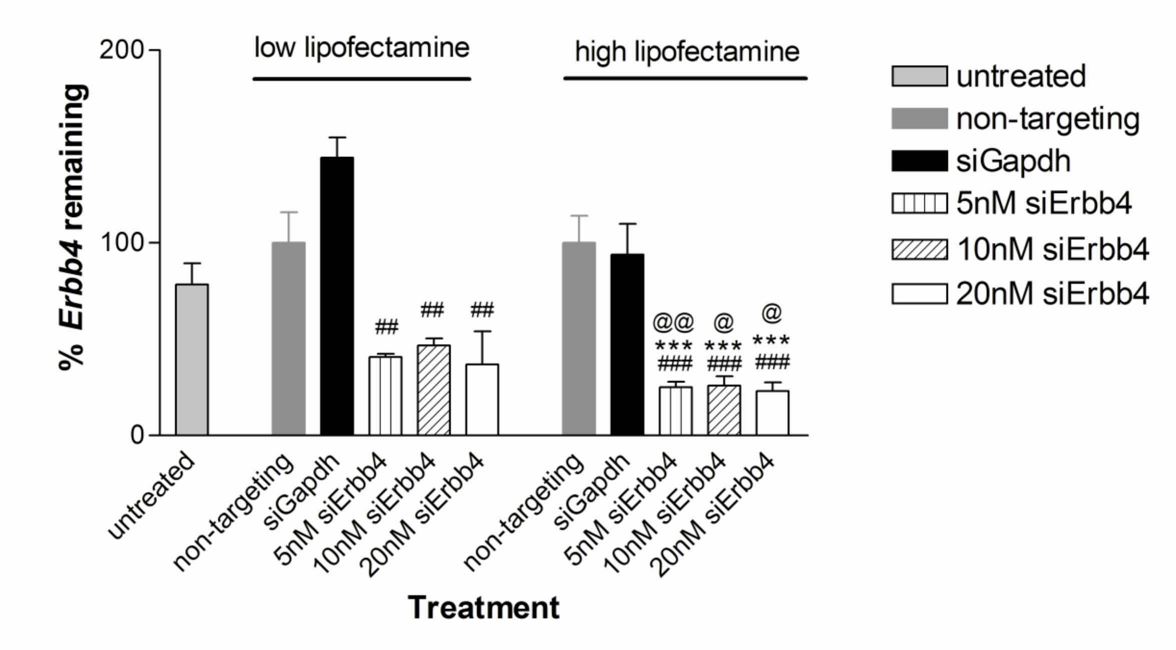


Figure 3.8 Comparison of the expression of Nrg1 protein in NG108-15 cells with neuronal tissue Representative western blots of 45μg NG108-15 cell (lane 1), 15μg rat brain (lane 2) and 15μg mouse brain (lane 3) total protein lysates depicting immunoreactive Nrg1 protein.

3.3.4 Optimisation of siRNA mediated Erbb4 knockdown

Confirmation of the expression of Erbb4 at the mRNA and protein levels indicated that this cell line could be used as an *in vitro* tool for validating Erbb4 function in a critical signalling pathway for schizophrenia by utilising siRNA (section 2.3.1) to knockdown expression and for validating viral vectors for *in vivo* analyses (methods section 2.3.2 and results section 4.3.1). Successful gene expression knockdown requires optimisation of key factors such as concentration of the siRNA and the cell transfection reagent. Optimisation experiments were conducted in which three concentrations of mouse *Erbb4* siRNA (5nM, 10nM and 20nM) were tested in conjunction with two volumes of transfection reagent (3.75μl and 7.5μl) (section 2.3.1). Knockdown of *Erbb4* mRNA was measured in the NG108-15 cells 24 hours post-siRNA application using qRT-PCR (section 2.11). Treatment of the cells with all three concentrations of *Erbb4* siRNA and both volumes of transfection reagent resulted in *Erbb4* mRNA knockdown (Figure 3.9A). Although there was no overall significant effect of transfection reagent volume on *Erbb4* expression (F (1, 33) =0.15 p>0.05), cells transfected with the *Erbb4* siRNA and the higher volume of transfection reagent (7.5μl Lipofectamine™ RNAiMAX), reduced *Erbb4* expression to a greater extent than those transfected with the lower volume (3.75μl Lipofectamine™ RNAiMAX) when compared to cells treated with non-targeting siRNA (Figure3.9A). NG108-15 cells had up to 77% knockdown in *Erbb4* expression following Erbb4 siRNA treatment.

All concentrations of *Erbb4* siRNA with 7.5μl transfection reagent significantly reduced *Erbb4* expression compared to untreated cells, cells treated with non-targeting siRNA and cells treated with *Gapdh* siRNA. Importantly there was no difference in *Erbb4* expression between the control treatment groups (untreated cells, non-targeting siRNA treated cells and *Gapdh* siRNA treated cells). Measurement of the expression of a house-keeping gene, *β-2-microglobulin*, was utilised to determine if the volume of transfection reagent or the *Erbb4* siRNA had off-target effects. There was no significant effect of treatment (F (10, 39) =0.33 p>0.05) or transfection reagent volume (F (1, 33) =0.01 p>0.05) on *β-2-microglobulin* expression. (Figure 3.9B)


low lipofectamine
high lipofectamine
% Erbb4 remaining
200
100
0
##
##
##
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untreated
non-targeting
siGapdh
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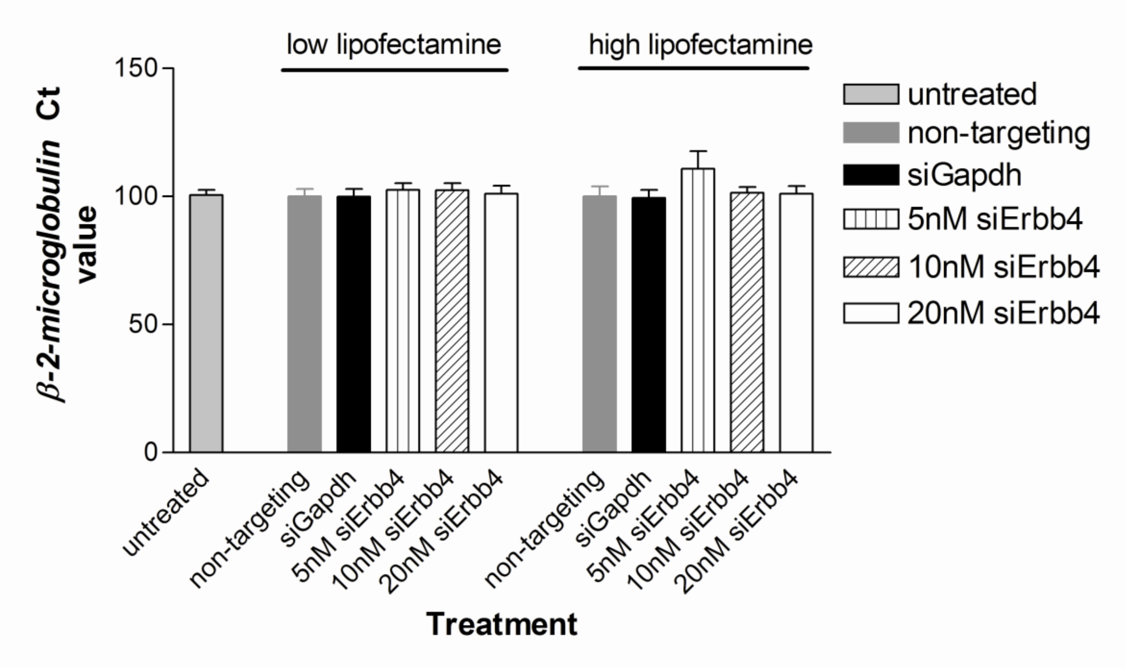
low lipofectamine
high lipofectamine
β-2-microglobulin Ct value
150
100
50
0
untreated
non-targeting
siGapdh
5nM siErbb4
10nM siErbb4
20nM siErbb4
non-targeting
siGapdh
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10nM siErbb4
20nM siErbb4
Treatment
untreated
non-targeting
siGapdh
5nM siErbb4
10nM siErbb4
20nM siErbb4

Figure 3.9 Optimisation of the concentration of *Erbb4* siRNA and transfection reagent volume to produce significant knockdown of *Erbb4* in NG108-15 cell transfections. (A) % expression remaining of *Erbb4* relative to *Erbb4* expression in non-targeting siRNA treated NG108-15 cells (B) % expression remaining of *β-2-microglobulin* relative to *β-2-microglobulin* expression in non-targeting siRNA treated NG108-15 cells. low lipofectamine = 3.75μl Lipofectamine™ RNAiMAX transfection reagent, high lipofectamine = 7.5μl Lipofectamine™ RNAiMAX transfection reagent. qRT-PCR was performed on total RNA isolated from Untreated = untreated NG108-15 cells, non-targeting = NG108-15 cells transfected with non-targeting siRNA, siGapdh = NG108-15 cells transfected with a *Gapdh* siRNA, siErbb4 = NG108-15 cells transfected with the *Erbb4* siRNA at a concentration of 5nM, 10nM and 20nM. Data represent mean ± SE expressed as a percentage of the *Erbb4 or β-2-microglobulin* expression remaining in cells transfected with non-targeting siRNA at equivalent lipofectamine volumes. n=3-6/treatment group. ### $p<0.001$ vs. *Erbb4* expression in NG108-15 cells transfected with *Gapdh* siRNA at equivalent lipofectamine volume, *** $p<0.001$ vs. *Erbb4* expression in NG108-15 cells transfected with non-targeting siRNA treated samples at equivalent lipofectamine volume, @ $p<0.05$, @@$p<0.01$ vs. *Erbb4* expression in untreated NG108-15 cells using two-way ANOVA followed by Tukey’s test for multiple comparisons.

Concurrently NG108-15 cells were transfected with a siRNA for the house-keeping gene *Gapdh*, to act as a positive control for the procedure. Knockdown of *Gapdh* mRNA was measured in the NG108-15 cells 24 hours post-siRNA application using qRT-PCR. *Gapdh* expression was significantly affected by treatment (F (5,17)=41.59 p<0.001) with *post hoc* comparisons revealing cells transfected with *Gapdh* siRNA had significantly less *Gapdh* expression than all other treatment groups (p<0.001) (Figure 3.10). *Gapdh* expression was also measured to check for off-target effects of the *Erbb4* siRNA, in addition to the measurement of *β-2-microglobulin*. Importantly *Gapdh* expression did not change under any of the conditions that the cells were transfected with *Erbb4* siRNA (Figure 3.10).

In summary, transfection of NG108-15 cells with the *Erbb4* siRNA produced significant knockdown of *Erbb4* mRNA but not *Gapdh* or *β-2-microglobulin,* suggesting knockdown specificity.

These data showed that transfection of NG108-15 cells with 20nM *Erbb4* siRNA using 7.5μl Lipofectamine™ RNAiMAX led to a 77% knockdown of *Erbb4* mRNA without affecting the expression of *Gapdh* or *β-2-microglobulin*. Therefore these conditions were chosen to investigate if reduced Erbb4 protein levels could be achieved and detected.

Although real time PCR analysis showed that at 24 hours post-*Erbb4* siRNA treatment there was significant *Erbb4* mRNA knockdown, protein levels of Erbb4 at 24 hours post-*Erbb4* siRNA treatment remain comparable to untreated cells and non-targeting siRNA treated cells. Erbb4 protein levels were also not knocked down using 20nM *Erbb4* siRNA treatment 48 hours, 72 hours or 96 hours post-siRNA application (Figure 3.11 top panel). *Gapdh* siRNA application however resulted in Gapdh protein knockdown 48 hours post-siRNA application (Figure 3.11 middle panel). Unfortunately, Erbb4 expression in NG108-15 cells was below the detection levels of the ERBB4 ELISA, therefore Erbb4 levels post-siRNA treatment could not be quantified using this method.

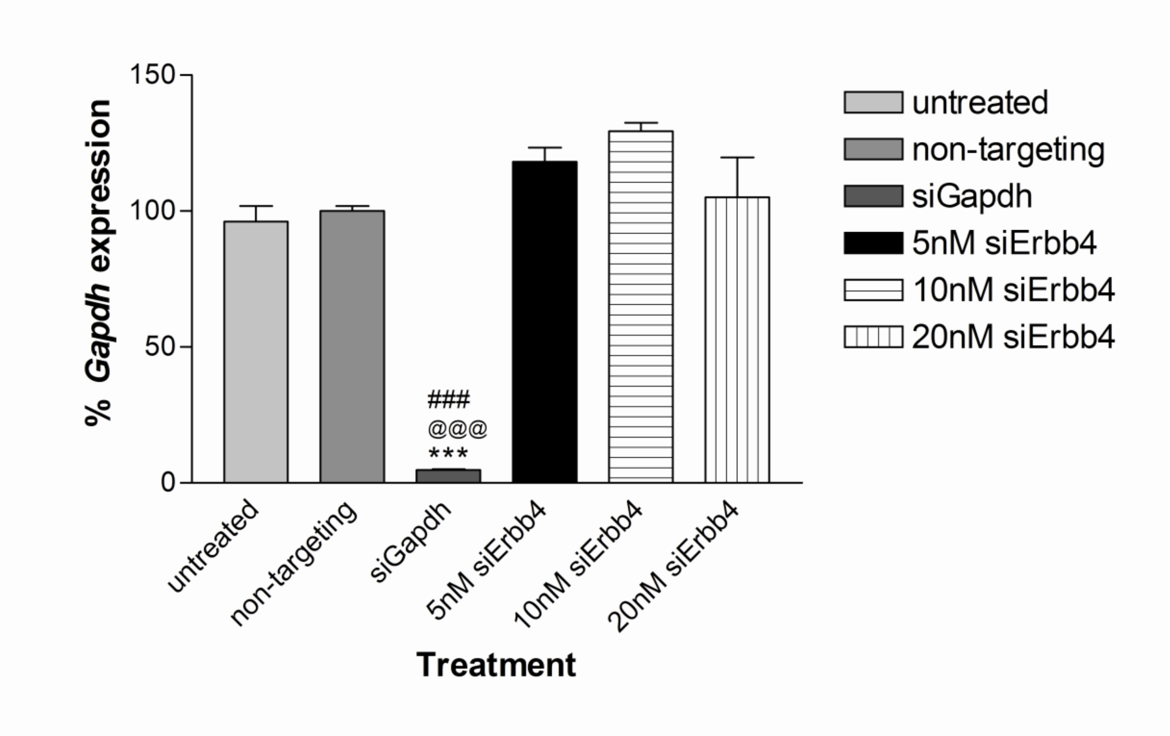


Figure 3.10 Effects on *Gapdh* expression in NG108-15 cells transfected with siRNA. % expression remaining of *Gapdh*. qRT-PCR was performed on total RNA isolated from Untreated = untreated NG108-15 cells, non-targeting = NG108-15 cells transfected with non-targeting siRNA, siGapdh = NG108-15 cells transfected with a *Gapdh* siRNA, siErbb4 = NG108-15 cells transfected with the *Erbb4* siRNA at a concentration of 5nM, 10nM and 20nM. Data represent mean ± SE expressed as a percentage of the *Gapdh* expression remaining in cells transfected with non-targeting siRNA. n=3/treatment group. ### $p<0.001$ vs. *Gapdh* expression in NG108-15 cells transfected with *Erbb4* siRNA at all concentrations tested, *** $p<0.001$ vs. *Gapdh* expression in NG108-15 cells transfected with non-targeting siRNA, @@@ $p<0.001$ vs. *Gapdh* expression in untreated NG108-15 cells using two-way ANOVA followed by Tukey’s test for multiple comparisons.

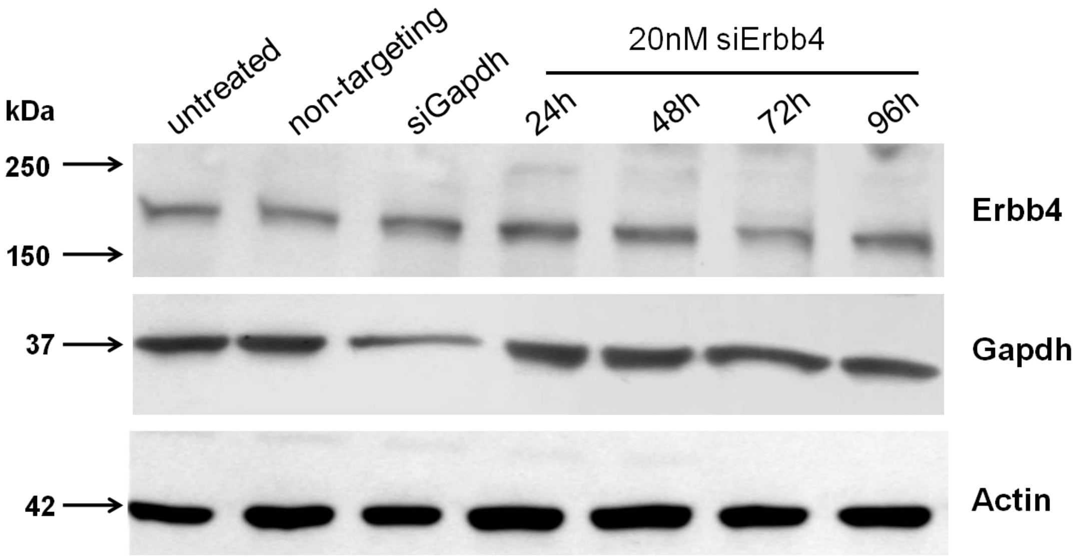


Figure 3.11 Erbb4 protein expression in NG108-15 cells following siRNA treatment. Representative western blots of 45μg NG108-15 cell lysates depicting immunoreactive protein levels of Erbb4 at 180kDa, Gapdh at 37kDa and Actin at 42kDa at 24 hours, 48 hours, 72 hours and 96 hours following application of siRNA. Untreated= untreated NG108-15 cells, non-targeting= NG108-15 cells transfected with non-targeting siRNA, siGapdh= NG108-15 cell transfected with *Gapdh* siRNA, siErbb4= NG108-15 cells transfected with *Erbb4* siRNA. Immunoblots are representative results of n=4 at each condition.

3.3.5 Effect of siRNA-mediated knockdown of Erbb4 in NG-108-15 cells on the expression of related genes

Having obtained *Erbb4* knockdown in NG108-15, expression of genes in the Erbb4 signalling pathway was assessed. Real-time PCR was used to quantify the expression of *Akt* and *Grin1*. *Akt* expression was not significantly affected by transfection of the cells with siRNA (F (5, 32) =2.19 $p>0.05$) (Figure 3.12). Conversely, *Grin1* expression was significantly affected in cells transfected with *non-targeting* siRNA (F (5, 32) =11.22 $p<0.001$) (Figure 3.13). However, there were no differences in *Grin1* expression in cells transfected with *Erbb4* siRNA.

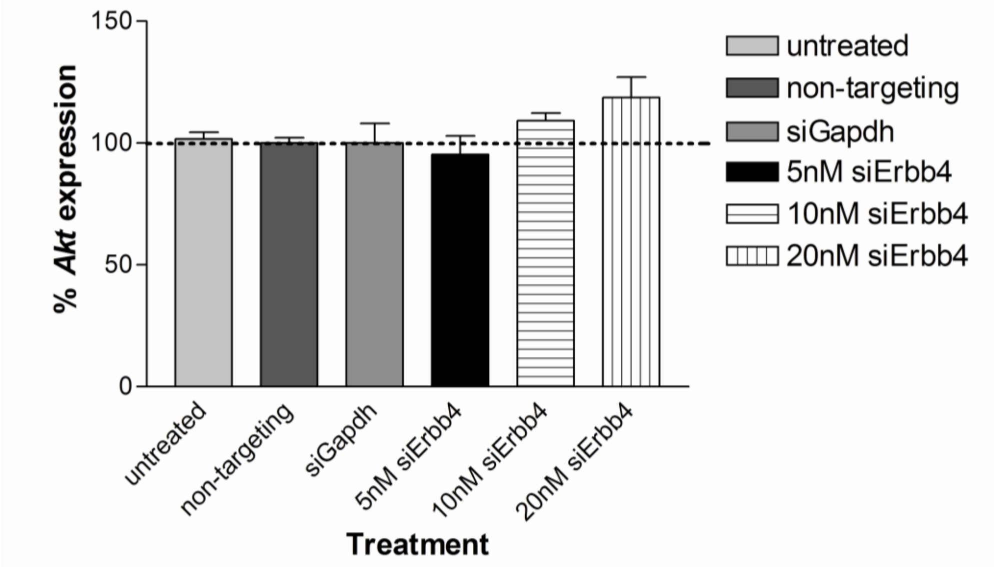


Figure 3.12 Effects on *Akt* expression in NG108-15 cells transfected with siRNA. % expression remaining of *Akt*. qRT-PCR was performed on total RNA isolated from Untreated = untreated NG108-15 cells, non-targeting = NG108-15 cells transfected with non-targeting siRNA, siGapdh = NG108-15 cells transfected with a *Gapdh* siRNA, siErbb4 = NG108-15 cells transfected with the *Erbb4* siRNA at a concentration of 5nM, 10nM and 20nM. Data represent mean ± SE expressed as a percentage of the *Akt* expression remaining in cells transfected with non-targeting siRNA. n=3/treatment group.

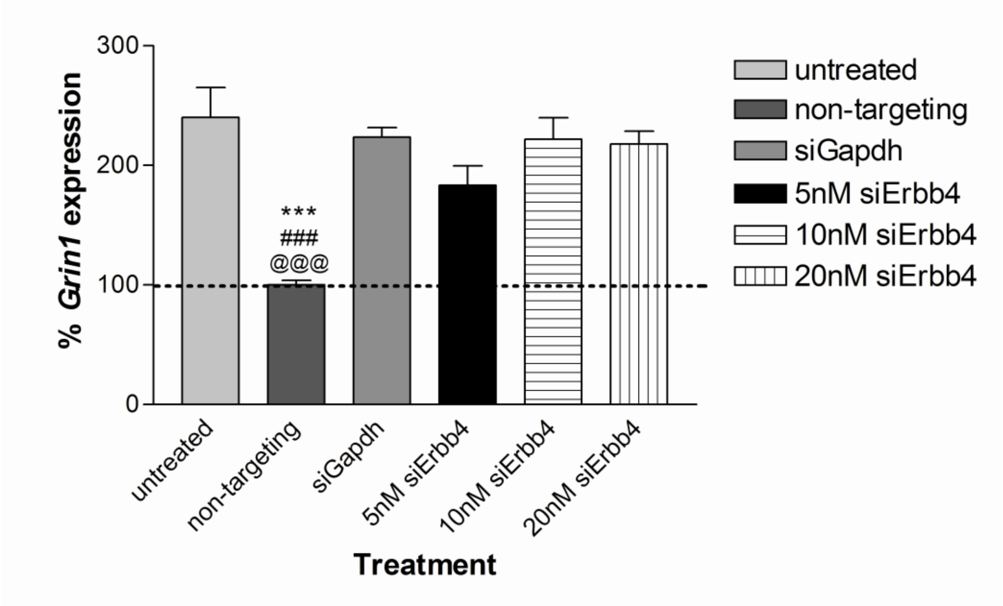


Figure 3.13 Effects on *Grin1* expression in NG108-15 cells transfected with siRNA. % expression remaining of *Grin1*. qRT-PCR was performed on total RNA isolated from Untreated = untreated NG108-15 cells, non-targeting = NG108-15 cells transfected with non-targeting siRNA, siGapdh = NG108-15 cells transfected with a *Gapdh* siRNA, siErbb4 = NG108-15 cells transfected with the *Erbb4* siRNA at a concentration of 5nM, 10nM and 20nM. Data represent mean ± SE expressed as a percentage of the *Grin1* expression remaining in cells transfected with non-targeting siRNA n=3/treatment group. ### $p<0.001$ vs. *Grin1* expression in NG108-15 cells transfected with *Erbb4* siRNA at all concentrations tested, *** $p<0.001$ vs. *Grin1* expression in NG108-15 cells transfected with *Gapdh* siRNA, @@@ $p<0.001$ vs. *Grin1* expression in untreated NG108-15 cells using two-way ANOVA followed by Tukey’s test for multiple comparisons.

3.3.6 Assessment of the functionality of Erbb4 receptors in NG108-15 cells

Having shown that Erbb4 receptors are expressed in NG108-15 cells, it was of great interest to assess if the receptors were actually functional. Therefore, Erbb4 phosphorylation following NRG1β application (section 2.2.3) was measured as a marker of activity and hence functionality. Phosphorylated Erbb4 (pERBB4) was determined using the phosphoERBB4 ELISA (section 2.14) following the application of a range of NRG1β concentrations (10ng/ml-200ng/ml) or vehicle treatment (PBS/0.1% (w/v) BSA) for varying amounts of time (10, 30 or 60 minutes) on NG108-15 cells (Figure 3.14). NRG1β treatment had a very variable effect on NG108-15 cells, which is seen as quite large error bars in each group. Although individual Mann-Whitney tests comparing the effects of NRG1β treatments compared to vehicle treatment at any particular time-point did not reveal any significant changes in pERBB4 levels following NRG1β treatment, there was a general trend that 10 minute stimulation with NRG1β resulted in higher phosphorylation levels than 30 minute or 60 minute stimulation durations. When the levels of pERBB4 in NRG1β treated cells relative to vehicle treated cells were analysed by Wilcoxon Rank Test, 10 minute NRG1β stimulation at the highest concentration (200ng/ml) significantly increased pERBB4 levels ($p<0.05$) (Figure 3.14).

To assess if the minimal change in pERBB4 expression observed in NG108-15 cells was due to a sensitivity problem with the pERBB4 ELISA kit, primary cortical cultures were treated with 200ng/ml NRG1β or vehicle for 10 minutes then pERBB4 levels assessed by ELISA. NRG1β stimulation led to a 5 fold increase in pERBB4 expression compared to vehicle treated cells ($p<0.001$) (Figure 3.15).

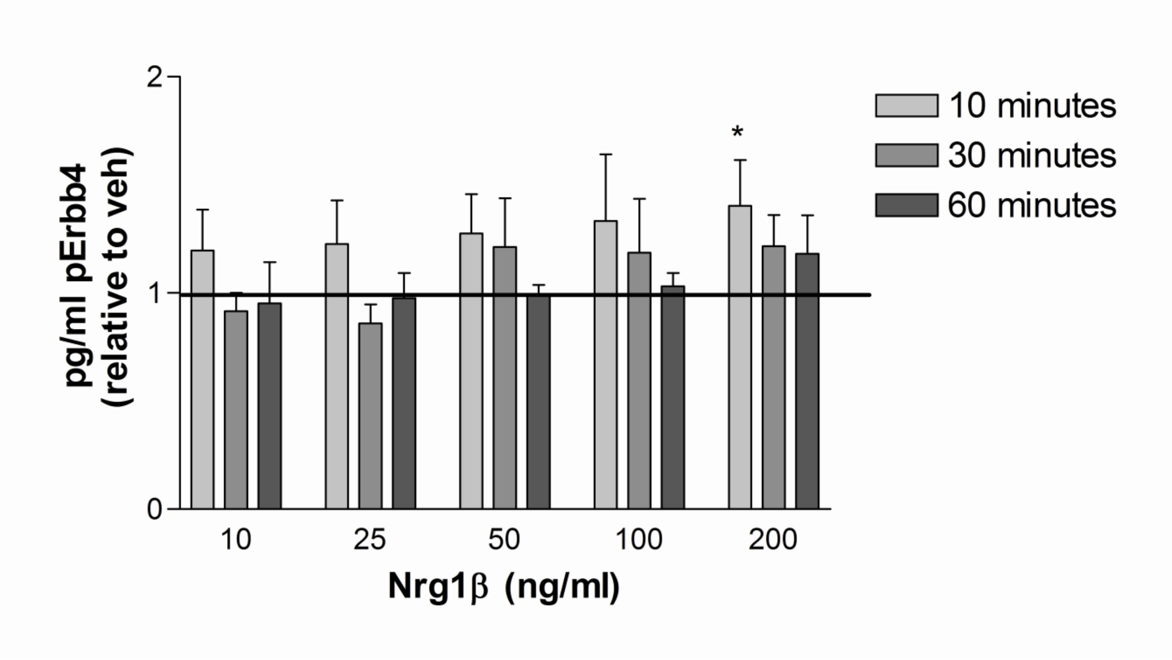


Figure 3.14 Effect of NRG1β stimulation on pERBB4 levels in NG108-15 cells. pERBB4 expression in NG108-15 cells analysed by ELISA following 10, 30 or 60 minute stimulation with vehicle (PBS/0.1% (w/v) BSA), 10ng/ml, 25ng/ml, 50ng/ml, 100ng/ml or 200ng/ml NRG1β. Data represent mean optical density ± SE relative to vehicle treated NG108-15 cells. n=2-5/treatment group. * $p<0.05$ greater than 1 using Wilcoxon Rank Sum Test.

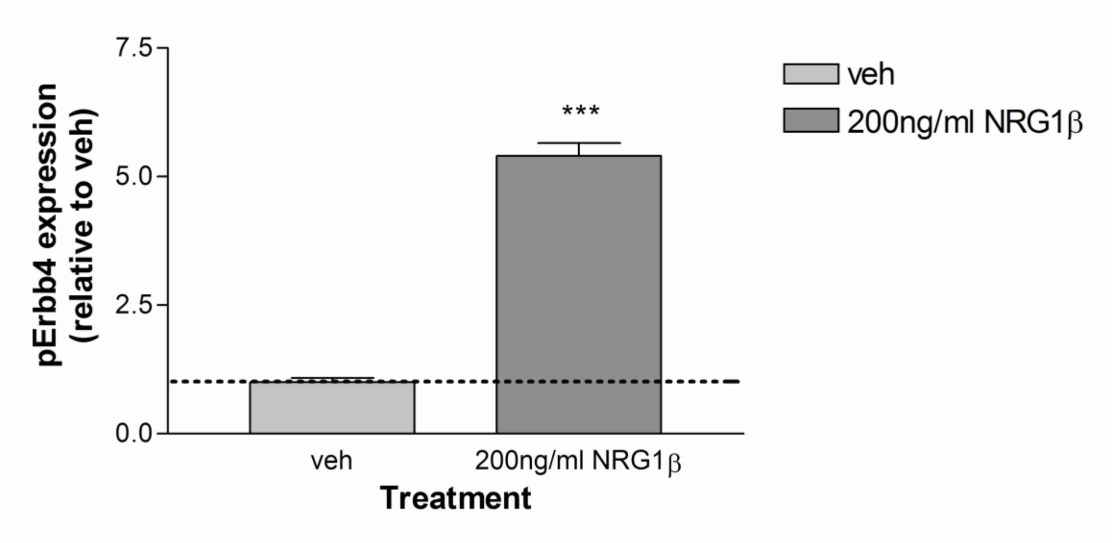


Figure 3.15 Effect of NRG1β stimulation on pERBB4 levels in primary cortical cells. pERBB4 expression in primary cortical cells analysed by ELISA following 10 minute stimulation with vehicle (veh) (PBS/0.1% (w/v) BSA), or 200ng/ml NRG1β. Data represent mean optical density ± SE relative to vehicle treated primary cortical cells. n=4-5/treatment group. *** $p<0.001$ vs. vehicle treated cells using unpaired Students *t* test.

To assess if NRG1β stimulation of Erbb4 in NG108-15 cells resulted in activation of downstream Nrg1-Erbb4 signalling pathways, levels of phosphorylated Gsk3β, Akt and Erk2 were measured by western blot analysis (Figure 3.16). As with pERBB4 levels, levels of phosphorylated Gsk3β, Akt and Erk2 in NG108-15 cells were very variable following NRG1β stimulation. However, Wilcoxon Rank Sum Tests revealed applications of 10ng/ml and 25ng/ml NRG1β on NG108-15 cells for 10 minutes, significantly increased pGSK3β levels compared to vehicle treated cells ($p<0.05$) (Figure 3.16). Phosphorylation of Akt or Erk2 in NG108-15 cells was not significantly changed at any concentration of NRG1β..

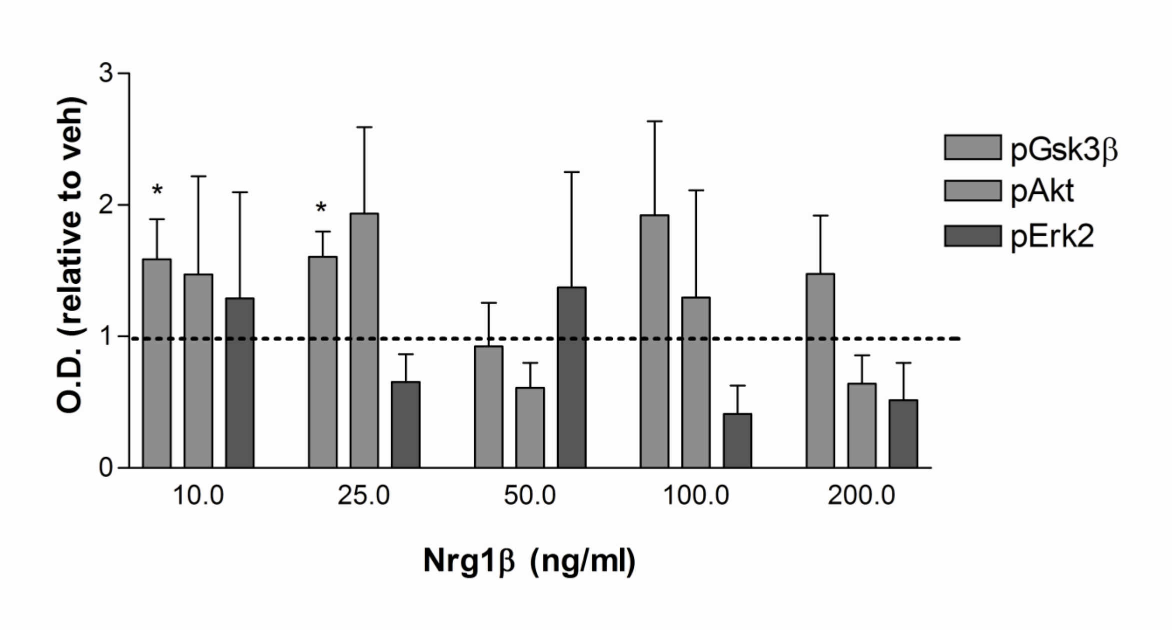


Figure 3.16 Effect of NRG1β stimulation on the activation of Nrg1-Erbb4 signalling pathways. pGSK3β, pAkt and pErk expression normalised to Actin levels in NG108-15 cells as analysed by western blot following 10 minute stimulation with vehicle (PBS/0.1% (w/v) BSA) or 10ng/ml, 25ng/ml, 50ng/ml, 100ng/ml or 200ng/ml NRG1β. Data represent mean optical density ± SE relative to vehicle treated NG108-15 cells. n=2-5/treatment group. * $p<0.05$ greater than 1 using Wilcoxon Rank Sum Test.

3.4 Discussion

3.4.1 Expression of Erbb4 in NG108-15 cells

A pinnacle finding of this study was that Erbb4 mRNA and protein are expressed in NG108-15 cells, as shown by RT-PCR and western blotting. This is the first study which has specifically looked at Erbb4 expression in this neuronal cell line and is in agreement with a previous study which showed that NG108-15 cells expressed the Erbb4 ligand Nrg1 (Pun *et al.,* 1997), which is also confirmed at the protein level in this current study. This suggests that Nrg1-Erbb4 signalling is present in NG108-15 cells and that this cell line may serve as a good *in vitro* model for investigating this signalling pathway.

A previous study by Schulz and colleagues (2005) suggested that Erbb receptors are not endogenously expressed by NG108-15 cells. The conflict in findings between this study and that of Schulz *et al.*, (2005) may be due to differences in methodology, as an antibody for Erbb1 and a non-specific Erbb inhibitor were used by Schulz *et al.*,. However, in the present study primers and antibodies specific for Erbb4 were used. In addition, previous studies of Erbb4 expression in other cell lines have led to contradictory results. For example, endogenous Erbb4 expression has been shown in HEK293 cells (Yang *et al.,* 2005); whereas other studies have concluded there is no endogenous Erbb4 expression in this cell line (Deb *et al.,* 2008). Other neuronal cell lines have been shown to express Erbb4 endogenously including the human SK-N-MC neuroblastoma, rat C6 glioma and human U251 glioma cell lines (Ritch *et al.,* 2003; Fallon *et al.,* 2004). Interestingly, many neuronal cell lines express either or both Erbb2 and Erbb3, but lack endogenous expression of Erbb4 (Vaskovsky *et al.,* 2000; Frohnert *et al.*, 2003; Gambarotta *et al.,* 2004; Fallon *et al.,* 2004; Croslan *et al.,* 2008). The specificity of the RT-PCR primers designed for *Erbb4* in this study was thoroughly checked and bioinformatic testing confirmed no other transcripts were detected by these primers. It is therefore unlikely that they detected any of the other *Erbb* receptor mRNAs.

From the RT-PCR results seen with “pan” *Erbb4* primers and isoform specific *Erbb4* primers, endogenous *Erbb4* expression in NG108-15 cells is low but detectable, when compared to the levels of *Erbb4* expression in mouse and rat brain. Other studies of endogenous *Erbb4* expression in cell lines have found similar low levels, such as in the mouse epithelial cell line YAMC (Frey *et al.,* 2009). In addition, many studies of endogenous *Erbb4* expression in cell lines may wrongly assume lack of expression if the same amount of template RNA or cell lysate is used for quantification of multiple Erbb family members, as it has been shown that in some cell lines such as L6 skeletal muscle cell line Erbb4 expression is 50-fold less than that of Erbb3 (Zhu *et al.,* 1995). The amount of NG108-15 cell lysate used in this study is consistent with a previous study in which similar amounts of cell lysate as used to visualise Erbb4 immunoreactive bands (Fallon *et al.,* 2004). However it should be noted that faint PCR products or western blots may be partly due to technical artefacts, such as a poorly designed PCR primer. However, it is unlikely that the low levels of Erbb4 mRNA and protein detected in the present study are due to technical artefacts as qRT-PCR experiments showed the cycle number at which the level of fluorescence overcame threshold levels was much higher in NG108-15 cell samples than that of samples from brain tissue (data not shown) emphasising a lower expression in the cell line than neuronal tissue.

As NG108-15 cells are a hybrid cell line derived from mouse and rat cell clones, it was of interest to determine the origin of Erbb4, in order to specify which sequence should be the target of downstream applications i.e. siRNA target sequence and qRT-PCR assay specificity. As NG108-15 cell cDNA only amplified with PCR primers specifically designed to recognise mouse *Erbb4*, and resulting sequencing confirmed NG108-15 PCR products were mouse *Erbb4* it can be concluded that the endogenous *Erbb4* in NG108-15 cells is expressed from the mouse genome. This finding is perhaps not surprising as a previous study found NG108-15 cells predominantly consist of mouse chromosomes, with an expected ratio of mouse:rat chromosomes of 1.9:1 (Ajiro *et al.,* 1982). Other studies have also found that their genes of interest were endogenously expressed in NG108-15 cells and originate from the mouse genome (LaFlamme *et al.,* 1998; Gorzalka *et al.,* 2005). On the other hand, other studies investigating NG108-15 cells have found genes deriving from the rat genome (LaFlamme *et al.,* 1998). Moreover, NG108-15 cells can express both mouse and rat cDNA transcripts of the same gene (Ajiro *et al.*, 1982; Ho & Zhao, 1996). It can't be ruled out therefore that other Erbb receptors or Nrg1 in NG108-15 cells derive from the rat genome.

It should be noted that the NG108-15 cells used here were not differentiated. These cells can be differentiated into a more "neuron-like" phenotype using a variety of agents, resulting in the appearance of neuron-specific markers, and the extensions of neurites (Ma *et al.*, 1998; Nagasawa *et al.*, 2009). Had more time been available it would be of interest to determine how Nrg1/Erbb4 expression alters during the process of differentiation.

3.4.2 Isoform specific expression of Erbb4 in NG108-15 cells

Following the finding that NG108-15 cells express mouse *Erbb4* mRNA, further exploration using RT-PCR primers designed to specifically recognise selected isoforms of *Erbb4* showed that NG108-15 cells express *JMa, CYT1 and CYT2* containing isoforms of *Erbb4,* which are also abundantly expressed in the human and rodent prefrontal cortex (Law *et al.*, 2007). The expression of *CYT1* and *CYT2* isoforms in NG108-15 cells suggests that activation of Erbb4 in NG108-15 cells could lead to the cascade of Mapk or PI3k signalling. The expression of the *JMa* but not *JMb* containing isoforms in NG108-15 cells suggests that all of the Erbb4 receptors present in this cell line are susceptible to cleavage by presenilin γ-secretase. The presence of the expression of *JMa* and *CYT1* containing isoforms in the NG108-15 cells is of great interest in terms of the validity of this cell line in investigating the role of Erbb4 in schizophrenia, as it is specifically the *JMa/CYT1* isoform which has been implicated in the pathophysiology of schizophrenia (*Silberberg et al.*, 2006; Law *et al.*, 2007). Unfortunately, it is not possible to compare this isoform specific expression of *Erbb4* in NG108-15 cells with other cell lines as the investigation of endogenous *Erbb4* isoform specific expression has not been carried out.

Also of great interest is that, like NG108-15 cells, human B lymphoblastoid cells which are widely used to study receptor tyrosine kinase signalling and as a model of investigating schizophrenia genes, exclusively express the *JMa* transcripts i.e. *JMa/CYT1* and *JMa/CYT2* (Law *et al.*, in press).

3.4.3 Protein expression of Erbb4 and Nrg1 in NG108-15 cells

Subsequent to finding *Erbb4* mRNA expression, protein expression of Erbb4 and Nrg1 was investigated in NG108-15 cells. Furthermore to validate the neuronal-like properties of the cell line the expression of Nrg1 and Erbb4 in NG108-15 cells was compared to that of neuronal and non-neuronal tissues. The immunoreactive banding pattern produced from the Nrg1 and Erbb4 antibodies was very similar to that of previous studies of human, non-primate and rodent that have utilised the same antibody as in this present study (Thompson *et al.,* 2007; Chong *et al.,* 2008; Feng *et al.,* 2010). Multiple immunoreactive bands were observed in all of these studies and the lower molecular weight bands are thought to represent cleavage products. The observation that there was a very strong immunoreactive band at ~60kDa in NG108-15 cells is in keeping with the finding that NG108-15 cells express the *JMa* isoform which is susceptible to cleavage by proteases.

Furthermore, the immunoreactive banding pattern in NG108-15 cells for both Nrg1 and Erbb4 are very similar to that observed for the rat and mouse brain samples, further validating the NG108-15 cell line as a good *in vitro* neuronal model. NG108-15 cell, rat and mouse brain samples did have a different immunoreactive banding pattern than rat heart which could be explained by cardiac tissue lacking expression of *JMa* isoform of *Erbb4* (Elenius *et al.,* 1997a).

3.4.4 Optimisation of Erbb4 knockdown in NG108-15 cells

Knockdown of genes utilising siRNA mediated RNA interference has been widely used to investigate the function of genes/proteins of interest in many different cell lines and also *in vivo.* It was therefore of great interest to determine whether Erbb4 could be knocked down in NG108-15 cells to validate the use of this cell line further to investigate the Nrg1-Erbb4 signalling pathway. Moreover, knockdown of *Erbb4* expression utilising this *Erbb4* siRNA which has the same sequence as that of the rAAV particles validates that this 19 nucleotide sequence complementary to *Erbb4* is proficient in activating the RNAi pathway and generating significant knockdown of *Erbb4* expression. Although *Erbb4* mRNA expression was low it was still possible to detect up to a 77% knockdown in

NG108-15 cells, following optimisation of the concentration of siRNA and transfection volume.

The knockdown of *Erbb4* and *Gapdh* in NG108-15 cells following the application of siRNAs targeting the respective genes suggests that the transfection reagent used (Lipofectamine™ RNA_{iMAX}) was efficient in transducing NG108-15 cells. Lipofectamine based transfection reagent has been previously shown to be very effective in transducing NG108-15 cells (Martin-Montanez *et al.,* 2010). In addition, the approach taken to optimise siRNA mediated knockdown was similar to previous studies utilising siRNAs to knockdown *Erbb4* (Zscheppang *et al.*, 2007). Normally multiple target sequences are tested for siRNA studies. However, only one was tested in the present study as the target sequence of the siRNA was designed to be the same sequence as that of the shRNA used in the following viral mediated *Erbb4* knockdown studies. As the knockdown of *Erbb4* appeared not to change level of *Gapdh*, *Akt* or *β-2-microglobulin* it is unlikely the knockdown of *Erbb4* following *Erbb4* siRNA is an off target effect.

As Erbb4 signals through the PI3k signalling cascade which involves Akt, the expression of *Akt* following Erbb4 knockdown was assessed. There appeared to be no knockdown of *Akt* expression even though *Erbb4* in these samples was significantly knocked down. This might be explained by the fact that previous studies have shown that when Erbb4 signalling is altered it is Akt activity rather than Akt expression that is altered (Hahn *et al.,* 2006; Horie *et al.,* 2010).

Furthermore, the expression of another gene which has also been linked to Erbb4 signalling was tested following *Erbb4* knockdown. Both the Erbb4 receptor and the NMDA receptor are associated with the PSD95 complex, and therefore the signalling of the two receptors has been linked. For that reason expression of the NMDA receptor gene *Grin1* was measured. Unfortunately, *Grin1* expression appeared to be affected by the non-targeting siRNA. Although the sequence of the non-targeting siRNA was unknown, the supplier ensures the sequence of this siRNA is does not have sequence identity to any known gene. As there appears to be no effect of either *Gapdh* or *Erbb4* siRNAs on *Grin1* expression, partial recognition of the non-targeting siRNA to *Grin1* is the most likely explanation of this unexpected knockdown. In all of the previous experiments the level of the gene expression in untreated cells was very similar to non-targeting siRNA treated cells; therefore comparison of *Erbb4* siRNA treated cells to untreated should give an indication of changes in *Grin1* expression following *Erbb4* knockdown. Previous studies have shown that changes in Erbb4 signalling capabilities result in altered NMDA receptor activity rather than NMDA receptor expression (Hahn *et al.,* 2006; Li *et al.,* 2007).

In addition, although non-targeting siRNA was the most appropriate negative control of the treatments used in this study as it encompasses effects of both transfection reagent and siRNA, the best negative control to use in such studies is an siRNA that contains a scrambled version of the gene of interest. Nonetheless, non-targeting siRNAs are widely and successfully used in siRNA studies.

Although there was a significant knockdown of *Erbb4* at the mRNA level in NG108-15 cells at 24 hours post-siRNA application, there was no quantifiable Erbb4 protein knockdown at this time point. Furthermore, there was lack of Erbb4 protein knockdown at 48, 72 and 96 hours post-siRNA application, however, since *Erbb4* mRNA expression was not assessed at these additional time points it cannot be determined if the *Erbb4* mRNA knockdown quantified at 24 hours was still present at the later time points. The lack of protein knockdown may be due to several reasons including further optimisation of concentration and time point post-siRNA application needed. The NG108-15 cells were successfully transduced and the quantification of protein valid as *Gapdh* expression was successfully knocked down. Other studies have been more successful in achieving Erbb4 protein knockdown (albeit not in NG108-15 cells) (Sharif *et al.,* 2009; Frey *et al.,* 2010; Horie *et al.,* 2010). This divergence of results may be due to several technical differences, such as the *Erbb4* siRNA target sequence, cell type transfected and transfection reagent used. Interestingly, one study utilised a double transfection protocol to achieve significant Erbb4 protein knockdown, and thus an alternative transfection protocol in NG08-15 cells may achieve significant Erbb4 knockdown (Zscheppang *et al.,* 2007). An important point to note is that the half-life of the Erbb4 receptor in NG108-15 cells is unknown therefore it is very difficult to interpret at which time-point to measure Erbb4 expression. The half-life of Erbb4 has been shown to dependent upon ligand stimulation (Yang *et al.,* 2005), therefore it is possible that if in NG108-15 cells the expression of Nrg1 is endogenously very high, the half-life of the Erbb4 receptor may be very short and thus difficult to measure changes in expression.

3.4.5 Functionality of Erbb4 receptors in NG108-15 cells

Although the expression of Erbb4 in NG108-15 cells was not within the range of detection by the total ERBB4 ELISA kit, the levels of phosphorylated Erbb4 were detectable by the phophoERBB4 ELISA kit. To determine if Erbb4 receptor expressed in NG108-15 cells was functional and thus provides a valid *in vitro* model in which to investigate Nrg1-Erbb4 signalling, the NG108-15 cells were stimulated with NRG1β and the activity of Erbb4 and downstream signalling partners were measured. Even though multiple concentrations of NRG1β were used (ranging from 10ng/ml to 200ng/ml) and stimulation durations of 15 minutes, 30 minutes and 60 minutes were analysed, very minimal activation of any of the molecules was observed. PErbb4 levels were significantly higher in NG108-15 cells following 10 minute stimulation with 200ng/ml NRG1β; however, in general the phosphorylation levels of Erbb4 (and the other molecules measured) were very variable. This variability may be due to changes in levels of Erbb4 or Nrg1 in different passages of the NG108-15 cells. Moreover this variability was not observed in rat primary cortical cultures which were used as a positive control. Also it was clear from the cortical cultures that the pERBB4 ELISA was able to detect larger changes in pErbb4 expression than that observed in the NG108-15 levels. Similar concentrations of Nrg1 isoforms have been used in previous studies to increase phosphorylation of Erbb4 or other Erbb receptors significantly (Hahn *et al.,* 2006; Lok *et al.,* 2009).

Since NG108-15 cells expressed both CYT1 and CYT2 isoforms of Erbb4, stimulation of the cells with NRG1β could possibly lead to the activation of PI3k and MAPK signalling cascades. NRG1β application however only led to minimal activation of Gsk3β and had no effect on pAkt or pErk. pAkt and pErk expression has been shown to be increased following Nrg1α or β application in multiple studies, utilising multiple cell types (Flores *et al.,* 2000; Li *et al.,* 2001; Chae *et al.,* 2005; Hahn *et al.,* 2006; Hellyer *et al.,* 2006; Sei *et al.,* 2007; Keri *et al.,* 2009). In addition previous studies utilising NG108-15 cells have shown that activation of Akt and Erk is achievable with other ligands such as opioids (Heiss *et al.,* 2009).

Schulz *et al.* (2005) found that in NG108-15 cells application of epidermal growth factor did not result in activation of Erk, but a reason for the lack of phosphorylation may be the low level of Erbb4 expression in the cell line. Conversely, another explanation for the low response in NG108-15 cell to NRG1β is that NG108-15 cells already express NRG1β (Pun *et al.,* 1997) and therefore the basal levels of activation and thus phosphorylation of Erbb4 and its downstream signalling partners are constitutively high and cannot be further increased. Interestingly, one study has showed that levels of Erbb4 phosphorylation are dependent upon the ratio of Erbb4 isoforms expressed; with JMa/CYT2 isoforms of Erbb4 being highly constitutively phosphorylated (Määttä *et al.,* 2006). Although the ratio of Erbb4 isoforms were not quantified in the present study, it was confirmed *JMa* and *CYT2* were expressed, so it is plausible phosphorylation of Erbb4 was at its maximum pre-NRG1β application. Furthermore, JMa expression was confirmed by RT-PCR and a high expression of Erbb4 cleavage products observed by western blot suggesting Erbb4 in NG108-15 cells may be predominantly in the form of intracellular forms. Therefore NRG1β may not be able to bind to the receptor and activate dimerisation and thus phosphorylation.

In addition, the minimal effect of NRG1β on NG108-15 cell activation of Erbb4 signalling should not be hastily interpreted as lack of functionality of the Erbb4 receptor in this cell line for the reasons described above, and also because there are multiple other ligands of the Erbb4 receptor that have not been investigated in this study such as Nrg3 and betacellulin (Zhang *et al.,* 1997). Finally as noted above, it should be noted that these studies have been carried out in undifferentiated NG108-15 cells, and therefore the activity or expression of the Erbb4 receptor in the cell line may change following differentiation. Previous studies have shown that receptor number increases following NG108-15 cell differentiation, such as the muscarinic acetylcholine receptors (Ghahary & Cheng, 1989).

