Genetic and functional investigation of FXYD6 and MAP2K7 as risk factors in schizophrenia

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Abstract

Schizophrenia is a debilitating psychiatric disorder with a prevalence of around 1% worldwide. It is an extraordinarily complex syndrome, which encompasses multiple psychological domains leading to the impairment of a range of symptoms. These symptoms are categorised as positive symptoms, negative symptoms and cognitive deficits. The profile of cognitive deficits is broad and severe, and is likely to be present in most, if not all patients. Despite cognitive enhancement being recognised as an important treatment target in schizophrenia, the discovery of an effective treatment has been met with difficulty.

The degree of psychosis is subject to numerous genetic and environmental factors. Family, twin and adoption studies show schizophrenia is unequivocally a genetic disorder, however the genetics behind schizophrenia are indisputably complex as it is not characterised by a single causative gene. A number of candidate genes have been implicated in schizophrenia. Recent genetic association studies have found an association for two genes, *FXYD6* and *MAP2K7,* as risk factors in the susceptibility to schizophrenia. However the roles of these genes in the underlying mechanisms of the symptoms of schizophrenia are unknown. To address this I utilise two mouse models, one containing homozygous disruption of *Fxyd6* (*Fxyd6*$^{-/-}$) and one heterozygous for *Map2k7* (*Map2k7*$^{+/-}$). I employ a range of molecular and behavioural tests to investigate the roles FXYD6 and MAP2K7 in schizophrenia- like phenotypes in these mice.

FXYD proteins are a family of seven single-span transmembrane proteins, all thought to be regulators of the Na^+ K^+ ATPase pump in a tissue-specific fashion. Up until now, FXYD6 function and its role in the risk to schizophrenia remain unclear. To address this I firstly investigated the association between *FXYD6* and schizophrenia in a Northern European population using a genetic association study. However from this study I was unable to confirm an allelic or haplotypic association between *FXYD6* and schizophrenia. Furthermore there was also no evidence for a role of epistatic interactions between *FXYD6* and *MAP2K7* in the risk of schizophrenia.

A putative functional link for FXYD6 in schizophrenia was explored further using *Fxyd6*$^{-/-}$ mice. The *in situ* hybridisation technique was utilised to reveal the expression of *Fxyd6* in the mouse brain. *Fxyd6* is interestingly expressed in the prefrontal cortex and hippocampus, two brain regions associated with schizophrenia and learning and memory. In addition, I have shown for the first time that disruption of *Fxyd6* results in a significant deficit in Na^+ K^+ ATPase activity in the forebrain, confirming that FXYD6 is a modulator of mouse brain Na^+ K^+ ATPase activity. Anxiety- like behaviours and hyperlocomotion were explored *Fxyd6*$^{-/-}$ mice. However activity in plus maze and open field tests, and response to amphetamine or ketamine was not altered in comparison to wildtype mice. Nonetheless subtle deficits observed in prepulse inhibition suggest potential deficits in neurotransmission in *Fxyd6*$^{-/-}$ mice may be present. Interestingly, *Fxyd6*$^{-/-}$ mice displayed deficits in working memory at delays of 5 seconds, indicating cognitive deficits. The molecular characterisation and insight into the phenotype of *Fxyd6*$^{-/-}$ mice are encouraging to investigate the role of FXYD6 in underlying mechanisms of schizophrenia-like symptoms further.

MAP2K7 belongs to the family of Map Kinases which have key roles in the regulation of a diverse range of cellular processes such as gene expression, apoptosis and synaptic plasticity. The brain expression of *Map2k7* was previously unknown, however this study utilised the *in situ* hybridisation technique to show expression in regions associated with schizophrenia, including the PFC and the hippocampus.

For the reason that the homozygous disruption of *Map2k7* is embryonically lethal, mice heterozygous for the disruption *Map2k7* were used to explore the role of *MAP2K7* in the susceptibility to schizophrenia. RTqPCR confirmed a modest but significant reduction of *Map2k7* in these mice. The heterozygous deletion of *Map2k7* results in alteration of glutamate receptor *Grin1* expression, a receptor reported to have altered expression in schizophrenia. Furthermore, *Map2k7*$^{+/-}$ mice display cognitive deficits, as observed by increased perseverative responding in the working memory task. Despite not exhibiting deficits in PPI, social behaviours or neurochemical deficits in GABAergic markers, *Map2k7*$^{+/-}$ mice revealed altered sensitivity to amphetamine, suggesting alterations in dopaminergic circuitry.

In conclusion, this study provides an insight in to the functional roles of FXYD6 and MAP2K7. Although the roles of *FXYD6* and *MAP2K7* as risk factors in schizophrenia still requires further elucidation, these results provide evidence of a putative role for both genes in some areas of the underlying neuronal activity associated with schizophrenia-associated symptoms. Furthermore, results from this study suggest both strains of mice are potential rodent models of cognitive impairments.

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Declaration of originality

I declare that the work presented in this thesis is entirely my own work, unless reference is made to the contribution of others, and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature……

Rhiannon Thompson

List of abbreviations

5HT	Serotonin
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ASST	Attentional set shifting
ATP	Adenosine triphosphate
bp	Base pairs
Ca+	Calcium
CAT	Computer axial tomography
CDCA	Common disease, common alleles
cDNA	Complementary DNA
CHIF	Corticosteroid-induced factor
CNS	Central nervous system
CNV	Copy number variant
COMT	Catechol-O-methyl transferase
Ct	Cycle threshold
DA	Dopamine
dB	Decibel
DEPC	Diethylpyrocarbonate
DISC1	Disrupted in schizophrenia 1
DNA	Deoxyribonucleic acid
DSM IV	Diagnostic and Statistical Manual of Mental Disorders IV
DTMBP1	dystrobrevin-binding protein 1 (dysbindin)
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
EPS	Extrapyramidal side effects
Erk	Extracellular signal-regulated kinase
GABA	Gamma-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT1	GABA transporter 1
gDNA	Genomic Deoxyribonucleic acid
GM	Genetically modified
GWAS	Genome wide association study
HRC1	Human Random Control DNA Panel 1
HRP	Horse radish peroxidase
ICD-10	International Classification of Diseases 10th revision
ip	Intraperitoneal
JNK	C Jun amino-terminal kinase
K^+	Potassium
Kb	Kilobase
kDa	Kilo Dalton
LD	Linkage disequilibrium
LSD	Lysergic acid diethylamide
MAF	Minor allele frequency
MAP2K	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MDMA	Methylenedioxymethamphetamine
mGluR	Metabotropic glutamate receptor

MRI	Magnetic resonance imaging
mRNA	Messenger RNA
Na^+	Sodium
Na^+ K^+ ATPase	Sodium potassium adenosine triphosphatase
NMDA	N-methyl-D-aspartic acid
NTC	No template control
PCP	Phencyclidine
PFA	Paraformaldehyde
PKA	Protein kinase A
PKC	Protein kinase C
PLM	Phospolemman
PPI	Prepulse inhibition
Pvalb	Parvalbumin
RNA	Ribonucleic acid
RTqPCR	Quantitative real time reverse transcriptase polymerase chain reaction
S.E.M	Standard error of mean
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SSTRs	Simple sequence tandem repeats
THC	Δ-9-tetrahydrocannabinol
UCL	The University College London

Chapter 1. Introduction

1.1 General Introduction

Schizophrenia is an extraordinarily complex syndrome, which encompasses multiple psychological domains leading to the impairment of a range of positive symptoms, negative symptoms and cognitive deficits. The schizophrenic phenotype is subject to numerous genetic and environmental factors however the genetics behind schizophrenia are indisputably complex as it is not characterised by a single causative gene. Recently, genetic association studies have found an association for two genes, *FXYD6* and *MAP2K7,* as risk factors in the susceptibility to schizophrenia. However the roles of these genes in the underlying mechanisms of the symptoms of schizophrenia are unknown.

We set out to investigate the roles of these genes in schizophrenia by firstly investigating the association between *FXYD6* and schizophrenia in a Northern European population using a genetic association study. Epistatic interactions between *FXYD6* and *MAP2K7* were also explored within these samples. The functional roles of FXYD6 and MAP2K7 in cognitive and schizophrenia- related molecular pathways and behaviours were further investigated using two separate mouse models; one homozygous for disrupted *Fxyd6* (*Fxyd6*$^{-/-}$) and one heterozygous for *Map2k7* (*Map2k7*$^{+/-}$).

1.2 Background

Schizophrenia is a debilitating psychiatric disorder with a prevalence of around 1% worldwide (Andreasen., 2000). It is an extraordinarily complex syndrome, which encompasses multiple psychological domains leading to the impairment of a range of cognitive and emotional functions, such as social interaction and the ability to form close relationships, executive function, imaginative thought, cognition and the expression of emotions (American Psychiatric Association., 2000). The course and outcome of schizophrenia present great variability from patient to patient however symptoms generally begin in late adolescence or early adulthood and usually progress throughout life, with males being at a slightly higher risk compared to females (risk ratio 1.3:1) (Aleman et al., 2003;McGrath et al., 2004). A huge economic burden is associated with

schizophrenia; this was estimated to be £6.7 billion in 2004/05, covering costs for treatment and care, lost productivity from employment, costs to the criminal justice system and benefit payments (Mangalore and Knapp., 2007).

1.3 Clinical Diagnosis

Schizophrenia has been described in the DSM-IV as a "disturbance that lasts for at least 6 months and includes at least 1 month of active phase-symptoms" (American Psychiatric Association., 2000). Due to the vast heterogeneity of schizophrenia, several criterions have been introduced to distinguish between the symptomology of this disorder, allowing standardised clinical diagnosis; criterions are based on both type and duration of symptoms and include the concurrent presence of 2 symptoms from the following list: Delusions, hallucinations, disorganised speech, grossly disorganised or catatonic behaviour and negative symptoms (American Psychiatric Association., 2000). The types of symptoms are as follows:

1.3.1 Positive Symptoms

Positive symptoms are additional to normal experiences and are usually absent in the general population. Positive symptoms can be classified under two dimensions: the "psychotic dimension" which includes hallucinations, the most prevalent being auditory ('hearing voices') and visual, however, frequent occurrences of tactile, gustatory and olfactory delusions are also known to occur and also the "disorganisation dimension" which includes disorganised speech and grossly disorganised or catatonic behaviour (American Psychiatric Association., 2000).

1.3.2 Negative Symptoms

Negative symptoms include those that could be seen as a diminution of normal functioning and are largely responsible for the morbidity associated with schizophrenia. Affective flattening (reduced emotional expression), alogia (reduced ability in fluency of thought and speech) and avolition (reduced motivation in goal-directed behaviour) are listed as negative symptoms of schizophrenia. However anhedonia (reduced ability to experience pleasure) and social withdrawal are associated with negative symptoms. These symptoms are

generally the first to arise in schizophrenic patients, followed by cognitive deficits and positive symptoms.

1.3.3 Cognitive Dysfunction

The profile of cognitive deficits is broad and severe, and is likely to be present in most, if not all patients. Marked cognitive deficits can be detected in first episode schizophrenia and include attention deficits, impairments in normal use of language and impairments in memory (Mohamed et al., 1999;Gold et al., 1999;Addington and Addington., 2002). The most prominent cognitive deficits occur in the domains of memory, attention, working memory, executive function, speed of processing, and social cognition (Nuechterlein et al., 2004;Fioravanti et al., 2005). Despite cognitive enhancement being recognised as an important treatment target in schizophrenia, the discovery of an effective treatment has met with difficulty.

1.4 Treatment

There are two main classifications of medication associated with the treatment of schizophrenia; the first generation typical antipsychotics (chlorpromazine, loxapine, haloperidol), and the newer atypical antipsychotics (clozapine, olanzapine, sertindole, asenapine). Administration of early typical antipsychotics at clinically effective doses were found to elicit severe side effects such as seizures, agranulocytosis, and long term administration induced extrapyramidal symptoms (EPS) akin to parkinsonian-like behaviour. The mechanism of action of typical antipsychotics was subsequently found to be inhibition of the DA D_2 receptor (Burt et al., 1976;Creese et al., 1976), inducing undesirable EPS symptoms as the result of attenuated DA transmission in the nigrostriatal pathway (Bunney et al., 1973;Clement-Cormier et al., 1974).

Soon after, atypical antipsychotics were developed and favoured. Positive, negative and some cognitive symptoms were found to respond following treatment with atypical antipsychotics, compared to the treatment of only positive symptoms with typical antipsychotics. Atypical antipsychotics often have a strong affinity for 5-HT receptors as well as the DA D_1 and D_2 receptors

(Leysen et al., 1994;Meller et al., 1985), resulting in low EPS at clinically effective doses (Leucht et al., 1999).

1.5 Neuropathology of Schizophrenia

Though the neuropathology of schizophrenia is somewhat elusive, there is considerable evidence suggesting it is fundamentally a disorder of subtle aberrations of brain development and plasticity. Despite some remaining inconsistencies, which may be a result of factors such as poor controls in early studies, differences in diagnostic criteria and changes caused by long term use of antipsychotics, technologies such as computer axial tomography (CAT) scans and magnetic resonance imaging (MRI) scans have revealed morphological and structural abnormalities in brains of patients with schizophrenia (Sigmundsson et al., 2001;Shenton et al., 1992). Key findings include enlarged lateral and third ventricles, decreased brain size, decreased cortical volume (temporal lobes) and decreased hippocampal volume (Daniel et al., 1991;Degreef et al., 1992;Lawrie and Abukmeil., 1998;Vita et al., 2006;Lawrie et al., 1999;Jeste and Lohr., 1989).

One of the initial studies associating structural abnormalities with schizophrenia was carried out by (Johnstone *et al.*, 1976), who observed a dilation of ventricles in chronic schizophrenics through the implementation of CAT scans. A number of studies have since followed and a comprehensive review estimates an average increase of 40% in ventricular size from MRI studies with a loss of around 3% brain tissue (Lawrie and Abukmeil., 1998) however a correlation has not been found between ventricular size and brain loss (Harrison., 1999).

Early evidence of gliosis (Stevens., 1982), a marker of neural scarring and inflammation, suggested schizophrenia was a neurodegenerative disorder, however subsequent studies failed to support this (Falkai *et al.*, 1999;Roberts *et al.*, 1986). A range of studies further dissociate schizophrenia from a neurodegenerative disorder; the failure to find discrete lesions such as amyloid plaques and neurofibrillary tangles has now led to the prevailing idea of schizophrenia being a neurodevelopmental disorder (Arnold *et al.*, 1998;Falkai *et al.*, 1999;Casanova *et al.*, 1993).

It has been suggested structural abnormalities may be a result from medication. However first-episode patients show ventricular enlargement and cortical volume reduction, and brain pathology in young adults at high risk of developing schizophrenia shows ventricular enlargement and smaller medial temporal lobes (Lawrie *et al.*, 1999;Shihabuddin *et al.*, 1998;Cannon *et al.*, 1993;Szeszko *et al.*, 2003). Following the start of symptoms, abnormalities correlate with disease progression (Ho *et al.*, 2003;Giedd *et al.*, 1999). MRI studies of monozygotic twins discordant for schizophrenia reveal the affected twin has larger ventricles, smaller cortical size and smaller hippocampal size (McNeil et al., 2000;Thomas et al., 1996;Goldman et al., 2008;Thomann et al., 2009), suggesting ventricle size is not only associated with schizophrenia phenotype, but is also subject to factors other than genetics, such as environmental factors (refer to section 1.7).

1.6 Neurochemical hypotheses of schizophrenia

Prominent aberrations of the neurotransmission systems in brain circuitry have been associated with the aetiology behind schizophrenia. In particular, dopaminergic, glutamatergic and serotoninergic systems have been postulated to contribute to the neurobiology of this disorder.

1.6.1 Dopamine (DA) hypothesis of schizophrenia:

The DA hypothesis is the preeminent hypothesis of schizophrenia; proposing that the dysfunction of the DA system underlies the behavioural and cognitive abnormalities that are associated with this disorder (Howes and Kapur., 2009). Dopaminergic receptors can be categorised into two main subtypes; the D_1-like receptor family (D_1 and D_5 receptors) and the D_2-like receptor family (D_2, D_3, and D_4 receptors). The DA hypothesis originated from correlations between the clinical potency and affinity of antipsychotics for the DA D_2 receptor (Seeman and Lee., 1975;Creese et al., 1976). Studies also found hyperfunction of D_2 receptors (Abi-Dargham et al., 2000) and increased D_2 receptor densities in mesolimbic regions of schizophrenia brains and *post mortem* schizophrenia brains (Abi-Dargham et al., 2000;Seeman., 1987;Cross et al., 1981;Laruelle., 1998). Positive symptoms of schizophrenia can be successfully controlled with D_2 antagonists, however, these typical antipsychotics prove less effective in the treatment of negative and cognitive symptoms (Meltzer and McGurk.,

1999;Harvey et al., 2005). This suggests D_2 receptors have a role in the underlying mechanisms of positive but not negative or cognitive symptoms.

D_1 receptors are the most abundant DA receptors in the neocortex (Meador-Woodruff et al., 1996;Lidow et al., 1991;Hurd et al., 2001), a brain region associated with cognition. It has been hypothesised that abnormalities in the activity of these receptors may be responsible for cognitive deficits associated with schizophrenia. Decreased densities of D_1 receptors have been observed in brains from non-medicated schizophrenia patients (Okubo et al., 1997;Hess et al., 1987). D_1 and D_2 receptors have been shown to have impaired molecular interaction in schizophrenia (Seeman et al., 1989), the reduction in D_1 activity may therefore contribute to hyperfunction of D_2 receptors. Together the abnormal activity of D_1 and D_2 receptors may be responsible for a wide range of schizophrenia symptoms. Thus the DA hypothesis was reformed to encompass both the hyperfunction of D_2 receptors in the underlying mechanisms of positive symptoms, and the hypofunction of D_1 receptors with the onset of negative and cognitive symptoms (Howes and Kapur., 2009).

Supporting the DA hypothesis, it is well known that administration of the DA-transmission enhancer, amphetamine, induces schizophrenic-like behaviour in both healthy volunteers and animals (Breier et al., 1997;Angrist and Gershon., 1970;Bell., 1965;Gambill and Kornetsky., 1976) and many of these behaviours can be reversed via the administration of anti-psychotics clozapine and haloperidol (Arnt., 1995;Warburton et al., 1994). Often genetic animal models of psychiatric disorders, such as schizophrenia, will be tested with amphetamine in order to investigate impairments to the Dopaminergic circuitry.

Glutamate hypothesis of schizophrenia:

The glutamate hypothesis of schizophrenia arose primarily from the observation that abuse of ‘angel dust’ (phencyclidine (PCP)) led to a psychosis similar to that observed in schizophrenia (Allen and Young., 1978). When PCP or ketamine, both NMDA receptor antagonists, are given to healthy subjects, positive symptoms, negative symptoms and cognitive deficits are induced (Adler et al., 1999;Lahti et al., 2001;Bakker and Amini., 1961), whereas administration of PCP to schizophrenia patients exacerbates the symptoms (Lahti et al., 2001;Allen and

Young., 1978). The ability of these compounds to transiently reproduce key features of schizophrenia led to the assumption that glutamatergic dysfunction has an underlying role in the onset of some of the symptoms associated with schizophrenia.

Glutamate receptors can be categorised into either ionotropic ligand-gated ion channels or metabotropic G-protein coupled glutamate receptors (mGluRs). The ionotropic glutamate receptors include N-methyl-D-aspartic acid (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainite receptors, named due to their selective responses to the respective agonists (reviewed by Traynelis *et al.,* 2010).

NMDA receptors are responsible for the mediation of the vast majority of excitatory neurotransmission in the brain, hypofunction of these receptors form the basis of the glutamate hypothesis of schizophrenia. Three subunits, termed NR1 (GRIN1), NR2 (GRIN2) and NR3 (GRIN3) make up the NMDA receptor, and these subunits and splice variants have been identified and associated with schizophrenia. For example, decreased expression of NMDA subunit splice variant NR2A has been observed in the PFC of schizophrenia brains (Bitanihirwe et al., 2009). In further support of the glutamate hypothesis, clinical studies have found an association between the NR1 subunit and schizophrenia (Georgi *et al.*, 2007;Galehdari., 2009;Qin *et al.*, 2005). This link is further supported by the behavioural characterisation of a mouse model expressing 5% of normal levels of the NR1 subunit. These mice display behavioural abnormalities reflective of those associated with schizophrenia symptoms, such as elevated motor activity and stereotypy and deficiencies in social and sexual interactions, furthermore, these behaviours are reversed with haloperidol or clozapine (Mohn et al., 1999).

Despite studies focussing mainly on the role of NMDA receptors in schizophrenia, AMPA receptors have also been implicated in the aetiology of schizophrenia. The AMPA receptor subunits are derived from a family of four genes termed gluR1–gluR4. Analysis of *post mortem* brains of schizophrenic patients revealed decreased expression of a range of AMPA receptors Glur1 and Glur2 in the medial temporal lobe and prefrontal cortex (PFC) (Eastwood et al., 1995;Beneyto et al., 2007;Mirnics et al., 2000), whilst mouse models haplosufficient for AMPA receptor subunits display behaviours similar to those associated with

schizophrenia, some of which are be reversed following antipsychotic administration (Chourbaji et al., 2008;Gray et al., 2009; Wiedholz et al., 2006)

Due to their role in the mediation of molecular correlates of neuroplasticity, such as long-term potentiation, via the modulation of the release and reuptake of synaptic glutamate, it is not surprising that mGluRs have also been implicated in schizophrenia. mGluRs are seven transmembrane domain, G-protein coupled receptors that can be categorised into group I, group and group III subtypes. Group I are responsible for increasing presynaptic glutamate release and consists of mGlur1 and mGlur5, and group II, which is responsible for the inhibition of presynaptic glutamate release, consists of mGlur2 and mGlur3 and group III consist of mGlur4, mGlur6, mGlur7, mGlur8 (reviewed by Traynelis *et al.,* 2010). mGluRs show strong expression in the prefrontal cortex and striatum, two regions associated with schizophrenia. Interestingly, altered immunoreactivity for receptors belonging to both mGluR group I and II have been observed in schizophrenia brains (Gupta et al., 2004; Volk et al., 2010) whilst animal studies and genetic association studies further support a role of mGluRs in schizophrenia (Bolanna et al., 2007; Devon et al., 2001; Moghaddam et al., 1998; Profaci et al., 2011).

1.6.2 GABAergic hypothesis of schizophrenia

The role of NMDA receptors in schizophrenia has also led to the implication of GABAergic dysfunction. Excitatory input derived from NMDA receptors acts as a mediator of the inhibitory activity of parvalbumin GABA (γ-amino-butyric acid) interneurons (Kinney et al., 2006). Studies have shown pharmacologically-induced impairments in NMDA receptor activity results in a dose-dependent decrease in the density of neurons immunoreactive for parvalbumin (Kinney et al., 2006;Abekawa et al., 2007). The impact of NMDA dysfunction on parvalbumin neurons is further observed in mice homozygous for the disrupted expression of the NMDA receptor NR1 subunit in parvalbumin-positive interneurons, these mice exhibit abnormal gamma oscillations and deficits in working memory and associative learning (Korotkova et al., 2010;Carlen et al., 2011). These studies, in addition to evidence of reduced expression of messenger ribonucleic acid (mRNA) for *PVALB* in schizophrenia brains (Beasley et al., 2002;Lewis et al., 1999), supports the use of parvalbumin as a marker of

potential molecular aberrations associated with schizophrenia pathophysiology. For this reason, *Pvalb* expression is explored in the molecular characterisation of two potential mouse models of schizophrenia (*Fxyd6* and *Map2k7)* later in these thesis (refer to Chapter 4 & Chapter 6).

Further evidence of GABAergic dysfunction in schizophrenia comes from decreased expression of mRNA for glutamic acid decarboxylase (*GAD*), the enzyme responsible for the conversion of glutamate to GABA (Beasley et al., 2002;Lewis et al., 1999). Furthermore, a decrease in the expression of the GABA transporter 1 (*GAT1*), is also observed in *post mortem* schizophrenia brain (Woo et al., 1998), which may be reflective of decreased GABA transmission due to altered GAD expression.

1.6.3 Serotoninergic hypothesis of schizophrenia

The serotoninergic hypothesis of schizophrenia proposes that hyperfunction of serotonin (5HT) receptors has a role in the symptoms of schizophrenia (Breier., 1995). The induction of psychosis-like symptoms following administration of the potent 5HT agonist D-lysergic acid diethylamide (LSD) (Woolley and Shaw., 1954) lead to the initial suggestion of a role of 5HT in the pathology of schizophrenia. Despite recent positron emission tomography studies failing to find alterations in 5-HT receptor expressions (Trichard et al., 1998;Lewis et al., 1999), evidence of a serotoninergic dysfunction in schizophrenia comes from reports that 5-HT_{1A} and 5-HT_{2A} receptor expressions are increased by up to 80% in the PFC (Lopez-Figueroa et al., 2004;Burnet et al., 1996;Sumiyoshi et al., 1996;Hashimoto et al., 1991) and atypical antipsychotics such as clozapine, olanzapine, and risperidone, have a stronger affinity for the 5-HT_{2a} receptor in comparison to DA D_2 antagonists (Leysen et al., 1994). It has been suggested these antipsychotics may elicit their effects through a DA-5HT interaction and accounts for the reduction in EPS observed in atypical antipsychotics (Meltzer., 1989;Meltzer., 1992;Leysen et al., 1994).

1.7 Risk factors for Schizophrenia

Family, twin and adoption studies show schizophrenia is unequivocally a genetic disorder, with studies estimating around 80% chance of heritability (Riley and

Kendler., 2006;Cardno and Gottesman., 2000;Gottesman et al., 1987). Despite running in families, schizophrenia is also sporadic. In the general population, the risk of developing schizophrenia is 1%, increasing to 2% if a first cousin or sibling has schizophrenia (Gottesman et al., 1987). This risk rises further to 15% in dizygotic twins and reaches 50% in monozygotic twins (Gottesman et al., 1987). Despite a high degree of genetic risk, genetic factors alone do not determine risk to schizophrenia; otherwise a 100% concordance rate would be observed between monozygotic twins. Instead schizophrenia is a multifaceted disorder subject to complex interactions of numerous risk factors.

Factors early on in life, such as intrauterine complications, viral infections during early childhood and/or parental loss all appear to be factors in the development of schizophrenia (Hultman et al., 1999;Dalman et al., 2001;Rantakallio et al., 1997). Environmental factors such as winter births or urban living are also thought to attribute to the risk (Mortensen et al., 1999). The recreational use of illicit substances such as cannabis has also been given much attention.

Studies have shown adolescence abuse of cannabis can result in an increased incidence of psychiatric diagnosis later in life (Arseneault et al., 2002;Andreasson et al., 1987;Arendt et al., 2005). Despite this, the association between cannabis and the development of schizophrenia remains controversial. It has been suggested that cannabis use hastens the development of schizophrenia in individuals predisposed to the disorder, or worsens the course and outcome of the disorder, rather than being a causal factor (Degenhardt et al., 2003). Furthermore, some studies have shown the association between cannabis and schizophrenia can also be influenced by a genetic interaction dependent on the allelic composition of the catechol-O-methyltransferase (*COMT*) gene, however this association has not been observed in all studies (Caspi et al., 2005;Henquet et al., 2009). COMT catabolism is the primary mechanism for catecholamine signal deactivation, such as with DA transmission, and *COMT* and has been genetically associated with schizophrenia by a single nucleotide polymorphism (SNP) located in exon 4 at codon 158. This results in a shift of methionine (Met) to valine (Val), resulting in increased enzymatic activity, and therefore decreased DA transmission (Chen et al., 2004; Lotta et

al., 1995). Carriers of the *COMT Val^{158}/Met* alleles had shown an increased risk of developing schizophrenia symptoms following cannabis use, unlike carriers homozygous for *met158* alleles (Caspi et al., 2005;Henquet et al., 2009); suggesting a modulating effect of *COMT* on the risk of developing schizophrenia. These studies show that not only is the risk of developing schizophrenia influenced by genetic and environmental risk factors, but it is also potentially susceptible to genetic and environmental interactions.

1.8 Genetic risk factors

The high heritability rate of schizophrenia has led to a search for genes that confer risk. However, the genetic architecture and mode of inheritance of schizophrenia is indisputably complex as it is not characterised by a single causative gene. Risk genes are likely to vary from case to case and models predict individual genes only confer a small risk which is mediated further by genetic and environmental interactions (refer to section 1.7).

It has been thought that in the general population, schizophrenia arose from the accumulation of modest effects from inherited common polymorphisms (the common disease, common alleles model (CDCA model) (Chakravarti., 1999;Gottesman and Shields., 1982). Genome wide association studies (GWAS) and candidate gene association studies have provided some evidence suggesting a role of common variants in the genetic architecture of schizophrenia in unrelated schizophrenia patients, (McClellan et al., 2007;Owen., 2010). Despite these variants being limited in number, these studies along with the increasingly high prevalence rate of schizophrenia and the lack of Mendelian inheritance associated with schizophrenia and linkage studies, has lead to the polygenic CDCA model receiving wide acceptance. However, this model does not explain why mutations are often specific to families, or why schizophrenia, an illness associated with reduced fertility, has not had reduced frequency over a long period of time (McGrath et al., 1999;Nimgaonkar et al., 1997). It is now being argued schizophrenia is likely to derive from rare but highly penetrant highly penetrant mutations (McClellan et al., 2007;Mitchell and Porteous., 2011).

Several features of schizophrenia support the view that it is a ‘common disease caused by multiple rare alleles’. For example, mutations are often specific to a

subset of families or an individual. This may explain why only a small fraction of genetic risk factors have been identified and consistently replicated in association and linkage studies (McGrath et al., 1999;Nimgaonkar et al., 1997). These studies assume unaffected individuals sharing the same SNP-defined haplotypes will share common risk variants and present a common phenotype, however rare but highly penetrant mutations causing schizophrenia may vary within the same haplotype block resulting in a lack, or reduced association which could be worsened with high sample numbers (reviewed by McClellan et al., 2007;Mitchell and Porteous., 2011).

The genetic variants are also subject to effects from other genetic and environmental factors (refer to section 1.7). Many processes such as gene regulation, signal transduction and biochemical networks are influenced and regulated by the interaction of multiple genes (Moore., 2003). The modification of a gene's output by the actions of another gene (known as epistatic interactions) results in differences in phenotype and has implications in disease phenotype (Cloninger., 1997;Moore., 2003). Epistatic interactions in combination with influencing risk factors make the identification of putative susceptibility genes in schizophrenia complex and very few methods have been developed to model epistasis.

Epigenetic mechanisms, which regulate chromatin structure and gene expression including deoxyribonucleic acid (DNA) methylation and histone modification, without changing the genetic code, have been suggested to be responsible for some of the genetic anomalies associated with schizophrenia (Matrisciano et al., 2012). A recent study conducted in monozygotic twins discordant for schizophrenia revealed numerous loci demonstrating differences in disease-associated DNA methylation (Dempster et al., 2011) suggesting environmental factors may exert their effects by influencing epigenetic mechanisms. Further support for a role of DNA methylation in modifying schizophrenia risk comes from studies that have observed increased expression of methylation enzymes in GABAergic neurons in the PFC of schizophrenia brains (Veldic et al., 2007;Ruzicka et al., 2007;Matrisciano et al., 2012).

1.9 Investigating the genetic basis of schizophrenia

1.9.1 Types of variants

Different types of DNA variants are associated with the risk of schizophrenia. CNVs are the submicroscopic deletion or duplication of segments of DNA, ranging from kilobases (kb) to megabases (Mb) (Redon et al., 2006;Sebat et al., 2004). Genes present in this segment will therefore have a lower or higher copy number than the standard two copies per genome, and may be present in one, three or more copies. CNVs associated with schizophrenia are not just used as markers but are risk variants. Despite the majority of CNVs not contributing towards an altered phenotype, genome-wide SNP arrays have shown aberrations in CNVs are a risk factor in schizophrenia (Nuechterlein et al., 2004;Fioravanti et al., 2005;Stefansson et al., 2008).

Microsatellites are di-, tri- or tetra- tandem repeats in short sequences of DNA e.g. GTGTGT. They are often referred to as simple sequence tandem repeats (SSTRs) and tend to occur in non-coding DNA. Microsatellites have been useful in associating genes as risk factors of schizophrenia, such as Neuroregulin 1 (Stefansson et al., 2003;Stefansson et al., 2002;Li et al., 2006;Allen et al., 2008).

SNPs are DNA sequence variations occurring when a single nucleotide base is altered at a specific locus between members of the same species. This alteration is usually a substitution of one nucleotide with another, deletions or insertions may occur. In a polymorphic locus, the frequency of the least commonly occurring allele (DNA variant) is termed the minor allele frequency (MAF), and must occur in at least 1% of the population to be classified a SNP. If a suspected DNA variant occurs more frequently in cases than healthy controls, this constitutes evidence of genetic association. SNPs can occur throughout the gene, with the majority occurring within non-coding introns; these SNPs are generally phenotypically silent (silent mutation) however some mutations can result in splice site mutation and lead to errors in intron splicing, protein formation and also the binding of transcription factors. Silent mutations in the coding regions of the gene (exons) are termed synonymous mutations; however mutations in

exons are often non-synonymous and can result in the alteration of a codon leading to a different amino acid and subsequent protein (missense mutation).

SNPs are thought to occur as often as once every 600 bp (Lander et al., 2001), implying there could be as many as 10 to 30 million potential SNPs. A combination of SNPs with high linkage disequilibrium (LD) are inherited together and is regarded as a haplotype block (Gabriel et al., 2002). Many haplotype blocks can be transmitted through generations with little or no recombination, making it possible to track a mutation (Gabriel et al., 2002).

1.9.2 Linkage disequilibrium (LD)

LD also known as gamete phase equilibrium or allelic association, reflects the non-random association of two or more alleles at different loci on the same chromosome (Cardon and Bell., 2001). Identification of a candidate region using LD is dependent on the association between the causal mutation and the marker allele (Cardon and Bell., 2001;Carlson et al., 2004;Li and Li., 2008).

1.9.3 Linkage studies

Linkage studies assess genetic loci in familial- based samples; providing the ability to investigate a trait with different alleles in different families (Cordell and Clayton., 2005). Linkage studies are effective for the detection of small but powerful causative polymorphisms and thereby support the concept that schizophrenia is a common disease with rare but highly penetrant alleles. Many loci have been associated with schizophrenia following linkage studies, however very few of those fulfilled criteria for statistical significance and subsequent studies often failed to repeat positive findings (Jurewicz et al., 2001). Linkage studies found an association between Disrupted-in-Schizophrenia-1 (*DISC1*) and cognitive disruption and psychosis in a Scottish pedigree (Millar et al., 2001), however, mixed results were found in several independent studies in multiple populations (Millar et al., 2001;Ekelund et al., 2004;Sachs et al., 2005), providing further evidence that schizophrenia is a disorder of rare but highly penetrant alleles. Due to the lack of recombination, linkage studies are a great way of analysing the interaction of environmental and lifestyle factors with genetic risk factors in the induction of a disease, however, they hold less power

than a genetic association study (Cordell and Clayton., 2005). Unlike linkage studies, genetic association studies aim to assess correlations between a genetic polymorphism and a disease phenotype across the whole population.

1.9.4Genetic association studies

Genetic association studies aim to investigate links between DNA variance and the general population, and include genome wide association studies (GWAS) and candidate gene association studies.

Association between a phenotype and a haplotype of SNPs can be determined by identifying and genotyping a SNP which is representative of the whole set of polymorphisms belonging to the haplotype. These SNPs are called 'Tag SNPs'. Haplotype analysis exposed significant associations between dystrobrevin-binding protein 1 (dysbindin or *DTNBP1*) and schizophrenia, but despite further evidence such as reduced *DTNBP1* mRNA expression in frontal brain areas of schizophrenia patients, implicating *DTNBP1* in schizophrenia a single susceptibility marker is yet to be found (Straub et al., 2002). Associations can also exist between markers which do not share the same chromosome, this is called epistatic interaction.

An association study requires the genotyping of many SNPs located throughout the gene to ensure the indirect association is captured. As associations are generally not directly linked to the disease, they can hold low power and can present difficulty in analysis (Cordell and Clayton., 2005). Population stratification can also be a problem in association studies. Common markers for a certain phenotype may be more common in certain populations despite there being no true causal relationship. If present it can be responsible for concealed associations or the generation of false-positive results. Control subjects need to be carefully matched to the cases by ethnicity to avoid population stratification. It may be required to select samples from mixed populations or match geographical location and ethnicity of cases and controls (Cardon and Bell., 2001;Carlson et al., 2004;Cordell and Clayton., 2005) and in complex disorders, such as schizophrenia, the accuracy of diagnostic criteria is important if diagnosis is performed by different medical practitioners.

1.9.5 Genome-wide association studies (GWAS)

GWAS are pivotal in the discovery of genetic components implicated in the susceptibility of disease. With the ability to scan hundreds of thousands of SNPs per sample, GWAS prove to be an efficient process for associating common DNA variations in the population with a phenotype. Since the introduction of GWAS in genetic studies for a range of disorders, over 1 million causative SNPs have now been discovered (szgene.org). A further advantage of GWAS is that no assumption is required about the location or function of a causal SNP prior to the study, providing unbiased results. However, GWAS is an expensive process to run, hindering the quantity of studies performed, also the SNPs have to be present on the chip for them to be genotyped in samples, so the sampling of genetic variation may not be complete. Furthermore, despite the large sample numbers, only a few risk loci have reached statistical significance (reviewed by Mitchell and Porteous., 2011).

1.9.6 Candidate gene association studies

Genetic association studies aim to identify polymorphisms within genes that vary between individuals with different disease states such as schizophrenia cases and healthy controls. Similar to GWAS, candidate gene association studies involve the genotyping of SNPs that are generally not causative but are located in a region inherited with a causative SNP.

1.9.7 Candidate gene criteria

Biological evidence is required to identify good candidate genes for genetic association studies. For example, confirmation of the gene's role in pathways associated with the disease, positional information or positive association from a previous association study is often required to consider the gene for a study (Hattersley and McCarthy., 2005). A number of candidate genes have been implicated in schizophrenia, a comprehensive database of these genes can be accessed at SZgene (http://www.szgene.org/). Though many putative susceptibility genes, such as *COMT, DISC1* and *Dysbindin*, have been implicated in schizophrenia, biological evidence of their role and association is still required to implicate a gene as a definitive risk factor of schizophrenia.

One of the most extensive and readily available sources of such evidence is provided by rodent models. Almost all mouse genes have orthologs in humans and further genetic manipulation provides an effective model to elucidate the roles and functions of putative candidate genes identified in association studies.

1.10 Animal Models of Schizophrenia

Animal models provide a unique way of analysing and understanding genetic, molecular and environmental influences implicated in the pathophysiology of schizophrenia. *In vitro* manipulation of cell biology can elucidate information surrounding the biochemical and molecular interactions involved in schizophrenia, yet it does not take into consideration the complex networks, interactions and potential regulatory and compensatory mechanisms which only an animal model can provide. However, schizophrenia is evidently a divergent multifactoral disorder with a wide range of symptoms and therefore difficulty arises in reaching a coherent animal model.

The generation of an animal model which fully encompasses all symptoms of schizophrenia has been met with failure and as a result, animal models are focussed on one subset of features of schizophrenia. These models will possess a convergence of a variety of domains; behavioural, neurochemical and genetics and allow investigation into the impact of environmental factors and pharmacological interventions. Behavioural tests alone cannot be used to validate an animal model, as phenotypes such as deficits in sensorimotor gating or impaired learning cannot be said to be solely indicative of schizophrenia as they are often present in other psychiatric disorders (Perry et al., 2007;Perry et al., 2001;Bennetto et al., 1996). Therefore it is important animal models are tested for analogy to symptoms of schizophrenia through a variety of molecular, biochemical and behavioural measures. Animal models of schizophrenia have been generated through a number of methods.

1.10.1 Pharmacological Models

Alterations in neurotransmitters and neuropeptides are a major factor in schizophrenia, and attempts to replicate this in an animal model have been made using pharmacological intervention. The use of pharmacological agents to

create animal models generally aim to explore the neurotransmission hypotheses of schizophrenia. NMDA receptor antagonists provide a model to explore the glutamatergic hypofunction hypothesis of schizophrenia (reviewed by Morris et al., 2005). In healthy human volunteers, administration of non-competitive NMDA receptor antagonists phencyclidine (PCP, also known as angel dust) or its analogue ketamine induces positive, negative and cognitive symptoms of schizophrenia, whereas administration to schizophrenia patients exacerbates symptoms (Lahti et al., 2001;Malhotra et al., 1997). Animal models of symptoms derived from glutamate dysfunction can be achieved via the administration of PCP or ketamine, and generally result in a phenotype reminiscent of the negative and cognitive symptoms associated with schizophrenia, such as impairments in cognitive flexibility and sensorimotor gating (Egerton et al., 2008;Sams-Dodd., 1996). Administration of ketamine and PCP in rats also reduces glucose utilisation in the PFC (hypofrontality) and network connectivity, providing evidence for a model of not only the symptoms of schizophrenia but also to investigate the underlying mechanisms and neural circuitry of schizophrenia (Cochran et al., 2003;Dawson et al., 2010;Dawson et al., 2011). The effectiveness with which ketamine and PCP models schizophrenia in rodents differs dependent of treatment regime, dose and testing end point. Administration of high doses of PCP results in neurotoxic effects; therefore low doses are generally administered to ensure any effects are reflective of schizophrenic pathology (Egerton et al., 2008).

Another highly documented animal model of schizophrenia is based on the DA hypothesis (refer to 1.6.1); this is replicated in animal models via the administration of amphetamine and amphetamine-like compounds such as 3,4-methylenedioxymethamphetamine (MDMA). Amphetamine and its analogs increase synaptic DA levels through a number of different mechanisms. These compounds act on transporters located in the plasma membrane, including the DA transporter (DAT), serotonin (5-hydroxytryptamine, 5HT) and norepinephrine transporter. Interaction with the DAT inhibits the reuptake of DA from the synapse into the nerve terminal. Amphetamine also can also induce the release of DA from synaptic vesicles back into the synaptic cleft, further increasing DA levels (reviewed by: (Fleckenstein et al., 2007;McMillen., 1983). Excess DA levels result in increased activation of DA D_1 and D2 receptors, and administration of

amphetamine and its analogs consistently results in behaviour stereotypic of schizophrenia, such as hyperlocomotion and deficits in sensorimotor gating in rodents (Sharp et al., 1987;Tenn et al., 2003;Mansbach et al., 1988;Pijnenburg et al., 1975). Administration of amphetamine and MDMA act as psychostimulants in healthy volunteers and exacerbate psychotic symptoms in schizophrenic patients, even at doses which are sub-psychotic in healthy volunteers, these symptoms are reversed by the administration of antipsychotics (Angrist et al., 1985;Angrist et al., 1980;Angrist and Gershon., 1970;Harris and Batki., 2000)

In addition to animal models based on the glutamate and DA hypotheses of schizophrenia, models also exist based on the 5HT hypothesis (refer to 0). LSD induces sensory distortions, such as hallucinations and euphoria. Despite the mechanisms of action being unclear, LSD is structurally similar to 5HT and has been suggested to exert its actions through the 5HT receptor (5-HT_{2A}) (Titeler et al., 1988;Marek and Aghajanian, 1996). Following administration of LSD to rats, sensorimotor deficits are induced, (Geyer and Braff., 1987;Braff and Geyer., 1990;Farid et al., 2000) however using LSD to model symptoms of schizophrenia in rodents has been met with controversy due to lack of evidence for prominent aberrations in the serotoninergic system in schizophrenia.

1.10.2 Developmental Models

The induction of a lesion via a toxin or an environmental insult at an early stage of development provides valuable animal models of the neurodevelopmental hypothesis of schizophrenia.

Lesion models are useful in evaluating and modelling the long term pathological changes associated with schizophrenia, which pharmacological models may not represent. Lesion models, usually created by the introduction of a toxin during early development, may not reflect the aetiology of schizophrenia. However they provide a model encapsulating the neurodevelopmental and circuitry disruption associated with schizophrenia (Lipska., 2004). A robust lesion model involves neonatal exocitoxic lesions of the rat ventral hippocampus, which results in deficits in spatial alternation learning, recognition memory, locomotor

activity, social interaction and sensitivity to amphetamine (Lipska., 2004;Lipska et al., 1995;Kruger et al., 2012;Wilkinson et al., 1993;Lipska et al., 1995).

The introduction of environmental insults at an early stage of development provides a non-invasive animal model of schizophrenia-related behaviours. Rats are highly social animals, and studies have shown that in social isolation models, where pups are removed from their home cages into an isolated cage following weaning, results in altered neural development and behavioural changes reminiscent to those associated with schizophrenia, such as sensitivity to amphetamine and deficits in sensorimotor gating (Geyer et al., 1993, Wilkinson et al., 1994). However, it must be noted that these effects are strain-specific and can be observed in hooded Listar rats but not Wistar rats (Geyer et al., 1993;Hall et al., 1997; Wilkinson et al., 1994).

The loss of a parent during childhood has been associated with the increased risk of depression and psychosis (Kendler et al., 2002; Morgan et al., 2007). The disruption of usual mother–infant interactions (maternal deprivation model), where a pup is removed from it's mother often for a short period of time (either a single 24 hours episode or brief period of 3-6 hours) results in changes in brain neurobiology such as sensitivity to dopaminergic drugs (Ellenbroek and Cools., 1995; Hall et al., 1999) and behavioural changes associated with psychosis, including deficits in sensorimotor gating and latent inhibition (Geyer and Braff., 1987). For these reasons, animal models of maternal deprivation are often used to investigate the neurodevelopmental hypothesis of schizophrenia.

1.10.3 Genetic Models of schizophrenia

Despite genetic association studies linking numerous genes to the pathology of schizophrenia, biological evidence is still required to explore and confirm the involvement of a candidate gene. Genetic animal models allow characterisation of genes of biological importance that are often implicated in disease-susceptibility and provide several advantages over lesion and pharmacological models, such as exploring gene x environment and gene x drug interactions.

The mouse genome can be altered by several different methods, such as random integration of transgenes or homologous recombination to change the

endogenous gene. Using the 'cre/ loxp system' allows tissue specific knockout or mutation of a gene in response to an external stimulus, and can therefore be induced at a specific time (Nagy., 2000). This is a particularly useful method to investigate the knockout of genes required for embryo development. Genes of interest can also be 'knocked in' and 'knocked out' via the insertion of DNA constructs, such as a 'gene trap vectors', which are accepted into the desired locus through homologous recombination (Evans and Kaufman., 1981). The insertion of a gene trap vector disrupts the gene of interest and will generally result in an inactivated (null) allele. Gene targeting and insertion of DNA constructs are predominately carried out in 129 mice, which are a proven germline competent embryonic stem cell strain, and have an impressive record of success in generation of knockouts (Melton., 1994;Simpson et al.,1997). Embryonic stem cells containing the inserted/recombined DNA locus are injected into mouse blastocysts and transferred into a pseudopregnant surrogate mother, however due to the poor phenotype of 129 mice, litters are often backcrossed onto a mouse strain expressing a more desired behavioural phenotype. The resultant phenotype of a mouse containing a targeted gene disruption is not only derived from the disruption created from the insert, but also from the cumulative effects and interactions elicited from background genes. It is therefore important that experimental mice are fully backcrossed onto one strain to eliminate variations in phenotype resulting from a mixed genetic background.