3.5 Conclusions

In this chapter data has been presented that validates NG108-15 cells as a suitable in vitro model for studying Erbb4. Confirmation of the expression of Erbb4 in NG108-15 cells was carried out using RT-PCR and western blot. mRNA analysis displayed that NG108-15 cells express a low but detectable level of *Erbb4* that originates from the mouse genome, specifically the *JMa/CYT1* and *JMa/CYT2* isoforms. Furthermore this mRNA expression of *Erbb4* in NG108-15 cells can be knocked down by the use of an optimised *Erbb4* siRNA.

Akin to these findings Erbb4 is expressed at the protein level in NG108-15 cells and the resultant receptors can be activated by NRG1β and become phosphorylated, activating selected downstream molecules.

Not only is the finding that NG108-15 cells express Erbb4 endogenously in terms of a novel model of investigating Nrg1-Erbb4 signalling, but also because the validation of this cell line confirms its potential use for the initial in vitro validation of adeno-associated viral particle studies.

4 Validation of rAAV particles for the knock down of Erbb4 *in vitro* and *in vivo*

4.1 Introduction

4.1.1 Viral mediated gene manipulation

Manipulation of gene expression *in vivo* has become an increasingly popular method for investigating gene function, to allow a greater knowledge of the pathophysiology of diseases and identification of potential therapeutic targets, since the production of the first transgenic mouse in 1989. Nonetheless, there are several limitations to the use of genetically modified rodents for translational research; often gross manipulation of genes is lethal during embryonic development, and the gene manipulation is not spatially controlled and therefore behavioural phenotypes can arise that are due to manipulation of the genes in tissues other than the region of interest. In addition gene manipulation is present during neurodevelopment and thus can lead to compensatory changes resulting in altered behavioural and/or pharmacology in adulthood that are not representative of the actual gene function in adulthood. However, recent technological advances have allowed the production of "conditional" transgenics through which the advanced knowledge of systems such as Cre-Lox recombinase and cell type specific promoters have led to control of gene expression at specific time-points in the animals development and in which cells this gene manipulation will occur. Yet the production of these rodents is very expensive and generation of a stable colony of genetically manipulated rodents can take years depending on the strain used and gene targeted.

In vivo viral mediated gene manipulation allows superior control of gene manipulation to that of transgenic laboratory animals due to strict spatial and temporal regulation of over expression or knockdown of genes of interest. In particular in the brain where no cell types are definitively specific to a distinct brain region, viral mediated gene manipulation allows spatial control of viral injection and thus gene manipulation in specific brain regions. Furthermore, multiple genes can be targeted over several brain regions simultaneously and this can be carried out at any time point throughout the rodent's life-span from *in utero* to adulthood. Moreover, although this technique requires a skilled surgical procedure and the production and optimisation of viral particles can be problematic, genetically manipulated animals can be quickly generated (Seshadri *et al.,* 2009).

Several types of viruses are commonly utilised for viral mediated gene manipulation *in vivo* including adenovirus, lentivirus and adeno associated virus (AAV).

4.1.2 Adeno-associated gene manipulation

The discovery of the human AAV was fortuitous as it was found as a contaminant of an adenovirus preparation (Atchison, 1965). Since their discovery AAV research has established that the viral particles are small, non-enveloped with a single stranded DNA (ssDNA) genome. AAVs are highly suited for use in viral mediated gene manipulation as they are not associated with any known disease in humans and moreover they cannot replicate independently, but instead rely on co-infection with adenovirus or exposure to genotoxic or UV radiation to remove the virus from its latent to lytic phase (Meyers et al., 2000). The wild type AAV genome contains *rep* and *cap* genes which encode proteins responsible for viral replication and encapsulation, respectively. These genes are flanked by inverted terminal repeats (ITR) and in recombinant AAVs (rAAV) used for viral mediated gene manipulation these are the only viral genetic component remaining, since the *rep* and *cap* genes are removed to produce a “gutless vector”, thus rendering the rAAVs replication incompetent. The ssDNA genome is instead manipulated to express or knockdown the expression of genes of interest. Often other elements are additionally engineered into the genome, for example for ease of identification of transduced cells a reporter gene is also commonly co-expressed. The woodchuck posttranscriptional regulatory element (WPRE) is often included in rAAVs as it is thought to enhance mRNA processing and increase the efficiency of mRNA translation (Xu *et al.,* 2001; Lipshutz *et al.,* 2003). A study showed inclusion of WPRE in the vector increased expression of a reporter gene by 6 fold in cortical cultures and by up to 35 fold in the hippocampus (Xu *et al.,* 2001).

RAAVs were first used as a vehicle for gene transfer in 1984 (Hermonat *et al.,* 1984) and since then have been widely used to investigate gene function due to their ability to transduce terminally differentiated cells such as photoreceptor cells, hepatocytes, bronchial epithelial cells and neurons (Kaplitt *et al.,* 1994; Conrad *et al,.* 1996; Flannery *et al.,* 1997; Mingozzi *et al.,* 2002). Unlike lentiviruses which readily integrate into multiple sites in the host genome, wild type AAVs have a specific integration site in the human genome on chromosome 19 termed *AAVS1* (Kotin *et al.,* 1990). However integration is dependent upon the *rep* gene and therefore rAAVs cannot integrate at this site and instead evidence suggests that rAAVs generate their second strand DNA episomally. There have been studies suggesting integration of AAVs does occur, but it is thought that these integrations occur at pre-existing DNA breaks (Miller *et al.,* 2004) and very infrequently. Two studies estimated that integration of AAV occurred at one integration event per 10^3-10^4 vector particles (Russell *et al.,* 1994; Rutledge *et al.,* 1997).

RAAV transduction is dependent upon binding of the viral particle to a cell surface receptor. The serotype of AAV defines which cell surface receptor it binds to and therefore which cell types the AAV can transduce, explaining the altered tropism observed amongst the AAV serotypes. To date 12 AAV serotypes have been identified (Muramatsu *et al.,* 1996; Rutledge *et al.,* 1998; Chiorini *et al.,* 1999a; Chiorini *et al.,* 1999b; Xiao *et al.,* 1999; Gao *et al.,* 2002; Gao *et al.,* 2004; Mori *et al.,* 2004; Schmidt *et al.,* 2008). AAV9 is thought to bind to the laminin receptor, as do AAVs 2, 3 and 8 (Akache *et al.,* 2006). Many studies are taking advantage of the varying tropism of AAV serotypes by producing pseudotyped rAAVs which package AAV2 genomes into capsids of other serotypes (Grimm *et al.,* 2003). This approach may be of benefit in reducing antibody mediated response to rAAVs, especially in human trials utilising rAAVs for gene therapy as approximately 80% of the population have antibodies against AAV2 (Erles *et al.*, 1999).

The mechanism of how rAAVs mediate gene manipulation is a multi-step process including internalisation of the viral particle into the cell, followed by trafficking to the nucleus where the rAAV genome is translated, to over expression of genes of interest or hairpin sequences resulting in knockdown of genes of interest. A summary of this process is shown schematically in Figure 4.1.

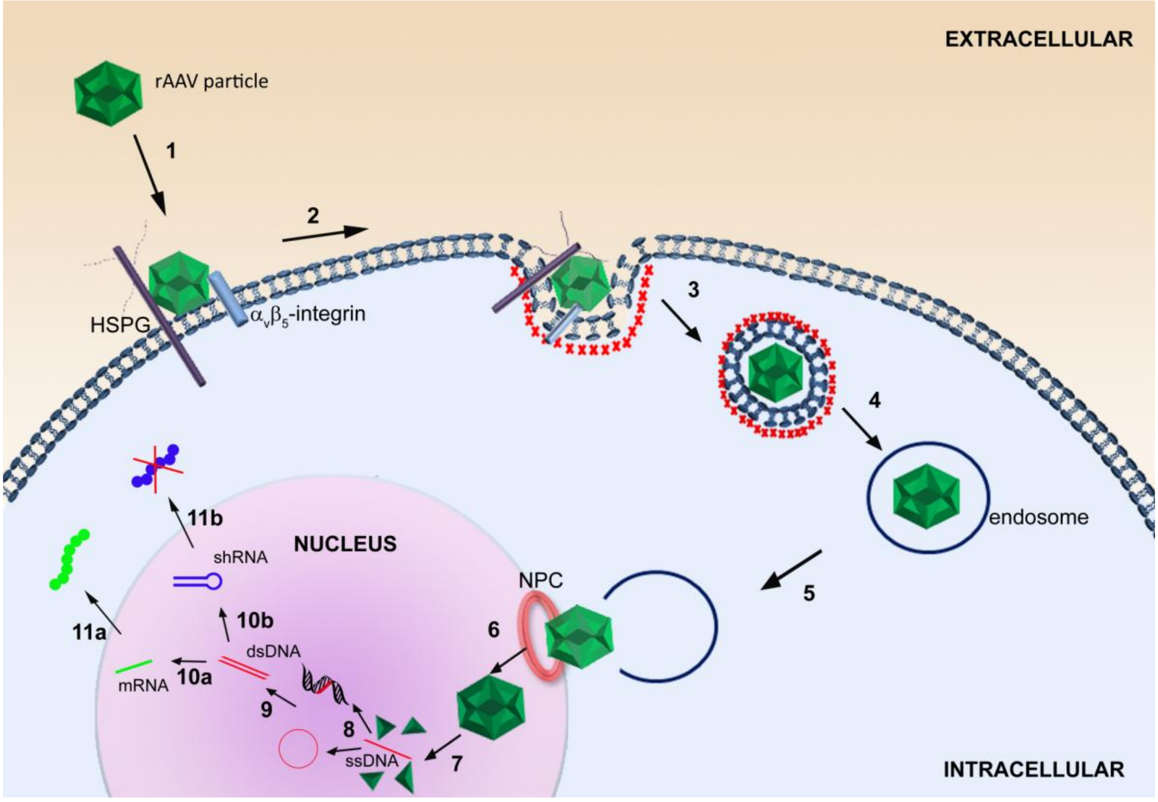


Figure 4.1 Schematic summary of the mechanism of adeno-association viral mediated gene manipulation. 1. rAAV binds to specific cells surface receptor. 2. rAAV binding and co-receptor activation initiates endocytosis. 3. rAAV is internalised via clathrin coated pit. 4. rAAV is transported to the nucleus by endosomal trafficking. 5. rAAV undergoes endosomal release. 6. rAAV enters the nucleus via the nuclear pore complex. 7. Viral uncoating releases rAAV ssDNA genome. 8. Viral ssDNA remains episomally or integrates into the host genome (rare). 9. Second strand cDNA synthesis. 10a. mRNA transcription. 11a. Protein translation. 10b. shRNA transcription. 11b. Activation of RNAi pathway – protein knockdown.

Image produced from information from Schultz & Chamberlain, 2008; Goncalves, 2005 and Ding *et al.,* 2005.

4.1.3 AAV mediated gene manipulation in the CNS

RAAVs have been repeatedly shown to be able to transduce brain tissue following intravenous injection of the virus (Inagaki *et al.,* 2006; Foust *et al.,* 2009). Local injection of rAAV into distinct brain regions has shown successful transduction of cells specifically within the rodent striatum (Xue *et al.,* 2009), hippocampus (Klein *et al.,* 2007; de Backer e*t al.,* 2010), cortex (Nathanson *et al.,* 2009) and amygdala (de Backer e*t al.,* 2010). The majority of rAAV studies in the CNS have made use of promoters for RNA Polymerase II or III promoters (*Pol II* or *Pol III*, respectively) however, gene promoters specific for the neuronal system such as the neuron-specific enolase (*NSE*), glial fibrillary acidic protein (*GFAP*) have been shown to have increased efficiency and effectiveness in transducing CNS tissue *in vitro* and *in vivo* (Xu *et al.,* 2001). Since the first knockdown study using rAAVs in 2003 (Tomar *et al.,* 2003), shRNA expression by rAAVs have led to many interesting findings regarding genes implicated in disorders of the CNS.

Many neurodegenerative disorders have been investigated using viral mediated gene manipulation and rAAVs have been successfully utilised for investigating the functions of genes in these disorders due to their capability of expressing transgenes for long periods of time, with reporter genes being observed up to 5 months after systemic AAV9 delivery (Duque *et al.,* 2009). Localised knockdown of the *ataxin* gene in the cerebellum of a mouse model of spinocerebellar ataxia type 1 (SCA1) resulted in the reversal of motor deficits in these mice (Xia *et al.,* 2004). In a study of another polyglutamine expansion disease, Huntington's disease, suppression of the *Htt* gene in the striatum of R6/1 mice which display behavioural phenotypes resembling those of Huntington's disease resulted in delayed onset of these behaviour phenotypes (Rodriguez-Lebron *et al.,* 2005). As well as reversing behavioural deficits, studies have made use of rAAVs to knockdown genes to induce phenotypes analogous to neurodegenerative disorders. Hommel and colleagues (2003) induced motor deficits and biochemical changes related to Parkinson's disease by reducing expression of the *Th* gene which encodes the enzyme tyrosine hydroxylase essential for dopamine synthesis.

4.1.4 Viral mediated gene manipulation to investigate potential schizophrenia candidate genes

As well as investigating genes associated with the pathophysiology of neurodegenerative disorders, rAAVs have been successfully employed to investigate genes that have biological relevance to aspects of schizophrenia. Polymorphisms in the *HOMER1* gene have been associated with increased risk for schizophrenia (Norton *et al.,* 2003; Spellman *et al.,* 2010) and the biological function of HOMER1 in regulating glutamate neurotransmission make this an interesting target to investigate its role of being potentially involved in the pathophysiology of schizophrenia. RAAV mediated over-expression of specific isoforms of *Homer 1* within the PFC of *Homer 1* knock-out mice resulted in isoform specific reversal of working memory and sensorimotor gating deficits as well as reversing glutamate deficits seen in the knock-out mice (Lominac *et al.,* 2005). RAAV mediated over-expression of another gene with biological relevance to schizophrenia, *Snap25* which regulates glutamate-dependent excitatory synaptic transmission within the dorsal hippocampus of rats, resulted in impaired performance in the water maze and fear conditioning, both of which are thought to reflect decreased cognition as seen in schizophrenia (McKee *et al.,* 2010). *Camk2a* knockdown in the rat hippocampus produced impairments in performance of the open field and water maze tasks reflective of the important roles Camk2a endogenously plays in spatial learning and maintaining neuronal plasticity (Babcock *et al.,* 2005).

As well as AAVs other virus types have been exploited to investigate genes potentially implicated in schizophrenia. Over the past few years investigations into the schizophrenia candidate gene *DISC1,* using *in vivo* viral mediated gene manipulation, have shed some light into the roles this gene may play within specific brain regions and how these may relate to the patholophysiology of schizophrenia. Retroviral-based introduction of sh*Disc1* into the hippocampus of mice has revealed that Disc1 is important in several aspects of cell morphology such as soma size and dendritic initiation, as well as maturation and migration of newborn neurons in the adult brain (Duan *et al.,* 2007; Faulkner *et al.,* 2008). A more recent study has been the first to use viral mediated investigation of a well known schizophrenia candidate gene to investigate behavioural outcomes of the gene manipulation. Mao and colleagues (2009) targeted the knockdown of *Disc1* in the dentate gyrus of adult mice using lentivirus based technology and subsequently found that cells from this brain region transduced with lentivirus had reduced BrdU incorporation indicative of decreased cell proliferation, an effect that was reversed by application of an inhibitor of GSK3. Moreover the authors found that Disc1 knockdown affected selected behaviours that may be relevant to schizophrenia, such as alterations in locomotor activity to a novel environment and depressive like behaviour in the forced swim test but no effect on anxiety related behaviour as measured by performance in the elevated plus maze. Interestingly performance in the affected behavioural tasks was normalised back to control level by application of the GSK3 inhibitor, suggesting that Disc1 functioning through the GSK3 pathway is important in regulating cell proliferation and subsequent behaviours involving the dentate gyrus (Mao *et al.*, 2009).

In a very recent study, lentiviral mediated knockdown specifically within the mouse cerebellum was carried out to target D-amino acid oxidase (Dao), a glial enzyme important in the degradation of D-amino acids. These amino-acids including D-serine, which is a co-activator of NMDA receptor transmission and therefore potentially of importance in the NMDA receptor hypofunction hypothesis of schizophrenia. Not only were protein and mRNA levels of Dao quantifiably reduced, but expression of several other components of the pathway Dao is involved in were altered, including D-serine and Grin2a (NR2A) levels specifically within the cerebellum (Burnet *et al.*, 2011). This study emphasised that not only is viral mediated gene manipulation a valid method of investigating the functions of a single gene within a distinct brain region, but also to investigate downstream effects on dysregulation of discrete pathways which are much more likely to have a greater pathophysiological impact than dysregulation of a single gene in such a complex disorder as schizophrenia.

4.2 Aims

The aims of the validation study described in this chapter were three-fold:

1) To test the capability of rAAV particles (eGFP.rAAV, scr.rAAV and shErbb4.rAAV) to transduce neuronal-like (NG108-15) and neuronal cells (primary cortical cultures), to use eGFP expression as a marker of cell transduction and to assess if there was quantifiable knockdown of *Erbb4* following transduction. This would validate the functionality of the viral particles before using them in *in vivo* experiments.

2) To validate the use of rAAV particles (eGFP.rAAV) *in vivo* by confirming their ability to transduce cells within the designated brain region of the rodent mPFC at specific time points post-surgery, and to determine which cell types of the mPFC are transduced by the rAAVs. This would confirm that, if genetic modification took place following viral injection, it would be within the correct region of the brain and that knockdown could occur within these cell types.

3) To quantify the effects of stereotaxic injection of shErbb4.rAAV in the rodent mPFC on mRNA and protein levels of Erbb4 and related signalling partners. This would assess whether injection of sh*Erbb4* into the mPFC could knockdown Erbb4 levels, and determine if this knockdown had secondary effects on the expression of members of the Nrg1-Erbb4 signalling pathways.

4.3 Results

4.3.1 Validation of the functionality of rAAV particles in vitro

Prior to using the rAAV particles *in vivo*, assessment was carried out to determine firstly if the viral particles were capable of transducing neuronal like cells (NG108-15) and secondly if they were capable of expressing their inserted transgenes (eGFP) in these cells. As the number of genome copies and length of time required for eGFP expression were unknown a range of titres of the eGFP.rAAV particles (section 2.1) were used. EGFP protein expression was assessed at multiple time points by epifluorescence with and without the use of an eGFP antibody (section 2.15.1).

This *in vitro* analysis showed that the eGFP.rAAV particles were indeed capable of transducing NG108-15 cells and that the expression of eGFP was visible in a time and titre dependent manner (summarised in Table 1). No eGFP expression was visible 1 day after rAAV application with any titre but low levels of eGFP expression were visible without the use of an eGFP antibody from 2 days post-transduction using the highest titre (10^{11}gc/ml). With the use of an antibody for eGFP more eGFP positive cells were visible at lower titres (10^{9}gc/ml and 10^{10}gc/ml) at 2 days post-viral application (Figure 4.2 A1-E1). At 7 days post-application, all titres showed eGFP positive cells, with the highest titre of eGFP.rAAV particles (10^{11}gc/ml) resulting in very high eGFP expression (Figure 4.2 A2-E2).

Table 4-1 Summary of eGFP expression at specific time points following transduction of NG108-15 cells with increasing titres of eGFP.rAAV.

– no eGFP expression, + low eGFP expression, ++ moderate eGFP expression, +++ high eGFP expression.

gc/ml **Day**	**10^{7}**	**10^{8}**	**10^{9}**	**10^{10}**	**10^{11}**
1	-	-	-	-	-
2	-	-	+	++	++
3	+	+	+	++	++
7	+	+	++	++	+++

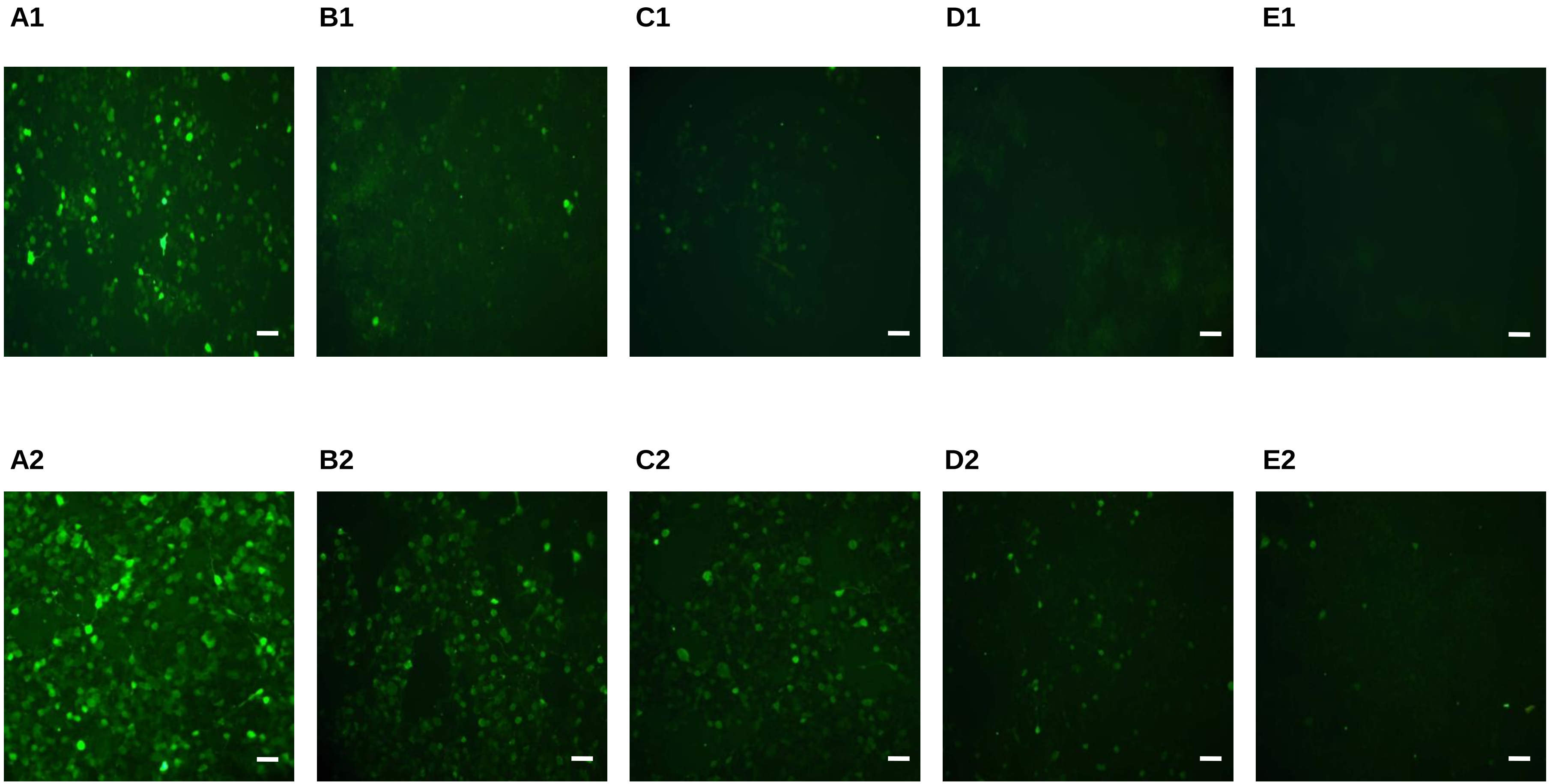


Figure 4.2 eGFP expression in NG108-15 cells transduced with eGFP.rAAV. eGFP expression in NG 108-15 cells transduced with eGFP.rAAV at titres of 1x10^{11} gc/ml (A), 1x10^{10} gc/ml (B), 1x10^{9} gc/ml (C), 1x10^{8} gc/ml (D), 1x10^{7} gc/ml (E). Images represent positive eGFP immunoreactivity 2 days (1) and 7 days (2) post-viral application. Scale bars represent 20μm.

To validate further the use of the viral particles *in vitro* prior to carrying out *in vivo* injections, the functionality of the viral particles (scr.rAAV and shErbb4.rAAV, section 2.1) was assessed further in primary dissociated cortical cultures from p1/2 rat pups to investigate a model system closer to *in vivo* conditions (section 2.2.2). This showed that application of scr.rAAV and shErbb4.rAAV particles at $2.5x10^{10}$gc/ml resulted in prominent eGFP expression and at 7 days post-viral application eGFP could clearly be seen without the use of an eGFP antibody. Both scr.rAAV particles (Figure 4.3A) and shErbb4.rAAV particles (Figure 4.3B) were capable of transducing cortical cells and producing the expression of eGFP in cell bodies and processes.

In addition, analysis of the capability of the shErbb4.rAAV particles to knockdown the expression of *Erbb4* mRNA in primary cortical cells was assessed by qRt-PCR (section 2.11). Again, different titres of viral particles were tested to assess this as it was unknown how many viral particles were necessary to affect mRNA levels. qRT-PCR at 4 days post-viral application revealed that $5x10^9$ gc/ml shErbb4.rAAV particles were sufficient to knockdown the expression of *Erbb4* significantly (F(2,14)= 8.64 $p<0.01$) (Figure 4.4A). *Post hoc* comparisons exposed there was significantly less *Erbb4* mRNA in cortical cells following shErbb4.rAAV treatment than cells treated with vehicle ($p<0.05$) or scr.rAAV ($p<0.01$). Furthermore, treating the cells with slightly more viral particles resulted in an even greater *Erbb4* knockdown (Figure 4.4B), with $2.5x10^{10}$ gc/ml shErbb4.rAAV particles knocking down *Erbb4* mRNA levels in cortical cells by 42% compared to cells treated with the same amount of scr.rAAV particles. There was no significant difference in *Erbb4* expression between vehicle treated cells and cells transduced with scr.rAAV at either titre of viral particles tested.

As there was a significant effect of the shErbb4.rAAV viral particles on *Erbb4* expression in primary cortical cells the mRNA expression of Erbb4 ligand *Nrg1* was also assessed. Interestingly, there was a significant effect of treatment on *Nrg1* expression (F (2,11)=10.73 $p<0.01$), with $2.5x10^{10}$ gc/ml shErbb4.rAAV treatment increasing the expression of *Nrg1* in cortical cells compared to vehicle treated cells ($p<0.01$) and $2.5x10^{10}$ gc/ml scr.rAAV ($p<0.05$) 4 days post-treatment (Figure 4.5).

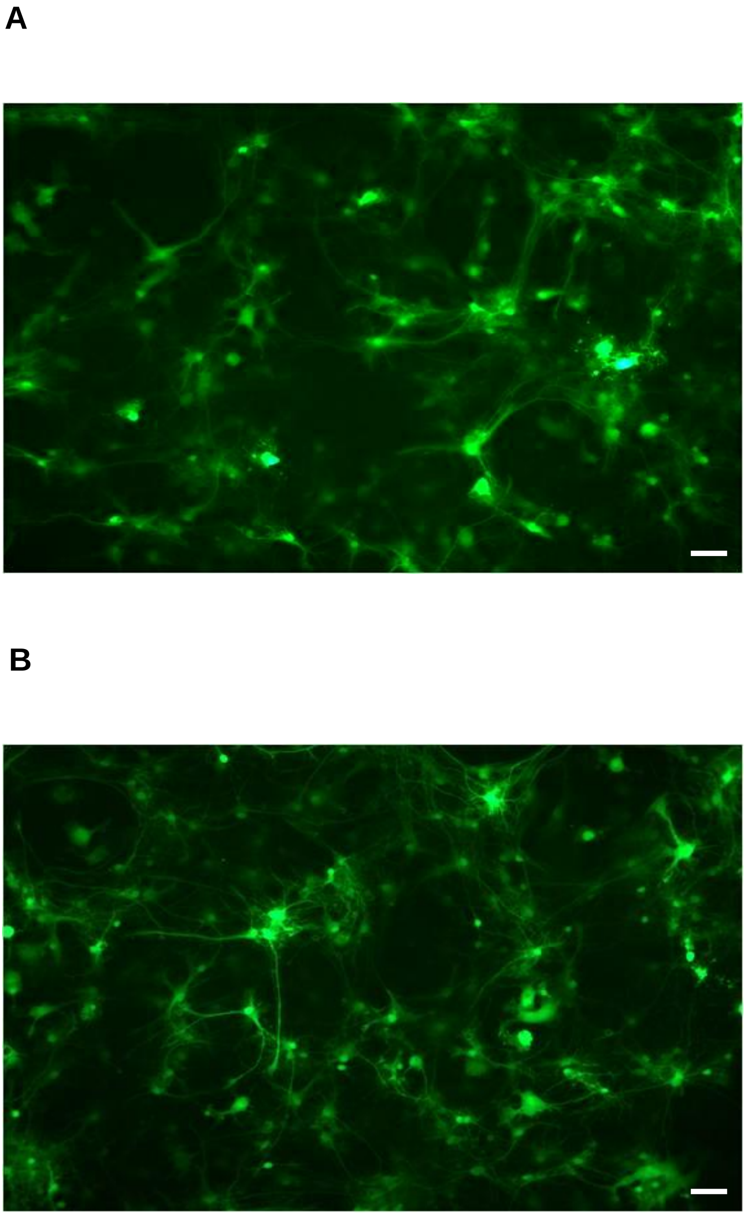
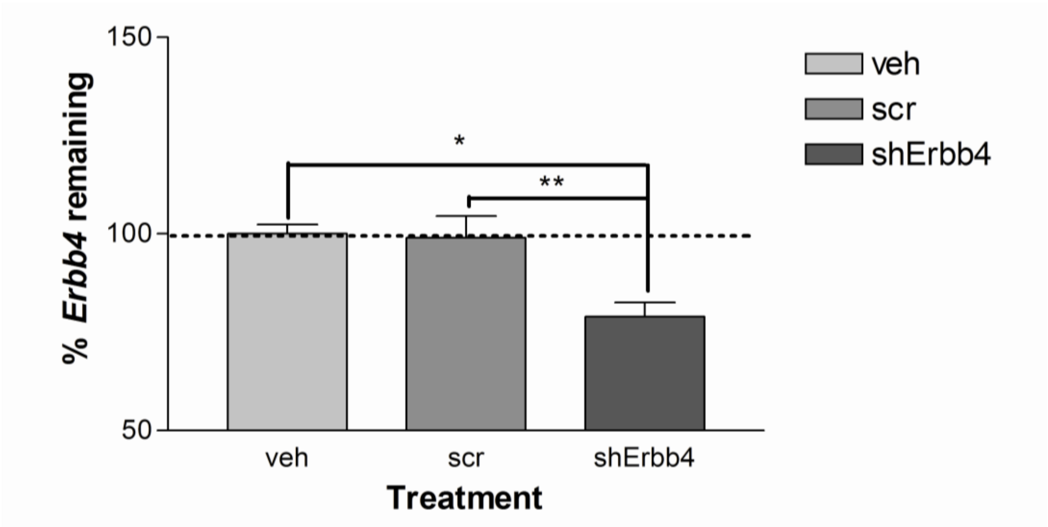


Figure 4.3 eGFP expression in P1/2 rat cortical cells. eGFP expression in dissociated rat cortical cultures visualised by epifluorescence 7days post-application of 2.5×10^{10}gc/ml scr.rAAV (A) or shErbb4.rAAV (B) viral particles. Scale bar represents 20μm.

A



B

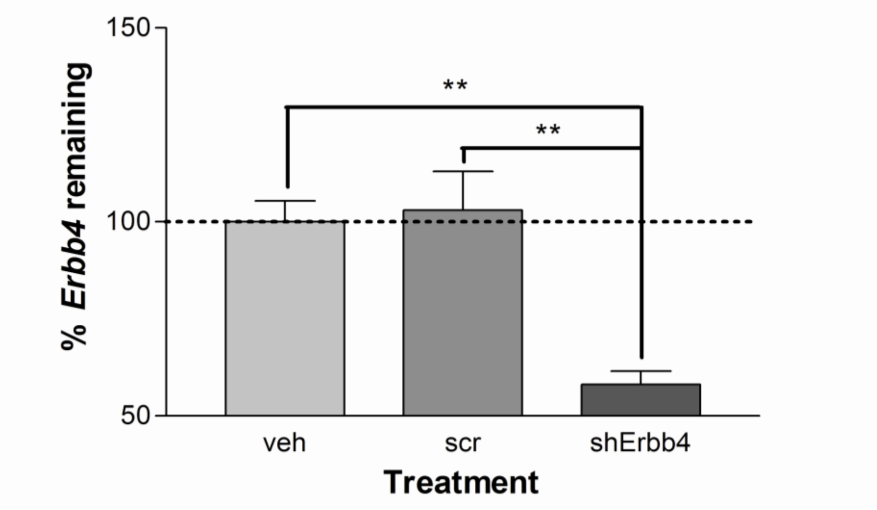


Figure 4.4 Effect of rAAV treatment on *Erbb4* expression in primary cortical cells. % *ErbB4* mRNA expression remaining in primary cortical cells 4 days post-transduction of vehicle = veh (n=4), scr = scr.rAAV (n=4-5) or shErbb4 = shErbb4.rAAV (n=4-6) at titres of 5×10^{9}gc/ml (A) or 2.5×10^{10}gc/ml (B) Quantified by qRT-PCR using the $^{2-}\Delta\Delta$CT method (section 2.11.2). Data represent mean ± S.E. relative to vehicle treated samples. * $p<0.05$, ** $p<0.01$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

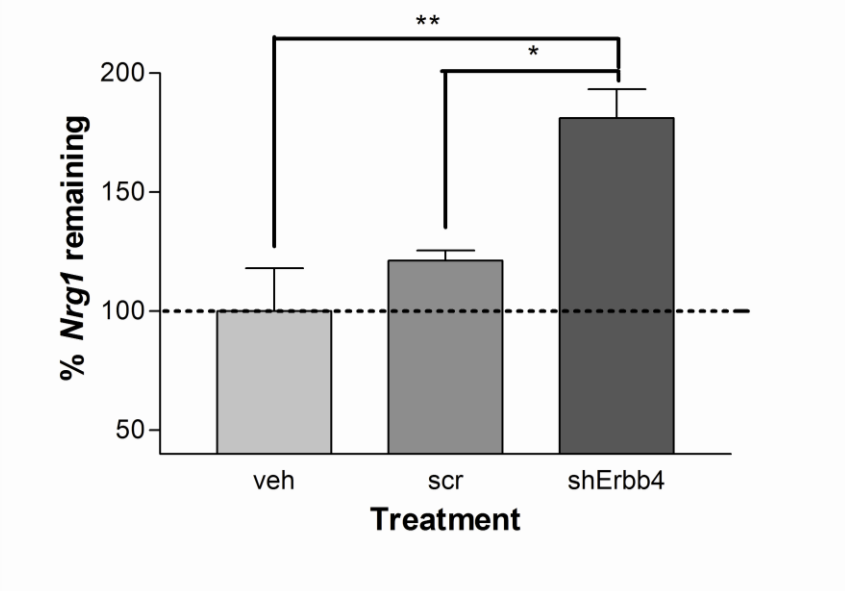


Figure 4.5 Effect of rAAV treatment on *Nrg1* expression in primary cortical cells. % *Nrg1* mRNA expression remaining in primary cortical cells 4 days post-transduction of vehicle = veh (n=4), scr = scr.rAAV (n=4) or shErbb4 = shErbb4.rAAV (n=4) at a titre of 2.5×10^{10}gc/ml quantified by qRT-PCR using the $^{2-}\Delta\Delta$CT method. Data represent mean ± S.E. relative to vehicle treated samples. * $p<0.05$, ** $p<0.01$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

4.3.2 Validation of the injection of rAAV particles in vivo

Following the *in vitro* validation of the eGFP.rAAV, scr.rAAV and shErbb4.rAAV rAAV particles, the location of the injections to confirm targeting of the rodent mPFC, along with the number of viral particles, and the length of time post-surgery necessary for eGFP expression were assessed. First the injection site to be used for targeting Erbb4 knockdown specifically to the mPFC was confirmed using injections of cresyl violet dye into the brain at co-ordinates according to Paxinos & Watson (2007). Injection of 2μl of cresyl violet dye at a 20° angle 4mm into the brain at 3.2mm anterior and 2mm lateral to Bregma resulted in localised dye remaining within the region of the prelimbic area of the mPFC (Figure 4.6A, left hemisphere), whereas insertion of the needle without injection resulted in very localised tissue damage at the cortex surface at the entrance site of the injection (see 2.4.2) (Figure 4.6A, right hemisphere). The region where cresyl violet dye was observed was equivalent to the prelimbic (PrL) area (Figure 4.6B).

After validating the injection site, injections of 2μl of eGFP.rAAV at $1x10^{11}$gc/ml or $1x10^{12}$gc/ml or vehicle were carried out and eGFP expression was analysed at 1 week, 3 weeks and 8 weeks post-surgery to confirm a) how many viral particles (titre) were required to cause eGFP expression, b) what length of time was required for eGFP expression and c) how much spread of eGFP positive cells were observed at these time points. Injections of vehicle at all time points resulted in a visible needle tract with very little tissue damage (as indicated by yellow auto-fluorescing cells localised to the needle tract) and no eGFP expression (Figure 4.7A). At one week post-surgery injection of both titres of eGFP.rAAV produced localised eGFP expression in cells close to the needle tract, this level of eGFP expression was comparable to that observed at 8 weeks post-surgery. Peak expression of eGFP was observed at 3 weeks post-surgery with injection of $1x10^{11}$gc/ml eGFP.rAAV resulting in slightly more eGFP positive cells than seen with the higher titre at 1 week post-surgery. $1x10^{12}$gc/ml eGFP.rAAV injection produced a spread of eGFP positive cells of approximately 0.65mm from the needle tract both in the medial-lateral plane (Figure 4.7B). At this time point eGFP positive cells were routinely observed over a series of 25-30 20μm coronal sections indicating an approximate rostro-caudal spread of 0.6mm.

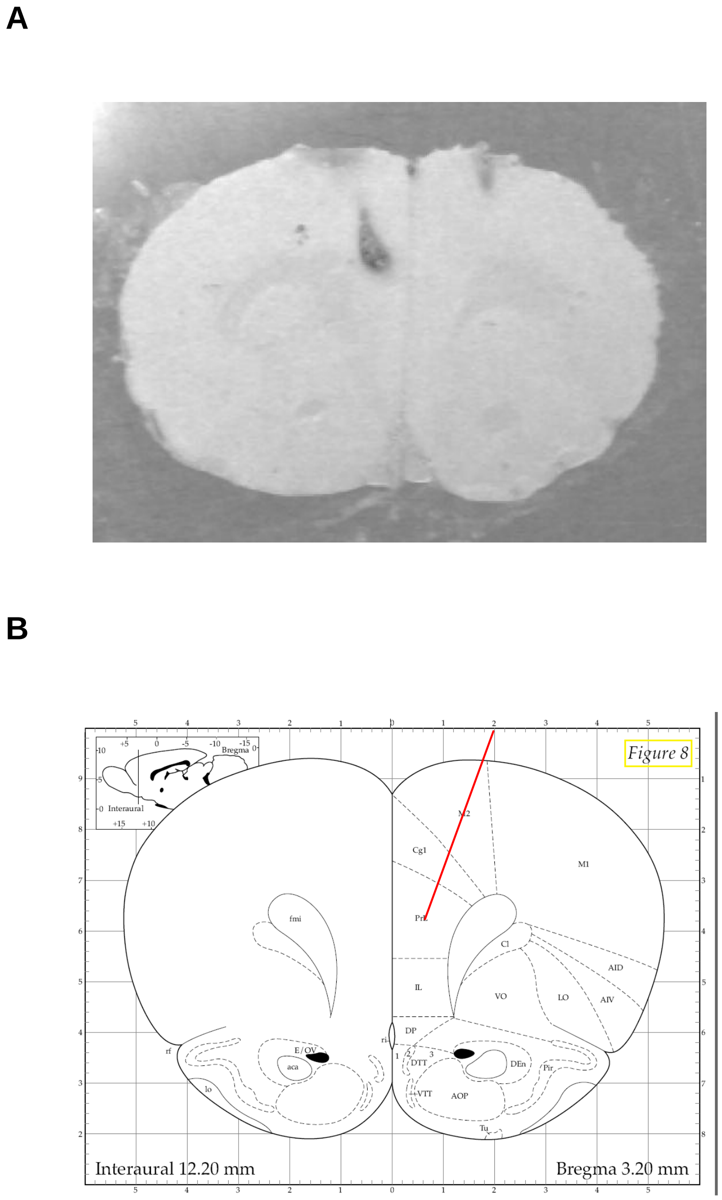


Figure 4.6 Validation of prL injection site in the rat brain. Representative brain section cut at the level of the PrL showing the effects of 2μl injection of cresyl violet dye into the left hemisphere compared to insertion of a Hamilton syringe without injection into the right hemisphere (A). The co-ordinates to the site of injection as indicated by the red line at a 20° angle into the mPFC 3.2mm anterior to bregma (B) (Image taken from Paxinos & Watson, 2007).

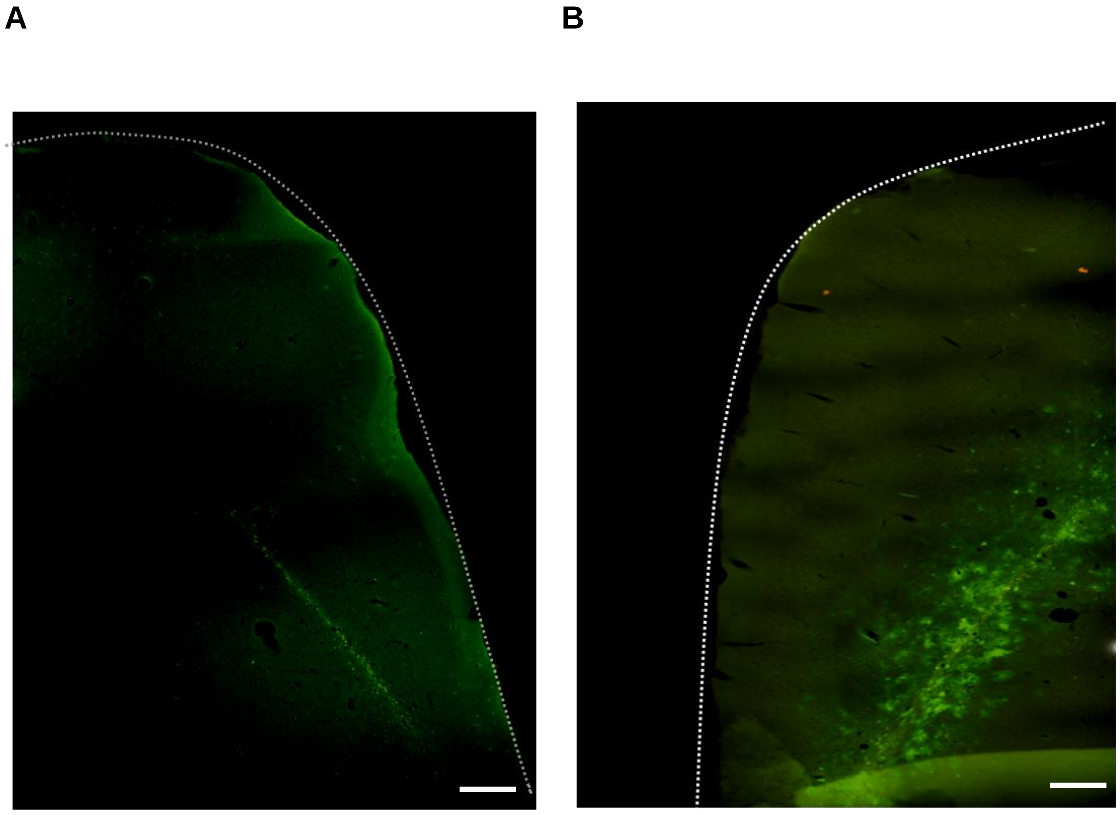


Figure 4.7 *In vivo* expression of eGFP following eGFP.rAAV injection. Representative 20μm coronal brain sections showing eGFP expression 5 weeks post-injection of 2μl vehicle (A) or 2μl 1x10^{12}gc/ml eGFP.rAAV (B) into the mPFC. Immunofluorescent staining was carried out to detect eGFP expression (section 2.15.2). The cortical edge of the brain sections are bordered by a dashed white line. Scale bar represents 200μm.

4.3.3 Identification of cell types transduced by rAAV particles in vivo

To identify which cell types were transduced, sections from hemispheres injected with 1x10^{12}gc/ml eGFP.rAAV 3 weeks post-surgery were analysed with antibodies for eGFP, Gfap (glial fibrillary acidic protein), N200 (Neurofilament 200) and Pvalb (parvalbumin) (section 2.15.2). eGFP positive cells were again prominent at this time point with this titre of virus (Figures 4.8, 4.9 and 4.10 top left panels). Glial cells were identified by immunoreactivity with the Gfap antibody and a proportion of the eGFP expressing cells were also reactive for Gfap (Figure 4.8, right panel). Pyramidal cells were identified using the N200 antibody and a fraction of the eGFP expressing cells were also reactive for N200 (Figure 4.9, right panel). Finally eGFP expressing cells were also seen in a subgroup of Pvalb containing interneurons (Figure 4.10, right panel).

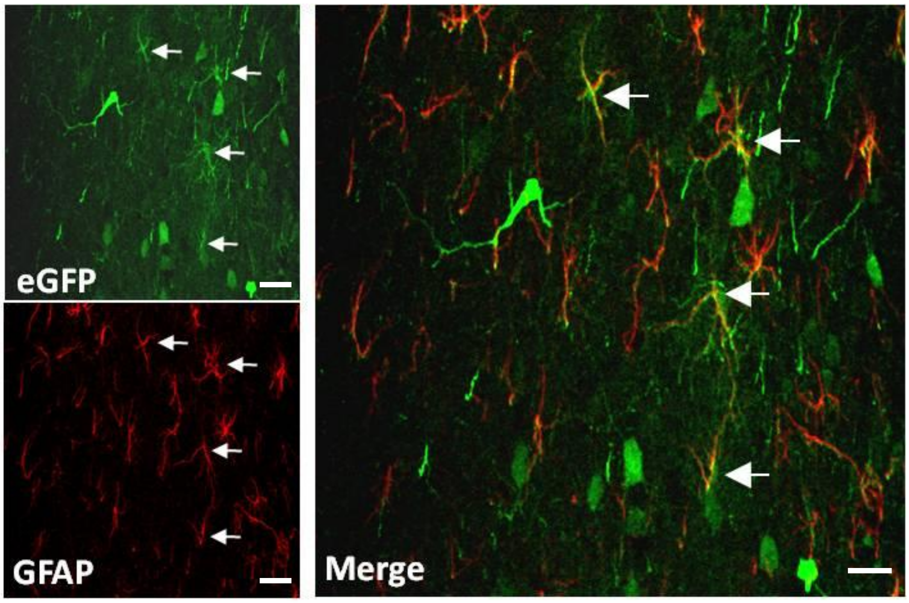


Figure 4.8 Co-localisation of eGFP and Gfap in eGFP.rAAV transduced cells. Immunohistological staining of brain sections cut at the level of the mPFC 3 weeks post-injection of 2μl $1x10^{12}$gc/ml eGFP.rAAV. Immunofluorescence shows cortical cells positive for either eGFP (green, top left panel) or Gfap (red, bottom left panel), merging of the two images shows co-localisation of eGFP and Gfap in some cortical glial cells (yellow, right panel). Arrows indicate eGFP+Gfap positive cells. Scale bar represents 20μm.

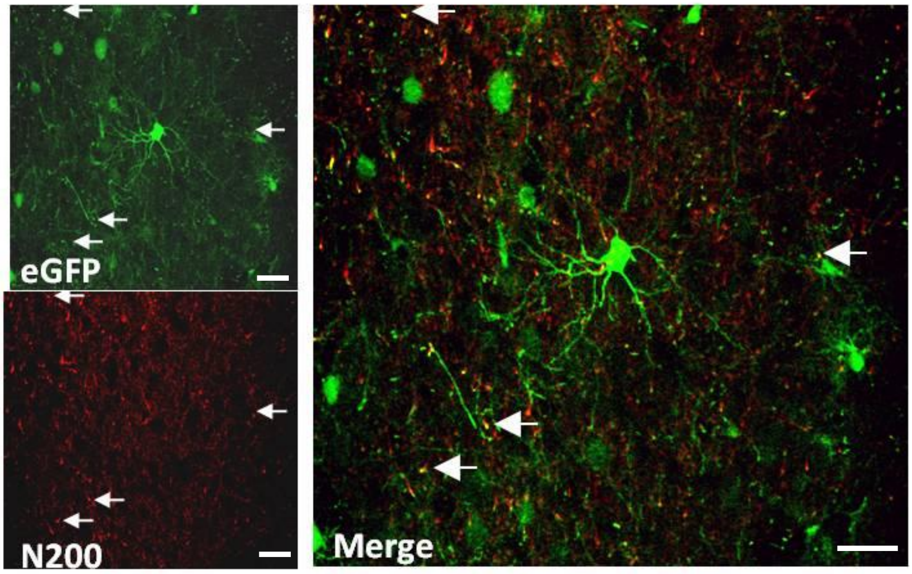


Figure 4.9 Co-localisation of eGFP and N200 in eGFP.rAAV transduced cells. Immunohistological staining of brain sections cut at the level of the mPFC 3 weeks post-injection of 2μl $1x10^{12}$gc/ml eGFP.rAAV. Immunofluorescence shows cortical dendritic processes of pyramidal cells positive for either eGFP (green, top left panel) or N200 (red, bottom left panel), merging of the two images shows co-localisation of eGFP and N200 in some dendritic processes (yellow, right panel). Arrows indicate eGFP+N200 positive processes. Scale bar represents 20μm.

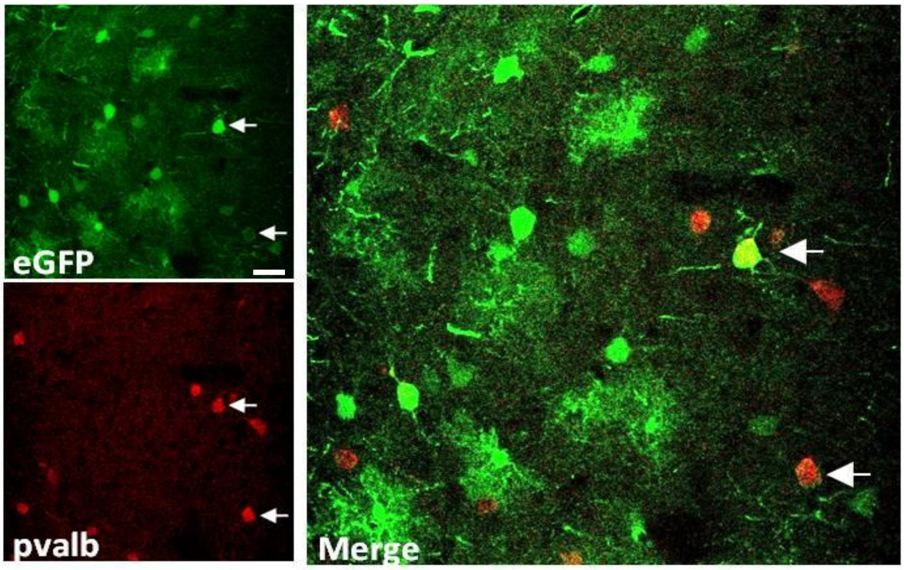


Figure 4.10 Co-localisation of eGFP and Pvalb in eGFP.rAAV transduced cells. Immunohistological staining of brain sections cut at the level of the mPFC 3 weeks post-injection of 2μl 1x10^{12}gc/ml eGFP.rAAV. Immunofluorescence shows cortical cells positive for either eGFP (green, top left panel) or Pvalb (red, bottom left panel), merging of the two images shows co-localisation of eGFP and Pvalb in some cortical cells (yellow, right panel). Arrows indicate eGFP+Pvalb positive cells. Scale bar represents 20μm.

4.3.4 Quantification of mRNA expression following rAAV injection in the mPFC

To ensure that any changes in mRNA expression following viral injection were not due to degradation of RNA, RNA quality was assessed using the Agilent Bioanalyser 2100 (section 2.8.3). Analysis of total RNA isolated from one PFC hemisphere of each rat 5 weeks post-stereotaxic surgery (vehicle, scr.RAAV and shErbb4.rAAV) showed that the RNA quality did not differ between surgical groups and therefore any subsequent changes in mRNA expression were unlikely to be due to RNA degradation. The average RIN values of vehicle, scr.RAAV and shErbb4.rAAV treated rats were 8.65 ± 0.59, 8.4 ± 0.46 and 8.78 ± 0.29, respectively.