The background strains must be carefully considered due to variations in areas such as locomotor activity, anxiety and sensorimotor gating (Bouwknecht and Paylor., 2002;Contet et al., 2001;Paulus et al., 1999;Rodgers et al., 2002). For example, 129 mice have a poor phenotype which may make subtle changes difficult to detect, whereas C57BL/6 strain of mouse portray high levels of open field (OF) locomotion and low levels of anxiety-related behaviours. Therefore subtle changes in a mouse model of anxiety would be easier to detect in a C57BL/6 strain, rather than a 129 strain. For this reason C57BL/6 are commonly used for backcrossing (Bouwknecht and Paylor., 2002;Contet et al., 2001;Paulus et al., 1999;Rodgers et al., 2002). In order to characterise a novel genetically modified (GM) mouse, a range of molecular, biochemical and behavioural tests are required.

1.10.4 Behavioural tests

Phenotypes of GM mice derive from the effects of the disrupted gene or over-expressed transgene in pathways and can therefore be influenced by a number of factors such as age, hormones and the background strain, in addition to familiarity to the testing apparatus, level of illumination in testing rooms, odours, housing conditions and motivation (in food restricted animals). The function of a gene cannot be elucidated from a single behavioural test, and in general a battery of tests encompassing a wide range of behaviours are utilised.

The elevated plus maze (EPM) and OF apparatus are designed to test locomotor activity and anxiety levels in the mouse. These paradigms are investigated by exploiting the mouse's innate conflict between their natural tendency to explore and the need to be cautious when exposed to a new environment (Denenberg, 1969). The OF behavioural test is widely used to test the efficacy of anxiolytic treatment or antipsychotics on hyperlocomotor activity.

Deficits in sensorimotor gating often derive from an impaired response to sensory information in the CNS, resulting in the inability to filter important information from 'noise'. It is a complex process involving multiple interactions with multiple neurotransmitters including DA, 5HT and glutamate (Ojima et al., 2004;Quednow et al., 2004;Egerton et al., 2008) and is also thought to involve the prefrontal cortex and the hippocampus (Swerdlow et al., 1995; Japha and Koch., 1999). Deficits are a core symptom of schizophrenia and can be investigated in rodent models through prepulse inhibition (PPI) of the startle response. PPI assesses the ability of the nervous system to adapt to a strong sensory stimulus, following a weaker signal. If the startle response is not attenuated in response to a preceding weaker signal, it is likely deficits in sensorimotor manifest (Braff et al., 2001;Powell et al., 2009).

Much attention has been focussed on the cognitive deficits in schizophrenia and a number of paradigms have been designed to assess different forms of cognition and memory in rodents. For example, spatial learning and memory can be tested using the Morris water maze; visual cues are used to help a rat or mouse learn and navigate a route to a hidden platform in a water arena (Morris et al., 2005). Executive function and cognitive flexibility can also be assessed through a rodent

version of the Wisconsin Card Sorting Task. The rodent attentional set shifting task (ASST) requires rodents to solve a series of discriminations containing an intra-dimensional to an extra-dimensional shift (Birrell and Brown. 2000). Tasks such as the 'n-back task' or 'delayed non-match to sample' have been designed to assess working memory, which requires synchrony between the prefrontal cortex and hippocampus (Sigurdsson *et al.*, 2010;Yoon *et al.*, 2008). During these tasks, rodents manipulate short term memory in order to reach a goal or reward, and involve delayed alteration in either a T maze or 8-arm radial maze.

1.11 Na^+ K^+ ATPase pump

The sodium potassium adenosine triphospatase (Na^+ K^+ ATPase) pump Na^+ K^+ ATPase pump is an integral membrane-bound enzyme present in the plasma membrane of all mammalian cells. It belongs to a family of P-type ATPases that includes the gastric H^+ K^+ ATPase and the Ca^{2+} ATPase (Lutsenko and Kaplan., 1995). The Na^+ K^+ ATPase pump is essential for the normal functioning of all excitable tissues by establishing and maintaining resting membrane potential following depolarisation, it is therefore imperative in neuronal functioning (Moseley et al., 2003). The pump maintains low internal sodium (Na^+) and high internal potassium (K^+) cellular concentrations through the active transport of 3 Na^+ ions out of the cell in exchange for 2 K^+ ions moving in (Figure 1.1), and is of paramount importance for establishing and maintaining resting membrane potential and cellular volume (Blanco and Mercer., 1998). This process requires free energy released from the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP); the release of inorganic phosphate from this reaction is often used as a marker of pump activity (Sarkar, 2002;Esmann, 1988). The activity of the Na^+ K^+ ATPase pump consumes approximately 30% of total energy in the body, whilst high expression in the brain consumes about 40-50% of brain ATP (Erecinska et al., 2004).

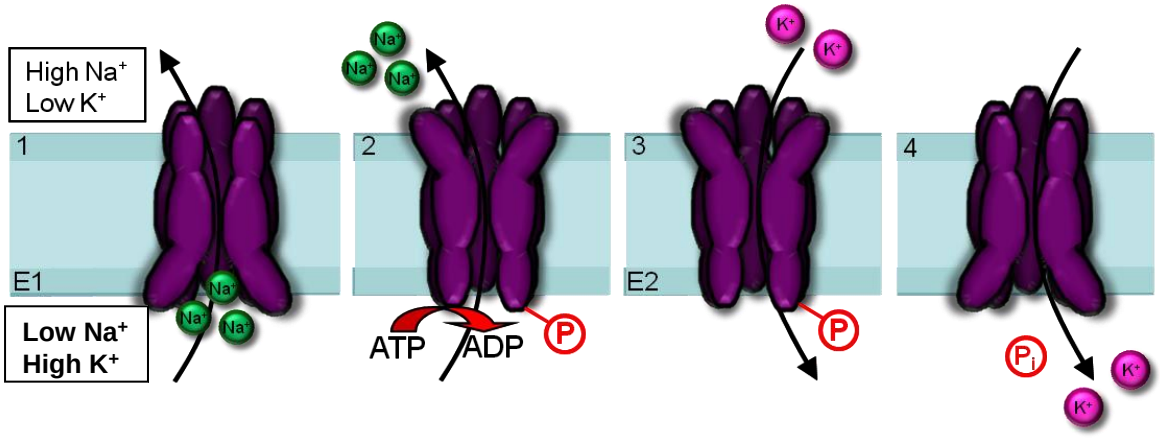


Figure 1.1 The Na^+ K^+ ATPase pump creates an electrochemical gradient across cell membranes by maintaining low internal Na^+ and high internal K^+ cellular concentrations. This is an active process involving the following steps:

1. In conformation E1, the pump has a high affinity for sodium. Three sodium ions bind to the pump from the cytoplasmic side of the phospholipid bilayer membrane, which has a low sodium (Na^+) concentration and a high potassium (K^+) concentration.

2. ATP phosphorylates the enzyme (represented by the P attached to the cytoplasmic side of the pump). This results in a change in pump conformation from E1 to E2, reducing the pumps affinity for sodium. The three sodium ion are released.

3. Two potassium ions enter and bind to the pump in the E2 conformation.

4. The pump becomes dephosphorylated, returning to conformation E1 and in the process transporting and releasing the potassium ions into the cell. The pump is ready to start again.

1.11.1 Structure and isoforms of Na^+ K^+ ATPase

The Na^+ K^+ ATPase pump is a heterodimer comprising of two essential subunits; the catalytic α subunit which is responsible for ATP hydrolysation and Na^+ and K^+ transportation, and the regulatory ß subunit required for protein folding and modulation of substrate affinity (Blanco and Mercer., 1998). Very little is known about the specific roles of the α/ß heterodimers.

The α subunit is a multi-spanning membrane protein, with a molecular mass of about 112 kilo Dalton (kDa), and contains binding sites for ATP and the Na^+ K^+ ATPase pump-specific inhibitor, ouabain (Jorgensen et al., 2003;Pedemonte and Kaplan, 1990). Four α isoforms exist (α1, α2, α3, α4) and three are abundant in the brain (α1, α2, α3) (Blanco and Mercer., 1998;Urayama et al., 1989;Lingrel et al., 2007;Orlowski and Lingrel., 1988). All four isoforms have a high degree of amino acid identity, however studies have shown isoforms differ in the kinetic properties and affinities for sodium, with α1 and α2 showing the most similarity (Jewell and Lingrel., 1991;Segall et al., 2001). The α isoforms are responsible for the tissue specific mechanisms of the Na^+ K^+ ATPase pump (Lingrel et al., 2007). The α1 subunit is present in neurons and glia and is thought to be essential for neurodevelopment as mice lacking α1 die at embryonic stage (Moseley et al., 2007;Lingrel et al., 2003). The α2 isoform is found mainly in skeletal muscle, heart and brain, and may be involved in the regulation of the Na^+/calcium (Ca^+) exchanger, whilst α3 is found in neurons of the brain and central nervous system (CNS), and plays a role in neurotransmission (Moseley et al., 2007;James et al., 1999;Lingrel et al., 2003;McGrail et al., 1991).

The glycosylated ß-subunit is 40-60 kDa in size and only crosses the membrane once. It is responsible for the normal activity of the membrane by maturation of the Na^+ K^+ ATPase pump and the transportation to the plasma membrane and (Lingrel et al., 2007;Noguchi et al., 1990;Geering et al., 1985). Moreover, the ß-subunit modulates the pump's affinity for sodium and potassium (Eakle et al., 1994;Jaisser et al., 1992). Without the ß subunit, the α subunit is unable to adopt the conformation of the mature proteins and is quickly degraded (Geering et al., 1985). Three isoforms of the ß subunit exist and they are all glycosylated

(ß1, ß2, ß3) (Orlowski and Lingrel., 1988;Martin-Vasallo et al., 1989). The ß1 isoform is ubiquitous (in almost all tissues), whilst ß2 is expressed in skeletal muscle and nervous tissues and ß3 in the testis, retina, liver and lung (Lingrel., 1992).

A third, non-obligatory, small protein of around 6.5 kDa has also been identified. The γ subunit is thought to not be essential for Na^+ K^+ ATPase normal functioning however does have a regulatory role through the modulation of the α/ß complexes (Mercer et al., 1993;Beguin et al., 1997;Therien et al., 1997). The γ subunit belongs to a family of small membrane proteins, the FXYD family (proline-phenylalanine-X-tyrosine-aspartate (PFXYD) refer to 1.12) (Sweadner and Rael., 2000;Crambert and Geering., 2003;Garty et al., 2002).

1.11.2 Modulation of the Na^+ K^+ ATPase pump

The exact mechanisms involved in the regulation and facilitation of the Na^+ K^+ ATPase pump remain largely unknown, however it is thought to be prominently maintained via the control of phosphorylation, after which a conformational transition occurs ($E_1 \rightarrow E_2$). The main rate-limiting step of the Na^+ K^+ ATPase comes from the modulation of the conformational change of the phosphorylated enzyme from $E_1 \rightarrow E_2$, and the conformational transition of the unphosphorylated enzyme from $E_2 \rightarrow E_1$ (Lupfert et al., 2001;Jorgensen and Andersen., 1988) (Figure 1.1). The γ subunit and other members of the FXYD family are not essential for the functioning of the Na^+ K^+ ATPase pump; however have a role in the modulation of its activity (Beguin et al., 2001;Bibert et al., 2008;Crambert et al., 2005;Delprat et al., 2007). With the exception of the γ subunit (FXYD2), FXYD proteins do not form an integral part of the Na^+ K^+ ATPase pump, and therefore interaction and modulation must take place at the molecular level. It is thought the pump is regulated via the interaction of FXYD protein and the α/ß heterodimer; however the specific interactions remain elusive. Beguin et al., (1997) have shown the γ subunit modulates the K^+ activation of Na^+ K^+ ATPase pump by associating with α/ß heterodimers.

1.11.3 Association between Na^+ K^+ ATPase activity and psychiatric disorders

Reduced Na^+ K^+ ATPase activity has been observed in various disorders affecting the brain (Ischemia, epilepsy, depression and bipolar disorder) (Looney and El-Mallakh., 1997;Mintorovitch et al., 1994;Renkawek et al., 1992). Ouabain-induced inhibition of the pump in rats has been shown to result in increased spontaneous locomotor activity and reduced spatial learning in the rat which may model some aspects of symptoms associated schizophrenia and similar disorders (El-Mallakh et al., 2003;Riegel et al., 2009;Zhan et al., 2004). However, despite studies suggesting that Na^+ K^+ ATPase activity modulates neurotransmission of DA, GABA, norepinephrine and glutamate (Levi et al., 1976;Rose et al., 2009;Westerink et al., 1989), little is known about the exact functioning of Na^+ K^+ ATPase in these disorders and further investigations are required to elucidate information about the role of Na^+ K^+ ATPase in psychiatric disorders.

1.12 FXYD Family

FXYD proteins are a family of single-span transmembrane proteins. So far, a total of 7 FXYD proteins have been identified at the mammalian level, all of which contribute to a variety of apparently unrelated physiological processes (summarised in Table 1.1). They are, however, all known to interact with the Na^+ K^+ ATPase pump in a tissue-specific fashion, altering the kinetics of the pump to meet specific tissue requirements (Crambert and Geering., 2003;Geering., 2006;Li et al., 2004;Cornelius and Mahmmoud., 2003;Geering et al., 2003;Therien et al., 1997). The most highly characterised FXYD protein is the γ subunit of the Na^+ K^+ ATPase pump (FXYD2) (Bibert et al., 2008;Crambert et al., 2002;Lifshitz et al., 2006). FXYD2 is expressed in the kidney and is thought to regulate electrolyte homeostasis (Crambert and Geering., 2003;Goldschmidt et al., 2004;Shustin et al., 1998). Protein expression and physiological roles of the FXYD family are summarised in Table 1.1.

Protein	Gene	Protein expression	Physiological role	References
Phospholemman (PLM)	*FXYD1*	Heart, brain, kidney	Substrate for proteinase A and C , suspected role in heart contractility	Sweadner and Rael, 2000;Crambert et al., 2002;Moshitzky et al., 2012;Chen et al., 1997
The γ subunit of the Na^+ K^+ ATPase pump	*FXYD2*	Kidney	Regulatory role in α/β complex activity and electrolyte homeostasis	Beguin et al., 2001;Bibert et al., 2008; Crambert et al., 2005;Delprat et al., 2007
Mat-8	*FXYD3*	Stomach, colon, cancer cells	Role in tumorogenesis	Bibert et al., 2009;Arimochi et al., 2007; Kayed et al., 2006
CHIF	*FXYD4*	Kidney, colon	regulatory role in electrolyte homeostasis through mediating sodium absorption	Shustin et al., 1998;Goldschmidt et al., 2004
RIC, dysadherin	*FXYD5*	Kidney, intestine, heart, lung, spleen	Regulates E-cadherin, promotes metastasis	Lubarski et al., 2005;Ino et al., 2002
Phosphohippolin	*FXYD6*	CNS, brain, inner ear	Endolymph homestasis, role in brain currently unknown	Delprat et al., 2007a;Delprat et al., 2007b; Kadowaki et al., 2004
	FXYD7	Brain, cerebellum	Suscepted role in neuronal excitability	Beguin et al., 2002;Crambert et al., 2003

Table 1.1 Summary of *FXYD* family. FXYD proteins all modulate the Na^+ K^+ ATPase pump in a tissue-specific manner.

1.12.1 Structure

The FXYD family consists of small hydrophobic proteins. Each protein has an extracellular NH_2 terminus and contains a marked homology in a 35 amino acid sequence located in the transmembrane domain, this sequence is made up of 3 exons and contains 7 invariant amino acids located within, and surrounding, the membrane span (Sweadner and Rael., 2000). With the exception of the 35 amino acid stretch, the structure of FXYD proteins has not been well conserved, around 11 % of the amino acids of phospholemman (PLM) and (corticosteroid-induced factor) CHIF vary between the rat and mouse sequence (Garty and Karlish., 2006). The short sequence proline-phenylalanine-X-tyrosine-aspartate (PFXYD) gives rise to the motif and is invariant among the proteins. It has been suggested that it is likely these residues are involved in a function common to FXYD proteins, such as interaction with the Na^+ K^+ ATPase pump (Beguin et al., 2001). FXYD proteins are modulatory proteins of the Na^+ K^+ ATPase pump, with the exception of PLM activity which has been shown to be modulated by protein kinase (PK) A (PKA)- and PK C (PKC)- dependent phosphorylation (Mahmmoud et al., 2000;Fuller et al., 2004).

1.12.2 FXYD modulation of Na^+ K^+ ATPase

Association between FXYD proteins and the Na^+ K^+ ATPase was first noted in 1978 when a short polypeptide (later to be identified as the γ subunit) was characterised and shown to be directly associated with the renal Na^+ K^+ ATPase (Forbush et al., 1978;Collins and Leszyk., 1987). Further studies confirmed co-immunoprecipitation and co-purification of the γ subunit with the Na^+ K^+ ATPase (Mercer et al., 1993;Arystarkhova et al., 2002). Studies investigating the functional relationship between the γ subunit and Na^+ K^+ ATPase have shown the pump is modulated by a change in confirmation of the enzyme from E_2 to E_1, increasing the affinity for ATP and increasing K^+ antagonism of cytoplasmic Na^+ activation (Beguin et al., 1997;Pu et al., 2002;Therien et al., 1999).

More recently, the functional relationship between the FXYD proteins and the Na^+ K^+ ATPase pump has shown to be a non-essential association between FXYD

and the α/ß complex (Cornelius and Mahmmoud., 2003;Crambert and Geering., 2003;Therien et al., 1997;Crambert et al., 2005;Geering et al., 2003). FXYD proteins associate with the α/ß complex, inducing changes in K^+ and Na^+ apparent affinity. The modulation of pump kinetics differs depending on the FXYD protein and the α/ß complex with which it associates. This suggests FXYD proteins modulate the kinetics of the pump specific to each tissue requirement (Table 1.2, Crambert and Geering., 2003;Geering., 2006;Li et al., 2004;Cornelius and Mahmmoud., 2003;Geering et al., 2003;Therien et al., 1997).

Protein	Gene	Role in Na^+ K^+ ATPase pump kinetics	References
Phospholemman (PLM)	*FXYD1*	Increases Na^+ binding affinity by 30- 50%,	Crambert et al., 2002;Cirri et al., 2011
The γ subunit of the Na^+ K^+ ATPase pump	*FXYD2*	Reduces apparent Na^+ affinity, increases K^+ affinity during hyperpolarisation, however decreases K+ affinity in high levels of Na^+, it also increases ATP affinity	Cirri et al., 2011;Li et al., 2004; Pu et al., 2002
Mat-8	*FXYD3*	Decreases apparent affinity for both Na^+ and K^+	Crambert et al., 2005
	FXYD4	Induces a two- to threefold increase in apparent affinity for Na^+ but does not affect affinity for K^+	Garty et al., 2002
CHIF	*FXYD5*	Increases the apparent affinity for Na^+ 2-fold, and decreased the apparent affinity for K^+ by 60%	Miller and Davis, 2008
RIC, dysadherin	*FXYD6*	Association of FXYD6 with Na^+ K^+ ATPase α1/β1 slightly decreases the apparent K^+ affinity and significantly decreases the apparent Na^+ affinity. Association with the α1/β2 heterodimer increases their apparent K^+ and Na^+ affinity.	Delprat et al., 2007
Phosphohippolin	*FXYD7*	Association with Na^+ K^+ ATPase increases K^+ affinity of α1/ß1 and α2/ß1, but not of α3/ß1 heterodimers	Beguin et al., 2002

Table 1.2 The *FXYD* family each modulate the kinetics of the Na^+ K^+ ATPase in tissue- specific ways

1.12.3 FXYD6 and schizophrenia

A number of SNPs within the *FXYD6* gene have been shown to be associated with schizophrenia in a Caucasian population, implicated this as a risk factor for schizophrenia (Choudhury *et al.*, 2006;Choudhury *et al.*, 2007). Despite the inability to confirm this association in Japanese and Chinese populations, several forms of evidence suggest a role for *FXYD6* in the susceptibility to schizophrenia. *FXYD6* is located on chromosome 11q23.3, a chromosomal region which has been previously linked with both schizophrenia and bipolar disorder (Grandy *et al.*, 1989;Egeland *et al.*, 1987). Furthermore, i*n situ* hybridization and western blotting have both reveal the expression of FXYD6 in regions of the brain associated with schizophrenia, in particular the PFC and hippocampus (Kadowaki *et al.*, 2004). The PFC and hippocampus both have several roles in memory and cognition; they also share pathways to regulate working memory. It is therefore possible that impairments in *FXYD6* may result in symptoms similar to schizophrenia, such as deficits in working memory.

As of yet, FXYD6 has not been fully characterized, however, it has been revealed to co-localise, and associate, with the Na^+ K^+ ATPase pump in the inner ear, suggesting it has a role in the modulation of the pump (Delprat et al., 2007). This demonstrates a theorized mechanism of action by which FXYD6 and the Na^+ K^+ ATPase pump interact, based on the interaction of other FXYD members with this pump (Figure 1.2) (Geering., 2006). It is possible that aberrations in *FXYD6* may induce impairments in Na^+ K^+ ATPase activity. Impairments in Na^+ K^+ ATPase pump activity have been previously associated with psychiatric disorders (El-Mallakh and Wyatt., 1995;Kurup and Kurup., 2003;Looney and El-Mallakh., 1997) and it would therefore be interesting to investigate further a role of Na^+ K^+ ATPase in schizophrenia. A mouse model expressing homozygous disruption of *Fxyd6* has been created, however it has yet to be characterised and assessed for biochemical, molecular and phenotypic similarities to schizophrenia.

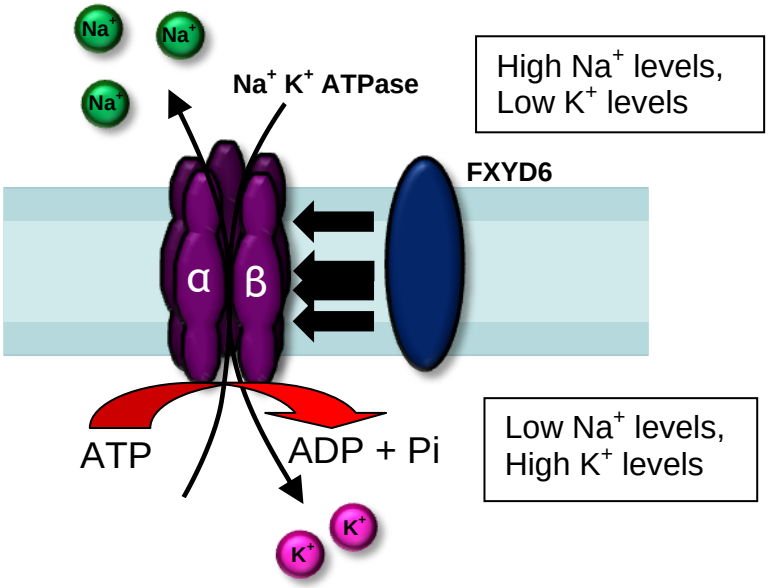


Figure 1.2 Proposed mechanism of the interaction between FXYD6 and Na^+ K^+ ATPase pump.

Despite FXYD6 showing co-localisation and association with the Na^+ K^+ ATPase pump in the inner ear, the functional relationship between FXYD6 and the pump is currently unclear. It has been suggested the Na^+ K^+ ATPase pump is modulated by a non-essential association between FXYD proteins and the α/ß complex, inducing changes in K^+ and Na^+ apparent affinity (Cornelius and Mahmmoud, 2003;Crambert and Geering, 2003;Therien et al., 1997;Crambert et al., 2005;Geering et al., 2003). ⬅ Represents the potential association between FXYD6 and the Na^+ K^+ ATPase pump.

1.13 Mitogen activated protein kinase (MAPK) cascades

MAPKs are serine/threonine specific protein kinases that respond to extracellular stimuli. They form part of the MAPK cascade; an evolutionarily conserved signal transduction cascade, responsible for the mediation of adaption and survival of eukaryotic cells' in response to physical and chemical stress triggers (Johnson and Lapadat., 2002;Robinson and Cobb., 1997). Despite the majority of information on MAPK cascades deriving from yeast studies, more information is now becoming elucidated on the role of mammalian MAPKs. An important role of the MAPK cascades in the regulation of a diverse range of cellular processes such as gene expression, growth factors and hormones and cell survival/ apoptosis is apparent.

MAPK cascades typically consist of a three kinase model that is activated through the sequential phosphorylation of MAPK by MAPK-kinases (MAP2Ks) (also known as MAPK/extracellular signal-regulated kinase (ERK)-kinases (MEKs), which are phosphorylated and activated by MAPKK-kinases (MAP3Ks) (Figure 1.3) (English et al., 1999). A number of distinct, but not mutually exclusive, mechanisms ensures specific MAPK activation in response to diverse cellular processes and extracellular stimuli (reviewed by (Johnson and Lapadat., 2002;Cobb and Goldsmith., 1995).

1.13.1 Mammalian MAPK cascades

In the MAPK cascades (reviewed by (Cobb., 1999), MAPKs are activated by MAP2Ks through common mechanisms at two regulatory phosphorylation sites located in the activation lip. This motif contains the sequence 'threonine-X-tyrosine', both residues require phosphorylation, but can be phosphorylated independently of one another, and result in a conformation change. Once activated, MAPKs need to find their targets. Although it is necessary to limit the phosphorylation of irrelevant substrates, it is also important that each MAPK recognises a number of substrates to allow the regulation of many processes. Mechanisms ensuring specific MAPK cascade activations include scaffold proteins responsible for the organisation of specific cascades to specific activators, the regulation of physical interactions between members of a given cascade and the

indirect regulation of both ligands and inhibitors for cell-surface receptors that feed into MAPK cascades (Pierce et al., 2001;Su and Karin., 1996).

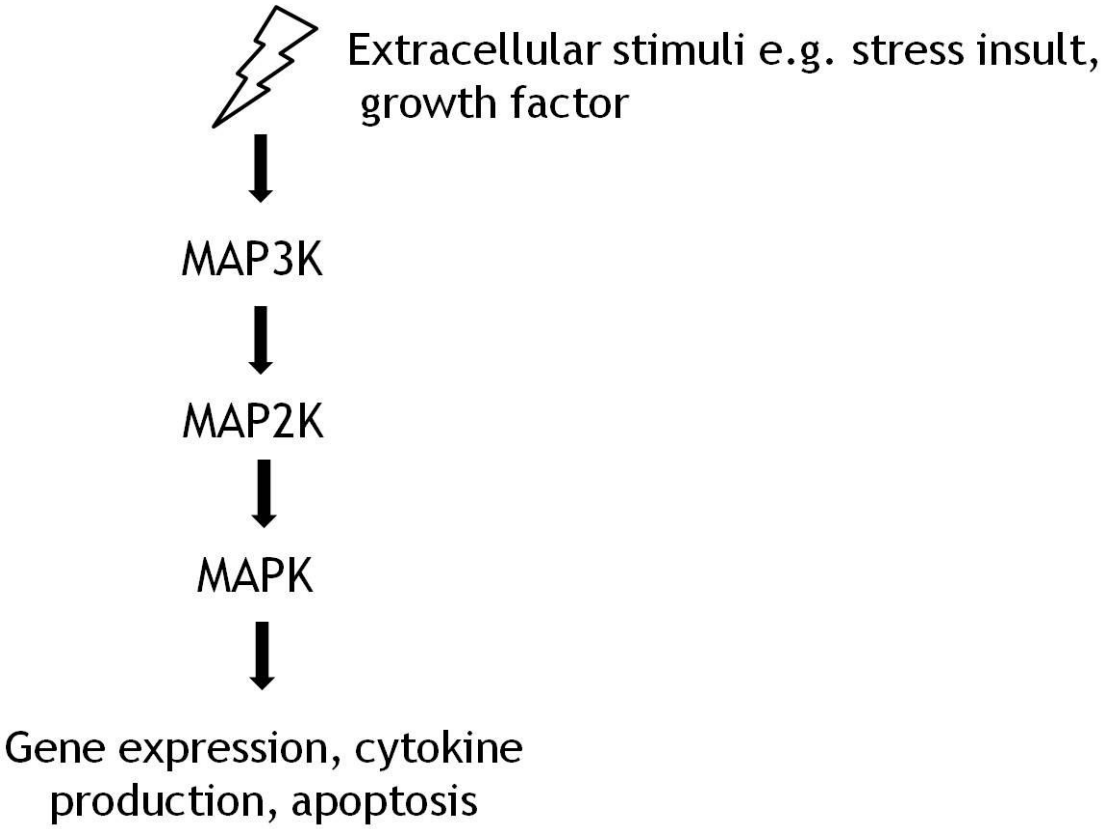


Figure 1.3 schematic diagram representing MAPK cascade. MAPK= mitogen activated protein kinase, MAP2K= mitogen activated protein kinase kinase, MAP3K= mitogen activated protein kinase kinase kinase.

The MAPK cascades are typically initiated by the activation of MAP3Ks in response to diverse cellular processes and extracellular stimuli. MAP3Ks sequentially phosphorylate and activate MAP2Ks through common mechanisms at two regulatory phosphorylation sites. MAP2Ks proceed to phosphorylate and activate MAPKs in a similar manner. Once activated, MAPKs recognise a number of substrates to allow the regulation of many processes such as gene expression, cytokine production and apoptosis.

1.13.2 Different classes of mammalian Map kinases

In mammalians, 4 distinct MAPK modules have been characterised (reviewed by (Johnson and Lapadat., 2002)). The four major MAPK members are: extracellular signal-regulated kinases (ERKs 1 and 2), C Jun amino- terminal Kinase (JNK1, 2, 3), also known as stress activated protein kinases (SAPK), p38 (α, ß, γ, δ) and the most recent to be identified, ERK5.

ERKs

The ERK signalling pathway was the first Mammalian MAPK pathway to be identified and is preferentially activated by growth- related signals. ERK1 and ERK2 are both activated by MKK1 (MEK1) and MKK2 (MEK2) through the concomitant phosphorylation of tyrosine and threonine residues, and subsequently undergo a conformational change. The activation of MKK1 and MKK2 is required for ERK activation, this occurs following phosphorylation by protein kinase Raf, which has in turn been activated by GTP-bound Ras.

ERKs act to regulate cell proliferation and cell differentiation. Interestingly, it was found ERKs are involved in signalling pathways of mature neurons that no longer require differentiation (Sava et al., 2011). Further studies revealed ERKs are involved in long-lasting neuronal plasticity, including long-term potentiation and memory consolidation, controlled by excitatory glutamatergic signalling through AMPA and NMDA receptors (Winder et al., 1999;English and Sweatt., 1997;Xia et al., 1996). These processes are crucial in the underlying mechanisms of complex behaviours in the brain such as learning and memory and a role of ERK in memory retention and fear conditioning has been confirmed in animal studies.

JNKs

JNK (also known as SAPKs) are important in controlling programmed cell death or apoptosis in response to stress stimuli such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. The JNK protein kinases are encoded by three

genes; JNK1 (also known as MAPK8), JNK2 (MAPK9) and JNK3 (MAPK10) and like the other map kinases, JNKs are also activated through the phosphorylation of tyrosine and threonine by inflammatory cytokines such as interleukin by MAP2Ks. Similar to ERKS, studies have revealed JNKs have a role in the mediation of cortical and hippocampal synaptic plasticity (Yang et al., 2011).

P38 MAP kinase

P38 MAP kinases have four isoforms and play an important role in the production of cytokines such as IL-1, tumour necrosis factor alpha (TNFa) following activation. Because the p38 MAPKs are key regulators of inflammatory cytokine expression, they appear to be involved in human diseases such as asthma and autoimmunity (Irusen *et al.*, 2002;Wilms *et al.*, 2003).

MAP2K and MAP3K

Unlike MAPKs which are specifically recognised by their corresponding MAP2Ks, each of the MAP2Ks can be phosphorylated and activated by several different MAP3Ks. These MAP3Ks recognise different MAP2Ks, enabling diversity in the activation of MAPK pathways upstream of MAP2K. JNK2 is activated by either of the two dual specificity kinases, MAP2K4 and MAP2K7. MAP3Ks (c-Raf, MEKK4, MLK3) are more complex and require multiple steps for activation. They are allosterically controlled enzymes residing in an inactive state. Small ligands such as (ras) partially activate MAP3Ks, resulting in the formation of dimers, which act to phosphorylate and activate one another (Cheng *et al.*, 2005).

1.13.3 MAPKs and schizophrenia

Due to the pivotal role of MAPK in the integration, amplification and regulation of signal transduction and synaptic plasticity, it is not surprising that alterations in the expression and/or function of various intermediates MAP kinase cascades are involved in the neuropathophysiological events occurring in the brain in schizophrenia. Because of the role in glutamatergic signalling, many studies have focussed on the role of ERK in schizophrenia. However, recently, our lab found evidence of a putative role of the JNK2-*MAP2K7* pathway in schizophrenia as a risk factor in schizophrenia. An expression study revealed *MAP2K7* mRNA levels

are reduced in the PFC of *post mortem* schizophrenia brains. The role of *MAP2K7* as a risk factor in schizophrenia was strengthened further by a positive genetic association in a UK population (Winchester et al., 2012). The MAP2K7 protein is an upstream activator of JNK2 which mediates signal transduction and regulates many cellular processes such as cell growth and proliferation, transcription and apoptosis (Tournier *et al.*, 2001;Holland *et al.*, 1997;Mayer *et al.*, 2005). JNK2 has also been shown to have decreased expression and phosphorylation in the anterior cingulate cortex (Funk et al., 2011). However, further biological and functional evidence of a role for *MAP2K7* in schizophrenia is required.

1.14 Hypotheses and Aim

The genetic interactions underlying the pathophysiology of schizophrenia still remain ambiguous. Many genes have been implicated as risk factors in this disorder however these genes still require biological evidence in order to confirm an association. Recent genetic association studies have implicated both *FXYD6* and *MAP2K7* as risk factors in the susceptibility to schizophrenia; these associations have been strengthened further by the expression of FXYD6 in schizophrenia-related brain regions, such as the PFC and the involvement of MAP2K7 in signalling cascades used in cognitive processes. We sought to investigate the functional roles of FXYD6 and MAP2K7 and explore whether these genes are risk factors in the susceptibility to schizophrenia.

This study initially aims to investigate whether *FXYD6* and *MAP2K7* individually propose a risk to schizophrenia susceptibility in a north European population. Epistatic interactions between *FXYD6* and *MAP2K7* are also investigated in order to explore genetic interactions between these two genes in the risk to schizophrenia.

The role of *FXYD6* in psychiatric disorders is further explored by using mice homozygous for disrupted *Fxyd6*. FXYD proteins are known modulators of Na^+ K^+ ATPase activity and disruption of FXYD6 is hypothesised to result in altered activity of brain Na^+ K^+ ATPase. A molecular characterisation of these mice aims to confirm whether FXYD6 plays a modulatory role in brain Na^+ K^+ ATPase activity, and explores the role of FXYD6 in the neurochemical pathways associated with schizophrenia.

The expression of FXYD6 in the PFC and the hippocampus suggests this protein may be involved in the underlying mechanisms of cognition, such as working memory. Therefore the disruption of this gene could result in cognitive impairments in *Fxyd6*$^{-/-}$ mice. In order to investigate the role of FXYD6 in cognitive impairments, as well as behaviours associated with psychiatric disorders, such as sensorimotor gating, this study aims to explore a number of phenotypes in *Fxyd6*$^{-/-}$ mice in order to provide further insight into the functional role of FXYD6 in areas such as locomotor activity, cognition, anhedonia and in DA and glutamatergic circuitry.

Similarly to *FXYD6*, the functional role of *MAP2K7* is explored using mice haploinsufficient for *Map2k7* (*Map2k7*$^{+/-}$ mice). Due to the recent association of *MAP2K7* with schizophrenia and the role in MAPK cascades and glutamate signalling and synaptic plasticity, it is hypothesised the disruption of *Map2k7* in mice will result in impairments to neurochemical circuitry associated with schizophrenia and psychiatric disorders, as well as impaired cognition. This aim is explored via the molecular and phenotypic characterisation of *Map2k7*$^{+/-}$ mice.

Chapter 2. Materials and Methods

2.1 Materials

Routinely used chemicals and buffers are listed in Table 2.1 and Table 2.2 respectively. Sources for chemicals not listed in these tables are detailed in the relevant methods section.

Chemical	Formula	Source
Tris Base	$C_4H_{11}NO_3$	BDH, UK
Potassium chloride	KCl	
Potassium phospate	KH_2PO_4	
Sodium chloride	NaCl	
Sodium hydrogen phosphate	Na_2HPO_4	
Sucrose	$C_{12}H_{22}O_{11}$	
Adenosine 5'-triphosphate disodium	$C_{10}H_{14}N_5Na_2O_{13}P_3$	Sigma-Aldrich, UK
Ethanol	C_2H_6O	
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	
Imidazile.hcl	$C_3H_4N_2 \cdot HCl$	
L-histidine	$C_6H_9N_3O_2$	
Magnesium Chloride	$MgCl_2$	
Methanol	CH_4O	
Ouabain octahydrate	$C_{29}H_{44}O_{12} \cdot 8H_2O$	
Saponin	$C_{27}H_{42}O_3$	
Sodium citrate	$Na_3C_6H_5O_7$	
Sodium phosphate dihydrate	$NaH_2PO_4.2H_2O$	
Tween®20	$C_{58}H_{114}O_{26}$	
All oligo primers		

Table 2.1 Routinely used chemicals and their source.

Buffer	Recipe/ source
10x phosphate buffered saline	1.3M NaCl, 30mM $NaH_2PO_4.2H_2O$, 70mM Na2HPO4, pH 7.4. Diluted to 1x using dH_20
20x Saline sodium citrate (SSC) buffer	3 M NaCl, 0.3 M $Na_3C_6H_5O_7$
10x TBS (forumla)	200 mM Tris base, 1.37 M NaCl, pH 7.6. Diluted to 1x using dH_20

Table 2.2 Recipes for routinely used buffers

2.2 Genetic association study

The genomic DNA (gDNA) sample set used for the genetic association study consists of The University College London (UCL) Schizophrenia Case Control samples (Pimm et al., 2005) and West of Scotland DNA samples.

2.2.1 Power Calculation

Power of sample size was calculated using G*Power 3.1.2, the study had a power level of 0.95.

2.2.2 UCL schizophrenia case control samples

The UCL schizophrenia case control gDNA samples were obtained from Professor Hugh Gurling (Pimm et al., 2005) and are comprised of 300 unrelated, gender mixed gender healthy controls (age- information unavailable) and 293 mixed gender schizophrenia participants (mean age 44, age range 19-91, mean age of onset 23) from Greater London (Pimm et al., 2005). Samples were included if both parents were of English, Irish, Scottish or Welsh descent and had at least 3 grandparents of British Ancestry. Samples did not contain pedigrees. All schizophrenia participants have an International Classification of Diseases version 10 (ICD10) diagnosis of F20 Schizophrenia (World Health Organization., 1992) and Schedule for Affective Disorders and Schizophrenia- Lifetime version (SADS-L) (Spitzer and Endicott., 1978) interview was completed for all case and control participants. Research subjects with short-term drug-induced psychoses,

learning disabilities, head injuries, and other symptomatic psychoses were excluded from the present study. The UCL schizophrenia Case Control samples have been shown not to have significant population stratification by ancestry (Pimm et al., 2005). The UCL samples were collected with full ethical consent, and the approval of the local ethical committee.

2.2.3 West of Scotland samples

The West of Scotland cohort was recruited mainly from in or around Glasgow. It consists of 198 healthy, unrelated mixed gender healthy controls (mean age 32) recruited from local higher education facilities and matched as a group for age and sex and DNA from 211 schizophrenia participants (mean age 44 (range 18-78)) with an ICD10 F20 diagnosis, ascertained by a consultant psychiatrist and verified by researchers using structured interview and case note review; meeting ICD10 criteria for schizophrenia spectrum disorders. gDNA was isolated from blood by Demetrius Vouyouiklis and Catherine Winchester (PsyRING) using the DNAce Spin Blood Kit (BIOLINE., London, UK). These samples have been shown not to have significant population stratification. A multicentre Research Ethics Committee (MREC) approval letter (reference 03/0/093) was obtained for the West of Scotland samples. All participants gave informed consent.

2.2.4 SNP selection

Six tagging SNPs representing polymorphic loci in *FXYD6* were selected using publicly available genetic recombination data from Utah residents with ancestry from northern and western Europe (CEU) ((The International HapMap Consortium., 2003) and SNPbrowser™ Software v4.0, ABI Life Technologies). The criteria for inclusion in the study were: 1:MAF was not lower than 4-10%, 2: SNPs were in an area of high LD and 3: SNP genotyping assays were commercially available. SNP loci and LD plot for *FXYD6* can be observed in Figure 2.1 and SNP allele information in Table 2.3. TaqMan® SNP genotyping assays for each selected tagSNP were purchased from ABI Life Technologies.

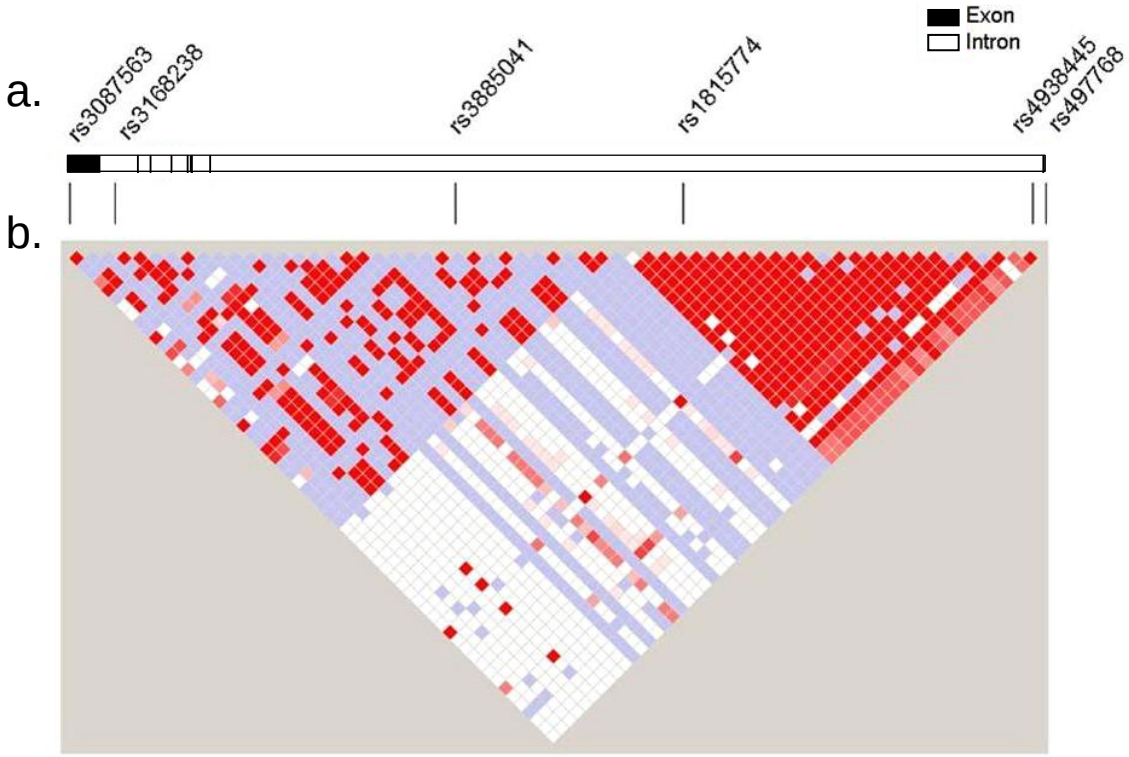


Figure 2.1 Tagging SNP positions on a) *FXYD6* locus and b) *FXYD6* linkage disequilibrium plot .

a) Six tag SNPs were selected for their ability to represent the polymorphic loci across the *FXYD6* gene. They are located within introns and exons

b) *FXYD6* linkage disequilibrium plot (The International HapMap Consortium, 2003). Red squares indicate high linkage disequilibrium between two loci (LOD≥ 2, D'=1), followed by pink (LOD≥ 2, D'<1), blue < 2, D'=1) and white <1, D'<1. Large areas of red squares indicate haplotype blocks of loci likely to be inherited together. SNPs were chosen by their ability to represent different haplotypes.

SNP ID	Assay ID	Location	Alleles	Ancestral allele	MAF	Allele x (SNP_VIC)	Allele y (SNP_FAM)	Polymorphism
rs30087563	2120467_10	Chr.11: 117707937	C:T	T	0.465	C	T	Transition Substitution
rs3168238	32368298_10	Chr.11:117709645	G:T	T	0.047	G	T	Transversion substitution
rs3885041	27877757_10	Chr.11: 117722742	C:T	C	0.221	C	T	Transition Substitution
rs1815774	11910811_10	Chr.11: 117731439	G:C	C	0.393	C	G	Transversion substitution
rs4938445	3236508_10	Chr.11:117745003	A:G	G	0.336	A	G	Transition substitution
rs497768	1048679_10	Chr.11:117750740	C:G	C	0.241	C	G	Transversion substitution

Table 2.3 *FXYD6* SNP assay information.

All SNP assays chosen for genotyping had a Minor Allele Frequency (MAF) higher than 4%.

2.2.5 Genotyping quality control

To confirm that the selected tagSNPs were polymorphic in European populations, the Human Random Control DNA panel 1 (HRC1) (Sigma- Aldrich) was genotyped using *FXYD6* TaqMan® SNP genotyping assays. Genotyping data quality was assessed using sample replicates across 96 well plates and not template control (NTC) samples were used to detect contamination. Failed and ambiguous samples were repeated.

2.2.6 Genotyping

Genotyping was done blind to sample diagnosis. Genotyping of the HRC1 gDNA sample set was carried out in 96 well plates using TaqMan® SNP Genotyping Assay 5' nuclease technology (ABI Life Technologies) according to the manufacturer's protocol. Briefly, a genotyping reaction mix (1x TaqMan® Genotyping master mix part no. 4371355, 1x SNP genotyping assay, dH_2O in a final volume of 15µl) was added to 5ng gDNA from each sample in a 96 well plate. Fluorescent amplicons were generated using standard thermal cycling conditions (Table 2.5 PCR conditions used for SNP genotyping.) using an ABI Prism Sequence Detection 7000 hardware and ABI Prism Sequence Detection 7000 software. Ambiguous samples were repeated. An end point read of the fluorescent signals generated a fluorescent cluster plot (Figure 2.2).

SNP ID	SNP VIC allele	SNP FAM allele
rs3087563	C	T
rs3168238	G	T
rs3885041	G	A
rs1815774	C	G
rs4938445	A	G
rs497768	C	G

Table 2.4 SNP genotypes

PCR Cycle			
Stage	Temperature	Time	Cycles
1	50°C	2 minutes	1
2	95°C	10 minutes	1
3	95°C	15 seconds	40
4	60°C	10 minutes	1

Table 2.5 PCR conditions used for SNP genotyping.