At 5 weeks post-injection of 2μl of 1×10^{12} gc/ml shErbb4.rAAV there was no significant effect on *Erbb4* mRNA expression in the PFC compared to the other surgical groups, with no effect of surgical group (F (2,22)=0.38 $p>0.05$) (Figure 4.11A), although there appeared to be a trend towards decreased *Erbb4* mRNA levels after shErbb4.rAAV injection. In concordance with this result there was also no effect of surgical group on *Nrg1* mRNA expression in these samples at this time point (F (2, 22) =0.72 $p>0.05$) (Figure 4.11B). However, when the ratio of ligand mRNA to receptor mRNA was quantified there was a significant effect of surgical group (F (2, 22) =11.13 $p<0.001$) with *post hoc* comparisons revealing rats treated with shErbb4.rAAV had significantly higher *Nrg1*/*Erbb4* mRNA ratio than scr.rAAV treated rats ($p<0.01$) and vehicle treated rats ($p<0.001$) (Figure 4.12).

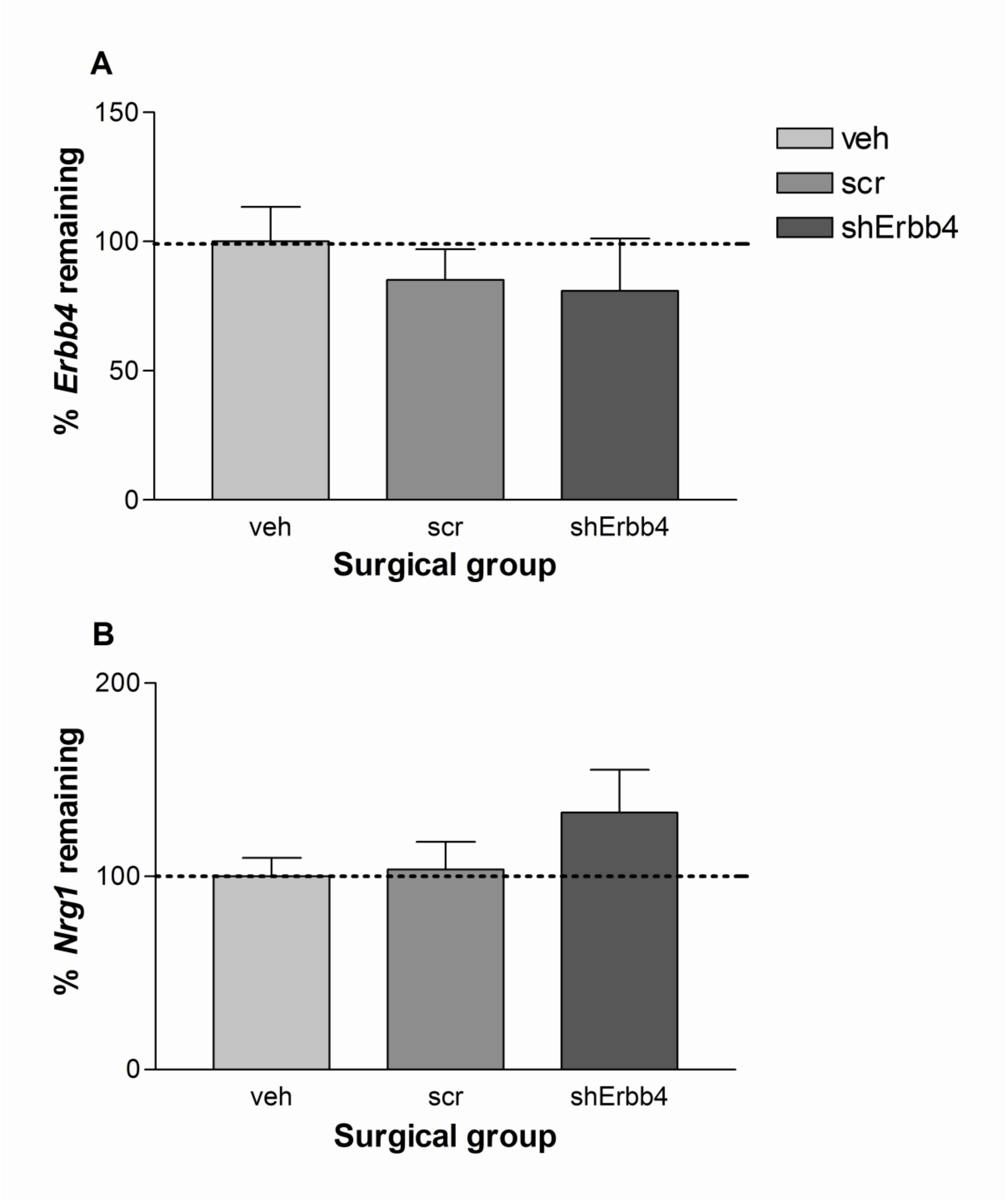


Figure 4.11 Effect of *in vivo* viral-mediated targeting of *Erbb4* on *Erbb4* and *Nrg1* mRNA expression. % *Erbb4* (A) and *Nrg1* (B) mRNA expression remaining in one hemisphere of the mPFC dissected from rats 5 weeks post 2μl injection of vehicle = veh (n=7), $1x10^{12}$gc/ml scr.rAAV = scr (n=8) or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (n=8) quantified by qRT-PCR using the $^{2-}\Delta\Delta$CT method. Data represent mean ± S.E. relative to vehicle treated samples.

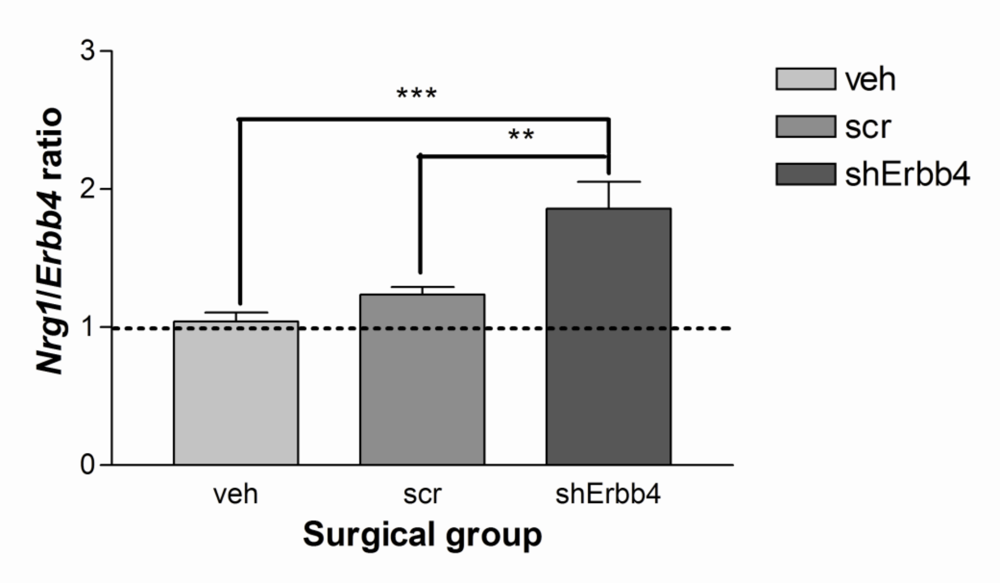


Figure 4.12 Effect of *in vivo* viral-mediated targeting of *Erbb4* on the ratio of *Nrg1/Erbb4* mRNA expression. The ratio of *Nrg1* mRNA to *Erbb4* mRNA expression in one hemisphere of the mPFC dissected from rats 5 weeks post 2μl injection of vehicle = veh (n=7), $1x10^{12}$gc/ml scr.rAAV = scr (n=8) or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (n=8) quantified by qRT-PCR using the $^{2-}\Delta\Delta$CT method. Data represent mean ± S.E. relative to vehicle treated samples. ** $p<0.01$, *** $p<0.001$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

4.3.5 Quantification of protein expression following rAAV injection in the mPFC

Erbb4 protein quantification using an ELISA (section 2.14) showed that surgical group had a significant effect on Erbb4 protein levels (F(2,22)=11.2 $p<0.001$) with rats injected with 2μl of $1\text{x}10^{12}$ gc/ml shErbb4.rAAV having significantly reduced Erbb4 protein expression in the PFC compared to vehicle ($p<0.001$) and scr.rAAV injected rats ($p<0.01$) (Figure 4.13A). However, the expression of phosphorylated Erbb4 was not significantly affected by surgical group (F (2, 2)0.98 $p>0.05$) (Figure 4.13B), although there was a non-significant decrease in pErbb4 levels in shErbb4.rAAV treated samples. Furthermore the ratio of phosphorylated Erbb4 to total Erbb4 was also not significantly affected by surgical group (F (2, 22) =0.01 $p>0.05$) (Figure 4.13C).

To assess if injection of viral particles caused glial infiltration within the PFC, levels of Gfap were quantified by western blotting in each sample at 5 weeks post-surgery (section 2.13). There was no significant effect of surgical group on Gfap expression (F (2, 21) =0.55 $p>0.05$). Although both viral groups did appear to have slightly heightened Gfap expression, this was not significantly higher than vehicle treated rats and most importantly not different between viral groups (Figure 4.14). To ensure there was eGFP expression in all protein lysates isolated from viral treated PFC tissue 5 weeks post-surgery (as this was not a time point assessed in the previous validation study (section 4.3.2), eGFP protein levels were assessed by western blot analysis (section 2.13). All scr.rAAV and shErbb4.rAAV treated samples showed eGFP expression, although levels were somewhat variable. Importantly no eGFP was visible in vehicle treated samples (Figure 4.15).

The weight of the rats post-stereotaxic surgery was monitored to assess for adverse effects of surgery or viral infection. There was no difference in the weight of rats in different surgical groups (F (2, 68) = 2.80 $p>0.05$) (Figure 4.16A) or 5-CSRTT (F (2, 98) = 2.5 $p>0.05$) (Figure 4.16B)).

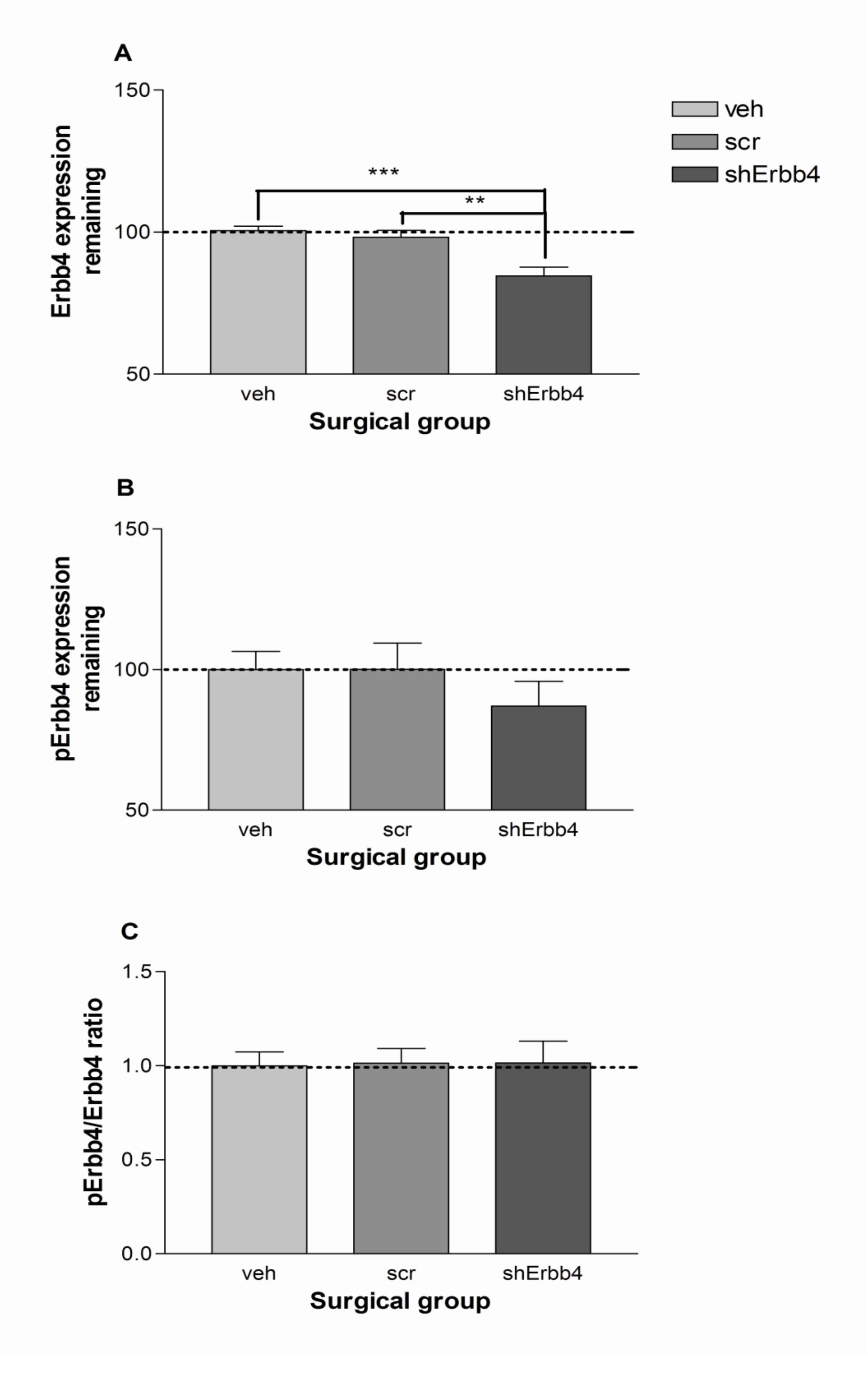


Figure 4.13 Effect of *in vivo* viral-mediated targeting of *Erbb4* on Erbb4 and pErbb4 protein expression. % Erbb4 (A), phosphorylated Erbb4 (pErbb4) (B) and the ratio of pErbb4 to total Erbb4 (C) protein expression remaining in one hemisphere of the mPFC dissected from rats 5 weeks post 2μl injection of vehicle = veh (n=7), $1x10^{12}$gc/ml scr.rAAV = scr (n=8) or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (n=8) quantified by Erbb4 or phosphoErbb4 ELISA. Data represent mean ± S.E. relative to vehicle treated samples. ** $p<0.01$, *** $p<0.001$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

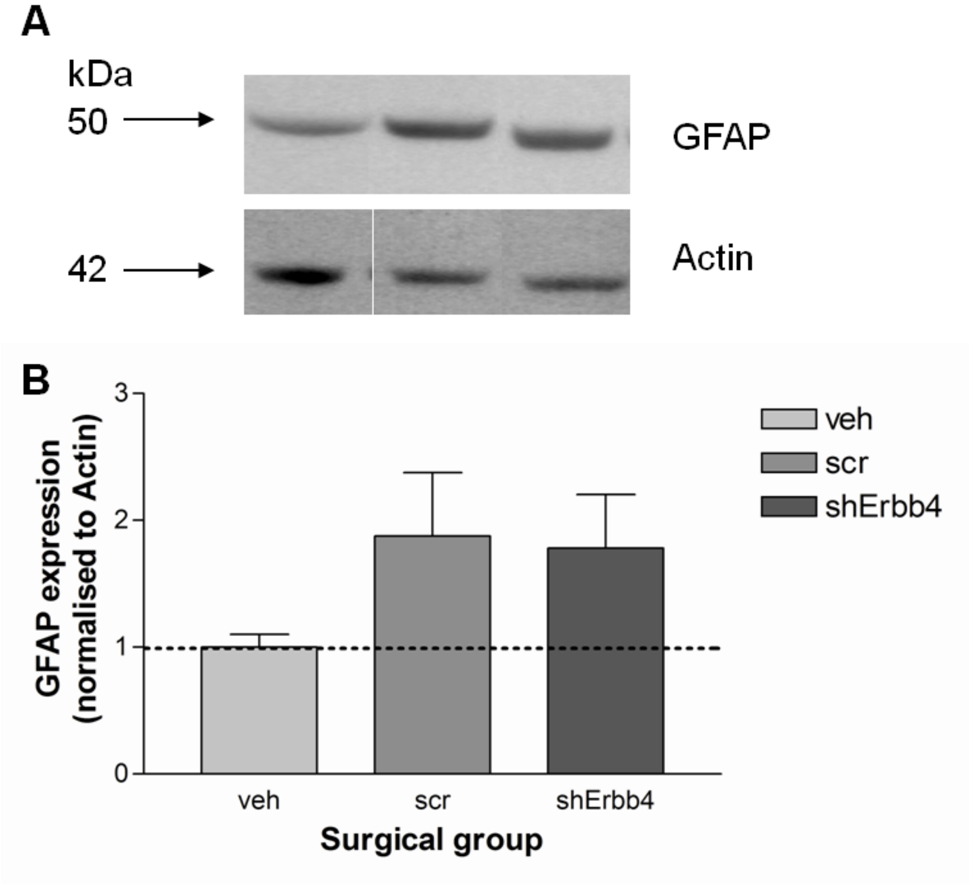


Figure 4.14 Effect of *in vivo* viral-mediated targeting of *Erbb4* on Gfap protein expression. Representative immunoblots of Gfap (top panel) and Actin (lower panel) in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh, $1x10^{12}$gc/ml scr.rAAV = scr or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (A). Quantification of Gfap protein expression in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh (n=7), $1x10^{12}$gc/ml scr.rAAV = scr (n=8) or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (n=8) normalised to the expression of Actin (B). Data represent mean ± S.E. relative to vehicle treated samples.

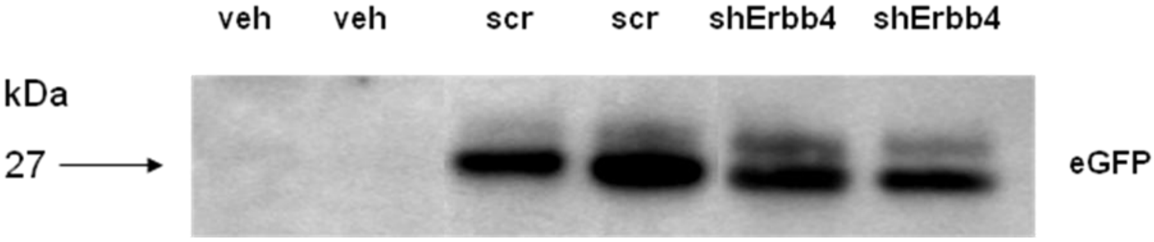


Figure 4.15 *in vivo* eGFP protein expression 5 weeks post-stereotaxic surgery. Representative immunoblots of eGFP in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle= veh, $1x10^{12}$gc/ml scr.rAAV = scr or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4.

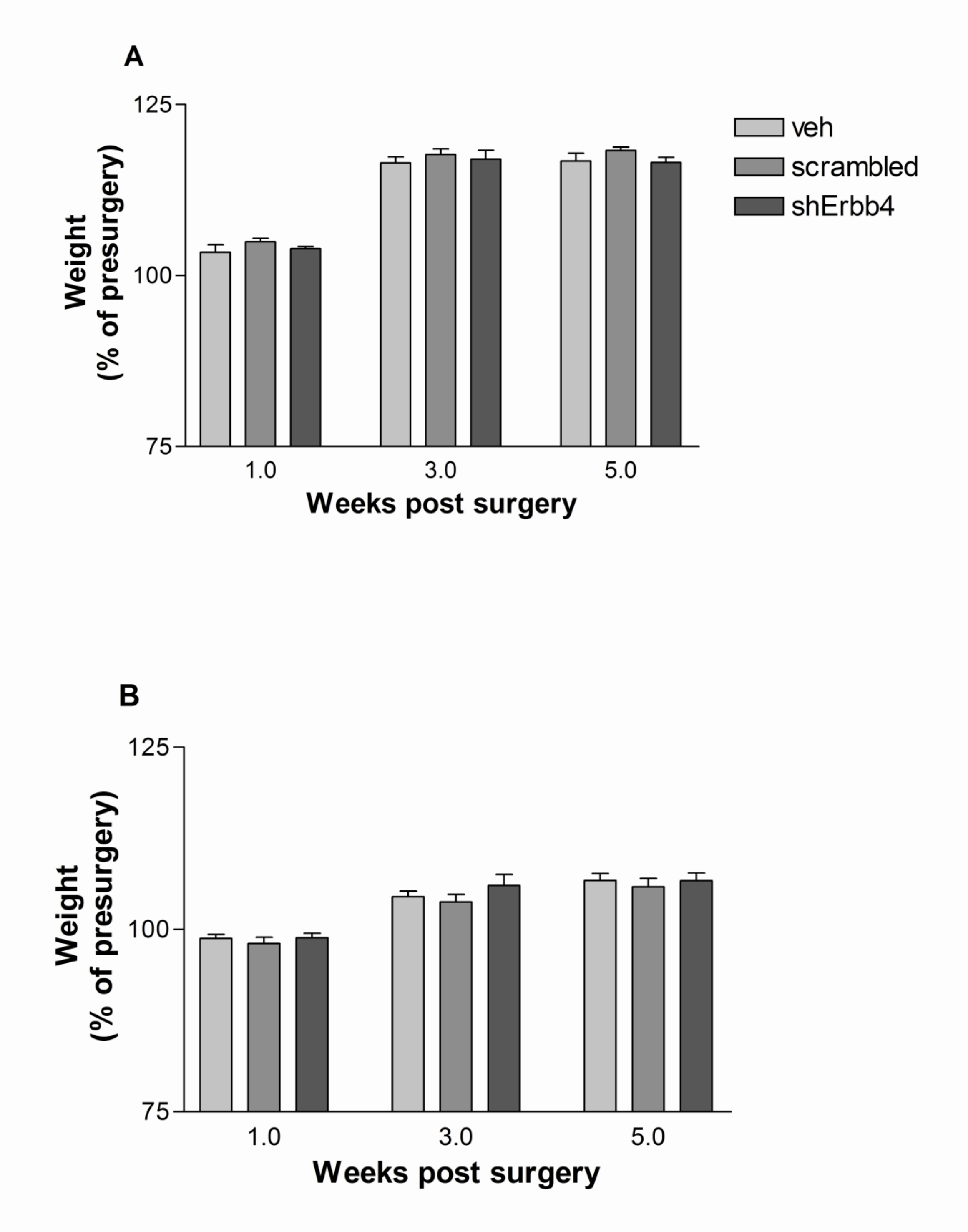


Figure 4.16 Effect of stereotaxic surgery on weight. Weight gain as measured by % of pre-surgery weight of rats in prepulse inhibition and locomotor activity study (A) or 5-CSRTT study (B) at 1, 3 and 5 weeks post-injection of 2μl injection of vehicle = veh (n=7-11), 1x10^{12}gc/ml scr.rAAV = scrambled (n=8-12) or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (n=8-11).

4.3.6 Effects of rAAV injection in the mPFC on protein levels of the Nrg1-Erbb4 signalling pathway

As there were significant reductions in Erbb4 protein levels 5 weeks post-surgery in the PFC of rats treated with shErbb4.rAAV particles, activity levels of the two downstream signalling pathways of Erbb4 were assessed. The activity of the Erk signalling pathway as measured by the ratio of pErk expression to total Erk (tErk) expression was not significantly affected by surgical group ($F(2,21)=0.39$ $p>0.05$) (Figure 4.17). In comparison, the activity of the Akt signalling pathway as measured by the ratio of pAkt expression to total Akt (tAkt) expression was significantly affected by surgical group ($F(2,20)=3.82$ $p<0.05$). *Post hoc* comparisons revealed shErbb4.rAAV treated rats had significantly less pAkt/tAkt than vehicle treated rats ($p<0.05$) and to some extent scr.rAAV treated rats (although not to a significant level) (Figure 4.18).

In agreement with mRNA results *in vitro* and to a certain degree *in vivo,* surgical group had a significant effect on Nrg1 protein expression ($F\ (2,22)=8.82$ $p<0.01$) with shErbb4.rAAV treatment resulting in approximately a 70% increase full length Nrg1 (100 kDa) expression compared to the scr.rAAV group ($p<0.05$) and vehicle group ($p<0.01$) (Figure 4.19). There were no effects on the expression of Nrg1 cleavage products (data not shown). Pearson's correlation analysis revealed a significant negative correlation between Erbb4 and Nrg1 protein levels remaining 5 weeks after surgery ($p<0.05$) (Figure 4.20).

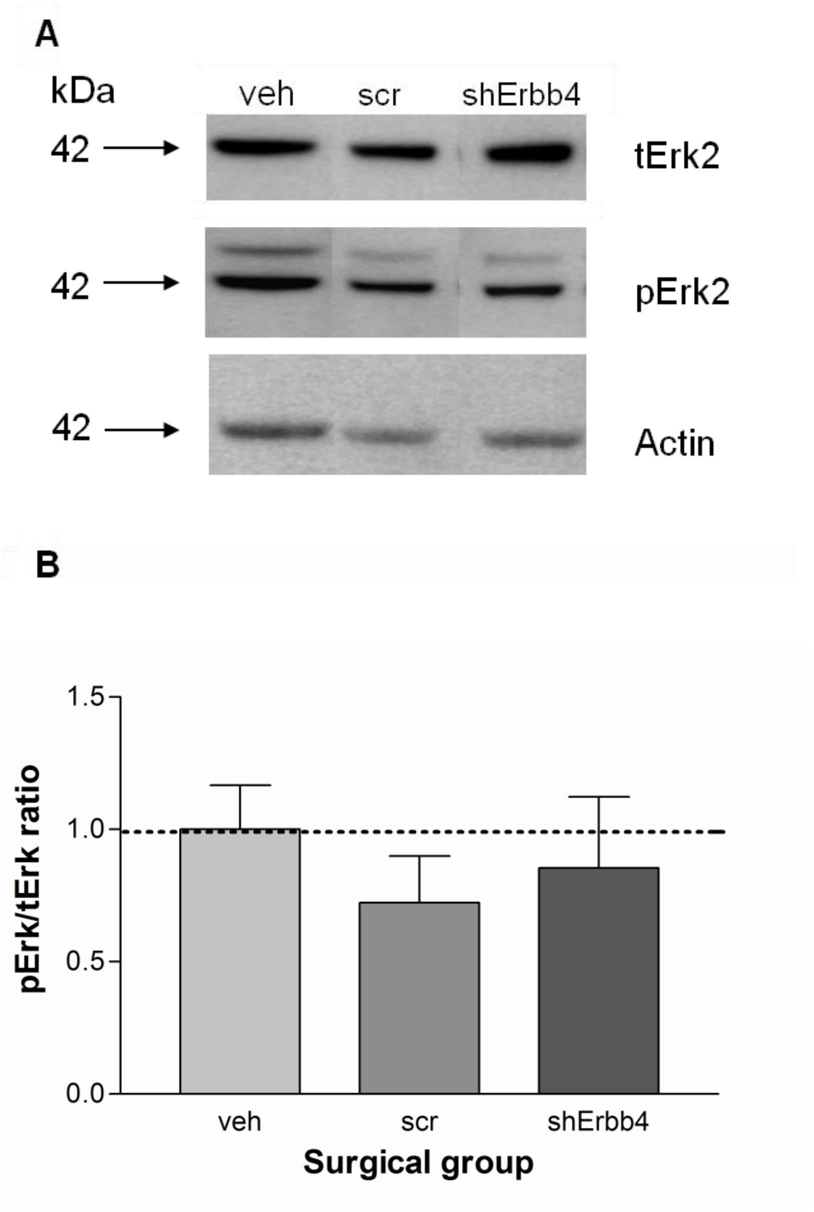


Figure 4.17 Effect of *in vivo* viral-mediated targeting of *Erbb4* on Erk signalling. Representative immunoblots of tErk2 (top panel), pErk2 (middle panel) and Actin (lower panel) in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh, 1x10^{12}gc/ml scr.rAAV = scr or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (A). Quantification of the ratio of pErk2 to tErk2 protein expression in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh (n=7), 1x10^{12}gc/ml scr.rAAV = scr (n=8) or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (n=8) normalised to the expression of Actin (B). Data represent mean ± S.E. relative to vehicle treated samples.

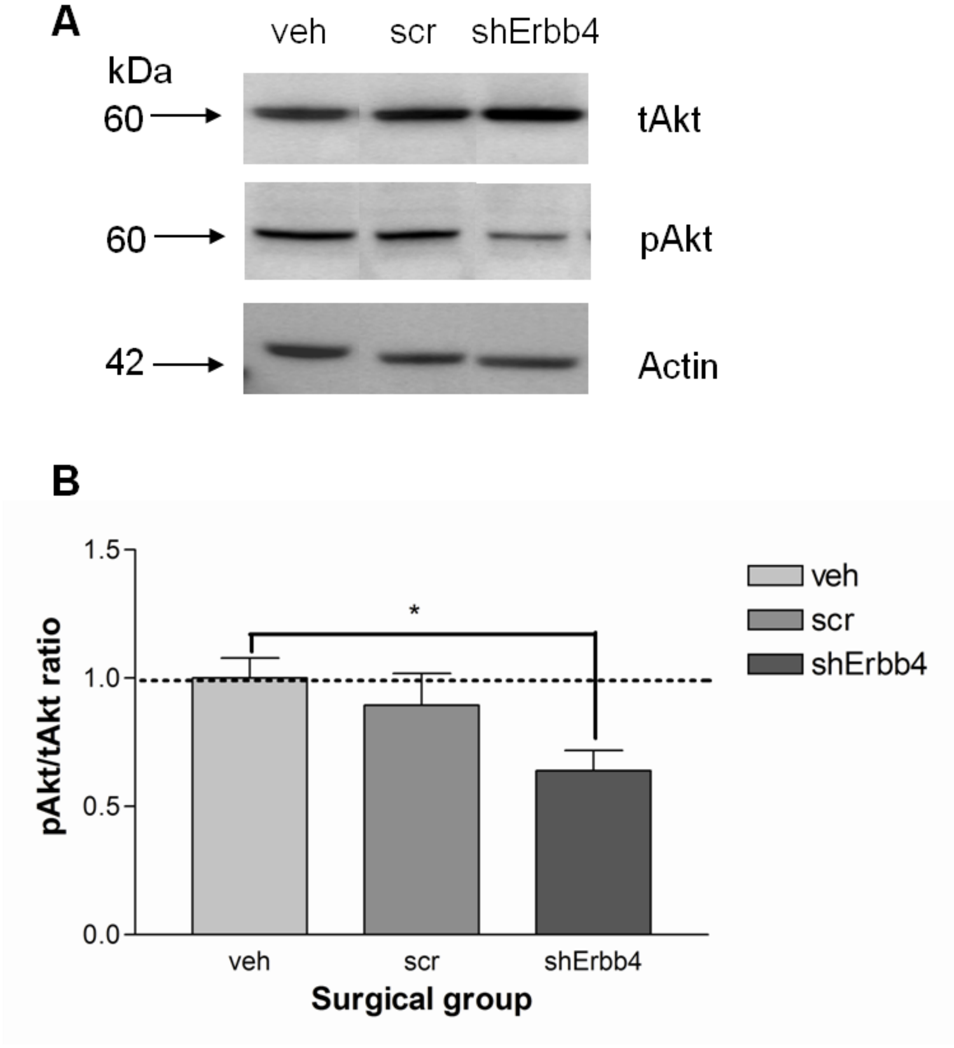


Figure 4.18 Effect of *in vivo* viral-mediated targeting of *Erbb4* on Akt signalling. Representative immunoblots of tAkt (top panel), pAkt (middle panel) and Actin (lower panel) in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh, $1x10^{12}$gc/ml scr.rAAV = scr or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (A). Quantification of the ratio of pAkt to tAkt protein expression in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh (n=7), $1x10^{12}$gc/ml scr.rAAV = scr (n=8) or $1x10^{12}$gc/ml shErbb4.rAAV = shErbb4 (n=8) normalised to the expression of Actin (B). Data represent mean ± S.E. relative to vehicle treated samples. * $p<0.05$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

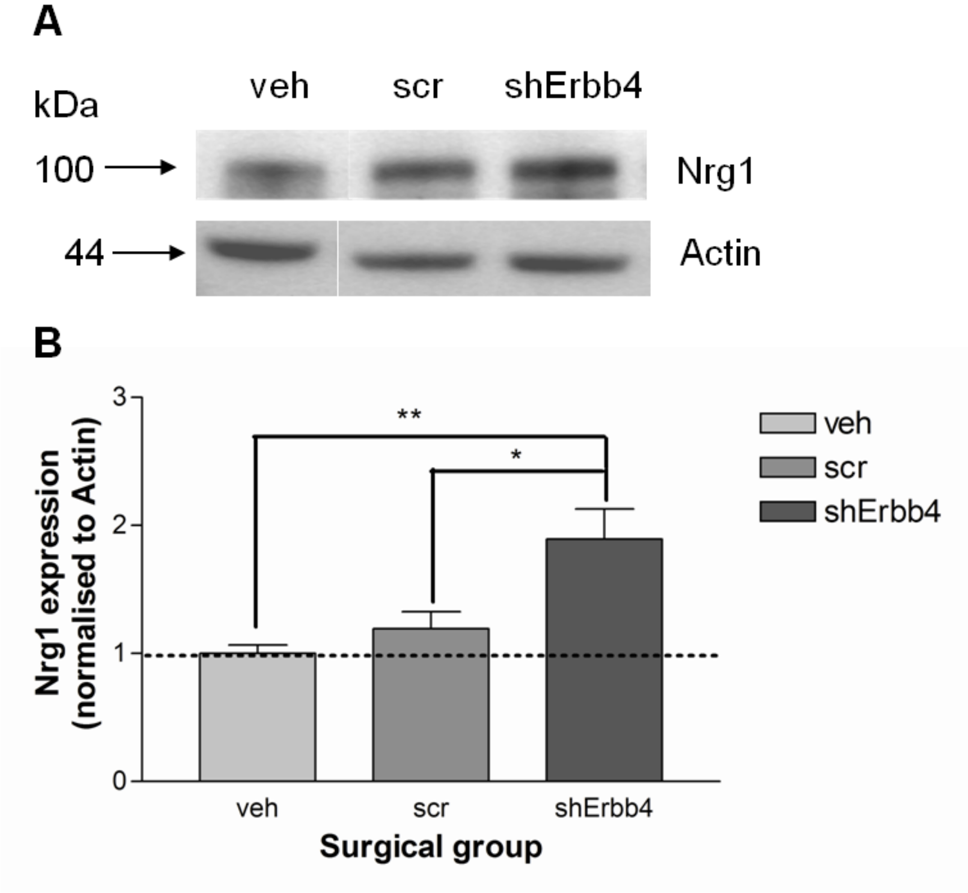


Figure 4.19 Effect of *in vivo* viral-mediated targeting of *Erbb4* on Nrg1 protein expression. Representative immunoblots of Nrg1 (top panel) and Actin (lower panel) in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh, 1x10^{12}gc/ml scr.rAAV = scr or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (A). Quantification of Nrg1 protein expression in lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh (n=7), 1x10^{12}gc/ml scr.rAAV = scr (n=8) or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (n=8) normalised to the expression of Actin (B). Data represent mean ± S.E. relative to vehicle treated samples. * $p<0.05$, ** $p<0.01$ analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

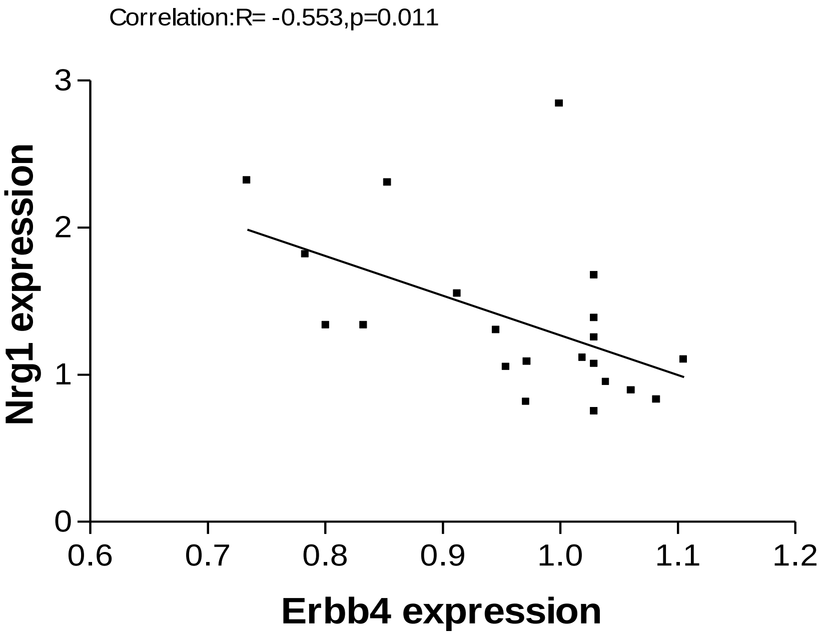


Figure 4.20 Correlation analysis of Erbb4 and Nrg1 protein levels. Correlation between Erbb4 protein expression and full length Nrg1 (100 kDa) protein expression in PFC lysates prepared from one hemisphere of the mPFC dissected from rats 5 weeks post-2μl injection of vehicle = veh, 1x10^{12}gc/ml (n=7), scr.rAAV = scr (n=8) or 1x10^{12}gc/ml shErbb4.rAAV = shErbb4 (n=7). Solid line represents the equation of best fit. Pearson's correlation co-efficient (R) and p value indicate significant negative correlation between Erbb4 and Nrg1 protein expression.

4.4 Discussion

4.4.1 Validation of rAAV functionality in vitro

Validation of the functionality of the rAAV particles was essential in this study as the particles themselves were a gift from an outside source, and evidence of their functionality *in vitro* or *in vivo* was not readily available. The aim of this *in vitro* validation study was not to calculate a titre for *in vivo* work. This preliminary analysis however, demonstrated that the viral particles were capable of transducing neuronal-like (NG108-15) and neuronal cells (primary cortical cultures) and that they drove the expression of eGFP, indicative of likely co-expression of the short-hairpin sequences in the other viral particles used in this study (scr.rAAV and shErbb4.rAAV). Additionally the *in vitro* validation study was successful in confirming that the hairpin sequence of the shErbb4.rAAV particles could elicit the knockdown of *Erbb4* expression in primary cortical cultures, to a significant level compared to treatment with scr.rAAV particles. This effect seems to be specific to the hairpin sequence as importantly even at the highest titre of viral particles used, there was no significant difference in *Erbb4* expression between vehicle treatment and scr.rAAV treatment, suggesting that the viral particles themselves were not overtly toxic. This validation study established the confidence required to progress to utilising these viral particles *in vivo*.

4.4.2 Validation of rAAV particles in vivo

The eGFP.rAAV viral particles were validated *in vivo* using 2 different titres and at 3 time points to assess viral tropism and expression of the eGFP transgene. The titres of viral particles used in this *in vivo* validation study were in line with multiple *in vivo* studies using rAAV particles within the CNS of rodents (Xia *et al.,* 2004; Babcock *et al.,* 2005; Ulusoy *et al.,* 2009) and importantly were below the threshold of those shown to be too toxic within the CNS (Ulusoy *et al.,* 2009). As expected the higher number of viral particles resulted in the transduction of more cells in the area surrounding the needle tract. The presence of eGFP expressing cells at 1 week following surgery is in accordance with a study which showed eGFP expression within the brain from 4 days onwards following rAAV treatment (Reimsnider *et al.,* 2007). In the present study peak eGFP expression was observed at 3 weeks post-surgery. Akin to this a study by Reimsnider *et al.,* (2007) confirmed that peak eGFP expression following rAAV injection into the striatum was by 4 weeks. As eGFP immunofluorescence analysis was not performed at 4 or 5 weeks post-surgery, it is unclear whether eGFP expression increased from week 3 to week 5. Interestingly, behavioural phenotypes emerged at 5 weeks post-surgery which may suggest that peak eGFP expression and thus hairpin expression will have already occurred by 5 weeks post-surgery. Although in the present study eGFP expression appeared to have diminished by 8 weeks post-surgery many studies have shown strong eGFP expression under the control of the same or similar promoters as this study at time points beyond 8 weeks (Kaplitt *et al.,* 1994; Xu *et al.,* 2001; Xue *et al.,* 2010). This may reflect region specific differences in factors which limit the expression of rAAV transgenes such as impaired intracellular trafficking (Ding *et al.,* 2005), uncoating of the viral capsid (Hauck *et al.,* 2004) or second strand DNA synthesis as single stranded rAAV genomes are only very transiently expressed in target cells before degradation (Hauck *et al.,* 2004). Since at 8 weeks post-surgery eGFP levels had returned to a similar level of that of 1 week post surgery, this would suggest that target cells surrounding the needle tract were transduced by multiple rAAV particles resulting in longer term eGFP expression than cells further from the needle tract that were presumably transduced by fewer rAAV particles owing to the more transient eGFP expression in cells out with the needle tract.

Peak spread of eGFP expression at 3 weeks post-surgery was approximately 0.65mm^2. This level of transduction is very similar to that of previous studies (Hommel *et al.,* 2003; Reimsnider *et al.,* 2007; Burnet *et al.,* 2011) and although this level of transduction has been sufficient in this study to produce both downstream behavioural and molecular alterations, it is of interest that previous studies have successfully reported behavioural and biochemical changes following injections of viral particles at multiple brain sites simultaneously (Winstanley *et al.,* 2009). Moreover, it has been shown that co-administration of viral particles along with agents that aid vasodilation such as mannitol increase spread of AAV transduction (Mastakov *et al.,* 2001).

Co-localisation of eGFP expression with multiple cell type markers revealed that within the mPFC rAAV serotype 9 is capable of transducing neurons and glia. Systemic injection of rAAV 9 has previously been reported to result in neuronal (Inagaki *et al.,* 2006) and neuronal and glial transduction (Foust *et al.,* 2009). However, intra-striatal injection of rAAV 9 was only efficient in transducing neurons but not glia (Xue *et al.,* 2010). This is in keeping with multiple reports of rAAVs having preference over neuronal than glial transduction (Cearly & Wolfe, 2006; Lawlor *et al.,* 2009). Although quantification was not carried out in the present study it was evident that the ratio of co-localised eGFP/Gfap expressing cells to un-colocalised Gfap expressing cells was very low. However, the presence of glial transduction within the mPFC in the present study compared to the lack of this observation in previous studies could be due to the suggestion that the tropism of rAAVs may vary dramatically between brain regions due to the expression of the specific serotypes and cell surface receptors or co-receptors with the distinct brain region, and also regional differences in the regulation of *Gfap* expression (Xu *et al.,* 2001). eGFP co-localisation in selected parvalbumin-expressing cells suggests the rAAVs were capable of transducing parvalbumin-positive GABAergic interneurons; this is in agreement with a previous study which found AAV 1/2 were capable of transducing both cortical inhibitory and excitatory interneurons (Nathanson *et al.,* 2009).

It is not surprising that the rAAVs used in this study transduced multiple cell types since the laminin receptor (thought to be the cell surface receptor for rAAV 9) is widely expressed throughout the CNS and is expressed in glial cells and neuronal cells including parvalbumin-containing interneurons (Baloui *et al.,* 2004). The wide tropism of eGFP expression observed in this study is indicative that the shRNA sequence of scr.rAAV and shErbb4.rAAV particles will also be expressed in these multiple cells types, and although it would be interesting to investigate the effects of knockdown of Erbb4 in specific cell types it is unlikely that the pathophysiology of schizophrenia is due to gene changes in gene expression in one individual cell type. In addition, in the rodent brain Erbb4 is expressed in glial cells (Gereck *et al.,* 2001), parvalbumin-containing intereurons (Yau *et al.,* 2003; Vullhorst *et al.,* 2009; Neddens & Buonanno, 2010) and also pyramidal cells (Gereck *et al.,* 2001; Mechawar *et al.,* 2007). However, this has recently been under debate (Vullhorst *et al.,* 2009; Neddens & Buonanno, 2010).

One very recent study has successfully utilised viral mediated technology to selectively target cortical inhibitory interneurons expressing Erbb4 in the mouse brain, but no functional measurements of behaviour or signalling were performed (Choi *et al.,* 2010).

4.4.3 Erbb4 mRNA and protein knockdown in vivo

Knockdown of *Erbb4* mRNA after injection of shErbb4.rAAV was not observed in the PFC at 5 weeks post-surgery, despite there being a significant decrease in the expression of Erbb4 protein. There are a number of reasons for why this is likely including that that the mRNA levels of *Erbb4* within shErbb4.rAAV transduced cells were successfully knocked down at 5 weeks post-surgery but this effect was diluted out by the large amount of tissue not transduced in the dissected PFC tissue analysed. MRNA and protein knockdown via viral mediated shRNA expression have been previously shown to occur at different time points post-viral injection (Burnet *et al.,* 2011). In the present study protein knockdown of Erbb4 within the PFC was on average 18%, suggesting that the transduced cells within the transduced region of the mPFC had a very low level of Erbb4 expression remaining. Although significant, the overall level of knockdown was subtle, suggesting that non-transduced cells within the dissected PFC tissue may have diluted out the Erbb4 knockdown levels. Some studies have shown greater knockdown levels using similar titres as in the present study however, it should be noted that many studies use techniques that quantify more defined areas than a gross tissue dissection, such as fluorescence-activated cell sorting (FACS) to analyse only GFP positive cells or laser capture dissection to dissect out only the precise area transduced by the virus (Hommel *et al.,* 2003; Mao *et al.,* 2009; McKee *et al.,* 2010).

4.4.4 The effect of Erbb4 knockdown on Nrg1-Erbb4 signalling

Erbb4 knockdown led to increased *Nrg1* mRNA levels *in vitro* and protein levels *in vivo* suggesting that the increase in the ligand expression compensates for the reduction in receptor number following shErbb4.rAAV treatment. This may explain why even though there is a significant knockdown in total Erbb4 the activity of the receptor as assessed by the ratio of pErbb4/tErbb4 is not changed, presumably caused by the remaining Erbb4 receptors being over-stimulated by the increased Nrg1. Moreover, this is strengthened by Pearson's correlation analysis revealing that the compensatory increase in Nrg1 is even more evident with the decreasing levels of Erbb4. Interestingly, although there have been numerous transgenic mouse lines produced with mutations in *Nrg1* or *Erbb4,* none of these studies have analysed whether there is a secondary effect on Erbb4 or Nrg1 levels, respectively. Similarly, many studies have measured Erbb4 and Nrg1 levels in the brains of patients with schizophrenia. However, these studies rarely measure both the ligand and receptor instead solely showing a change in levels of Nrg1 or Erbb4 (Hashimoto *et al.*, 2004; Law *et al.,* 2006; Law *et al.,* 2007; Silberberg *et al.,* 2007). One study correlated the level of both proteins in the PFC of patients with schizophrenia and found that increased NRG1 was correlated with increased ERBB4 levels (Chong *et al.*, 2008). Although these results are in opposition with what is seen in the present study, it should be noted that Chong and colleagues found a positive correlation with a cleaved form of NRG1 whereas here full length Nrg1 is changed and there was no effect of Erbb4 knockdown on any Nrg1 cleavage products. This suggests that Erbb4 knockdown in the mPFC did not affect the enzymatic cleavage of Nrg1. In a recent study, knock-out of the gene *bace* which transcribes an enzyme important in the cleavage of Nrg1 resulted in decreased Nrg1 but no changes in the expression levels of Erbb4 (Savonenko *et al.,* 2008).

As there was no significant change in the activity of the Erbb4 receptor following Erbb4 knockdown it was not expected that there would be changes in the activity of either of the signalling pathways initiated by Erbb4. In line with this hypothesis there was no effect of Erbb4 knockdown on Erk signalling. However, shErbb4.rAAV treatment appeared to reduce Akt signalling. Fascinatingly, in the PFC of brains from patients with schizophrenia the decrease in Erbb4 expression is limited to the CYT1 isoform, which signals through the Akt pathway (Law *et al.,* 2007). Moreover, Nrg1 induced changes in Akt activity in schizophrenia have been replicated in several studies, where although Nrg1 consistently increases activity of Akt in schizophrenia this increase in activity is attenuated (Sei *et al.*, 2007; Keri *et al.*, 2009) whereas Erk activity appears to be unaltered (Sei *et al.*, 2007). Studies looking at basal activity of Akt are less consistent with both decreases (Emamian *et al.*, 2004) and no changes (Ide *et al.,* 2006; Sei *et al.,* 2010) in Akt activity being identified.

Whether the change in Akt activity is a direct effect of Erbb4 knockdown or an effect of the secondary change in Nrg1 levels is unclear, and although the changes in levels of Erbb4 activity and Akt activity are not significantly different between the scr.rAAV and shErbb4.rAAV treatment groups, it is known that subtle changes in activity as measured by phosphorylation are less robust than changes in total protein levels. Phosphorylation states are rapid and reversible modifications that are dependent on cell type (Emamian *et al.,* 2004). Reductions in Akt activity have been associated with dopamine receptor D2 (Drd2) over-activity (Beaulieu *et al.*, 2004; Beaulieu *et al.*, 2007). Akt activity has also been associated with GABAergic (Wang *et al.,* 2003; Oishi *et al.,* 2009) and glutamatergic neurotransmission (Chalecka-Franaszek & Chuang, 1999). The finding that Akt activity is altered following Erbb4 knockdown is of great interest as it is consistent with the growing opinion that Akt is a key mediator of Nrg1-Erbb4 signalling within the prefrontal cortex, dysregulation of which may potentially lead to symptoms that appear to be dependant upon regulated function of this brain region.

4.4.5 Potential off target effects and toxicity of rAAV injection

GFAP expression is an indicator of astrocytic activation (gliosis) prior to visible anatomical neural tissue damage (O'Callaghan, 1988). There was no significant increase in Gfap levels in viral treated samples (scr.rAAV or shErbb4.rAAV) indicating that the virus itself had no overt adverse effects on the tissue surrounding the injection site. Moreover, since there is no difference in Gfap levels between the viral treatment groups the shErbb4 sequence and thus Erbb4 knockdown did not cause gliosis. These findings are in agreement with injection of similar titres of rAAV than that used in the present study did not increase levels of gliosis (Klein *et al.,* 2007). The weights of the rats in either of the studies did not change after injection of rAAV, indicating there were no gross effects on the rat's post-operative health which would lead to reduced eating and loss of body weight. Finally, there were several measures where there was no change between treatment groups on *Erbb4* and *Nrg1* mRNA levels, as well as the levels of housekeeping gene *r18s* and Erk protein levels, suggestive that Erbb4 knockdown and the rAVV particles themselves did not have broad off-target effects. This finding is in concordance with a study that found only rAAVs used at titres above 3.7 x 10^{12}gc/ml had some toxicity and off target effects (Ulusoy *et al.*, 2009).

4.5 Conclusions

The data in this chapter present a host of data validating the knockdown of schizophrenia candidate *Erbb4*.

I have shown in this chapter that following *in vitro* validation of rAAV particles expressing a hairpin sequence to rat *Erbb4* in NG108-15 cells and dissociated cortical cultures, it was possible to achieve a quantifiable knockdown of Erbb4 protein expression in the rat PFC at 5 weeks post-surgery. This knockdown of Erbb4 could be in pyramidal neurons, parvalbumin neurons and glial cells of the prefrontal cortex, as all of these cells types were capable of transduction by the rAAV particles.

There was no quantifiable *Erbb4* mRNA knockdown within the PFC at the time point assessed, however the ratio of *Nrg1* to *Erbb4* mRNA was significantly increased, which was in accordance with the finding that Nrg1 protein expression increased in response to Erbb4 knockdown. Furthermore, the activity of the Akt signalling pathway was significantly reduced by Erbb4 knockdown, whereas the Erk signalling pathway was unaffected.

Overall this chapter provides supporting evidence that viral-mediated knockdown in *vivo* is achievable, and validates the view that any behavioural changes due to shErbb4.rAAV injection will be due to subtle but significant knockdown of Erbb4 within the PFC.

5 Viral-mediated Erbb4 knockdown produces differential effects on schizophrenia-related behaviours

5.1 Introduction

Schizophrenia is characterised by several behaviour phenotypes, many of which have been induced in rodent models of schizophrenia by pharmacological agents, environmental insults and also by genetic insults (section 1.7). Rodent models with manipulations of the schizophrenia candidate genes *Nrg1* and *Erbb4* show a diverse range of schizophrenia-related behavioural phenotypes (section 1.9.4); however it is unclear if these phenotypes are due to the function of Nrg1-Erbb4 signalling in distinct brain regions. It is therefore of great interest to determine region specific functionality of these molecules in mediating schizophrenia-related behaviours in order to potentially dissect the roles of these candidate genes in schizophrenia.

5.1.1 Prepulse inhibition of the startle response

Prepulse inhibition of the startle response is a cross-species phenomenon whereby a non-startling “prepulse” stimulus suppresses the magnitude of the startle reflex to a startling stimulus. This test is widely used to measure sensorimotor gating and deficits in the performance of this task have been observed in patients with schizophrenia and also their unaffected relatives (Braff *et al.,* 2001). Decreased PPI is also apparent in multiple other neurological disorders such as Huntington’s disease, obsessive compulsive disorder (OCD), Tourette Syndrome and some forms of Bipolar disorder (Swerdlow et al., 1995; Castellanos *et al.,* 1996; Hoenig et al., 2005; Gogos *et al.,* 2009).

5.1.2 Modalities of cognition assessed by PPI

PPI deficits in schizophrenia representing sensorimotor gating dysfunction are thought to correspond to the inability of patients to filter out unimportant external stimuli leading to loss of focus and disorganised behaviour. Although the exact functional consequence of deficits in PPI in schizophrenia is unknown PPI has been termed as a promising endophenotype for schizophrenia by the CNTRICS program as a measure of “gain control” (Green *et al.,* 2009). Whether PPI deficits in schizophrenia are related to cognitive dysfunction is debatable, with some studies showing a positive correlation of PPI performance with performance in the CANTAB battery in healthy controls (Bitsios *et al.,* 2006) and Global Assessment of Function and Independent Living Scales in patients (Swerdlow *et al.,* 2006) . However, there appears to be no association of poor PPI with poor performance in tasks such as WCST in schizophrenia (Swerdlow *et al.,* 2006). Therefore PPI deficits may be important in being an early signal to consequential deficits in cognition. However, there is much more research needed to identify how deficits in PPI in schizophrenia patients manifest into cognitive deficits, and indeed which modality of cognition PPI deficits represent.

5.1.3 Neuronal circuitry and neurotransmitters involved in PPI

Studies in rats have helped elucidate the brain circuitry involved in the maintenance of PPI; this complex circuitry involves connections between the limbic cortex, ventral striatum, ventral pallidum and the pontine tegmentum, termed the CSPP circuit (Swerdlow & Geyer, 1999). Lesioning or direct infusion of multiple substances directly into regions of the limbic cortex, including the mPFC, hippocampus and amygdala result in PPI disruption (Swerdlow, 2001).

Pharmacological studies have highlighted some of the neurotransmitter systems that disrupt PPI which in turn may elucidate the aberrant neurotransmission occurring in schizophrenia resulting in dysfunctional PPI performance in patients. PPI is thought to be heavily dependant upon regulated dopaminergic signalling, as over-stimulation of the dopamine system by direct or indirect dopamine agonists such as amphetamine disrupts PPI in rodents (Mansbach 1998, Larrauri *et al.,* 2010). Moreover rats with genetic disruption of the dopamine transporter gene show deficits in PPI, namely due to the constant hyperdopaminergic state in the synaptic cleft via lack of dopamine clearance (Ralph *et al.,* 2001; Barr *et al.,* 2004).

Blockade of glutamatergic neurotransmission also results in PPI deficits in rodents with previous work from our laboratory showing that acute and sub-chronic PCP administration result in robust PPI deficits (Egerton *et al.,* 2008). In addition, mice with a reduction in the expression of the NMDA receptor subunit, NR1, show PPI deficits (Duncan *et al.,* 2004). Other neurotransmitter systems also appear to play an important role in regulating PPI, with nicotine injection in rats resulting in increased PPI (Levin *et al.,* 2005; Curzon 1994). It is therefore clear that PPI is a behaviour regulated by a distinct but complex circuitry involving multiple neurotransmitter systems.

5.1.4 Locomotor activity in response to a novel environment

Although positive symptoms such as hallucinations and delusions cannot be readily assessed in rodents other positive symptoms such as psychomotor agitation, which is apparent in patients with schizophrenia, can be characterised by increased locomotor activity in response to a novel environment (Arguello & Gogos, 2006). The open field task is an ideal way to measure locomotor activity utilising the inbuilt characteristic of rodents to be naturally explorative but also cautious to a novel environment. Locomotor activity is widely used to study behavioural phenotypes similar to positive symptoms in schizophrenia as it is relatively easy and inexpensive to perform.

Locomotor activity is a dopamine dependent process and in some studies increased locomotor activity to a novel environment can be reversed or dampened by antipsychotic treatment (which generally have high affinity for D2 receptors)(Stefansson *et al.,* 2002). Moreover, mice that lack D2 receptors perform at approximately half the locomotor activity of control mice (Kelly *et al.,* 2008).

5.1.5 Sensitivity to amphetamine

In addition to assessing locomotor activity to a novel environment, many studies have employed the additional challenge of the psychostimulant amphetamine on locomotor activity. This has been employed as it is known that amphetamine has psychosis- like effects in non-schizophrenic individuals (Carlsson & Lindqvist, 1963) and also exacerbates positive symptoms in patients with schizophrenia. Moreover, amphetamine challenge in patients with schizophrenia results in a much greater amphetamine-induced dopamine release than that seen in healthy controls (Laruelle *et al.,* 1996; Breier *et al.,* 1997; Abi-Dargham *et al.,* 1998; Laruelle *et al.,* 1999) and therefore, changes in response to amphetamine in rodents may indicate a schizophrenia-like dopaminergic imbalance due to pharmacological challenge or gene manipulation. Amphetamine acts primarily by increasing dopamine levels at the synaptic cleft (Seiden *et al.,* 1993).

However, amphetamine not only increases dopamine levels but also levels of monoamines such as noradrenaline and serotonin (Kuczenski & Segal, 1997).

Amphetamine challenge in rodents has also been commonly used to test the intactness of the cortico-striatal loop which is essential in mediating the effects of amphetamine (Burns *et al.,* 1993). Therefore a heightened response to amphetamine in response to a pharmacological agent or genetic modification may indicate a disturbance in dopaminergic signalling resulting from a dysfunction in the cortico-striatal loop circuitry.

5.2 Aims

The aims of this study were to test the hypothesis that viral mediated knockdown of Erbb4 within the mPFC in rats produces schizophrenia-related behaviour in rats. This was achieved by examining Erbb4 knockdown on sensorimotor gating (PPI) and hyperactivity to a novel environment (open field), thus determining if Erbb4 within this specific brain region was important in mediating or regulating these behaviours. In addition, the effect of an amphetamine challenge on locomotor activity was investigated with the aim to determine if altering Erbb4 expression within the mPFC affected dopaminergic transmission and the integrity of the cortico-striatal loop.

5.3 *Results*

5.3.1 *Effect of viral-mediated gene manipulation on pre-pulse inhibition*

To assess if Erbb4 within the prefrontal cortex of the adult brain is important in regulation of sensorimotor gating rats underwent stereotaxic surgery receiving bilateral injections of either vehicle (veh)n=7, rAAV encoding a scrambled hairpin sequence (scr) n=8 or rAAV encoding a hairpin sequence complementarily to rat *Erbb4* (shErbb4) n=8 (section 2.4.2). One, three and five weeks post-surgery sensorimotor gating was assessed via measuring prepulse inhibition of the startle response (section 2.6.3).

Startle response to 120dB startle alone pulses (i.e. those not preceded by pre-pulse stimuli) was not affected by surgical group (*F* (2, 68) =1.19 p>0.05) (Figure 5.1). Startle response levels did not significantly change over the three time points assessed post-surgery, with no significant effect of week on startle response (*F*(2,68)=1.64 p>0.05) and moreover there were no significant differences in startle levels between surgical groups at individual time points (surgical group x week interaction *F*(4, 68)=1 p>0.05).

Mean % PPI (mean % PPI of 4, 8 and 16dB prepulse amplitudes) was significantly affected by surgical group (*F* (2, 68) =9.10 p<0.001), with *post hoc* analysis revealing that scrambled hairpin virus (scr.rAAV) treated rats (p<0.01) and shErbb4 virus (shErbb4.rAAV) treated rats (p<0.001) had a significantly lower mean %PPI than vehicle treated rats. This effect was a general observation over all time points with no significant interaction between surgical group and week being found (*F* (4, 68) =1.1 p>0.05). Interestingly, there was a significant effect of week on mean % PPI (*F* (2, 68) =11.46 p<0.001) with mean % PPI being significantly higher at week 5 than at weeks 1 (p<0.001) and 3 (p<0.05) (Figure 5.2).

To probe the results observed with mean % PPI further, the effects of viral manipulation on % PPI were analysed by individual prepulse amplitudes. The most striking finding was that prepulse amplitude had a very significant effect on % PPI (*F* (2,206) =213.05 p<0.001), with % PPI increasing with increasing prepulse amplitude level. % PPI at a prepulse amplitude of 16dB was significantly higher than at amplitudes of 8dB (p<0.001) and 4dB (p<0.001) and prepulse amplitude of 8dB resulting in higher % PPI than 4dB (p<0.001). The analysis of % PPI by individual prepulse amplitude yielded very similar findings to those of mean % PPI. Again surgical group had a significant effect on % PPI (*F* (2, 206) =13.07 p<0.001) with both viral groups showing reduced %PPI than vehicle treated rats. In agreement with mean % PPI, week also had a significant effect on % PPI (*F* (2,206)=15.61 p<0.001) with % PPI being higher at week 5 than at weeks 1 and 3, however this effect was observed over all prepulse amplitudes with no prepulse amplitude x week interaction being found (*F* (4,206)=0.41 p>0.05).

Unlike the findings of mean % PPI, % PPI analysed by individual prepulse amplitudes showed there was a significant interaction between surgical group and week (*F* (4, 206) =2.57 p<0.05) owing to shErbb4.rAAV treated rats having a significantly lower % PPI than vehicle treated rats at 1 week post-surgery (p<0.05) and scr.rAAV treated rats having significantly lower % PPI than vehicle treated rats at 3 weeks post-surgery (p<0.001). These observations were general findings over all prepulse amplitudes as there was no significant interaction between surgical group x prepulse amplitude x week (*F* (8, 206) =0.95 p>0.05) (Figure 5.3).

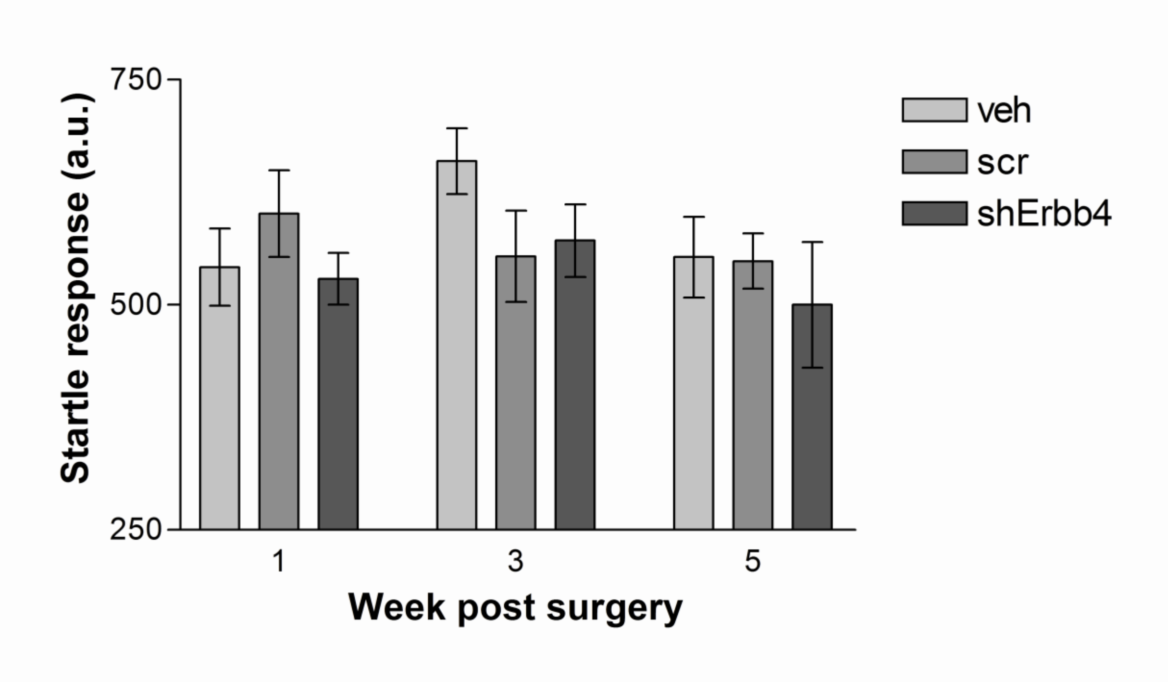


Figure 5.1 Effect of viral mediated gene manipulation on startle response. The startle response to 120dB startle only trials at 1, 3 and 5 weeks following bilateral injections of vehicle (veh) (n=7), scr.rAAV (scr) (n=8) or shErbb4.rAAV (shErbb4) (n=8) into the mPFC. There was no effect of surgical group on startle response. Data represents mean startle response (measured in arbitrary units (a.u.)) ± SE.

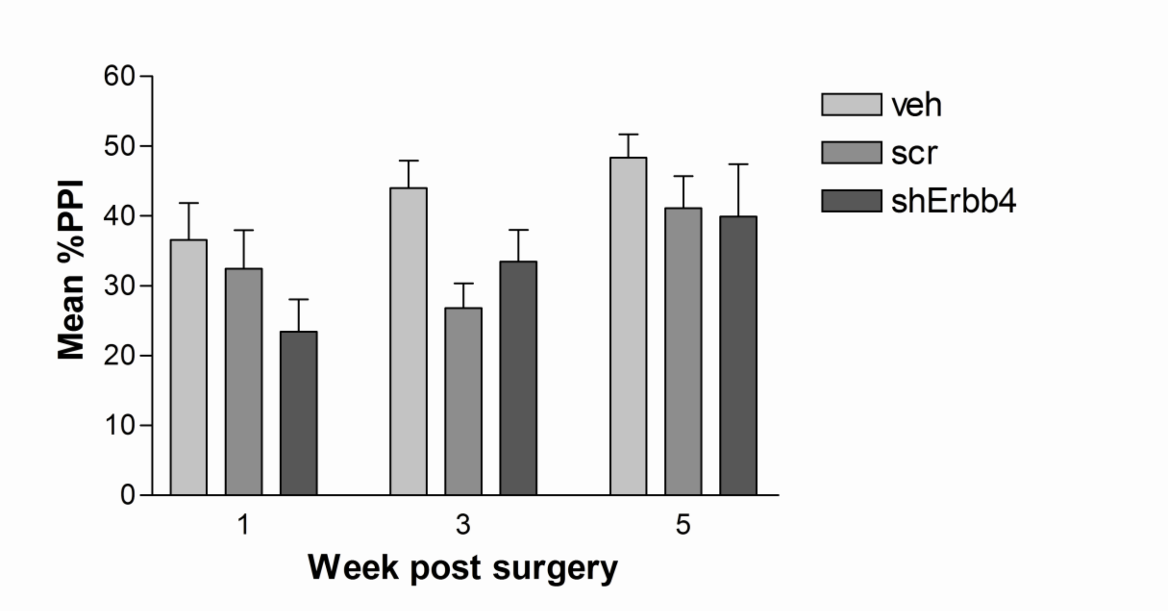


Figure 5.2 Effect of viral mediated gene manipulation on mean % PPI. % PPI (mean of %PPI at prepulse amplitudes of 4dB, 8dB and 16dB) was measured at 1, 3 and 5 weeks following bilateral injections of vehicle (veh) (n=7), scr.rAAV (scr) (n=8) or shErbb4.rAAV (shErbb4) (n=8) into the mPFC. Data represents mean ± SE. Data were analysed using two-way repeated measures ANOVA followed by Tukey's test for multiple comparisons. Mean %PPI was overall significantly lower in scr.rAAV ($p<0.01$) and shErbb4.rAAV ($p<0.001$) treated rats compared to vehicle treated rats.

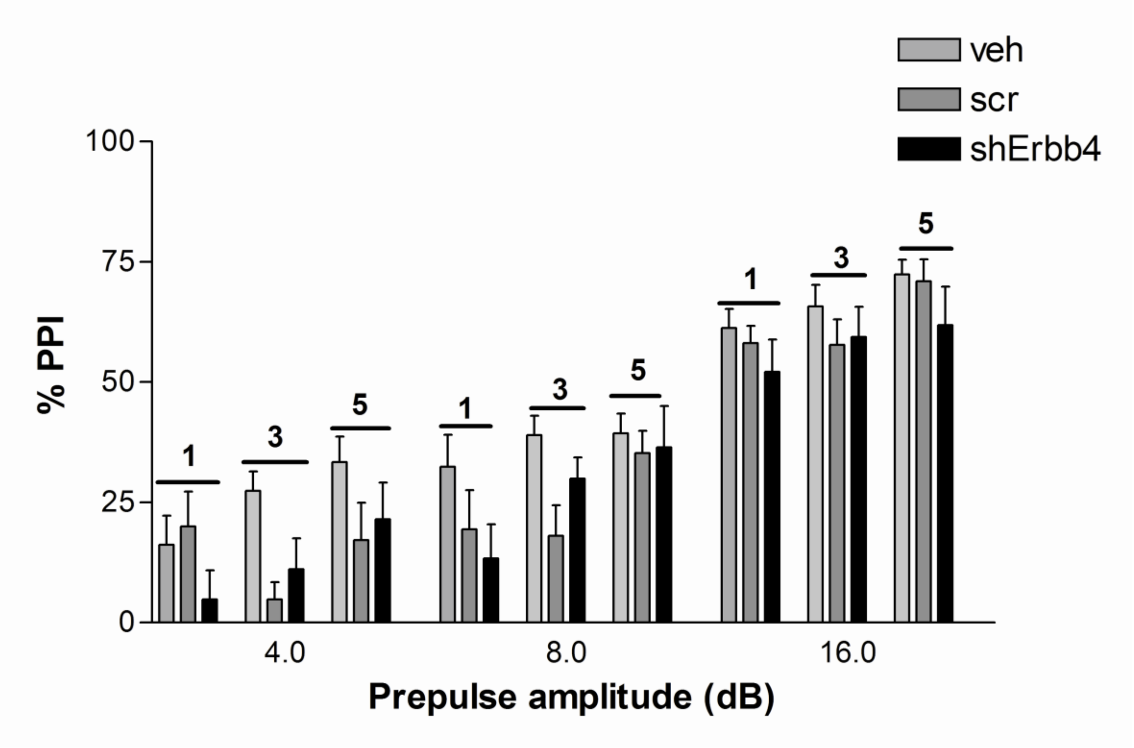


Figure 5.3 Effect of viral-mediated gene manipulation on % PPI at prepulse amplitudes of 4dB, 8dB or 16dB. % PPI was assessed at prepulse amplitudes of 4, 8 and 16dB at 1, 3 and 5 weeks following bilateral injections of vehicle (veh) (n=7), scr.rAAV (scr) (n=8) or shErbb4.rAAV (shErbb4) (n=8) into the mPFC. Data represents mean ± SE. Data were analysed using two-way repeated measures ANOVA followed by Tukey's test for multiple comparisons. %PPI was overall significantly lower in scr.rAAV ($p<0.001$) and shErbb4.rAAV ($p<0.001$) treated rats compared to vehicle treated rats

5.3.2 Effect of viral mediated gene manipulation on locomotor activity

In the same rats that were being assessed for sensorimotor gating deficits, locomotor activity in response to amphetamine was also measured to assess if Erbb4 within the prefrontal cortex of the adult brain is important in regulation of cortico-striatal dopamine regulation. 23 rats that had received intra-mPFC injections of vehicle (veh) n=7, rAAV encoding a scrambled hairpin sequence (scr) n=8 or rAAV encoding a hairpin sequence directed against rat *Erbb4* (shErbb4) n=8 (section 2.4.2) were assessed in the open field one, three and five weeks post-surgery (section 2.5). In the open field assessments rats were monitored for a 30 minute habituation period (2.5.2), and injected with vehicle (0.9% w/v saline) or 1mg/ml amphetamine before being monitored for a test period of 60 minutes (section 2.6).

Analysis of locomotor activity during the 30 minute habituation phase to the open field showed that there was no significant difference in the distance travelled during this habituation period on the first or second exposure to the open field apparatus at each week that the locomotor activity was assessed (*F* (1, 827) =1.75 p>0.05). Therefore any further effects seen during the habituation period are not due to differences by day. There was an overall significant effect of surgical group on distance travelled during the habituation phase (*F* (2, 827)=7.08 p=0.001) with *post hoc* analysis showing vehicle treated rats travelled less distance than scrambled hairpin virus treated rats (p<0.01) and shErbb4 virus treated rats (p<0.01). However these significant differences between surgical groups were not observed at any individual week assessed (surgical group x week interaction *F* (4, 827) =1.13 p>0.05)

The rats in general travelled a significantly longer distance at week 5 than at weeks 1 (p<0.01) or 3 (p<0.001) explaining the significant effect of week on distance travelled during the habituation phase (*F* (2,827) =7.08 p<0.001). There was also a significant effect of time on distance travelled during the habituation phase (*F* (5, 827)=280.91 p<0.001) with distance travelled dropping over time. Each time bin was significantly different from the previous time bin until 20 minutes into the habituation phase where final three time bins were not statistically significant from each other. This drop in distance travelled over time during the habituation phase was observed over all surgical groups at all weeks owing to no significant surgical group x week x time interaction (*F* (20, 827)=0.38 p>0.05) (Figures 5.4A-C).

5.3.3 Effect of viral mediated gene manipulation on sensitivity to amphetamine

Concurrent with the results of the locomotor activity habituation phase, there was a significant effect of surgical group on distance travelled during the test phase (*F* (2, 1655) =6.02 p<0.01). Again, this was due to scr.rAAV treated rats (p<0.05) and shErbb4.rAAV treated rats (p<0.01) travelling longer distances than vehicle treated rats. Distance travelled was significantly affected by the drug administered (*F* (1, 1655) =323.6 p<0.001) with 1mg/kg amphetamine causing hyperlocomotor activity in all surgical groups in all weeks assessed. Importantly, distance travelled was not affected by the cross-over design implemented in the experiment as the day amphetamine was administered (day 1 or 2 at each week) did not have a significant effect on distance travelled in the test phase ((*F* (1, 1655)=0.25 p>0.05).

Importantly at 5 weeks post-surgery rats with Erbb4 knockdown within the mPFC travelled significantly longer distance under the influence of amphetamine than vehicle (p<0.05) or scr.rAAV treated rats (p<0.001). This led to a significant group x week x drug interaction ((*F* (4, 1655)=5.3 p<0.001) on distance travelled in the test phase. *Post hoc* comparisons revealed that although there was an effect at 5 weeks post-surgery, there was no significant difference in the effect of amphetamine challenge between surgical groups at weeks 1 or 3 post-surgery.

Some similarities between the results of the habituation phase and test phase were apparent with saline administered scr.rAAV treated rat travelling longer distances than saline administered vehicle treated rats at weeks 3 and 5 post-surgery.

Time had a significant effect on distance travelled in the test phase ((*F* (11, 1655)=7.22 p<0.001) where it was observed that at 30 minutes after saline injection the rats in all surgical groups were travelling only very short distances.

Amphetamine treated rats’ peak distance travelled was observed at 15 minutes post-injection.

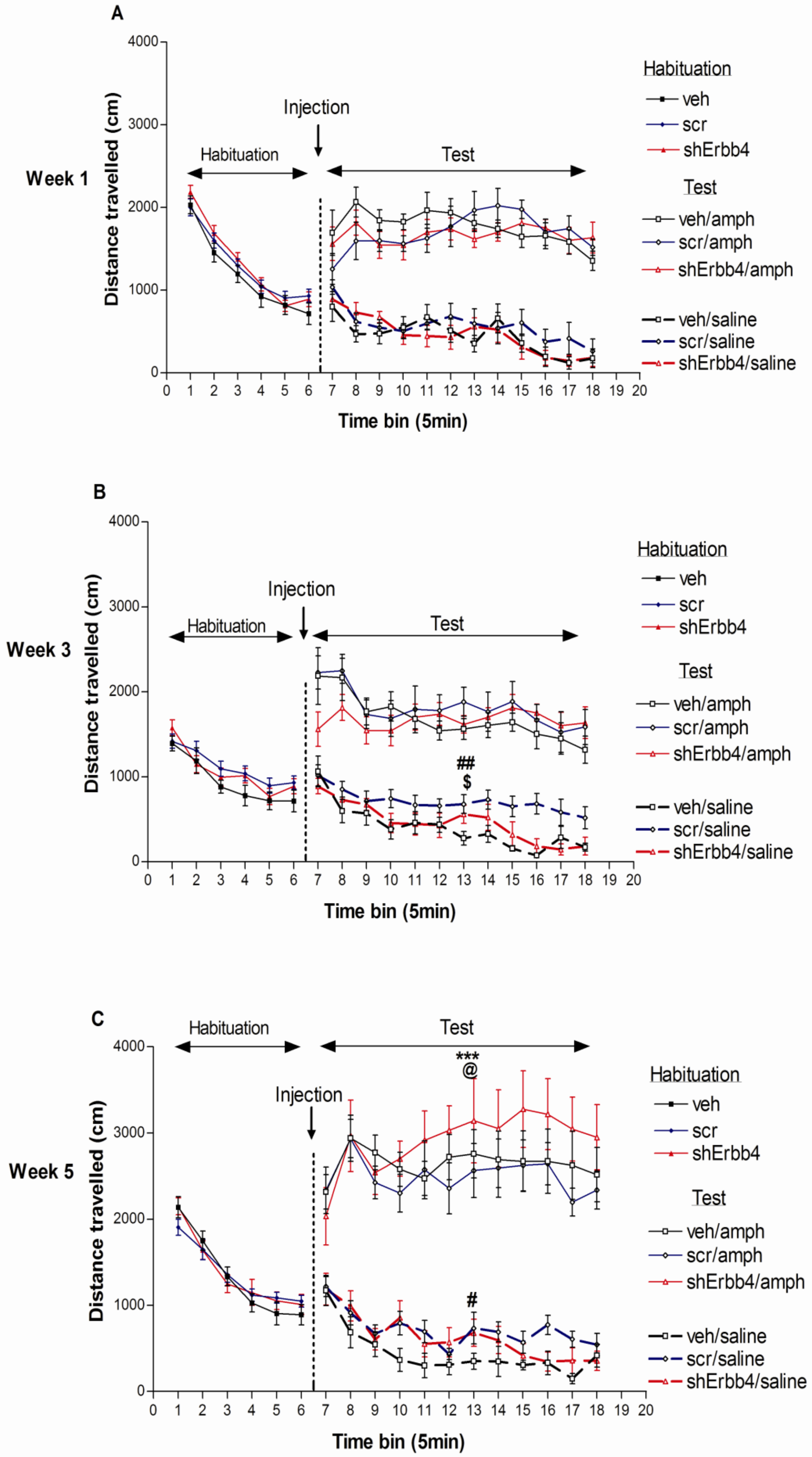

A
Week 1
B
Week 3
C
Week 5
Distance travelled (cm)
0
1000
2000
3000
4000
Time bin (5min)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Habituation
Injection
Test
##
$
***
@
#
Habituation
veh
scr
shErbb4
Test
veh/amph
scr/amph
shErbb4/amph
veh/saline
scr/saline
shErbb4/saline

Figure 5.4 Effects of viral mediated gene manipulation on amphetamine induced hyperlocomotor activity. Locomotor activity was assessed 1 (A), 3(B) and 5(C) weeks post-bilateral injections of vehicle (veh) (n=7), scr.rAAV (scr) (n=8) or shErbb4.rAAV (shErbb4) (n=8) into the mPFC of rats. Activity was measured during a 30 minute habituation phase (habituation, left panel of graphs) and a 1 hour testing phase (test) immediately following s.c. injection of 0.9% saline (saline, lower data sets on graph) or 1mg/kg amphetamine (amph, upper data sets on graphs). Data represents distance travelled in 5 minute time bins ±SE mean analysed using three-way repeated measures ANOVA followed by Tukey’s test for multiple comparisons. ANOVA analysis showed an overall significant effect of drug administration on locomotor activity with amphetamine significantly increasing distance travelled compared to saline ($p<0.001$). *Post hoc* comparisons showed shErbb4.rAAV treated rats had a significantly heightened locomotor response to amphetamine at 5 weeks post-surgery compared to vehicle ($p<0.05$=@) and scr.rAAV ($p<0.001$=***) treated rats. Saline injected vehicle treated rats travelled significantly shorter distances than saline injected scr.rAAV treated rats at 3 weeks (##=$p<0.01$) and 5 weeks (#=$p<0.05$) post-surgery and saline injected shErbb4.rAAV treated rats 3 weeks post-surgery ($=$p<0.05$).

5.4 Discussion

5.4.1 Erbb4 knockdown does not disrupt PPI

The lack of changes in the amplitude of startle response between surgical groups may suggest that there is no difference in the levels of anxiety following viral injection or Erbb4 knockdown, as increases in the startle levels *per se* have been previously associated with increased anxiety (Deakin *et al.,* 2009*).* The finding that there is no difference in % PPI between the rAAV.scr and rAAV.shErbb4 treatments groups suggests that decreasing Erbb4 specifically in the mPFC does not affect sensorimotor gating. This is in agreement with a previous study using heterozygous null *Erbb4* transgenic mice (Stefansson *et al.,* 2002). However, several studies have also shown deficits in PPI following CNS specific knock-out of Erbb2/4 (Barros *et al.,* 2009) or knock-out of *Erbb4* in parvalbumin-positive interneurons (Wen *et al.,* 2010). Variation in the PPI results obtained from transgenic *Erbb4* mice may reflect differences in which isoforms are affected by the gene manipulation and in addition which background strains of mice are used for backcrossing.

This is highlighted when reviewing the literature of *Nrg1* mutant mice and PPI performance. The effect on PPI appears to be dependant upon which isoform of *Nrg1* is mutated: deficits in PPI have been reported in transmembrane domain knock-out mice (Stefansson *et al.,* 2002) and type III *Nrg1* knock-out mice, but no changes in PPI in epidermal growth factor containing isoforms (Ehrlichman *et al.,* 2009). Moreover, *Nrg1* type I over-expression also results in deficits in PPI (Deakin *et al.,* 2009), conflicting with results of this study, as an increase of Nrg1 in the mPFC, secondary to Erbb4 knockdown results in no change in PPI. As well as differences in which isoforms are being manipulated, these studies are manipulating the expression of Erbb4/Nrg1 signalling in multiple brain regions. Therefore, it is plausible that manipulation of Nrg1-Erbb4 signalling simultaneously in multiple brain regions disrupts PPI, as PPI depends on cortical, limbic and striatal systems (Swerdlow *et al.,* 2001).

Additionally, it is also possible that the level of Erbb4 expression changes in this study were too subtle to cause a gross effect on PPI. However, a recent study using antisense oligodeoxynucleotides to knockdown the expression of *mortalin* within the mPFC showed that even though there was only a trend towards knockdown of mortalin protein within the PFC there was still a significant deficit in PPI in *mortalin* knockdown rats (Gabriele *et al.,* 2010). Interestingly, recent studies utilising viral-mediated gene manipulation to knock down the expression of *Disc1* in the prefrontal cortex of mice *in utero* led to PPI deficits at postnatal day 56 (Niwa *et al.,* 2010) suggesting *Disc1* expression in the PFC is essential for intact sensorimotor gating. A subsequent study has showed that Disc1 expression is regulated by Nrg-Erbb signalling but it is not dependent on Erbb4 or the Erbb4-specific ligand, Nrg3 (Seshadri *et al.,* 2010), further emphasising that Erbb4 may not be important in sensorimotor gating.

Another pertinent point is that all of the studies of the effect of Nrg1 and Erbb4 on PPI have been carried out in mice, whereas this present study has utilised rats. Although the neuroanatomical systems that underpin PPI are similar in mice and rats, there is some evidence that PPI differs between rat and mice with 5-HT-1A agonists (Gogos *et al.,* 2008) and D2 receptor agonists (Mohr *et al.,* 2007) having differing roles between the species. In further support of the lack of effect on PPI in the present study, a recent study reported that although polymorphisms in the *5HT2R* and *COMT* genes elicited changes in PPI performance, a polymorphism in *NRG1* had no effect on PPI levels (Quednow *et al.,* 2009). However, another study did show an effect of NRG1 genotype on PPI performance (Hong *et al.,* 2008), albeit this study was investigating a different SNP. This strengthens the idea that the isoform or exon targeted for gene manipulation may have a strong impact in the behavioural outcome.

Although there was no difference in %PPI between both the viral treatment groups, the viral groups did have overall a reduced %PPI compared to vehicle treated rats. This suggests that the viral particles themselves had a slight effect on PPI, in keeping with the findings that rats treated with viral particles were different from vehicle treated rats in terms of locomotor activity (see sections 5.3.2 and 5.3.3) and response speeds in the 5-CSRTT (section 6.3). These “off-target” effects of the viral particles themselves are difficult to explain as the majority of other studies using viral particles in conjunction with behavioural analysis do not include comparison of data from viral-manipulated rats with vehicle treated rats and so therefore these off target effects may actually be routinely occurring. As the main focus of the present study was to investigate the role of Erbb4 on behaviour the effects between viral treatment groups (or lack of effect) is the key observation.

As a final point it is also significant to highlight that the lack of effects seen in this study on PPI performance is unlikely due to experimental limitations as the values obtained were very similar to that of another study in rats in our laboratory where %PPI was altered by a pharmacological challenge (Egerton *et al.,* 2008). Also in concordance with this study %PPI increases with repetition of the task, suggesting sensitisation/habituation of the rats to the anxiolytic nature of the task. Moreover, the quality of the experimental set-up is confirmed by %PPI values increasing with the increasing amplitude of the pre-pulse. Furthermore, this also validates that the hearing of the rats was not impaired by the atraumatic earbars that were in place during stereotaxic surgery.

5.4.2 Erbb4 knockdown has no effect on general locomotor activity

As with PPI, knockdown of Erbb4 specifically within the mPFC did not result in a change in response to a novel environment. Thus there were no differences between scrambled hairpin virus treated rats and rats treated with shErbb4 virus. All groups of rats appeared to habituate to the novel environment of the open field apparatus and this appeared to take around 20 minutes. The increase in distance travelled during the habituation period at week 5 compared to weeks 1 and 3 may be due to the rats becoming desensitised to the novelty of the open field arenas, and therefore less anxious to their surrounding environment, resulting in the rats moving more freely around the arena.

The hypolocomotor state of the vehicle treated group of rats compared to the virus treated groups reflects the increase in response speeds observed in the 5-CSRTT in the virus treated rats (section 6.3). In general, tissue damage caused by injection of the virus would be hypothesised to cause a motor retardation rather than an increase in motor activity. As seen in the PPI task, where differences were observed between vehicle and virus treated rats there may be some minor off-target effect of the rAAV in general. Yet again, this may be a common occurrence not revealed in previous studies utilising rAAV by the exclusion of “non-virus” behavioural control group. For example many studies have used viral-mediated gene manipulation to target genes involved in Parkinson’s disease, with gene manipulation causing a blatant motor deficit (measured by lever pressing or open field) however the effect of viral injection alone on these measures is not assessed (Hommel *et al.,* 2003). Moreover, this hyperlocomotor effect by viral injection in the present study is not significant at individual time points and so is clearly not a gross deficit while the vehicle treated rats are still travelling an average of 7.4 meters in the 30 minute habituation phase. Interestingly, Xue and collegues (2009) found an altered response to amphetamine in mice injected with rAAV expressing GFP compared to saline treated rats. The authors suggest this was an effect of the viral particles themselves, and propose that the GFP expressed from the rAAV may have built up within the transduced cells, and led to neurotoxicity (Xue *et al.,* 2009).

As with PPI there have been many studies of basal locomotor activity in Erbb4 and Nrg1 transgenic mouse lines, and akin to the PPI data many of the studies of locomotor activity in these mice are contradictory. Studies of Erbb4 transgenic mice have showed locomotor phenotypes of hyperactivity (Stefansson *et al.,* 2002; Wen *et al.,* 2010), hypoactivity (Golub *et al.,* 2004) and in concordance with the present study locomotor activity akin to wildtype controls (Barros *et al.,* 2009). Neuregulin knock-out mice also show a varied response to novelty induced locomotor activity with multiple studies showing a hyperactive response compared to wildtypes (Gerlai *et al.,* 2000; Stefansson *et al.,* 2002; Karl *et al.,* 2007; O’Tuathaigh *et al.,* 2007). However some studies have also shown a motor response in Nrg1 knockouts that is not different from wildtypes (Rimer *et al.,* 2005; Chen *et al.,* 2008; Ehrlichman *et al.,* 2009). Over expression of type I Nrg1 appears to result in a slight hypolocomotor activity phenotype which is more evident in female transgenic mice (Deakin *et al.,* 2009).

Divergence of the results in this study compared to previous studies again may be due to the region specific focus of the manipulation of gene expression in this study compared to global gene manipulation, due to the isoforms or ratio of heterodimers affected by the gene manipulation.

5.4.3 Erbb4 knockdown induces hypersensitivity to amphetamine

Firstly, the hyperlocomotor behaviour observed in the viral treated rats during the habituation phase was carried through to the testing phase to some extent in saline injected rats. This effect was apparent at multiple time points (unlike the response to amphetamine which was present only at 5 weeks post-surgery) suggesting that this motor effect was not due to gene manipulation. Moreover this general virus vs. vehicle hyperactivity was not observed when the rats were injected with amphetamine, this may highlight again that the potential “off target” effect of rAAV injection on motor activity is not a gross motor effect as under the influence of amphetamine the rats’ motor systems are greatly challenged and motor deficits should thus be more apparent under amphetamine challenge than with saline.

The effect of amphetamine increasing locomotor activity was in complete agreement with previous studies and pharmacological evidence of amphetamine increasing dopamine levels in the synaptic cleft highlighting the soundness of the experimental set-up and behavioural analysis. There was a clear distinction in the locomotor response to amphetamine between the shErbb4.rAAV treated rats and vehicle and scr.rAAV treated rats at 5 weeks post-surgery, although there were no changes in the response to amphetamine between surgical groups at 1 or 3 weeks post-surgery. Thus, at 5 weeks post-viral injection there was confirmed Erbb4 knockdown and also effects on multiple sites of the Nrg1-Erbb4 signalling pathway (section 4.3.5), as well as the manifestation of heightened locomotor response to amphetamine. The behavioural response at this time point also correlates with the eGFP expression analysis where peak viral expression appeared to be somewhere between 3 and 8 weeks post-surgery. It is unclear whether peak knockdown of Erbb4 was also at 5 weeks post-surgery as the expression profiling was only carried out at one time point. However, it is also conceivable that the behavioural response occurred following peak Erbb4 knockdown as it is likely behavioural changes are due to changes in multiple aspects of neuronal circuitry which, in all probability, would not be an instantaneous occurrence.

Hypersensitivity to amphetamine solely in shErbb4.rAAV treated rats suggests that Nrg1-Erbb4 signalling within the mPFC is important in regulating the cortico-striatal loop functionality and modulating dopaminergic neurotransmission. To my knowledge only one previous study has utilised amphetamine challenge in Erbb4 manipulated animals and the results are strikingly similar to the present study. Roy *et al.* (2007) showed that mice expressing a dominant negative form of Erbb4 in oligodendrocytes to eliminate Erbb4 function in white matter had a potentiated locomotor response to amphetamine. Furthermore, this study also showed that these mice had increased D1-like receptor binding and dopamine transporter levels in multiple brain regions including the cortex and have increased evoked dopamine release (Roy *et al.*, 2007). Taking the results of this previous study into consideration may suggest that knockdown of Erbb4 in the mPFC may result in similar dopaminergic changes.

In addition, there is evidence to suggest that Nrg1 function is important in dopamine regulation specifically in the mPFC, as Nrg1 administration directly into this brain region results in a dopaminergic overflow in the mPFC of mice (Kato *et al.*, 2010). Increased Nrg1 expression and decreased Erbb4 expression in the mPFC of the rats in this study following shErbb4.rAAV injection may therefore have an increased release of dopamine within their mPFC which would lead to an increased sensitivity to the locomotor effects of amphetamine, but this increased response to amphetamine may also be due to changes in dopamine re-uptake, changes in dopamine receptor number, altered dopamine transporter function/expression or reduced metabolism of amphetamine itself.

Although the main region thought to elicit the hyperdopaminergic effect of hypersensitivity to amphetamine challenge is the striatum, it has been noted that the hyperdopaminergic state of this brain region is most likely caused by over activation of glutamatergic tracts of the PFC and hippocampus connecting to the mesolimbic pathway (Grace, 1991). It is therefore plausible that manipulation of neurotransmission specifically in the mPFC would be capable of affecting the cortico-striatal circuitry resulting in the observed behavioural changes.

Interestingly, manipulation of genes related to Nrg1-Erbb4 signalling results in altered response to amphetamine. For example, mice with knock-out of genes encoding enzymes known to cleave Nrg1 and/or Erbb4 (and therefore aid their signalling capabilities) show heightened response to amphetamine (Dejaegere *et al.,* 2008). It is worthy to note, that manipulation of other schizophrenia candidate genes results in altered response to amphetamine with viral mediated knockdown of *Disc1* within the mPFC resulting in hypersensitivity to the hyperlocomotor effects of amphetamine. It has been shown that amphetamine challenge dephosphorylates Akt, via increased dopamine signalling through D2 receptors, enabling *Gsk3b* activity which is in part responsible for the development of the hyperlomotor response (Beaulieu *et al.,* 2004; Beaulieu *et al.,* 2005). Therefore, if as a result of Erbb4 knockdown Akt signalling in the mPFC is somewhat already reduced (section 4.3.5) a challenge of amphetamine would have an even greater effect on these rats due to further reduced Akt activity. The data of the present study are therefore consistent with Erbb4 knockdown potentiating the ability of amphetamine to reduce Akt signalling in the prefrontal cortex, and this resulting in hyperlocomotion.

5.5 Conclusions

The data in this chapter present multiple interesting findings regarding the role Nrg1-Erbb4 signalling in schizophrenia-related behaviours including PPI and locomotor activity in response to a novel environment and an amphetamine challenge.

Lack of response of the manipulation of Nrg1-Erbb4 signalling in the mPFC on PPI and novelty-induced hyperactivity strengthens the hypothesis that Erbb4 signalling in the specific brain region of the mPFC does not play a key role in these rather general behaviours and instead may be important in modulating more cognitive based behaviours. Although multiple previous studies have shown effects of Nrg1-Erbb4 manipulations on PPI and novelty induced hyperactivity, the results of this present study further emphasise the need to investigate gene functions in specific brain regions and the validity of using viral mediated gene manipulation to accomplish this.

Overall, a key finding of this study was that Erbb4 knockdown specifically within the mPFC results in hypersensitivity to amphetamine. This suggests that, Nrg1-Erbb4 signalling is important in regulating dopaminergic neurotransmission in the cortico-striatal loop.

6 Effect of Erbb4 knockdown on performance in 5-CSRTT

6.1 Introduction

Deficits in prefrontal cortex function are thought to be highly contributory to the cognitive deficits of schizophrenia; however the physiological origins of these deficits are still unknown. Initiatives to identify potential therapeutic targets of the poorly treated cognitive deficits in schizophrenia are ongoing. To progress pre-clinical translational animal models of cognitive deficits of schizophrenia, the cognitive domains that encompass this symptom group have been identified and suitable rodent behavioural paradigms recommended (Young *et al.,* 2009). Of these the 5-choice serial reaction time task (5-CSRTT) has high validity for assessing cognitive modalities dependent on regulated prefrontal cortical function. Other rodent behavioural tasks are known to measure PFC-dependent behaviour and have been previously used to assess the effect of mutations in schizophrenia candidate genes, such as the attentional set-shifting task to measure COMT function (Tunbridge *et al.*, 2004). However, the 5-CSRTT was the most appropriate task to use in the present study due to the large number of animals used. We therefore required a behavioural task that is high throughput and automated, such as the 5-CSRTT. The attentional set-shifting task requires manual monitoring of the rats behaviour, resulting in a maximum of 4 rats being able to be tested per day.

Several potential risk genes for schizophrenia have been identified, with the NRG1-ERBB4 genes strong candidates. Their location within the prefrontal cortex and expression in neuronal subtypes that regulate neurotransmission within this brain region make them attractive molecules to study in terms of cognition (sections 1.1.2 and 1.1.4). However previous animal models of these genes have failed to assess prefrontal cortex dependent cognitive behaviours.

6.1.1 Aspects of cognition assessed by 5-CSRTT

The 5-CSRTT was originally developed to investigate attentional deficit hyperactivity disorder (ADHD) as an assessment of sustained attention in rodents (Carli *et al.*, 1983). This basic form of the 5-CSRTT parallels that of the continuous performance task (CPT) which is routinely used in the clinic to assess attentional deficits in humans with ADHD or schizophrenia (Rosvold *et al.*, 1956).

Although originally developed to quantify sustained attention, the 5-CSRTT simultaneously assesses multiple attributes of cognition including: sustained, divided and selective attention, impulse control, cognitive flexibility, motivation and processing speed (Robbins *et al.*, 2002). It is well known that many of these aspects of cognition measured by the 5-CSRTT are dysfunctional in schizophrenia, adding to the validity this task has in serving as a good pre-clinical assessment of schizophrenia-related cognitions following pharmacological, neural or genetic manipulations. As with the CPT task, the 5-CSRTT can be modified by altering inter-trial interval or stimulus duration to further challenge the sensory and attentional demand on the rodents.

6.1.2 Neural systems involved in 5-CSRTT performance

The neuroanatomical involvement of brain regions in 5-CSRTT performance has been thoroughly assessed (in rats) by lesion studies. The frontal cortical systems are of critical importance to 5-CSRTT performance with excitotoxic lesions of the mPFC producing significant deficits in performance of the task including increases in perseverative responses and response latencies simultaneous with a reduction in accuracy (Muir *et al.*, 1996). In addition, studies quantifying neuronal activity support the importance of cortical areas on 5-CSRTT performance, with increased [^{14}C]deoxyglucose uptake correlating with increased accuracy in regions of the forebrain (Barbelivien *et al.*, 2001). Furthermore, discrete areas of the prefrontal cortex have been shown to have distinct roles in 5-CSRTT performance, with lesions of the dorsal mPFC producing decreases in accuracy (Passetti *et al.*, 2002; Chudasama *et al.*, 2003), whereas lesions of more ventral regions of the mPFC, such as the prelimbic and orbitofrontal regions, result in increased perseverative responses (Chudasama & Muir, 2001; Passetti *et al.*, 2002). Concurrent with findings in human CPT performance (Cohen *et al.*, 1998), the hippocampus does not appear to have a major role in 5-CSRTT performance as bilateral lesions have little effect on performance (Kirkby & Higgins, 1998). The medial striatum however, is thought to play a very important role in 5-CSRTT performance with the same disruptions in performance observed in rats with bilateral lesions of either mPFC or striatum as in rats with a unilateral lesion of the mPFC in one hemisphere and a lesion of the medial striatum in the contra-lateral hemisphere (Christakou *et al.*, 2001).

Together these data suggest that an intact cortico-striatal system is necessary for task performance.

6.1.3 Neurotransmitter systems involved in 5-CSRTT performance

Pharmacological studies have allowed some elucidation of which neurotransmitter systems are involved in aspects of 5-CSRTT performance. Studies using the 5-CSRTT have shown involvement of several monoaminergic neurotransmitter systems. The regulation of the dopaminergic system is of great importance to 5-CSRTT performance, as dopamine depletion by 6-OHDA lesions of the mPFC result in decreased accuracy when the task parameters are manipulated to challenge the rats further (Robbins *et al.,* 1998). Moreover, D1 receptor activation in the PFC of rats increased accuracy only in rats performing sub-optimally (Granon *et al.*, 2000). As well as the dopaminergic system, the noradrenergic system is also vital in 5-CSRTT performance: the noradrenaline re-uptake inhibitor, atomoxetine, reduces premature responses in the 5-CSRTT in rats (Navarra *et al.,* 2008; Robinson *et al.,* 2008) paralleling the improvement in attention and impulsivity seen with noradrenaline re-uptake inhibitors in clinical studies in patients with ADHD (Chamberlain *et al.,* 2007). However clinical studies in patients with schizophrenia show little effect of atomoxetine on cognition (Kelly *et al.,* 2009) or add-on therapies with other noradrenaline re-uptake inhibitors (Poyurovsky *et al.*, 2010). However, as atomoxetine stimulates the overflow of both dopamine and noradrenaline in PFC it is unclear whether the procognitive effects are due to increases in dopamine or noradrenaline (Bymaster *et al.,* 2002).

Other drugs which are non-selective for their monoaminergic site of action also have effects on 5-CSRTT performance. For example methylphenidate is a non-selective dopamine/noradrenaline re-uptake inhibitor which has been previously shown to increase accuracy in the 5-CSRTT along with increasing premature responding (Paine *et al.,* 2007). Conversely, d-amphetamine, although it induces release of monoamines, has an inhibitory effect on 5-CSRTT performance, increasing premature and perseverative responses (Cole & Robbins, 1987; Paterson *et al.,* 2011). The multiple action site of this drug including, dopamine, 5-HT and noradrenaline make its actions hard to interpret. The

COMT inhibitor tolcapone, which has previously been shown to enhance performance in set-shifting thought to be by increased PFC dopamine levels (Tunbridge *et al.*, 2004) has no effect in 5-CSRTT performance (Paterson *et al.*, 2011).

Serotonergic systems also play a central role in 5-CSRTT and are thought to mediate key effects on premature responding (Robbins *et al.*, 2002). However, the importance of the PFC in mediating these effects is under enquiry as direct infusions of 5-HT receptor antagonists into the infra-limbic or pre-limbic cortex had no effect on impulsivity in 5-CSRTT, but infusion of a 5-HT2A antagonist into the nucleus accumbens reduced impulsivity (Robinson, *et al.*, 2008). This may explain why newer atypical antipsychotic treatments, which generally have affinity for 5-HT receptors as well as dopamine receptors, have had disappointing results in alleviating cognitive deficits in schizophrenia.

In line with the opinion that the high rate of smokers among schizophrenia patients is for self-medicating purposes, nicotine administration to rats has been shown to increase performance in the 5-CSRTT (Young *et al.*, 2004; Pattij *et al.*, 2007; Semenova *et al.*, 2007). However, many of these studies have shown that the subtle effect of nicotine on accuracy is only detectable after chronic/sub-chronic nicotine in sub-optimally performing rats or mice in 5-CSRTT paradigms where the task parameters were manipulated.

It is important to take into consideration the gross amount of interplay between these neurotransmitter types and also interplay between the brain regions that appear to be affected by these pharmacological agents. It is most likely that it is a strict control between the levels of all these neurotransmitters that lead to optimal performance in the 5-CSRTT.

6.1.4 The effects of genetic manipulation on 5-CSRTT performance

The majority of studies utilising the 5-CSRTT have been to characterise the involvement of neuronal networks in attentional functions or to serve as a translational pre-clinical test for drug functions. However, there have been some studies utilising the task to measure cognitive effects of gene manipulation allowing further dissection of the function of the gene product. For example homozygous knock-out of the serotonin transporter gene *Sert* in rats results in decreased premature responses (Homberg *et al.*, 2007). These findings support the findings of studies showing systemic application of 5-HT antagonists or 5-HT re-uptake inhibitors reduce premature responses (Robinson *et al.,* 2008).

To date there have been very limited studies employing the 5-CSRTT to investigate the roles of genes related to the cognitive deficits in schizophrenia. This is probably due to genetic manipulations being mainly carried out in mice, and although the 5-CSRTT is suitable for mice (albeit slightly modified from the rat version, Humby *et* al., 2005) the majority of groups routinely using 5-CSRTT assess rats only. However, with the recent commercialisation of genetically modified rats, this lack of studies will undoubtedly change within the near future. Interestingly, with respect to schizophrenia, transgenic mice lacking the α-7 nicotinic acetylcholine receptor show deficits in the 5-CSRTT task (Young *et al.*, 2004; Hoyle *et al.*, 2006; Young *et al.*, 2007). Furthermore, there has been a huge amount of interest in recent years in α-7 nicotinic acetylcholine receptor agonists as cognitive enhancers. For example, the drug DMXBA has shown some promising procognitive effects in the 5-CSRTT. However, when the drug was taken forward to clinical trials there have been problems with side-effects and desensitisation (see review by Thomsen *et al.,* 2010).