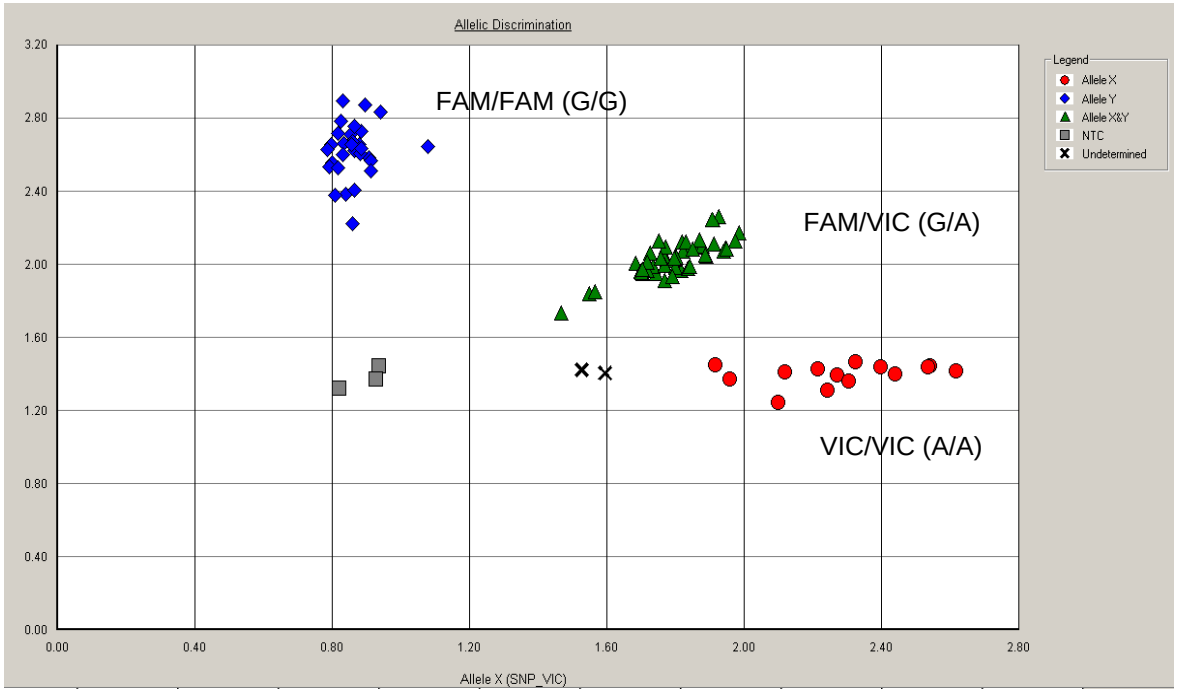


Figure 2.2 Example of end point read cluster plot and genotype calling.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (alleles A/A), ◆ = samples homozygous for SNP_FAM™ (alleles G/G), ▲ = samples heterozygous for SNP_VIC® and SNP_FAM™ (alleles G/A).

2.2.7 Haploview

All genotyping data were uploaded to Haploview v4.2 (www.broad.mit.edu/mpg/haploview) in Linkage Format as a .txt file. All data and statistical analyses were performed using Haploview v4.2.

2.2.8 Quality Control

Data quality was assessed using 'check markers' in Haploview. Genotyping call rate was assessed; this was taken as the percentage of samples which were successfully genotyped by each SNP genotyping assay and was used to give indication of quality of genotyping and power of the study. Hardy-Weinberg equilibrium (HWE) *p*-value ($p<0.001$) was determined for controls and cases for each SNP genotyping assay. This provided an indication of any deviation from genetic equilibrium and is used to primarily detect errors in genotyping. The output file also provides the following information for each SNP genotyping assay: expected heterozygote genotype numbers, observed heterozygote genotype numbers, MAF and alleles detected.

2.2.9 Case/control association test

Case/ control association tests were performed on allele frequencies for all SNP genotyping data. The output file provided information on the dominant allele, the allele frequency for case and controls, the Chi Square value and the *p* value.

2.2.10 Linkage Disequilibrium blocks

Linkage disequilibrium was investigated in all data; blocks sharing high levels of linkage disequilibrium were defined using Haploview's 'solid spine of LD' method using *D'* values greater than 0.8. Haplotype blocks were analysed for association. Output data provided information on frequency of haplotype associations, case/control ratios, chi square value and the derived *p* value. MAF and LD data from this study were compared to published CEU HapMap Data.

2.2.11 Epistatic interactions with Map2k7

Epistatic interactions between *FXYD6* and *MAP2K7* (*MAP2K7* human data generated from C. Winchester) were investigated using SNPStats using a co-dominant model (www.bioinfo.iconologia.net). Data were formatted under the following headings: ID, sex (1 male, 2 female), status (Ca (control), Co (case)), SNP ID (alleles) and analysis of interaction of covariates was used to indicate epistasis.

2.3 Mouse lines.

2.3.1 Fxyd6 Founder Mice

F1 heterozygote founder mice (*Fxyd6*$^{+/-}$) were obtained from (Lexicon Pharmaceuticals Incorporated, 2007). The *Fxyd6* knockout mice were generated via a gene trap mutation disrupting the *Fxyd6* gene upstream of the first coding exon (Figure 2.3a). The F1 heterozygote founder mice had a mixed genetic background of *C57BL/6J* and *129SvEvBr* strain.

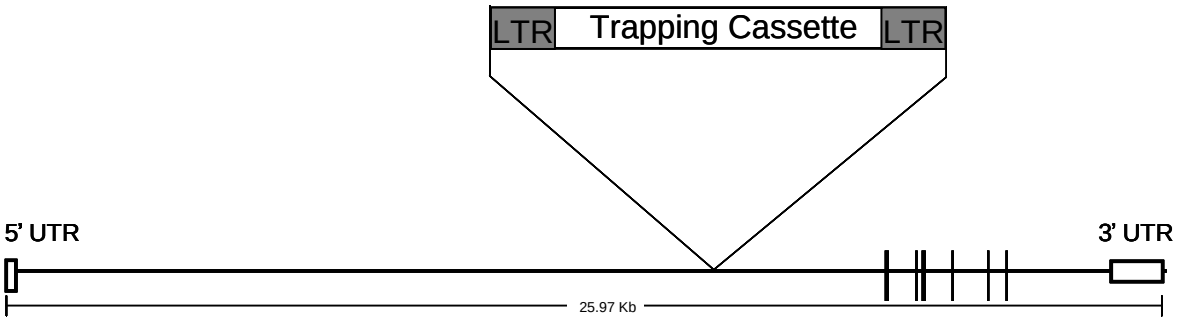
2.3.2 Map2k7 Founder Mice

Founder *Map2k7* mice were a gift from Professor. J. Penninger (Institute of Molecular Biology of the Austrian Academy of Science, Vienna, Austria). The *Map2k7* knockout mouse was generated by replacement of a portion of exon 9 with a PGK-Neo cassette (Figure 2.3b). Homozygous disruption of *Map2k7* (*Map2k7*$^{-/-}$) results in embryonic lethality, thus only wildtype (*Map2k7*$^{+/+}$) and heterozygous (*Map2k7*$^{+/-}$) mice for *Map2k7* were used in the following studies. The founder Map2k7 Mouse background comprised of at least 87.5% *C57BL/6J* (the rest is *129SvEvBrd* (129) strain).

2.3.3 Backcrosses

All *Fxyd6* and *Map2k7* mice used in procedures were backcrossed onto C57b6/j_rcchsd strain to eliminate variability which may arise from a mixed genetic background. Mice used in chapter 4 were backcrossed for 3 generations, whilst mice used in chapter 5 were backcrossed for 4 generations, and mice used in chapter 6 were backcrossed for at least 5 generations. Mice used for generating backcrosses were aged 6-8 weeks, pair housed in a temperature and humidity regulated room with a 12-h dark/light cycle. Animals were given access to food and water *ad libitum*.

a



b

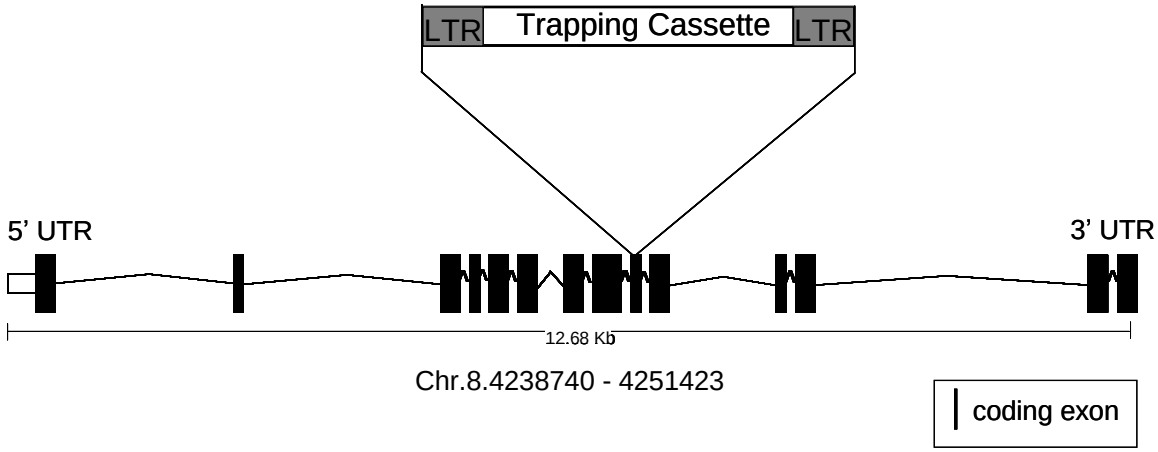


Figure 2.3 Schematic diagram representing disruption of

a) *Fxyd6* gene in *Fxyd6*$^{-/-}$ mice showing the insertion of a trapping cassette up stream of the first coding exon. Adapted from (Lexicon Pharmaceuticals Incorporated, 2007)

b) Disruption of *Map2k7* in *Map2k7* $^{+/-}$ mice showing the insertion of a PGK neo cassette into exon 9. Not drawn to scale. UTR= untranslated region. LTR= long terminal repeat.

2.4 Genotyping

2.4.1 DNA extraction

Ear clips were taken from all *Fxyd6* and *Map2k7* mice at 3 weeks of age. Genomic DNA was extracted by digesting ear clips in lysis buffer (0.8x cell lysis solution (Promega, Cat no A7933), 0.1M EDTA, 140 µg Proteinase K (Sigma-Aldrich, UK) at a total volume of 200 µl) overnight at 50°C with agitation at 1000 rpm for a duration of 10 seconds once every minute. The following day Proteinase K was deactivated by heating samples to 99°C for 15 minutes. 2x volume of 100% ethanol was added to all samples prior to centrifugation at 13,000 rpm for 5 minutes. Supernatant was discarded and 1 ml of 70% ethanol (w/v) was added to all samples prior to centrifugation at 13,000 rpm for 2 minutes. Supernatant was discarded and samples were centrifuged at 13,000 rpm for 5 seconds. Remaining supernatant was discarded and DNA pellet samples were air-dried for 30 minutes. DNA was re-suspended in 100 µl dH_20 by incubation at 50°C for 20 minutes with agitation at 1000 rpm for 10 seconds per minute. DNA samples were stored at -20°C until use.

2.4.2 Quantification of DNA

The concentration DNA determined using a spectrophotometer (Gene Quant, RNA/DNA Calculator, Amersham Pharmcia Biotech) set to measure absorbance at wavelength 260 nm and path length 5 mm. Measuring the wavelength at 260 nm gives a direct indication of nucleic acid quantity. The following calculation was used to derive the exact concentration of DNA in each sample:

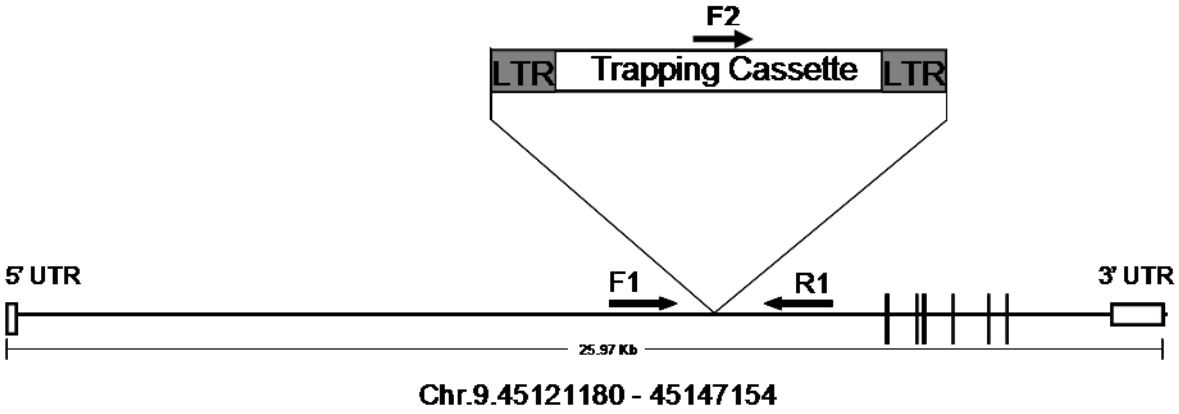
OD260 x dsDNA co-efficient x path length correction = concentration of total DNA (µg/µL)

2.4.3 Polymerase Chain Reaction (PCR)

PCR was used to amplify regions of mouse genomic DNA extracted from *Fxyd6* and *Map2k7* mice in order to determine their genotype using KOD Hotstart DNA polymerase (Merck Millipore, UK). Reaction components used in PCR reaction are outlined in Table 2.6 and conditions in Table 2.7. PCR primers specific for *Fxyd6* and *Map2k7* were designed using OligoPerfect™ Designer (Invitrogen, UK). Proposed forward and reverse primers were 'blasted' using Nucleotide Blast

(NCBI) to ensure primers were specific to gene of interest. The wildtype forward primer for *Fxyd6* targets the region upstream of the trapping cassette insert (intron 1) and the reverse immediately following the insertion site (intron 1) (Figure 2.4). To identify mice with disrupted copies of *Fxyd6*, a forward primer was designed to recognise the trapping cassette. *Map2k7* wildtype mice were distinguished from *Map2k7* heterozygote mice by primers designed to target regions either side of the trapping cassette insertion site (exon 9). A primer was designed to anneal to the trapping cassette in order to identify heterozygote mice (Figure 2.4). Primer sequences are displayed in Table 2.8 and example genotype results can be observed in Figure 2.4b and Figure 2.5b.

a



B

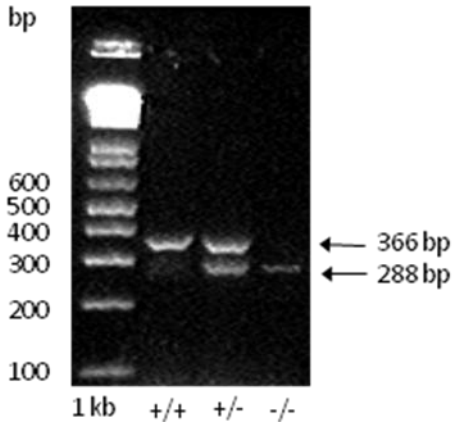
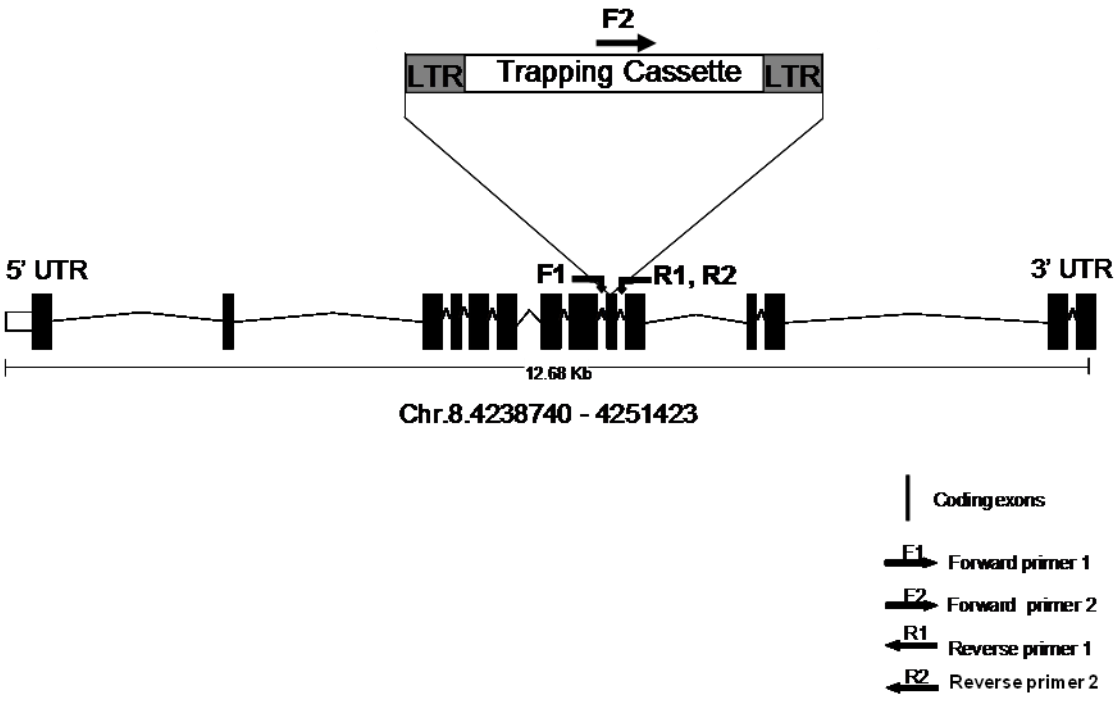


Figure 2.4 *Fxyd6* Genotyping PCR primer locations

a) *Fxyd6*. Wildtype primers (F1, R2) are adjacent to cassette insert site (upstream of the first coding exon), the presence of the cassette will inhibit the annealing of the wildtype primers. F2 primer has been designed to target the insert, identifying heterozygous and knockout mice.

b) Representative PCR gel used to identify genotypes. *Fxyd6*$^{+/+}$ mice $^{(+/+)}$ have a single band at 366 bp, *Fxyd6*$^{+/-}$ mice $^{(+/-)}$ have two bands at 288 bp and 366 bp, *Fxyd6*$^{-/-}$ mice $^{(-/-)}$ have a single band at 288 bp. 1 kb= 1 kb pair ladder.

a



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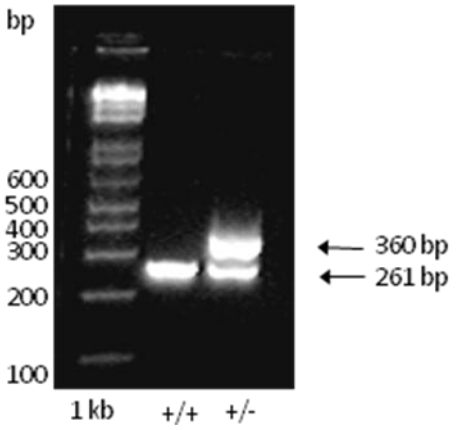


Figure 2.5 *Map2k7* Genotyping PCR primer locations

a) *Map2k7*. Wildtype mice are identified by primers F1 and R1, located in introns flanking exon 9, the site of trapping cassette insertion. The disruption of *Map2k7* by the insertion of the trapping cassette is identified by primers F2 and R2, located along and immediately after the trapping cassette.

b) Representative PCR gel used to identify genotypes. *Map2k7*$^{+/+}$ mice (+/+) have a single band at 360 bp, *Map2k7*$^{+/-}$ mice (+/-) have two bands at 261 bp and 360 bp, *Map2k7*$^{-/-}$ mice are embryonically lethal. 1 kb= 1 kb pair ladder.

Component	Final Concentration
KOD Hot Start DNA Polymerase buffer (Merck Millipore, UK)	1X
$MgSO_4$	1.5 mM
dNTPs	0.2 mM (each)
PCR Grade Water	
Primers	0.3 uM each
gDNA	0.1 ug
KOD Hot Start DNA Polymerase	1 µl
DMSO	2%

Table 2.6 PCR master mix components and concentrations optimised for use with primers designed to genotype Fxyd6 and *Map2k7* mice.

Step	Conditions
1. Polymerase Activation	95 °C for 2 min
2. Denature	95 °C for 20 s
3. Annealing	
Fxyd6	58 °C for 10 s
Map2k7	66 °C for 10 s
4. Extension	70 °C for 10 s
Repeat steps 2-4	30 Cycles

Table 2.7 PCR reaction conditions optimised for primers targeted to *Fxyd6* and *Map2k7*.

Gene	Primer	Sequence 5' → 3'
Fxyd6	Wildtype forward	TACCTCACACCTCAGTTCCAAGTGG
	Cassette forward	AATGGCGTTACTTAAGCTAGCTTGC
	Reverse	GGGTGGGAGTTCTCGCCTATCACAG
Map2k7	Wildtype Forward	GCTCTGTGACTTTGGCATCA
	Wildtype Reverse	GCCCCAACTAACCAGTGAGA
	Cassette Forward	GGATGTGGAATGTGTGCGAG
	Cassette Reverse	TCCCCAGTAGCATGAGGACAC

Table 2.8 Sequences of forward and reverse primers used to target and amplify regions of *Fxyd6 (*refer *to* Figure 2.4a)Figure 2.4 *Fxyd6* Genotyping PCR primer locations and *Map2k7* (refer to Figure 2.4b).

2.5 Na^+ K^+ ATPase activity in Fxyd6 mice

2.5.1 Preparation of crude plasma membrane

Mice (male, 12 weeks old) were killed by cervical dislocation, the brains removed rapidly on ice and the prefrontal cortex and hippocampus dissected and immediately frozen on dry ice. Brain regions were stored at -80°C until use and stored overnight at - 20°C prior to testing. Each frozen brain region was homogenised using a plastic Dounce in 300 µl ice cold 0.32 M sucrose solution buffer and further homogenised by being passed through a 26g needle 10 times. Homogenates were centrifuged at 1000 x g for 10 minutes at 4°C (Sigma 2K15 rotor Nr. 12148) and supernatants were extracted into a new eppendorf. Crude plasma membranes from supernatants were obtained via differential centrifugation using sucrose gradients, briefly supernatants were centrifuged at 1000 x g for 10 minutes at 4°C (Sigma 2K15 rotor Nr. 12148). The resultant supernatants were layered over a sucrose gradient of 300 µl 0.32 M ice-cold sucrose and 700 µl 1.2 M ice-cold sucrose in Thinwall Polyallomer tubes (Beckman Coulter) and centrifuged at 34,000 x g for 50 minutes at 4°C in a TLS55 Beckman rotor. The fraction collected between 0.32 M and 1.2 M sucrose was diluted 1:3 with ice-cold bi-distilled water, then layered over 500 µl ice cold 0.8 M sucrose and centrifuged at 34,000 x g for 30 minutes. The pellet was re-suspended in 300 µl ice cold 5 mM imidazole- HCl buffer (pH 7.4), an aliquot was taken for protein quantification, and the rest stored over night at -20°C.

2.5.2 Protein quantification

Protein concentration of plasma membrane fractions were determined by an adapted version of the method of Bradford (Bradford, 1976). Briefly, a standard curve of bovine serum albumin (BSA, Sigma-Aldrich, UK) ranging from 0 µg/ml to 250 µg/ml was prepared in respective homogenisation buffer. Samples, depending on size of tissue region, were either used neat or diluted in homogenisation buffer to ensure protein concentration is within the range of the standard curve. 10 µl of each sample or BSA standard was pipetted, in triplicate, into a flat-bottomed 96 well plate and 200 µl of 1:4 protein assay dye reagent

concentrate (BIO-RAD, Bio-Rad laboratories, UK) (diluted with ddH_20) was added to each well and incubated at room temperature for 10 minutes to allow colour development. Absorbance was read at OD 630nm using Revelation software on a DYNEX MRX plate reader. A linear regression of optical density vs. protein concentration was calculated from the known standard concentrations and the concentration of each sample was calculated from the standard curve.

2.5.3 Na^+ K^+ ATPase activity assay

The day following the preparation of crude plasma membrane, Na^+ K^+ ATPase activity was tested in samples by a method adapted from (Sarkar., 2002).

20 µg/ml protein suspended in imidazole.HCl buffer (pH 7.4) was added 1:1 to 2x ATPase buffer (60 mM imidazole-HCl, 280 mM NaCl, 60 mM histidine, 20 mM $MgCl_2$, pH 7.4) and 1:1 to ouabain-ATPase buffer (60 mM imidazole-HCl, 50 mM KCl, 20 mM ouabain, 280 mM NaCl, 60 mM L-histidine, 20 mM $MgCl_2$, pH 7.4) (total volumes 100 µl). Samples were incubated in the dark, on ice for 60 minutes. 20 mM Na.ATP was made up in the respective buffer and 100 µl was added to all samples to give a final concentration of 10 mM. Samples were incubated for 5 minutes at 37°C with agitation at 1000 rpm for a duration of 10 seconds per minute. Reactions were stopped via the addition of 400 µl BioMol Green (Enzo Life Sciences, Inc, Exeter UK) and the optical density was read 25 minutes later at 630 nm wavelength on Dynex Technologies MRX plate reader. Background was deducted using tissue-free control samples. Na^+ K^+ ATPase - specific activity was calculated by deducting the signal from samples incubated in ouabain- ATPase buffer from samples incubated in ATPase buffer.

2.6 Confirmation of protein expression by western blotting

2.6.1 Samples

Samples used for western blotting were either prepared as crude plasma membranes (section 2.5.1) or brain regions were dissected on ice and homogenised with a plastic Dounce in 300- 500 µl ice cold 0.32 M sucrose solution buffer. The homogenates were further homogenised by being passed through a 26g needle 10 times.

2.6.2 Sample preparation

32.5 µg of crude plasma membrane or 16 µg of protein homogenate (determined from the protein concentration (see section 2.5.2)) was added to 4x NuPAGE® LDS sample buffer (Invitrogen, UK) and 10x NuPAGE® reducing agent (Invitrogen, UK), and denatured at 70°C for 10 minutes.

2.6.3 SDS-polyacrylamide gel electrophoresis (-PAGE)

Proteins were separated by molecular weight using SDS-PAGE gel electrophoresis in the XCell *SureLock* Mini-Cell system (Invitrogen, UK). 10 µl of pre-stained Novex® Sharp Protein Standard ladder (Invitrogen, UK) was added to the first well of a 10% NuPAGE® Novex® Bis-Tris gel (Invitrogen, UK). 20 µl of prepared samples were added to the subsequent wells. The gel was run in 1x NuPAGE® MOPs SDS running buffer (Invitrogen, UK) at 200 V for 45 minutes, or until the dye had run to the bottom of the gel, with a Gibco BRL PS304 Electrophoresis power supply (Gibco BRL Life Technologies).

2.6.4 Transfer of protein to nitrocellulose

Following the running of samples, proteins were transferred onto an Invitrolon™ Polyvinylidene fluoride (PVDF) membrane (0.45 µm pore size, Invitrogen, UK). Membranes were submerged in 100% methanol (>1 minute) prior to being soaked in 1x NuPAGE® transfer buffer. All filter papers and sponges were soaked in 1x NuPAGE® transfer buffer. The gel was carefully removed from the gel plates and the transfer cassette was assembled in the following order: cassette back, sponges x3, filter paper, gel, PVDF membrane, filter paper, sponges x4, cassette front and inserted into the MiniCell System. The system was filled with 1x NuPAGE® transfer buffer and proteins were transferred at 30V for 2 hours using a Gibco BRL PS304 Electrophoresis power supply (Gibco BRL Life Technologies).

2.6.5 Protein detection

Following protein transfer, membranes were rinsed twice in ddH_20 and blocked with 3% (w/v) milk powder (Marvel) in TBS/Tween (0.05% (w/v) for 2 hours at

room temperature with gentle agitation. Membranes were further incubated overnight in the relevant blocking buffer and primary antibody (Table 2.9).

After overnight incubation, the membranes were washed in TBS/Tween (0.05% (w/v)) followed by 2hr incubation with the appropriate secondary antibody in 1% milk powder in TBS/Tween (0.05%) at room temperature (see Table 2.10).

Antibody	Species	Working dilution	Supplier
α-Fxyd6	Rabbit	1:1000	Gift from B. Delprat
α-Actin-HRP	Rabbit	1:2000	Santa Cruz
α-Actin-HRP	Goat	1:5000	Santa Cruz
α-Na^+ K^+ ATPase α1 s	Mouse	1:1000	Abcam
α-Map2k7	Rabbit	1:1000	Calbiochem
α-GluR2	Rabbit	1:1000	Sigma
α-GAD	Rabbit	1:500	Sigma

Table 2.9 The primary antibodies and their working dilutions used for western blotting

Antibody	Working dilution	Supplier
α- Mouse HRP	1:5000-1:10,000	Santa Cruz
α- Mouse HRP	1:5000-1:10,000	

Table 2.10 The secondary antibodies and their working dilutions used for western blotting

2.6.6 Protein Visualisation

Enhanced chemiluminescence (ECL) used for protein visualisation was dependent on primary antibody. FXYD6 required homemade ECL (1:1 solution A (33% (w/v) DMSO, 2.5 mM luminol (Fluka analytica, UK), 0.9M coumaric acid (Sigma- Aldrich, UK), 0.1M Tris) solution B (0.02% (v/v) H_2O_2 in 0.1M Tris)) applied for 1 minute. All other antibodies required Millipore Immoblin (Fischer Scientific, UK), applied for 5 minutes and exposed to X-ray film for 15 seconds- 20 minutes and developed using an automatic developer (HyperProcessor, Amersham)

The film images were scanned to obtain a digital image and the amount of protein in samples was estimated using image analysis (Image J).

2.7 Gene transcript expression RTq-PCR

2.7.1 Total RNA isolation

PFC samples were homogenised in Buffer RLT with (% (v/v) ß-mercaptoethanol) using Hybaid Ribolyser tubes (Lysing Matrix D, Qbiogene., UK) and total RNA was isolated using silica-based membrane spin columns in RNEasy mini Kit (Qiagen., UK), according to the manufacturer's protocol. Total RNA was eluted using DNase/RNase free water (Ambion., UK) and contaminating DNA removed by treatment with DNaseI (Invitrogen., UK) according to manufacturers protocol. The quality of total RNA was analysed using the Nano chip Bioanalyser 2100 (Agilent Technologies., UK) and quantified using Nanodrop™ 1000 (Thermo Scientific., UK). Samples were stored at -80°C until use.

2.7.2 First Strand CDNA Synthesis

First strand cDNA was synthesised from 1 µg of total RNA using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies., UK) according to the manufacturers protocol. In order to detect contamination, samples with no template and no enzyme were included. Samples were stored at -20°C until use.

2.8 Quantitative real time reverse transcriptase PCR (RTq-PCR)

2.8.1 Primers

Gene expression assays were purchased from Applied Biosystems (refer to Table 2.11). B2M, ribosomal 18s and GAPDH were used to create a geometric mean to normalise C_T values generated from genes of interest (Fxyd6_m1, Fxyd6_g1, Map2k7 10/11, Map2k7 1a/1b, PVALB, Grin1).

Gene expression assay	Gene	Amplification region	Status	Source
B2M	*B2M*		Housekeeping gene	Applied Biosystems
18s	*18s*		Housekeeping gene	Applied Biosystems
GAPDH	*GAPDH*		Housekeeping gene	Applied Biosystems
Fxyd6_m1	*Fxyd6*	*E*xons 1 & 2	Gene of interest	Applied Biosystems
Fxyd6_g1	*Fxyd6*	Exons 3 & 4	Gene of interest	Applied Biosystems
Map2k7 10/11	*Map2k7*	Exons 10 & 11	Gene of interest	Applied Biosystems
Map2k7 1a/1b	*Map2k7*	Exons 1a & 1b	Gene of interest	Applied Biosystems
PVALB	*PVALB*		Gene of interest	Applied Biosystems
Grin1	*Grin1*		Gene of interest	Applied Biosystems

Table 2.11 Gene expression assays were purchased from Applied Biosystems. B2M, ribosomal 18s (18s) and GAPDH were used to create a geometric mean to normalise C_T values generated from genes of interest.

2.8.2 RTqPCR

500 ng of RNA equivalent 1st strand cDNA and mastermix (1x TaqMan®Gene Expression Master Mix (Applied Biosystems), 1x Gene Expression Assay (Applied Biosystems) and nuclease-free H_2O to a final volume of 25 µl) was aliquoted, in triplicates, into a 96 well MicroAmp™ optical reaction plate (Applied Biosystems) and sealed with MicroAmp™ optical adhesion film (Applied Biosystems). To detect contamination, cDNA samples of known genotype were used as controls controls (section 2.7.2) and no template controls were included. PCR thermal cycling conditions were ran for 50°C for 2 minutes, 90°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 10 minutes on the ABI SDS7000.

2.8.3 Gene expression quantification

C_T values (cycle threshold) were exported for each well. The C_T value is defined as the number of cycles required for the fluorescent signal to exceed reach an arbitrary set fluorescent threshold in the linear phase of the amplification level, this level is set as a threshold of 0.3. As C_T levels are inversely proportional to the amount of target nucleic acid in the sample, values can be used to quantify gene expression using LinRegPCR. No template controls were checked for non-amplification. Triplicates were checked for outliers using the Grubbs test (GraphPad, http://graphpad.com/quickcalcs/Grubbs1.cfm) and sample averages were calculated. REST V2.0.13 (Qiagen., 2009) was used to calculate relative expression of each gene of interest, values were normalised by using a geometric

mean from the three reference genes using amplification efficiencies obtained from LinReg. 2000 iterations were used and a hypothesis test was performed with 10,000 random reallocations of samples and controls between the groups, significance was set at $p<0.05$.

2.9 *In situ* Hybridisation

2.9.1 Labelling Oligonucleotide probes

A 7 µl mastermix containing 5ng oligonucleotide, 1x TdT buffer, 3.6 µl α^{33}P-dATP and 1.5 µl TdT enzyme was incubated at 32 °C for 60 minutes (Amersham Biosciences, UK). 50 µl DEPC-treated water was added to the mastermix and labelled nucleotide probes were separated from excess radioactivity using Biospin 6 Tris Columns (Biorad, 732- 6227, UK), according to manufacturer's instructions. The radioactivity counts of probes were calculated using a liquid scintillation analyser (2200CA, Tricarb-Packard). Probes with a radioactivity count between 100-500 x 10^3 dpm/ µl in a total volume of 50 µl were considered successfully labelled.

2.9.2 Slide preparation and fixation

Brains were removed from -80°C and left to equilibrate to -20°C for at least 1 hour prior to sectioning. 20 µm coronal sections containing the prefrontal cortex, striatum/ nucleus accumbens, hippocampus, substantia nigra on the cerebellum were collected using a -20°C cryostat (CM1850, Leica). Sections were thaw-mounted onto Poly-L-Lysine coated slides (0.01% w/v) and air-dried for around one %hour prior to fixation in 4% (w/v) paraformaldehyde (in PBS) for 5 minutes. Slides were then transferred into a sterile trough containing 1 x PBS then dehydrated in 70 % (v/v) ethanol followed by 95% (v/v) ethanol for 5 minutes each and stored under 100% ethanol at 4°C until use.

2.9.3 Hybridisation

Sections were removed from ethanol and air-dried for ~30 minutes. Experimental sections were incubated in 'Hot' probe buffer (4 µl labelled probe, 200 µl hybridisation buffer (4x SSC, 50% deionised formamide, 10% dextran sulphate, 0.5M sodium phosphate, 0.1M sodium pyrophosphate, 54 mM polyadenylic acid and covered with Parafilm® to ensure sections and probes do no dry out. Controls sections were incubated in 'Cold' probe buffer (4 µl labelled probe, 4.5 µl unlabelled probe, 200 µl hybridisation buffer) and covered with Parafilm®. All sections were placed in petri- dishes containing SSC (150 mM NaCl, 15 mM Na.Citrate) -soaked tissue to maintain humidity and incubated overnight at 42°C.

2.9.4 Hybridisation- washing

Slides were removed from the oven and submerged in 1x SSC at 55°C. Parafilm® covers were carefully removed and slides were transferred into a fresh trough of 1x SSC at 55°C for 30 minutes with slight agitation. Slides underwent a dehydration step by immersion in 0.1 x SCC, 70% (v/v) ethanol and 95% (v/v) ethanol for 20 seconds each with slight agitation. Slides were air-dried for ~ one hour and placed under Biomax™MR films (Kodak) for 1 to 3 weeks.

2.9.5 Film Development and analysis

Biomax™MR films (Kodak) were developed using an automatic developer (HyperProcessor, Amersham, Piscataway, NJ, USA). mRNA levels were analysed using Image J software. Non-specific labelling (slides labelled with 'cold probe') was deducted from total labelling (slides labelled with 'hot probe') to quantify mRNA levels.

2.10 Phenotyping

2.10.1 Primary phenotype screen

A primary phenotype screen was carried out on *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice and *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice as a first screen for phenotype abnormalities. This consisted of a modified version of the Smithkline Beecham, MRC Harwell, Imperial College, the Royal London Hospital Phenotype Assessment (SHIRPA) assessment. PPS was carried out as outlined in Table 2.12. Weekly weight measurements were catalogued and a growth curve for each strain was produced. *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice n=10 per group, aged 7 weeks. *Map2k7*$^{+/+}$ mice n=9 and *Map2k7*$^{+/-}$ mice n=7 per group, aged 6 weeks. Data analysed by Student's t-test.

Testing location	Measure	Response score
In arena	Body position	0= Inactive, 1= Active, 2= Excessive Activity
	Spontaneous activity	0= Absent, 1=Intermittent rapid darts, 2= Excessive rapid darts
	Tremor	0= Absent, 1= Present
	Urination	Quantity
	Defecation	Quantity
	Bizarre behaviours	0= Absent, 1= Present
	Convulsions	0= Absent, 1= Present
	Palpebral closure	0= Eyes fully open, 1= Eyes mid closed, 2= Eyes fully closed
	Gait	0= Fluid movement, 1=Lack of fluidity in movement
	Pelvic Elevation	0= Flattened, 1=Normal (3 mm elevated), 2= Elevated
	Coat appearance	0= Tidy and well groomed, 1= Ungroomed
	Piloerection	0= Absent, 1= Present
	Tail Elevation	0=Dragging, 1= Horizontal extension, 2= Elevated/ straub tail
Restrained	Pinna reflex	0= Present, 1= Absent
	Cornea reflex	0= Present, 1= Absent
	Lacrimation	0= Absent, 1= Present
	Evidence of biting	0= Absent, 1= When handled
	Vocalisation	0= Absent, 1= Present
	Trunk curl	0= Absent, 1= Present
Other	Hanging wire n=3	mean latency to fall from wire (three 120s trials)
	Contact righting reflex	0= Present, 1= Absent

Table 2.12 Primary phenotype screen testing parameters.

The battery of tests is designed to reveal any basic phenotype abnormalities in a new mouse line. Phenotyping is carried out either with a free moving mouse in an arena or by restraining the mouse.

2.10.2 Rotorod

The rotorod assesses motor coordination and consisted of a rotating drum set to accelerate gradually. Latencies for mice either to fall from the rod or to passively rotate with the rod were recorded. The speed was initiated at 4 RPM, and incrementally increased until 40 RPM was reached within a 5 minute time period. Testing was repeated three times with a 15 minute resting interval between tests. *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice n=10 per group, aged 7 weeks. *Map2k7*$^{+/+}$ mice n=9 and *Map2k7*$^{+/-}$ mice n=7 per group, aged 6 weeks. Data analysed by Student's t-test.

2.10.3 Plus Maze

Plus maze apparatus consisted of two opposing enclosed, dark arms (30 cm x 6 cm) and two opposing open light arms (30 cm x 6 cm). The mouse was placed in centre of the maze and allowed to freely move for 5 minutes. An overhead infra-red detecting camera tracked and analysed activity. Data was analysed using EthoVision Video Tracking System (Noldus Information Technology, Leesburg, VA). Four zones were created in order to analyse behaviour and admittance to open and enclosed arms, these zones are out lined in Figure 2.6. *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice n=10 per group, aged 7 weeks. *Map2k7*$^{+/+}$ mice n=9 and *Map2k7*$^{+/-}$ mice n=7 per group, aged 6 weeks. Duration and frequency analysed by analysis of variance (ANOVA) followed by Tukey's *post hoc* for multiple comparisons. Distance and mean velocity analysed by Student's t-test.

2.10.4 Open field (OF)

The OF apparatus consisted of four identical plastic arenas (dimensions 40 cm x 40 cm x 40 cm) situated on an infra-red light box. Movement within the arenas was tracked by an overhead infra-red detecting camera using Ethovision XT (Noldus) video tracking software. In order to habituate mice to arenas, one mouse was placed in each arena for 30 minutes prior to locomotor assessment and allowed to move freely.

Locomotor activity was assessed immediately following habituation to the arenas. Mice were removed from arenas for around five minutes and during this time received subcutaneous injections of vehicle (0.9% (w/v) saline) or d-amphetamine sulphate (Sigma-Aldrich., UK) (3 mg/ kg/ 5 ml) and immediately placed back in the arena and allowed to move freely for 1 hour. Mice received both treatments over 3 days, with one day washout between drug administrations. Treatment groups were fully counterbalanced. Data were analysed using Ethovision software. An inner (20 cm X 20 cm) and outer zone were created (Figure 2.7) to assess anxiety and exploratory like behaviour. Velocity was measured by distance (cm) moved per second. *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice n=12 per group, aged 8 weeks. *Map2k7*$^{+/+}$ mice n=9 and *Map2k7*$^{+/-}$ mice n=7 per group, aged 7 weeks. Duration and frequency analysed by ANOVA followed by Tukey's *post hoc* for multiple comparisons. Distance and mean velocity analysed by Student's t-test.

2.10.5 Prepulse inhibition

Prepulse inhibition (PPI) was assessed following a 7 day washout period after the open field amphetamine challenge. PPI apparatus consisted of four identical sound-attenuated chambers. Immediately prior to PPI testing, mice received intraperitoneal (ip) injections of vehicle (0.9% (w/v) saline) or ketamine (10 mg/ kg/ 2 ml and 30 mg/ kg/ 2 ml). Mice was subsequently placed in restrainers and secured in the PPI chamber. Mice were exposed to 120 dB noise (pulse), with varying levels of prepulse preceding the pulse. Prepulses are of values 4, 8 and 16 dB above background white noise of 65 dB. Mouse startle response to the pulses were recorded. *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice n=10 per group, aged 9 weeks. *Map2k7*$^{+/+}$ mice n=9 and *Map2k7*$^{+/-}$ mice n=7 per group, aged 8 weeks. Duration and frequency analysed by ANOVA followed by Tukey's *post hoc* for multiple comparisons.

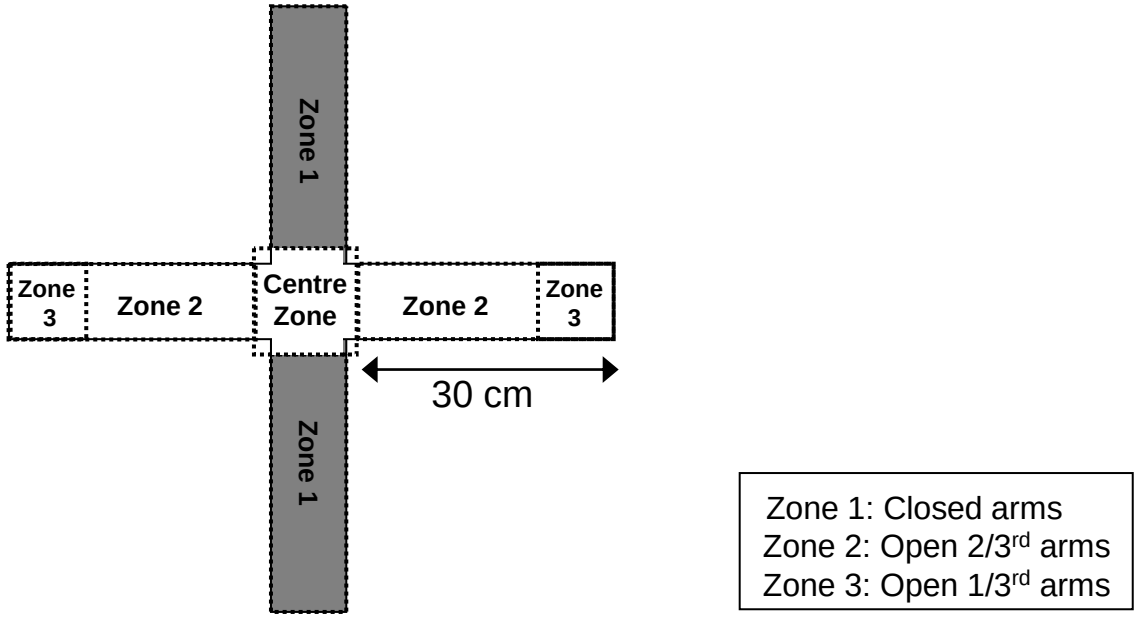


Figure 2.6 Schematic diagram illustrating plus maze zones and corresponding arms.

Total activity in closed arms was analysed by collating data from both arms to form zone 1, similarly activity from open 2/3rd arms (zone 2) and open 1/3rd arms (zone 3) were collated to give indication of anxiety levels.

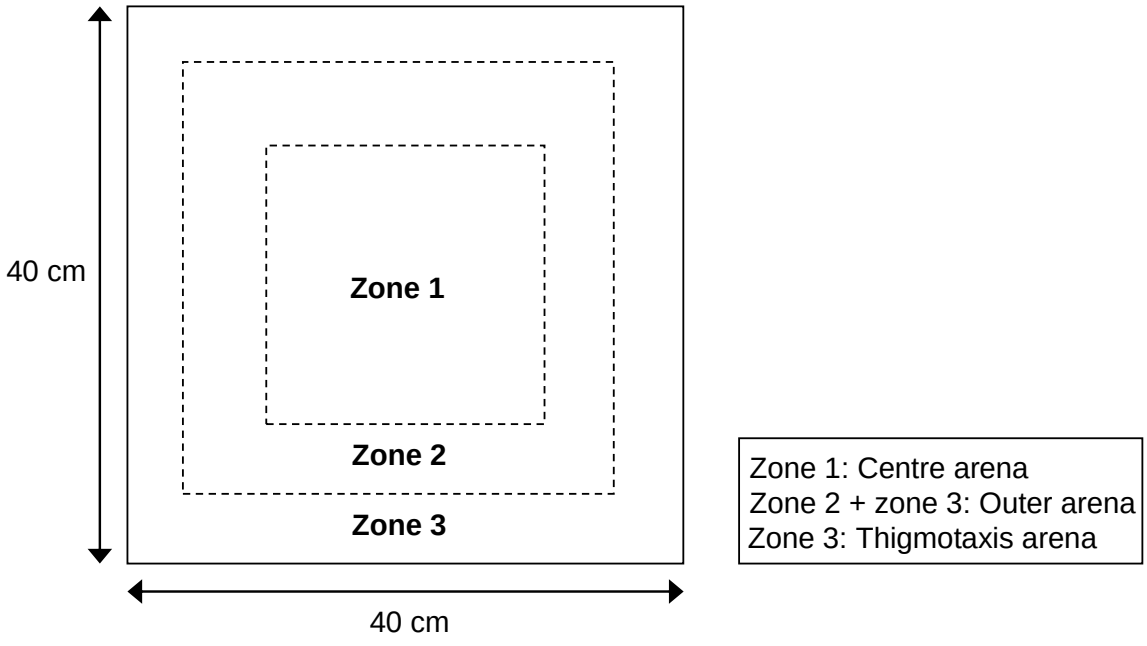


Figure 2.7 Schematic diagram illustrating open field zones in order to analyse anxiety levels and locomotor behaviours.

Anxious mice would be expected to spend least time in zone 1, centre of the arena, whilst mice with high levels of anxiety may spend more time in the very outer arena, zone 3.