To my knowledge there have been no studies of 5-CSRTT performance in mice with genetic modifications of the most popular schizophrenia candidate genes such as *Disc1, Nrg1* and *Comt.* This is most likely due to the necessity of specific equipment to perform the task and also because the training and testing schedule is somewhat long compared to other behavioural tests.

Given the localisation of Nrg1 and Erbb4 in the prefrontal cortex, previous studies showing regulation of several neurotransmitter systems that are involved in optimal 5-CSRTT performance and evidence from schizophrenia patients identifying variants in these genes lead to impaired cognitive ability, it is reasonable to propose that knockdown of Erbb4 in the rat prefrontal cortex will elicit measurable changes in cognition as assessed by the 5-CSRTT.

6.2 Aims

The aim of this study was to test the hypothesis that viral mediated gene manipulation of Erbb4, specifically within the mPFC, would affect performance in the 5-CSRTT in adult rats, thereby determining if Erbb4 within this distinct brain region is important in cognitive functions known to be dysfunctional in schizophrenia. In addition, the effect of manipulating test parameters was investigated with the aim to explore if any changes in 5-CSRTT performance following Erbb4 knockdown were present in conditions of increased inhibitory or cognitive load.

6.3 Results

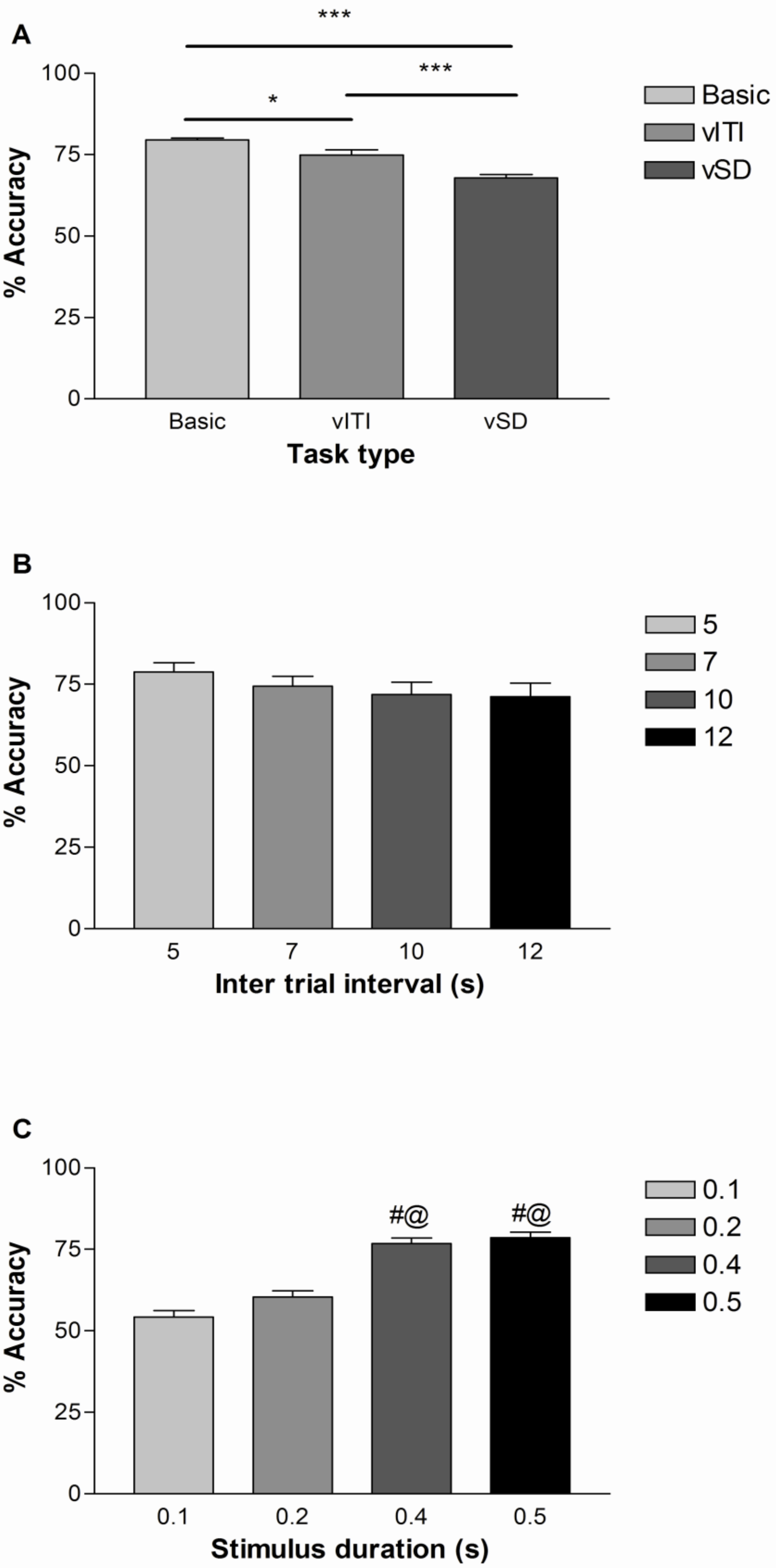
6.3.1 Baseline performance of rats in three variations of the 5-Choice Serial Reaction Time Task

Prior to surgery, the rats were habituated to the 5-CSRTT (section 2.7.2), then trained in the task under basic parameters until the rats perform at a criterion of at least 80% accuracy with less than 20% omissions at a stimulus duration and inter-trial interval of 0.6 seconds and 5 seconds, respectively (section 2.7.3). The baseline performance of the rats in three variations of the 5-CSRTT was assessed (n=35; one rat failed to meet criteria above training level 9 and so was discarded from the study). The performance of the rats was investigated in the basic task, the vITI task and the vSD task where attentional load is increased (section 2.7.4). Baseline performance in the 5-CSRTT was performed prior to surgery to ensure that when the rats were split into surgical groups, each group was performing to an equal level, therefore any post-surgical effects would be due to the agent injected during surgery. In addition, baseline testing was performed to confirm that manipulating the task parameters of the 5-CSRTT resulted in an alteration in the rats behaviour, reflective of the challenge in cognition.

Percentage accuracy in the basic task remained at a stable level of performance from the previous training level at 79.5% ±3.6%. As predicted, percentage accuracy was overall significantly affected by manipulating task parameters (*F* (2,104) =20.33, $p<0.001$). Tukey’s *post hoc* analysis revealed that percentage accuracy was significantly reduced in the vITI task ($p<0.05$) and the vSD task ($p<0.001$) compared to the basic task (Figure 6.1A). Further analysis of these tasks showed that individual inter-trial interval duration was not responsible for the drop in percentage accuracy in the vITI task (*F* (3,139)=0.99, $p>0.05$, Figure 6.1B) Conversely, the duration of stimulus was very significantly associated with a decrease in percentage accuracy observed in the vSD task (*F* (3,139)=42.25, $p<0.001$), with *post hoc* analysis showing that this reduction in percentage accuracy was mainly attributable to stimulus durations of 0.1 and 0.2 seconds (Figure 6.1C).

In addition to percentage accuracy, percentage omissions and the number of premature responses were significantly affected by altering task type ((*F* (2, 104) =25.22, $p<0.001$) and (*F* (2, 104) =391.44, $p<0.001$) respectively) (Figures 6.2A and 6.3A). Percentage omissions was essentially doubled in the vITI task compared to the basic task and vSD task types ($p<0.001$) where the level of omissions was very low at around 7%. More in depth analysis by individual Mann-Whitney tests of each ITI of the vITI manipulation confirmed this significant increase in omissions was a result of increasing inter-trial interval to 10 seconds or more (Figure 6.2B). The number of premature responses was also significantly increased with increasing inter-trial duration (Figure 6.3B).

Despite task type initiating predicted changes in other parameters measured in the 5-choice serial reaction time task, task type had no effect on latency to correct response (*F* (2,104)=0.07, $p>0.05$) or latency to collect reward (*F* (2,104)=2.65, $p>0.05$) (Figures 6.4A and 6.4B).


A
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Task type
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25
0
% Accuracy
5
7
10
12
Inter trial interval (s)
5
7
10
12
C
100
75
50
25
0
% Accuracy
#@
#@
0.1
0.2
0.4
0.5
Stimulus duration (s)
0.1
0.2
0.4
0.5

Figure 6.1 Effects of manipulation of stimulus duration and inter-trial interval duration upon percentage accuracy in the 5-CSRTT. Figure A illustrates the effects of task type on percentage accuracy in rats prior to surgery. vITI and vSD data represents cumulative percentage accuracy values of all ITI and SD durations. Varying ITI or stimulus duration decreased percentage accuracy. Figure B illustrates the effect of increasing inter-trial interval duration from 5 seconds to 7, 10 and 12 seconds on percentage accuracy. Increasing ITI duration did not alter percentage accuracy. Figure C illustrates the effect of decreasing stimulus duration from 0.5 seconds to 0.4, 0.2 and 0.1 seconds on percentage accuracy. As predicted, decreasing stimulus duration below 0.4 seconds decreased percentage accuracy. Data represent mean ± SE (n=35) analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. *p<0.05, ***p<0.001 between task types indicated. # p<0.001 vs. 0.1s SD, @p<0.001 vs. 0.2s SD. ITI= inter-trial interval, SD= stimulus duration

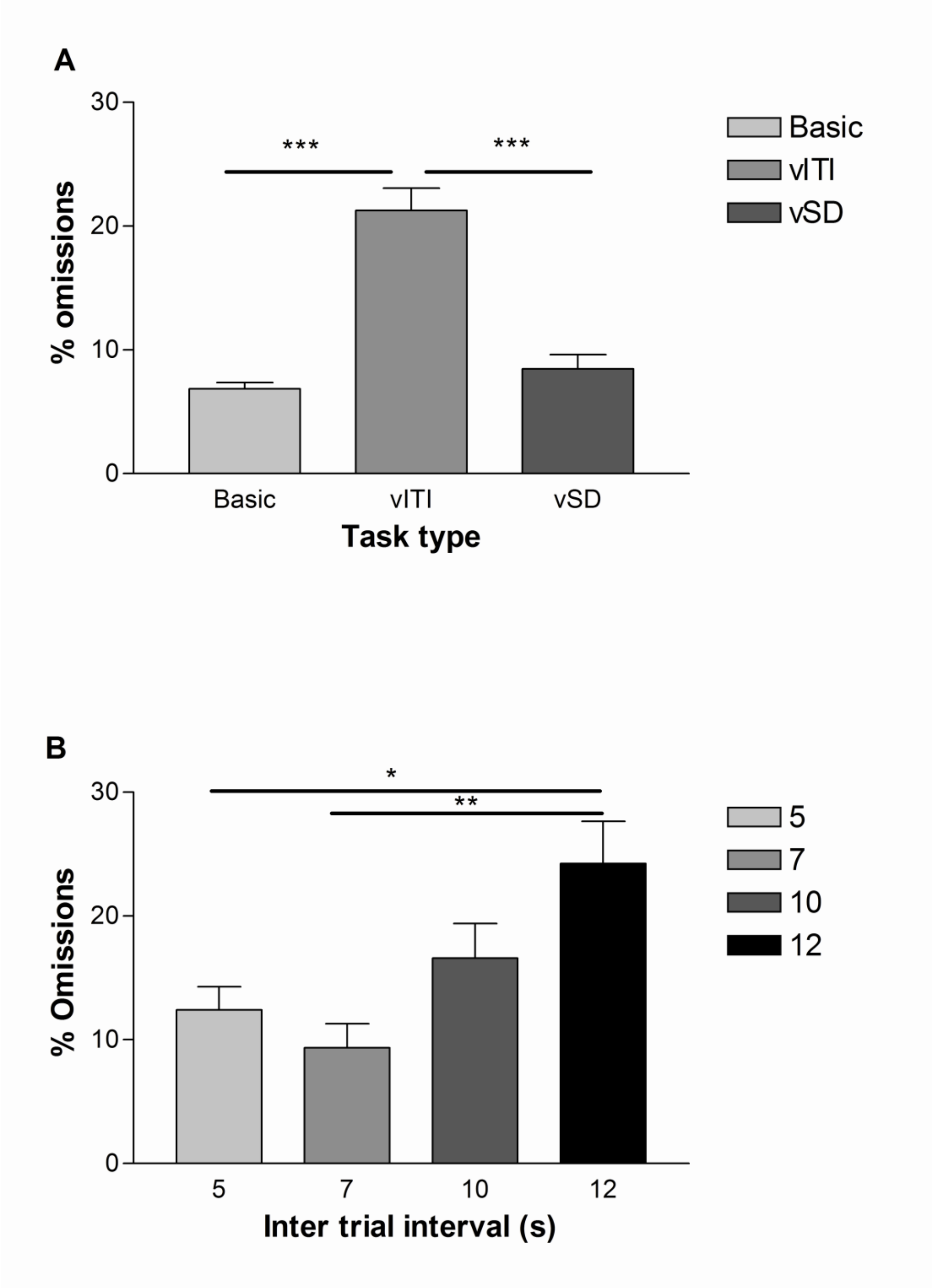


Figure 6.2 Effects of manipulation of stimulus duration and inter-trial interval duration upon percentage omissions in the 5-CSRTT. Figure A illustrates the effects of task type on percentage omissions in rats prior to surgery, as predicted varying ITI duration increased percentage omissions. Figure B illustrates the effect of increasing inter-trial interval duration from 5 seconds to 7, 10 and 12 seconds on percentage omissions, increasing ITI duration above 7seconds increased percentage omissions. Data represent mean ± SE (n=35). *** p<0.001 vs. Basic or vSD task types analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. * p<0.05 vs. 5s ITI **p<0.01 vs. 7s ITI analysed using Mann-Whitney non-parametric U tests. vITI=variable inter-trial interval, vSD=variable stimulus duration.

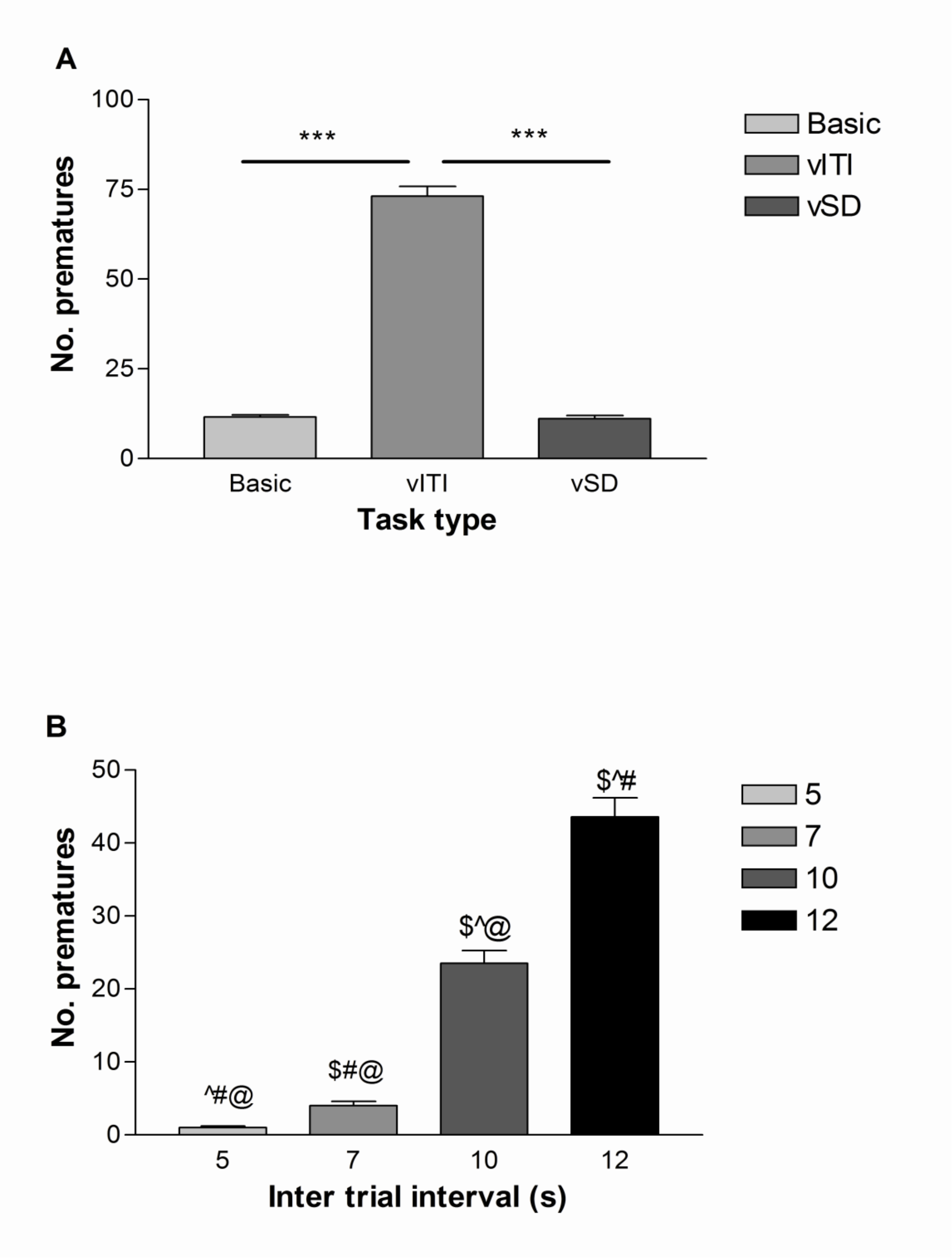


Figure 6.3 Effects of manipulation of stimulus duration and inter-trial interval duration upon the number of premature responses in the 5-CSRTT. Figure A illustrates the effects of task type on premature responding in rats prior to surgery. As predicted varying ITI duration increased premature responding. Figure B illustrates the effect of increasing inter-trial interval duration from 5 seconds to 7, 10 and 12 seconds on the number of premature responses. The number of premature responses increased with longer ITI durations. Data represent mean ± SE (n=35). *** p<0.001 vs. Basic or vSD task types analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. $ p<0.001 vs. 5s ITI, ^ p<0.001 vs. 7s ITI, # p<0.001 vs. 10s ITI, @ p<0.001 vs. 12s ITI using Mann-Whitney non-parametric U tests. vITI=variable inter-trial interval, vSD=variable stimulus duration

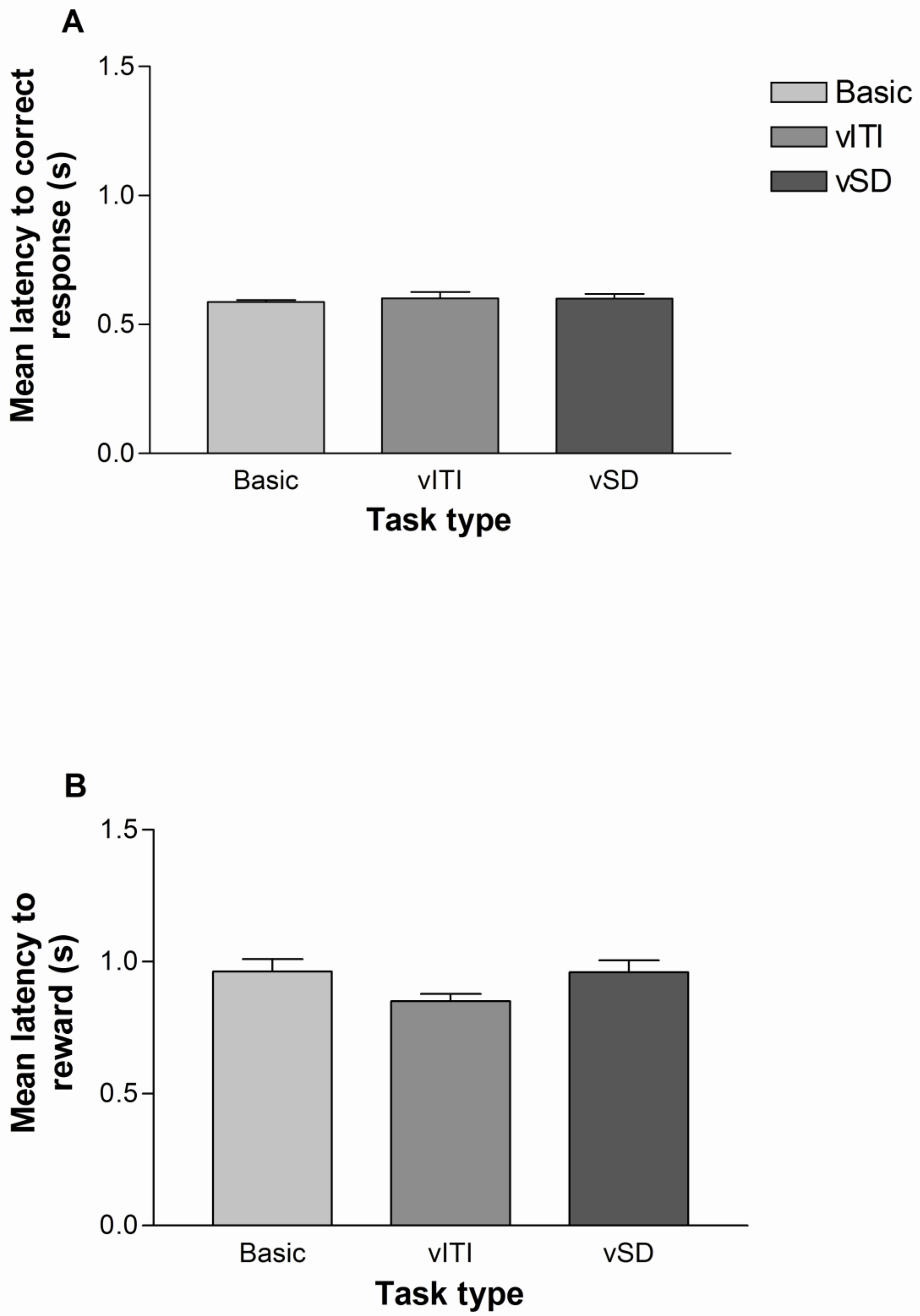


Figure 6.4 Effects of manipulation of stimulus duration and inter-trial interval duration upon latencies in the 5-CSRTT. Figure A illustrates the effects of task type on latency to correct response in rats prior to surgery. Varying ITI or SD did not affect the latency to correct response. Figure B illustrates the effects of task type on latency to collect reward following correct response in rats prior to surgery. Varying ITI or SD did not affect the latency to collect reward. Data represent mean ± SE (n=35).

6.3.2 Post-surgical testing in the basic 5-CSRTT performance

Based on baseline performance in the basic, vITI and vSD task the rats were split into three equally performing groups (section 2.7.4). Stereotaxic surgery was then carried out on the rats, with bilateral injections of either vehicle, rAAV encoding a scrambled hairpin sequence (scr.rAAV) or rAAV encoding a short hairpin sequence corresponding to rat *Erbb4* into the rat mPFC (section2.4.2). One, three and five weeks post-surgery the rats' performance in the basic 5-CSRTT was assessed (section 2.7.5). Five variables were analysed: percentage accuracy, percentage omissions, the number of premature responses, latency to collect reward and latency to make a correct response. Two rats died during surgery reducing group sizes to vehicle n=10, scr.rAAV n=12, shErbb4.rAAV n=11.

In the basic task the main effect of Erbb4 knockdown was apparent at 5 weeks post-surgery where rats with Erbb4 knockdown within the PFC performed at a higher percentage accuracy that scr.rAAV treated rats ($p<0.05$)(Figure 6.5). Importantly, percentage accuracy was equal between surgical groups at baseline level (pre-surgery) (F (2, 32) =0.03, $p>0.05$) (Figure 6.5A) permitting the interpretation that any changes in this parameter after surgery were due to the type of agent injected. Surgical group had a significant effect on percentage accuracy overall (F (2, 131) =4.26), $p<0.05$). Following a two-way ANOVA the *post hoc* Tukey's analysis indicated a significant difference in the performance of scr.rAAV treated rats and shErbb4.rAAV treated rats (p=0.01).

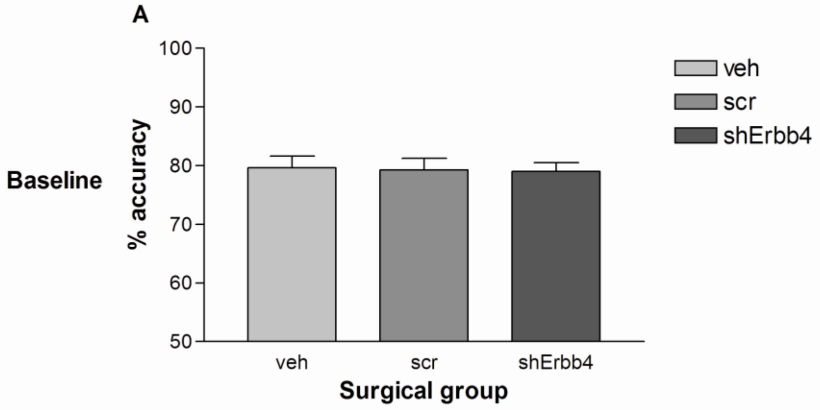
There was also a significant effect of week on percentage accuracy (F (3,131) =3.21, $p<0.05$) with percentage accuracy dropping slightly at 1 week post-surgery compared to the baseline performance (p=0.066) and being significantly higher at week 5 compared to week 1 ($p<0.05$).

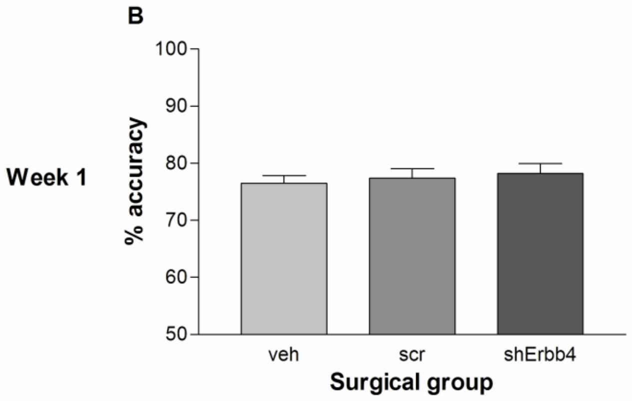
Subsequent to this analysis the percentage accuracy relative to the rats' baseline performance was carried out to analyse whether the change in percentage accuracy post-surgery was significantly different between surgical groups. Similar to the previous analysis, surgical group had a significant effect on relative percentage accuracy (F (2, 98) =9.23), $p<0.001$) this time with the performance of rats that had received virus expressing shErbb4 being significantly different from scrambled hairpin virus treated rats ($p<0.001$) and also vehicle treated rats ($p<0.01$) Furthermore, *post hoc* comparisons strengthened the previous finding at 5 weeks post-surgery with rats with Erbb4 knockdown within the PFC performing the basic task with higher accuracy than scrambled virus treated rats ($p=0.0025$) (Figure 6.6).

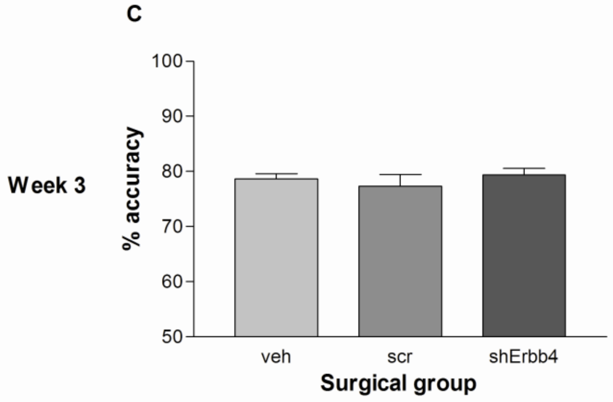
As with percentage accuracy, in the basic task percentage omissions, number of premature responses, latency to reward and latency to correct response were also equal between groups at baseline (Figures 6.7A, 6.8A, 6.9A and 6.10A). Compared to baseline, percentage omissions were significantly increased in all surgical groups at one week post-surgery ($p<0.001$). This level of omissions returned back to baseline level, with omissions at weeks 3 and 5 not being significantly different from baseline but significantly lower than at week 1. This pattern is reflected by a significant effect of week on percentage omissions (F (3, 131) =10.58, $p<0.001$). In addition, surgical group had a significant effect on percentage omissions (F (2, 131) =8.96, $p<0.001$) with rats treated with scr.rAAV performing significantly more omissions over all weeks than vehicle treated rats ($p<0.01$) and rats with Erbb4 knockdown ($p<0.001$). These significant differences between surgical groups are reflected by a significant interaction between surgical group and week at 3 weeks where scr.rAAV treated rats performed significantly more omissions that shErbb4.rAAV treated rats. At 5 weeks this effect was no longer apparent but scr.rAAV treated rats performed significantly more omissions than vehicle treated rats at this time point.

Akin to percentage omissions and percentage accuracy, surgical group did have a significant effect on the number of premature responses (F (2, 131) =3.52, $p<0.05$) with shErbb4 virus treated rats performing a significantly different amount of premature responses over all weeks than vehicle treated rats ($p<0.05$). Again there was also a significant effect of week on this parameter (F (3, 131) =3.90, $p=0.01$) with week three approaching significance compared to week one ($p=0.059$). This effect was a consequence of vehicle treated rats performing significantly fewer premature responses than scrambled hairpin virus-treated rats at week 3 ($p<0.05$) (Figure 6.8).

Latency to reward was significantly affected by surgical group (F (2, 131) =3.58, $p<0.05$), with rats with Erbb4 knockdown retrieving rewards faster than vehicle treated rats. This effect was a general observation with no significant effect of week (*F* (3. 131) =2.27, p>0.05) or surgical group x week interactions (Figure 6.9). Analysis of latency to correct response showed some results paralleling these of latency to reward, with there being no significant effect of week (*F* (2, 131) =1.18, p>0.05) or surgical group x week interactions. There was however a significant effect of surgical group on this parameter ((*F* (2, 131) =7.81, p<0.001) with scr.rAAV treated rats being slower to make a correct response than the vehicle treated rats (p<0.001) (Figure 6.10).


A
Baseline
% accuracy
100
90
80
70
60
50
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4


B
Week 1
% accuracy
100
90
80
70
60
50
veh
scr
shErbb4
Surgical group


C
Week 3
% accuracy
100
90
80
70
60
50
veh
scr
shErbb4
Surgical group

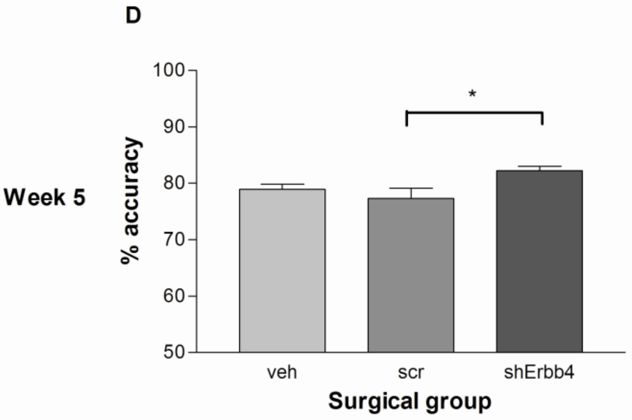

D
Week 5
% accuracy
100
90
80
70
60
50
*
veh
scr
shErbb4
Surgical group

Figure 6.5 Effect of viral mediated gene manipulation on percentage accuracy in basic 5-CSRTT. Rats were split into three surgical groups based on baseline percentage accuracy performance (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage accuracy was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE (n=10-12). * $p<0.05$ analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed main effects of surgical group on percentage accuracy ($p<0.05$) with shErbb4.rAAV treated rats performing over all significantly higher percentage accuracy than scr.rAAV treated rats ($p<0.01$). A main effect of week was also found ($p<0.05$) with percentage accuracy being significantly lower at 1 week post-surgery than 5 weeks post-surgery ($p<0.05$). *Post hoc* analysis revealed that at 5 weeks post-surgery shErbb4.rAAV treated rats performed more accurately than scr.rAAV treated rats ($p<0.05$).

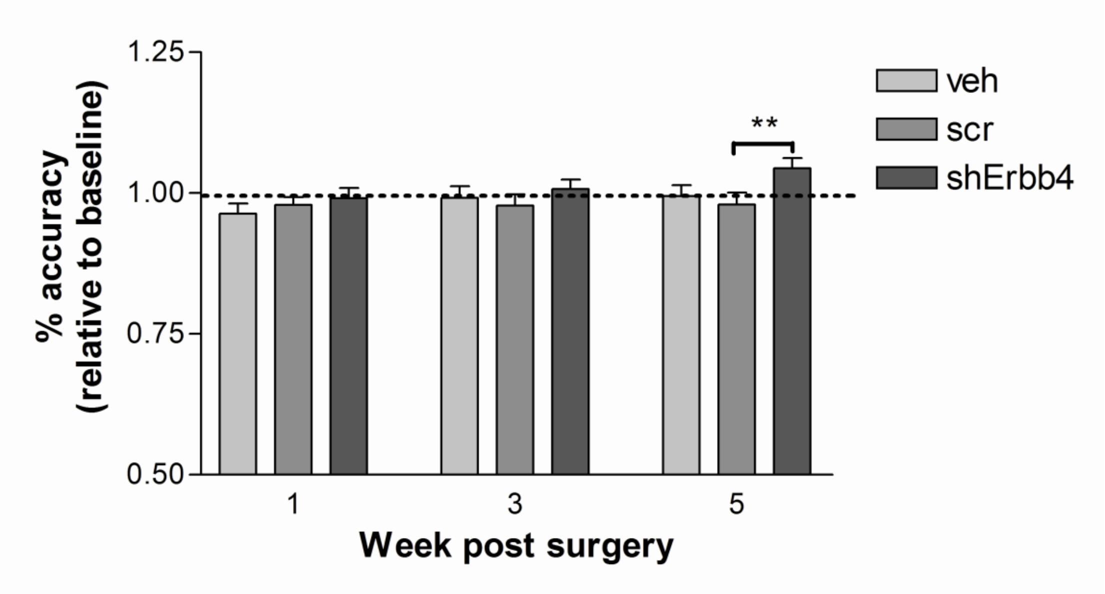


Figure 6.6 Effect of viral mediated gene manipulation on percentage accuracy relative to performance at baseline in basic 5-CSRTT. percentage accuracy was determined relative to baseline performance at 1, 3 and 5 weeks following bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. ** p<0.01 between surgical groups analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way repeated measures ANOVA found main effect of surgical group on percentage accuracy with shErbb4.rAAV treated rats performing more accurately than scr.rAAV (p<0.001) and vehicle treated rats (p<0.01). *Post* hoc comparisons revealed at 5 weeks post-surgery shErbb4.rAAV treated rats performed with increased percentage accuracy than scr.rAAV treated rats (p<0.01).

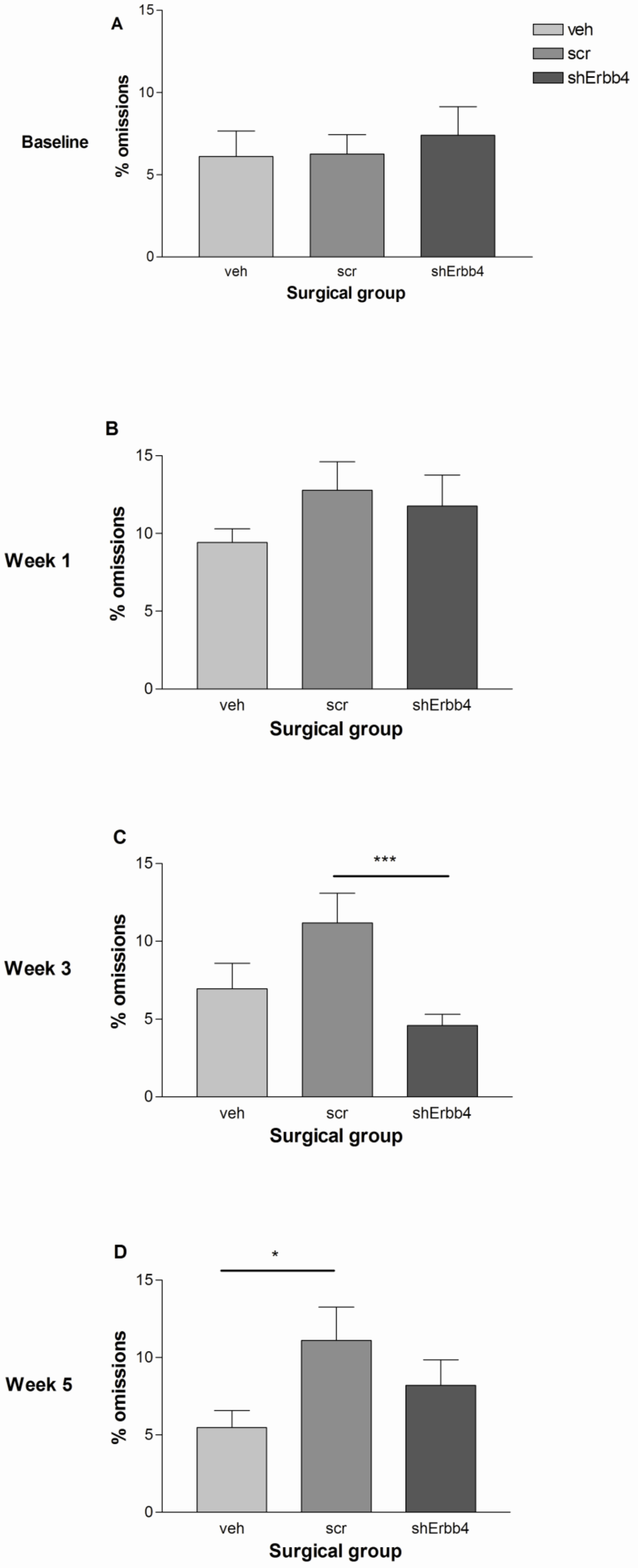

A
Baseline
veh
scr
shErbb4
% omissions
15
10
5
0
veh
scr
shErbb4
Surgical group
B
Week 1
% omissions
15
10
5
0
veh
scr
shErbb4
Surgical group
C
Week 3
***
% omissions
15
10
5
0
veh
scr
shErbb4
Surgical group
D
Week 5
*
% omissions
15
10
5
0
veh
scr
shErbb4
Surgical group

Figure 6.7 Effect of viral mediated gene manipulation on percentage omissions in basic 5-CSRTT. Rats were split into three surgical groups based on baseline percentage omissions (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage omissions were assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE (n=10-12). * $p<0.05$, ***$p<0.001$ analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed an over all effect of surgical group on percentage omissions with rats treated with scr.rAAV performing significantly more omissions than vehicle ($p<0.01$) or shErbb4.rAAV ($p<0.001$) treated rats. There was also an over all effect of week on percentage omissions with significantly higher omissions performed at 1 week post-surgery compared to all other time points. *Post hoc* comparisons revealed scr.rAAV treated rats performed significantly more omissions than shErbb4.rAAV treated rats at 3 weeks post-surgery ($p<0.001$), and vehicle treated rats at 5 weeks post-surgery ($p<0.05$).

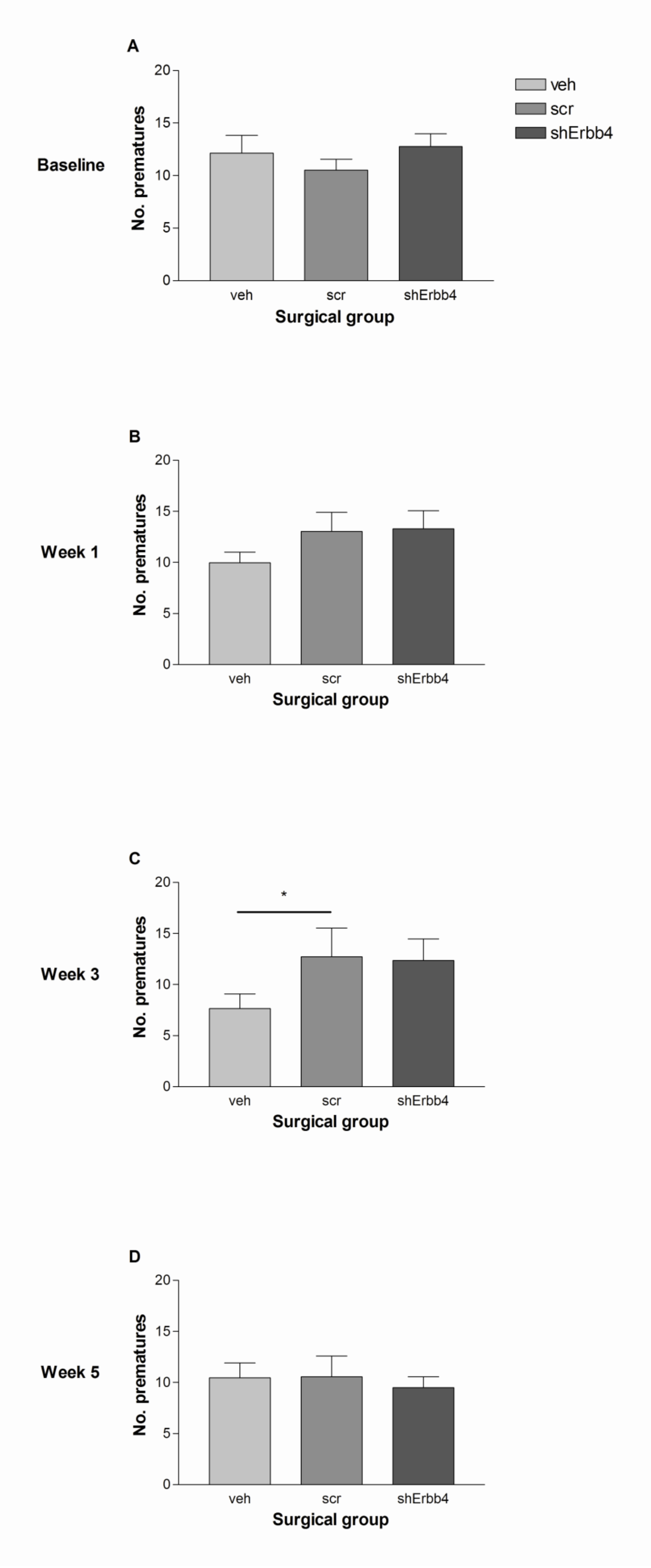

A
Baseline
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
▭ veh
▭ scr
■ shErbb4
B
Week 1
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
C
Week 3
*
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
D
Week 5
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group

Figure 6.8 Effect of viral-mediated gene manipulation on the number of premature responses in the basic 5-CSRTT. Rats were split into three surgical groups based on baseline premature responding (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. The number of premature responses was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE (n=10-12). * $p<0.05$, analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed an over all effect of surgical group on premature responding with shErbb4.rAAV treated rats performing significantly more premature responses than vehicle treated rats overall ($p<0.05$). *Post hoc* comparisons revealed that scr.rAAV treated rats performed more premature responses than vehicle treated rats at 3 weeks post-surgery ($p<0.05$).

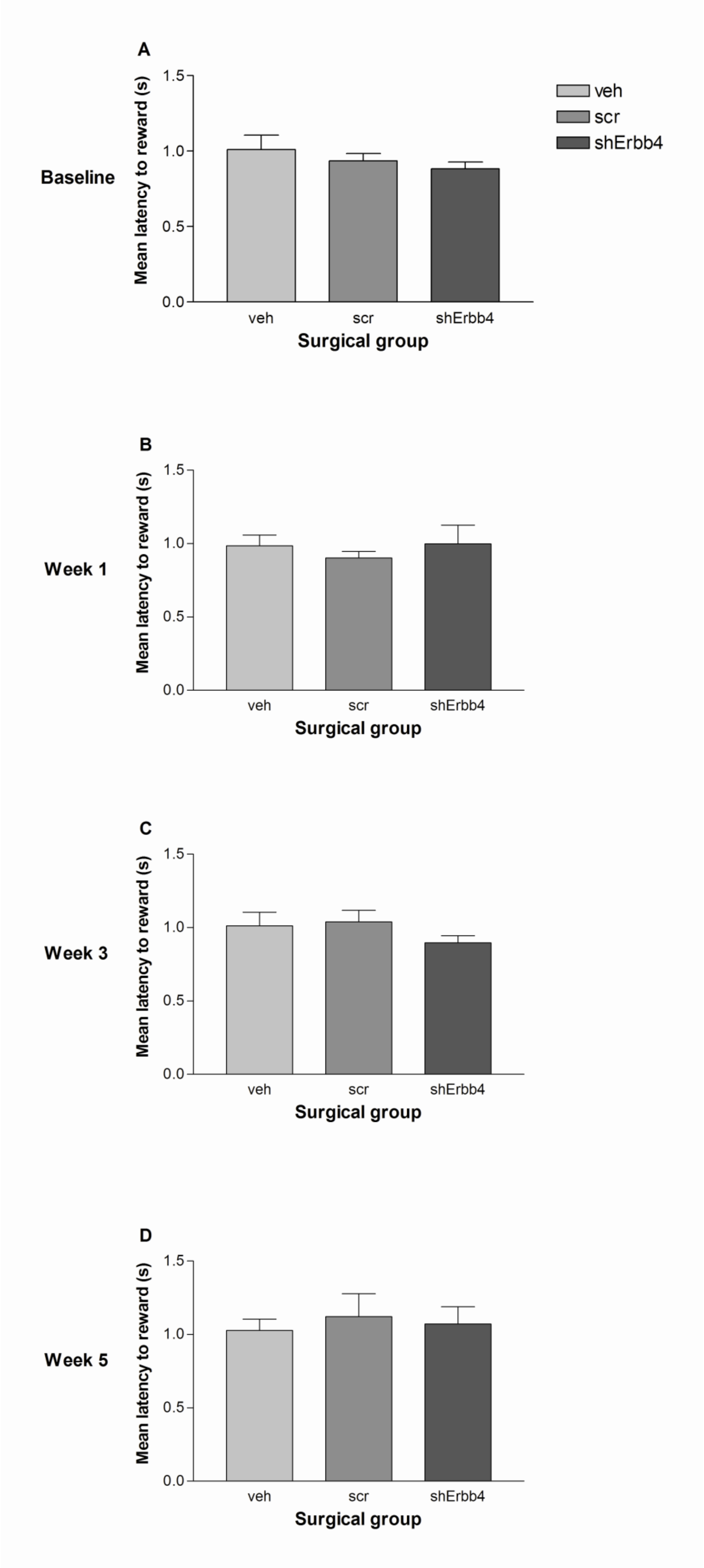

A
Baseline
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4
B
Week 1
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
C
Week 3
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
D
Week 5
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group

Figure 6.9 Effect of viral-mediated gene manipulation on latency to collect reward in the basic 5-CSRTT. Rats were split into three surgical groups based on baseline reward latency (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Latency to reward was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE (n=10-12). Data were analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed an over all effect of surgical group on reward latency with shErbb4.rAAV treated rats having significantly lower latencies to collect reward than vehicle treated rats ($p<0.05$).

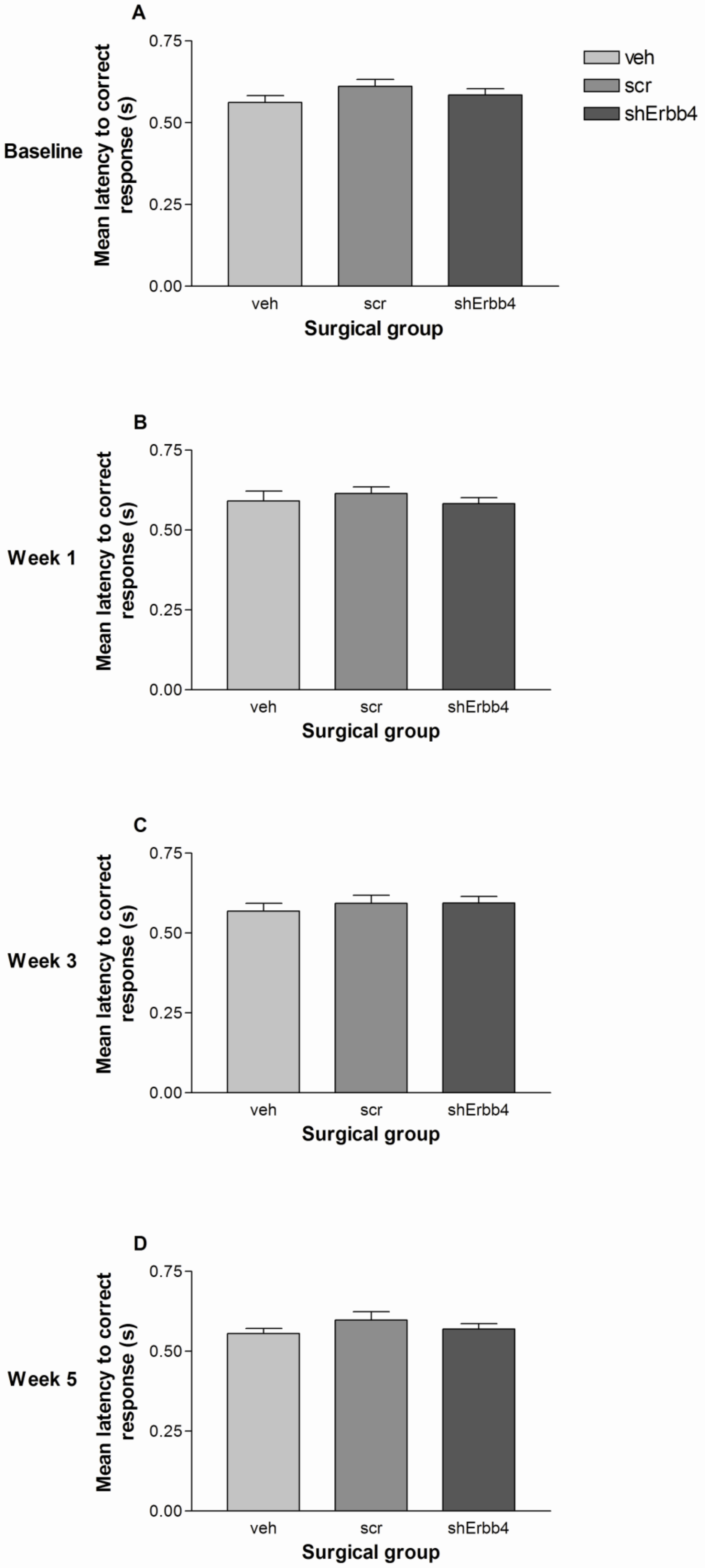

A
Baseline
B
Week 1
C
Week 3
D
Week 5
Mean latency to correct response (s)
0.00
0.25
0.50
0.75
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4

Figure 6.10 Effect of viral-mediated gene manipulation on latency to correct response in the basic 5-CSRTT. Rats were split into three surgical groups based on baseline response latency (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Latency to correct response was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE (n=10-12). Data were analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test multiple comparisons. Two-way ANOVA showed a significant effect of surgical group on latency to response with scr.rAAV treated rats having an overall significantly higher latency to make a correct response than vehicle treated rats ($p<0.001$).

6.3.3 Effect of Erbb4 knockdown on performance in vITI task

To assess if viral-mediated gene knockdown of Erbb4 within the mPFC affected the performance of rats in a manipulated form of the 5-CSRTT, where the inter-trial interval is made variable, rats who had undergone stereotaxic injection of vehicle, scr.rAAV or shErbb4.rAAV (2.4.2) were tested in the vITI version of the 5-CSRTT 1, 3 and 5 weeks post-surgery (2.7.5).

Rats in all three surgical groups performed at equal percentage accuracy in the vITI task at baseline (*F* (2, 32) =0.05, p>0.05) (Figure 6.11A) and this level of accuracy did not change significantly between groups at any time point post-surgery with no significant effect of surgical group (*F* (2, 131) =0.43, p>0.05) or week (*F* (3,131) =0.51, p>0.05) on percentage accuracy (Figure 6.11 B-D). Similarly, there was no effect of surgical group (*F* (3, 131) =2.66, p>0.05) or week (*F* (3, 131) =0.16, p>0.05) on percentage omissions in the vITI task (Figure 6.12). Number of premature responses in the vITI task was not affected by surgical group (*F* (2, 131)=1.33, p>0.05), however, there was a highly significant effect of week on premature responding (*F* (3, 131)=5.39,p<0.01) with *post hoc* comparisons indicating this effect was a result of significantly less premature responses being performed at 3 weeks (p<0.01) and 5 weeks (p<0.01) post-surgery compared to the number of premature responses made prior to surgery (Figure 6.13).