2.10.6 Working memory in delayed non-match to sample

Animals

Mouse littermates were littermate pair housed with free access to water and received a restricted diet of 3g feed pellet/ mouse/ day. Weights were recorded daily throughout the study to ensure weights did not fall below 85% of expected *ad libitum* feed weight.

Apparatus

An elevated cross- maze (elevated off the ground by 1 metre) was adapted to be used as a T-Maze using 5 cm by 15.5 cm sliding doors, creating three arms 30 cm by 15.5 cm by 5 cm,. The maze was constructed from grey Plexiglas. The arm used as the start alley was varied for each trial to prevent the use of visual cues. During inter- and intra- trial periods, mice were placed into a holding cage located adjacent to the maze.

Habituation to handler and reward

In order to reduce handler-mediated anxiety within the mice, all mice were handled for 5 minutes each for 3 days prior to habituation to the maze. Each day, following handling, mice were introduced to 50% (v/v H_20) condensed milk by placing a small plastic tray into their home cage containing 8x 70 µl drops. The tray was removed following the consumption of all condensed milk drops.

Habituation to Maze

Around 1 hour following handling on the 3rd day, mice were exposed to the maze in pairs with their cage mate. Mice were placed in the maze for 5 minutes. The two 'goal' arms were both baited with 140 µl of 50% condensed milk. This process was repeated so all mice were exposed to the maze twice on the 3rd day.

Forced alternations

On the 4th day, mice were exposed to 10 forced alternations. Both goal arms were baited with a food reward of 70 µl 50% condensed milk. One goal arm of the maze (arm A) was blocked off using a sliding door and mice were placed in the starting arm. Mice were required to run down the open goal arm (arm B) and consume the food reward. This is referred to as a forced-choice run. Following the consumption of the reward, or a 2 minute time period, mice were removed from maze and placed in the holding cage adjacent to the maze for 10 seconds. During this time, access to arm B was blocked using the sliding door and access to arm A and its reward was made available. The mouse was placed back on the starting arm and was now required to run down arm A and consume the reward. This is repeated for 10 trials. Mice have a limit of 2 minutes to find the reward for all trials. Forced alternations were continued for 3 days by which point the mice were actively running to the goal arm.

Training

In the training stage, the mouse performed a (pseudorandom) forced-choice run, followed by a 10 seconds intra-trial delay in the holding cage. The mouse was placed back into the starting arm of the maze now with access to both goal arms; however, the arm which contained the reward for the forced choice run was not re-baited and only the previously closed arm remained baited. The aim was for the mouse to remember which arm previously contained a reward and to avoid this arm, and explore the previously closed off arm. A different pattern of randomly chosen forced runs were used each day, with inter-trial delays of 40 seconds. Training phase was repeated until animals reach a criterion of 7 correct trials out of 10 trials in 3 consecutive days.

Testing

Mice were tested the day following the completion of training. This consisted of the mice performing 3 days of 12 pseudorandom forced choice runs, followed by a 5, 15 or 30 second intra-trial delay in the holding cage. Mice were then placed back in the starting arm with access to both arms. The mouse had to correctly identify the unexplored goal arm to be included as a correct trial. The order of the forced choice runs was kept constant for all mice over the three testing days. Trials were separated by an inter-trial delay of 40 seconds. *Fxyd6*$^{+/+}$ mice

n= 7, *Fxyd6*$^{-/-}$ mice n=6, *Map2k7*$^{+/+}$ mice n=5 , *Map2k7*$^{+/-}$ mice=5. Data analysed by ANOVA followed by Tukey's *post hoc* for multiple comparisons.

2.10.7 Sociability

Sociability was performed on mice one week following the working memory assessment.

Apparatus

Apparatus consisted of a Plexiglas test box separated by sliding doors into 3 chambers. The two outer chambers contained a cylindrical wire cage. A clear Plexiglas lid was placed on top of the test box. All movement within the arenas was tracked Ethovision XT (Noldus).

Habituation

To habituate mice to apparatus, mice individually freely explored the test box and chambers, including the cylindrical wire cages, for 5 minutes, before being placed into a holding cage adjacent to the arena for around 3 minutes.

Sociability

Immediately following habituation, an unfamiliar mouse was placed in one of the cylindrical wire cages and the test mouse was placed back into the centre chamber of the arena and allowed to explore the whole arena, including the cylindrical wire cages, freely for 10 minutes, before being placed into a holding cage adjacent to the arena for around 3 minutes.

Social Novelty

Immediately following from sociability, the 'unfamiliar' mouse was kept in the wire cage and was termed 'familiar mouse'. An novel unfamiliar mouse, termed 'stranger mouse' was placed into the empty wire cage. The test mouse was placed back into the centre chamber of the arena and allowed to explore the whole arena, including the cylindrical wire cages, freely for 10 minutes.

Activity analysis

Activity within the arena was analysed using Ethovison XT (Noldus). Time spent and entries into each chamber, and time spent exploring each wire cage were analysed. *Fxyd6*$^{+/+}$ mice n= 7, *Fxyd6*$^{-/-}$ mice n=6 , *Map2k7*$^{+/+}$ mice n=5 , *Map2k7*$^{+/-}$ mice=5. Data analysed by ANOVA followed by Tukey's *post hoc* for multiple comparisons.

2.10.8 Sucrose Preference

Anhedonia was investigated in mice by sucrose preference testing. Following sociability testing, mice were singly housed. Water bottles were removed from cages and replaced with 2 bottles containing known volumes of either water or 1% sucrose solution (dissolved in H_20). Bottles were counterbalanced across the left and right sides of the feeding compartments and positions were alternated daily. For 5 consecutive days both bottles were weighed every 24 hours and preference for sucrose was presented as the percentage of total fluid consumption using the following equation:

Sucrose preference (%) = sucrose solution consumption (mg)/ total fluid intake (mg)

Fxyd6$^{+/+}$ mice n= 7, *Fxyd6*$^{-/-}$ mice n=6 , *Map2k7*$^{+/+}$ mice n=5 , *Map2k7*$^{+/-}$ mice=5. Data analysed by ANOVA followed by Tukey's *post hoc* for multiple comparisons.

2.11 Statistical Analysis

All statistical analyses were carried out in Minitab version 16. Data were assessed for normal distribution using the Anderson Darling normality test, data which did not follow a normal distribution were transformed using Johnson transformation and tested using either Student's t-test or ANOVA with Tukey's post hoc comparison test. Significance set at $p \leq 0.05$.

Chapter 3. A genetic association study of the *FXYD6* gene and epistatic interactions in susceptibility to schizophrenia in a British population.

3.1 Introduction

The high heritability rate of schizophrenia (refer to 1.7) has led to a search for genes that confer disease susceptibility. The two prevailing ideas behind most genetic research on schizophrenia are that schizophrenia is a combination of multiple common polymorphisms of modest effect (The 'common disease – common alleles' model, refer to 1.8) (Chakravarti., 1999;Gottesman and Shields., 1982) and more recently, a common disease caused by multiple rare alleles (refer to 1.8, McClellan et al., 2007).

GWAS and candidate gene genetic association studies have provided evidence suggesting a role of common variants in the genetic background of schizophrenia in unrelated schizophrenia patients (refer to 1.9.4 (McClellan et al., 2007;Owen., 2010)). These studies are thought to strengthen the CDCA model of schizophrenia, which implies that schizophrenia is the result of the effects of multiple inherited genetic mutations (McClellan et al., 2007). However, the mode of inheritance is unclear and the genetic architecture of schizophrenia remains complex and poorly understood. Recent studies providing contradicting results to current literature have led to the re-evaluation of the 'common disease – common alleles' model as the primary hypothesis of the genetic architecture of schizophrenia (reviewed by Mitchell and Porteous., 2011). GWAS studies with large sample sizes have only found a few common variants associated with the increased risk of schizophrenia (Jia et al., 2010;Purcell et al., 2009;Stefansson et al., 2009) challenging the CDCA model which would require a larger quantity of common variants to be a valid hypothesis. Furthermore, in depth analysis of these studies revealed variance is not due to polygenic common variants, and instead the risk of schizophrenia is more likely to stem from rare but highly penetrant mutations (Purcell et al., 2009). Several features of schizophrenia support the view that it is a 'common disease caused by multiple rare alleles', such as mutations are often specific to a subset of families, or why schizophrenia, an illness associated with reduced fertility, has not had reduced frequency over a long period of time (McGrath et al., 1999;Nimgaonkar et al., 1997). Furthermore, this model may offer an explanation to why only a small fraction of genetic risk factors have been

identified and consistently replicated in association and linkage studies. These studies assume unaffected individuals sharing the same SNP-defined haplotypes will share common risk variants and present a common phenotype, however rare but highly penetrant mutations causing schizophrenia may vary within the same haplotype block resulting in a lack, or reduced association which could be worsened with high sample numbers (reviewed by McClellan et al., 2007).

Over a thousand genes and polymorphisms have been implicated in schizophrenia as a result of gene discovery strategies, such as GWAS studies, genetic association studies and meta-analysis (Allen et al., 2008). However schizophrenia is subject to environmental and genetic factors, factors such as pre- and post-natal environmental insults and genetic interactions (refer to 1.7), increasing the difficulty of identifying rare but highly penetrant susceptibility genes that often only confer a fractional. Processes such as gene expression can also be mediated by epigenetic mechanisms, which occur without changing the genetic code (refer to 1.8) and would therefore go undetected in genetic association investigations. A gene's output can also be modified by the actions of another gene (known as epistatic interactions) (refer to 1.8), resulting in differences in phenotype between individuals sharing a risk variant (Cloninger., 1997;Moore., 2003).

Reduced association can also stem from the variations in the classification and diagnoses of disorders. The imprecise nature of the schizophrenic phenotype and lack of relevant biological markers lead to variability in the classification of schizophrenia. Rare but highly penetrant alleles may be responsible for a small range of phenotypes associated with schizophrenia; genetic associations can be missed if association studies are performed in a high number sample encompassing a wide variety of symptoms.

Recently a genetic association study has implicated 5 genetic variations from *FXYD6* in the aetiology of schizophrenia in samples from the United Kingdom (Choudhury et al., 2006) with two variants further confirmed in an Aberdeen sample (Choudhury et al., 2007). The association between *FXYD6* and schizophrenia has been investigated in different populations with mixed results. Allelic, genotypic or haplotypic associations were not observed in similar studies carried out in a Japanese population (Ito et al., 2008;Iwata et al., 2010) or a

Chinese Han population (Zhang et al., 2010), however (Zhong et al., 2011) found significant associations between a Chinese Han population and schizophrenia suggesting *FXYD6* gene as a risk factor in schizophrenia across populations is subject to other risk factors.

FXYD6 is located on 11q23.3 (chromosome 11, locus 23.3). 11q22-24 has one of the strongest genetic associations with schizophrenia, as revealed by meta-analysis of 20 genome scans in combination with a number of genome studies (Lewis et al., 2003). The FXYD6 protein belongs to the FXYD family, members of which are modulators of the Na^+ K^+ ATPase pump (Garty and Karlish., 2006;Sweadner and Rael., 2000) (refer to 1.11 and 1.12). As of yet, FXYD6 has not been fully characterized, however, it has been revealed to modulate the Na^+ K^+ ATPase pump in the inner ear (Delprat et al., 2007). Several potential links to schizophrenia have been found, such as strong mRNA and protein expression in the rat brain (Kadowaki et al., 2004, personal correspondance) and in particular the forebrain (Kadowaki et al., 2004, personal correspondance); a region that is responsible for cognitive, sensory and motor function and is highly associated with schizophrenia. In addition FXYD6 protein has been shown to have highest levels of expression in the post natal rat brain during development (Kadowaki et al., 2004), suggesting it may be involved in neurodevelopment. Furthermore, co-localisation with the GABAergic interneuron marker parvalbumin in the inner ear suggests a potential role of FXYD6 in the GABAergic circuitry (Delprat et al., 2007) strengthening the argument for a role of *FXYD6* in schizophrenia.

3.2 Hypothesis and aim

FXYD6 has been shown to be associated with schizophrenia in a North European population (Scotland and England); however despite molecular evidence further supporting a link with schizophrenia, difficulty in replicating this genetic association has been encountered.

The aim of this genetic association study was to genotype alleles of six SNPs in *FXYD6* and to look for association of single SNPs and haplotypes to determine if *FXYD6* is a risk factor in schizophrenia in a North European population (the West of Scotland and London samples). Epistatic interactions between *FXYD6* and schizophrenia- associated candidate gene, *MAP2K7* (refer to Chapter 6), in the

samples were also investigated in order to explore whether the combined effects of these genes add to the risk of schizophrenia.

3.3 Results

3.3.1Data Quality

Six tagging SNPs were selected to analyse genetic association of *FXYD6* with schizophrenia (refer to 2.2.4) to capture variation across the entire gene. Prior to testing for association with schizophrenia, the data were assessed for quality (refer to 2.2.5) to eliminate the chance of false data. Ambiguous samples were repeated and replication samples that were included throughout the study confirmed the accuracy of the data. NTC within the study did not produce a signal, confirming samples were contamination free. The allele frequency (in all controls and samples) was used to confirm that genotyping was likely to be accurate by being comparable to the expected frequency and with control data sets in databases too and a call rate of over 99% of all samples confirmed lack of missing data (Table 3.1). Representative allelic discrimination plots can be observed in Figure 3.1-Figure 3.6.

SNP ID	Location	Call rate (%)	SNP VIC allele	SNP FAM allele
rs3087563	Chr.11: 117707937	99.6	C	T
rs3168238	Chr.11:117709645	99.8	G	T
rs3885041	Chr.11: 117722742	99.8	G	A
rs1815774	Chr.11: 117731439	99.8	C	G
rs4938445	Chr.11:117745003	99.4	A	G
rs497768	Chr.11:117750740	99.6	C	G

Table 3.1 Summary of SNP assays.

A high percentage of samples were genotyped in all assays (98.5% - 99%). SNPs were determined using reporter dyes SNP VIC and SNP FAM to detect the presence of the major and minor alleles.

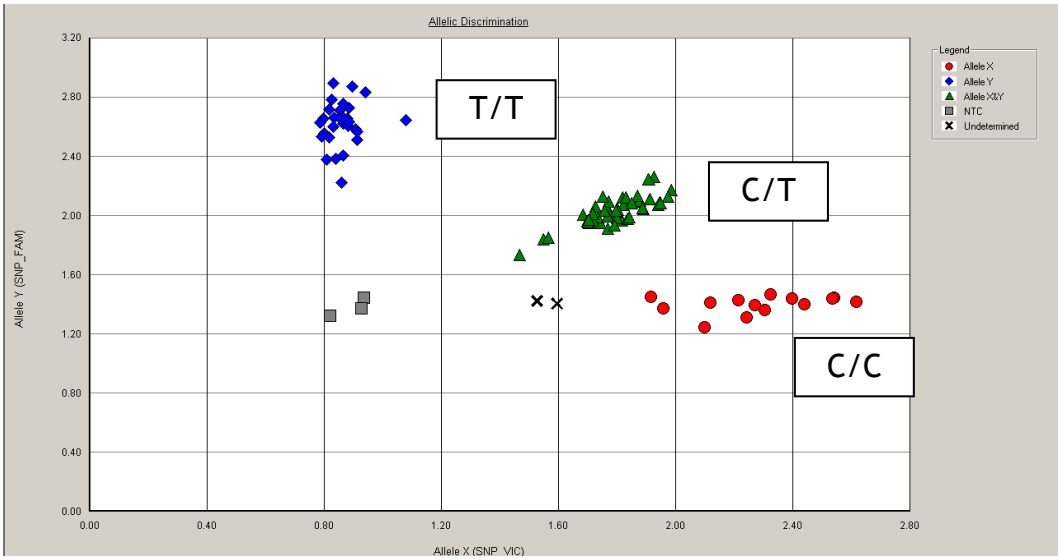


Figure 3.1 Representative allelic discrimination plot for rs3087563.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (C/C alleles), ◆ = samples homozygous for SNP_FAM™ (T/T alleles), ▲= samples heterozygous for SNP_VIC® and SNP_FAM™ (C/T alleles).

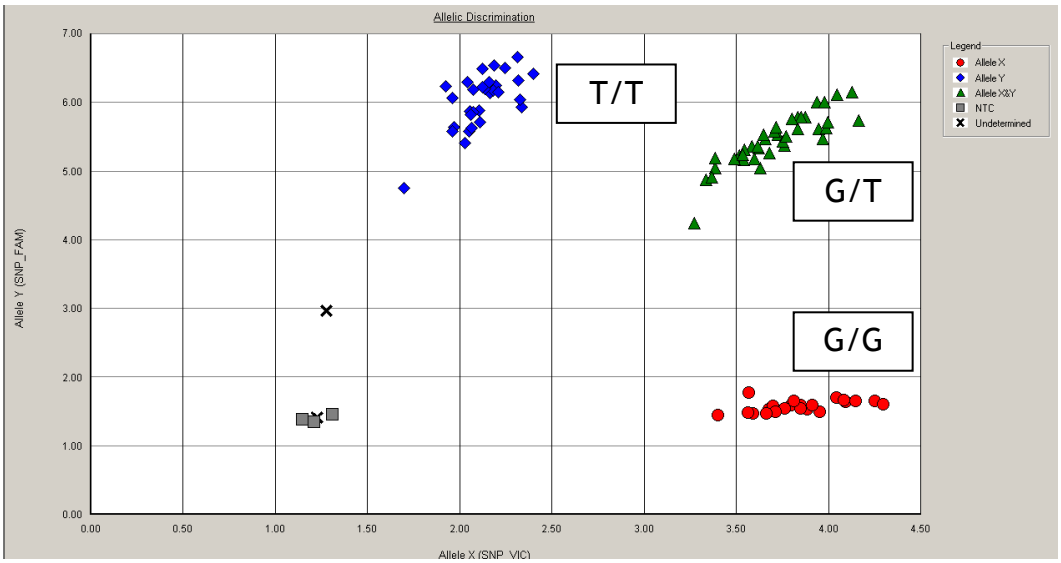


Figure 3.2 Representative allelic discrimination plot for rs3168238.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (G/G alleles), ◆ = samples homozygous for SNP_FAM™ (T/T alleles), ▲= samples heterozygous for SNP_VIC® and SNP_FAM™ (G/T alleles).

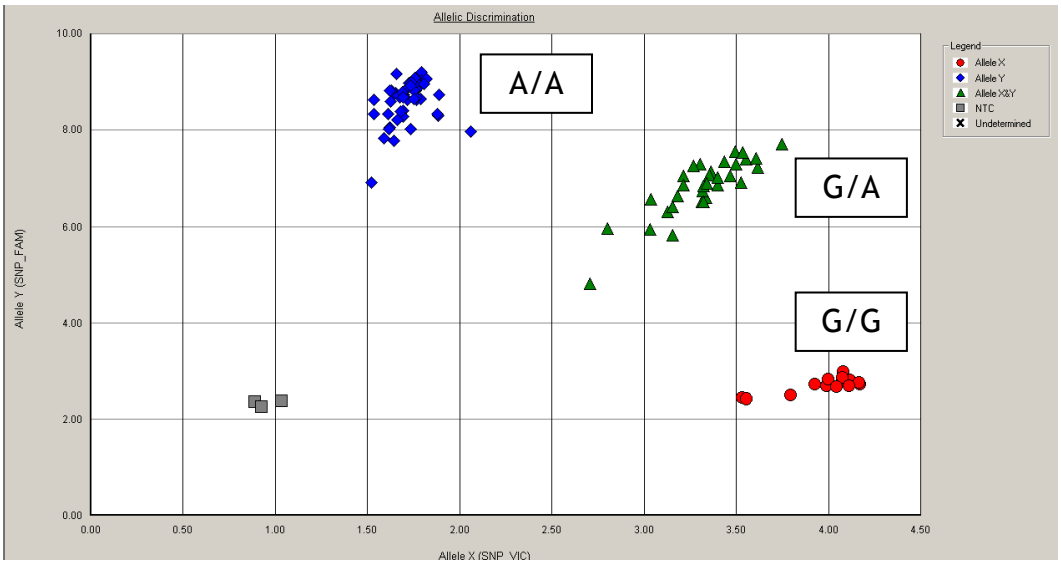


Figure 3.3 Representative allelic discrimination plot for rs3885041.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (G/G alleles), ◆ = samples homozygous for SNP_FAM™ (A/A alleles), ▲ = samples heterozygous for SNP_VIC® and SNP_FAM™ (G/A alleles).

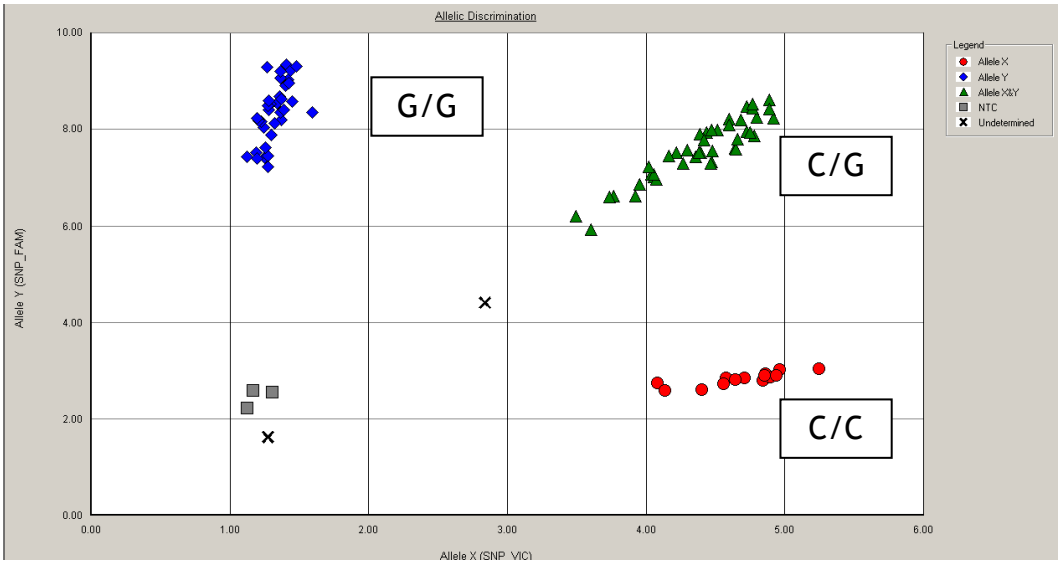


Figure 3.4 Representative allelic discrimination plot for rs1815774.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (C/C alleles), ◆ = samples homozygous for SNP_FAM™ (G/G alleles), ▲ = samples heterozygous for SNP_VIC® and SNP_FAM™ (C/G alleles).

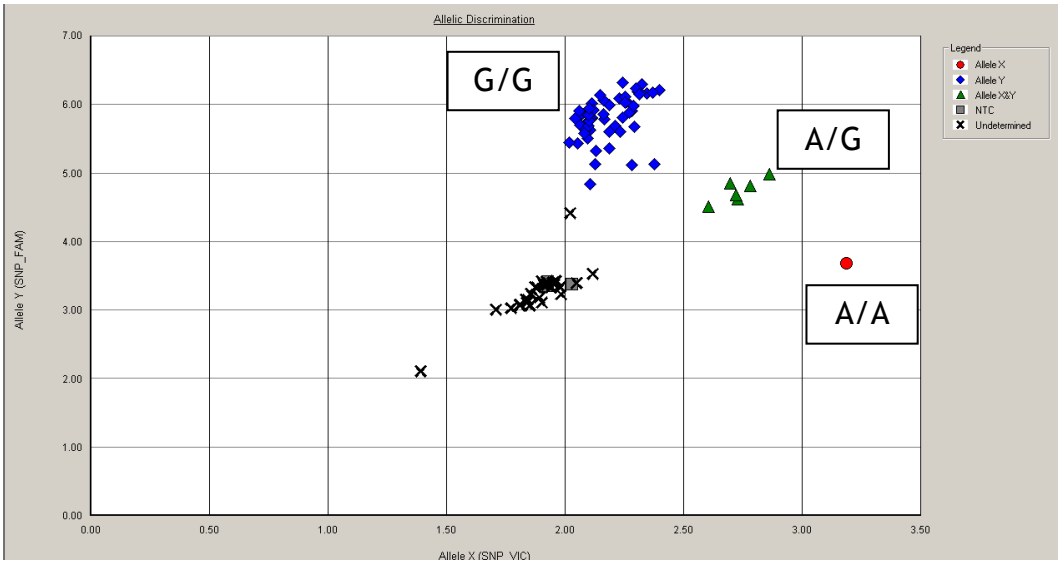


Figure 3.5 Representative allelic discrimination plot for rs4938445.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (A/A alleles), ◆ = samples homozygous for SNP_FAM™ (G/G alleles), ▲= samples heterozygous for SNP_VIC® and SNP_FAM™ (A/G alleles).

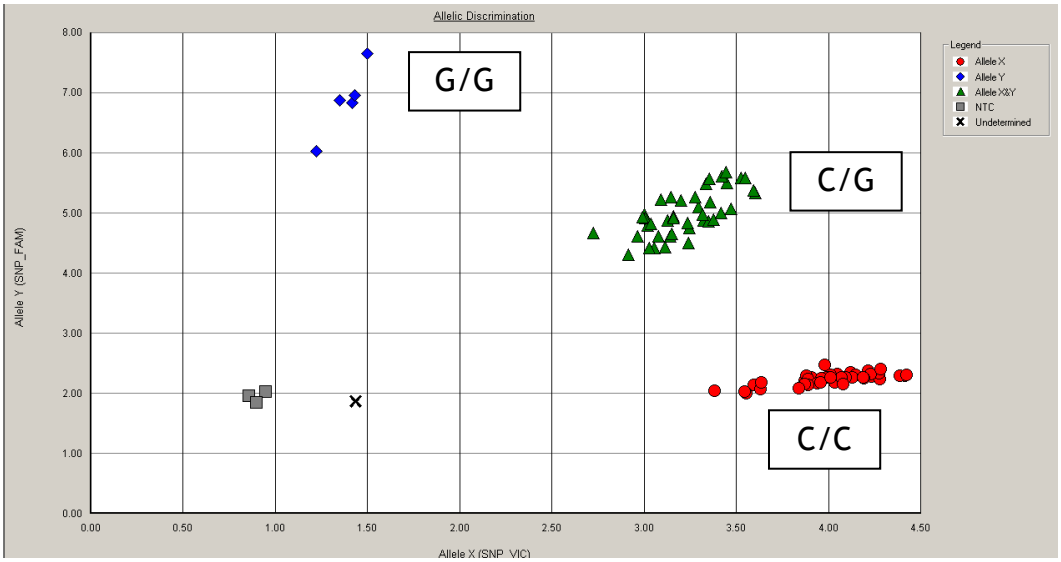


Figure 3.6 Representative allelic discrimination plot for rs497768.

Genotype of samples are based on arbitrary intensity values for SNP_VIC® (axis x) and SNP_FAM™ (axis y). Samples where genotype could not be determined are represented by an 'X', ■ = no template controls, ● = samples homozygous for SNP_VIC® (C/C alleles), ◆ = samples homozygous for SNP_FAM™ (G/G alleles), ▲= samples heterozygous for SNP_VIC® and SNP_FAM™ (C/G alleles).

3.3.2 Allele Frequencies

The allele frequencies for all SNPs were in Hardy-Weinberg equilibrium (HWE) (Table 3.2). However when the data sets were combined, one SNP, rs3885041 did not follow the expected distribution and therefore deviated from HWE (p= $8x10^{-4}$, Table 3.2). This is unlikely to be due to genotyping error due to the stringent quality control measures (refer to 2.2.8 & 3.3.1) and may be representative of disease association in the samples, when these data were separated into cases and controls, the allele frequency was in HWE (p<0.001) (Table 3.3) confirming rs3885041 follows the expected allelic distribution pattern and can therefore be used in this study.

SNP ID	Exp.HET	Obs.HET	Call rate (%)	MAF	Alleles	HWpval
rs3087563	0.498	0.505	99.6	0.465	C:T	0.6524
rs3168238	0.49	0.482	99.8	0.428	G:T	0.9337
rs3885041	0.449	0.4	99.8	0.341	G:A	8.00E-04
rs1815774	0.48	0.438	99.8	0.399	C:G	0.0124
rs4938445	0.096	0.097	99.4	0.051	A:G	0.6263
rs497768	0.365	0.384	99.6	0.241	C:G	0.1365

Table 3.2 Summary of genetic frequency in the sample set combining the West of Scotland and the London controls and cases samples. Rs3885041 is the only SNP assay in which samples are not in HWE (p=$8x10^{-4}$). Exp.HET= expected heterozygous allele frequencies, Obs.HET= observed heterozygous allele frequencies, MAF= minor allele frequency. HWpval= Hardy-Weinberg p value (Significance set at p<0.001, refer to 2.2.8). Case n=498, controls=504.

	Control samples			Case samples		
SNP ID	Obs.HET	%Genotype	HWpval	Obs.HET	%Genotype	HWpval
rs3087563	0.499	100	0.652	0.505	98.5	0.9515
rs3168238	0.483	100	0.7837	0.482	99.2	0.9594
rs3885041	0.38	100	0.0655	0.4	99.6	5.20E-03
rs1815774	0.416	100	0.2363	0.438	99.5	0.0244
rs4938445	0.087	100	0.5186	0.097	98.8	1
rs497768	0.394	99.8	0.3833	0.384	99	0.2784

Table 3.3 Summary of control and case genetic frequency in the sample set combining the West of Scotland and the London samples. Rs3885041 is now in HWE in both control and case samples (p>0.001). Exp.HET= expected heterozygous allele frequencies, Obs.HET= observed heterozygous allele frequencies, MAF= minor allele frequency. HWpval= Hardy-Weinberg p value. Case n=498, controls=504.

3.3.3 Genetic Association

Association tests were carried out on allele frequencies in control vs. case data in the combined West of Scotland and London data set to give indication of whether the SNPs in *FXYD6* are associated with increased risk to schizophrenia (Table 3.4). Chi square values revealed observed results do not significantly deviate from expected results, whilst *p* value confirms there is no significant association between the SNPs and schizophrenia cases.

SNP ID	Assoc.allele	Case, control ratio	Chi Sq.	*p* value
rs3087563	T	543:467, 521:475	0.425	0.5144
rs3168238	G	587:427, 563:433	0.382	0.5368
rs3885041	G	684:330, 644:352	1.753	0.1854
rs1815774	G	625:389, 584:412	1.89	0.1692
rs4938445	A	959:47, 936:60	1.809	0.1787
rs497768	G	251:761, 232:762	0.587	0.4437

Table 3.4 Allelic association analyses in the combined West of Scotland and London sample set. There is no significant association of SNPs rs30087563, rs3168238, rs3885041, rs1815774, rs4938445 or rs497768 with schizophrenia in the combined West of Scotland and London data set ($p>0.05$), as observed by no significant difference in case samples frequencies compared to control sample frequencies (as represented by Case, control ratio column) chi Sq > 0.05 Case n=498, controls=504

3.3.4 Linkage disequilibrium associate and haplotype association

Data were explored for haplotype blocks of adjacent SNPs (refer to 2.2.10) to investigate association of genetic loci with schizophrenia. Two haplotype blocks of associated SNPs were observed in the data set using the solid spine of LD method implemented in the Haploview software (refer to 2.2.7), however analysis did not reveal any SNP or haplotype associations with schizophrenia. Block 1 indicates linkage disequilibrium between rs3885041 and rs1815774 (region size = 8kb, D'= 91, LOD score>2.0, indicated by red blocks (Figure 3.7) whilst block 2 indicates linkage disequilibrium between rs4938845 and rs497768 (region size= 5 kb, D'=100, LOD score>2.0, indicated by red block, (Figure 3.7), ($p>0.05$) (Table 3.5).

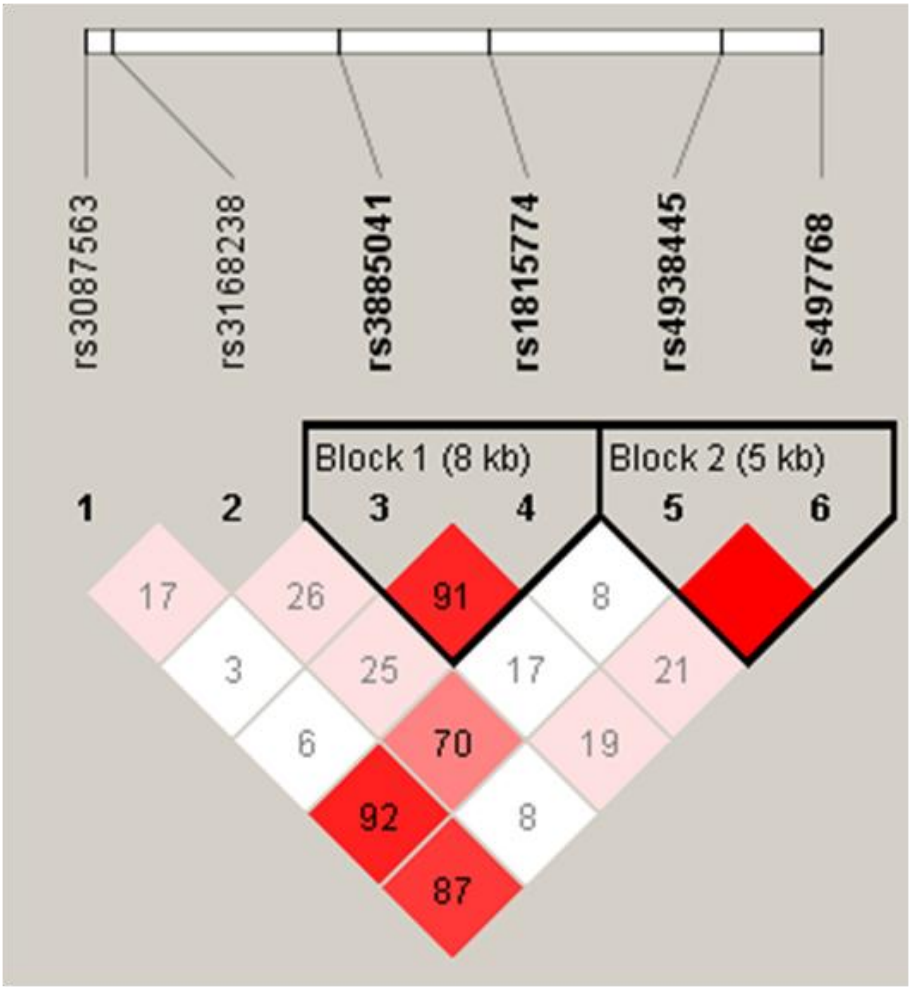


Figure 3.7 Linkage disequilibrium (LD) was measured using samples combined from the West of Scotland and London sample sets.

Each box provides D' values (1-100) with darker shades of red representing stronger LD and an LOD score of >2. The haplotype blocks were determined by the solid spine of LD method implemented in the Haploview software (refer to 2.2.7). 2 haplotype blocks were determined in this sample set. Block 1 show LD between rs3885041 and rs1815774 (region size = 8kb, D' = 91, LOD score >2.0) and block 2 rs4938845 and rs497768 (region size = 5 kb, D' = 100 LOD score >2.0). Case n=498, controls=504

Haplotype	Case frequency : control frequency	P value
Block1		
GG	611.9, 402.1 : 562.8, 433.2	0.081
AC	316.9, 697.1 : 330.8, 665.2	0.346
GC	72.1, 941.9 : 81.2, 914.8	0.38
AG	13.1,1000.9 : 21.2, 974.8	0.149
Block 2		
AC	715.1, 298.9 : 703.5, 292.5	0.9588
AG	251.5, 762.5 : 232.5, 763.5	0.4442
GC	47.4, 966.6 : 60.0, 936.0	0.177

Table 3.5 Haplotypic analyses in the combined sample set. Two haplotype blocks were observed in the sample set and are referred to as Block 1 and Block 2. The frequency of allele combinations observed in both block 1 and block 2 did not significantly differ between case samples (case frequency) and control samples (control frequency) in the sample set (*p*>0.05). Case n=498, controls=504.

3.3.5FXYD6 and MAP2K7 epistasis

Although there was no association of *FXYD* variants with schizophrenia, we investigated whether there was an epistatic relationship between *FXYD6* and *MAP2K7*. *MAP2K7* has recently been found to be associated with susceptibility to schizophrenia however this study reveals that interactions between these two genes do not contribute to the risk of schizophrenia (refer to 2.2.11). Analysis of epistatic interactions between *MAP2K7* SNPs rs3679 and rs4804833 and *FXYD6* SNPs rs3087563, rs497768, rs4938445, rs1815774, rs3168238, rs3885041 did not reveal any significant interactions in case compared to control samples in West of Scotland, London and combined data set (Table 3.6).

Model	codominant	codominant	codominant	codominant	codominant	codominant
rs3087563	0.77	0.77	0.92	0.12	0.98	0.82
rs3168238	0.96	0.9	0.25	0.21	0.39	0.3
rs3885041	0.73	0.92	0.19	0.14	0.92	0.51
rs1815774	0.51	0.74	0.16	0.9	0.34	0.8
rs4938445	0.37	0.31	0.46	0.63	0.65	0.45
rs497768	0.87	0.38	0.7	0.53	0.72	0.19

Table 3.6 Epistatic interaction *p* values. *P* values representing epistatic interactions between *MAP2K7* SNPs rs3679 and rs4804833 and *FXYD6* SNPs rs3087563, rs497768, rs4938445, rs1815774, rs3168238, rs3885041 suggest epistatic interactions between these two genes do not contribute a risk to schizophrenia. Case n=498, controls=504.

3.4 Discussion

Genetic association studies investigating whether *FXYD6* is a risk factor in the susceptibility to schizophrenia have yielded mixed results, with evidence for an association (Choudhury et al., 2006; Choudhury et al., 2007; Zhong et al., 2011) and against (Ito et al., 2008;Iwata et al., 2010; Zhang et al., 2010).

This genetic association study genotyped alleles of six SNPs in *FXYD6* and explored both single SNPs and haplotypes to determine if *FXYD6* is a risk factor in schizophrenia in a North European population (the West of Scotland and London samples). Furthermore, epistatic interactions between *FXYD6* and *MAP2K7*, a gene recently shown to be genetically associated with schizophrenia (Winchester et al., 2012), were investigated to see if genetic interactions between these two genes contribute to the risk of schizophrenia.

3.4.1 SNP criteria and data quality

SNPs investigated in this study were selected based upon the following criteria: MAF was at least 5-10%, SNPs provided a full coverage of the haplotype diversity in *FXYD6* and SNP genotyping assays were commercially available. This criterion suggests SNPs are polymorphic and span the *FXYD6* locus. Five SNPs out of the six selected (rs3168238 p=0.009, odds ratio 1.64; rs1815774 p=0.049, odds ratio 1.21; rs4938445 p=0.010, odds ratio 1.31; rs4938446 p=0.025, odds ratio 1.26 and rs497768 p=0.023, odds ratio 1.24 (Choudhury et al., 2006) were found to have a positive association with Schizophrenia in Choudhury et al's studies (Choudhury et al., 2006;Choudhury et al., 2007). In data samples, no contamination was detected and all SNPs were confirmed polymorphic and allele frequency followed a similar distribution pattern to those published in HapMap (www.hapmap.org). Assay rs3885041 deviated from HWE in London samples. HWE is often used as a primary data quality check in association studies (Wittke-Thompson et al., 2005) and can be investigated through a goodness of fit chi-squared (X^2) test, which assesses the difference between the expected and observed allele frequencies. The HWE principle states that in a randomly mating population, genetic frequency remains in a state of equilibrium, assuming no mutations (Hardy, 1908;Weinberg, 1908), and thus allele frequency is conserved from generation to generation. Hardy-Weinberg assumes both alleles are equally

selective and so does not take into account evolution. Samples are said to deviate from HWE when they exhibit a HWE *p* value of <0.001 (Guo and Thompson., 1992;Wigginton et al., 2005). Deviation may indicate an error in genotyping techniques; however it may also be a result of disease-association or poor study design (Balding., 2006;Wittke-Thompson et al., 2005). When control and case samples tested with rs3885041 were separated, both sample sets were in HWE, and so the data were not excluded from the study.

3.4.2Allelic and haplotypic association

Haplotype blocks were determined using Haploview's 'Solid spine of LD method'; a method which searches for a "spine" of strong LD amongst markers within the haplotype block. The first and last markers in the haplotype block are in strong LD with the other markers, however adjacent SNPs within the haplotype block may not be in strong LD with each other. LD, also known as gamete phase equilibrium or allelic association, reflects the relationship between two alleles at different loci on the same chromosome, indicating the likeness of these alleles being inherited together (Cardon and Bell., 2001). LD association is dependent on the association between the causal SNP and the marker SNP (Cardon and Bell., 2001;Carlson et al., 2004;Li and Li., 2008). Levels of LD between two markers in the data sets were represented by D' values. D' gives an indication of high levels of association and inheritance between two loci- D' values range from 0 to 100, with D' = 100 showing complete LD, and 0 no LD (Lander et al., 2001;Wall and Pritchard., 2003). A combination of SNPs at the same locus likely to be inherited together is regarded as a haplotype block (Gabriel et al., 2002). Many haplotype blocks can be transmitted through generations with little or no recombination, making it is possible to track a mutation (Gabriel et al., 2002).

Allelic analysis of the six SNPs did not reveal any significant differences in variant frequencies between case and control data, suggesting these SNPs do not contribute towards the risk of schizophrenia phenotype in this sample set. In the data set 2 haplotype blocks were identified with LOD scores of >2. LOD scores indicate if the SNPs are linked and are likely to be inherited together or whether the association observed is through chance (Lander et al., 2001;Wall and Pritchard., 2003). The frequency of allele combinations forming haplotypes did

not significantly differ in either block between case and control samples, suggesting the inheritance of these sets of loci are not a risk factor in schizophrenia.

The London data sample set from this study was a subset of samples from (Choudhury et al., 2007) study in which rs3087563, rs3168238, rs1815774, rs4938445, rs497768 were significantly associated with schizophrenia. When compared, data reveal a very similar frequency to those observed in Choudhury et al's study, and it is a possibility that despite the study having a high statistical power (power level 0.95, refer to 2.2.1), it is not high enough to produce a significant association (control n=498, case n=504). Alternatively, it is possible that data from the Choudury et al studies (Choudhury et al., 2006;Choudhury et al., 2007), have generated a false- positive association with schizophrenia. False-positive associations in genetic association studies may arise for a number of reasons including population stratification, which can arise when case-control samples are poorly matched for genetic ancestry. Allele frequencies are known to vary amongst different populations, these differences in allele frequencies between cases and controls can be wrongly interpreted as an association of genes with disease.

Population stratification can be tested by the genotyping of unlinked marker loci in both cases and controls (Pritchard & Rosenberg., 1999). However, the power to detect population stratification is often inadequate if only a few dozen markers are tested or if the markers are not informative of ancestry. The samples used in the Choudhury studies have been tested for population stratification, making this an unlikely reason behind the generation of false-positive results. However, other factors such as the wrong use of statistical analysis model and human error could have generated false- positive results in the Choudhury studies.

Human error either during the testing procedure or bias during analysis can also lead to the production of false results. Efforts can be made in order to detect human errors, such as using genotyping repeat samples to ensure the same results are reached, including no- template controls in experiments, using automated genotype results calling, or having results called by two independent researchers. Re-genotyping using a technology different from that originally

employed could also provide reassurance that the results are not due to genotyping error.

Given the concern for false-positive findings in studies implicating genes to a disease state, replication of results in new patient samples is crucial to determining the significance of a reported genetic association. However, lack of replication is not always indicative of false-positive results from previous sample sets, and can derive from a number of reasons, such as low power or low sample numbers, SNPs genotyped, poor study design and incorrect statistical models, variations in diagnosis, and assumptions or over interpretation of the data (Cardon and Bell., 2001). A number of reasons could be used to explain why this study did not replicate the Choudhury study in finding a significant association between *FXYD6* and schizophrenia. It could be argued that the addition of the West of Scotland sample set would be associated with new environmental factors (refer to 1.7), differences in epigenetic modification of gene expression (refer to 1.8), as well as the possibility of differences in diagnostic criteria used to assess Schizophrenic patients. In addition, the following hypotheses of schizophrenia offer further explanations as to why positive associations between a disease state and genes can be hard to detect.

The 'common disease- common variants' hypothesis of schizophrenia, which states 'the genetic risk for common diseases will often be due to disease producing alleles found at relatively high frequency' (Pritchard and Cox., 2002;Becker., 2004) suggests it would be expected that genetic association studies with high enough sample numbers will detect causative alleles. If the 'common disease, common variants' model was applied to this study, where it is proposed that schizophrenia is a result of multiple common variants of modest effect, the effect of *FXYD6* may contribute to schizophrenia phenotype albeit with too small an effect size to reach significance. However, recent studies are also suggesting schizophrenia is a common disease caused by multiple rare alleles. The 'common disease- rare alleles' hypothesis proposes mutations are highly penetrant and individually rare (Pritchard., 2001;McClellan et al., 2007). These types of mutations may be specific to patients/families, thereby making them difficult to identify, resulting in many mutations going undetected due to low significance. If the 'rare but highly penetrant allele' model is applied to this study, it could be suggested that statistical significance was not reached due to

the large number of non-pedigree samples used. This would introduce a large number of different risk mutations, diluting associations of rare but highly penetrant variants with schizophrenia. Furthermore, genetic analysis of tagging SNPs or haplotypes, rather than analysis of fully sequenced DNA may not provide adequate scrutinisation of risk DNA and rare disease-related variants may go undetected.

Due to the lack of clinical data provided with these samples, it is not possible to confirm this study is age- matched and sex- matched. As a result of this, limitations may occur. Lack of clinical data also limits further analyses of these samples, for example, investigating whether *FXYD6* acts as a sex-specific risk factor, which would be required to evaluate fully whether *FXYD6* is a risk factor for schizophrenia.

3.4.3 FXYD6 and MAP2K7 epistasis

A gene's phenotype can be modified by the actions of a separate gene; association between SNPs which do not share the same chromosome can act as a biomarker for schizophrenia and similar disorders. It is possible *FXYD6* becomes a risk factor for schizophrenia following epistatic interactions. Recently a genetic association between *MAP2K7* and schizophrenia has been discovered (odds ratio ~1.9) (Winchester et al., 2012). *MAP2K7* is located on chromosome 19 and the protein has been associated with the activation of JNK2, which has decreased expression in the frontal cortex of patients with schizophrenia (Funk., 2012). There are several different inheritance models which can be used to assess epistatic interactions to account for the number of copies of alleles required to alter the risk for a disease. For example, a polymorphism with two alleles, T and a risk allele C, could be tested as a 'recessive model', where two copies of C would be necessary to cause a risk, or a 'dominant mode' where a single copy of C could cause a risk, or a 'co-dominant model' which is the most generalised model and assumes alleles do not present an additive risk (Solé et al., 2006). A 'co-dominant model' was used in this study. Haplotypic analysis did not reveal an association between selected markers in *FXYD6* and *MAP2K7*. *MAP2K7* SNPs rs3679 and rs4804833 and *FXYD6* SNPs rs3087563, rs497768, rs4938445, rs1815774, rs3168238, rs3885041 did not reveal any significant interactions

between *MAP2K7* and *FXYD6,* suggesting interaction of these genes is not a risk factor in susceptibility to schizophrenia.

3.4.4 Conclusion

In conclusion, this study did not find an allelic or haplotypic association between *FXYD6* and schizophrenia in a north European population. A number of influencing factors, such as environmental factors or genetic interactions may have contributed to the lack of association with schizophrenia, which has previously been observed in a similar genetic study. Epistatic interactions between *FXYD6* and *MAP2K7* are not a risk factor in susceptibility to schizophrenia in a North European population.

Chapter 4. Molecular characterisation of FXYD6

4.1 Introduction

Despite three recent genetic association studies finding an association between *FXYD6* and schizophrenia in a Caucasian population (Choudhury *et al.*, 2006;Choudhury *et al.*, 2007), and a Chinese Han population (Zhong et al., 2011), subsequent genetic association studies have failed to confirm an association in a similar Caucasian population, a Japanese population (Ito et al., 2008;Iwata et al., 2010) and a further Chinese Han population (Zhang et al., 2010). Therefore it remains ambiguous as to whether *FXYD6* is a risk factor in developing schizophrenia and what role it plays.

FXYD6 is known to be expressed in the brain (Kadowaki *et al.*, 2004;Stansberg *et al.*, 2011;Yamaguchi *et al.*, 2001) and several studies suggest a molecular association between *FXYD6* and schizophrenia may exist. For example, it has recently been revealed that *Fxyd6* is strongly expressed in the rat forebrain (Ferra., 2006); a region that is responsible for cognitive, sensory and motor function and is highly associated with schizophrenia. In addition, the protein encoded by *Fxyd6* (often referred to as phosphohippolin), has been discovered by means of western blot analysis to have highest levels of expression in the postnatal rat brain during development (Kadowaki et al., 2004); a finding which may correlate with the theory that schizophrenia is a neurodevelopmental disorder.

FXYD6 has been shown to co-localise with the GABAergic interneuron marker parvalbumin in the inner ear (Delprat et al., 2007). Loss of parvalbumin cells has been associated with GABAergic signalling deficits in schizophrenia (Kadowaki *et al.*, 2004;Zhang & Reynolds, 2002;Beasley & Reynolds, 1997;Lewis *et al.*, 2005) and it is thought the inhibitory activity of parvalbumin GABAergic signalling can be mediated by the excitatory input derived from NMDA receptors on parvalbumin interneurons (Kinney et al., 2006). As deficits in glutamate and GABAergic neurotransmitters are leading neurochemical hypotheses of schizophrenia (refer to 1.6), it would therefore be interesting to investigate the role of *Fxyd6* in both GABAergic and glutamatergic circuitry in brain regions associated with memory and cognition. This can be explored using markers of GABAergic and glutamatergic neurons and neurotransmisson such as parvalbumin

protein markers and glutamate receptor markers in regions such as the prefrontal cortex and hippocampus.

FXYD6 has also been shown to co-localise with Na^+ K^+ ATPase α1 subunit in the inner ear and act as a modulator of the inner ear Na^+ K^+ ATPase pump (Delprat *et al.*, 2006;Delprat *et al.*, 2007). It is currently unknown whether FXYD6 also acts as a modulator of brain Na^+ K^+ ATPase pump activity however if this is the case it may provide a further link with schizophrenia. Not only is brain Na^+ K^+ ATPase activity associated with glutamate transport (Rose *et al.*, 2009;Pellerin & Magistretti., 1997), but it has also been implicated in several psychiatric disorders, with low Na^+ K^+ ATPase activity reported in schizophrenic and bipolar mood disorder patients (Goldstein *et al.*, 2006;Kurup *et al.*, 2001;Petronijevic *et al.*, 2003).

4.2 Aim

This chapter aims to determine if *Fxyd6 acts* as a modulator of the Na^+ K^+ ATPase pump and to investigate the molecular association between FXYD6 and schizophrenia-associated neurochemical markers. The following aims were explored in this chapter:

- Evaluation of the expression of *Fxyd6* and *Na^+ K^+ ATPase* were investigated in the mouse brain using an *in situ* hybridisation technique. Mice homozygous for disrupted *Fxyd6* were utilised to evaluate the impact of lack of *Fxyd6* on and Na^+ K^+ ATPase α1 expression.

- Characterisation of the relationship between FXYD6 and Na^+ K^+ ATPase pump was further explored by investigating the impact of the lack of *Fxyd6* on Na^+ K^+ ATPase pump activity in brain regions associated with schizophrenia.

- The impact of the lack of *Fxyd6* on glutamatergic and GABAergic pathways were investigated to explore whether neurochemical changes associated with schizophrenia are present in *Fxyd6*$^{-/-}$ mice.

4.3 Results

4.3.1 Expression of Fxyd6 in the mouse brain

Hybridisation of a 33P- labelled probe designed to target *Fxyd6* mRNA confirmed the expression of *Fxyd6* in 20 µm coronal adult mouse brain sections. Non-specific binding was determined using excess of 50x unlabelled probe (refer to 2.9.1) and was deducted from total binding to calculate the specific binding. Qualitative assessment of specific binding revealed *Fxyd6* has higher levels of expression in the following brain regions: secondary motor cortex, agranular insular cortex and prelimbic (PrL) area of the prefrontal cortex, cingulate cortex (Cg), claustrum (Cl), perirhinal cortex (PRh), dentate gyrus and the CA3 region of the hippocampus and cerebellar lobules of the cerebellum (Figure 4.1).

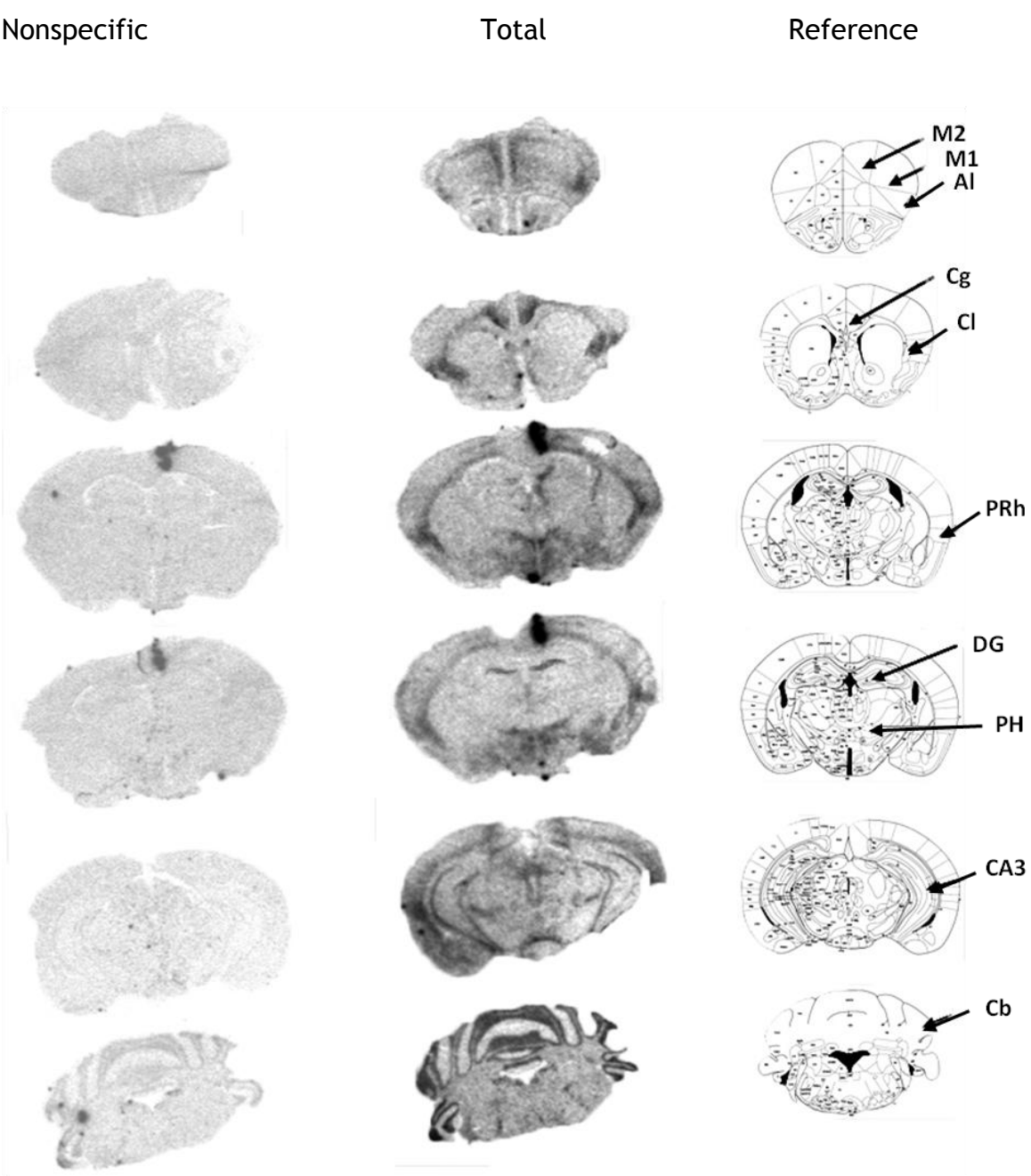


Figure 4.1 Autoradiographic film showing *Fxyd6* distribution in 20 µm coronal mouse brain sections, as detected by *in situ* hybridisation.

Qualitative assessment revealed *Fxyd6* is expressed in a) the secondary motor cortex (M2) and the agranular insular (AI) cortex in the prefrontal cortex (bregma 2.10 mm) b) cingulate cortex (Cg) and claustrum (Cl) (bregma 1.18 mm) c) perirhinal cortex (PRh) (bregma -1.22 mm) d) posterior hypothalamic area (PH) dentate gyrus in the hippocampus (DG) (bregma -1.82), e) CA3 region of the hippocampus (bregma -2.92 mm) f) cerebellar lobules of the cerebellum (bregma -6.00). Non-specific binding= 50x excess of unlabelled probe to indicate non-specific binding, total binding= antisense probe only, reference= brain regions according to the Allen Mouse Brain Atlas (Lein et al., 2006). Scale bar = 2mm. Arrows indicate relevant brain region.

4.3.2 Fxyd6 total RNA and protein is significantly reduced in mice homozygous for Fxyd6 disruption

To investigate the potential functional link between *FXYD6* and schizophrenia, a novel strain of mouse containing two disrupted copies of *Fxyd6* was obtained from Lexicon Pharmaceuticals Incorporated (refer to 2.3.1). Prior to molecular characterisation of these mice, validation of disruption of *Fxyd6* total RNA and protein was required. Quantitative real-time polymerase chain reaction (RTq-PCR) (refer to 2.7), *in situ* hybridisation (refer to 2.9) and western blotting techniques (refer to 2.6) were employed to confirm that homozygous disruption of *Fxyd6* in mice results in lack of *Fxyd6* total RNA and protein. 500 ng of total RNA was extracted from the frontal cortex and converted to cDNA from 3 adult mice homozygous for functioning *Fxyd6* (referred to as $Fxyd6^{+/+}$ mice) and 3 age-matched littermate mice homozygous for disrupted *Fxyd6* (referred to as $Fxyd6^{-/-}$ mice) were studied. RTq-PCR primers were designed to target *Fxyd6* at two sites in the gene (refer to 2.8.1). Fxyd6 m1 is designed to span the targeting vector insertion point across exon 1 and exon 2, and Fxyd6 g1 is designed to detect splice variants by targeting exon 3 and 4 (Fxyd6 g1). Using a geometric mean from three housekeeping genes (*18s ribosomal RNA*, *B2M* and *GAPDH*) analysis of cycle threshold values (CT values) revealed Fxyd6 m1 expression in $Fxyd6^{-/-}$ mice is significantly reduced by a ratio of 0.001 (standard error of 0.000 - 0.003, $p<0.001$) and Fxyd6 g1 significantly reduced by a ratio of 0.001 (standard error 0.001- 0.00, $p<0.001$) in comparison to $Fxyd6^{+/+}$ mice, a ratio of 1 shows no effect, thus confirming null expression of *Fxyd6* RNA following *Fxyd6* disruption (Figure 4.2). The *in situ* hybridisation probe used in section 4.3.1 confirmed *Fxyd6* homozygous disruption results in null expression of *Fxyd6* RNA in the adult brain (Figure 4.3a-d), as observed in the following brain regions; prelimbic cortex, agranular insular cortex, cingulate cortex, claustrum, medial orbital cortex, primary and secondary motor cortex, striatum, nucleus accumbens, CA1, CA2, CA3 and dentate gyrus of the hippocampus and cerebellum. White matter in the cerebellum was used to normalise results (Genotype ($F_{1, 95} = 31.89$, $p<0.001$).

To confirm that FXYD6 protein is disrupted in parallel with *Fxyd6* RNA reduction, western blotting analysis was utilised to establish *Fxyd6*$^{-/-}$ mice also lack FXYD6 protein (refer to 2.6). An antibody designed to target FXYD6 protein specifically produced a band of the correct size (15 kDa) in *Fxyd6*$^{+/+}$ mice. This band was absent in *Fxyd6*$^{-/-}$ mice, indicating null expression of FXYD6 protein (Figure 4.5e).

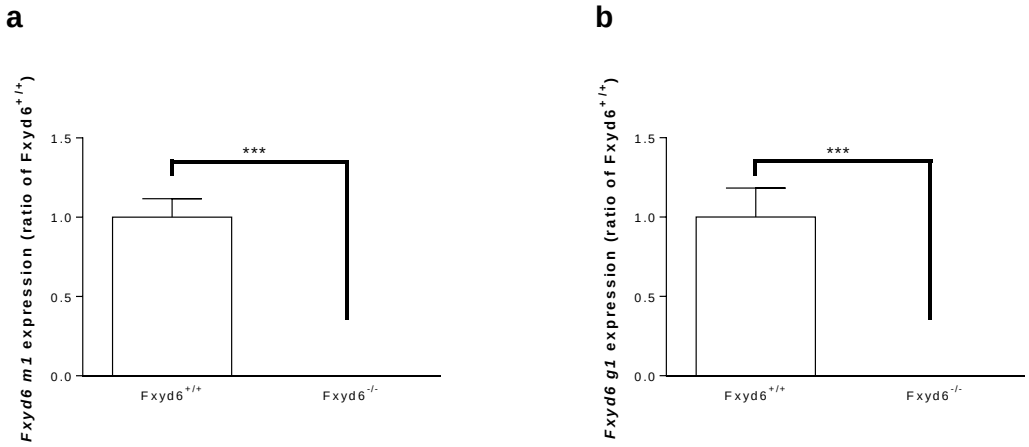


Figure 4.2 *Fxyd6* cDNA relative expression is significantly reduced in *Fxyd6*$^{-/-}$ mice.

Cycle threshold (CT) values for a) Fxyd6 g1 and b) Fxyd6 m1 are significantly reduced in *Fxyd6*$^{-/-}$ mice in comparison to *Fxyd6*$^{+/+}$ mice ($p < 0.001$). Data represent expression ratio ± S.E.M. relative to *Fxyd6*$^{+/+}$ mice samples. *Fxyd6*$^{+/+}$ mice n=3, *Fxyd6*$^{-/-}$ mice n=3

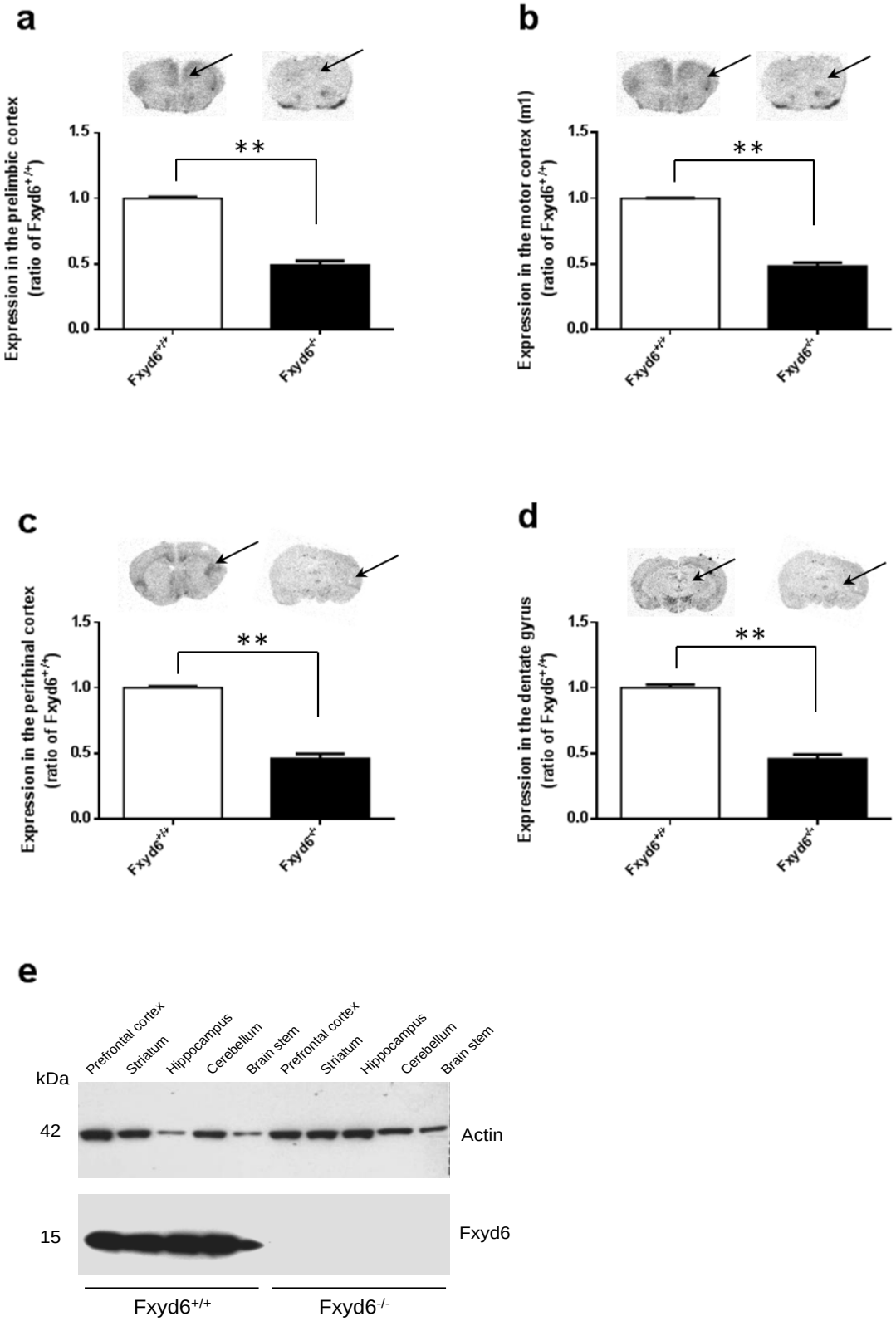

a
Expression in the prelimbic cortex (ratio of Fxyd6+/+)
1.5
1.0
0.5
0.0
**
Fxyd6+/+
Fxyd6-/-
b
Expression in the motor cortex (m1) (ratio of Fxyd6+/+)
1.5
1.0
0.5
0.0
**
Fxyd6+/+
Fxyd6-/-
c
Expression in the perirhinal cortex (ratio of Fxyd6+/+)
1.5
1.0
0.5
0.0
**
Fxyd6+/+
Fxyd6-/-
d
Expression in the dentate gyrus (ratio of Fxyd6+/+)
1.5
1.0
0.5
0.0
**
Fxyd6+/+
Fxyd6-/-
e
Prefrontal cortex
Striatum
Hippocampus
Cerebellum
Brain stem
Prefrontal cortex
Striatum
Hippocampus
Cerebellum
Brain stem
kDa
42
Actin
15
Fxyd6
Fxyd6+/+
Fxyd6-/-

Figure 4.3 *Fxyd6*$^{-/-}$ mice display disrupted *Fxyd6* RNA and protein expression.

***In situ* hybridisation in 20 µm sections reveals *Fxyd6*$^{-/-}$ mice have significantly lower *Fxyd6* RNA expression, in comparison to *Fxyd6*$^{+/+}$ mice, a) prelimbic cortex ($F_{1,7} = 15.47$, $p=0.008$), b) motor cortex ($F_{1,7} = 16.37$, $p=0.007$), c) perirhinal cortex ($F_{1,7} = 16.96$, $p<0.006$) d) dentate gyrus ($F_{1,7} = 14.54$, $p<0.009$). Data represented as expression ratio of *Fxyd6*$^{+/+}$ mice ± S.E.M**

e) Western blotting confirms the disruption of *Fxyd6*, in *Fxyd6*$^{-/-}$ mice, results in the lack of FXYD6 protein expression, in comparison to *Fxyd6*$^{+/+}$ mice. Lack of protein observed in 16 µg protein prefrontal cortex, striatum, hippocampus, cerebellum and brain stem samples. *Fxyd6*$^{+/+}$ mice n=3, *Fxyd6*$^{-/-}$ mice n=3

4.3.3 Fxyd6 null expression increases Pvalb but not Map2k7 and GRIN1 RNA expression

To investigate if the disruption of *Fxyd6* impacts on the expression of *Pvalb* and also schizophrenia-associated gene *Map2k7* and the NMDA receptor gene *GRIN1*, RTqPCR (using probes designed to target these genes) was performed simultaneously with Fxyd6 m1 and Fxyd6 g1 probes (refer to 4.3.2). Using a geometric mean from three housekeeping genes (*18s ribosomal RNA*, *B2M* and *GAPDH*) analysis of cycle threshold values (C_T values) revealed *Pvalb* expression in *Fxyd6*$^{-/-}$ mice is significantly increased by a ratio of 2.987 (standard error 1.431 – 5.598, $p<0.05$) in comparison to *Fxyd6*$^{+/+}$ mice (Figure 4.4). Since a ratio of 1 shows no effect, thus revealing an interaction between *Fxyd6* and *Pvalb*.

Analysis of C_T values also revealed *Map2k7* and *Grin1* expression in *Fxyd6*$^{-/-}$ mice does not significantly deviate from *Fxyd6*$^{+/+}$ mice (*Map2k7* expression ratio = 0.901, standard error ranging 0.681- 1.146, *Grin1* expression ratio= 0.541, standard error ranging 0.184 – 1.111) (Figure 4.4).

4.3.4Fxyd6 null expression does not impact on GLUR2, NR2A and GAD 65/67 protein expression

The involvement of *FXYD6* in the glutamatergic and GABAergic hypothesis of schizophrenia was explored further by western blotting technique. Antibodies designed to target glutamatergic receptor subunit proteins; AMPA receptor subunit GLUR1 and the NMDA receptor subunit NR2A together with the GABA synthesising enzyme glutamate decarboxylase 65/ 67, produced bands of the correct size (GluR1 102 kDa, NR2A 170 kDa, GAD 65/67 produced a doublet band at 65 kDa and 67 kDa) in both *Fxyd6*$^{+/+}$ mice and *Fxyd6*$^{-/-}$ mice. Disruption of *Fxyd6* did not have an impact on expression on any of the proteins investigated (Figure 4.5).

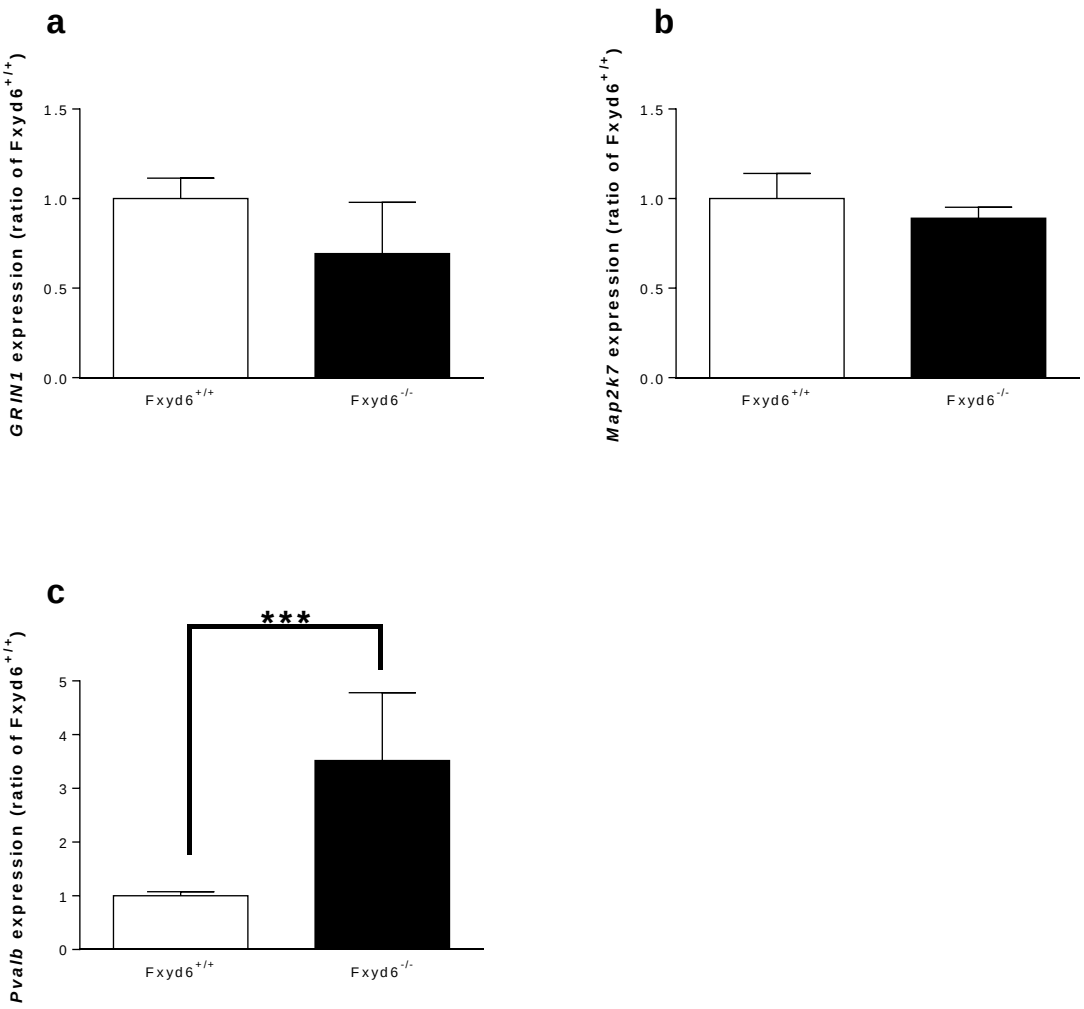


Figure 4.4 *Pvalb* RNA relative expression is significantly increased in *Fxyd6*$^{-/-}$ mice.

The disruption

n of *Fxyd6* in *Fxyd6*$^{-/-}$ mice, in comparison to *Fxyd6*$^{+/+}$ mice a) does not alter *Grin1* expression or b) *Map2k7* expression however c) significantly increases *Pvalb* expression (*$p < 0.001$), data represent C_T values as a ratio of *Fxyd6*$^{+/+}$ mouse expression. *Fxyd6*$^{+/+}$ mice n=3, *Fxyd6*$^{-/-}$ mice n=3**

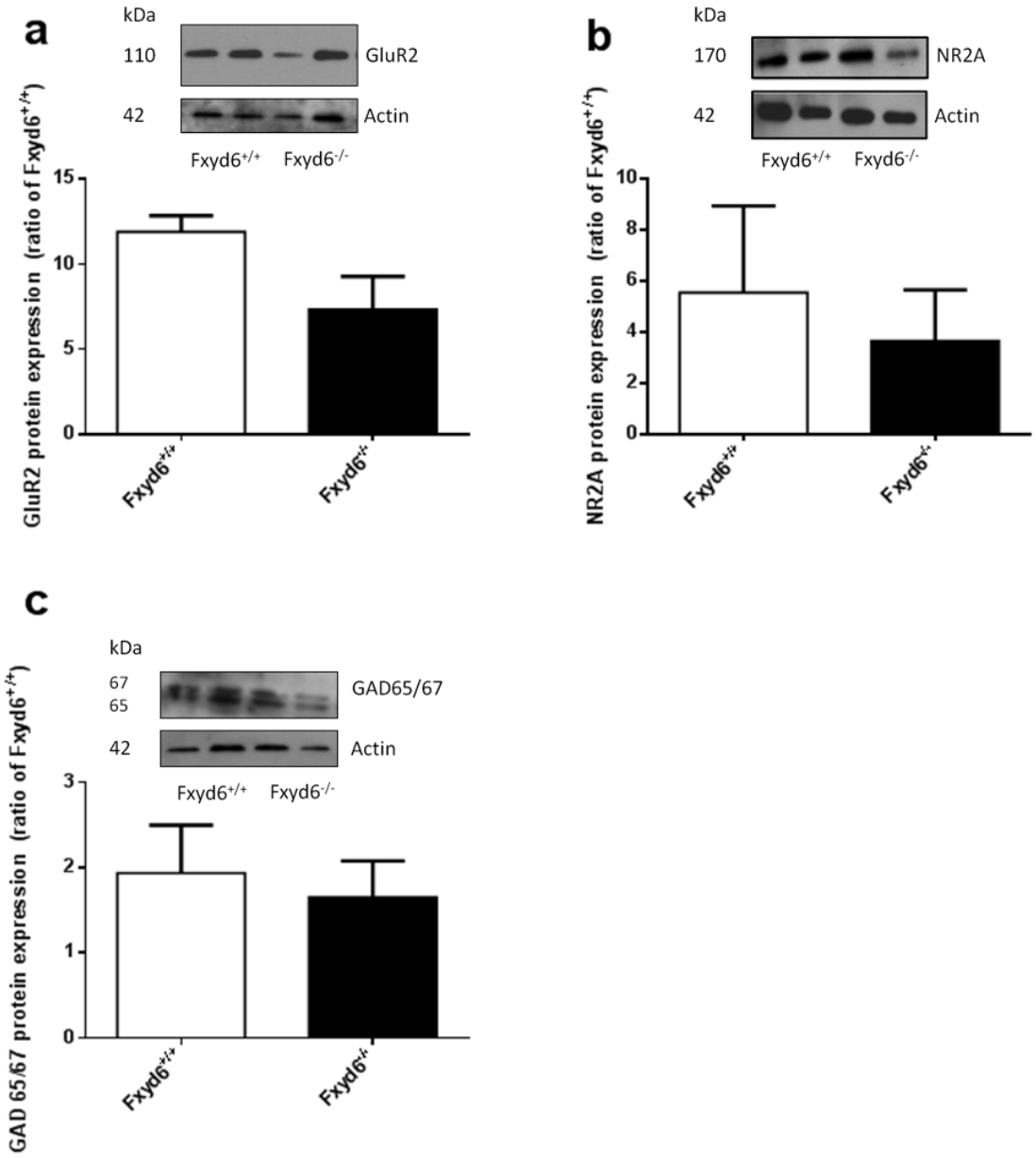


Figure 4.5 Western blotting analysis reveals *Fxyd6* disruption in *Fxyd6*$^{-/-}$ mice does not impact on the expression of

a) GLUR2 ($F_{(1,8)}$ = 0.15), *p*>0.05.

b) NR2A ($F_{(1,8)}$ = 0.15), *p*>0.05.

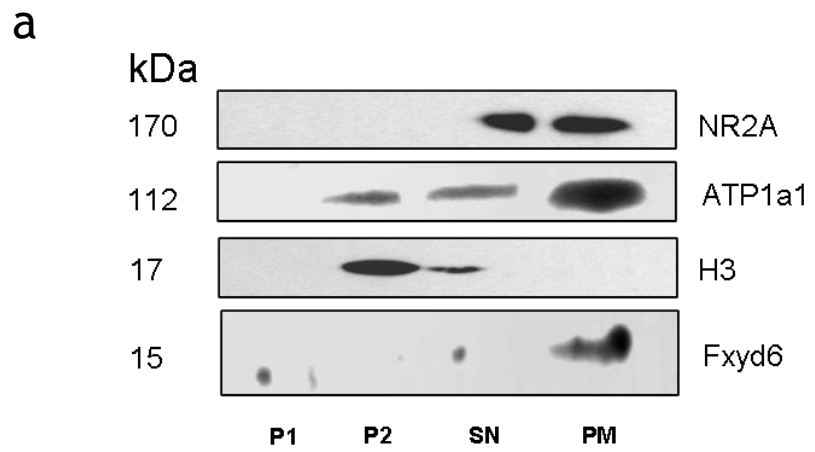
c) GAD 65/67. ($F_{(1,8)}$ = 3.76), *p*>0.05. Data represent mean ± S.E.M. Data analysed using Student's t-test. N=4

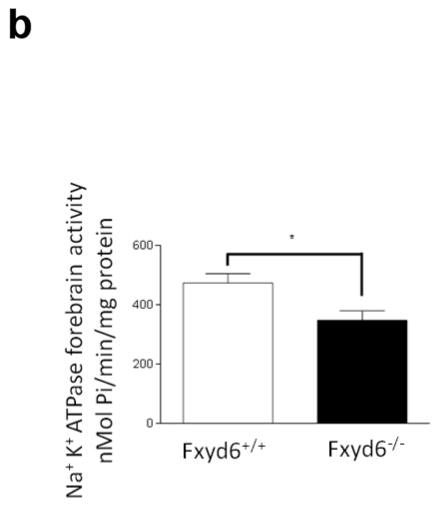
4.3.5 Impact of Fxyd6 null expression on Na^+ K^+ ATPase activity in the forebrain and hippocampus

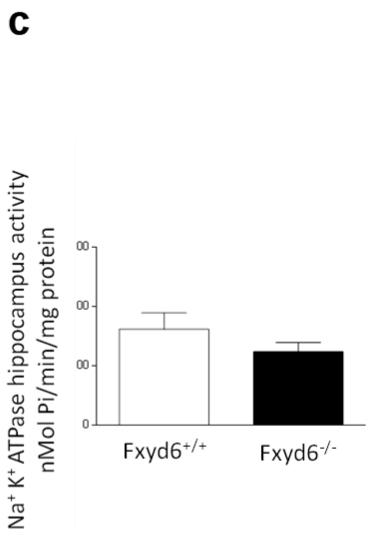
The Na^+ K^+ ATPase activity in adult mice homozygous for *Fxyd6* deletion was investigated in 16 µg forebrain and hippocampus plasma membrane using an assay optimised to detect Na^+ K^+ ATPase pump activity via phosphate release (refer to 2.5). Crude plasma membrane extraction by sucrose fractionation was confirmed using 3 different antibodies by western blot (Figure 4.8a). NR2A antibody produces a band of the correct size of 170 kDa and shows highest expression in the fraction taken as crude plasma membrane fraction, confirming the fraction contains the plasma membrane (Figure 4.8a). H3 antibody is used as an indicator of nuclear fractions, and produces a band of the size at 17 kDa in fractions pellet2 and supernatant (Figure 4.8a). FXYD6 and Na^+ K^+ ATPase α1 subunit antibodies both show highest expression in the crude plasma membrane fraction (Figure 4.8a). In comparison to *Fxyd6*$^{+/+}$ mice, Na^+ K^+ ATPase activity was significantly decreased in the forebrain of the *Fxyd6*$^{-/-}$ mice ($F_{(1,12)}$= 7.90, p= 0.017, Figure 4.8b). However, despite a trend towards decreased Na^+ K^+ ATPase activity in the hippocampus, the decrease in activity was not found to be significant ($F_{(1,14}$= 2.61, p= 0.130 Figure 4.8c). Following the finding that *Fxyd6* disruption results in a significant decrease of Na^+ K^+ ATPase activity, protein expression of adult mouse Na^+ K^+ ATPase α1 subunit was subsequently examined in forebrain homogenates. Western blotting results revealed protein expression of Na^+ K^+ ATPase α subunit does not alter with the deletion of *Fxyd6* ($F_{(1,9}$= 2.61, p= 0.130 Figure 4.8d, Figure 4.8e).

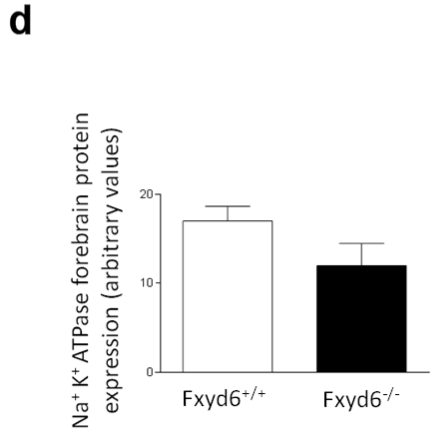
The impact of lack of *Fxyd6* on Na^+ K^+ ATPase expression was further explored using *in situ* hybridisation technique with a probe designed to target *Na^+ K^+ ATPase a1* subunit (refer to 2.9). Quantitative analysis of autoradiogram films revealed *Na^+ K^+ ATPase a1* subunit expression is not changed in the following brain regions in *Fxyd6*$^{-/-}$ compared to *Fxyd6*$^{+/+}$ mice : prelimbic cortex, medial orbital cortex, primary and secondary motor cortex, striatum, nucleus accumbens, CA1, CA2, CA3 and dentate gyrus of the hippocampus and cerebellum (genotype interaction ($F_{1,82}$ = 0.09, p> 0.05, (representative

autoradiographic films (Figure 4.9), there was also no affect of genotype on specific regions ($F_{11,82}$ = 0.29, p> 0.05).


a
kDa
170
112
17
15
NR2A
ATP1a1
H3
Fxyd6
P1
P2
SN
PM


b
Na+ K+ ATPase forebrain activity
nMol Pi/min/mg protein
600
400
200
0
*
Fxyd6+/+
Fxyd6-/-


c
Na+ K+ ATPase hippocampus activity
nMol Pi/min/mg protein
Fxyd6+/+
Fxyd6-/-


d
Na+ K+ ATPase forebrain protein
expression (arbitrary values)
20
10
0
Fxyd6+/+
Fxyd6-/-

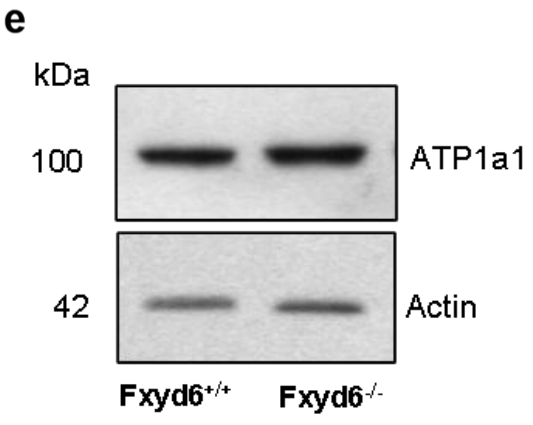

e
kDa
100
42
ATP1a1
Actin
Fxyd6+/+
Fxyd6-/-

Figure 4.6 Confirmation of Na^+ K^+ ATPase expression in the plasma membrane and impact of *Fxyd6* disruption on Na^+ K^+ ATPase pump activity and protein

a) Expression of NR2A confirms the fraction in which FXYD6 and Na^+ K^+ ATPase are expressed in is the plasma membrane (PM), marker histone H3 (H3) confirms pellet 2 (P2) and supernatant (SN) contain nuclear components P1= pellet 1 (refer to 2.5.1).

b) Forebrain Na^+ K^+ ATPase activity is significantly decreased following the disruption of *Fxyd6* in *Fxyd6*$^{-/-}$ mice compared to *Fxyd6*$^{+/+}$ mice $F_{(1,12)}$= 7.90, * *p*= 0.017

c) Hippocampal Na^+ K^+ ATPase activity in *Fxyd6*$^{-/-}$ mice does not significantly deviate from *Fxyd6*$^{+/+}$ mice $F_{(1,14)}$=2.61, p=0.130.

d) Na^+ K^+ ATPase α1 subunit protein expression is not affected by *Fxyd6* disruption in *Fxyd6*$^{-/-}$ mice, compared to *Fxyd6*$^{+/+}$ mice. Data are expressed as mean ± S.E.M nMol Pi/min/mg protein.

e) Representative western blot images showing Na^+ K^+ ATPase α1 subunit protein expression is not affected by *Fxyd6* disruption in *Fxyd6*$^{-/-}$ mice, compared to *Fxyd6*$^{+/+}$ mice.. Data analysed using Student's t-test.

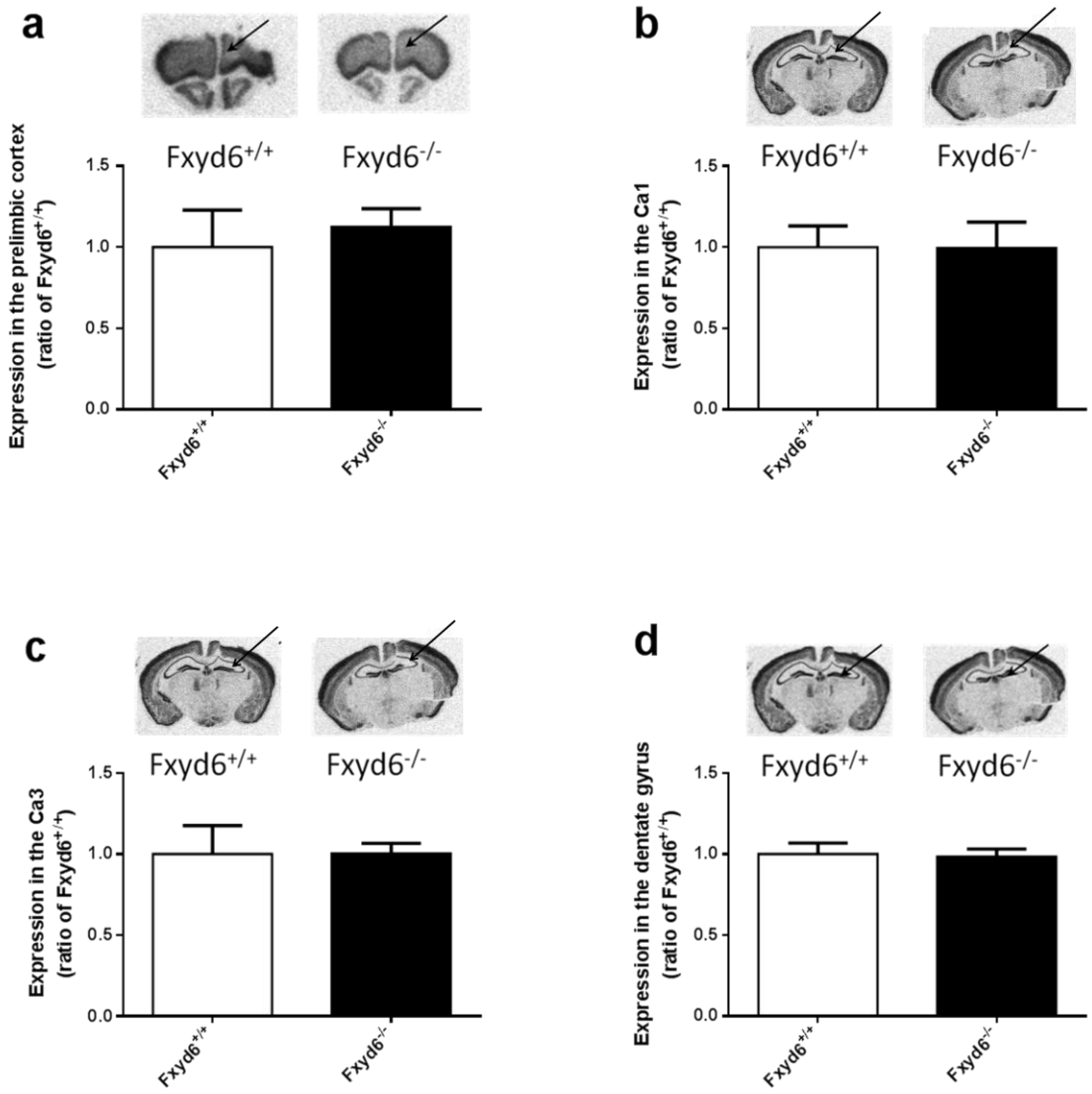


Figure 4.7 Representative autoradiographic film and expression levels of *Na^+ K^+ ATPase*. Arrows indicate relevant brain region. Levels do not differ between *Fxyd6$^{+/+}$* (n=3) and *Fxyd6$^{-/-}$* mice (n=4) in:

a) The prelimbic cortex ($F_{1,6}$=0.29, p>0.05)

b) The CA1 region of the hippocampus ($F_{1,6}$=0.08, p>0.05)

c) The CA2 region of the hippocampus ($F_{1,6}$=0.03, p>0.05)

d) The dentate gyrus ($F_{1,6}$=0.03, p>0.05)

4.4 Discussion

4.4.1 Fxyd6 mRNA brain expression

In this present work, I have confirmed *Fxyd6* mRNA is expressed in various regions of the mouse brain. Two regions which are of particular interest that show strong *Fxyd6* expression are the prefrontal cortex and the hippocampus. These two regions have been repeatedly associated with the symptoms of schizophrenia (refer to 4.3.1) and are therefore of significance in mouse models of this disorder. Decreased functioning and metabolism in the prefrontal cortex (hypofrontality) has been associated with schizophrenia symptoms, furthermore, impaired cognitive tasks such as decision making are also attributed to the altered functioning of this region. Impairments in the functioning of the hippocampus have also been associated with deficits in spatial learning, and connectivity networks between the prefrontal cortex and the hippocampus are thought to be responsible for many areas of cognition and memory consolidation, in particular working memory (Laroche *et al.*, 2000;Yoon *et al.*, 2008). Although *Fxyd6* mRNA is strongly expressed in these regions, qualitative western blot analysis of FXYD6 protein expression would be required to investigate whether there is also a potential functional role of FXYD6 in these regions and associated pathways. If FXYD6 showed expression in these regions, it would suggest *Fxyd6*$^{-/-}$ mice may exhibit impairments in cognition and related phenotypes.

4.4.2 Fxyd6 DNA and protein is significantly reduced in mice homozygous for Fxyd6 disruption

In order to further characterise *Fxyd6* and evaluate the impact of *Fxyd6* in the modulation of Na^+ K^+ ATPase pump activity modulation I utilised a mouse homozygous for the disruption of *Fxyd6* (*Fxyd6*$^{-/-}$ mice) *(Lexicon Pharmaceuticals Incorporated., 2007).* In these mice I also investigated the role of FXYD6 in pathways associated with schizophrenia, such as the GABAergic and glutamatergic pathways. *Fxyd6*$^{-/-}$ mice were created via the insertion of a trapping cassette upstream of exon1 in *Fxyd6 (Lexicon Pharmaceuticals Incorporated., 2007).* To confirm that insertion of the cassette results in the full disruption of *Fxyd6* and FXYD6 protein transcripts, two RTqPCR primers were designed to target *Fxyd6* across exon 1 and 2 and exon 3 and 4. This ensures that

if alternative splicing has occurred following the insertion of the trapping vector upstream of exon1, short transcripts expressing exons 1 and 2 and longer transcripts also expressing exons 3 and 4, will be detected. *Fxyd6* expression levels were quantified using cDNA converted from *Fxyd6*$^{-/-}$ mouse- derived RNA. Results revealed significant disruption of both splice variants of *Fxyd6* in *Fxyd6*$^{-/-}$ mice in comparison to *Fxyd6*$^{+/+}$ mice. To ensure results are truly representative of physiological RNA expression, a geometric mean of 3 housekeeping genes was used as reference to normalise samples. This method is better than using only one housekeeping gene as it reduces the risk of genotype-related changes in expression of any individual housekeeping gene.