Comparable to the basic task, mean latency to reward was significantly affected by surgical group in the vITI task (*F* (2,131) =14.63, p<0.001) again with shErbb4.rAAV treated rats being overall significantly quicker to collect reward following a correct response than vehicle treated rats (p<0.001) (Figure 6.14). In addition, in the vITI task scr.rAAV treated rats also had a significantly lower mean latency to reward than vehicle treated rats (p<0.001). These effects were a general observation with no significant interaction between surgical group and week being identified. Furthermore, there was no significant effect of week on mean latency to reward (*F* (3, 131) =0.75,>0.05) (Figure 6.14). Mean latency to correct response was not significantly affected by surgical group (*F* (2,131) =0.19, p>0.05) or week (*F* (3,131) =0.71, 0>0.05) in the vITI task (Figure 6.15).

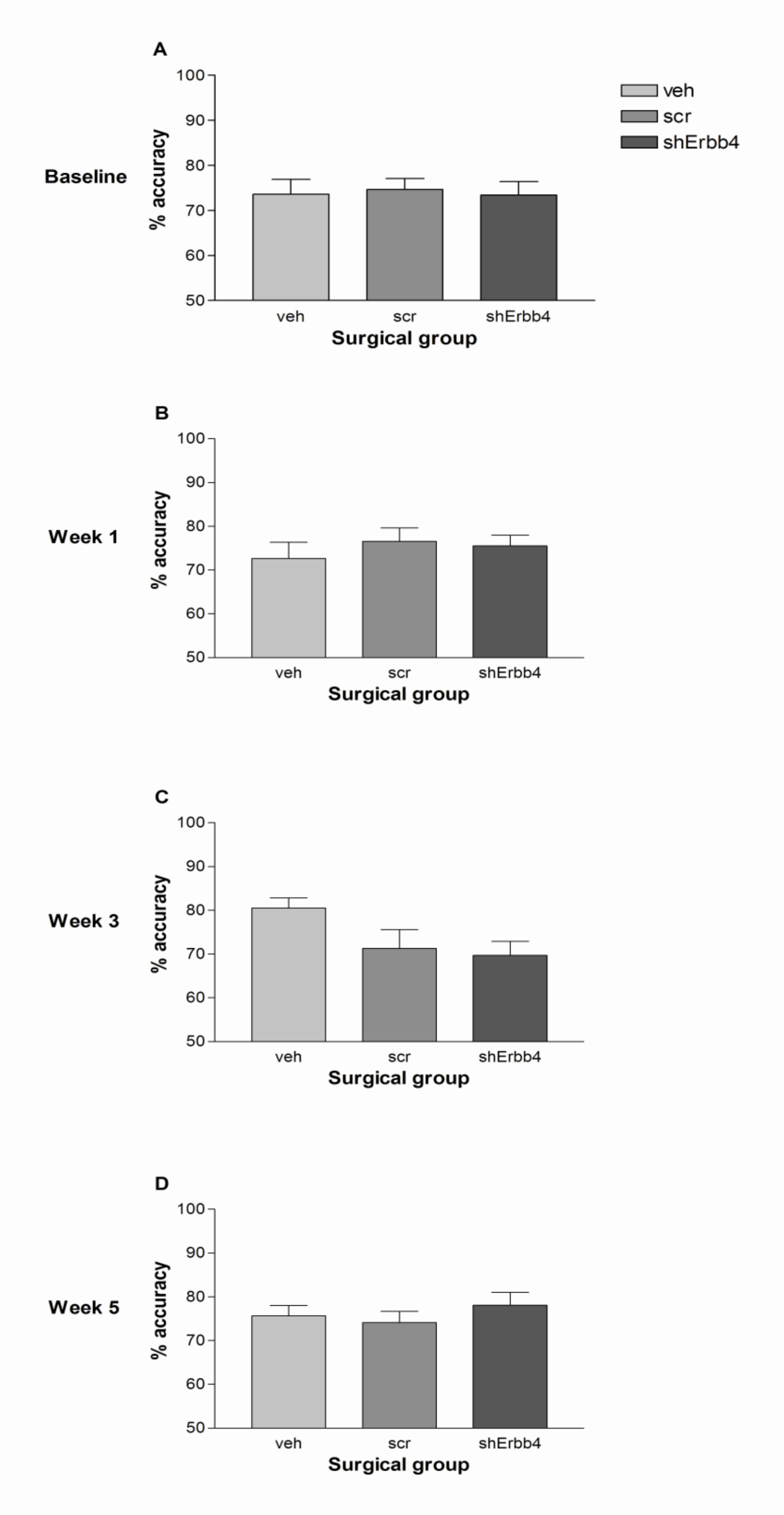


Figure 6.11 Effect of viral-mediated gene manipulation on percentage accuracy in the vITI task. Rats were split into three surgical groups based on baseline percentage accuracy (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage accuracy was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total percentage accuracy from trials with inter-trial intervals of 5, 7, 10 and 12 seconds (n=10-12). Two-way ANOVA showed there was no effect of surgical group or week on percentage accuracy.

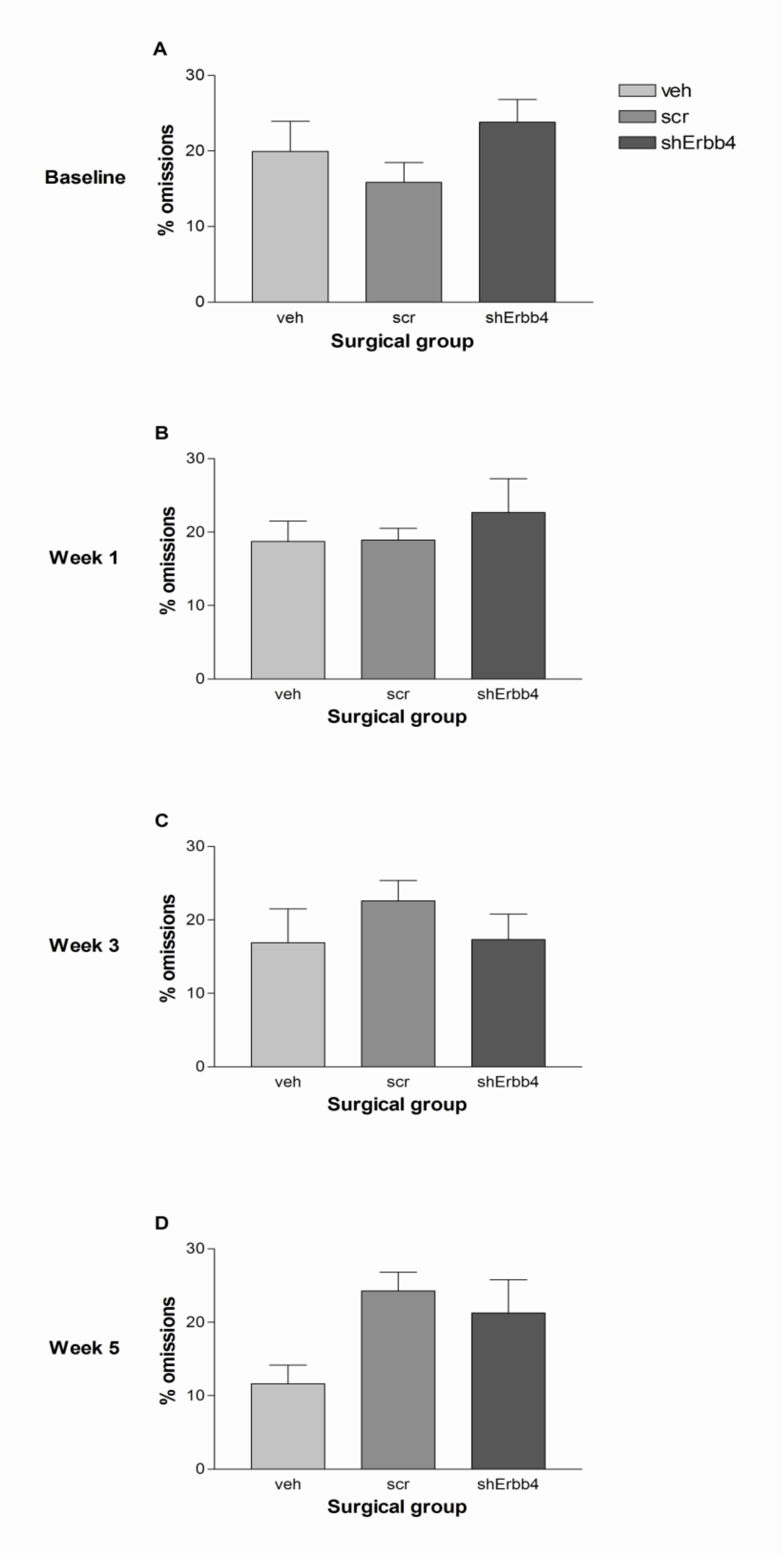


Figure 6.12 Effect of viral-mediated gene manipulation on percentage omissions in the vITI task. Rats were split into three surgical groups based on baseline percentage omissions (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage omissions were assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total percentage omissions from trials with inter-trial intervals of 5, 7, 10 and 12 seconds (n=10-12). Two-way ANOVA showed there was no significant effect of surgical type or week on percentage omissions.

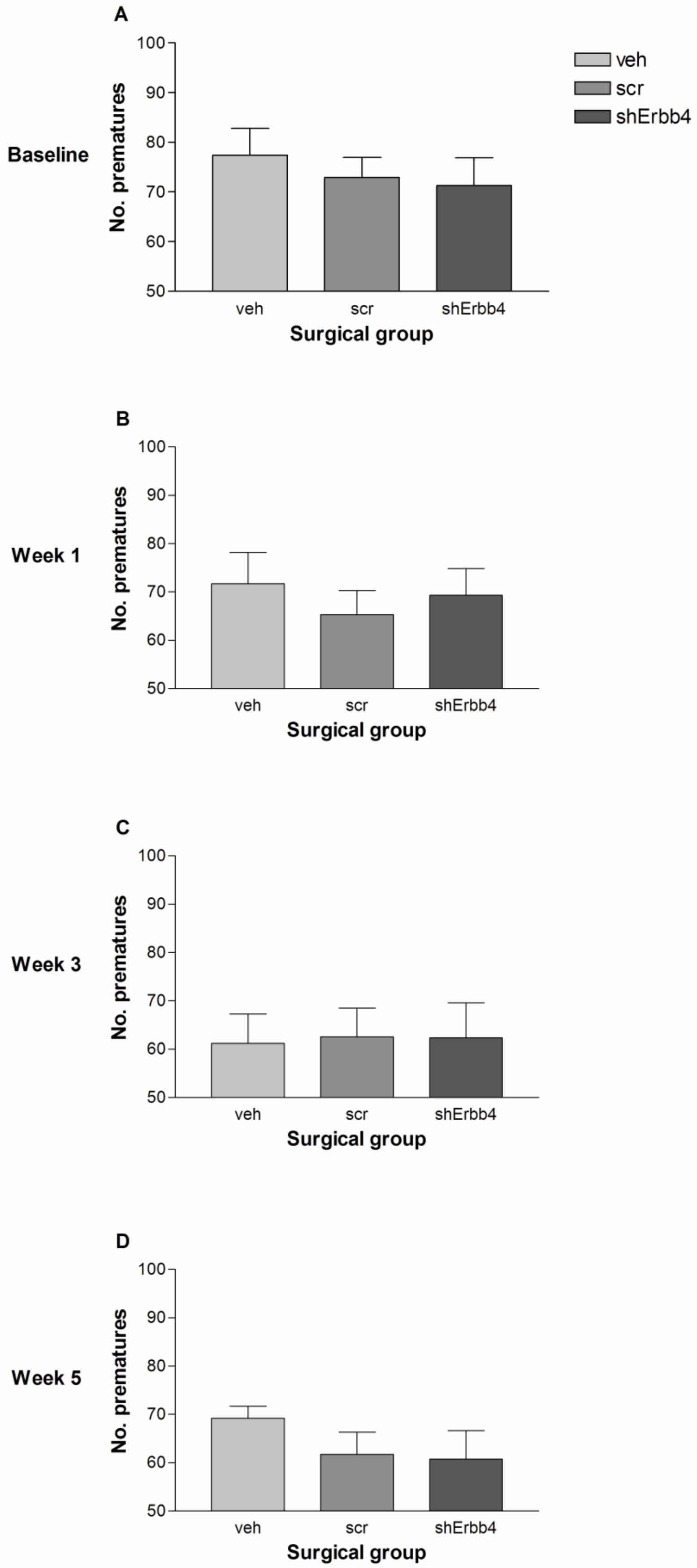

A
Baseline
B
Week 1
C
Week 3
D
Week 5
No. prematures
100
90
80
70
60
50
veh
scr
shErbb4
Surgical group

Figure 6.13 Effect of viral-mediated gene manipulation on the number of premature responses in the vITI task. Rats were split into three surgical groups based on baseline premature responding (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. The number of premature responses were assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total number of premature responses from trials with inter-trial intervals of 5, 7, 10 and 12 seconds (n=10-12). Two-way ANOVA showed the number of premature responses was not affected by surgical group, however, there was an overall effect of week on this parameter with more premature responses being made over all surgical groups at baseline compared to 3 ($p<0.01$) and 5 ($p<0.01$) weeks post-surgery

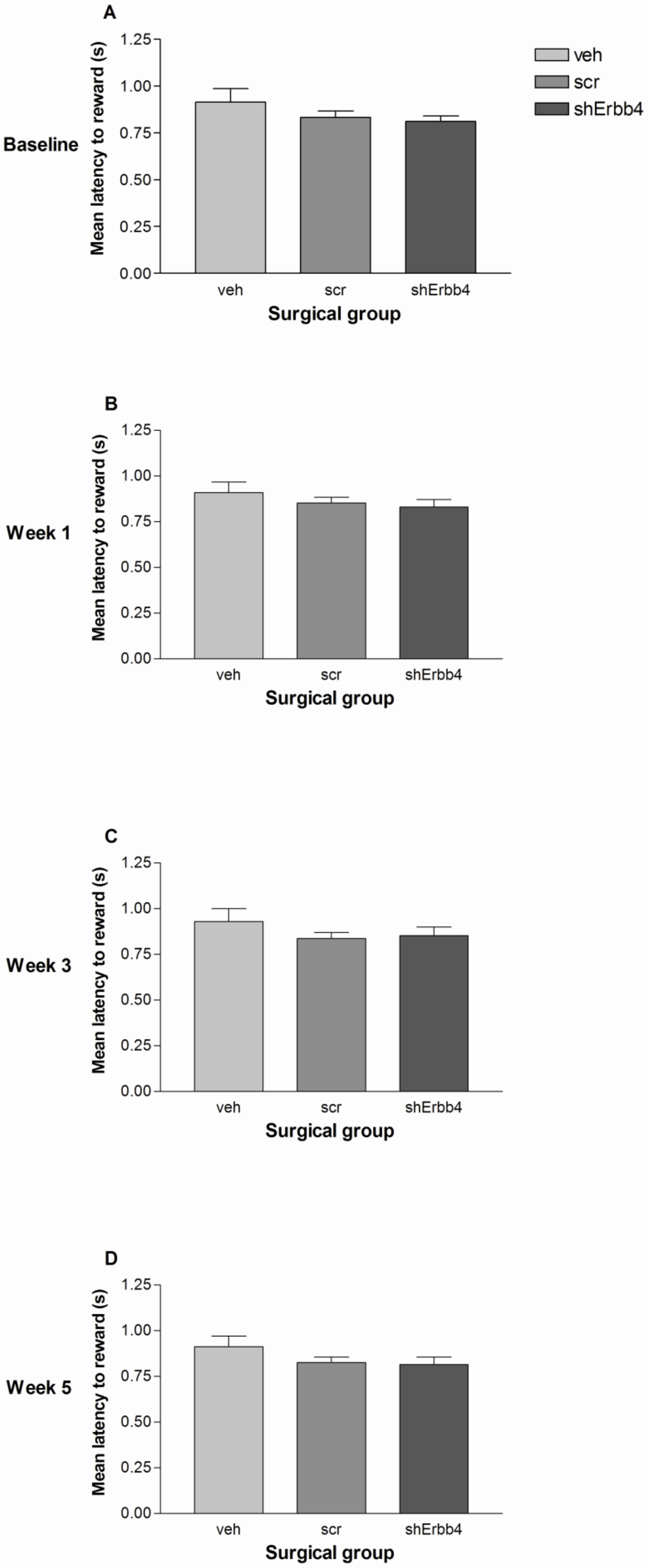

A
Baseline
Mean latency to reward (s)
1.25
1.00
0.75
0.50
0.25
0.00
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4
B
Week 1
Mean latency to reward (s)
1.25
1.00
0.75
0.50
0.25
0.00
veh
scr
shErbb4
Surgical group
C
Week 3
Mean latency to reward (s)
1.25
1.00
0.75
0.50
0.25
0.00
veh
scr
shErbb4
Surgical group
D
Week 5
Mean latency to reward (s)
1.25
1.00
0.75
0.50
0.25
0.00
veh
scr
shErbb4
Surgical group

Figure 6.14 Effect of viral-mediated gene manipulation on latency to reward in the vITI task. Rats were split into three surgical groups based on baseline latency to reward (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Mean reward latency was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total mean reward latency from trials with inter-trial intervals of 5, 7, 10 and 12 seconds (n=10-12). Data were analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed an overall effect of surgical group on latency to reward with vehicle treated rats having an overall significantly higher latency to collect reward than scr.rAAV ($p<0.001$) and shErbb4.rAAV treated rats ($p<0.001$).

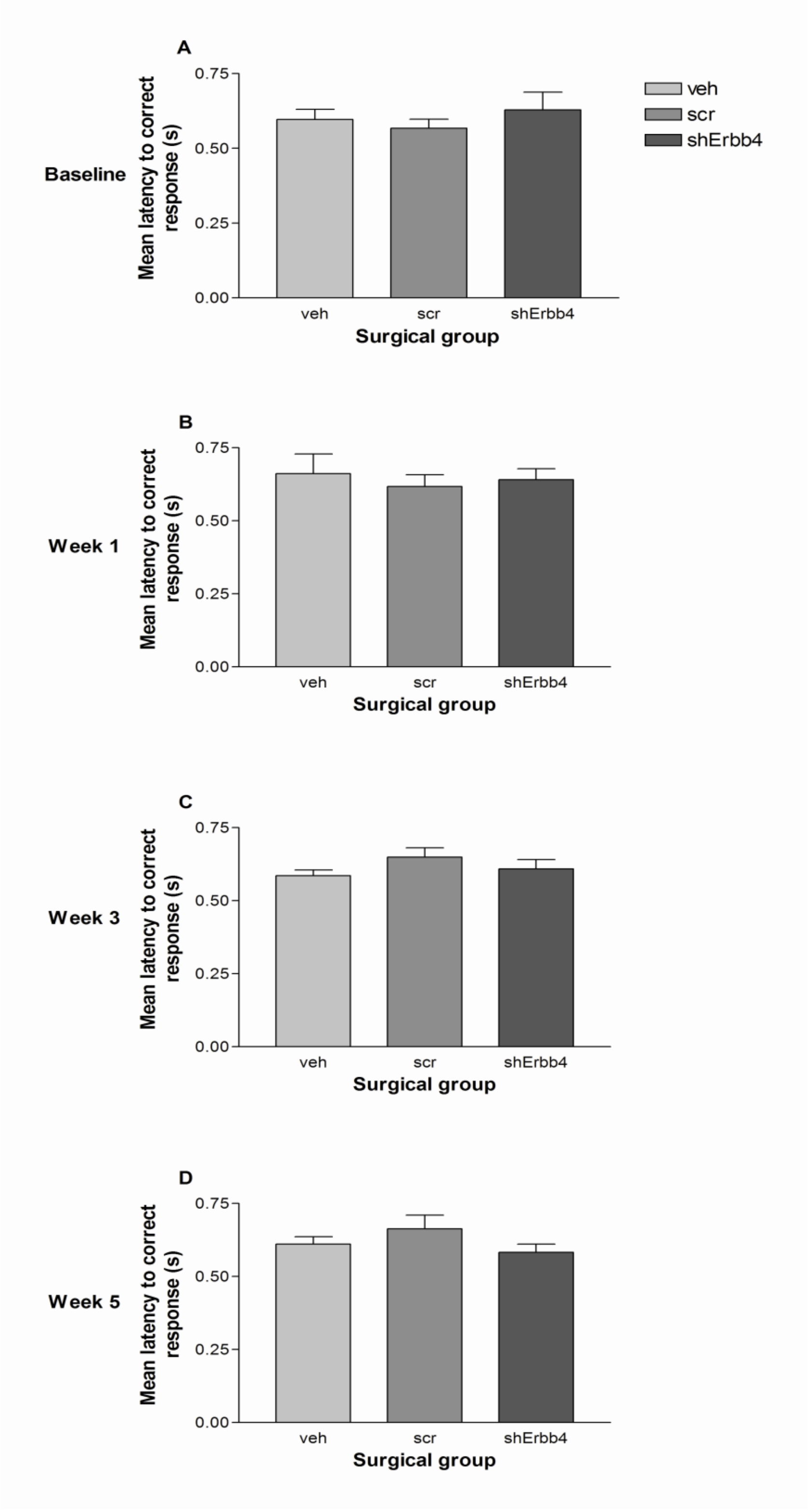


Figure 6.15 Effect of viral-mediated gene manipulation on latency to correct response in the vITI task. Rats were split into three surgical groups based on baseline response latency (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Mean latency to correct response was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total mean response latency from trials with inter-trial intervals of 5, 7, 10 and 12 seconds (n=10-12). Two-way ANOVA showed there was no effect of surgical group or week on latency to correct response.

6.3.4 Effect of Erbb4 knockdown on performance in the vSD task

To assess if viral-mediated gene knockdown of Erbb4 within the mPFC affected the performance of rats in a manipulated form of the 5-CSRTT, where the stimulus duration is made variable, rats who had undergone stereotaxic injection of vehicle, scr.rAAV or shErbb4.rAAV (2.4.2) were tested in the vSD version of the 5-CSRTT 1, 3 and 5 weeks post-surgery (2.7.5).

At baseline each group of rats were performing at an approximately equal level of 67% accuracy in the vSD task (Figure 6.16A). Two-way repeated measures ANOVA revealed there was no significant effect of surgical group (*F* (2, 131) = 0.71, p>0.05) or week (*F* (3, 131) = 1.95, p>0.05) on percentage accuracy in the vSD task. Convergent with baseline data, this level of percentage accuracy achieved in the vSD task was mainly due to poor performance at stimulus durations of 0.1 and 0.2 seconds at all time points where three way repeated measures ANOVA showed that although there was no significant effect of treatment or week on percentage accuracy, stimulus duration was highly significant (*F* (3,527) = 200.89, p<0.001).

There was an overall significant effect of surgical group on percentage omissions (*F* (2,131) = 5.63, p<0.01) in the vSD task with vehicle treated rats performing significantly differently from scrambled hairpin virus treated rats (p<0.01). There were no significant week by surgical group interaction however, there was a significant effect of week on percentage omissions (*F* (3, 131) = 5.19, p<0.01). *Post hoc* comparisons revealed this was due to more percentage omissions occurring in week three than at baseline (p<0.001) (Figure 6.17).

Similar to premature responding in the vITI task, the number of premature responses was significantly lower at week 3 (p<0.01) and week 5 (p<0.01) compared to baseline levels in the vSD task. This contributed to “week” having a statistically significant effect on the number of premature responses (*F* (3,131) =5.99, p<0.001). Surgical group had no effect on the number of premature responses performed (Figure 6.18) in the vSD task. Surgical group did however have an effect on mean latency to reward (*F* (2,131)=5.86, p<0.01) with *post hoc* analysis identifying that, identically to the results of the vITI task, vehicle treated rats were significantly slower to collect rewards after making a correct response compared to scr.rAAV ($p<0.05$) and shErbb4.rAAV ($p<0.01$) treated rats. There were no significant surgical group by week interactions and week itself did not have a significant effect on mean latency to reward in the vSD task (*F* (3,131) =1.09, $p>0.05$) (Figure 6.19).

Unfortunately, despite best efforts of equally splitting the rats into three groups, at baseline mean latency to correct response was not equal between surgical groups prior to surgery (*F* (2, 32) = 4.60, $p<0.05$) (Figure 6.20). Therefore, further analysis of this data was performed as mean latency to reward expressed as relative to baseline performance. Interestingly, very similar results to latency to reward were found with a significant effect of surgical group (*F* (2, 98) = 6.92, $p<0.01$). Subsequent *post hoc* comparisons showed that, as with reward latency, vehicle treated rats correctly responded at a significantly slower speed than rats that received scrambled virus ($p<0.01$) and rats that received virus expressing shErbb4 ($p<0.01$). In addition no effect of week on this parameter was found (*F* (2, 98) =0.02, $p>0.05$) (Figure 6.21).

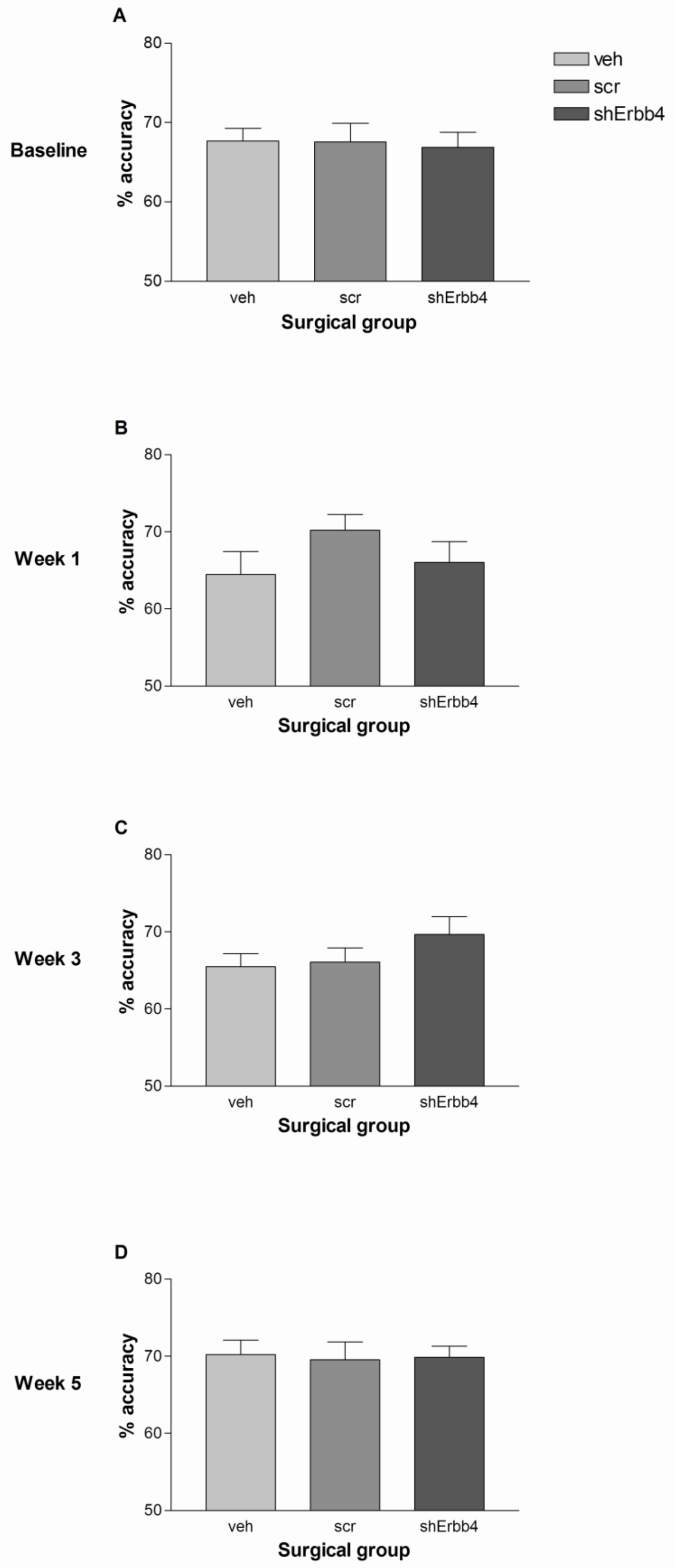

A
Baseline
veh
scr
shErbb4
80
70
60
50
% accuracy
veh
scr
shErbb4
Surgical group
B
Week 1
80
70
60
50
% accuracy
veh
scr
shErbb4
Surgical group
C
Week 3
80
70
60
50
% accuracy
veh
scr
shErbb4
Surgical group
D
Week 5
80
70
60
50
% accuracy
veh
scr
shErbb4
Surgical group

Figure 6.16 Effect of viral-mediated gene manipulation on percentage accuracy in the vSD task. Rats were split into three surgical groups based on baseline percentage accuracy (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage accuracy was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total percentage accuracy from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds (n=10-12). Two-way ANOVA showed percentage accuracy was not affected by surgical group or week in the vSD task.

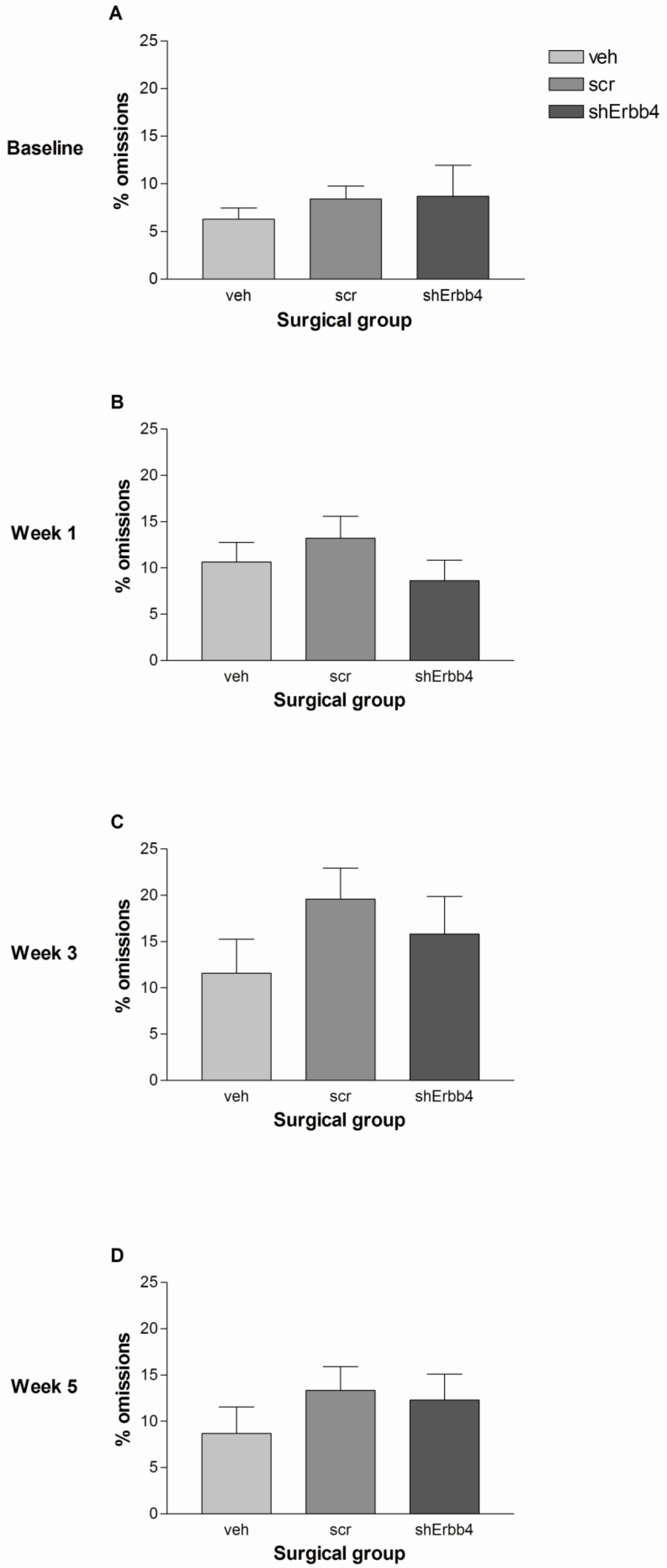

A
Baseline
B
Week 1
C
Week 3
D
Week 5
% omissions
0
5
10
15
20
25
veh
scr
shErbb4
Surgical group

Figure 6.17 Effect of viral-mediated gene manipulation on percentage omissions in the vSD task. Rats were split into three surgical groups based on baseline percentage omissions (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Percentage omissions were assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total percentage omissions from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds. Data were analysed using two-way repeated measures ANOVA followed by Tukey’s *post hoc* test for multiple comparisons. Two-way ANOVA showed a significant overall effect of surgical group on percentage omissions in the vSD task with scr.rAAV treated rats performing more omissions than vehicle treated rats ($p<0.01$). There was also an over all effect of week on percentage omissions with significantly more omissions being made over all surgical groups at 3 weeks post-surgery compared to baseline ($p<0.001$).

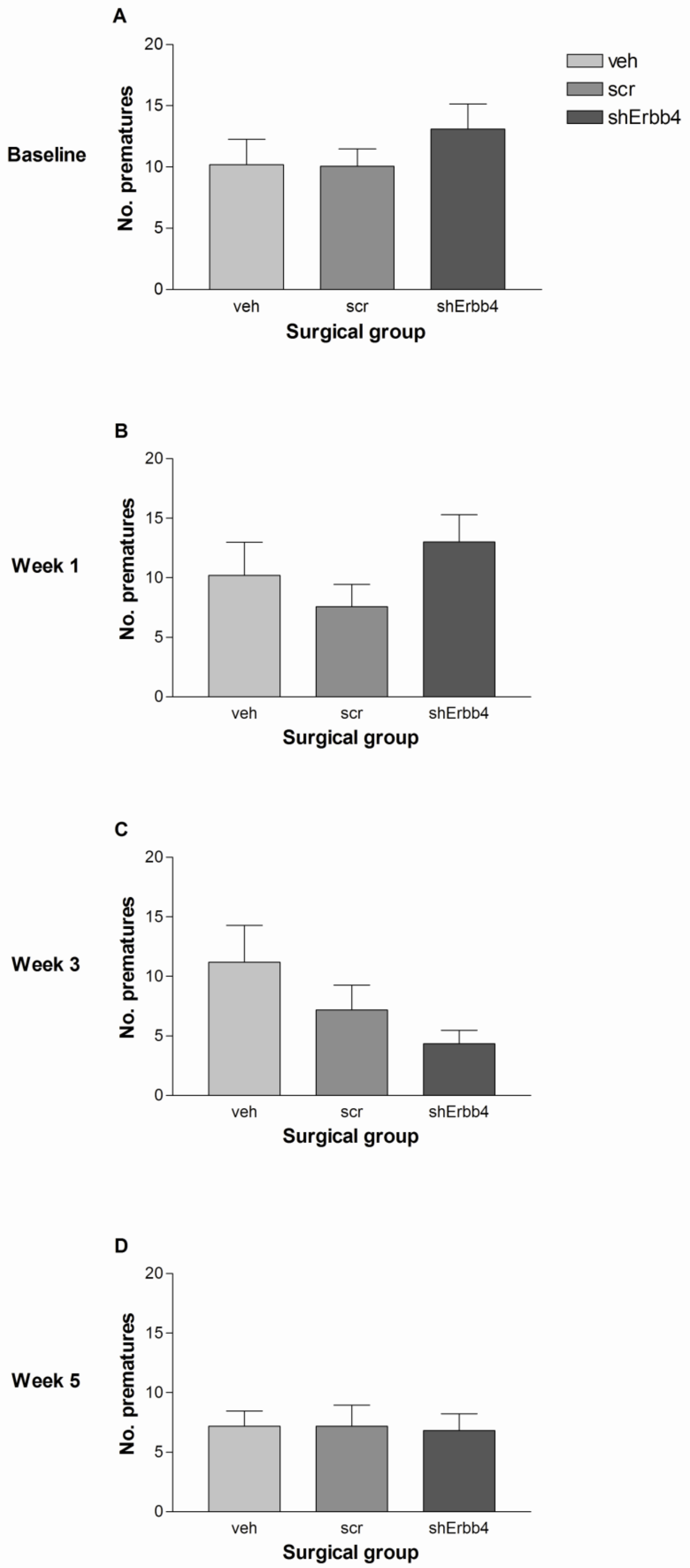

A
Baseline
No. premitures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4
B
Week 1
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
C
Week 3
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group
D
Week 5
No. prematures
20
15
10
5
0
veh
scr
shErbb4
Surgical group

Figure 6.18 Effect of viral-mediated gene manipulation on the number of premature response in the vSD task. Rats were split into three surgical groups based on baseline premature responding (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. The number of premature responses were assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total number of premature responses from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds. Two-way ANOVA showed premature responding was not affected by surgical group, however there was a significant effect of week on premature responses with less premature responses being made at 3 ($p<0.01$) and 5 ($p<0.01$) weeks post-surgery compared to baseline.

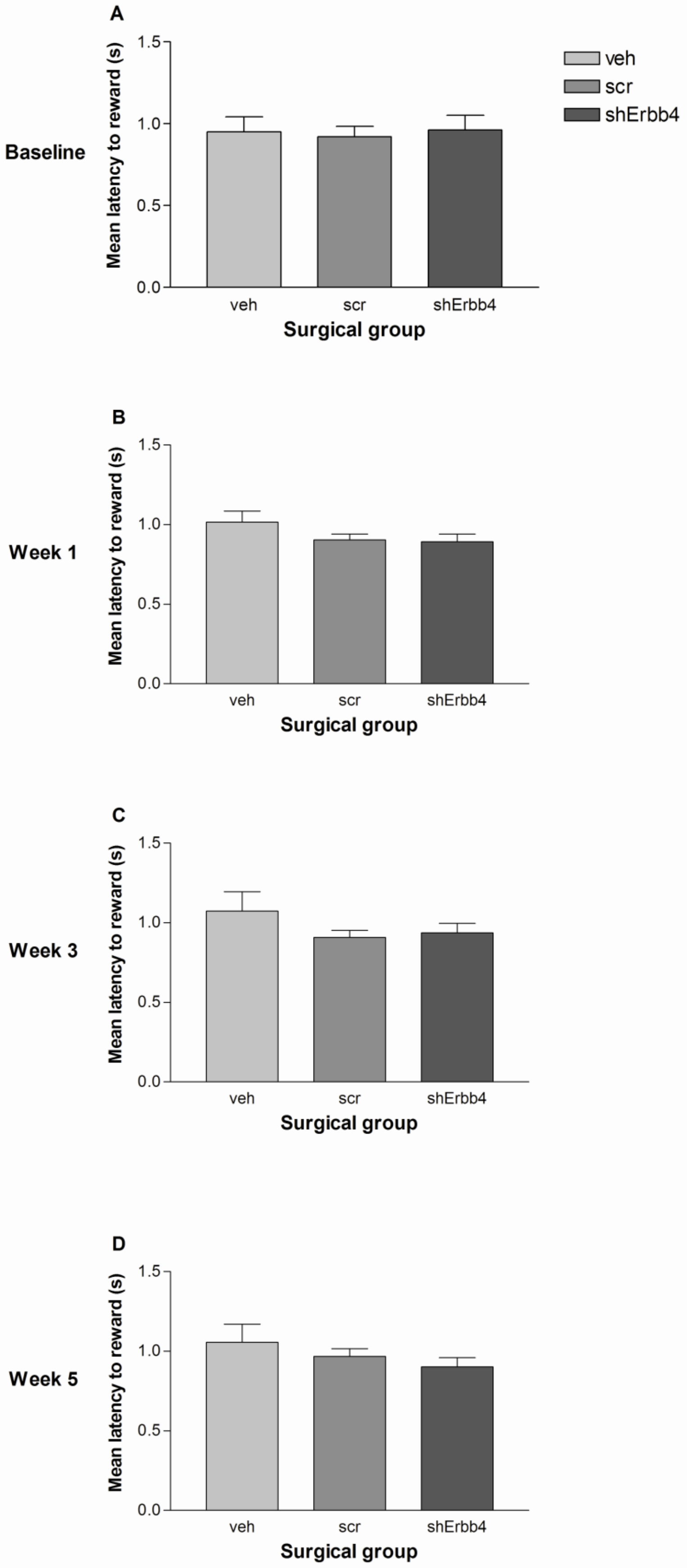

A
Baseline
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
veh
scr
shErbb4
B
Week 1
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
C
Week 3
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group
D
Week 5
Mean latency to reward (s)
1.5
1.0
0.5
0.0
veh
scr
shErbb4
Surgical group

Figure 6.19 Effect of viral-mediated gene manipulation on mean latency to reward in the vSD task. Rats were split into three surgical groups based on baseline reward latency (A) then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Mean reward latency was assessed in these rats at 1, 3 and 5 weeks post-surgery (B-D). Data represent mean ± SE of the total mean latency to response from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds. Data were analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showd an overall effect of surgical group on reward latency in the vSD task with vehicle treated rats having significantly higher latencies to collect reward than scr.rAAV ($p<0.05$) and shErbb4.rAAV ($p<0.01$) treated rats.

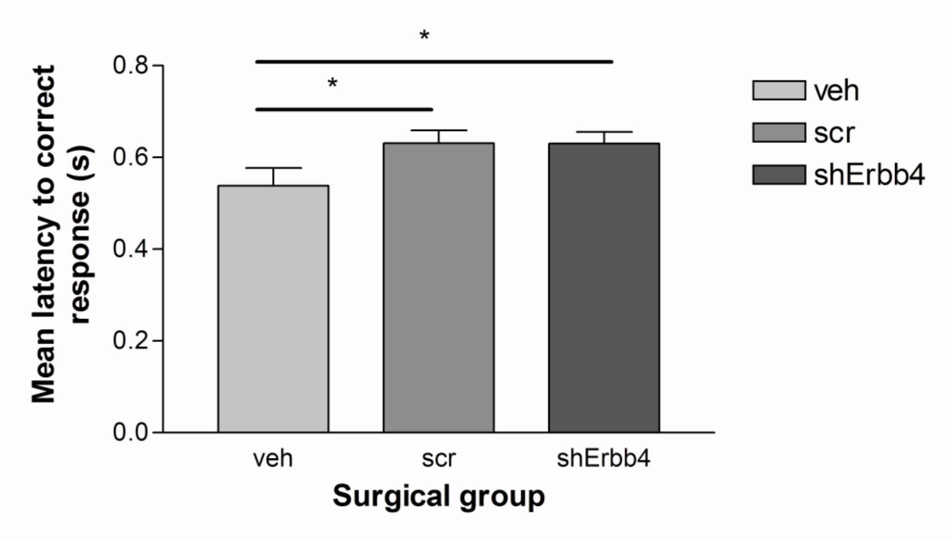


Figure 6.20 Baseline mean latency to correct response in the vITI task. Baseline mean latency to correct response in rats prior to surgical procedure of bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11). Data represent mean ± SE of the total mean latency to response from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds. Scr.rAAV and shErbb4 treated rats had significantly higher latency to correct response than vehicle treated rats at baseline. * $p<0.05$, analysed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

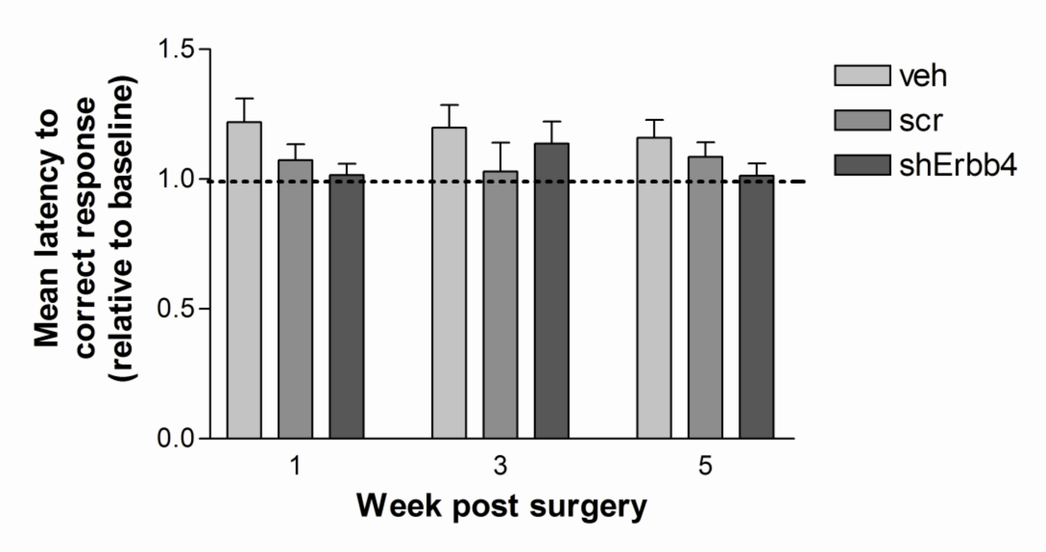


Figure 6.21 Effect of viral-mediated gene manipulation on mean latency to correct response in the vSD task relative to baseline mean latency to reward performance. Rats were split into three surgical groups then received bilateral injections of vehicle (veh) (n=10), scr.rAAV (scr) (n=12) or shErbb4.rAAV (shErbb4) (n=11) into the mPFC. Mean response latency was assessed in these rats at 1, 3 and 5 weeks post-surgery. Data represent mean ± SE of the total mean latency to response relative to baseline from trials with stimulus durations of 0.1, 0.2, 0.4 and 0.5 seconds, analysed using two-way repeated measures ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Two-way ANOVA showed a significant effect of surgical group on respinse latency with scr.rAAV (p<0.01) and shErbb4.rAAV (p<0.01) treated rats having significantly lower latencies to correct response compared to vehicle treated rats.

6.4 Discussion

6.4.1 Manipulation of 5-CSRTT parameters alters behaviour

The present results show that altering the parameters of stimulus duration and inter-trial interval duration had the profound predicted effects on many measurements of the 5-CSRTT. This further highlights the validity of the task in a pre-clinical model of cognition as it known that when parameters of the CPT in humans are altered the performance is significantly affected (Elvevåg *et al.*, 2000).

Manipulation of stimulus duration

Percentage accuracy was decreased by an average of 12% in the vSD task compared to the basic task, and this was undoubtedly due to changes in the stimulus duration; this is in agreement with Bari *et al.*, (2008). Moreover the trend seen for decreasing percentage accuracy with stimulus duration has been previously reported (Hille *et al.*, 2008) and supports the notion that reducing the stimulus duration further challenges the rats' cognitive ability by increasing the need for sustained attention. As this change in percentage accuracy was the only change in the rats' behaviour following reduction of the stimulus duration, the vSD task appears to be a good model system for assessing the specific effect of genetic manipulations/pharmacological agents on percentage accuracy.

Manipulation of inter -trial interval

The number of premature responses were hugely affected by increasing ITI duration, in agreement with Bari *et al.*, (2008), and this increase was highly correlated with increased ITI duration. This has been previously reported (Navarra *et al.*, 2008; Paterson *et al.*, 2011) and is a clear indication of increased impulsivity. Percentage accuracy was also significantly reduced when the inter-trial interval was made variable. However, this decrease in accuracy was a relatively small decrease with an average of 4.6% decrease, in comparison to the change in accuracy observed in the vSD task. Furthermore, the fact that percentage accuracy was not significantly associated with increased ITI duration suggests that the drop in percentage accuracy was not due to the rats having to wait longer between trials, but more than likely was due to the novelty of the task, as this was the first time the rats were exposed to the vITI paradigm. Moreover, it is important to note that in the vITI task the amount of “time outs” being given to the rats is significantly increased due to increased number of premature responses and omissions being performed, and therefore the amount of time remaining in the 30 minute task time is decreased. Therefore it is possible that the drop in percentage accuracy observed in the vITI task may be an overrepresentation, as fewer corrects and incorrects may be performed and thus small changes in percentage accuracy may be calculated as bigger changes than if 90 corrects and incorrects were performed as in the basic task.

In comparison to the vSD task, the vITI task appeared to give a more broad manipulation to the rats’ behaviours. As increasing ITI duration also increased percentage omissions, especially at ITIs of 10 seconds or more, indicating that in all probability the rats, when waiting this extended amount of time for the light to appear, had resorted to nose poking in the food magazine to initiate another trial, and missed the stimulus when it did appear

Again, the results seen when ITI or SD is manipulated are in agreement with the results stated by Bari *et al.,* (2008) in that neither response latency or reward latency were affected.

Overall, these results suggest that the 5-CSRTT is flexible, and can be manipulated to tease apart specific modalities of cognition and will thus allow easier identification of agents which affect specific parameters of the task i.e. to test a drug that reduces impulsivity in the vITI task where premature responses is the main parameter being controlled. Moreover, the changes in behaviour of the rats in the vSD and vITI task in comparison to the basic task imply that in the basic task the rats may have learned to rely on automatic processes whereas in the variable tasks the rats have continually to sustain attention and adapt their responses.

6.4.2 Effects of Erbb4 knockdown on 5-CSRTT performance

Surgery/training-related deficits

The general finding that several of the parameters of the 5-CSRTT changed over the testing period was not unexpected. One week following surgery it was observed that in the basic task percentage accuracy dropped and percentage omissions increased suggesting that there was an overall deficit in task performance at this time point. This deficit was observed over all surgical groups and thus was not an effect of the agent injected *per se* but was most likely a general effect of stereotaxic surgery in combination with the fact that the rats had not received training for one week. Moreover the deficits in percentage accuracy and percentage omissions were short lived and performance returned to at least baseline levels by week 3, supporting the previous findings that there was very little tissue damage at the site of injection (section 4.3.2) and in addition that viral injection did not have an undesired global effect (supported by the findings that body weight and levels of glial infiltration did not alter following viral injections) (sections 4.3.5).

The other trend of week having an effect on 5-CSRTT performance was evident in the vITI and vSD tasks where the number of premature responses was lower at weeks 3 and 5 compared to baseline performance. This finding is in concordance with previous literature suggesting that rats become quickly adapted to manipulated forms of the 5-CSRTT (Bari *et al.,* 2008). Although percentage accuracy appeared to be the most affected parameter by altering the stimulus duration, there was no evidence that the rats adapted to this parameter in the same way as they did with premature responding. This may suggest that impulsivity is a more adaptable process than that of sustained attention.

Specific effects of *Erbb4* gene manipulation on task performance

The increase in percentage accuracy in the basic task detected at 5 weeks post-surgery in rats that received shErbb4.rAAV is a very interesting result, as this corresponds to the previous finding that Erbb4 protein levels were confirmed to be decreased in the PFC at this specific time point (section 4.3.5). In addition, the time point post-surgery at which this increase in percentage accuracy became evident in shErbb4.rAAV treated rats (5 weeks) was the same time point post-surgery that the change in locomotor response to amphetamine manifested in shErbb4.rAAV treated rats in the locomotor activity task (section 5.3.3). Again this emphasises the suggestion that whatever effects Erbb4 knockdown is having on behaviour, it is taking 5 weeks to become apparent; this may be because this is when the optimal amount of hairpin is being expressed and thus when maximal gene knockdown is occurring, or it may be due to it taking 5 weeks for Erbb4 knockdown to affect the prefrontal circuitry and manifest as behaviour changes.

The finding of an increase in cognitive ability following Erbb4 knockdown, although subtle, is in accordance with previous literature in schizophrenia patients where the levels of ERBB4 within the dorsolateral prefrontal cortex are significantly increased, and expression is directly associated with a haplotype consisting of three intronic SNPs in linkage disequilibrium near exon 3 (Silberberg *et al.,* 2006; Law *et al.,* 2007; Chong *et al.,* 2008). It is therefore feasible to consider that decreasing Erbb4 expression within the PFC (thought to be a homolog of some aspects of the human DLPFC (Uylings *et al.,* 2003; Seamans *et al.,* 2008) would result in increased cognitive ability. Interestingly one of the SNP's from the risk haplotype, *rs839523*, has also been associated with poorer performance in the oddball task (in terms of reaction time) in healthy individuals homozygous for the risk allele (Konrad *et al.,* 2009). In addition a very recent study showed an epistatic interaction of SNPs in *NRG1*, *ERBB4* and *AKT1* resulting in reduced pre-frontal cortical engagement during the n-back task (Nicodemus *et al.,* 2010).