Fxyd6 expression in *Fxyd6*$^{-/-}$ mice was examined further using *in situ* hybridisation. Dramatically reduced expression of *Fxyd6* was apparent throughout the brain. Despite these results in conjunction with RTqPCR results clearly indicating *Fxyd6* RNA has been reduced in *Fxyd6*$^{-/-}$ mice, and therefore suggesting protein expression will also be disrupted, it is still important to evaluate FXYD6 protein expression in the mice, to exclude residual alternative splicing, or remaining low levels of RNA transcripts. I have shown that FXYD6 protein was also knocked out in the mice using western blotting. These results confirm *Fxyd6*$^{-/-}$ mice are suitable to be used as a tool for investigating the molecular properties of FXYD6.

4.4.3 Fxyd6 null expression increases Pvalb but not Map2k7 and GRIN1 RNA expression

In situ hybridisation interestingly revealed an up-regulation of *Pvalb* expression in the frontal cortex of *Fxyd6*$^{-/-}$ mice in comparison to *Fxyd6*$^{+/+}$ mice. *Pvalb* is a marker of a subtype of GABAergic interneurons and reduced parvalbumin-expressing interneuron density has been previously associated with schizophrenia (Kinney *et al.*, 2006;Abekawa *et al.*, 2007);(Korotkova *et al.*, 2010). It would therefore be interesting to futher investigate whether the expression in *Fxyd6*$^{-/-}$ mice is due to reduced *Pvalb* expression in neurons, or a reduction in the number of neurons expressing *Pvalb*. Although this is an interesting avenue to explore, further confirmation of altered parvalbumin expression in these mice is required, such as increased sample numbers (n = 3 in this study) and a

confirmation of a functional association between FXYD6 and parvalbumin proteins in *Fxyd6*$^{-/-}$ mice would also be needed.

Reduced expression of *MAP2K7* has recently been reported in post-mortem prefrontal cortex brain samples from schizophrenic patients (Winchester *et al.*, 2012). However change in expression was not observed in *Fxyd6*$^{-/-}$ mice, suggesting there is no interaction between these two genes. Glutamate dysfunction is also associated with the pathophysiology of schizophrenia and glutamate transport has also been linked to the Na^+ K^+ ATPase pump (Nanitsos *et al.*, 2004;Nanitsos *et al.*, 2005), and so the role of FXYD6 in this circuitry was explored by investigating RNA expression of *Grin1*, a NMDA glutamate receptor subunit reported to be involved in memory and learning (Bannerman., 2008), however this was also not affected by *Fxyd6* disruption, which may be due to small n numbers (n=3).

4.4.4FXYD6 disruption does not impact on GLUR2, NR2A and GAD 65/67 protein expression

As previously mentioned (refer to 4.4.3), parvalbumin and GABAergic neurotransmission is reduced in schizophrenia. In addition, the activity of glutamic-acid-decarboxylase (GAD), an enzyme which differentially contributes to GABA synthesis, is also dysregulated (Dracheva *et al.*, 2004;Addington *et al.*, 2004). GAD 65/67 protein expression was investigated in *Fxyd6*$^{-/-}$ mice, using the western blotting technique, to further investigate an interaction between FXYD6 and GABAergic neurotransmission. However *Fxyd6* disruption does not have a significant impact on expression in *Fxyd6*$^{-/-}$ mice.

Glutamate dysfunction in *Fxyd6*$^{-/-}$ mice was further explored by investigating NR2A and AMPA subunit expressions. The role NMDA glutamate receptor subunit NR2A (GluN2A) in the PFC in schizophrenia is not well characterised, however decreased expression of NR2A in parvalbumin neurons has been observed in the PFC of schizophrenia brains (Bitanihirwe et al., 2009). The key AMPA subunit, GLUR2 (GluA2), also has altered expression in schizophrenia brains (Eastwood et al., 1995;Eastwood et al., 1997), however both of these receptor subunits do not have altered protein expression in *Fxyd6*$^{-/-}$ mice compared to *Fxyd6*$^{+/+}$ mice,

suggesting the disruption of *Fxyd6* does not impact on these subunits in the glutamatergic system.

4.4.5 FXYD6 modulates Na^+ K^+ ATPase pump activity in the forebrain

To investigate whether FXYD6 plays a modulatory role of Na^+ K^+ ATPase activity, plasma membrane was extracted from the frontal cortex and hippocampus of mice using a sucrose fractionation method. The fraction containing plasma membrane was confirmed using antibodies targeting NR2A and Na^+ K^+ ATPase α1 as both are present in the plasma membrane of mammalian cells.

The Na^+ K^+ ATPase-specific inhibitor ouabain was used as a means of detecting Na^+ K^+ ATPase specific activity in plasma membrane. The results revealed a decrease in activity in the forebrain of *Fxyd6*$^{-/-}$ mice. This is the first time that FXYD6 has been shown to be a modulator of brain Na^+ K^+ ATPase activity. A non-significant trend towards decreased Na^+ K^+ ATPase activity was observed in the hippocampus, an increase in n number may be required to reach statistical power. Despite the altered activity of forebrain Na^+ K^+ ATPase pump activity, Na^+ K^+ ATPase α1 RNA and protein expression were not affected by *Fxyd6* homozygous disruption. The mechanism behind how FXYD6 modulates the Na^+ K^+ ATPase pump activity is unclear however it has been suggested that the FXYD proteins are non-essential modulators of the α/β complex of the Na^+ K^+ ATPase pump and act to regulate the kinetics of the pump in a tissue-specific manner (refer to 1.12.2). To further investigate the interaction and modulation of the Na^+ K^+ ATPase pump, it would be interesting to use immunocytochemistry technique using antibodies targeted to the relevant proteins to investigate the cellular expression of these proteins in relation to each other. Immunoprecipitation would also be a good technique to employ to investigate interactions between these proteins. These methods would allow further insight in to whether the Na^+ K^+ ATPase pump and FXYD6 proteins interact by directly associating with one another.

4.5 Conclusion

This chapter has provided an insight in the molecular profile of FXYD6. *Fxyd6*$^{-/-}$ mice have been validated as having *Fxyd6* DNA, RNA and protein disruption and utilised as a tool to elucidate information on the molecular functioning of FXYD6.

Fxyd6 was shown to be expressed in a number of regions of the brain including the forebrain and hippocampus, two regions of interest in schizophrenia. Furthermore, although the disruption of *Fxyd6* does not appear to have an impact on glutamatergic receptor expression, *Pvalb* was found to have increased expression in *Fxyd6*$^{-/-}$ mice, this suggests a potential interaction between FXYD6 and GABAergic neuronal activity however this area requires further evidence to confirm an interaction exists.

We have confirmed FXYD6 modulates brain Na^+ K^+ ATPase activity in the frontal cortex, whilst the interaction between FXYD6 and Na^+ K^+ ATPase does not manifest through alteration of protein expression; further experiments could be conducted to elucidate the means of interaction between FXYD6 and Na^+ K^+ ATPase.

Due to the expression of *Fxyd6* in the prefrontal cortex, the impairment of *Fxyd6* in *Fxyd6*$^{-/-}$ mice could have the potential to disrupt memory and learning. The phenotype of these mice would therefore be an interesting area to investigate.

Chapter 5. Phenotype of mice containing disrupted Fxyd6 and similarities to cognitive symptoms of schizophrenia.

5.1 Introduction

The molecular characterisation of FXYD6 (refer to Chapter 4) provides novel evidence which further supports a role of *FXYD6* in schizophrenia and similar disorders associated with impaired cognition. *Fxyd6* was shown to be expressed in the prefrontal cortex and hippocampus, two brain regions highly implicated in schizophrenia. The targeted disruption of a gene in an animal model provides an excellent tool to research the role of a desired gene in disease pathology, however molecular and behavioural characterisation are both required to validate the model as being representative of a disorder.

The expression pattern of *Fxyd6* in the brain (refer to 4.3.1) indicates it may be involved in cognition and memory. The prefrontal cortex plays a major role in working and executive memory. Dysfunction in associated circuitry are thought to underlie many of the cognitive symptoms of schizophrenia such as deficits in working and executive memory, task switching and decision making (Kim et al., 2009;Heerey et al., 2008). Clinical, neuropsychological and neuroimaging studies have all implicated dysfunction of the prefrontal cortex in the pathology of schizophrenia (Chai et al., 2011;Pomarol-Clotet et al., 2010). The hippocampus is also a key structure implicated in the symptoms of schizophrenia. It is responsible for memory and spatial learning (Gaffan., 1985;Olton and Paras., 1979). Furthermore, connectivity networks between the prefrontal cortex and the hippocampus are thought to be responsible for many areas of cognition and memory consolidation, in particular working memory (Laroche *et al.*, 2000;Yoon *et al.*, 2008). The presence of *Fxyd6* in these brain regions alone make this an interesting mouse model to investigate as a model of schizophrenia. However the potential interaction of *Fxyd6* in GABAergic neuronal activity (refer to 4.4.3), as observed by increased *Pvalb* expression in *Fxyd6*$^{-/-}$ mice , further suggests *Fxyd6*$^{-/-}$ mice may exhibit a phenotype analogous to symptoms observed in schizophrenia.

Currently not much information is known on the role of Na^+ K^+ ATPase activity in behaviour. Mice homozygous for the genetic disruption of Na^+ K^+ ATPase subunits α1, α2, or α3 are all embryonically lethal, however heterozygous disruption of each subunit results in altered locomotor activity in response to amphetamine, impaired memory and spatial learning, and increased anxiety (Moseley et al.,

2007). Furthermore, it has been suggested Na^+ K^+ ATPase is required for memory consolidation (Wyse et al., 2004;Sato et al., 2004). *Fxyd6*$^{-/-}$ mice exhibit decreased Na^+ K^+ ATPase activity in the prefrontal cortex (refer to 4.3.5) and may therefore have deficits in memory, locomotor and/or increased anxiety as a result of Na^+ K^+ ATPase dysfunction.

5.2 Hypothesis and aim

FXYD6 has been shown to be expressed in brain regions associated with working and executive memory, and consolidation of memory; therefore the targeted disruption of *Fxyd6* may result in cognitive deficits, a symptom associated with schizophrenia. The targeted genetic disruption of *Fxyd6* may reveal further association with symptoms of schizophrenia as the disruption Na^+ K^+ ATPase α subunit has been shown to induce deficits in locomotor activity and increase anxiety-like behaviours. The genetic disruption of *Fxyd6* results in decreased Na^+ K^+ ATPase activity and *Fxyd6*$^{-/-}$ mice may therefore show similar phenotypes.

The aim of this chapter is to provide a phenotypic characterisation of mice homozygous for disrupted *Fxyd6* (*Fxyd6*$^{-/-}$ mice), in order to elucidate the *in vivo* role of *Fxyd6* and determine if *Fxyd6*$^{-/-}$ mice represent a rodent model of schizophrenia-related behaviours. The strain's basic phenotype is investigated using a battery of assessments, followed by more challenging behavioural tests designed to investigate specific traits such as anxiety, locomotor activity, sensorimotor gating and cognitive deficits.

5.3 Results

5.3.1 Fxyd6$^{-/-}$ mice have a normal primary phenotype

In comparison to *Fxyd6*$^{+/+}$ mice (n= 10) the disruption of *Fxyd6* in *Fxyd6*$^{-/-}$ mice (n=10) did not induce significant abnormalities in basic behaviours tested by the primary phenotype screen (Table.5.1) and motor coordination and balance, as tested by rotarod, both appeared unaffected by the disruption of *Fxyd6* (Table 5.1, refer to 2.10.2).

5.3.2 Fxyd6$^{-/-}$ mice do not differ from Fxyd6$^{+/+}$ in plus maze performance

In order to investigate the impact of lack of *Fxyd6* on anxiety related behaviours, *Fxyd6*$^{-/-}$ mouse performance in the elevated plus maze was analysed (refer to 2.10.3). In comparison to *Fxyd6*$^{+/+}$ mice (n=10), *Fxyd6*$^{-/-}$ mice (n=10) did not reveal any significant changes in anxiety related behaviours, as detected by no significant differences in zone duration ($F_{1,79}$=0.299), p>0.05), zone entry frequency ($F_{1,\ 79}$= 0.228, p>0.05), distance travelled ($F_{1,19}$=0.99, p>0.05) and mean velocity ($F_{1,19}$=0.99, p>0.05,Table 5.2) n=10 per group.

Location	Phenotype	Genotype		Significance
		$Fxyd6^{+/+}$	$Fxyd6^{-/-}$	
In Arena	Body position	1 ± 0	1 ± 0	p >0.05
	Spontaneous activity	0 ± 0	0 ± 0	p >0.05
	Tremor	0 ± 0	0 ± 0	p >0.05
	Urination	0.4	0.6	p >0.05
	Defecation	1.2	1	p >0.05
	Bizarre behaviours	0 ± 0	0 ± 0	p >0.05
	Convulsions	0 ± 0	0 ± 0	p >0.05
	Palebral Closure	0 ± 0	0 ± 0	p >0.05
	Piloerection	0 ± 0	0 ± 0	p >0.05
	Gait	0 ± 0	0 ± 0	p >0.05
	Pelvic elevation	1 ± 0	1 ± 0	p >0.05
	Tail elevation	1 ± 0	1 ± 0	p >0.05
	Pinna reflex	0 ± 0	0 ± 0	p >0.05
Restrained	Cornea reflex	0 ± 0	0 ± 0	p >0.05
	Provoked biting	0 ± 0	0.1 ± 0.1	p >0.05
	Trunk curl	1 ± 0	1 ± 0	p >0.05
	Visual placing	1 ± 0	1 ± 0	p >0.05
	Hanging wire 1	55.0 ± 5	56.5 ± 3.5	p >0.05
	Hanging wire 2	60.0 ± 0	51.5 ± 5.7	p >0.05
	Hanging wire 3	60.0 ± 0	54.9 ± 5.1	p >0.05
	Hanging wire average	58.3 ± 1.7	54.3 ± 4.4	p >0.05
	Rotarod 1	85.9 ± 15.6	131.2 ± 19.9	p >0.05
	Rotarod 2	130.7 ± 21.6	107.7 ± 14.1	p >0.05
	Rotarod 3	155.6 ± 25.0	144.4 ± 18.2	p >0.05
	Rotarod average	124.1 ± 16.8	127.8 ± 14.9	p >0.05

Table.5.1 Summary of primary phenotype screen performed on mice homozygous for *Fxyd6* (*Fxyd6*$^{+/+}$) and mice homozygous for *Fxyd6* deletion (*Fxyd6*$^{-/-}$).

***Fxyd6*$^{-/-}$ mice do not significantly differ from *Fxyd6*$^{+/+}$ mice in any parameters tested in the primary phenotype screen. Data are presented as mean ± SEM, n = 10. Data analysed by Student's t-test.**

A

	Duration		
Zone	Fxyd6+/+	Fxyd6-/-	Significance
Centre	101.1 ± 9.4	93.6 ± 6.3	*p* =0.99, *ns*
Zone 1	166.7 ± 14.8	163.7 ± 10.9	*p* =0.99, *ns*
Zone 2	26.9 ± 7.7	32.2 ± 7.2	*p* =0.67, *ns*
Zone 3	4.9 ± 2.7	10.1 ± 4.5	*p* =0.89 *ns*

B

	Frequency		
Zone	Fxyd6+/+	Fxyd6-/-	Significance
Centre	24.8 ± 2.0	25.7 ± 1.8	*p* =0.99, *ns*
Zone 1	19.4 ± 1.8	19.3 ± 2.0	*p* =1.00, *ns*
Zone 2	7.1 ± 1.8	10.3 ± 1.3	*p* =0.99, *ns*
Zone 3	0.8 ± 0.4	2.5 ± 0.9	*p* =0.84, *ns*

C

Parameter	Fxyd6+/+	Fxyd6-/-	Significance
Distance travelled (cm)	1218.2 ± 57.0	1297.6 ± 45.7	*p* =0.28, *ns*
Mean velocity (cm/s)	4.1 ± 0.2	4.3 ± 0.2	*p* =0.28, *ns*

Table 5.2 *Fxyd6*$^{-/-}$ mice do not display abnormal anxiety-related behaviours in the elevated plus maze, in comparison to *Fxyd6*$^{+/+}$ mice.

Performance in the elevated plus maze did not differ between *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice in the following parameters

a) duration (seconds) ($F_{1,79}$=0.299, *p*> 0.05)

b) frequency of entering zones in the EPM ($F_{1,79}$=0.228, *p*> 0.05 and

c) Distance travelled (T=0.99, *p*> 0.05) and mean velocity (T=0.99, *p*> 0.05) travelled in all zones. Zone 1= closed arm total, zone 2= 2/3^{rds} open arm, zone 3= 1/3rd open arm (refer to 2.10.4) Data are presented as mean ± SEM. N=10 per group. Duration and frequency analysed using ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Distance and mean velocity analysed by Student's t-test.

5.3.3 Fxyd6$^{-/-}$ mouse performance in open field with pharmacological challenges

Anxiety-like behaviours in *Fxyd6*$^{-/-}$ mice were further evaluated using the open field behavioural test (refer to 2.10.42.10.5). During analysis of data, the open field arena was split into three zones, inner, thigmotaxis and outer (refer to 2.10.4). In addition, normal functioning of the mesolimbic dopamine pathway was investigated by assessing hyperlocomotor activity following administration of amphetamine and similarly assessing ability of NMDA neurotransmission following the administration of ketamine.

Amphetamine challenge

Fxyd6$^{-/-}$ mice did not differ from *Fxyd6*$^{+/+}$ mice in the duration of time spent in individual zones, and did not show altered sensitivity to amphetamine in hyperlocomotor activity, as described below:

Genotype did not significantly affect the duration of time spent in zones ($F_{1,119}$=0.01, p>0.05) and there was no overall interaction between treatment and genotype ($F_{1,119}$=0.07, p>0.05) (Figure 5.1a). Administration of 3 mg/kg amphetamine had a significant overall interaction with zone ($F_{2,119}$= 17.63, p<0.001). Tukey's *post hoc* analysis reveals that amphetamine treated mice spend significantly less time in the centre duration compared to mice administered saline (p<0.001) and more time in the outer zone (p<0.001). Time spent in the thigmotaxis zone does not significantly differ in response to amphetamine (p>0.05). n= 10 per group (Figure 5.1a).

Fxyd6$^{-/-}$ mice do not significantly differ from *Fxyd6*$^{+/+}$ mice in velocity ($F_{1,479}$=306, p=0.08). Administration of 3 mg/kg amphetamine significantly increases velocity in both genotypes ($F_{1,39}$=4126, p<0.001), however there is no significant interaction between amphetamine and genotype ($F_{1,39}$=1.59, p>0.05, Figure 5.1b).

Total distance travelled during the testing stage is not affected by genotype ($F_{1,39}$=0.32, p>0.05), however overall amphetamine significantly increases

distance travelled ($F_{1,39}$=43.68, p<0.001) but does not have a significant interaction with genotype ($F_{1,39}$=0.17, p>0.05) Figure 5.2. n= 10 per group.

Ketamine challenge

Fxyd6$^{-/-}$ mice did not differ from *Fxyd6*$^{+/+}$ mice in the duration of time spent in individual zones, despite one group revealing a higher baseline velocity (*Fxyd6*$^{-/-}$ mice prior to receiving 10 mg/kg ketamine in comparison to *Fxyd6*$^{+/+}$ mice) no significant differences were observed following ketamine treatment, as described below:

Administration of treatment (saline, 10 mg/kg ketamine or 25 mg/kg ketamine) does not have an overall significant effect on duration in zones in the open field ($F_{2,152}$= 0.84, p>0.05, Figure 5.3a). There was not an overall effect of genotype on duration spent in zones ($F_{1,152}$=0.14, p>0.05) or interaction between drug and genotype ($F_{1,152}$=0.07, p>0.05). Duration spent in zones significantly differed from one another ($F_{2,152}$=191.20, p<0.001) n= 10 per group.

Mean velocity of movement of *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice was analysed during the habituation period and following ketamine administration. During the habituation period, treatment (saline, ketamine 10mg/kg and 25 mg/kg) did not influence velocity ($F_{2,305}$=0.24, p>0.05), however genotype did have a significant effect ($F_{1,305}$=10.23, p=0.002). Tukey's *post hoc* analysis revealed *Fxyd6*$^{-/-}$ mice in treatment group 10 mg/kg ketamine have a higher basal velocity, prior to receiving treatment, compared to *Fxyd6*$^{+/+}$ mice (p=0.02), this is not observed between any of the other groups (p>0.05). During the test stage, treatment does not have a significant effect on velocity over the 60 minute period ($F_{2,566}$=0.20, p>0.05) however during the 10 minutes succeeding treatment administration (time point 35-45 minutes) treatment has an overall significant impact ($F_{2,140}$=26.33, p<0.001), this effect does not differ between *Fxyd6*$^{-/-}$ mice *Fxyd6*$^{+/+}$ mice ($F_{2,140}$=2.27, p>0.05) (Figure 5.3b). Tukey's *post hoc* analysis demonstrates 10 mg/kg ketamine and 25 mg/kg both induce an overall significant effect in comparison to saline (10 mg/kg p=0.03, 25 mg/kg p<0.001). *Fxyd6*$^{+/+}$ n=10, *Fxyd6*$^{-/-}$ n=7.

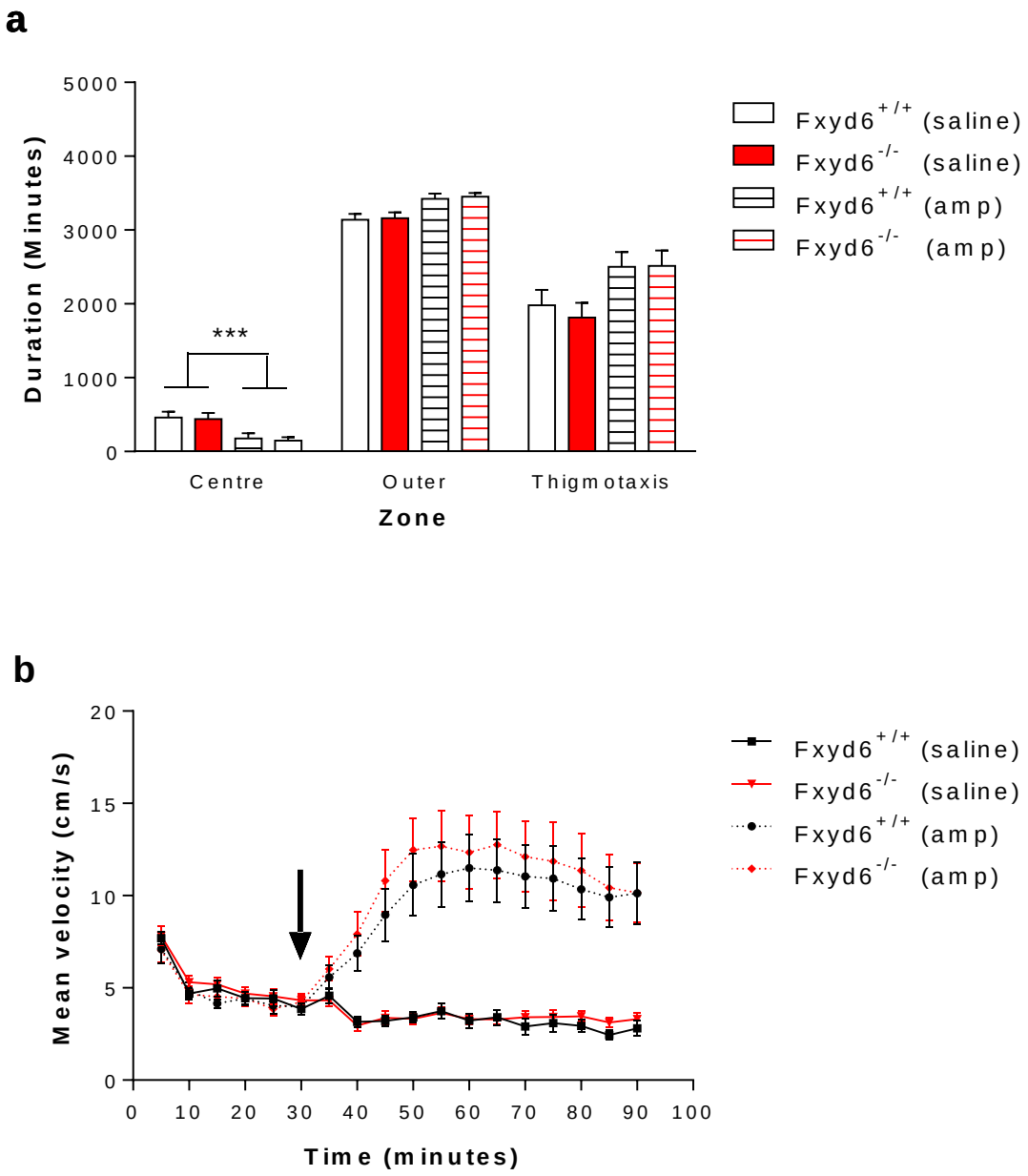


Figure 5.1 *Fxyd6*$^{-/-}$ mice do not exhibit altered locomotor activity responses to amphetamine in comparison to *Fxyd6*$^{+/+}$ mice in the open field test.

a) Analysis of duration spent in centre, inner and outer zones of the open field arena reveal *Fxyd6*$^{-/-}$ mice do not significantly differ from *Fxyd6*$^{+/+}$ mice, suggesting the homozygous disruption of *Fxyd6* does not impact on anxiety-related behaviours ($F_{1,119}$=1.08, p>0.05). Administration of 3mg/kg of amphetamine (amp) overall significantly increases anxiety-related behaviours in *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice, as observed by a decrease in time spent in the centre of the arena in both genotypes ($F_{2,119}$= 17.63, p<0.001). In comparison to *Fxyd6*$^{+/+}$ mice, *Fxyd6*$^{-/-}$ mice do not show hypersensitivity to amphetamine challenge ($F_{1,59}$= 0.04, p>0.05). *** p<0.001 overall amphetamine vs. saline affect on duration

b) Mean velocity in *Fxyd6*$^{+/+}$ mice and *Fxyd6*$^{-/-}$ are both increased following the administration of amphetamine (amp) after a 30 minute habituation period ($F_{1,479}$=4126, p<0.001), *Fxyd6*$^{-/-}$ mice do not differ from their *Fxyd6*$^{+/+}$ littermates in their response to amphetamine ($F_{1,479}$=306, p>0.05). n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. ↓ represents time of treatment administration

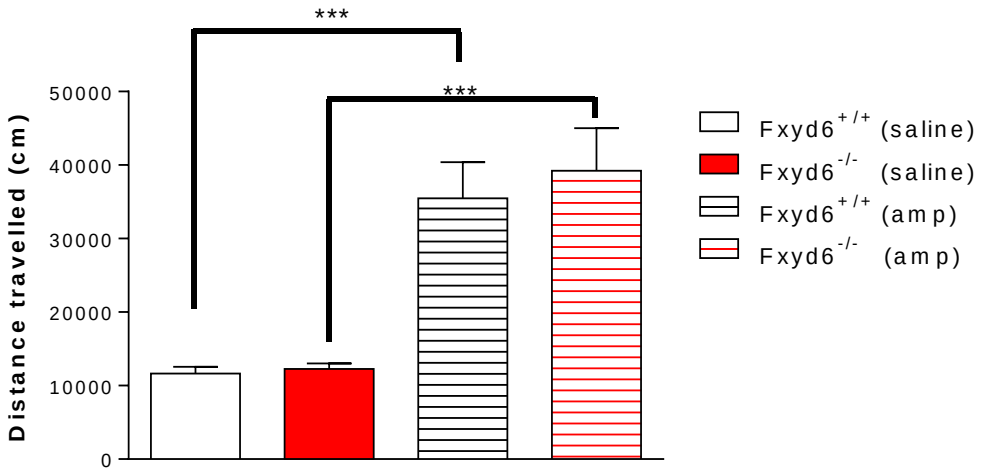
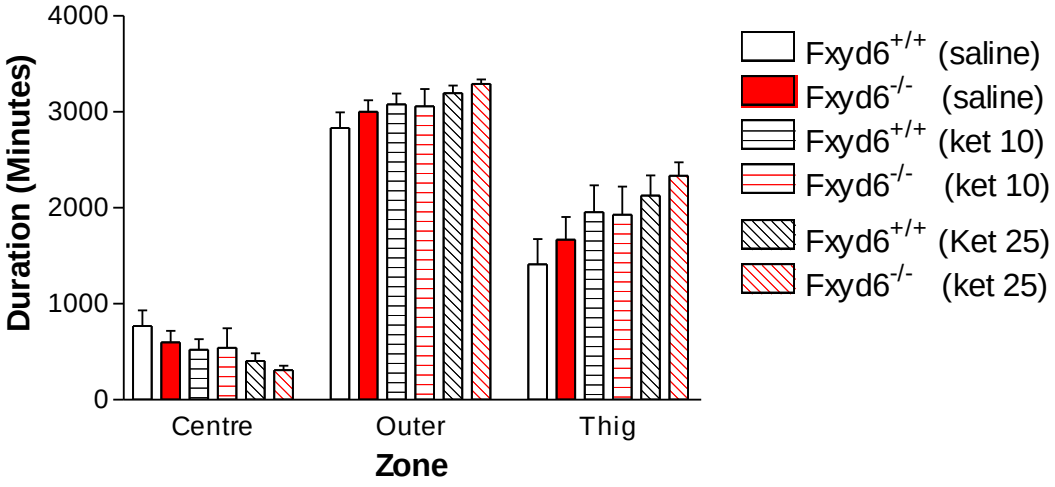


Figure 5.2 *Fxyd6*$^{-/-}$ mice do not show altered sensitivity to amphetamine in the open field test

Analysis of distance travelled during the testing stage of open field did not reveal a significance difference between *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice ($F_{1,39}$=0.32, p>0.05) . Administration of 3mg/kg of amphetamine significantly increases the overall distance travelled ($F_{1,39}$=43.68, p<0.001), the effect of amphetamine is not influenced by genotype ($F_{1,39}$=0.17, p>0.05) Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. ***p<0.001.

a



b

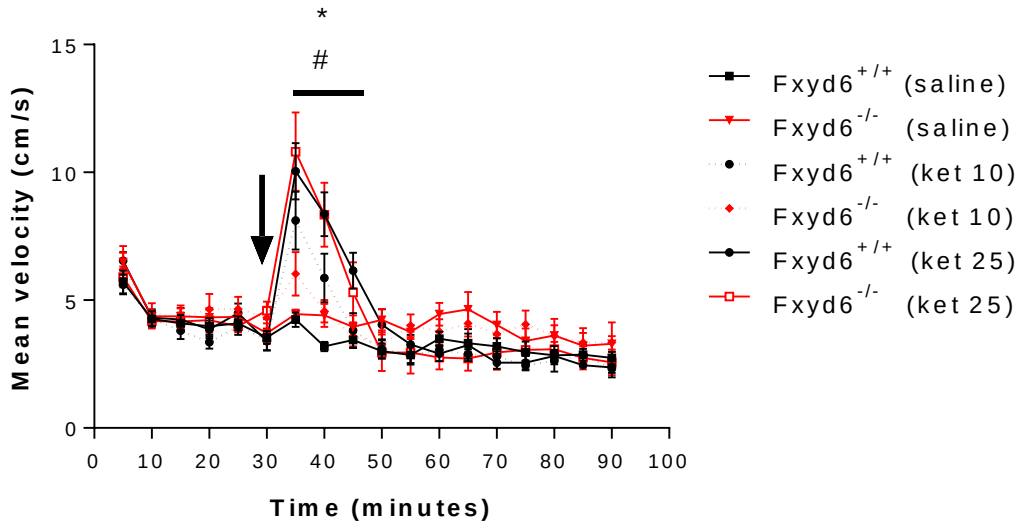


Figure 5.3 *Fxyd6*$^{-/-}$ do not reveal sensitivity to ketamine in the open field test.

a) Analysis of duration spent in centre, inner and outer zones of the open field arena reveal *Fxyd6*$^{-/-}$ mice do not significantly differ from *Fxyd6*$^{+/+}$ mice ($F_{1,152}$=0.14, p>0.05). Administration of 10 mg/kg and 25 mg/kg ketamine (ket), using a cross-over treatment regime, does not significantly affect anxiety- like behaviours in both *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice, as observed by no change in time spent in the arena zones ($F_{2,152}$= 0.84, p>0.05).

b) Velocity of *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice were both significantly increased in response to ketamine over time points 35-45 minutes ($F_{2,140}$=26.33, p<0.001) however there is not a drug x genotype interaction ($F_{2,140}$=2.27, p>0.05). Key: * 10 mg/kg ketamine (ket 10) vs. saline p=0.03, # 25 mg/kg ketamine (ket 25) vs. saline p<0.001). *Fxyd6*$^{+/+}$ n=10, *Fxyd6*$^{-/-}$ n=7. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. ↓ represents time of treatment administration

5.3.4Fxyd6$^{-/-}$ acoustic startle

Prior to conducting PPI, acoustic startle was examined to ensure PPI results were not confounded by alterations in hearing (refer to 2.10.5). Startle responses from mice homozygous for *Fxyd6* null expression did not significantly differ from mice homozygous for *Fxyd6* expression ($F_{1,143}$=0.08, p> 0.05, Figure 6.7) suggesting lack of *Fxyd6* does not affect hearing. n=10 per group.

5.3.5Fxyd6$^{-/-}$ sensorimotor gating

As predicted ketamine induced overall deficits in sensorimotor gating. Deficits in sensorimotor gating in *Fxyd6*$^{-/-}$ mice compared to *Fxyd6*$^{+/+}$ mice are only apparent following administration of 10 mg/kg ketamine, but not saline or 25 mg/kg ketamine, as described below (refer to 2.10.5):

As expected, prepulse levels also had a significant impact on sensorimotor gating ($F_{2,206}$=45.86, p<0.001). Overall PPI results reveal *Fxyd6*$^{-/-}$ mice have significantly altered PPI compared to *Fxyd6*$^{+/+}$ mice ($F_{1,206}$=6.15, p=0.01). There is also an overall effect of treatment (saline, 10 mg/kg ketamine, 25 mg/kg ketamine) ($F_{2,206}$=8.75, p<0.001) and a significant interaction between genotype and treatment ($F_{2,206}$=4.94, p=0.01). The response to the size of prepulse is not affected by genotype ($F_{2,206}$=0.22, p>0.05), or by treatment ($F_{4,1.12,}$ p>0.05). Tukey's *post hoc* exposes *Fxyd6*$^{-/-}$ mice only reveal PPI deficits, in comparison to *Fxyd6*$^{+/+}$ mice, following treatment with 10 mg/kg ketamine (p=0.03), but not saline (p>0.05) or 25 mg/kg ketamine (p>0.05). However, *post hoc* analysis revealed there is no deficits at individual prepulses; at prepulse 4, both genotype ($F_{1,68}$=2.32, p>0.05) and treatment ($F_{2,68}$=0.341) do not significantly impact on PPI responses. Similarly, at prepulse 8 both genotype ($F_{1,68}$=0.78, p>0.05) and treatment ($F_{2,68}$=2.04) also do alter PPI response. At prepulse 16, treatment significantly inhibits PPI overall ($F_{1,68}$=6.54, p=0.003) but not genotype does not affect the response ($F_{1,68}$=6.54, p>0.05). n=10 per group.

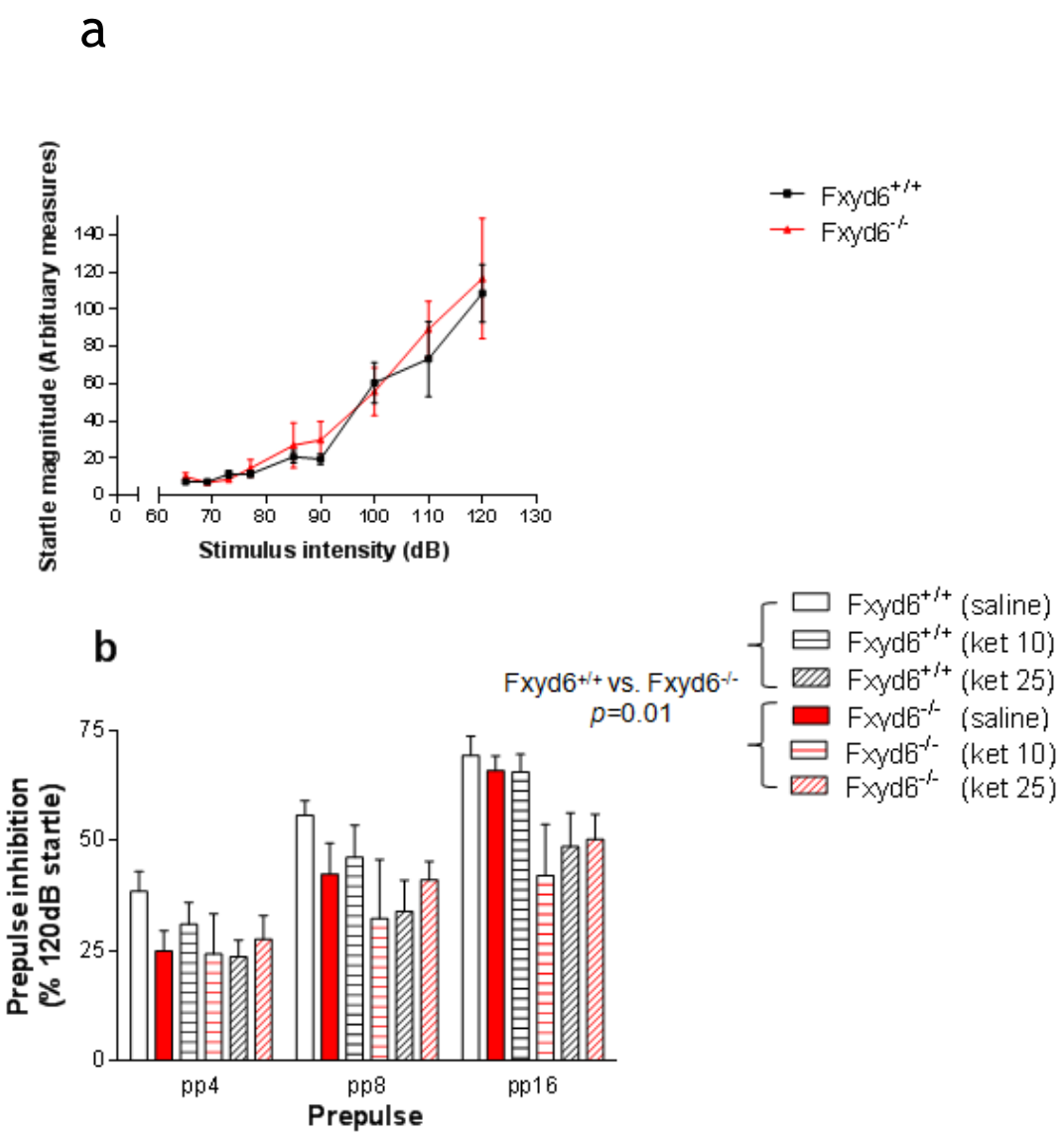


Figure 5.4 *Fxyd6*$^{-/-}$ mice do not display hearing loss but do have prepulse inhibition deficits, compared to *Fxyd6*$^{+/+}$ mice.

a) Startle magnitude to auditory stimuli of 65 dB, 69 dB, 73 dB, 77 dB, 85 dB, 90 dB, 100 dB, 110 dB, 120 dB intensities did not differ between *Fxyd6*$^{+/+}$ mice and *Fxyd6*$^{-/-}$ mice ($F_{1,143}$ = 0.08, p> 0.05) suggesting disruption of *Fxyd6* does not impact on hearing. n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

b) Effect of ketamine treatment (10 or 25 mg/kg) on PPI in *Fxyd6*$^{-/-}$ mice compared to *Fxyd6*$^{+/+}$ mice. Prepulse inhibition of the startle response was overall significantly lowered in *Fxyd6*$^{-/-}$ mice compared to *Fxyd6*$^{+/+}$ mice ($F_{1,206}$=6.15, p=0.01) and 10 mg/kg ketamine had less effect on *Fxyd6*$^{-/-}$ mice overall ($F_{1,206}$=6.15, p=0.01). However, genotype and treatment individually did not have a significant effect at each prepulse level (p>0.05). n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Data shown as mean ±S.E.M.

5.3.6Fxyd6$^{-/-}$ mouse sociability and social novelty.

Fxyd6$^{+/+}$ and *Fxyd6*$^{-/-}$ mice both demonstrated a strong preference for exploring the chamber containing a strange mouse, in comparison to the empty chamber ($F_{2,29}$=48.36, p<0.001), demonstrating similar levels of mouse sociability (Figure 5.5a). The disruption of *Fxyd6* in *Fxyd6*$^{-/-}$ mice did not appear to have an impact on levels of sociability, as observed by no differences in the duration of time spent exploring the chamber containing a mouse, in comparison to *Fxyd6*$^{+/+}$ mice ($F_{1,29}$=0.00, p>0.05), and also there was no genotype*zone interaction ($F_{2,29}$=0.12, p=0.884) (Figure 5.5a). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{+/+}$ n=6.

When a novel mouse was added to the arena in the social novelty aspect of this task (refer to 2.10.7), zones had a significant impact on duration ($F_{2,29}$=22.95, p<0.001), Tukey's *post hoc* analysis reveals only *Fxyd6*$^{+/+}$ mice display significantly more time investigating the novel mouse compared to the now familiar mouse (*Fxyd6*$^{+/+}$ p=0.003, *Fxyd6*$^{-/-}$ p<0.05) (Figure 5.5b). This suggests *Fxyd6*$^{-/-}$ mice may have deficits in social novelty. Time spent in each zone did not significantly differ between *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice (p>0.05). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{+/+}$ n=6.

5.3.7Fxyd6$^{-/-}$ mouse sucrose preference

A sucrose consumption test was performed as a measure of anhedonia in mice (refer to 2.10.8). Genotype did not have an overall impact on % of sucrose consumption over 5 days ($F_{1,50}$=2.40, p>0.05), there was a significant effect of day ($F_{4,50}$=3.31, p=0.02) however this was not affected by genotype ($F_{4,50}$=0.23, p>0.05) (Figure 5.6). The average % sucrose consumed over the 5 day period was also not significantly affect by genotype (p>0.05). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{+/+}$ n=6.

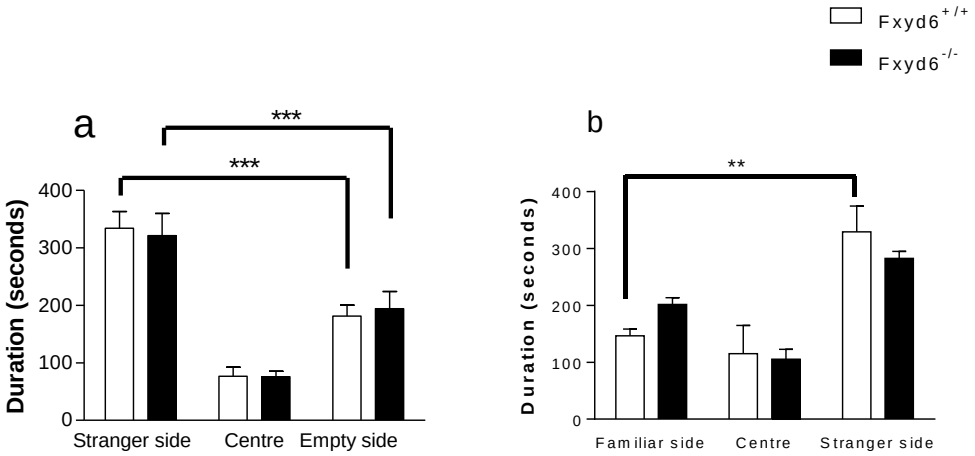


Figure 5.5 *Fxyd6*$^{-/-}$ mice do not have sociability deficits however do not show social novelty.

a) *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice both displayed sociability, as observed by significantly more time spent interacting with the stranger side of the arena, compared to the empty side ($F_{2,29}$=48.36, *p*<0.001). Genotype did not have an effect on levels of sociability ($F_{1,29}$=0.00, *p*>0.05) ***p<0.001. *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

b) Only *Fxyd6*$^{+/+}$ mice displayed significant sociability, as observed by significantly more time spent interacting with a novel mouse (stranger side), in comparison to the now familiar mouse (familiar side).= (*Fxyd6*$^{+/+}$ mice *p*=0.003, *Fxyd6*$^{-/-}$ mice *p*>0.05). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. ** *p*<0.01

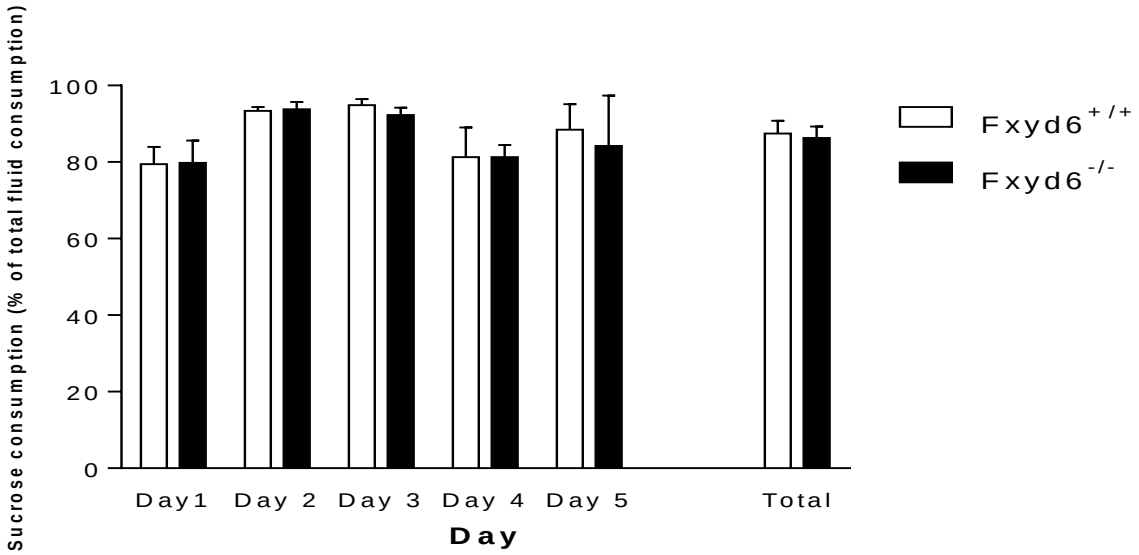
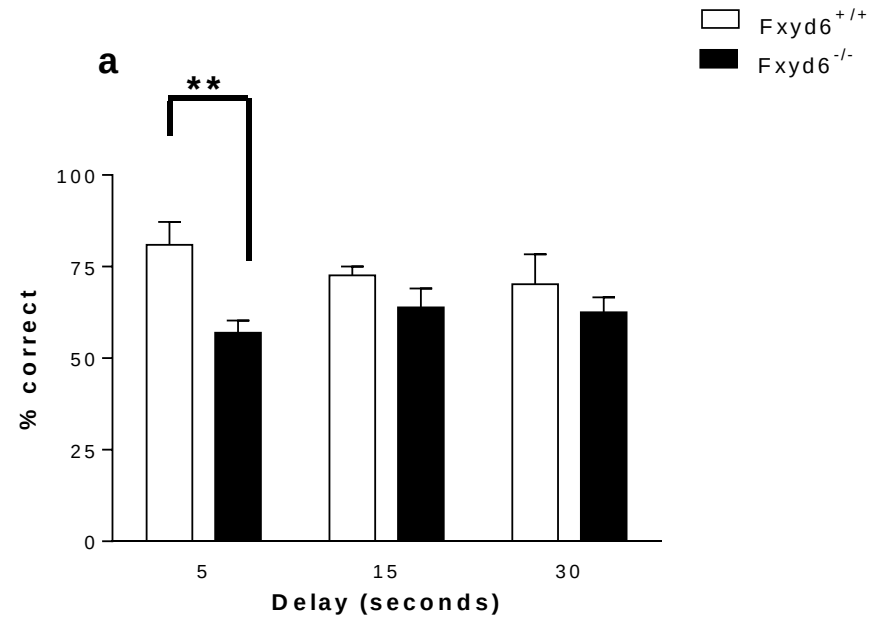


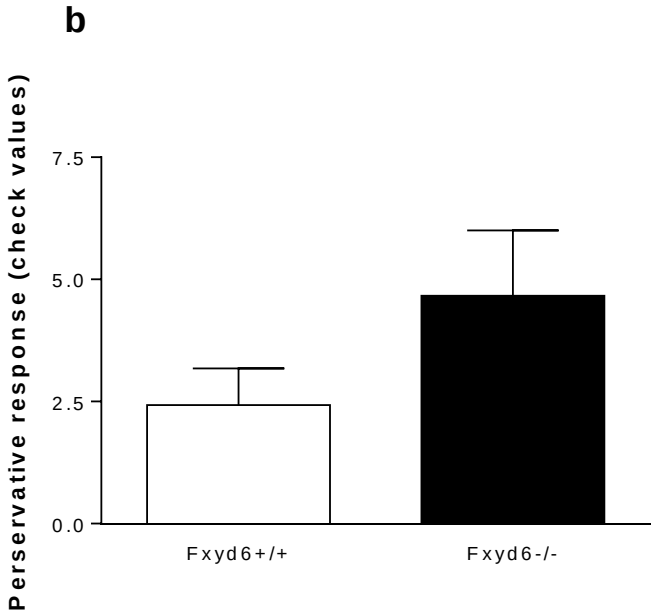
Figure 5.6 *Fxyd6*$^{-/-}$ mice also do not show deficits in a model to detect anhedonia.