At 5 weeks post-surgery the protein expression of Nrg1 is also altered. However, this is in the opposite direction to Erbb4, with increased expression being found (chapter 5). These behavioural effects on percentage accuracy could therefore also be due to a change in the expression of Nrg1. However, the studies showing differential expression of NRG1 in schizophrenia are somewhat unclear with type I NRG1 shown to be increased in DLPFC of patients (Hashimoto *et al.,* 2004; Law *et al.,* 2006; Chong *et al.,* 2008). However, studies have also shown decreased *NRG1* expression at mRNA level (Barakat *et al.,* 2010) and protein level in the prefrontal cortex in schizophrenia (Bertram *et al.,* 2007). Furthermore, studies have also showed no change in *NRG1* expression (Boer *et al.,* 2009). Despite this ambiguity in expression studies, NRG1 appears to be important in cognition. A variant in the *NRG1* gene which has shown association with increased risk to schizophrenia also shows association with decreased activity of the mPFC, and decreased premorbid IQ as assessed by the national adult reading test (Hall *et al.,* 2006).

Although there is no evidence of this cognitive enhancement effect of PFC Erbb4 knockdown at 5 weeks post-surgery in the vSD task, this may be due to a “ceiling effect” in that the rats cannot perform at a higher level of percentage accuracy in the vSD task than they achieved pre-surgery and thus no pro-cognitive effect could emerge. What is more, the cognitive enhancing effects seen by Erbb4 knockdown on percentage accuracy, although subtle, are similar to levels of cognitive enhancement previously shown in the 5-CSRTT by nicotine (Hahn *et al.,* 2002; Semenova *et al.,* 2007). Another explanation for not detecting changes in the vSD task is that the rats were not performing at a high enough level in the basic task before the task was manipulated as suggested by Semenova and colleagues. They state that for Sprague-Dawley rats performing at a baseline accuracy in the basic 5-CSRTT of 79.79±1.74% “*there was concern that in the modified task, SD rats’ low baseline performance would preclude meaningful evaluation of the effects of manipulations*”. Wistar rats performing at a baseline level of 87% accuracy in the basic task were, however, thought to be suitable for evaluation in the vSD task, where an effect of nicotine on accuracy was observed.

Nrg1/Erbb4 signalling affects multiple neurotransmitter systems that are known to play an important role in the performance of the 5-CSRTT and in cognition in general. For example Nrg1-Erbb4 signalling is important in maintaining glutamatergic neurotransmission, with Nrg1 mutant mice showing reduced NMDA receptor phosphorylation (Bjarnadottir *et al.,* 2007) and fewer functional NMDA receptors in the forebrain (Stefansson *et al.,* 2002). In addition, in a study by Hahn and colleagues in 2006, it was found that in brain tissue from patients that had schizophrenia the level of activity of Nrg1-Erbb4 signalling was enhanced and that this enhancement led to NMDA receptor hypofunction. Moreover, Nrg1 and Erbb4 mutants appear to have some behavioural similarities to NMDA receptor hypomorphs (Mohn *et al.,* 1999). This evidence is suggestive that disruption of Nrg1-Erbb4 signalling affects NMDA receptor functionality, interestingly; the NMDA antagonist phencyclidine has a marked effect in disrupting 5-CSRTT performance (Amitai *et al.,* 2008; Thomson *et al.,* 2010) with acute NMDA blockade leading to deficits in premature responding and accuracy, and repeated treatment affecting speed of processing.

Nrg1-Erbb4 signalling is also important in regulating dopaminergic neurotransmission, with a mouse line expressing a dominant negative form of Erbb4 in oligodendrocytes having significantly more dopamine transporter and D1-like receptor binding in the cortex, striatum and nucleus accumbens (Roy *et al.,* 2007). More recently a study showing peripheral Nrg1 injections in mice results in Erbb4 receptor activation and elevated dopamine levels (Kato *et al.,* 2010). Furthermore injection of Nrg1 into the dorsal substantia nigra stimulates striatal dopamine overflow (Yurek *et al.,* 2004). Therefore, in the current study it is possible that the knockdown of Erbb4 in the mPFC affected prefrontal dopamine levels and as a result increased percentage accuracy as seen with dopaminergic compounds (6.1.3).

Type I Nrg1 (initially termed ARIA: acetylcholine receptor inducing activity) was initially identified as a factor which was capable of accumulating AChRs at the neuromuscular junction (Usdin & Fischbach, 1986) and later in hippocampal interneurons (Liu *et al.,* 2001). Several recent studies have implicated Nrg1 signalling in α7-nicotinic acetylcholine receptor (α-7nAChR) transmission with Nrg1 affecting α-7nAChR surface expression (Chang & Fischbach, 2006; Hancock *et al.,* 2008) and fast inward currents (Chang & Fischbach, 2006). Additionally, cultures from Nrg1 type III heterozygous knock-out mice revealed these mice have reduced functional α-7nAChR expression (Zhong *et al.,* 2008). As described in section 6.1.4, α-7nAChR knock-out mice have reduced accuracy in the 5-CSRTT whereas α-7nAChR agonists have shown some promise as pro-cognitive agents in the 5-CSRTT. The manipulation of the Nrg1-Erbb4 signalling pathway leading to pro-cognitive effects, as seen in this study, could consequently be a result of elevated α-7nAChR expression or activity.

It is difficult to compare the results of this behavioural task with those studies previously carried out in transgenic *Erbb4* and *Nrg1* mice for several reasons. The primary comparative concern is that none of the previous studies have used 5-CSRTT to assess the effect of mutations in Erbb4 or Nrg1 on behaviour; instead many of the cognitive assessments in these previous studies have relied heavily on tests that are hippocampus based. Previous studies have shown that performance in spatial and reference working memory tasks (such as the radial arm maze and the Morris water maze) is impaired by knock-out of *Erbb4* in the CNS (Golub *et al.,* 2004; Wen *et al.,* 2010). Transgenic *Nrg1* mice also show deficits in tasks such as fear conditioning, novel object recognition, continuous T-maze alternation task and latent inhibition (Rimer *et al.*, 2005; Chen *et al.*, 2008; Ehrlichman *et al.,* 2009; Duffy *et al.,* 2010). However, some studies also show no effect of *Nrg1* manipulation on spatial learning and memory tasks (Gerlai *et al.,* 2000; O'Tuathaigh *et al.,* 2007; Duffy *et al.,* 2010). Care must be taken to interpret the wealth of studies utilising transgenic Nrg1 mice as they vary in which isoform of Nrg1 is being manipulated.

Interestingly, Nrg1 type I over-expression results in some of the same behavioural observations from *Nrg1* knock-out mice (Deakin *et al.,* 2009). This finding highlights the inverted U shaped hypothesis whereby either increased or decreased levels of Nrg1-Erbb4 signalling from the optimal levels result in deficits in synaptic plasticity (Role & Talmage, 2007). This may also hold true for behavioural dysfunction where in schizophrenia the directionality of change in expression of Nrg1/Erbb4 is not key for pathophysiology: what is important is that the signalling has shifted from the "norm".

Secondly, it is essential to take into account that the manipulation of Erbb4 in this study is region specific whereas previous *in vivo* studies of Erbb4 have opted for a more global depletion of the gene. One limitation to analysing results (in particular behavioural phenotypes) from transgenic mice is that the effects observed may be complicated by the interaction between abnormalities in multiple brain regions (Chen *et al.,* 1998). Furthermore, the evidence for altered Erbb4 expression in schizophrenia appears to be limited to the prefrontal cortex, as no change was quantified in the hippocampus (Law *et al.,* 2006).

Therefore, behavioural changes following Erbb4 manipulation specifically in the mPFC may be more representative of schizophrenia.

Small but significant changes in omissions and premature responses were observed in the present study; however many changes that were observed were not stable over time. The amount of premature responses and omissions performed in the 30 minute task period is very low, with on average in the basic task only 10 omissions and 12 premature responses being performed in the test period. Therefore, a change between surgical groups in premature responses and omissions is more likely to produce significant results as a few additional premature responses or omissions will result in a big difference. Moreover, there was no significant effect of surgical group on premature responses or omissions in the vITI task, despite these parameters being highlighted by extending the inter-trial interval. This task has been widely used to measure subtle changes in impulsivity and visuo-spatial attention, and has been successful in identifying changes in these parameters not clearly evident in the basic task (Paterson *et al.,* 2011).

Changes in latencies suggest that the vehicle treated rats were slower to collect rewards than rats with Erbb4 knockdown in all three task types and also slower than scr.rAAV treated rats in both the vITI and vSD tasks. Changes in latency to reward are generally thought to reflect changes in motivation (Robbins *et al.,* 2002). However, as both viral-treated groups were quicker to collect rewards following correct response this would suggest the viral particles themselves other than the gene manipulation were affecting motivation. There also appeared to be a slight effect of viral treatment on latency to make a correct response with viral treated rats responding quicker than the vehicle treated rats in the vSD task. This may suggest there was a slight effect on the motor system of vehicle treated rats. These changes in response times in viral-treated rats compared to vehicle treated rats are similar to the observations seen in the open field task where the vehicle treated rats travelled the least distance under vehicle treatment compared to the viral treatment groups (see chapter 5).

However, the results with latency to response were less consistent than those of the latency to reward as there was no effect of viral treatment on latency to response in the vITI task and in the basic task the scrambled hairpin treated rats were slower than those that received vehicle. It is important to note that the speed that these highly trained rats are performing at is very fast, with the speed of the vehicle treated rats in the basic task of 569ms ±16ms, vITI task of 613ms±34ms and vSD task of 605ms±46ms. These latencies are very similar to that of Bari *et al.*, (2008) who suggest male Hooded Lister rats should perform at a response latency of 571ms±41ms, 554ms±31ms, 598ms±41ms in the aforementioned tasks, respectively. Furthermore, it should be noted that the differences between the latencies performed by the surgical groups (either to reward or correct response) are very small with the biggest difference between groups being for reward latency a 100ms difference on average between shErbb4.rAAV treated rats and veh treated rats in the vSD task, and for latency to response a 97 ms difference on average between scr.rAAV treated rats and veh treated rats in the vSD task.

6.5 Conclusions

The data in this chapter present some interesting findings with respect to a) the utilisation of the 5-CSRTT in measuring cognitive changes following gene manipulation, b) the role of Erbb4 in the mPFC in terms of cognition.

The findings of the change in rats' performance in the 5-CSRTT when stimulus duration is shortened or inter-trial interval is extended are in complete agreement with the expected results previously reported (Bari *et al.*, 2008) with percentage accuracy decreasing in the vSD task and the number of premature responses increasing and slight effects on percentage accuracy and percentage omissions in the vITI task compared to that of the basic task set-up. These findings not only support that set-up in habituation, training and testing of the 5-CSRTT in our laboratory are accurate, but also support that the task is versatile and can be manipulated to suit the experimental requirements.

With respect to viral mediated gene manipulation, this study emphasises that this technique is viable in investigating schizophrenia genes and will allow further knowledge of what part these genes, which are generally very poorly understood in terms of their potential pathophysiological roles, may play in cognition in specific brain regions at specific stages of life.

The main finding of this chapter was that viral-mediated Erbb4 knockdown in the mPFC resulted in an increase in percentage accuracy at 5 weeks post-surgery without having a great impact on any of the other parameters assessed in the task. This would suggest that Erbb4 knockdown or Nrg1 over-expression within the mPFC is pro-cognitive and that the pre-frontal dependant cognitive deficits observed in schizophrenia may be partly due to an imbalance in this signalling pathway in this brain region. Although no specific neurotransmitter system was analysed in depth following Erbb4 knockdown, it is most likely that this pro-cognitive effect is a result of changes in glutamatergic, cholinergic and dopaminergic neurotransmission as Nrg1-Erbb4 are known to have important roles in all these systems (and most likely others too). As the effect is subtle, and is not emphasised by decreasing SD, these data support the general consensus that schizophrenia is not a single gene or network disorder but one that is a result of insults in multiple genes which can impact upon multiple networks.

The results of this study emphasise that further research should be invested in investigating the Nrg1-Erbb4 signalling pathway specifically in the mPFC in terms of cognition in order potentially to identify possible therapeutic drug targets to treat the cognitive deficits in schizophrenia or other disorders where Nrg1-Erbb4 signalling may be dysfunctional.

7 General discussion

The overall aims of this PhD study were to identify the roles of the schizophrenia candidate gene *Erbb4* in neuregulin signal pathway transduction, and schizophrenia-related behaviours dependent on prefrontal cortex functioning. I demonstrated that adeno-associated viral mediated knockdown of Erbb4 within the prefrontal cortex of rats resulted in changes in selected behaviours including measures of cognition, along with alterations in expression and activity of several components of the Nrg1-Erbb4 signalling cascade. These data allow a greater knowledge of how dysfunctional NRG1-ERBB4 signalling in schizophrenia may be potentially linked to the cognitive deficits observed in the disorder, and may prospectively lead to therapeutic targets for the treatments of these poorly managed symptoms.

7.1 Nrg1-Erbb4 signalling in NG108-15 cells

In initial experiments the expression and activity of endogenous Erbb4 in the neuronal cell line NG108-15 were studied, with the aim of potentially identifying a neuronal cell line that could be utilised to investigate Nrg1-Erbb4 signalling (chapter 3). Western blotting and RT-PCR techniques were used to show that NG108-15 cells express murine *Erbb4* mRNA and protein, and moreover selectively express the *JMa/CYT1* and *JMa/CYT2* isoforms of *Erbb4*. Although the levels of *Erbb4* endogenous expression appeared to be low in NG108-15 cells, optimised siRNA-mediated knockdown of *Erbb4* without overt off-target effects was achieved at the mRNA level. Moreover, there was subtle but significant activation of Erbb4 and GSK3β following the application of NRG1β to the cells, indicative that Erbb4 receptors in NG108-15 cells are functional. It has been previously reported that NG108-15 cells expressed Nrg1 (Pun *et al.,* 2007); however, this is, to my knowledge, the first study specifically investigating the expression of *Erbb4* in this cell line. As Erbb4 is expressed endogenously in NG108-15 cells they offer a superior model for investigating Nrg1-Erbb4 signalling as compared to cells manipulated to express these genes. Moreover since NG108-15 are a neuronal cell line, it is likely that the roles that these genes play in this cell line will be more representative of the brain than non-neuronal cell lines such as HEK-293 cells. Identifying cell lines, and specifically neuronal cell lines, that express schizophrenia candidate genes allowed preliminary investigations of the functions of these genes to be carried out prior to *in vivo* studies.

7.2 Erbb4 knockdown on schizophrenia-related signalling pathways and behaviours

The pinnacle finding of this PhD study was that knockdown of Erbb4 expression exclusively in the mPFC was sufficient for producing selective behavioural phenotypes that relate to schizophrenia, such as increasing accuracy in the 5-CSRTT which translates to increased sustained attention (chapter 6).

This study utilised the innovative technique of viral-mediated gene manipulation which to date has only been used by a small number of groups to study schizophrenia-related genes (Mao *et al.,* 2009; Burnet *et al.,* 2011). Although one very recent study has published viral-mediated targeting of Erbb4 (Choi *et al.,* 2010), this current project is the first demonstration of the functional effects of viral –mediated Erbb4 manipulation *in vivo.* Moreover, although several studies have elegantly shown behavioural and biochemical changes in genetically manipulated rodents that express altered levels of *Erbb4,* this present study is the first to identify the effects of altered Erbb4 expression within a distinct brain region.

By targeting a distinct brain region such as the mPFC, which has been highly implicated in the cognitive deficits of schizophrenia, we can identify the potential role Erbb4 may have distinctively within this brain region. These findings can then reflect how, if the function of this gene if perturbed in schizophrenia, can ultimately lead to these poorly treated symptoms. Remarkably Erbb4 knockdown within the mPFC led to an improvement in performance of the 5-CSRTT, a measure of attentional processes and processing speed (Young *et al.,* 2009).

These data are not only significant from a technical perspective but also from a biological standpoint. No previous animal models of altered Nrg1 or Erbb4 expression have utilised the 5-CSRTT or measures of attention and processing speed and therefore cannot be compared to the present study, however, the finding of increased accuracy following Erbb4 knockdown parallels human studies. In *post-mortem* dlPFC tissue from schizophrenia patients expression of ERBB4 has repeatedly been found to be increased (Silberberg *et al.,* 2006; Law *et al.,* 2007; Chong *et al.,* 2008), and that this increase is selective for this brain region (Law *et al.,* 2007). Moreover, patients with variations in ERBB4 have dysfunctional activity in this brain region (Nicodemus *et al.,* 2010). In rodents performance accuracy in the 5-CSRTT has been positively correlated with activity of the mPFC (Barbelivien *et al.,* 2001) and lesions of this region result in reduced accuracy (Muir *et al.,* 1996). It can therefore be interpreted that tight regulation of ERBB4 within the dlPFC is essential for optimal cognitive performance.

In addition, the reliability of the 5-CSRTT finding is emphasised by the parallel finding that Erbb4 knockdown within the mPFC heightens the sensitivity to locomotor response to amphetamine at exactly the same time point post-viral manipulation – 5 weeks. Not only does this finding illustrate that the behavioural responses occurred at the same time, but also that it is likely that they are both a result of altered dopamine transmission. The locomotor response to amphetamine is due to increased dopamine availability (Seiden *et al.,* 1993) and dopamine levels in the frontal cortex have been positively correlated with choice accuracy in the 5-CSRTT (Puumala & Sirvio, 1998). These findings are consistent with the hypothesis that reduced dopamine levels within the PFC of schizophrenia patients are responsible for cognitive deficits.

Moreover this link between prefrontal Erbb4 expression and dopamine regulation was highlighted by a change in the activity of Akt signalling within the PFC following Erbb4 knockdown. Akt signalling has been repeatedly associated with dopamine regulation (Beaulieu *et al.,* 2009), so this reduction in Akt signalling may reflect an altered dopaminergic modulation of Akt subsequent to Erbb4 knockdown. The directional of the change in Akt activity in the PFC following Erbb4 knockdown is inconsistent with the results of Emamian *et al.*, (2004) who reported decreased Akt activity in the PFC of patients with schizophrenia. However, the direction of the change is consistent with the data of Beaulieu *et al.* (2009), who reported that dopaminergic modulation of mouse behaviour, in behavioural assays such as locomotor activity, involves a suppression of Akt activity. It is tempting to speculate that a reduction in PFC Erbb4 levels facilitates dopaminergic suppression of Akt in the PFC, and thus enhances performance in behavioural tasks involving the PFC. Equally, it should be noted that these signalling cascades are very complex and have multiple inputs. Moreover, since dopamine has also been reported to stimulate Akt, the decreased Akt activity may reflect a compensatory effect to reverse the hyperdopaminergic state that is potentially producing the behavioural phenotypes.

This potential compensatory effect of decreased Akt activity is not unwarranted as we observed a compensatory increase in the expression of Nrg1 within the PFC following Erbb4 knockdown that led to a significant negative correlation between Nrg1 and Erbb4 expression. This finding leads to an interesting query that has not been addressed in previous animal models of altered Nrg1 and Erbb4 expression, of whether this balance of Nrg1 and Erbb4 expression is also affected. Again, increasing the credibility of the findings in this present study to the observations of human studies, it has been repetitively found that there is an interaction between components of the NRG1-ERBB4-AKT1 signalling cascade (Benzel *et al.,* 2007; Nicodemus *et al.,* 2010; Sei *et al.,* 2010). The findings from these previous studies along with those reported in the current study emphasise that with the complexity of schizophrenia, it is unlikely that expression changes in one gene or protein are solely pathogenic, but instead it is a combination of changes in multiple related genes. Indeed, *NRG1* and *ERBB4* have also been associated with other genes including *COMT*, *ESR1* (estrogen receptor 1α) and *DISC1* (Wong & Weickert, 2009; Sei *et al.,* 2010; Seshadri *et al.,* 2010).

As expected Erbb4 knockdown did not lead to widespread behavioural deficits. Thus it appears that Erbb4 within the prefrontal cortex does not impact upon sensorimotor gating, as there was no effect of Erbb4 knockdown on prepulse inhibition performance. This may reflect the more distributed neuronal network thought to be involved in PPI responses. In addition, the lack of effect of Erbb4 knockdown within the mPFC on PPI performance may also be due to the small area of mPFC transduced by the rAAV particles, or that the amount of Erbb4 knockdown achieved by the rAAVs was insufficient to cause an effect on PPI.

7.3 Further work

The data generated from this study are very thought-provoking, and if more time and funding were available several interesting follow-up studies could be carried out. Although the behavioural changes following Erbb4 knockdown within the mPFC provide promising insight into Erbb4 regulating cognitive behaviour which points towards altered dopaminergic transmission, it is uncertain as to which neurotransmitter systems are altered, as neurotransmitter levels were not measured in this study. It would therefore be of great interest to measure the levels of neurotransmitters related to Erbb4 function such as dopamine, glutamate, serotonin and GABA within the mPFC following Erbb4 knockdown by a technique such as *in vivo* microdialysis.

Due to time constraints, one limiting factor of the current study is that gene and protein expression were only quantified at one time point post-viral injection. Although we successfully identified significant knockdown in Erbb4 expression and alterations in the levels of expression several related proteins, it would be of great interest to determine when the peak level of knockdown or secondary effects occurred by performing a time-line experiment. However, to carry this out and to have a large enough sample size per time point, would require a substantial number of animals. Consequently, it would also be worthwhile to assess the behaviours measured in this study at time points beyond 5 weeks post-viral injection, to determine how long lasting these phenotypes were present and if they became more evident over time.

Since it has been shown that specifically within the PFC it is distinct isoforms of *ERBB4* that are over-expressed (Silberberg *et al.,* 2006; Law *et al.,* 2007), it would be fascinating to use the techniques mastered in this present study to knockdown specific isoforms of *Erbb4* within the mPFC. It would be hypothesised that knockdown of *JMa/CYT1* isoforms alone would produce a more distinct behavioural effect that knockdown of *JMb/CYT2* isoforms. Also, as it is unlikely that the complex range of symptoms which define schizophrenia result from dysfunction in one brain region alone, further studies akin to the present study where Erbb4 expression is manipulated in multiple brain regions simultaneously would identify behavioural and biochemical consequences of dysfunctional Erbb4 signalling within a complete circuit.

Finally, it would be appealing to repeat the current studies performed in NG108-15 cells in cells that had been differentiated, to determine whether the expression and activity of Erbb4 in this cell line increases in response to differentiation. This might further validate this cell line as a good *in vitro* model to study Nrg1-Erbb4 signalling.

7.4 Conclusions

This thesis presents a number of novel findings that impact on the understanding of Erbb4 function within the prefrontal cortex. The innovative technique of using adeno-associated viral-mediated manipulation of *Erbb4* with functional behavioural and biochemical analyses has been reported here for the first time. Following primary *in vitro* and *in vivo* validation, viral-mediated Erbb4 knockdown was achieved within the PFC, which led to increased accuracy performance in the 5-CSRTT and heightened sensitivity to the locomotor effects of amphetamine, without affecting sensorimotor gating function.

As well as impacting on behavioural phenotypes, Erbb4 knockdown within the rat PFC altered biochemical signalling, with an increase in Nrg1 expression and decreased Akt activity observed. These behavioural and biochemical findings point to a tentative link between prefrontal Erbb4 levels and dopamine regulation; however, it is likely that numerous other neurotransmitters may also be affected.

Importantly, the data in this study highlight the validity of viral-mediated gene manipulation to investigate schizophrenia candidate genes, with the advantage of having control over spatial and temporal gene manipulation. This innovative technique has enabled the investigation of the function of Erbb4 specifically within the prefrontal cortex in the adult rodent brain, and has led to the findings that 1) Erbb4 expression within the PFC is important in cognitive function 2) prefrontal Erbb4 is not essential in regulating sensorimotor gating and 3) alterations in Erbb4 expression affect multiple components of the Nrg1-Erbb4 signalling cascade.

These findings are a vital stepping stone to a greater understanding of how *ERBB4* may be potentially pathophysiological in schizophrenia, and with follow up studies can prospectively lead to new therapeutic targets for the treatment of the cognitive deficits of schizophrenia.

8 References

Abi-Dargham A, Laruelle M (2005) Mechanisms of action of second generation antipsychotic drugs in schizophrenia: insights from brain imaging studies. Eur Psychiatry 20:15-27.

Adler CM, Malhotra AK, Elman I, Goldberg T, Egan M, Pickar D, Breier A (1999) Comparison of ketamine-induced thought disorder in healthy volunteers and thought disorder in schizophrenia. Am J Psychiatry 156:1646-1649.

Ajiro K, Kano-Tanaka K, Higashida H, Tanaka T (1982) Coexpression of species-specific histone H1 and H2B genes in mouse-rat hybrid cells. Somatic Cell Genet 8:377-383.

Akache B, Grimm D, Pandey K, Yant SR, Xu H, Kay MA (2006) The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. J Virol 80:9831-9836.

Aleman A, Kahn RS, Selten JP (2003) Sex differences in the risk of schizophrenia: evidence from meta-analysis. Arch Gen Psychiatry 60:565-571.

Allen RM, Young SJ (1978) Phencyclidine-induced psychosis. Am J Psychiatry 135:1081-1084.

Amitai N, Markou A (2009) Increased impulsivity and disrupted attention induced by repeated phencyclidine are not attenuated by chronic quetiapine treatment. Pharmacol Biochem Behav 93:248-257.

Angrist B, Sathananthan G, Wilk S, Gershon S (1974) Amphetamine psychosis: behavioral and biochemical aspects. J Psychiatr Res 11:13-23.

Anton ES, Ghashghaei HT, Weber JL, McCann C, Fischer TM, Cheung ID, Gassmann M, Messing A, Klein R, Schwab MH, Lloyd KC, Lai C (2004) Receptor tyrosine kinase ErbB4 modulates neuroblast migration and placement in the adult forebrain. Nat Neurosci 7:1319-1328.

Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE (2002) Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. Bmj 325:1212-1213.

Atchison RW, Casto BC, Hammon WM (1965) Adenovirus-Associated Defective Virus Particles. Science 149:754-756.

Babcock AM, Standing D, Bullshields K, Schwartz E, Paden CM, Poulsen DJ (2005) In vivo inhibition of hippocampal Ca2+/calmodulin-dependent protein kinase II by RNA interference. Mol Ther 11:899-905.

Badner JA, Gershon ES (2002) Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. Mol Psychiatry 7:405-411.

Baldessarini RJ, Frankenburg FR (1991) Clozapine. A novel antipsychotic agent. N Engl J Med 324:746-754.

Baloui H, von Boxberg Y, Vinh J, Weiss S, Rossier J, Nothias F, Stettler O (2004) Cellular prion protein/laminin receptor: distribution in adult central nervous system and characterization of an isoform associated with a subtype of cortical neurons. Eur J Neurosci 20:2605-2616.

Bao J, Lin H, Ouyang Y, Lei D, Osman A, Kim TW, Mei L, Dai P, Ohlemiller KK, Ambron RT (2004) Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. Nat Neurosci 7:1250-1258.

Barakat A, Dean B, Scarr E, Evin G Decreased Neuregulin 1 C-terminal fragment in Brodmann's area 6 of patients with schizophrenia. Schizophr Res 124:200-207.

Barbelivien A, Ruotsalainen S, Sirvio J (2001) Metabolic alterations in the prefrontal and cingulate cortices are related to behavioral deficits in a rodent model of attention-deficit hyperactivity disorder. Cereb Cortex 11:1056-1063.

Bari A, Dalley JW, Robbins TW (2008) The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. Nat Protoc 3:759-767.

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG (2004) Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. Proc Natl Acad Sci U S A 101:5099-5104.

Beaulieu JM, Tirotta E, Sotnikova TD, Masri B, Salahpour A, Gainetdinov RR, Borrelli E, Caron MG (2007) Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. J Neurosci 27:881-885.

Beaulieu JM, Gainetdinov RR, Caron MG (2009) Akt/GSK3 signaling in the action of psychotropic drugs. Annu Rev Pharmacol Toxicol 49:327-347.

Beck LH, Bransome ED, Jr., Mirsky AF, Rosvold HE, Sarason I (1956) A continuous performance test of brain damage. J Consult Psychol 20:343-350.

Benes FM, Todtenkopf MS, Logiotatos P, Williams M (2000) Glutamate decarboxylase(65)-immunoreactive terminals in cingulate and prefrontal cortices of schizophrenic and bipolar brain. J Chem Neuroanat 20:259-269.

Benes FM, Berretta S (2001) GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. Neuropsychopharmacology 25:1-27.

Benzel I, Bansal A, Browning BL, Galwey NW, Maycox PR, McGinnis R, Smart D, St Clair D, Yates P, Purvis I (2007) Interactions among genes in the ErbB-Neuregulin signalling network are associated with increased susceptibility to schizophrenia. Behav Brain Funct 3:31.

Bernstein HG, Lendeckel U, Bertram I, Bukowska A, Kanakis D, Dobrowolny H, Stauch R, Krell D, Mawrin C, Budinger E, Keilhoff G, Bogerts B (2006) Localization of neuregulin-1alpha (heregulin-alpha) and one of its receptors, ErbB-4 tyrosine kinase, in developing and adult human brain. Brain Res Bull 69:546-559.

Bertram I, Bernstein HG, Lendeckel U, Bukowska A, Dobrowolny H, Keilhoff G, Kanakis D, Mawrin C, Bielau H, Falkai P, Bogerts B (2007) Immunohistochemical evidence for impaired neuregulin-1 signaling in the prefrontal cortex in schizophrenia and in unipolar depression. Ann N Y Acad Sci 1096:147-156.

Birrell JM, Brown VJ (2000) Medial frontal cortex mediates perceptual attentional set shifting in the rat. J Neurosci 20:4320-4324.

Bjarnadottir M, Misner DL, Haverfield-Gross S, Bruun S, Helgason VG, Stefansson H, Sigmundsson A, Firth DR, Nielsen B, Stefansdottir R, Novak TJ, Stefansson K, Gurney ME, Andresson T (2007) Neuregulin1 (NRG1) signaling through Fyn modulates NMDA receptor phosphorylation: differential synaptic function in NRG1+/- knock-outs compared with wild-type mice. J Neurosci 27:4519-4529.

Boer S, Berk M, Dean B (2009) Levels of neuregulin 1 and 3 proteins in Brodmann's area 46 from subjects with schizophrenia and bipolar disorder. Neurosci Lett 466:27-29.

Boucher AA, Arnold JC, Duffy L, Schofield PR, Micheau J, Karl T (2007) Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of Delta9-tetrahydrocannabinol. Psychopharmacology (Berl) 192:325-336.

Brown AS, Cohen P, Harkavy-Friedman J, Babulas V, Malaspina D, Gorman JM, Susser ES (2001) A.E. Bennett Research Award. Prenatal rubella, premorbid abnormalities, and adult schizophrenia. Biol Psychiatry 49:473-486.

Buka SL, Tsuang MT, Torrey EF, Klebanoff MA, Bernstein D, Yolken RH (2001) Maternal infections and subsequent psychosis among offspring. Arch Gen Psychiatry 58:1032-1037.

Burnet PW, Anderson PN, Chen L, Nikiforova N, Harrison PJ, Wood MJ (2011) D-Amino acid oxidase knockdown in the mouse cerebellum reduces NR2A mRNA. Mol Cell Neurosci 46(1):167-75.

Bymaster FP, Katner JS, Nelson DL, Hemrick-Luecke SK, Threlkeld PG, Heiligenstein JH, Morin SM, Gehlert DR, Perry KW (2002) Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder. Neuropsychopharmacology 27:699-711.

Cannella B, Pitt D, Marchionni M, Raine CS (1999) Neuregulin and erbB receptor expression in normal and diseased human white matter. J Neuroimmunol 100:233-242.

Cannon TD, Kaprio J, Lonnqvist J, Huttunen M, Koskenvuo M (1998) The genetic epidemiology of schizophrenia in a Finnish twin cohort. A population-based modeling study. Arch Gen Psychiatry 55:67-74.

Cannon M, Walsh E, Hollis C, Kargin M, Taylor E, Murray RM, Jones PB (2001) Predictors of later schizophrenia and affective psychosis among attendees at a child psychiatry department. Br J Psychiatry 178:420-426.

Cardno AG, Marshall EJ, Coid B, Macdonald AM, Ribchester TR, Davies NJ, Venturi P, Jones LA, Lewis SW, Sham PC, Gottesman, II, Farmer AE, McGuffin P, Reveley AM, Murray RM (1999) Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. Arch Gen Psychiatry 56:162-168.

Carli M, Robbins TW, Evenden JL, Everitt BJ (1983) Effects of lesions to ascending noradrenergic neurones on performance of a 5-choice serial reaction task in rats; implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal. Behav Brain Res 9:361-380.

Carlsson A, Lindqvist M (1963) Effect of Chlorpromazine or Haloperidol on Formation of 3methoxytyramine and Normetanephrine in Mouse Brain. Acta Pharmacol Toxicol (Copenh) 20:140-144.

Carraway KL, 3rd, Weber JL, Unger MJ, Ledesma J, Yu N, Gassmann M, Lai C (1997) Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. Nature 387:512-516.

Caspi A, Moffitt TE, Cannon M, McClay J, Murray R, Harrington H, Taylor A, Arseneault L, Williams B, Braithwaite A, Poulton R, Craig IW (2005) Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. Biol Psychiatry 57:1117-1127.

Cearley CN, Wolfe JH (2006) Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. Mol Ther 13:528-537.

Chae KS, Martin-Caraballo M, Anderson M, Dryer SE (2005) Akt activation is necessary for growth factor-induced trafficking of functional K(Ca) channels in developing parasympathetic neurons. J Neurophysiol 93:1174-1182.

Chalecka-Franaszek E, Chuang DM (1999) Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. Proc Natl Acad Sci U S A 96:8745-8750.

Chamberlain SR, Del Campo N, Dowson J, Muller U, Clark L, Robbins TW, Sahakian BJ (2007) Atomoxetine improved response inhibition in adults with attention deficit/hyperactivity disorder. Biol Psychiatry 62:977-984.

Chana G, Lucero G, Salaria S, Lozach J, Du P, Woelk C, Everall I (2009) Upregulation of NRG-1 and VAMP-1 in human brain aggregates exposed to clozapine. Schizophr Res 113:273-276.

Chang Q, Fischbach GD (2006) An acute effect of neuregulin 1 beta to suppress alpha 7-containing nicotinic acetylcholine receptors in hippocampal interneurons. J Neurosci 26:11295-11303.

Chaudhury AR, Gerecke KM, Wyss JM, Morgan DG, Gordon MN, Carroll SL (2003) Neuregulin-1 and erbB4 immunoreactivity is associated with neuritic plaques in Alzheimer disease brain and in a transgenic model of Alzheimer disease. J Neuropathol Exp Neurol 62:42-54.

Chen MS, Bermingham-McDonogh O, Danehy FT, Jr., Nolan C, Scherer SS, Lucas J, Gwynne D, Marchionni MA (1994) Expression of multiple neuregulin transcripts in postnatal rat brains. J Comp Neurol 349:389-400.

Chen J, Kelz MB, Zeng G, Sakai N, Steffen C, Shockett PE, Picciotto MR, Duman RS, Nestler EJ (1998) Transgenic animals with inducible, targeted gene expression in brain. Mol Pharmacol 54:495-503.

Chen YJ, Johnson MA, Lieberman MD, Goodchild RE, Schobel S, Lewandowski N, Rosoklija G, Liu RC, Gingrich JA, Small S, Moore H, Dwork AJ, Talmage DA, Role LW (2008) Type III neuregulin-1 is required for normal sensorimotor gating, memory-related behaviors, and corticostriatal circuit components. J Neurosci 28:6872-6883.

Cheng QC, Tikhomirov O, Zhou W, Carpenter G (2003) Ectodomain cleavage of ErbB-4: characterization of the cleavage site and m80 fragment. J Biol Chem 278:38421-38427.

Chiorini JA, Afione S, Kotin RM (1999) Adeno-associated virus (AAV) type 5 Rep protein cleaves a unique terminal resolution site compared with other AAV serotypes. J Virol 73:4293-4298.

Chiorini JA, Kim F, Yang L, Kotin RM (1999) Cloning and characterization of adeno-associated virus type 5. J Virol 73:1309-1319.

Choi J, Young JA, Callaway EM Selective viral vector transduction of ErbB4 expressing cortical interneurons in vivo with a viral receptor-ligand bridge protein. Proc Natl Acad Sci U S A 107:16703-16708.

Chong VZ, Thompson M, Beltaifa S, Webster MJ, Law AJ, Weickert CS (2008) Elevated neuregulin-1 and ErbB4 protein in the prefrontal cortex of schizophrenic patients. Schizophr Res 100:270-280.

Christakou A, Robbins TW, Everitt BJ (2001) Functional disconnection of a prefrontal cortical-dorsal striatal system disrupts choice reaction time performance: implications for attentional function. Behav Neurosci 115:812-825.

Chudasama Y, Muir JL (2001) Visual attention in the rat: a role for the prelimbic cortex and thalamic nuclei? Behav Neurosci 115:417-428.

Chudasama Y, Passetti F, Rhodes SE, Lopian D, Desai A, Robbins TW (2003) Dissociable aspects of performance on the 5-choice serial reaction time task following lesions of the dorsal anterior cingulate, infralimbic and orbitofrontal cortex in the rat: differential effects on selectivity, impulsivity and compulsivity. Behav Brain Res 146:105-119.

Cochran SM, Fujimura M, Morris BJ, Pratt JA (2002) Acute and delayed effects of phencyclidine upon mRNA levels of markers of glutamatergic and GABAergic neurotransmitter function in the rat brain. Synapse 46:206-214.

Cochran SM, Kennedy M, McKerchar CE, Steward LJ, Pratt JA, Morris BJ (2003) Induction of metabolic hypofunction and neurochemical deficits after chronic intermittent exposure to phencyclidine: differential modulation by antipsychotic drugs. Neuropsychopharmacology 28:265-275.

Cole BJ, Robbins TW (1987) Amphetamine impairs the discriminative performance of rats with dorsal noradrenergic bundle lesions on a 5-choice serial reaction time task: new evidence for central dopaminergic-noradrenergic interactions. Psychopharmacology (Berl) 91:458-466.

Comery TA, Shah R, Greenough WT (1995) Differential rearing alters spine density on medium-sized spiny neurons in the rat corpus striatum: evidence for association of morphological plasticity with early response gene expression. Neurobiol Learn Mem 63:217-219.

Conrad CK, Allen SS, Afione SA, Reynolds TC, Beck SE, Fee-Maki M, Barrazza-Ortiz X, Adams R, Askin FB, Carter BJ, Guggino WB, Flotte TR (1996) Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. Gene Ther 3:658-668.

Crawley JC, Owens DG, Crow TJ, Poulter M, Johnstone EC, Smith T, Oldland SR, Veall N, Owen F, Zanelli GD (1986) Dopamine D2 receptors in schizophrenia studied in vivo. Lancet 2:224-225.

Croslan DR, Schoell MC, Ford GD, Pulliam JV, Gates A, Clement CM, Harris AE, Ford BD (2008) Neuroprotective effects of neuregulin-1 on B35 neuronal cells following ischemia. Brain Res 1210:39-47.

Culouscou JM, Carlton GW, Aruffo A (1995) HER4 receptor activation and phosphorylation of Shc proteins by recombinant heregulin-Fc fusion proteins. J Biol Chem 270:12857-12863.

Davis KL, Kahn RS, Ko G, Davidson M (1991) Dopamine in schizophrenia: a review and reconceptualization. Am J Psychiatry 148:1474-1486.

Dawson N, Thompson RJ, McVie A, Thomson DM, Morris BJ, Pratt JA Modafinil Reverses Phencyclidine-Induced Deficits in Cognitive Flexibility, Cerebral Metabolism, and Functional Brain Connectivity. Schizophr Bull.

de Backer MW, Fitzsimons CP, Brans MA, Luijendijk MC, Garner KM, Vreugdenhil E, Adan RA An adeno-associated viral vector transduces the rat hypothalamus and amygdala more efficient than a lentiviral vector. BMC Neurosci 11:81.

Deakin IH, Law AJ, Oliver PL, Schwab MH, Nave KA, Harrison PJ, Bannerman DM (2009) Behavioural characterization of neuregulin 1 type I overexpressing transgenic mice. Neuroreport 20:1523-1528.

Dean B, Sundram S, Bradbury R, Scarr E, Copolov D (2001) Studies on [3H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use. Neuroscience 103:9-15.

Deb TB, Coticchia CM, Barndt R, Zuo H, Dickson RB, Johnson MD (2008) Pregnancy-upregulated nonubiquitous calmodulin kinase induces ligand-independent EGFR degradation. Am J Physiol Cell Physiol 295:C365-377.

Ding W, Zhang L, Yan Z, Engelhardt JF (2005) Intracellular trafficking of adeno-associated viral vectors. Gene Ther 12:873-880.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007) Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 130:1146-1158.

Duffy L, Cappas E, Lai D, Boucher AA, Karl T Cognition in transmembrane domain neuregulin 1 mutant mice. Neuroscience 170:800-807.

Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, Fyfe J, Moullier P, Colle MA, Barkats M (2009) Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther 17:1187-1196.

Egerton A, Reid L, McKerchar CE, Morris BJ, Pratt JA (2005) Impairment in perceptual attentional set-shifting following PCP administration: a rodent model of set-shifting deficits in schizophrenia. Psychopharmacology (Berl) 179:77-84.

Egerton A, Reid L, McGregor S, Cochran SM, Morris BJ, Pratt JA (2008) Subchronic and chronic PCP treatment produces temporally distinct deficits in attentional set shifting and prepulse inhibition in rats. Psychopharmacology (Berl) 198:37-49.

Ehrlichman RS, Luminais SN, White SL, Rudnick ND, Ma N, Dow HC, Kreibich AS, Abel T, Brodkin ES, Hahn CG, Siegel SJ (2009) Neuregulin 1 transgenic mice display reduced mismatch negativity, contextual fear conditioning and social interactions. Brain Res 1294:116-127.

Eisinger DA, Ammer H (2011) Epidermal Growth Factor Treatment switches {delta}-Opioid Receptor-stimulated ERK1/2 Signaling from an EGF to an IGF-1 Receptor-dependent Mechanism. Mol Pharmacol 79(2):326-335.

Elenius K, Corfas G, Paul S, Choi CJ, Rio C, Plowman GD, Klagsbrun M (1997a) A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. J Biol Chem 272:26761-26768.

Elenius K, Paul S, Allison G, Sun J, Klagsbrun M (1997b) Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. Embo J 16:1268-1278.

Elenius K, Choi CJ, Paul S, Santiestevan E, Nishi E, Klagsbrun M (1999) Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase. Oncogene 18:2607-2615.

Elvevag B, Weinberger DR, Suter JC, Goldberg TE (2000) Continuous performance test and schizophrenia: a test of stimulus-response compatibility, working memory, response readiness, or none of the above? Am J Psychiatry 157:772-780.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nat Genet 36:131-137.

Emerit MB, Martres MP, Miquel MC, el Mestikawy S, Hamon M (1995) Differentiation alters the expression of the two splice variants of the serotonin 5-HT3 receptor-A mRNA in NG108-15 cells. J Neurochem 65:1917-1925.

Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW (1997) ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. Development 124:4999-5011.

Erles K, Sebokova P, Schlehofer JR (1999) Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). J Med Virol 59:406-411.

Fallon KB, Havlioglu N, Hamilton LH, Cheng TP, Carroll SL (2004) Constitutive activation of the neuregulin-1/erbB signaling pathway promotes the proliferation of a human peripheral neuroepithelioma cell line. J Neurooncol 66:273-284.

Falls DL (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 284:14-30.

Faulkner RL, Jang MH, Liu XB, Duan X, Sailor KA, Kim JY, Ge S, Jones EG, Ming GL, Song H, Cheng HJ (2008) Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain. Proc Natl Acad Sci U S A 105:14157-14162.

Feng Y, Wang XD, Guo CM, Yang Y, Li JT, Su YA, Si TM Expressions of neuregulin 1beta and ErbB4 in prefrontal cortex and hippocampus of a rat schizophrenia model induced by chronic MK-801 administration. J Biomed Biotechnol 2010:859516.

Fiddes RJ, Campbell DH, Janes PW, Sivertsen SP, Sasaki H, Wallasch C, Daly RJ (1998) Analysis of Grb7 recruitment by heregulin-activated erbB receptors reveals a novel target selectivity for erbB3. J Biol Chem 273:7717-7724.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Fisahn A, Neddens J, Yan L, Buonanno A (2009) Neuregulin-1 modulates hippocampal gamma oscillations: implications for schizophrenia. Cereb Cortex 19:612-618.

Flames N, Long JE, Garratt AN, Fischer TM, Gassmann M, Birchmeier C, Lai C, Rubenstein JL, Marin O (2004) Short- and long-range attraction of cortical GABAergic interneurons by neuregulin-1. Neuron 44:251-261.

Flannery JG, Zolotukhin S, Vaquero MI, LaVail MM, Muzyczka N, Hauswirth WW (1997) Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus. Proc Natl Acad Sci U S A 94:6916-6921.

Flores AI, Mallon BS, Matsui T, Ogawa W, Rosenzweig A, Okamoto T, Macklin WB (2000) Akt-mediated survival of oligodendrocytes induced by neuregulins. J Neurosci 20:7622-7630.

Flynn SW, Lang DJ, Mackay AL, Goghari V, Vavasour IM, Whittall KP, Smith GN, Arango V, Mann JJ, Dwork AJ, Falkai P, Honer WG (2003) Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. Mol Psychiatry 8:811-820.

Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK (2009) Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol 27:59-65.

Frey MR, Hilliard VC, Mullane MT, Polk DB ErbB4 promotes cyclooxygenase-2 expression and cell survival in colon epithelial cells. Lab Invest 90:1415-1424.

Frey MR, Edelblum KL, Mullane MT, Liang D, Polk DB (2009) The ErbB4 growth factor receptor is required for colon epithelial cell survival in the presence of TNF. Gastroenterology 136:217-226.

Frohnert PW, Stonecypher MS, Carroll SL (2003) Constitutive activation of the neuregulin-1/ErbB receptor signaling pathway is essential for the proliferation of a neoplastic Schwann cell line. Glia 43:104-118.

Fujikawa A, Chow JP, Shimizu H, Fukada M, Suzuki R, Noda M (2007) Tyrosine phosphorylation of ErbB4 is enhanced by PSD95 and repressed by protein tyrosine phosphatase receptor type Z. J Biochem 142:343-350.

Gambarotta G, Garzotto D, Destro E, Mautino B, Giampietro C, Cutrupi S, Dati C, Cattaneo E, Fasolo A, Perroteau I (2004) ErbB4 expression in neural progenitor cells (ST14A) is necessary to mediate neuregulin-1beta1-induced migration. J Biol Chem 279:48808-48816.

Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 99:11854-11859.

Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM (2004) Clades of Adeno-associated viruses are widely disseminated in human tissues. J Virol 78:6381-6388.

Garcia RA, Vasudevan K, Buonanno A (2000) The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. Proc Natl Acad Sci U S A 97:3596-3601.

Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378:390-394.

Gendron L, Oligny JF, Payet MD, Gallo-Payet N (2003) Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells. J Biol Chem 278:3606-3614.

Gerecke KM, Wyss JM, Karavanova I, Buonanno A, Carroll SL (2001) ErbB transmembrane tyrosine kinase receptors are differentially expressed throughout the adult rat central nervous system. J Comp Neurol 433:86-100.

Gerlai R, Pisacane P, Erickson S (2000) Heregulin, but not ErbB2 or ErbB3, heterozygous mutant mice exhibit hyperactivity in multiple behavioral tasks. Behav Brain Res 109:219-227.

Geyer MA, Vollenweider FX (2008) Serotonin research: contributions to understanding psychoses. Trends Pharmacol Sci 29:445-453.

Ghahary A, Cheng KW (1989) Characterization of muscarinic acetylcholine receptors on intact neuroblastoma x glioma NG108-15 cell upon induced differentiation. Mol Cell Biochem 86:181-188.

Ghashghaei HT, Weber J, Pevny L, Schmid R, Schwab MH, Lloyd KC, Eisenstat DD, Lai C, Anton ES (2006) The role of neuregulin-ErbB4 interactions on the proliferation and organization of cells in the subventricular zone. Proc Natl Acad Sci U S A 103:1930-1935.

Gilbertson R, Hernan R, Pietsch T, Pinto L, Scotting P, Allibone R, Ellison D, Perry R, Pearson A, Lunec J (2001) Novel ERBB4 juxtamembrane splice variants are frequently expressed in childhood medulloblastoma. Genes Chromosomes Cancer 31:288-294.

Goldman-Rakic PS, Muly EC, 3rd, Williams GV (2000) D(1) receptors in prefrontal cells and circuits. Brain Res Brain Res Rev 31:295-301.

Golub MS, Germann SL, Lloyd KC (2004) Behavioral characteristics of a nervous system-specific erbB4 knock-out mouse. Behav Brain Res 153:159-170.

Gorzalka S, Vittori S, Volpini R, Cristalli G, von Kugelgen I, Muller CE (2005) Evidence for the functional expression and pharmacological characterization of adenine receptors in native cells and tissues. Mol Pharmacol 67:955-964.

Gothelf D, Feinstein C, Thompson T, Gu E, Penniman L, Van Stone E, Kwon H, Eliez S, Reiss AL (2007) Risk factors for the emergence of psychotic disorders in adolescents with 22q11.2 deletion syndrome. Am J Psychiatry 164:663-669.

Gottesman, II, McGuffin P, Farmer AE (1987) Clinical genetics as clues to the "real" genetics of schizophrenia (a decade of modest gains while playing for time). Schizophr Bull 13:23-47.

Granon S, Passetti F, Thomas KL, Dalley JW, Everitt BJ, Robbins TW (2000) Enhanced and impaired attentional performance after infusion of D1 dopaminergic receptor agents into rat prefrontal cortex. J Neurosci 20:1208-1215.

Graus-Porta D, Beerli RR, Daly JM, Hynes NE (1997) ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. Embo J 16:1647-1655.

Green MF (1996) What are the functional consequences of neurocognitive deficits in schizophrenia? Am J Psychiatry 153:321-330.

Green MF (2006) Cognitive impairment and functional outcome in schizophrenia and bipolar disorder. J Clin Psychiatry 67 Suppl 9:3-8; discussion 36-42.

Grimm D, Kay MA (2003) From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. Curr Gene Ther 3:281-304.

Gu Z, Jiang Q, Fu AK, Ip NY, Yan Z (2005) Regulation of NMDA receptors by neuregulin signaling in prefrontal cortex. J Neurosci 25:4974-4984.

Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL, 3rd (1994) Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A 91:8132-8136.

Hahn B, Shoaib M, Stolerman IP (2002) Nicotine-induced enhancement of attention in the five-choice serial reaction time task: the influence of task demands. Psychopharmacology (Berl) 162:129-137.

Hahn CG, Wang HY, Cho DS, Talbot K, Gur RE, Berrettini WH, Bakshi K, Kamins J, Borgmann-Winter KE, Siegel SJ, Gallop RJ, Arnold SE (2006) Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. Nat Med 12:824-828.

Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, Haroutunian V, Fienberg AA (2001) Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A 98:4746-4751.

Hall W, Solowij N (1998) Adverse effects of cannabis. Lancet 352:1611-1616.

Hall J, Whalley HC, Job DE, Baig BJ, McIntosh AM, Evans KL, Thomson PA, Porteous DJ, Cunningham-Owens DG, Johnstone EC, Lawrie SM (2006) A neuregulin 1 variant associated with abnormal cortical function and psychotic symptoms. Nat Neurosci 9:1477-1478.

Hamprecht B, Glaser T, Reiser G, Bayer E, Propst F (1985) Culture and characteristics of hormone-responsive neuroblastoma X glioma hybrid cells. Methods Enzymol 109:316-341.

Hancock ML, Canetta SE, Role LW, Talmage DA (2008) Presynaptic type III neuregulin1-ErbB signaling targets {alpha}7 nicotinic acetylcholine receptors to axons. J Cell Biol 181:511-521.

Harari D, Tzahar E, Romano J, Shelly M, Pierce JH, Andrews GC, Yarden Y (1999) Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. Oncogene 18:2681-2689.

Harrison PJ (1999) The neuropathology of schizophrenia. A critical review of the data and their interpretation. Brain 122 (Pt 4):593-624.

Harrison PJ, Weinberger DR (2005) Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol Psychiatry 10:40-68; image 45.

Harvey PD, Keefe RS (2001) Studies of cognitive change in patients with schizophrenia following novel antipsychotic treatment. Am J Psychiatry 158:176-184.

Hashimoto R, Straub RE, Weickert CS, Hyde TM, Kleinman JE, Weinberger DR (2004) Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. Mol Psychiatry 9:299-307.

Hauck B, Zhao W, High K, Xiao W (2004) Intracellular viral processing, not single-stranded DNA accumulation, is crucial for recombinant adeno-associated virus transduction. J Virol 78:13678-13686.

Heiss A, Ammer H, Eisinger DA (2009) delta-Opioid receptor-stimulated Akt signaling in neuroblastoma x glioma (NG108-15) hybrid cells involves receptor tyrosine kinase-mediated PI3K activation. Exp Cell Res 315:2115-2125.

Hellyer NJ, Mantilla CB, Park EW, Zhan WZ, Sieck GC (2006) Neuregulin-dependent protein synthesis in C2C12 myotubes and rat diaphragm muscle. Am J Physiol Cell Physiol 291:C1056-1061.

Henquet C, Murray R, Linszen D, van Os J (2005) The environment and schizophrenia: the role of cannabis use. Schizophr Bull 31:608-612.

Hermonat PL, Muzyczka N (1984) Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. Proc Natl Acad Sci U S A 81:6466-6470.

Hille C, Bate S, Davis J, Gonzalez MI (2008) 5-HT4 receptor agonism in the five-choice serial reaction time task. Behav Brain Res 195:180-186.

Ho BY, Zhao J (1996) Determination of the cannabinoid receptors in mouse x rat hybridoma NG108-15 cells and rat GH4C1 cells. Neurosci Lett 212:123-126.

Hoek HW, Brown AS, Susser E (1998) The Dutch famine and schizophrenia spectrum disorders. Soc Psychiatry Psychiatr Epidemiol 33:373-379.

Hof PR, Haroutunian V, Copland C, Davis KL, Buxbaum JD (2002) Molecular and cellular evidence for an oligodendrocyte abnormality in schizophrenia. Neurochem Res 27:1193-1200.

Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, et al. (1992) Identification of heregulin, a specific activator of p185erbB2. Science 256:1205-1210.

Homberg JR, Pattij T, Janssen MC, Ronken E, De Boer SF, Schoffelmeer AN, Cuppen E (2007) Serotonin transporter deficiency in rats improves inhibitory control but not behavioural flexibility. Eur J Neurosci 26:2066-2073.

Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ (2003) Local gene knockdown in the brain using viral-mediated RNA interference. Nat Med 9:1539-1544.

Horie T, Ono K, Nishi H, Nagao K, Kinoshita M, Watanabe S, Kuwabara Y, Nakashima Y, Takanabe-Mori R, Nishi E, Hasegawa K, Kita T, Kimura T Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. Cardiovasc Res 87:656-664.

Howes OD, Kapur S (2009) The dopamine hypothesis of schizophrenia: version III--the final common pathway. Schizophr Bull 35:549-562.

Hoyer D (2007) RNA interference for studying the molecular basis of neuropsychiatric disorders. Curr Opin Drug Discov Devel 10:122-129.

Hoyle E, Genn RF, Fernandes C, Stolerman IP (2006) Impaired performance of alpha7 nicotinic receptor knockout mice in the five-choice serial reaction time task. Psychopharmacology (Berl) 189:211-223.

Huang YZ, Won S, Ali DW, Wang Q, Tanowitz M, Du QS, Pelkey KA, Yang DJ, Xiong WC, Salter MW, Mei L (2000) Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. Neuron 26:443-455.

Humby T, Wilkinson L, Dawson G (2005) Assaying aspects of attention and impulse control in mice using the 5-choice serial reaction time task. Curr Protoc Neurosci Chapter 8:Unit 8 5H.

Ide M, Ohnishi T, Murayama M, Matsumoto I, Yamada K, Iwayama Y, Dedova I, Toyota T, Asada T, Takashima A, Yoshikawa T (2006) Failure to support a genetic contribution of AKT1 polymorphisms and altered AKT signaling in schizophrenia. J Neurochem 99:277-287.

Inagaki K, Fuess S, Storm TA, Gibson GA, McTiernan CF, Kay MA, Nakai H (2006) Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol Ther 14:45-53.

Janowsky DS, Risch C (1979) Amphetamine psychosis and psychotic symptoms. Psychopharmacology (Berl) 65:73-77.

Johnstone EC, Crow TJ, Frith CD, Husband J, Kreel L (1976) Cerebral ventricular size and cognitive impairment in chronic schizophrenia. Lancet 2:924-926.

Jones CA, Brown AM, Auer DP, Fone KC (2010) The mGluR2/3 agonist LY379268 reverses post-weaning social isolation-induced recognition memory deficits in the rat. Psychopharmacology (Berl). *In press*

Jones FE, Jerry DJ, Guarino BC, Andrews GC, Stern DF (1996) Heregulin induces in vivo proliferation and differentiation of mammary epithelium into secretory lobuloalveoli. Cell Growth Differ 7:1031-1038.

Jones JT, Akita RW, Sliwkowski MX (1999) Binding specificities and affinities of egf domains for ErbB receptors. FEBS Lett 447:227-231.

Jones PB, Barnes TR, Davies L, Dunn G, Lloyd H, Hayhurst KP, Murray RM, Markwick A, Lewis SW (2006) Randomized controlled trial of the effect on Quality of Life of second- vs first-generation antipsychotic drugs in schizophrenia: Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS 1). Arch Gen Psychiatry 63:1079-1087.

Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, Ozeki Y, Sawamura N, Park U, Kudo C, Okawa M, Ross CA, Hatten ME, Nakajima K, Sawa A (2005) A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. Nat Cell Biol 7:1167-1178.

Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (1994) Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat Genet 8:148-154.

Kato T, Abe Y, Sotoyama H, Kakita A, Kominami R, Hirokawa S, Ozaki M, Takahashi H, Nawa H (2010) Transient exposure of neonatal mice to neuregulin-1 results in hyperdopaminergic states in adulthood: implication in neurodevelopmental hypothesis for schizophrenia. Mol Psychiatry. *In press*

Keefe RS, Bilder RM, Davis SM, Harvey PD, Palmer BW, Gold JM, Meltzer HY, Green MF, Capuano G, Stroup TS, McEvoy JP, Swartz MS, Rosenheck RA, Perkins DO, Davis CE, Hsiao JK, Lieberman JA (2007) Neurocognitive effects of antipsychotic medications in patients with chronic schizophrenia in the CATIE Trial. Arch Gen Psychiatry 64:633-647.

Kelly DL, Buchanan RW, Boggs DL, McMahon RP, Dickinson D, Nelson M, Gold JM, Ball MP, Feldman S, Liu F, Conley RR (2009) A randomized double-blind trial of atomoxetine for cognitive impairments in 32 people with schizophrenia. J Clin Psychiatry 70:518-525.

Kerber G, Streif R, Schwaiger FW, Kreutzberg GW, Hager G (2003) Neuregulin-1 isoforms are differentially expressed in the intact and regenerating adult rat nervous system. J Mol Neurosci 21:149-165.

Keri S, Seres I, Kelemen O, Benedek G (2009) Neuregulin 1-stimulated phosphorylation of AKT in psychotic disorders and its relationship with neurocognitive functions. Neurochem Int 55:606-609.

Kilian P, Campbell S, Bilodeau L, Guimond MO, Roberge C, Gallo-Payet N, Payet MD (2008) Angiotensin II type 2 receptor stimulation increases the rate of NG108-15 cell migration via actin depolymerization. Endocrinology 149:2923-2933.

Kirkby DL, Higgins GA (1998) Characterization of perforant path lesions in rodent models of memory and attention. Eur J Neurosci 10:823-838.

Kirov G, Gumus D, Chen W, Norton N, Georgieva L, Sari M, O'Donovan MC, Erdogan F, Owen MJ, Ropers HH, Ullmann R (2008) Comparative genome hybridization suggests a role for NRXN1 and APBA2 in schizophrenia. Hum Mol Genet 17:458-465.

Klee WA, Nirenberg M (1974) A neuroblastoma times glioma hybrid cell line with morphine receptors. Proc Natl Acad Sci U S A 71:3474-3477.

Klein RL, Dayton RD, Tatom JB, Henderson KM, Henning PP (2008) AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. Mol Ther 16:89-96.

Komurasaki T, Toyoda H, Uchida D, Morimoto S (1997) Epiregulin binds to epidermal growth factor receptor and ErbB-4 and induces tyrosine phosphorylation of epidermal growth factor receptor, ErbB-2, ErbB-3 and ErbB-4. Oncogene 15:2841-2848.

Konrad A, Winterer G (2008) Disturbed structural connectivity in schizophrenia primary factor in pathology or epiphenomenon? Schizophr Bull 34:72-92.

Konrad A, Vucurevic G, Musso F, Stoeter P, Dahmen N, Winterer G (2009) ErbB4 genotype predicts left frontotemporal structural connectivity in human brain. Neuropsychopharmacology 34:641-650.

Kotin RM, Siniscalco M, Samulski RJ, Zhu XD, Hunter L, Laughlin CA, McLaughlin S, Muzyczka N, Rocchi M, Berns KI (1990) Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A 87:2211-2215.

Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA (1989) Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci U S A 86:9193-9197.

Krystal JH, Karper LP, Seibyl JP, Freeman GK, Delaney R, Bremner JD, Heninger GR, Bowers MB, Jr., Charney DS (1994) Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. Arch Gen Psychiatry 51:199-214.

Kuramochi Y, Cote GM, Guo X, Lebrasseur NK, Cui L, Liao R, Sawyer DB (2004) Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. J Biol Chem 279:51141-51147.

Kwon OB, Longart M, Vullhorst D, Hoffman DA, Buonanno A (2005) Neuregulin-1 reverses long-term potentiation at CA1 hippocampal synapses. J Neurosci 25:9378-9383.

Kwon OB, Paredes D, Gonzalez CM, Neddens J, Hernandez L, Vullhorst D, Buonanno A (2008) Neuregulin-1 regulates LTP at CA1 hippocampal synapses through activation of dopamine D4 receptors. Proc Natl Acad Sci U S A 105:15587-15592.

Laflamme L, Brechler V, Reudelhuber TL, Gallo-Payet N, Deschepper CF (1998) The renin-angiotensin system in hybrid NG108-15 cells. Renin gene is from mouse neuroblastoma, angiotensinogen and angiotensin-converting enzyme genes are of rat glioma origin. Regul Pept 77:9-15.

Lahti AC, Weiler MA, Tamara Michaelidis BA, Parwani A, Tamminga CA (2001) Effects of ketamine in normal and schizophrenic volunteers. Neuropsychopharmacology 25:455-467.

Law AJ, Deakin JF (2001) Asymmetrical reductions of hippocampal NMDAR1 glutamate receptor mRNA in the psychoses. Neuroreport 12:2971-2974.

Law AJ, Shannon Weickert C, Hyde TM, Kleinman JE, Harrison PJ (2004) Neuregulin-1 (NRG-1) mRNA and protein in the adult human brain. Neuroscience 127:125-136.

Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, Harrison PJ, Kleinman JE, Weinberger DR (2006) Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5' SNPs associated with the disease. Proc Natl Acad Sci U S A 103:6747-6752.

Law AJ, Kleinman JE, Weinberger DR, Weickert CS (2007) Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. Hum Mol Genet 16:129-141.

Law AJ, Wang Y, Sei Y, Papaleo F, Huang W, Thomas CJ, Harrison PJ, Lipska BK, Hyde TM, Kleinman JE, Weinberger DR NRG1-ErbB4-p110δ signaling in schizophrenia. Reversal of amphetamine-induced behaviors by selective p110δ inhibition. J Clin Invest. *In press*

Lawlor PA, Bland RJ, Mouravlev A, Young D, During MJ (2009) Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. Mol Ther 17:1692-1702.

Lawrie SM, Abukmeil SS (1998) Brain abnormality in schizophrenia. A systematic and quantitative review of volumetric magnetic resonance imaging studies. Br J Psychiatry 172:110-120.

Lewis DA (2000) GABAergic local circuit neurons and prefrontal cortical dysfunction in schizophrenia. Brain Res Brain Res Rev 31:270-276.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, Williams NM, Schwab SG, Pulver AE, Faraone SV, Brzustowicz LM, Kaufmann CA, Garver DL, Gurling HM, Lindholm E, Coon H, Moises HW, Byerley W, Shaw SH, Mesen A, Sherrington R, O'Neill FA, Walsh D, Kendler KS, Ekelund J, Paunio T, Lonnqvist J, Peltonen L, O'Donovan MC, Owen MJ, Wildenauer DB, Maier W, Nestadt G, Blouin JL, Antonarakis SE, Mowry BJ, Silverman JM, Crowe RR, Cloninger CR, Tsuang MT, Malaspina D, Harkavy-Friedman JM, Svrakic DM, Bassett AS, Holcomb J, Kalsi G, McQuillin A, Brynjolfson J, Sigmundsson T, Petursson H, Jazin E, Zoega T, Helgason T (2003) Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. Am J Hum Genet 73:34-48.

Li Y, Tennekoon GI, Birnbaum M, Marchionni MA, Rutkowski JL (2001) Neuregulin signaling through a PI3K/Akt/Bad pathway in Schwann cell survival. Mol Cell Neurosci 17:761-767.

Li L, Cleary S, Mandarano MA, Long W, Birchmeier C, Jones FE (2002) The breast proto-oncogene, HRGalpha regulates epithelial proliferation and lobuloalveolar development in the mouse mammary gland. Oncogene 21:4900-4907.

Li B, Woo RS, Mei L, Malinow R (2007) The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity. Neuron 54:583-597.

Lieberman JA, Stroup TS, McEvoy JP, Swartz MS, Rosenheck RA, Perkins DO, Keefe RS, Davis SM, Davis CE, Lebowitz BD, Severe J, Hsiao JK (2005) Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. N Engl J Med 353:1209-1223.

Limosin F, Rouillon F, Payan C, Cohen JM, Strub N (2003) Prenatal exposure to influenza as a risk factor for adult schizophrenia. Acta Psychiatr Scand 107:331-335.

Linggi B, Carpenter G (2006) ErbB-4 s80 intracellular domain abrogates ETO2-dependent transcriptional repression. J Biol Chem 281:25373-25380.

Lipshutz GS, Titre D, Brindle M, Bisconte AR, Contag CH, Gaensler KM (2003) Comparison of gene expression after intraperitoneal delivery of AAV2 or AAV5 in utero. Mol Ther 8:90-98.

Lipska BK, Lerman DN, Khaing ZZ, Weickert CS, Weinberger DR (2003) Gene expression in dopamine and GABA systems in an animal model of schizophrenia: effects of antipsychotic drugs. Eur J Neurosci 18:391-402.

Liu Y, Ford B, Mann MA, Fischbach GD (2001) Neuregulins increase alpha7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus. J Neurosci 21:5660-5669.

Loeb JA, Fischbach GD (1995) ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. J Cell Biol 130:127-135.

Lok J, Sardi SP, Guo S, Besancon E, Ha DM, Rosell A, Kim WJ, Corfas G, Lo EH (2009) Neuregulin-1 signaling in brain endothelial cells. J Cereb Blood Flow Metab 29:39-43.

Lominac KD, Oleson EB, Pava M, Klugmann M, Schwarz MK, Seeburg PH, During MJ, Worley PF, Kalivas PW, Szumlinski KK (2005) Distinct roles for different Homer1 isoforms in behaviors and associated prefrontal cortex function. J Neurosci 25:11586-11594.

Lopez-Bendito G, Cautinat A, Sanchez JA, Bielle F, Flames N, Garratt AN, Talmage DA, Role LW, Charnay P, Marin O, Garel S (2006) Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. Cell 125:127-142.

Luby ED, Cohen BD, Rosenbaum G, Gottlieb JS, Kelley R (1959) Study of a new schizophrenomimetic drug; sernyl. AMA Arch Neurol Psychiatry 81:363-369.

Ma W, Pancrazio JJ, Coulombe M, Dumm J, Sathanoori R, Barker JL, Kowtha VC, Stenger DA, Hickman JJ (1998) Neuronal and glial epitopes and transmitter-synthesizing enzymes appear in parallel with membrane excitability during neuroblastoma x glioma hybrid differentiation. Brain Res Dev Brain Res 106:155-163.

Ma YJ, Hill DF, Creswick KE, Costa ME, Cornea A, Lioubin MN, Plowman GD, Ojeda SR (1999) Neuregulins signaling via a glial erbB-2-erbB-4 receptor complex contribute to the neuroendocrine control of mammalian sexual development. J Neurosci 19:9913-9927.

Määttä JA, Sundvall M, Junttila TT, Peri L, Laine VJ, Isola J, Egeblad M, Elenius K (2006) Proteolytic cleavage and phosphorylation of a tumor-associated ErbB4 isoform promote ligand-independent survival and cancer cell growth. Mol Biol Cell 17:67-79.

Mackay AV, Iversen LL, Rossor M, Spokes E, Bird E, Arregui A, Creese I, Synder SH (1982) Increased brain dopamine and dopamine receptors in schizophrenia. Arch Gen Psychiatry 39:991-997.

Mangalore R, Knapp M (2007) Cost of schizophrenia in England. J Ment Health Policy Econ 10:23-41.

Mao Y, Ge X, Frank CL, Madison JM, Koehler AN, Doud MK, Tassa C, Berry EM, Soda T, Singh KK, Biechele T, Petryshen TL, Moon RT, Haggarty SJ, Tsai LH (2009) Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. Cell 136:1017-1031.

Marder SR, Fenton W, Youens K (2004) Schizophrenia, IX: Cognition in schizophrenia--the MATRICS initiative. Am J Psychiatry 161:25.

Martin-Montanez E, Lopez-Tellez JF, Acevedo MJ, Pavia J, Khan ZU Efficiency of gene transfection reagents in NG108-15, SH-SY5Y and CHO-K1 cell lines. Methods Find Exp Clin Pharmacol 32:291-297.

Mastakov MY, Baer K, Xu R, Fitzsimons H, During MJ (2001) Combined injection of rAAV with mannitol enhances gene expression in the rat brain. Mol Ther 3:225-232.

Matsushima K, Imanishi T, Asano H, Funakami Y, Wada T, Ichida S Changes in characteristics of the specific binding of [3H]LY-278584, a 5-HT3-receptor antagonist, on differentiated NG108-15 cells. J Pharmacol Sci 113:281-284.

McGrath J, Saha S, Chant D, Welham J (2008) Schizophrenia: a concise overview of incidence, prevalence, and mortality. Epidemiol Rev 30:67-76.

McKee AG, Loscher JS, O'Sullivan NC, Chadderton N, Palfi A, Batti L, Sheridan GK, O'Shea S, Moran M, McCabe O, Fernandez AB, Pangalos MN, O'Connor JJ, Regan CM, O'Connor WT, Humphries P, Farrar GJ, Murphy KJ AAV-mediated chronic over-expression of SNAP-25 in adult rat dorsal hippocampus impairs memory-associated synaptic plasticity. J Neurochem 112:991-1004.

Mechawar N, Lacoste B, Yu WF, Srivastava LK, Quirion R (2007) Developmental profile of neuregulin receptor ErbB4 in postnatal rat cerebral cortex and hippocampus. Neuroscience 148:126-139.

Mei L, Xiong WC (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nat Rev Neurosci 9:437-452.

Meltzer HY (2002) Suicidality in schizophrenia: a review of the evidence for risk factors and treatment options. Curr Psychiatry Rep 4:279-283.

Meyers C, Mane M, Kokorina N, Alam S, Hermonat PL (2000) Ubiquitous human adeno-associated virus type 2 autonomously replicates in differentiating keratinocytes of a normal skin model. Virology 272:338-346.

Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA, Devon RS, St Clair DM, Muir WJ, Blackwood DH, Porteous DJ (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. Hum Mol Genet 9:1415-1423.

Miller DG, Petek LM, Russell DW (2004) Adeno-associated virus vectors integrate at chromosome breakage sites. Nat Genet 36:767-773.

Mingozzi F, Schuttrumpf J, Arruda VR, Liu Y, Liu YL, High KA, Xiao W, Herzog RW (2002) Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. J Virol 76:10497-10502.

Mohn AR, Gainetdinov RR, Caron MG, Koller BH (1999) Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell 98:427-436.

Moore H, Jentsch JD, Ghajarnia M, Geyer MA, Grace AA (2006) A neurobehavioral systems analysis of adult rats exposed to methylazoxymethanol acetate on E17: implications for the neuropathology of schizophrenia. Biol Psychiatry 60:253-264.

Mori S, Wang L, Takeuchi T, Kanda T (2004) Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. Virology 330:375-383.

Morris JA, Kandpal G, Ma L, Austin CP (2003) DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation. Hum Mol Genet 12:1591-1608.

Muir JL, Everitt BJ, Robbins TW (1996) The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex lesions on a five-choice serial reaction time task. Cereb Cortex 6:470-481.

Muramatsu S, Mizukami H, Young NS, Brown KE (1996) Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology 221:208-217.

Murphy KC (2002) Schizophrenia and velo-cardio-facial syndrome. Lancet 359:426-430.

Nagasawa K, Tarui T, Yoshida S, Sekiguchi F, Matsunami M, Ohi A, Fukami K, Ichida S, Nishikawa H, Kawabata A (2009) Hydrogen sulfide evokes neurite outgrowth and expression of high-voltage-activated Ca2+ currents in NG108-15 cells: involvement of T-type Ca2+ channels. J Neurochem 108:676-684.

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell 2:279-289.

Nathanson JL, Yanagawa Y, Obata K, Callaway EM (2009) Preferential labeling of inhibitory and excitatory cortical neurons by endogenous tropism of adeno-associated virus and lentivirus vectors. Neuroscience 161:441-450.

Navarra R, Graf R, Huang Y, Logue S, Comery T, Hughes Z, Day M (2008) Effects of atomoxetine and methylphenidate on attention and impulsivity in the 5-choice serial reaction time test. Prog Neuropsychopharmacol Biol Psychiatry 32:34-41.

Neddens J, Buonanno A Selective populations of hippocampal interneurons express ErbB4 and their number and distribution is altered in ErbB4 knockout mice. Hippocampus 20:724-744.

Ni CY, Murphy MP, Golde TE, Carpenter G (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 294:2179-2181.

Nicodemus KK, Law AJ, Radulescu E, Luna A, Kolachana B, Vakkalanka R, Rujescu D, Giegling I, Straub RE, McGee K, Gold B, Dean M, Muglia P, Callicott JH, Tan HY, Weinberger DR Biological validation of increased schizophrenia risk with NRG1, ERBB4, and AKT1 epistasis via functional neuroimaging in healthy controls. Arch Gen Psychiatry 67:991-1001.

Norton N, Williams HJ, Williams NM, Spurlock G, Zammit S, Jones G, Jones S, Owen R, O'Donovan MC, Owen MJ (2003) Mutation screening of the Homer gene family and association analysis in schizophrenia. Am J Med Genet B Neuropsychiatr Genet 120B:18-21.

Norton N, Moskvina V, Morris DW, Bray NJ, Zammit S, Williams NM, Williams HJ, Preece AC, Dwyer S, Wilkinson JC, Spurlock G, Kirov G, Buckland P, Waddington JL, Gill M, Corvin AP, Owen MJ, O'Donovan MC (2006) Evidence that interaction between neuregulin 1 and its receptor erbB4 increases susceptibility to schizophrenia. Am J Med Genet B Neuropsychiatr Genet 141B:96-101.

Nuechterlein KH, Barch DM, Gold JM, Goldberg TE, Green MF, Heaton RK (2004) Identification of separable cognitive factors in schizophrenia. Schizophr Res 72:29-39.

Numakawa T, Yagasaki Y, Ishimoto T, Okada T, Suzuki T, Iwata N, Ozaki N, Taguchi T, Tatsumi M, Kamijima K, Straub RE, Weinberger DR, Kunugi H, Hashimoto R (2004) Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. Hum Mol Genet 13:2699-2708.

O'Callaghan JP (1988) Neurotypic and gliotypic proteins as biochemical markers of neurotoxicity. Neurotoxicol Teratol 10:445-452.

O'Donovan MC, Craddock N, Norton N, Williams H, Peirce T, Moskvina V, Nikolov I, Hamshere M, Carroll L, Georgieva L, Dwyer S, Holmans P, Marchini JL, Spencer CC, Howie B, Leung HT, Hartmann AM, Moller HJ, Morris DW, Shi Y, Feng G, Hoffmann P, Propping P, Vasilescu C, Maier W, Rietschel M, Zammit S, Schumacher J, Quinn EM, Schulze TG, Williams NM, Giegling I, Iwata N, Ikeda M, Darvasi A, Shifman S, He L, Duan J, Sanders AR, Levinson DF, Gejman PV, Cichon S, Nothen MM, Gill M, Corvin A, Rujescu D, Kirov G, Owen MJ, Buccola NG, Mowry BJ, Freedman R, Amin F, Black DW, Silverman JM, Byerley WF, Cloninger CR (2008) Identification of loci associated with schizophrenia by genome-wide association and follow-up. Nat Genet 40:1053-1055.

O'Tuathaigh CM, Babovic D, O'Sullivan GJ, Clifford JJ, Tighe O, Croke DT, Harvey R, Waddington JL (2007) Phenotypic characterization of spatial cognition and social behavior in mice with 'knockout' of the schizophrenia risk gene neuregulin 1. Neuroscience 147:18-27.

Ohkuma S, Katsura M, Chen DZ, Chen SH, Kuriyama K (1994) Presence of N-methyl-D-aspartate (NMDA) receptors in neuroblastoma x glioma hybrid NG108-15 cells-analysis using [45Ca2+]influx and [3H]MK-801 binding as functional measures. Brain Res Mol Brain Res 22:166-172.

Oishi K, Watatani K, Itoh Y, Okano H, Guillemot F, Nakajima K, Gotoh Y (2009) Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. Proc Natl Acad Sci U S A 106:13064-13069.

Okada M, Corfas G (2004) Neuregulin1 downregulates postsynaptic GABAA receptors at the hippocampal inhibitory synapse. Hippocampus 14:337-344.

Omerovic J, Puggioni EM, Napoletano S, Visco V, Fraioli R, Frati L, Gulino A, Alimandi M (2004) Ligand-regulated association of ErbB-4 to the transcriptional co-activator YAP65 controls transcription at the nuclear level. Exp Cell Res 294:469-479.

Ozaki M, Sasner M, Yano R, Lu HS, Buonanno A (1997) Neuregulin-beta induces expression of an NMDA-receptor subunit. Nature 390:691-694.

Paine TA, Tomasiewicz HC, Zhang K, Carlezon WA, Jr. (2007) Sensitivity of the five-choice serial reaction time task to the effects of various psychotropic drugs in Sprague-Dawley rats. Biol Psychiatry 62:687-693.

Passetti F, Chudasama Y, Robbins TW (2002) The frontal cortex of the rat and visual attentional performance: dissociable functions of distinct medial prefrontal subregions. Cereb Cortex 12:1254-1268.

Paterson NE, Ricciardi J, Wetzler C, Hanania (2011) T Sub-optimal performance in the 5-choice serial reaction time task in rats was sensitive to methylphenidate, atomoxetine and d-amphetamine, but unaffected by the COMT inhibitor tolcapone. Neurosci Res 69(1):41-50.

Pattij T, Janssen MC, Loos M, Smit AB, Schoffelmeer AN, van Gaalen MM (2007) Strain specificity and cholinergic modulation of visuospatial attention in three inbred mouse strains. Genes Brain Behav 6:579-587.

Paylor R, McIlwain KL, McAninch R, Nellis A, Yuva-Paylor LA, Baldini A, Lindsay EA (2001) Mice deleted for the DiGeorge/velocardiofacial syndrome region show abnormal sensorimotor gating and learning and memory impairments. Hum Mol Genet 10:2645-2650.

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates 6th Edition. Academic Press

Pedersen CB, Mortensen PB (2001) Evidence of a dose-response relationship between urbanicity during upbringing and schizophrenia risk. Arch Gen Psychiatry 58:1039-1046.

Pitcher GM, Beggs S, Woo RS, Mei L, Salter MW (2008) ErbB4 is a suppressor of long-term potentiation in the adult hippocampus. Neuroreport 19:139-143.

Plowman GD, Green JM, Culouscou JM, Carlton GW, Rothwell VM, Buckley S (1993) Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. Nature 366:473-475.

Poyurovsky M, Glick I, Koran LM Lamotrigine augmentation in schizophrenia and schizoaffective patients with obsessive-compulsive symptoms. J Psychopharmacol 24:861-866.

Prevot V, Rio C, Cho GJ, Lomniczi A, Heger S, Neville CM, Rosenthal NA, Ojeda SR, Corfas G (2003) Normal female sexual development requires neuregulin-erbB receptor signaling in hypothalamic astrocytes. J Neurosci 23:230-239.

Pun S, Yang JF, Ng YP, Tsim KW (1997) NG108-15 cells express neuregulin that induces AChR alpha-subunit synthesis in cultured myotubes. FEBS Lett 418:275-281.

Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, Sullivan PF, Sklar P (2009) Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. Nature 460:748-752.

Puricelli L, Proietti CJ, Labriola L, Salatino M, Balana ME, Aguirre Ghiso J, Lupu R, Pignataro OP, Charreau EH, Bal de Kier Joffe E, Elizalde PV (2002) Heregulin inhibits proliferation via ERKs and phosphatidyl-inositol 3-kinase activation but regulates urokinase plasminogen activator independently of these pathways in metastatic mammary tumor cells. Int J Cancer 100:642-653.

Puumala T, Sirvio J (1998) Changes in activities of dopamine and serotonin systems in the frontal cortex underlie poor choice accuracy and impulsivity of rats in an attention task. Neuroscience 83:489-499.

Reimsnider S, Manfredsson FP, Muzyczka N, Mandel RJ (2007) Time course of transgene expression after intrastriatal pseudotyped rAAV2/1, rAAV2/2, rAAV2/5, and rAAV2/8 transduction in the rat. Mol Ther 15:1504-1511.

Rentschler S, Zander J, Meyers K, France D, Levine R, Porter G, Rivkees SA, Morley GE, Fishman GI (2002) Neuregulin-1 promotes formation of the murine cardiac conduction system. Proc Natl Acad Sci U S A 99:10464-10469.

Reynolds GP, Abdul-Monim Z, Neill JC, Zhang ZJ (2004) Calcium binding protein markers of GABA deficits in schizophrenia--postmortem studies and animal models. Neurotox Res 6:57-61.

Reynolds GP, Harte MK (2007) The neuronal pathology of schizophrenia: molecules and mechanisms. Biochem Soc Trans 35:433-436.

Rieff HI, Raetzman LT, Sapp DW, Yeh HH, Siegel RE, Corfas G (1999) Neuregulin induces GABA(A) receptor subunit expression and neurite outgrowth in cerebellar granule cells. J Neurosci 19:10757-10766.

Riese DJ, 2nd, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, Stern DF (1996) Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. Oncogene 12:345-353.

Rimer M, Barrett DW, Maldonado MA, Vock VM, Gonzalez-Lima F (2005) Neuregulin-1 immunoglobulin-like domain mutant mice: clozapine sensitivity and impaired latent inhibition. Neuroreport 16:271-275.

Rio C, Rieff HI, Qi P, Khurana TS, Corfas G (1997) Neuregulin and erbB receptors play a critical role in neuronal migration. Neuron 19:39-50.

Ritch PA, Carroll SL, Sontheimer H (2003) Neuregulin-1 enhances motility and migration of human astrocytic glioma cells. J Biol Chem 278:20971-20978.

Robbins TW, Granon S, Muir JL, Durantou F, Harrison A, Everitt BJ (1998) Neural systems underlying arousal and attention. Implications for drug abuse. Ann N Y Acad Sci 846:222-237.

Robbins TW (2002) The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology (Berl) 163:362-380.

Robinson ES, Eagle DM, Mar AC, Bari A, Banerjee G, Jiang X, Dalley JW, Robbins TW (2008) Similar effects of the selective noradrenaline reuptake inhibitor atomoxetine on three distinct forms of impulsivity in the rat. Neuropsychopharmacology 33:1028-1037.

Rodriguez-Lebron E, Denovan-Wright EM, Nash K, Lewin AS, Mandel RJ (2005) Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. Mol Ther 12:618-633.

Role LW, Talmage DA (2007) Neurobiology: new order for thought disorders. Nature 448:263-265.

Romano N, Macino G (1992) Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol Microbiol 6:3343-3353.

Roy K, Murtie JC, El-Khodor BF, Edgar N, Sardi SP, Hooks BM, Benoit-Marand M, Chen C, Moore H, O'Donnell P, Brunner D, Corfas G (2007) Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders. Proc Natl Acad Sci U S A 104:8131-8136.

Russell DW, Miller AD, Alexander IE (1994) Adeno-associated virus vectors preferentially transduce cells in S phase. Proc Natl Acad Sci U S A 91:8915-8919.

Rutledge EA, Russell DW (1997) Adeno-associated virus vector integration junctions. J Virol 71:8429-8436.

Rutledge EA, Halbert CL, Russell DW (1998) Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J Virol 72:309-319.

Savonenko AV, Melnikova T, Laird FM, Stewart KA, Price DL, Wong PC (2008) Alteration of BACE1-dependent NRG1/ErbB4 signaling and schizophrenia-like phenotypes in BACE1-null mice. Proc Natl Acad Sci U S A 105:5585-5590.

Schmidt M, Katano H, Bossis I, Chiorini JA (2004) Cloning and characterization of a bovine adeno-associated virus. J Virol 78:6509-6516.

Schulz R, Wehmeyer A (2005) Opioid receptors activate extracellular signal-regulated MAPKs in a receptor tyrosine kinase independentmanner. Signal Transduction 4:184-194.

Seamans JK, Lapish CC, Durstewitz D (2008) Comparing the prefrontal cortex of rats and primates: insights from electrophysiology. Neurotox Res 14:249-262.

Searles CD, Singer HS (1988) The identification and characterization of a GABAergic system in the cholinergic neuroblastoma x glioma hybrid clone NG108-15. Brain Res 448:373-376.

Seeman P, Ulpian C, Bergeron C, Riederer P, Jellinger K, Gabriel E, Reynolds GP, Tourtellotte WW (1984) Bimodal distribution of dopamine receptor densities in brains of schizophrenics. Science 225:728-731.

Sei Y, Li Z, Song J, Ren-Patterson R, Tunbridge EM, Iizuka Y, Inoue M, Alfonso BT, Beltaifa S, Nakai Y, Kolachana BS, Chen J, Weinberger DR Epistatic and functional interactions of catechol-o-methyltransferase (COMT) and AKT1 on neuregulin1-ErbB signaling in cell models. PLoS One 5:e10789.

Sei Y, Ren-Patterson R, Li Z, Tunbridge EM, Egan MF, Kolachana BS, Weinberger DR (2007) Neuregulin1-induced cell migration is impaired in schizophrenia: association with neuregulin1 and catechol-o-methyltransferase gene polymorphisms. Mol Psychiatry 12:946-957.

Selemon LD, Goldman-Rakic PS (1999) The reduced neuropil hypothesis: a circuit based model of schizophrenia. Biol Psychiatry 45:17-25.

Semenova S, Stolerman IP, Markou A (2007) Chronic nicotine administration improves attention while nicotine withdrawal induces performance deficits in the 5-choice serial reaction time task in rats. Pharmacol Biochem Behav 87:360-368.

Seshadri AJ, Hayashi-Takagi A (2009) Gene manipulation with stereotaxic viral infection for psychiatric research: spatiotemporal components for schizophrenia. Prog Brain Res 179:17-27.

Sharif A, Duhem-Tonnelle V, Allet C, Baroncini M, Loyens A, Kerr-Conte J, Collier F, Blond S, Ojeda SR, Junier MP, Prevot V (2009) Differential erbB signaling in astrocytes from the cerebral cortex and the hypothalamus of the human brain. Glia 57:362-379.

Shenton ME, Kikinis R, Jolesz FA, Pollak SD, LeMay M, Wible CG, Hokama H, Martin J, Metcalf D, Coleman M, et al. (1992) Abnormalities of the left temporal lobe and thought disorder in schizophrenia. A quantitative magnetic resonance imaging study. N Engl J Med 327:604-612.

Shi J, Levinson DF, Duan J, Sanders AR, Zheng Y, Pe'er I, Dudbridge F, Holmans PA, Whittemore AS, Mowry BJ, Olincy A, Amin F, Cloninger CR, Silverman JM, Buccola NG, Byerley WF, Black DW, Crowe RR, Oksenberg JR, Mirel DB, Kendler KS, Freedman R, Gejman PV (2009) Common variants on chromosome 6p22.1 are associated with schizophrenia. Nature 460:753-757.

Silberberg G, Darvasi A, Pinkas-Kramarski R, Navon R (2006) The involvement of ErbB4 with schizophrenia: association and expression studies. Am J Med Genet B Neuropsychiatr Genet 141B:142-148.

Silva-Gomez AB, Rojas D, Juarez I, Flores G (2003) Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning social isolation rats. Brain Res 983:128-136.

Singer O, Marr RA, Rockenstein E, Crews L, Coufal NG, Gage FH, Verma IM, Masliah E (2005) Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. Nat Neurosci 8:1343-1349.

Spellmann I, Rujescu D, Musil R, Mayr A, Giegling I, Genius J, Zill P, Dehning S, Opgen-Rhein M, Cerovecki A, Hartmann AM, Schafer M, Bondy B, Muller N, Moller HJ, Riedel M Homer-1 polymorphisms are associated with psychopathology and response to treatment in schizophrenic patients. J Psychiatr Res.

St Clair D, Blackwood D, Muir W, Carothers A, Walker M, Spowart G, Gosden C, Evans HJ (1990) Association within a family of a balanced autosomal translocation with major mental illness. Lancet 336:13-16.

St Clair D, Xu M, Wang P, Yu Y, Fang Y, Zhang F, Zheng X, Gu N, Feng G, Sham P, He L (2005) Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. Jama 294:557-562.

St Clair D (2009) Copy number variation and schizophrenia. Schizophr Bull 35:9-12.

Steen RG, Mull C, McClure R, Hamer RM, Lieberman JA (2006) Brain volume in first-episode schizophrenia: systematic review and meta-analysis of magnetic resonance imaging studies. Br J Psychiatry 188:510-518.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, Birgisdottir B, Jonsson H, Gudnadottir VG, Gudmundsdottir E, Bjornsson A, Ingvarsson B, Ingason A, Sigfusson S, Hardardottir H, Harvey RP, Lai D, Zhou M, Brunner D, Mutel V, Gonzalo A, Lemke G, Sainz J, Johannesson G, Andresson T, Gudbjartsson D, Manolescu A, Frigge ML, Gurney ME, Kong A, Gulcher JR, Petursson H, Stefansson K (2002) Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 71:877-892.

Stefansson H, Ophoff RA, Steinberg S, Andreassen OA, Cichon S, Rujescu D, Werge T, Pietilainen OP, Mors O, Mortensen PB, Sigurdsson E, Gustafsson O, Nyegaard M, Tuulio-Henriksson A, Ingason A, Hansen T, Suvisaari J, Lonnqvist J, Paunio T, Borglum AD, Hartmann A, Fink-Jensen A, Nordentoft M, Hougaard D, Norgaard-Pedersen B, Bottcher Y, Olesen J, Breuer R, Moller HJ, Giegling I, Rasmussen HB, Timm S, Mattheisen M, Bitter I, Rethelyi JM, Magnusdottir BB, Sigmundsson T, Olason P, Masson G, Gulcher JR, Haraldsson M, Fossdal R, Thorgeirsson TE, Thorsteinsdottir U, Ruggeri M, Tosato S, Franke B, Strengman E, Kiemeney LA, Melle I, Djurovic S, Abramova L, Kaleda V, Sanjuan J, de Frutos R, Bramon E, Vassos E, Fraser G, Ettinger U, Picchioni M, Walker N, Toulopoulou T, Need AC, Ge D, Yoon JL, Shianna KV, Freimer NB, Cantor RM, Murray R, Kong A, Golimbet V, Carracedo A, Arango C, Costas J, Jonsson EG, Terenius L, Agartz I, Petursson H, Nothen MM, Rietschel M, Matthews PM, Muglia P, Peltonen L, St Clair D, Goldstein DB, Stefansson K, Collier DA (2009) Common variants conferring risk of schizophrenia. Nature 460:744-747.

Steiner H, Blum M, Kitai ST, Fedi P (1999) Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. Exp Neurol 159:494-503.

Steinthorsdottir V, Stefansson H, Ghosh S, Birgisdottir B, Bjornsdottir S, Fasquel AC, Olafsson O, Stefansson K, Gulcher JR (2004) Multiple novel transcription initiation sites for NRG1. Gene 342:97-105.

Straub RE, Jiang Y, MacLean CJ, Ma Y, Webb BT, Myakishev MV, Harris-Kerr C, Wormley B, Sadek H, Kadambi B, Cesare AJ, Gibberman A, Wang X, O'Neill FA, Walsh D, Kendler KS (2002) Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. Am J Hum Genet 71:337-348.

Sullivan PF, Kendler KS, Neale MC (2003) Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. Arch Gen Psychiatry 60:1187-1192.

Sunnarborg SW, Hinkle CL, Stevenson M, Russell WE, Raska CS, Peschon JJ, Castner BJ, Gerhart MJ, Paxton RJ, Black RA, Lee DC (2002) Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J Biol Chem 277:12838-12845.

Tallant EA, Diz DI, Khosla MC, Ferrario CM (1991) Identification and regulation of angiotensin II receptor subtypes on NG108-15 cells. Hypertension 17:1135-1143.

Tan W, Dean M, Law AJ Molecular cloning and characterization of the human ErbB4 gene: identification of novel splice isoforms in the developing and adult brain. PLoS One 5:e12924.

Tan PH, Yang LC, Shih HC, Lan KC, Cheng JT (2005) Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. Gene Ther 12:59-66.

Tan W, Wang Y, Gold B, Chen J, Dean M, Harrison PJ, Weinberger DR, Law AJ (2007) Molecular cloning of a brain-specific, developmentally regulated neuregulin 1 (NRG1) isoform and identification of a functional promoter variant associated with schizophrenia. J Biol Chem 282:24343-24351.

Tanaka T, Ohashi S, Funakoshi T, Kobayashi S YB-1 binds to GluR2 mRNA and CaM1 mRNA in the brain and regulates their translational levels in an activity-dependent manner. Cell Mol Neurobiol 30:1089-1100.

Thompson M, Lauderdale S, Webster MJ, Chong VZ, McClintock B, Saunders R, Weickert CS (2007) Widespread expression of ErbB2, ErbB3 and ErbB4 in non-human primate brain. Brain Res 1139:95-109.

Thomsen MS, Hansen HH, Timmerman DB, Mikkelsen JD Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology. Curr Pharm Des 16:323-343.

Thomson DM, McVie A, Morris BJ, Pratt JA Dissociation of acute and chronic intermittent phencyclidine-induced performance deficits in the 5-choice serial reaction time task: influence of clozapine. Psychopharmacology (Berl).

Threlkeld SW, McClure MM, Bai J, Wang Y, LoTurco JJ, Rosen GD, Fitch RH (2007) Developmental disruptions and behavioral impairments in rats following in utero RNAi of Dyx1c1. Brain Res Bull 71:508-514.

Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH, Bahn S (2003) Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet 362:798-805.

Tomar RS, Matta H, Chaudhary PM (2003) Use of adeno-associated viral vector for delivery of small interfering RNA. Oncogene 22:5712-5715.

Torrey EF, Miller J, Rawlings R, Yolken RH (1997) Seasonality of births in schizophrenia and bipolar disorder: a review of the literature. Schizophr Res 28:1-38.

Toyoda-Ohno H, Obinata M, Matsui Y (1999) Members of the ErbB receptor tyrosine kinases are involved in germ cell development in fetal mouse gonads. Dev Biol 215:399-406.

Tunbridge EM, Bannerman DM, Sharp T, Harrison PJ (2004) Catechol-o-methyltransferase inhibition improves set-shifting performance and elevates stimulated dopamine release in the rat prefrontal cortex. J Neurosci 24:5331-5335.

Tzahar E, Levkowitz G, Karunagaran D, Yi L, Peles E, Lavi S, Chang D, Liu N, Yayon A, Wen D, et al. (1994) ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. J Biol Chem 269:25226-25233.

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, et al. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418-425.

Ulusoy A, Sahin G, Bjorklund T, Aebischer P, Kirik D (2009) Dose optimization for long-term rAAV-mediated RNA interference in the nigrostriatal projection neurons. Mol Ther 17:1574-1584.

Usdin TB, Fischbach GD (1986) Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. J Cell Biol 103:493-507.

Uylings HB, Groenewegen HJ, Kolb B (2003) Do rats have a prefrontal cortex? Behav Brain Res 146:3-17.

Vaskovsky A, Lupowitz Z, Erlich S, Pinkas-Kramarski R (2000) ErbB-4 activation promotes neurite outgrowth in PC12 cells. J Neurochem 74:979-987.

Vidal GA, Naresh A, Marrero L, Jones FE (2005) Presenilin-dependent gamma-secretase processing regulates multiple ERBB4/HER4 activities. J Biol Chem 280:19777-19783.

Vita A, De Peri L, Silenzi C, Dieci M (2006) Brain morphology in first-episode schizophrenia: a meta-analysis of quantitative magnetic resonance imaging studies. Schizophr Res 82:75-88.

Vullhorst D, Neddens J, Karavanova I, Tricoire L, Petralia RS, McBain CJ, Buonanno A (2009) Selective expression of ErbB4 in interneurons, but not pyramidal cells, of the rodent hippocampus. J Neurosci 29:12255-12264.

Walsh T, McClellan JM, McCarthy SE, Addington AM, Pierce SB, Cooper GM, Nord AS, Kusenda M, Malhotra D, Bhandari A, Stray SM, Rippey CF, Roccanova P, Makarov V, Lakshmi B, Findling RL, Sikich L, Stromberg T, Merriman B, Gogtay N, Butler P, Eckstrand K, Noory L, Gochman P, Long R, Chen Z, Davis S, Baker C, Eichler EE, Meltzer PS, Nelson SF, Singleton AB, Lee MK, Rapoport JL, King MC, Sebat J (2008) Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. Science 320:539-543.

Wang LM, Kuo A, Alimandi M, Veri MC, Lee CC, Kapoor V, Ellmore N, Chen XH, Pierce JH (1998) ErbB2 expression increases the spectrum and potency of ligand-mediated signal transduction through ErbB4. Proc Natl Acad Sci U S A 95:6809-6814.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang YT (2003) Control of synaptic strength, a novel function of Akt. Neuron 38:915-928.

Wang XD, Su YA, Guo CM, Yang Y, Si TM (2008) Chronic antipsychotic drug administration alters the expression of neuregulin 1beta, ErbB2, ErbB3, and ErbB4 in the rat prefrontal cortex and hippocampus. Int J Neuropsychopharmacol 11:553-561.

Weinberger DR (1987) Implications of normal brain development for the pathogenesis of schizophrenia. Arch Gen Psychiatry 44:660-669.

Wen L, Lu YS, Zhu XH, Li XM, Woo RS, Chen YJ, Yin DM, Lai C, Terry AV, Jr., Vazdarjanova A, Xiong WC, Mei L Neuregulin 1 regulates pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons. Proc Natl Acad Sci U S A 107:1211-1216.

Wible CG, Shenton ME, Hokama H, Kikinis R, Jolesz FA, Metcalf D, McCarley RW (1995) Prefrontal cortex and schizophrenia. A quantitative magnetic resonance imaging study. Arch Gen Psychiatry 52:279-288.

Winchester CL, O’Donovan LH, Pratt JA, Morris BJ (2007) Schizophrenia: from gene arrays to novel drug targets. Schizophr Bull 33:305

Winstanley CA, Bachtell RK, Theobald DE, Laali S, Green TA, Kumar A, Chakravarty S, Self DW, Nestler EJ (2009) Increased impulsivity during withdrawal from cocaine self-administration: role for DeltaFosB in the orbitofrontal cortex. Cereb Cortex 19:435-444.

Wong J, Weickert CS (2009) Transcriptional interaction of an estrogen receptor splice variant and ErbB4 suggests convergence in gene susceptibility pathways in schizophrenia. J Biol Chem 284:18824-18832.

Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL (2004) RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med 10:816-820.

Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM (1999) Gene therapy vectors based on adeno-associated virus type 1. J Virol 73:3994-4003.

Xu R, Janson CG, Mastakov M, Lawlor P, Young D, Mouravlev A, Fitzsimons H, Choi KL, Ma H, Dragunow M, Leone P, Chen Q, Dicker B, During MJ (2001) Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. Gene Ther 8:1323-1332.

Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M (2008) Strong association of de novo copy number mutations with sporadic schizophrenia. Nat Genet 40:880-885.

Xue YQ, Ma BF, Zhao LR, Tatom JB, Li B, Jiang LX, Klein RL, Duan WM AAV9-mediated erythropoietin gene delivery into the brain protects nigral dopaminergic neurons in a rat model of Parkinson's disease. Gene Ther 17:83-94.

Yamamoto T, Akiyama T, Yokota J, Mori S, Toyoshima K (1986) Expression of the c-erbB-2 gene encoding a growth factor receptor. Princess Takamatsu Symp 17:203-210.

Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C, Birchmeier W (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol 131:215-226.

Yang X, Kuo Y, Devay P, Yu C, Role L (1998) A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis. Neuron 20:255-270.

Yang XL, Huang YZ, Xiong WC, Mei L (2005) Neuregulin-induced expression of the acetylcholine receptor requires endocytosis of ErbB receptors. Mol Cell Neurosci 28:335-346.

Yau HJ, Wang HF, Lai C, Liu FC (2003) Neural development of the neuregulin receptor ErbB4 in the cerebral cortex and the hippocampus: preferential expression by interneurons tangentially migrating from the ganglionic eminences. Cereb Cortex 13:252-264.

Yokozeki T, Wakatsuki S, Hatsuzawa K, Black RA, Wada I, Sehara-Fujisawa A (2007) Meltrin beta (ADAM19) mediates ectodomain shedding of Neuregulin beta1 in the Golgi apparatus: fluorescence correlation spectroscopic observation of the dynamics of ectodomain shedding in living cells. Genes Cells 12:329-343.

Young JW, Finlayson K, Spratt C, Marston HM, Crawford N, Kelly JS, Sharkey J (2004) Nicotine improves sustained attention in mice: evidence for involvement of the alpha7 nicotinic acetylcholine receptor. Neuropsychopharmacology 29:891-900.

Young JW, Powell SB, Risbrough V, Marston HM, Geyer MA (2009) Using the MATRICS to guide development of a preclinical cognitive test battery for research in schizophrenia. Pharmacol Ther 122:150-202.

Yurek DM, Zhang L, Fletcher-Turner A, Seroogy KB (2004) Supranigral injection of neuregulin1-beta induces striatal dopamine overflow. Brain Res 1028:116-119.

Zammit S, Spurlock G, Williams H, Norton N, Williams N, O'Donovan MC, Owen MJ (2007) Genotype effects of CHRNA7, CNR1 and COMT in schizophrenia: interactions with tobacco and cannabis use. Br J Psychiatry 191:402-407.

Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, Hillan K, Crowley C, Brush J, Godowski PJ (1997) Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. Proc Natl Acad Sci U S A 94:9562-9567.

Zhong C, Du C, Hancock M, Mertz M, Talmage DA, Role LW (2008) Presynaptic type III neuregulin 1 is required for sustained enhancement of hippocampal transmission by nicotine and for axonal targeting of alpha7 nicotinic acetylcholine receptors. J Neurosci 28:9111-9116.

Zhu X, Lai C, Thomas S, Burden SJ (1995) Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. Embo J 14:5842-5848.

Zscheppang K, Liu W, Volpe MV, Nielsen HC, Dammann CE (2007) ErbB4 regulates fetal surfactant phospholipid synthesis in primary fetal rat type II cells. Am J Physiol Lung Cell Mol Physiol 293:L429-435.