Genotype did not have an overall impact on % of sucrose consumption during single days and in total over a 5 day period, suggesting that *Fxyd6*$^{-/-}$ mice do not suffer from anhedonia ($F_{1,50}$=2.40, p>0.05), *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

5.3.8 Fxyd6$^{-/-}$ mouse performance in a delayed non-match to sample task

Results from the delayed non-match to sample task revealed that *Fxyd6*$^{-/-}$ mice have difficulty with working memory following a 5 second delay, as shown by significantly fewer correct trials ($F_{1,12}$=10.44, p=0.008, Figure 5.7a), inter trial delays were kept constant. There were no significant differences at intra-trial delays of 15 seconds (p>0.05) and 30 seconds ($F_{1,12}$=0.65, p>0.05). *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice do not differ in perseverative responding as measured by two or more consecutive incorrect trials ($F_{1,12}$=2.31, p>0.05, Figure 5.7b) or the % of overall trials correct ($F_{1,12}$=3.17, p>0.05, Figure 5.7c). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6.


a
Fxyd6+/+
Fxyd6-/-
**
100
75
50
25
0
% correct
5
15
30
Delay (seconds)


b
Perservative response (check values)
7.5
5.0
2.5
0.0
Fxyd6+/+
Fxyd6-/-

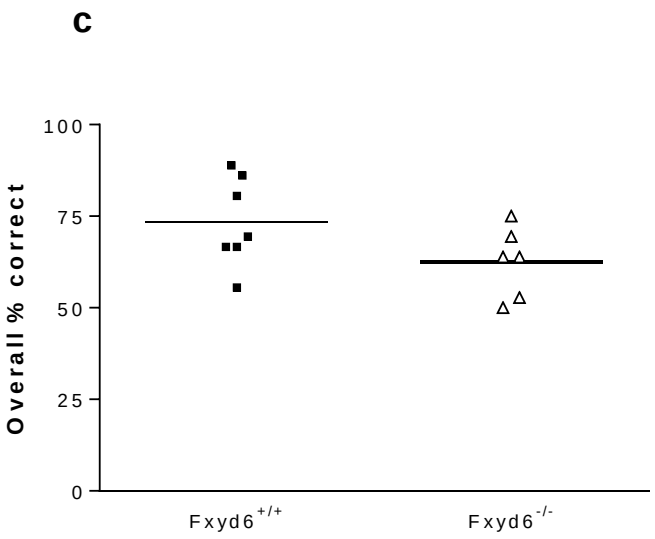

c
Overall % correct
100
75
50
25
0
Fxyd6+/+
Fxyd6-/-

Figure 5.7 *Fxyd6*$^{-/-}$ mice have deficits in working memory.

a) In comparison to *Fxyd6*$^{+/+}$ mice, *Fxyd6*$^{-/-}$ mice have deficits in working memory when encountering a delay of 5 seconds ($F_{1,12}$=10.44, *p*=0.008) but not 15 (p<0.05) and 30 seconds ($F_{1,12}$=0.65, *p*=0.438). *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6. Data analysed by Student's t-test with the exception of delay at 15 which was analysed by Mann-Whitney non-parametric test. ***p*<0.01

b) *Fxyd6*$^{-/-}$ do not have significantly increased perseverative response in comparison to *Fxyd6*$^{+/+}$ mice ($F_{1,12}$=2.31, *p*>0.05). ***p*<0.01

c) Overall % trials correct does not differ between *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice. *Fxyd6*$^{+/+}$ n=7, *Fxyd6*$^{-/-}$ n=6. Data analysed by Student's t-test . ***p*<0.01

5.4 Discussion

The effect of the homozygous disruption of *Fxyd6* on phenotype was investigated using a range of tests. These were no gross deficits however there is evidence for subtle phenotype alterations in particular tests.

5.4.1 Primary phenotype screen

The primary phenotype screen comprising of a battery of tests based on the SHIRPA screen (Rogers et al., 1997) was initially performed on *Fxyd6*$^{-/-}$ mice in order to detect manifestation of abnormal behaviours such as spontaneous activity, aggressiveness, neuromuscular abnormalities, convulsions general condition of coat and eye reflexes. For the full list of criteria refer to (refer to 2.10.1). The screen is initiated by viewing undisturbed mouse behaviour in a clear observation arena. The screening is an imperative process that should be carried out as a first line of phenotyping for any novel mouse strain as such effects could impact on subsequent behaviours and confound interpretation of results in more specific tests. In this study, *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice undergo various behavioural tests where results could be misinterpreted due to deficits in motor control or muscle fatigue, or tremors or convulsions. The primary phenotyping screen did not indicate abnormal behaviours in *Fxyd6*$^{-/-}$ mice in comparison to *Fxyd6*$^{+/+}$ mice, and rotarod and grip strength aimed to detect poor motor performance, co-ordination and balance also did not differ between mouse genotypes. Therefore deficits in subsequent behavioural tests such as locomotor activity, social interaction and working memory are likely to be a true indication of a role for *Fxyd6* in behaviour and cognition, rather than non-specific confounds.

5.4.2 Anxiety-related behaviours in Fxyd6$^{-/-}$ *mice*

Mouse performance in the elevated plus maze was analysed in order to assess the effect of *Fxyd6* disruption on anxiety-related behaviours. The elevated plus maze uses anxiety-related conflict that arises between the mouse's natural preference for dark enclosed space, compared to the drive to explore a novel environment (Lister., 1987). Mice with high levels of anxiety will avoid the bright open arms of the maze. *Fxyd6*$^{-/-}$ mice did not show altered anxiety-related

behaviours when compared to *Fxyd6*$^{+/+}$ mice during the 5 minutes allowed to explore the elevated plus maze.

Anxiety levels in *Fxyd6*$^{-/-}$ mice were further explored, along with locomotor activity, using the open field test. In order to analyse anxiety-related behaviours in the open field arena further, the arena was split into 3 zones during data analysis. The 'centre zone' indicates the amount of time the mouse spends in the centre of the arena, indicating increased exploratory behaviour in the mouse, while the outer zone represents the time spent in the rest of the arena. The outer zone was further split to analyse thigmotaxis. Similarly to the elevated plus maze, the open field test assesses anxiety arising from the mouse's urge to explore the open centre of the arena, compared to the anxiety-induced preference to stay close to the wall (thigmotaxis). In accordance with the plus maze, *Fxyd6*$^{-/-}$ mice did not reveal an increased anxiety phenotype in the open field arena. Mice heterozygous for the deletion of the Na^+ K^+ ATPase α1-subunit also do not display increased anxiety-related behaviours (Moseley et al., 2007) Since molecular characterisation of *Fxyd6*$^{+/+}$ mice revealed co-localisation between FXYD6 protein and Na^+ K^+ ATPase α1-subunit (refer Chapter 4), and functional studies link FXYD6 with the Na^+ K^+ ATPase subunit (Delprat et al., 2007a, Delprat et al., 2007b) this suggests that Na^+ K^+ ATPase modulation in the PFC or hippocampus is a key factor controlling anxiety.

The administration of 3mg/kg amphetamine significantly induced hyperlocomotion and as a result, distance travelled in both *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice was also increased. In comparison to *Fxyd6*$^{+/+}$ mice, *Fxyd6*$^{-/-}$ mice did not reveal any increased sensitivity to amphetamine. Amphetamine induced hyperlocomotion is an expected result and confirms the validity of the experiment (Ralph et al., 2001). Amphetamine increased anxiety levels in both genotypes; again the effect was not affected by genotype. Amphetamine-induced hyperlocomotion are a result of increased dopamine in the synapse in the mesolimbic system (Costall et al., 1987;Pijnenburg et al., 1976). The increase in dopamine results in the increased activation of dopamine D1 and D2 receptors, inducing hyperlocomotion (Sharp et al., 1987;Tenn et al., 2003;Mansbach et al., 1988;Pijnenburg et al., 1975 refer to 1.6.1), Administration of amphetamine in rodents is used to model hyperlocomotor activity in order to represent some of the positive symptoms of schizophrenia

(refer to 1.3.1, Knable and Weinberger., 1997;Mansbach et al., 1988;Pijnenburg et al., 1976;Thornburg and Moore., 1973). In mice containing genetic mutations or lesions, amphetamine administration can reveal dopamine dysfunction or subtle abnormalities in locomotor activity or anxiety which open field test alone may not show. Hence this evidence implies that *Fxyd6*$^{-/-}$ mice do not have abnormalities in dopamine network. To my knowledge there is no known involvement between FXYD proteins and the dopamine network function, however mice heterozygous for deletion of α isoforms of Na^+ K^+ ATPase show abnormally increased anxiety and locomotor activity in response to methamphetamine (Moseley et al., 2007).

Potential dysfunction of glutamatergic circuitry was explored in *Fxyd6*$^{-/-}$ mice by administration of a low dose of ketamine (10 mg/kg) and a higher but sub-anaesthetic dose (25 mg/kg) (Ralph et al., 2001;Imre et al., 2006;Kamiyama et al., 2011). Initial analysis of duration spent in zones and velocity of *Fxyd6*$^{+/+}$ and *Fxyd6*$^{-/-}$ mice did not reveal any differences following administration of 10 mg/kg ketamine and 25 mg/kg ketamine, however due to the short half-life of Ketamine (13 minutes, (McLean et al., 1996;Maxwell et al., 2006)) data were reanalysed for the first 10 minutes after ketamine administration only. Both doses of ketamine were then found to induce hyperlocomotion in both genotypes; however the effect was not genotype- dependent. Despite treatment order and genotype being pseudorandom and balanced amongst groups, one group (*Fxyd6*$^{-/-}$ mice receiving 10 mg/kg ketamine) displayed increased velocity baseline, in comparison to *Fxyd6*$^{+/+}$ mice. However, in this study it is unlikely this is a physiological response derived from the genetic disruption of *Fxyd6,* as it was not displayed in the *Fxyd6*$^{-/-}$ mice subsequently treated with 25 mg/kg ketamine or amphetamine, or *Fxyd6*$^{+/+}$ mice with treated 10 mg/kg ketamine, despite all groups being counterbalanced.

5.4.3 Sensorimotor gating in Fxyd6$^{-/-}$ *mice*

Prior to testing sensorimotor gating, hearing in *Fxyd6*$^{-/-}$ mice was tested to ensure the disruption of *Fxyd6* does not affect hearing, as FXYD6 is expressed in the inner ear (Delprat et al., 2007a;Delprat et al., 2007b). *Fxyd6*$^{-/-}$ mice do not have altered hearing. However they have overall deficits in prepulse inhibition, indicating deficits in sensorimotor gating. Sensorimotor gating is a complex

process involving multiple interactions with multiple neurotransmitters including dopamine, serotonin and glutamate (Ojima et al., 2004;Quednow et al., 2004;Egerton et al., 2008). Deficits in sensorimotor gating stems from problems in inhibition in the CNS and results in the inability to filter important sensory information from 'noise' and is associated with symptoms of schizophrenia. Deficits in PPI are often used as a rodent model of the sensorimotor gating deficits associated with schizophrenia (Geyer et al., 2001). Inhibition of Na^+ K^+ ATPase activity by ouabain has been associated with inhibition of dopamine and serotonin uptake (Steffens M and Feuerstein TJ., 2004) and glutamate transport (Nanitsos et al., 2005;Rose et al., 2009). It is possible impairments in neurotransmission as a result of impaired Na^+ K^+ ATPase activity are responsible for the impaired sensorimotor gating observed in *Fxyd6*$^{-/-}$ mice.

5.4.4Social interaction and social novelty in Fxyd6$^{-/-}$ *mice*

Social deficits are potentially associated with the negative symptoms of schizophrenia, as well as autism and related neurodevelopmental disorders (Lord et al., 2000;Aghevli et al., 2003;Wing and Gould., 1979). Mice are highly social animals and therefore make an excellent model to study deficits in sociability. Sociability testing in mice takes into account social approach of mice to a stranger mouse. A cylindrical wire cage containing the stranger mouse permits visual, olfactory and tactile interaction. In this study, sociability was defined as the tendency to approach and remain in the zone containing the stranger mouse. Disruption of *Fxyd6* did not cause deficits in sociability. However it appears to induce a trend towards reduced levels of social novelty- the preference to interact with a new stranger mouse, compared to the now familiar mouse, reached significance in *Fxyd6*$^{+/+}$ mice but not *Fxyd6*$^{-/-}$ mice. Despite this suggesting that *Fxyd6*$^{-/-}$ mice have reduced levels of social novelty, the levels did not significantly differ from *Fxyd6*$^{+/+}$ mice. An increase in 'n' numbers could determine whether this result is a true biological effect that reflects a lack of interest from *Fxyd6*$^{-/-}$ mice in the new mouse. Reduced social novelty could also reflect the inability to distinguish between the familiar and novel mouse which could be explored further by using the novel object recognition test, which explores recognition memory by assessing time spent exploring familiar and non-familiar objects. This test may provide further insight into novel object preference in *Fxyd6*$^{-/-}$ mice.

5.4.5 Sucrose preference

Anhedonia, the inability to experience pleasure, has been associated with the negative symptoms of schizophrenia (refer to 1.3.2). Mice have a preference for sweet sucrose water over standard water. It has been suggested that the presence of anhedonia in mice can be detected by attenuated sucrose preference over water (Papp et al., 1991). *Fxyd6*$^{-/-}$ mice consumed similar amounts of sucrose water compared to normal water, not only revealing a lack of anhedonia but also showing they do not have deficits in taste, which may have existed due to the expression of FXYD6 in taste cells (Shindo et al., 2011).

5.4.6 Working memory

Impaired working memory is a well recognised cognitive deficit associated with schizophrenia (Chai et al., 2011;Goldman-Rakic., 1994;Kim et al., 2009;Manoach., 2003;Manoach et al., 2000;Pomarol-Clotet et al., 2010). Working memory differs from short term memory and spatial memory because it requires the retention and manipulation of short term memory in order to reach an internal goal (e.g. food reward). A T maze is used in this task, and mice are required to remember which arm they visited following a forced arm run (refer to 2.10.6), following an intra-trial delay, mice need to retain and manipulate this information to choose to enter the previously unvisited arm in return for a reward. Delays of varying times are introduced into the intra-trial delays of the working memory tasks and the ability to correctly enter an unvisited arm is used as an assessment (Aultman and Moghaddam., 2001). Male mice were solely used in 'working memory' tasks due to female mice requiring considerably longer training to reach testing stage (personal communication, Dr. Thomson). This gender difference could be due to behaviour being affected by a number of factors such as circadian rhythm, menstrual cycle, environmental factors such as stimulating housing toys, littermates etc. It is therefore important that during behavioural tasks, such as those designed to test working memory, influencing factors are kept constant.

Fxyd6$^{-/-}$ mice displayed deficits in working memory with delays of 5 seconds, however not at long delay periods of 15 and 30 seconds. The working memory task showed a non-significant trend to becoming increasingly more difficult for

wildtype mice as delays are increased, reducing the average % of trials correct, significant time-dependent deficits have been in similar working memory studies (Aultman and Moghaddam., 2001;Zoubovsky et al., 2011) and it is possible these were not observed in this study due to the low n numbers reducing power and adding variability (*Fxyd6*$^{+/+}$ mice=7, *Fxyd6*$^{-/-}$ mice n=6) . Deficits in working memory in mice should reach a minimum of a 50% correct score by chance alone, it is possible *Fxyd6*$^{-/-}$ mice have deficits in working memory that are not apparent due to the low n numbers and variability in data, especially at the high delays of 15 and 30 seconds.

Hypofrontality (reduced glucose utilisation and blood flow in the prefrontal cortex) has been associated with deficits in working memory (Berman K et al., 1988;Carter et al., 1998;Glahn DC et al., 2005); it would therefore be interesting to carry out 2 deoxyglucose experiments in these mice to assess whether there is altered metabolism in the prefrontal cortex (Weinberger et al., 1986, Callicott et al., 2000., Manoach et al., 2000). The hippocampus has also been associated with deficits in working memory and memory consolidation (Lipska et al., 2002;Sato et al., 2004;Wyse et al., 2004) and it has been suggested that both the prefrontal cortex and the hippocampus are required for different components of working memory (Yoon et al., 2008;Laroche et al., 2000). Hence, since *Fxyd6* is prominently expressed in the PFC and hippocampus, impaired working memory in these mice is consistent with altered function in these areas.

5.4.7Conclusion

This chapter presents a behavioural characterisation of *Fxyd6*$^{-/-}$ mice and provides evidence that the disruption of *Fxyd6* in mice models some of the aspects of schizophrenia.

Fxyd6$^{-/-}$ mice do not exhibit altered sensitivity to ketamine or amphetamine, indicating no potential glutamatergic or dopaminergic circuitry dysfunction, respectively. However indication of deficits in neurotransmission in *Fxyd6*$^{-/-}$ mice comes from subtle deficits in prepulse inhibition.

The targeted disruption of *Fxyd6* also results in prefrontal cortex and hippocampal- related behaviour deficits, such as deficits in working memory.

The genetic disruption of *Fxyd6* in mice results in phenotypes similar to a range of symptoms associated with schizophrenia. This is encouraging to further investigate *Fxyd6*$^{-/-}$ a potential mouse model of schizophrenia and associated psychiatric and cognitive disorders. It also provides further evidence for a role of FXYD6 dysfunction in the aetiology of schizophrenia.

Chapter 6. Molecular and phenotypic characterisation of *Map2k7*, and potential as a mouse model of schizophrenia -like deficits

6.1 Introduction

The symptoms of schizophrenia are diverse and do not appear to be the result of a single neurobiological factor, instead it is often thought to be a result of several subtle anomalies including aberrations in cell signalling. Various studies have implicated impairments in neuronal circuitry and neurotransmission in the pathology of schizophrenia (Sumiyoshi et al., 1996;Lisman et al., 2008); however, the pathological mechanisms that result in impaired cell signalling and the signalling cascades involved remain unclear.

MAP kinases belong to the family of serine/ threonine kinases and are important mediators of signal transduction (refer to 1.13); playing a major role in all eukaryotic cells' response to extracellular stimuli and regulating a wide range of cellular processes that may lead to functional and morphological alterations of neurons (Kyosseva et al., 1999;Pearson et al., 2001;Kyosseva, 2004). The role of MAP kinase signal transduction cascades, and in particular MAP kinase kinase 7 (*MAP2K7)*, in major psychiatric disorders, including schizophrenia, is not well understood (refer to 1.13.3). However due to the abundant expression in the CNS and pivotal role in the regulation of signal transduction, synaptic plasticity and modification of gene expression in neurons (Kyosseva et al., 1999;Pearson et al., 2001;Kyosseva, 2004), it is not surprising various intermediates of MAP kinase cascades have been suggested to be involved in neurodevelopmental abnormalities of the brain in schizophrenia and psychiatric disorders (Kyosseva et al., 1999;Funk et al., 2011).

Recently, our lab found evidence of a role of *MAP2K7* as a susceptibility factor in schizophrenia. Not only was *MAP2K7* found to have attenuated expression in the PFC of *post mortem* schizophrenia brains, but a genetic association between *MAP2K7* and schizophrenia was found (Winchester et al., 2012). The MAP2K7 protein is primarily activated by cytokines in the immune system (e.g. TNF, IL-1), although it has also been shown to be activated by NMDA receptor activity in the CNS, a receptor often showing impaired activity in schizophrenia (Tournier et al., 2001;Centeno et al., 2006). Following activation, MAP2K7 is dually phosphorylated on a specific threonine and tyrosine residue of the

phosphorylation motif (refer to 1.13, (Tournier et al., 2001;Holland et al., 1997) prior to acting as an upstream JNK2 activator. JNK2 mediates signal transduction and regulates many cellular processes such as cell growth and proliferation, transcription and apoptosis (Tournier *et al.*, 2001;Holland *et al.*, 1997;Mayer *et al.*, 2005). JNK2 has also been shown to have decreased expression and phosphorylation in the anterior cingulate cortex (Funk et al., 2011) strengthening the hypothesis for a role of MAP2K7 and related pathways as a risk factor in schizophrenia.

Despite the genetic association implicating *MAP2K7* as a risk factor of schizophrenia, further biological evidence is required to ascertain a functional link between the gene and disease. Genetic association studies provide an indication of markers of disease; however they do not elucidate information on the causative variation or the exact biological mechanism underlying the risk factor. Animal models provide a unique way of analysing and understanding the functional role of genetic risk factors in the pathophysiology of schizophrenia (refer to 1.10). The heterozygous or homozygous disruption of a gene of interest allows investigation into signalling networks, interactions and potential regulatory and compensatory mechanisms, as well as the impact on behaviour. In addition to providing a model to investigate behaviours similar to those associated with schizophrenia, the neurochemical hypotheses of schizophrenia can be explored in the mice by investigating the expression of relevant markers such as the GABAergic interneuron marker parvalbumin or the expression of glutamate receptors.

A mouse heterozygous for *Map2k7* has been established (Wada et al., 2004). Heterozygous expression of *Map2k7* in these mice was confirmed using northern and southern blotting which revealed a clear reduction in *Map2k7* genomic mRNA and DNA (respectively) (Wada et al., 2004), however as of yet these mice have not been investigated for impairments in neural circuitry or behaviours associated with schizophrenia, such as cognitive deficits.

6.2 Aim and hypothesis

MAP2K7 is a putative risk factor in the susceptibility to schizophrenia; however biological evidence is still required to establish this link. Map kinases are

associated with the maintenance of signal transduction and it is hypothesised the heterozygous deletion of *Map2k7* will have implications in neurotransmission and memory consolidation. This chapter aims to elucidate information on the molecular and functional role of *Map2k7* by utilising *Map2k7* heterozygous mice (*Map2k7*$^{+/-}$) and investigating changes in schizophrenia-related proteins and behaviours, such as working memory.

6.3 Results

6.3.1 Expression of Map2k7 in the mouse brain

Expression of *Map2k7* in 20 µm coronal adult mouse brain sections was visualised using a 33P- labelled probe designed to target *Map2k7* RNA (refer to 2.9). Qualitative assessment from comparison of total staining to non-specific staining revealed *Map2k7* is expressed in the following brain regions: the prefrontal cortex, the granular cell layer of the olfactory bulb (GrO), the CA1 region of the hippocampus and the granule cell layer of the dentate gyrus (GrDG), and the cerebellar lobules of the cerebellum (Figure 6.1).

6.3.2 Evidence for a trend towards reduced Map2k7 RNA expression in Map2k7$^{+/-}$ mice

To investigate the potential functional link between MAP2K7 and schizophrenia, mice containing one targeted disruption of *Map2k7* were utilised (refer to 2.3.2). The *in situ* probe used to detect mouse brain expression of *Map2k7* (section 6.3.1) was further utilised to investigate the effect of heterozygous targeted disruption of *Map2k7* on RNA levels. Despite a trend towards reduced expression, no significant overall attenuation of *Map2k7* mRNA expression was observed in *Map2k7*$^{+/-}$ mice ($F_{1,38}$= 2.88, p>0.05), neither were there decreases in the following regions: CA1 ($T_{1,6}$=0.04, p>0.05), CA3 ($T_{1,6}$= 4.25, p>0.05), cerebellum ($T_{1,6}$= 0.55, p>0.05), DG ($T_{1,6}$= 0.3, p>0.05), or olfactory bulb (GrO) ($T_{1,6}$= 0.1, p>0.05) (Figure 6.2a-i). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=3.

RTq-PCR primers designed to target *Map2k7* transcripts across two sites: exon 1a and 1b and exons 10 and 11 (refer to 2.8.1), were employed to investigate the impact of heterozygous genetic knockdown of *Map2k7 upon Map2k7* mRNA expression. Using a geometric mean from three housekeeping genes (18s ribosomal RNA, *B2M* and *GAPDH*) analysis of C_T values revealed *Map2k7* expression in *Map2k7*$^{+/-}$ mice is reduced across exon 10/11 by 20% (Figure 6.3a) however this does not reach significance (ratio 1: 0.80, $F_{1,5}$=1.09, p>0.05). A modest reduction of *Map2k7* exon 1a and b mRNA expression of 32% was observed (Figure 6.3b). To further investigate *Map2k7* mRNA expression in *Map2k7*$^{+/-}$ mice, the effect of genotype on overall *Map2k7* expression was analysed using ANOVA with multiple comparisons, which showed a near

significant effect of genotype of *Map2k7* expression ($F_{1,11}$=5.47, *p*=0.06) n=3 per group.

6.3.3 *Map2k7*$^{+/-}$ mice have increased Grin1 RNA expression

RTqPCR revealed that the heterozygous targeted disruption of *Map2k7* significantly increased glutamate receptor *Grin1* mRNA expression (ratio 1: 4.3, ($F_{1,5}$=15.99, *p*=0.016, Figure 6.3e). However the disruption does not have a significant impact on *Pvalb* (ratio 1: 0.79, $F_{1,5}$=0.71, p>0.05, Figure 6.3c) or *Fxyd6* mRNA expression (ratio 1: 1.09, $F_{1,5}$=0.25, p>0.05, Figure 6.3d).

6.3.4 *Map2k7* reduced expression does not impact on GLUR2, NR2A and GAD 65/67 protein expression

The involvement of *MAP2K7* in the glutamatergic hypothesis of schizophrenia was explored by the western blotting technique. Antibodies designed to target glutamatergic receptor proteins GluR2 and NR2A and glutamate decarboxylase (GAD) 65/ 67 produced bands of correct size (GluR2 102 kDa, NR2A 170 kDa, GAD 65/67 produces a doublet band at 65 kDa and 67 kDa) in both *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice. Disruption of *Map2k7* did not impact on the expression of any of the proteins investigated (Figure 6.4). Despite attempts at the optimisation of two different MAP2K7 antibodies, neither was able to produce a clear band of correct size to confirm MAP2K7 protein knockdown.

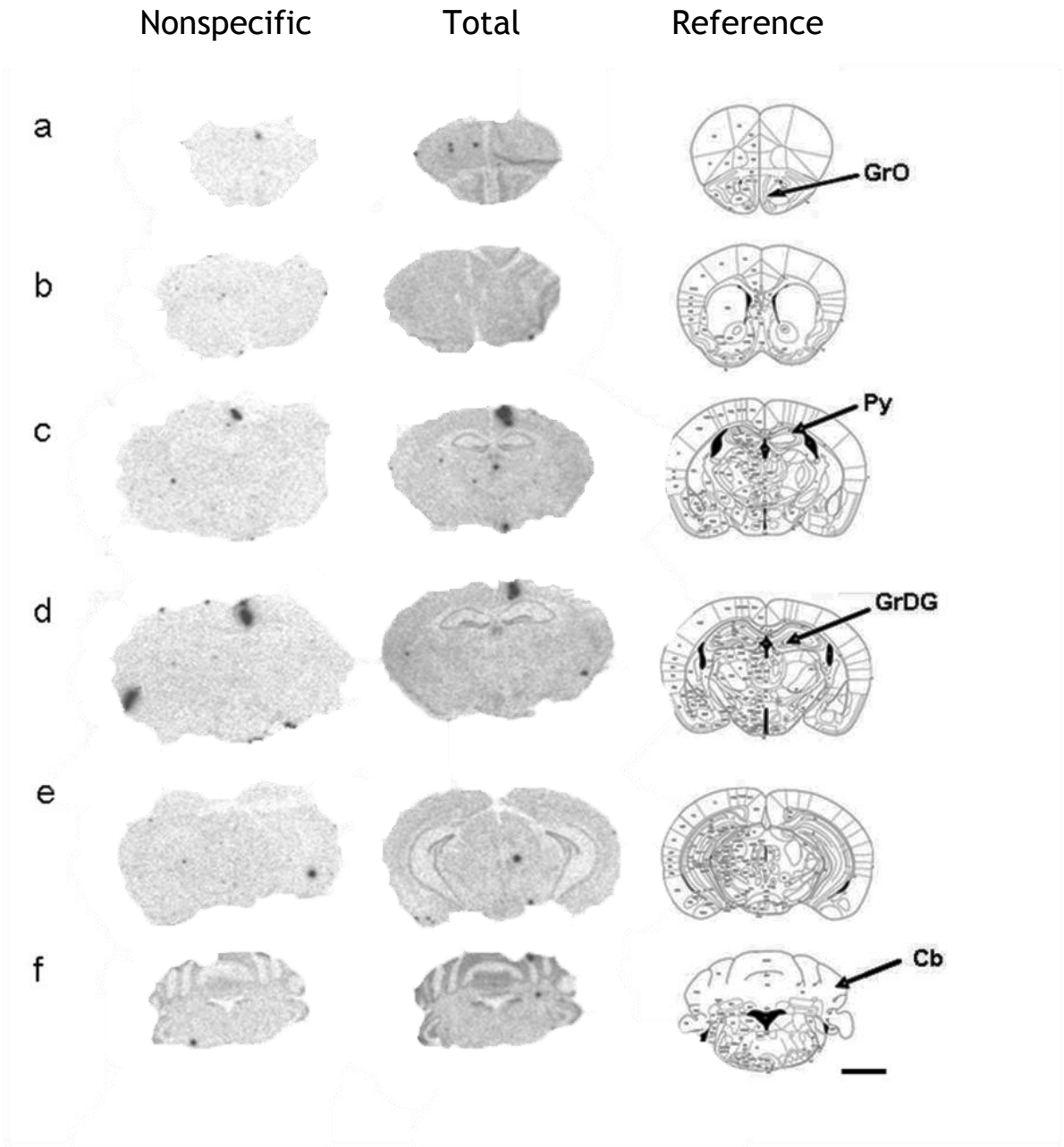
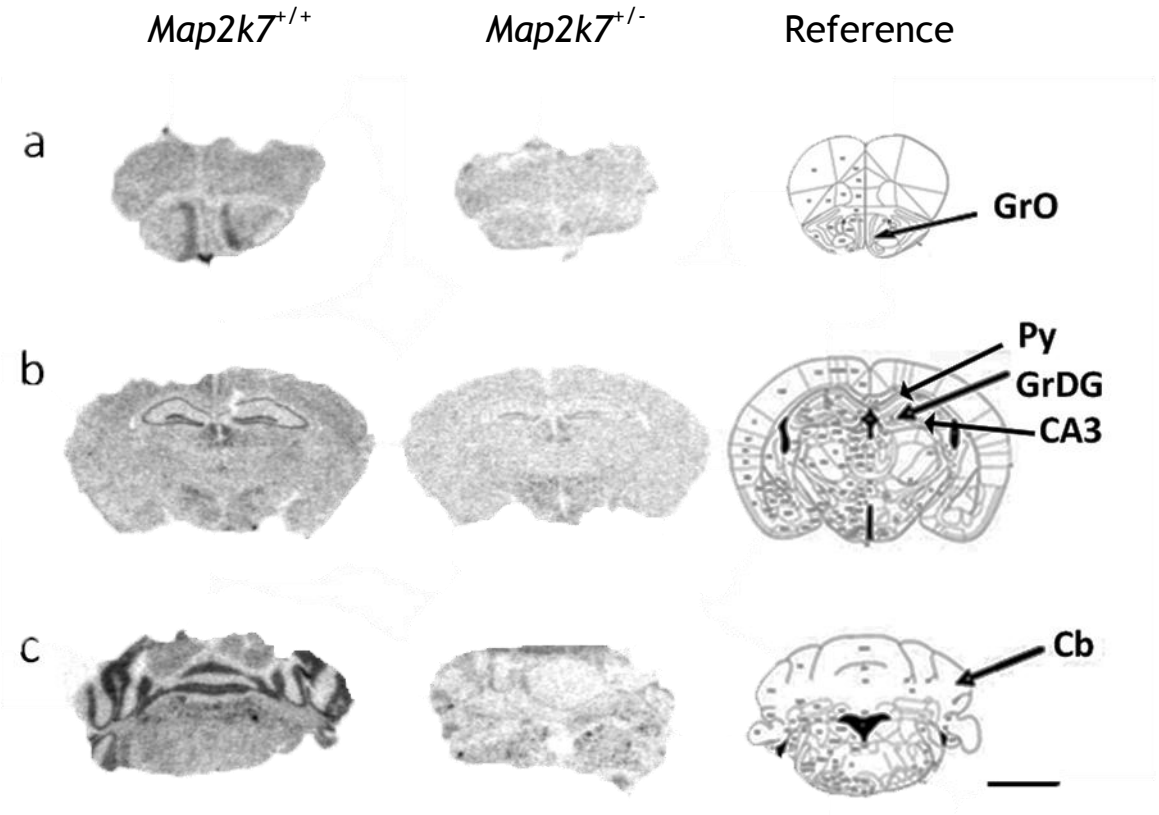


Figure 6.1 Autoradiogram images showing *Map2k7* mRNA distribution in coronal mouse brain sections, as detected by *in situ* hybridisation.

Qualitative assessment suggests *Map2k7* is expressed in a) the prefrontal cortex and in particular the granular cell layer of the olfactory bulb (GrO) (bregma 2.10 mm) b) (bregma 1.18 mm) c) pyramidal cell layer of the hippocampus (Py), granular layer of the dentate gyrus (GrDG) (bregma -1.22 mm) d) pyramidal cell layer of the hippocampus (Py), granular layer of the dentate gyrus (GrDG) (bregma -1.82), e) pyramidal cell layer of the hippocampus (Py), granular layer of the dentate gyrus (GrDG) (bregma -2.92 mm) f) cerebellar lobules of the cerebellum (bregma -6.00). Unlabelled= slides incubated with 'cold probe', indicates non specific labelling, total = slides incubated with 'hot probe' refer to 2.9.1. Scale bar = 2mm.


Map2k7+/+
Map2k7+/-
Reference
a
b
c
GrO
Py
GrDG
CA3
Cb

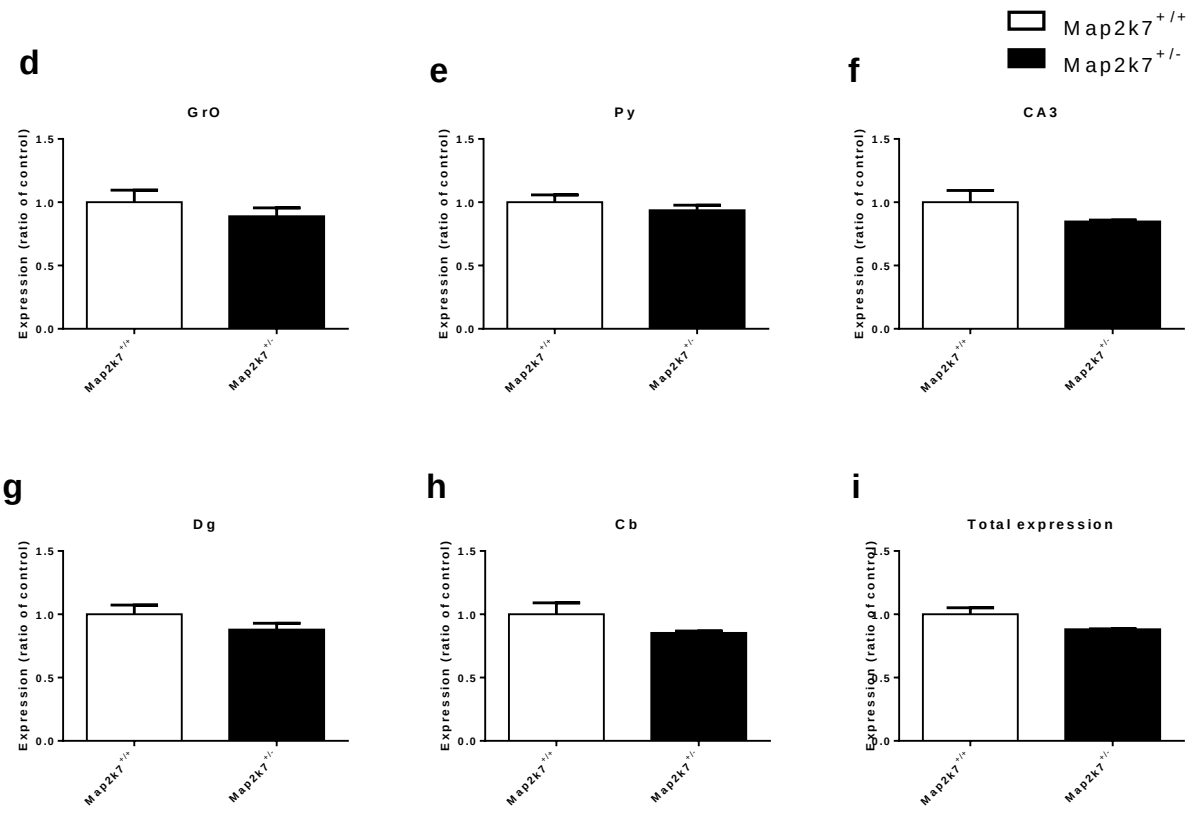

Map2k7+/+
Map2k7+/-
d
GrO
e
Py
f
CA3
g
Dg
h
Cb
i
Total expression
Expression (ratio of control)
0.0
0.5
1.0
1.5

Figure 6.2 *Map2k7* expression in *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mouse brain

Autoradiogram images showing *Map2k7* distribution in Map2k7$^{+/+}$ and Map2k7$^{+/-}$ coronal mouse brain sections in a) the prefrontal cortex and in particular the granular cell layer of the olfactory bulb (GrO) b) pyramidal cell layer (Py) and CA3 of the hippocampus, granular layer of the dentate gyrus (GrDG) c) cerebellar lobules of the cerebellum. Scale bar = 2mm.

Quantitation of autoradiographic film images reveals that *Map2k7* mRNA levels do not differ significantly in Map2k7$^{+/+}$ and Map2k7$^{+/-}$ mice in the following brain sections a) granular cell layer of the olfactory bulb (GrO) ($T_{1,7}$=0.18, p>0.05) b) pyramidal cell layer (Py) ($T_{1,7}$=0.46, p>0.05) c) CA3 of the hippocampus ($T_{1,7}$=4.19, p>0.05) d) granular layer of the dentate gyrus (GrDG) ($F_{1,7}$=0.92, p>0.05) e) cerebellar lobules of the cerebellum ($T_{1,7}$=0.62, p>0.05) f) total expression ($T_{1,7}$=2.32, p>0.05). Map2k7$^{+/+}$ n=5, Map2k7$^{+/-}$ n=3. Data analysed by Student's t-test.

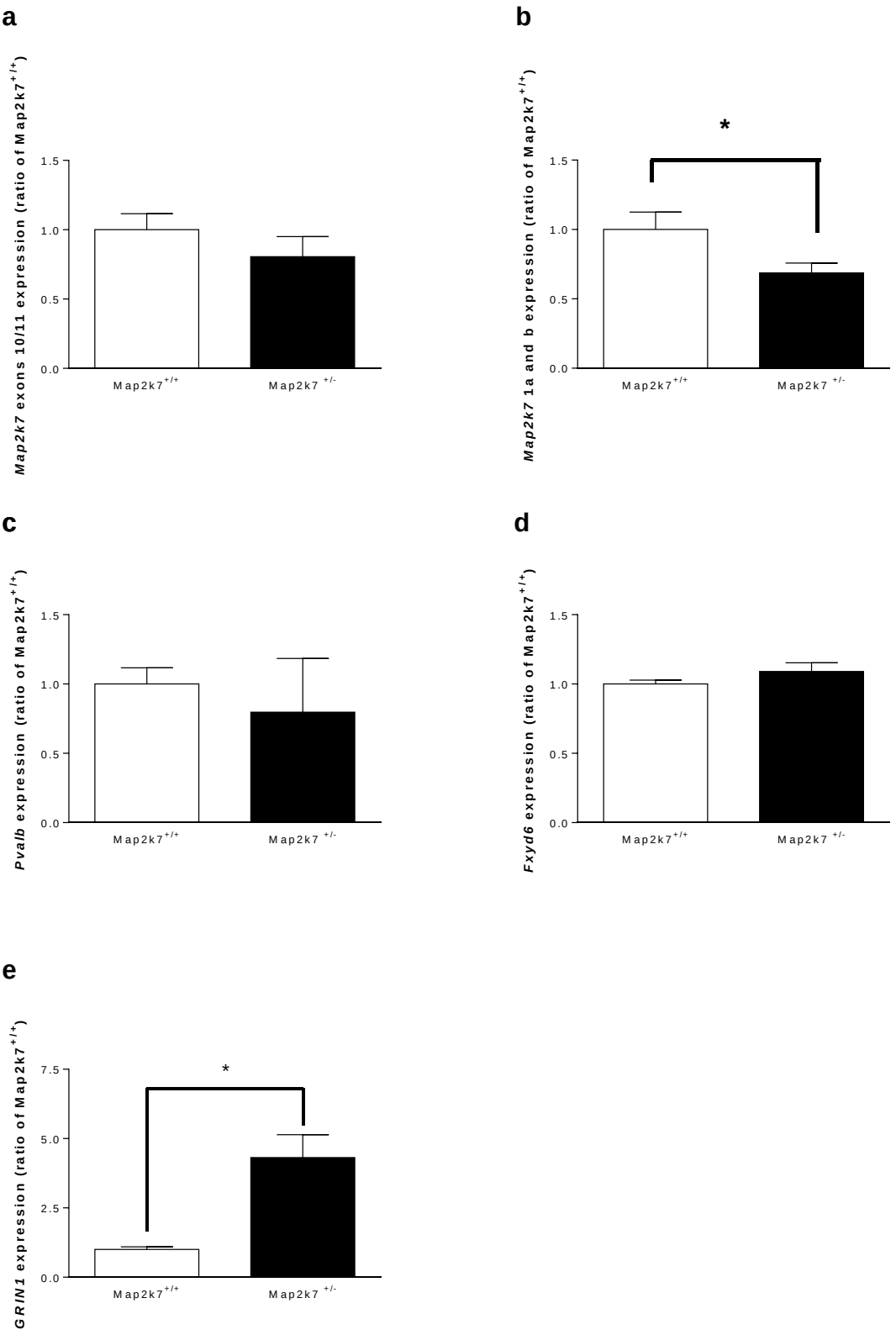


Figure 6.3 Map2k7$^{+/-}$ mice have increased GRIN1 mRNA expression.

Analysis of CT values revealed *Map2k7* heterozygous deletion results in a) a non-significant reduction of *Map2k7* exon 10/11 expression (ratio 0.80, $F_{1,5}$=1.09, *p*=0.18) b) a modest reduction of *Map2k7* exon 1a and b mRNA expression (ratio 0.68, $T_{1,5}$=2.17, *p*=0.05 c) no effect on parvalbumin (Pvalb) mRNA expression (ratio 1.79, $T_{1,5}$=0.71, *p*>0.05) and d) *Fxyd6* mRNA expression (ratio 1: 1.09, $T_{1,5}$=0.25, *p*>0.05) e) GRIN 1 expression (ratio 1: 4.3, $F_{1,5}$=15.99, *p*=0.016). **p*<0.05. n=3. Data analysed by Student's t-test.

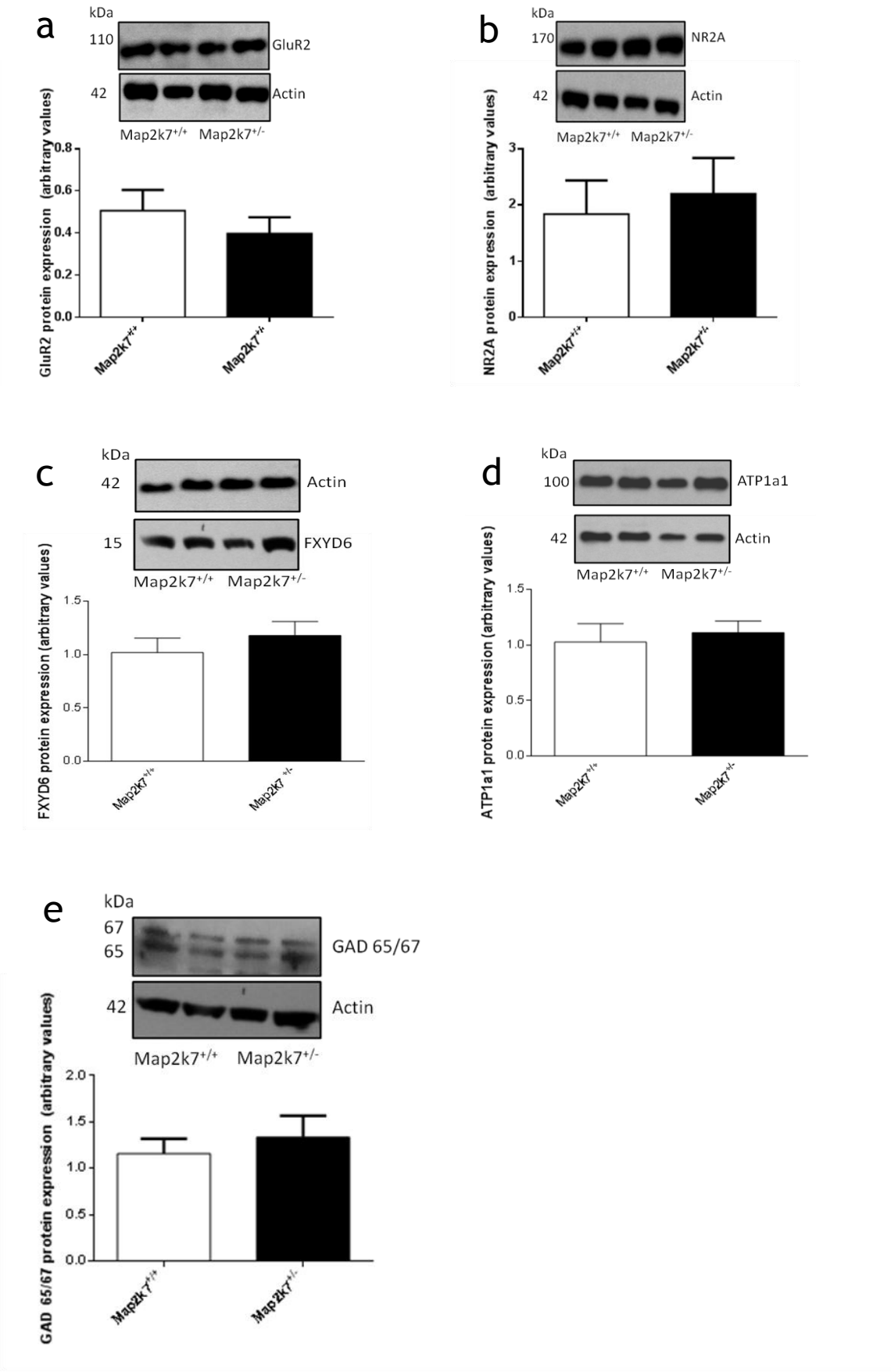


Figure 6.4 Western blotting analysis reveals *Map2k7* heterozygous disruption does not impact on the protein expression of a) GluR2 ($F_{1,7}$=2.61, p>0.05) b) NR2A ($F_{1,7}$=1.82, p>0.05) c) FXYD6 ($F_{1,7}$=0.29, p>0.05) d) ATP1a1 ($F_{1,7}$=0.09, p>0.05) e)GAD 65/67 ($F_{1,7}$=1.22, p>0.05). n=4

6.3.5 Map2k7$^{+/-}$ primary phenotype screen

Map2k7$^{+/-}$ mice (n=9) were not distinguishable from *Map2k7*$^{+/+}$ mice (n=7) in all parameters of the primary phenotype screen (Table 6.1). Neuromuscular function, as tested by hanging wire and motor coordination and balance, as tested by rotarod (refer to 2.10.1 & 2.10.2), were also unaffected by the heterozygous disruption of *Map2k7* (p>0.05, Table 6.1).

6.3.6 Map2k7$^{+/-}$ mice do not differ from Map2k7$^{+/+}$ in plus maze performance

In comparison to *Map2k7*$^{+/+}$ mice, *Map2k7*$^{+/-}$ mice did not appear to have increased levels of anxiety in the EPM (refer to 2.10.3). This was detected by no significant differences in zone duration ($F_{1,63}$=0.238 p> 0.05), zone entry frequency ($F_{1, 63}$=0.123, p> 0.05), distance travelled (T=3.84, p> 0.05) and mean velocity (T=3.29, p> 0.05) in the plus maze (Table 6.2) *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9.

Location	Phenotype	Genotype		Significance
		Map2k7$^{+/+}$	Map2k7$^{+/-}$	
In Arena	Body position	1 ± 0	1 ± 0	*p* >0.05
	Spontaneous activity	0 ± 0	0 ± 0	*p* >0.05
	Tremor	0 ± 0	0 ± 0	*p* >0.05
	Urination	0.5	0.7	*p* >0.05
	Defecation	1.4	1.2	*p* >0.05
	Bizarre behaviours	0 ± 0	0 ± 0	*p* >0.05
	Convulsions	0 ± 0	0 ± 0	*p* >0.05
	Palebral Closure	0 ± 0	0 ± 0	*p* >0.05
	Piloerection	0 ± 0	0 ± 0	*p* >0.05
	Gait	0 ± 0	0 ± 0	*p* >0.05
	Pelvic elevation	1 ± 0	1 ± 0	*p* >0.05
	Tail elevation	1 ± 0	1 ± 0	*p* >0.05
	Pinna reflex	0 ± 0	0 ± 0	*p* >0.05
Restrained	Cornea reflex	0 ± 0	0 ± 0	*p* >0.05
	Provoked biting	0 ± 0	0 ± 0	*p* >0.05
	Trunk curl	1 ± 0	1 ± 0	*p* >0.05
	Visual placing	1 ± 0	1 ± 0	*p* >0.05
	Hanging wire 1	55.7 ±4. 3	52.5- ± 5.2	*p* >0.05
	Hanging wire 2	49.7 ± 6.7	49.1 ± 5.3	*p* >0.05
	Hanging wire 3	59.4 ± 0.5	54.2 ± 5.7	*p* >0.05
	Hanging wire average	54.9 ± 3.3	52.0 ± 3.7	*p* >0.05
	Rotarod 1	96.7 ± 20.7	136.4 ± 17.6	*p* >0.05
	Rotarod 2	105.7 ± 17.8	122.8 ± 19.6	*p* >0.05
	Rotarod 3	132.1 ± 17.5	104.3 ± 17.2	*p* >0.05
	Rotarod average	111.5 ± 14.7	121.2 ± 12.6	*p* >0.05

Table 6.1 Summary of primary phenotype screen performed on mice heterozygous for the genetic disruption of *Map2k7* (*Map2k7*$^{+/-}$) and their wildtype littermates *Map2k7*$^{+/+}$ mice.

Heterozygous disruption of *Map2k7* did not significantly alter mice phenotype in parameters tested in the primary phenotype screen. Data is presented at mean ± SEM, *Map2k7*$^{+/+}$ n = 7, *Map2k7*$^{+/-}$ n=9. Data analysed by Student's t-test.

a

Genotype	Duration (seconds)				
	Centre	Closed arm	2/3rd open arm	1/3rd open arm	Significance
Map2k7$^{+/+}$	112.2 ± 33.3	132.1.7 ± 37.3	37.9 ± 22.5	17.12 ± 11.8	*p* >0.05, *ns*
Map2k7$^{+/-}$	118.2 ± 12.3	120.9 ± 15.6	44.8 ± 7.9	15.8 ± 6.2	*p* >0.05, *ns*

b

Genotype	Frequency				
	Centre	Closed arm	2/3rd open arm	1/3rd open arm	Significance
Map2k7$^{+/+}$	14.4 ± 2.2	9.9 ± 3.0	7.6 ± 2.9	1.6 ± 0.9	*p* >0.05, *ns*
Map2k7$^{+/-}$	23.9 ± 1.7	13.9 ± 1.6	12.9 ± 2.0	2.3 ± 0.8	*p* >0.05, *ns*

c

Genotype	Distance travelled (cm)	Mean velocity (cm/s)	Significance
Map2k7$^{+/+}$	961.0 ± 166.3	3.6 ± 0.3	*p* >0.05, *ns*
Map2k7$^{+/-}$	1302.6 ± 84.4	4.3 ± 0.3	*p* >0.05, *ns*

Table 6.2 *Map2k7*$^{+/-}$ mice do not display abnormal anxiety-related behaviours in the elevated plus maze, as indicated by no differences in performance in the following parameters

a) duration (seconds) ($F_{1,63}$=0.238 *p*> 0.05)

b) frequency of entering zones in the EPM ($F_{1, 63}$=0.123, *p*> 0.05) and c) Distance travelled (T=3.84, *p*> 0.05) and mean velocity (T=3.29, *p*> 0.05) travelled in all zones. Closed zone (non-aversive), 2/3rd open arm (mildly aversive), 1/3rd open arm (aversive) refer to 2.10.3) Data is presented as mean ± SEM. *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9. Duration and frequency analysed using ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Distance and mean velocity analysed by Student's t-test.

6.3.7Map2k7$^{+/-}$ mouse performance in open field with amphetamine challenge

Map2k7$^{+/-}$ mice did not show anxiety-like behaviours or hyperlocomotor activity in the open field test in comparison to *Map2k7*$^{+/+}$ mice, however slightly altered sensitivity to amphetamine was observed as described below.

Overall, genotype did not significantly affect the duration of time spent in zones during the habituation stage ($F_{,1,83}$=2.8, p>0.05) however it approached significance in the test stage ($F_{1,83}$=2.8, p=0.06). There was an overall effect of genotype on duration of time spent in each zone ($F_{1,83}$=4.07, p=0.02) and on further investigation, Tukey's *post hoc* analysis revealed *Map2k7*$^{+/-}$ mice spent significantly decreased duration of time in the thigmotaxis zone under saline treatment (p=0.02) Figure 6.5a). *Map2k7*$^{+/-}$ mice did not significantly differ from *Map2k7*$^{+/-}$ mice in velocity in both the habituation stage ($F_{1,179}$ =1.15, p>0.05) or testing stage ($F_{1,359}$=1.46, p>0.05) (Figure 6.5b). Administration of 3 mg/kg amphetamine significantly increases velocity in both genotypes ($F_{1,27}$=1.24, p<0.001), however there is no significant interaction between amphetamine and genotype ($F_{1,27}$=0.03, p>0.05, Figure 6.5b). Similar results were observed with distance travelled during testing stage, *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice did not differ from each other ($F_{1,39}$=0.32, p>0.05, Figure 6.6a), although the administration of 3mg/kg of amphetamine significantly increases the overall distance travelled for both genotypes ($F_{1,39}$=43.68, p<0.001 Figure 6.6a), the response was not influenced by genotype ($F_{1,39}$=0.17, p>0.05).

Comparing velocity to the mean baseline revealed an overall near significance effect of genotype ($F_{1,359}$=3.84, p=0.05), and the effect of amphetamine was influenced by genotype ($F_{1,359*}$=9.33, p=0.002, Figure 6.6b). Tukey's *post hoc* analysis revealed *Map2k7*$^{+/+}$ mice exhibited significantly increased velocity at time points 35 (p= 0.003), 40 (p=0.04) and 45 minutes (p=0.02), in comparison to *Map2k7*$^{+/-}$ following amphetamine treatment Figure 6.6b). *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9.

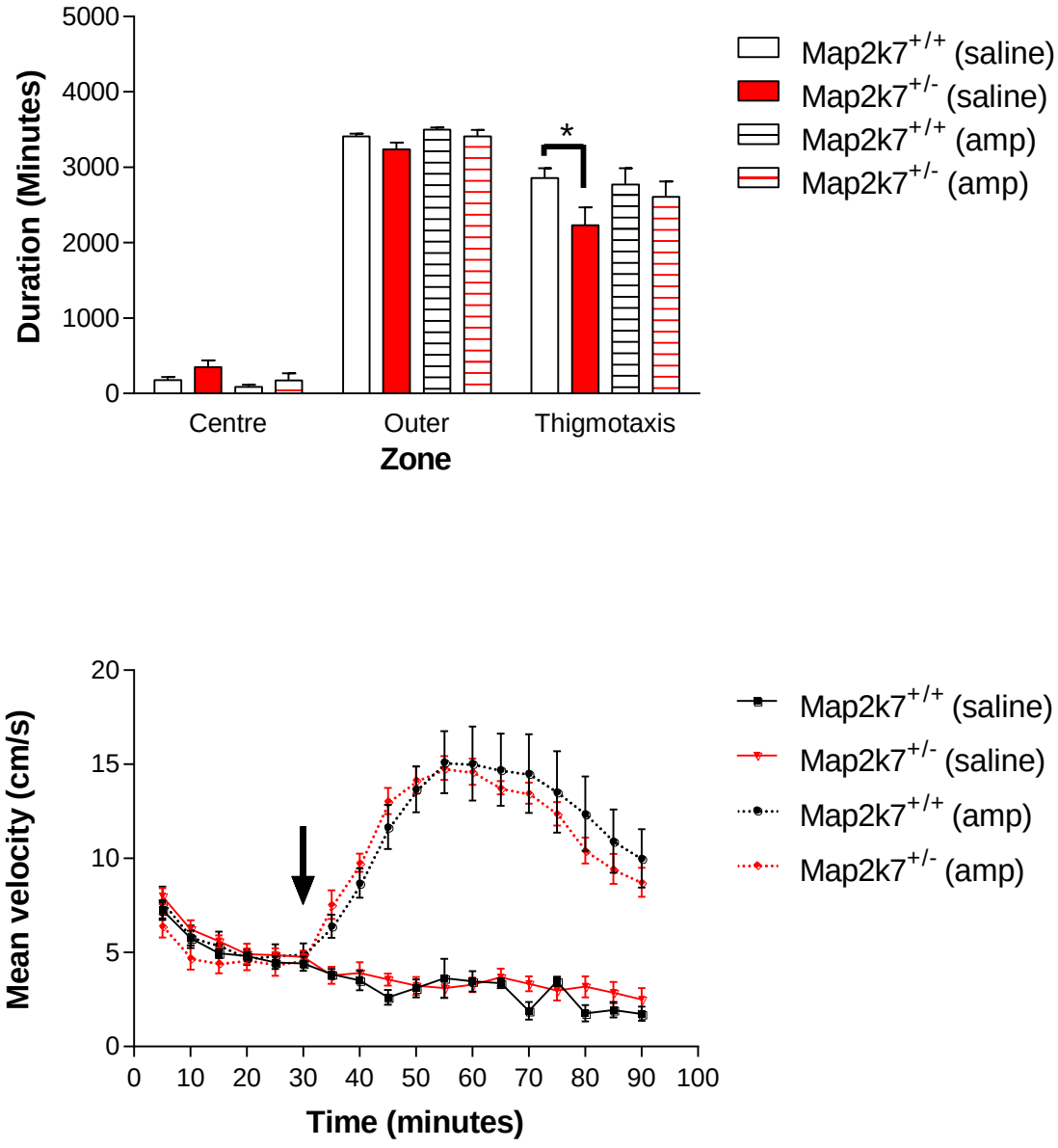


Figure 6.5 *Map2k7*$^{+/-}$ mice do not have altered locomotor activity however display decreased thigmotaxis in comparison to *Map2k7*$^{+/+}$ mice

a) Analysis of duration spent in centre, inner and outer zones of the open field arena reveal a significant interaction between genotype and zone duration ($F_{1,83}$=2.8, p=0.06) with *Map2k7*$^{+/-}$ mice spending less time in the thigmotaxis zone compared to *Map2k7*$^{+/+}$ mice during treatment with saline ($F_{1,119}$=1.08, p=0.02). Administration of 3mg/kg of amphetamine did not affect duration spent in zones for both genotypes ($F_{1,83}$=0.06, p>0.05.) n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9. *p=0.02 *Map2k7*$^{+/+}$ vs *Map2k7*$^{+/-}$

b) Mean velocity in *Map2k7*$^{+/+}$ mice and *Map2k7*$^{+/-}$ are both increased following the administration of amphetamine after a 30 minute habituation period ($F_{1,27}$=1.24, p<0.001). *Map2k7*$^{+/-}$ mice do not differ from their *Map2k7*$^{+/+}$ littermates in their response to amphetamine ($F_{1,27}$=0.03, p>0.05). n=10 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9. ↓ represents time of treatment administration

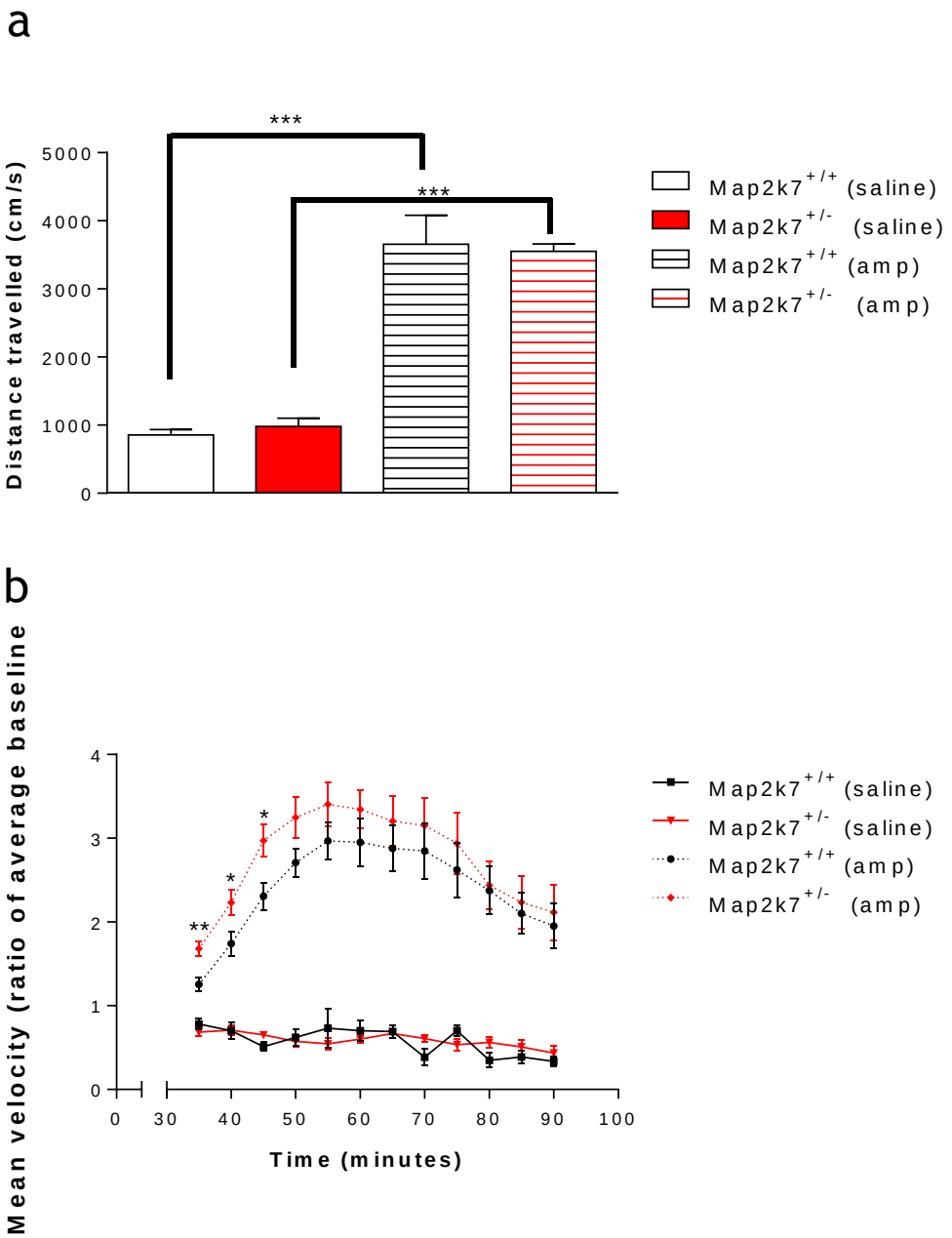


Figure 6.6 *Map2k7*$^{+/-}$ mice reveal altered sensitivity to amphetamine

a) Analysis of distance travelled following the administration of amphetamine or saline (time point 30 min) did not reveal a significance difference between *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice ($F_{1,39}$=0.32, p>0.05). Administration of 3mg/kg of amphetamine significantly increases the overall distance travelled ($F_{1,39}$=43.68, p<0.001), however the effect of amphetamine was not influenced by genotype ($F_{1,39}$=0.17, p>0.05). Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9. *p<0.001 amphetamine (amp) vs saline

b) Mean velocity as a ratio of average baseline in *Map2k7*$^{+/+}$ mice and *Map2k7*$^{+/-}$ are both increased following the administration of amphetamine (3 mg/kg) after a 30 minute habituation period ($F_{1,39}$=1.59, p<0.001), there is a near-significant overall effect of genotype ($F_{1,359}$=3.84, p=0.05) and a significant overall interaction between genotype and amphetamine ($F_{1,359*}$=9.33, p=0.002, Tukey's *post hoc* analysis revealed *Map2k7*$^{+/-}$ mice exhibited significantly increased velocity at time points 35 (p= 0.003), 40 (p=0.04) and 45 minutes (p=0.02), in comparison to *Map2k7*$^{+/+}$ following amphetamine treatment. *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9. *p<0.05, **p<0.01 significance observed between *Map2k7*$^{+/+}$ vs. *Map2k7*$^{+/-}$ with amphetamine (amp).

6.3.8 *Map2k7*$^{+/-}$ acoustic startle

Prior to conducting PPI, acoustic startle was examined to ensure *Map2k7*$^{+/-}$ mice do not exhibit deficits in hearing which may affect PPI results (refer to 2.10.5). Age-matched *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice were tested for their startle responses to a range of auditory stimuli (65 dB, 69 dB, 73 dB, 77 dB, 85 dB, 90 dB, 100 dB, 110 dB, 120 dB). Overall, the heterozygous targeted disruption of *Map2k7* did not significantly alter startle response in *Map2k7*$^{+/-}$ mice, compared to *Map2k7*$^{+/+}$ mice ($F_{[1, 89}$ = 2.20, p>0.05) suggesting that the lack of *Map2k7* does not affect hearing (Figure 6.7a). As predicted the size of auditory stimuli produces a decibel- dependent increase in startle amplitude ($F_{8,89}$=12.73, p<0.001) however is it not affected by genotype ($F_{8,89}$=0.76, p>0.05). N=5 per group.

6.3.9 *Map2k7*$^{+/-}$ mouse sensorimotor gating

Overall PPI results reveal *Map2k7*$^{+/-}$ mice do not significantly differ from *Map2k7*$^{+/+}$ mice, as described below.

Map2k7$^{+/-}$ mice do not have deficits in PPI, in comparison to *Map2k7*$^{+/+}$ mice ($F_{1,95}$=0.09, p>0.05). Ketamine and prepulse intensity both had an overall significant effect ($F_{1,95}$=6.43, p=0.01 & $F_{2,95}$=35.98, p<0.001 respectively) however effects were not influenced by genotype (genotype x treatment $F_{1,95}$=0.05, p>0.05, genotype x prepulse interaction $F_{2,95}$=1.16, p>0.05) (Figure 6.7b). *Post hoc* analysis did not identify which prepulse level were affected by ketamine (pp4 p= 0.54, pp8 p= 0.56, pp16 p=0.89). *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9.

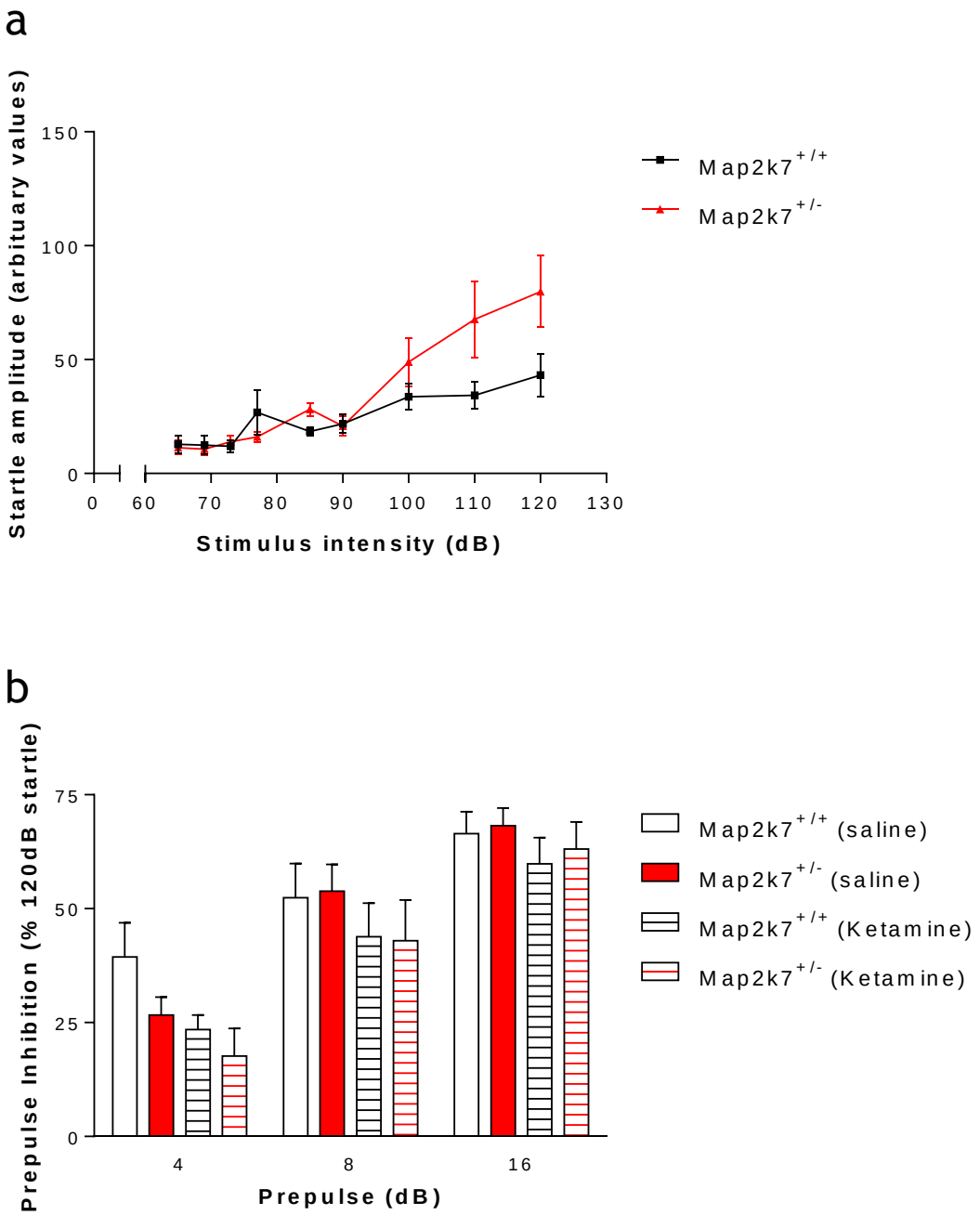


Figure 6.7 *Map2k7*$^{+/-}$ mice do not display hearing loss or sensorimotor gating deficits in comparison to *Map2k7*$^{+/+}$ mice.

a) Startle magnitude to auditory stimuli of 65 dB, 69 dB, 73 dB, 77 dB, 85 dB, 90 dB, 100 dB, 110 dB, 120 dB intensities did not differ between *Map2k7*$^{+/+}$ mice and *Map2k7*$^{+/-}$ mice ($F_{[1, 89}$ = 2.20, p>0.05) suggesting disruption of *Map2k7* does not impact on hearing. n=5 per group. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

b) Overall, prepulse inhibition of the startle response did not significantly differ between *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice ($F_{1,95}$=0.09, p>0.05). Ketamine (25 mg/kg) and prepulse both had an overall significant effect ($F_{1,95}$=6.43, p=0.01 & $F_{2,95}$=35.98, p<0.001 respectively) however there was no significant interaction between genotype and treatment ($F_{1,95}$=0.05, p>0.05) or genotype x prepulse interaction ($F_{2,95}$=1.16, p>0.05). *Map2k7*$^{+/+}$ n=7, *Map2k7*$^{+/-}$ n=9, data analysed using ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

6.3.10 *Map2k7*$^{+/-}$ mouse sociability and social novelty.

The heterozygous genetic disruption of *Map2k7* does not significantly impact on levels of sociability. A preference for social novelty was not observed in either *Map2k7*$^{+/+}$ or *Map2k7*$^{+/+}$ mice, as described below.

Map2k7$^{+/+}$ and *Map2k7*$^{+/-}$ mice both exhibit sociability as observed by prolonged duration of time spent exploring the chamber containing a strange mouse, in comparison to the empty chamber ($F_{2,29}$=39.13, p<0.001). The heterozygous genetic disruption of *Map2k7* does not significantly impact on levels of sociability ($F_{1,29}$=0.09, p>0.05) however there is a significant interaction between zone and genotype ($F_{2,29}$=3.33, p=0.05, Figure 6.8a).

Social novelty was investigated by assessing the duration of time spent investigating a novel mouse in this study (refer to 2.10.7), compared to the now familiar mouse. Genotype did not have a significant impact on duration ($F_{,29}$=0.23, p>0.05), however zone did have an overall impact ($F_{2,29}$=12.18, p<0.001), and there was an overall interaction between zone and genotype ($F_{2,29}$=4.07, p=0.03, Figure 6.8b). Tukey's *post hoc* analysis revealed neither *Map2k7*$^{+/+}$ or *Map2k7*$^{+/-}$ mice spend a longer duration of time interacting with the stranger mouse, compared to the now familiar mouse, suggesting social novelty is not present in these mice (p>0.05, Figure 6.8b). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=5, data analysed using ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

6.3.11 *Map2k7*$^{+/-}$ mouse sucrose preference

Anhedonia in *Map2k7*$^{+/-}$ mice was investigated using the sucrose preference test (refer to 5.3.7), results suggest the heterozygous deletion of *Map2k7*$^{+/-}$ mice does not cause significant levels of anhedonia. One *Map2k7*$^{+/-}$ mouse was removed from testing due to not displaying sucrose preference. *Map2k7*$^{+/-}$ mice were not significantly different from *Map2k7*$^{+/+}$ mice in levels of sucrose consumption over a 5 day period ($F_{1,44}$=1.23, p>0.05, Figure 6.9). There was a significant effect of day on the % of sucrose consumed ($F_{4,44}$=1.24, p=0.02) but not an impact of genotype on consumption of sucrose on individual days ($F_{4,44}$=0.49, p>0.05). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=4.

The overall average % sucrose consumed over the 5 day period was also not significantly affect by genotype ($F_{1,8}$=0.09, p>0.05, Figure 6.9). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=4.

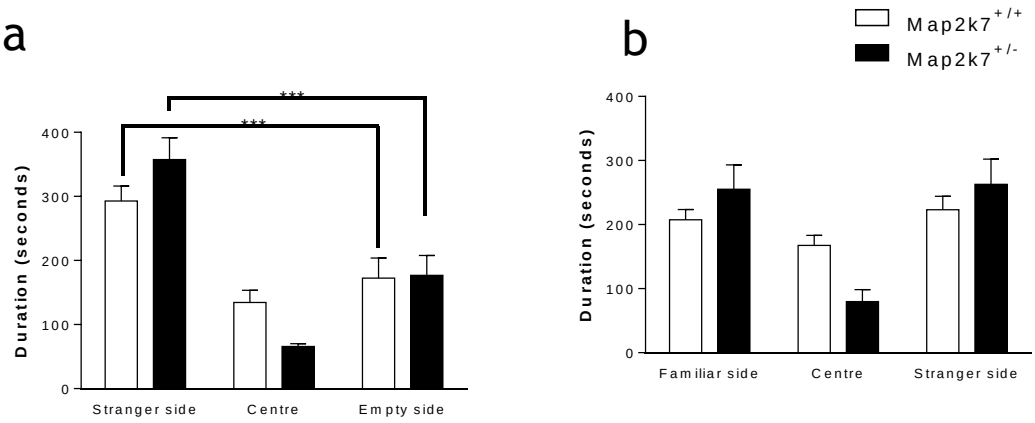


Figure 6.8 *Map2k7*$^{+/-}$ mice do not have deficits in sociability or suffer from anhedonia.

a) Levels of sociability; duration spent exploring and interacting with the stranger mouse in comparison to an empty chamber was used as an indication of mouse sociability levels. Both *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice showed levels of sociability ($F_{2,29}$=39.13, p<0.001). Genotype did not have an overall effect on levels of sociability ($F_{1,29}$=0.00, p>0.05), however there is a near significant interaction between zone and genotype ($F_{2,29}$=3.33, p=0.05). ***p<0.001

b) Preference for social novelty; There was an overall interaction between zone and genotype ($F_{2,29}$=4.07, p=0.03) however Tukey's *post hoc* analysis revealed that neither *Map2k7*$^{+/+}$ or *Map2k7*$^{+/-}$ mice spend a longer duration of time interacting with the stranger mouse, compared to the now familiar mouse, suggesting social novelty is not present in these mice (p>0.05). Genotype did not have an overall impact ($F_{,29}$=0.00, p>0.05). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=5.

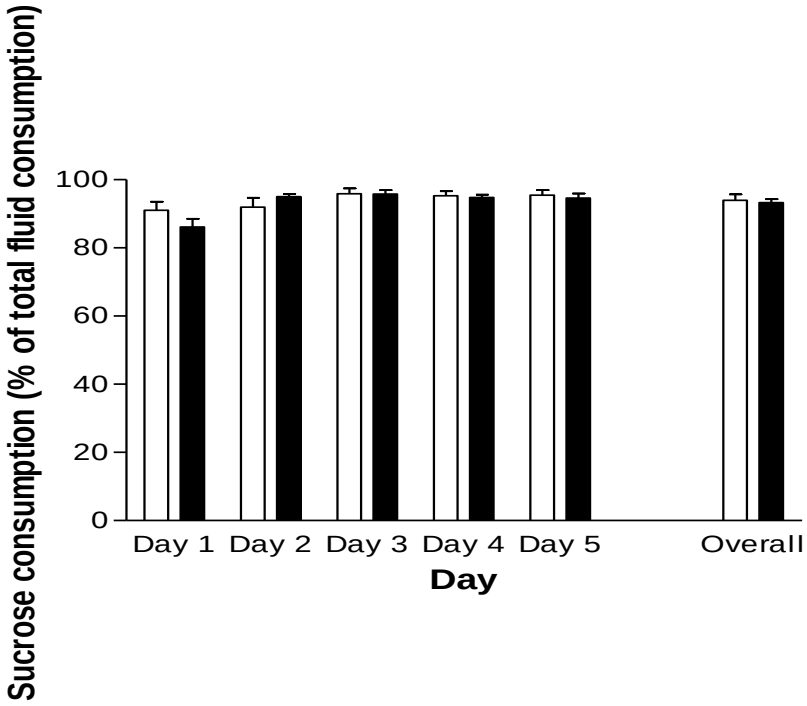


Figure 6.9 *Map2k7*$^{+/-}$ mice do not suffer from anhedonia.

Genotype did not have an overall impact on % of sucrose consumption during single days and in total over a 5 day period, revealing *Map2k7*$^{+/-}$ mice do not suffer from anhedonia ($F_{1,44}$=1.23, p>0.05), *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=4. Data analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

6.3.12 Map2k7$^{+/-}$ mouse performance in delayed non-match to sample

Results from the delayed non-match to sample T maze task (refer to 2.10.6) revealed that there was an overall effect of genotype on working memory ($F_{1,32}$=7.24, p=0.01). However *post hoc* analysis did not identify which delay the deficits occurred (Figure 6.10a) 5 seconds ($F_{1,10}$=2.31, p=0.22), 15 seconds ($F_{1,10}$=0.82, p=0.96 and 30 seconds ($F_{1,10}$=1.522, p=0.65). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=6.

Collapsing of the data in order to evaluate an average response, revealed that *Map2k7*$^{+/-}$ mice exhibit a trend towards reduced working memory overall ($F_{1,10}$=4.73, p=0.05 Figure 6.10b). *Map2k7*$^{+/-}$ mice also showed a significant increase in perseverative responding, in comparison to *Map2k7*$^{+/+}$ mice ($F_{1,10}$=13.29, p=0.005, Figure 6.10c) as measured by the number of 2 or more consecutive incorrect trials. *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=6.

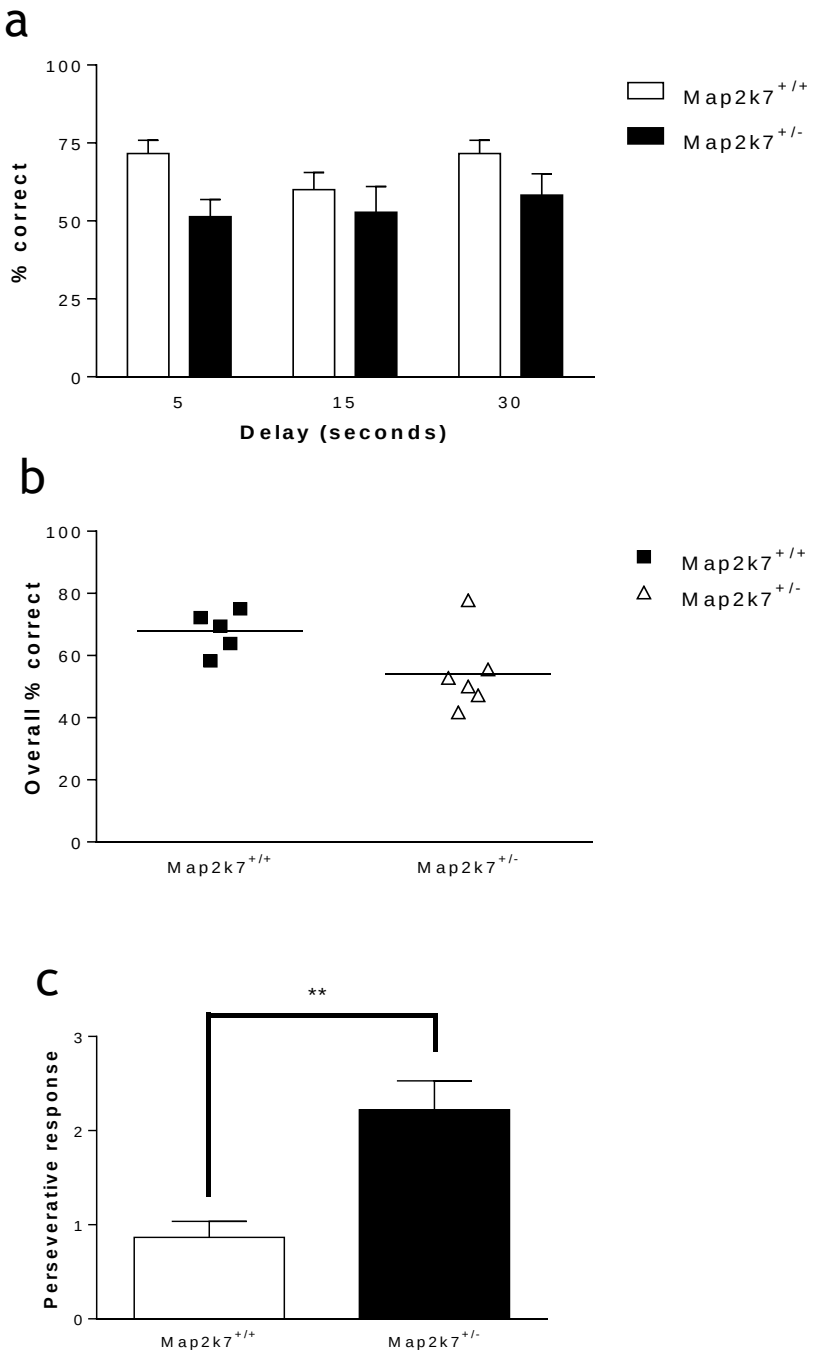


Figure 6.10 *Map2k7*$^{+/-}$ mice exhibit an overall deficit in working memory and increased perseverative errors in delayed non-match to sample test.

a) In comparison to *Map2k7*$^{+/+}$ mice, *Map2k7*$^{+/-}$ mice had an overall deficit in working memory ($F_{1,32}$=7.24, *p*=0.01) however no significant differences were observed at individual time delays, 5 seconds ($F_{1,10}$=2.31, *p*>0.05), 15 ($F_{1,10}$=0.82, p>0.05) and 30 seconds ($F_{1,10}$=1.522, *p*>0.05). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=6. Data analysed using ANOVA followed by Tukey's *post hoc* test for multiple comparisons

b) Overall % trials correct revealed *Map2k7*$^{+/-}$ mice have a trend towards reduced working memory ($T_{1,10}$=4.73, *p*=0.05). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=6, data analysed using Student's t'test. **p*=0.05

c) *Map2k7*$^{+/-}$ mice had significant increase in perseverative responding, in comparison to *Map2k7*$^{+/+}$ mice ($T_{1,10}$=13.29, *p*=0.005). *Map2k7*$^{+/+}$ n=5, *Map2k7*$^{+/-}$ n=6, data analysed using Student's t'test. ***p*<0.01

6.4 Discussion

6.4.1 Map2k7 is expressed in brain regions associated with schizophrenia

Qualitative assessment revealed *Map2k7* is expressed in regions of the mouse brain associated with schizophrenia; the prefrontal cortex, the granular cell layer of the olfactory bulb (GrO) and CA1 and dentate gyrus of the hippocampus. Altered prefrontal cortical function has been repeatedly implicated in the pathophysiology of schizophrenia. Decreased functioning and metabolism of the prefrontal cortex (hypofrontality) and dysfunction of prefrontal related tasks, such as working memory and decision making are associated with schizophrenia. The hippocampus, as part of the medial temporal lobe, is also a central region in the pathophysiology of schizophrenia, and is associated with tasks such as spatial working memory and learning. Connectivity networks between the prefrontal cortex and the hippocampus are thought to be responsible for many areas of cognition and memory consolidation, in particular working memory (Laroche *et al.*, 2000;Yoon *et al.*, 2008). The expression of *Map2k7* in these brain regions suggests a potential role in the underlying mechanisms of cognition and memory.

Animal models of genetic risk factors are likely to aid our understanding of the pathogenesis of schizophrenia, for this reason I utilised a mouse model containing heterozygous deletion of *Map2k7* to elucidate information on the functional role of MAP2K7 in schizophrenia. Homozygous deletion of murine *Map2k7* results in embryonic lethality (Wada *et al.*, 2004), suggesting *Map2k7* has a vital role in neurodevelopment; however the stage of development at which lethality occurs is unknown.

6.4.2 Mice heterozygous for Map2k7 disruption exhibit a trend towards reduced Map2k7 mRNA

RTqPCR detected a modest significant reduction of *Map2k7* RNA in *Map2k7*$^{+/-}$ mice, in comparison to *Map2k7*$^{+/+}$ mice. Primers designed to target exons 10/11 revealed a non-significant reduction of 20%, whilst primers designed to alternative splice exon 1a and b revealed a significant reduction of 32%. Exon b has been shown to have increased expression in subjects homozygous for

rs4804833; a *Map2k7*$^{+/-}$ SNP associated with schizophrenia (Winchester et al., 2012). It is important to use primers located at different loci to confirm that viral insertion disrupts the entire gene of interest and does not result in alternative transcripts.

In order to further investigate *Map2k7* expression in *Map2k7*$^{+/-}$ mice, RNA levels were quantified using *in situ* hybridisation technique. On average, a non-significant reduction of 12% *Map2k7* expression was observed. It is possible compensatory up-regulation of transcription from the single *Map2k7* allele has occurred. However, non-significance could also be due to the low 'n' number (n=3) used in this study, resulting in increased data variability and decreased statistical power. These findings are similar to those observed in other heterozygous mouse models that die early in development (Williamson et al., 2000;Graziotto et al., 2008), which suggest that if the homozygous knockout of a gene is embryonically lethal, the gene is likely to have an essential role in development and so compensatory up-regulation of related gene expressions will occur. The translation into protein was unable to be examined due to failed attempts at producing quantifiable protein results using western blotting, despite repeated attempts at optimisation of antibodies.

Wada et al., 2004 confirmed heterozygous expression of *Map2k7* in *Map2k7*$^{+/-}$ mice using northern and southern blotting which revealed a clear reduction in *Map2k7* genomic mRNA and DNA (respectively). These results combined with DNA and RNA analysis from this study suggest it is highly likely *Map2k7* has reduced expression in *Map2k7*$^{+/-}$ mice and thus are suitable to be used as a tool for investigating the functional properties of *Map2k7*.

6.4.3A trend towards Map2k7 reduced expression increases Grin1 but not Pvalb and Fxyd6 RNA expression

RTqPCR interestingly revealed that the heterozygous targeted genetic disruption of *Map2k7* significantly increases *Grin1* expression in the frontal cortex of *Map2k7*$^{+/-}$ mice. The *Grin1* gene encodes the NR1 (GluN1) subunit for the glutamatergic NMDA receptors (Sakurai *et al.*, 2000;Myers *et al.*, 1999). NMDA receptors play a crucial role in the glutamatergic excitatory neurotransmitter system and hypofunction of receptors are the basis of the glutamate hypothesis

of schizophrenia. Emerging evidence suggests a genetic role of *GRIN1* in schizophrenia. A genetic association has been found between *GRIN1* and schizophrenia (Georgi *et al.*, 2007;Galehdari, 2009;Qin *et al.*, 2005) and mice expressing 5% of normal levels of the *Grin1* subunit display behavioural abnormalities reflective of those associated with schizophrenia symptoms, such as elevated motor activity and stereotypy and deficiencies in social and sexual interactions, which were ameliorated by treatment with antipsychotics haloperidol or clozapine (Mohn *et al.*, 1999). It is possible that the up-regulation of GRIN1 expression is a compensatory mechanism employed to continue the normal functioning of synaptic transmission, following reduced expression of *Map2k7*. Interestingly, the NR1 and NR2B subunit of the NMDA receptor have been shown to have increased expression in *post mortem* schizophrenia brains (Grimwood et al., 1999;Clinton et al., 2006;Kristiansen et al., 2006;Dracheva et al., 2001), suggesting up-regulation of NMDA receptor subunits may be a feature of the neurobiology of schizophrenia. However, in order to fully confirm an association between *Map2k7* and *Grin1*, sample numbers would need to be increased as the significant interaction observed in this study was derived from an n of 3.

6.4.4 The modest reduction of Map2k7 does not impact on GLUR2, NR2A, GAD 65/67, FXYD6 and Na^+ K^+ ATPase protein expression

To investigate further the effect of heterozygous knockout of *Map2k7* on neurotransmission, GLUR2, NR2A and GAD 65/67 protein expression levels were investigated using the western blotting technique. The role of the NMDA glutamate receptor subunit NR2A (GluN2A) in the PFC in schizophrenia is not well characterised, however decreased expression of NR2A in parvalbumin neurons has been observed in the PFC of schizophrenia brains (Bitanihirwe et al., 2009). Nonetheless, NR2A protein expression was not altered in *Map2k7*$^{+/-}$ mice compared to *Map2k7*$^{+/+}$ mice, suggesting there is no compensatory regulation of the expression of these receptors, despite the increased expression of the NMDA receptor subunit *Grin1* as observed with RTqPCR (refer to 6.3.3). Furthermore, GLUR2 (GluA2), the key subunit of heteromeric glutamate AMPA receptors has altered expression in schizophrenia brains (Eastwood et al., 1995;Eastwood et

al., 1997), but is not altered in *Map2k7*$^{+/-}$ mice, suggesting signalling of these receptors is not altered by impairments in *Map2k7* expression.

GABAergic neurotransmission is also dysregulated in schizophrenia, glutamic-acid-decarboxylase (GAD), the enzyme which catalyses the synthesis of GABA from glutamate, has been found to be decreased in schizophrenia, however *Map2k7* disruption does not have a significant impact in expression in *Map2k7*$^{+/-}$ mice. It'd be interesting to carry out further analyses such as studies of expression of other related genes to further investigate whether *Map2k7* has an impact on GABAergic circuitry.

It is widely accepted that schizophrenia derives from the effects of multiple genes. Epistatic static interactions between *MAP2K7* and *FXYD6* in schizophrenia were investigated in a north European population (refer to Chapter 3), however interactions were not found be a risk factor. Lack of interaction between *Fxyd6* and *Map2k7* was further confirmed by no change in RNA or protein expression of FXYD6 and the Na^+ K^+ ATPase pump, which it co-localises with and modulates in *Map2k7* mice.

6.4.5Primary phenotype screen

The homozygous knockout of *Map2k7* results in embryonic lethality, which would suggest a role in neurodevelopment, however no abnormal neurological behaviours, such as spontaneous activity, aggressiveness, neuromuscular abnormalities, convulsions and general condition of coat and eye reflexes were detected in *Map2k7*$^{+/-}$ mice compared to *Map2k7*$^{+/-}$ mice.

From these results it was concluded that any phenotypes generated from subsequent behavioural tests designed to detect more complex behaviours, such as anxiety and sensorimotor gating, are more likely to be a true reflection of phenotype and not a result of poor motor skills or poor health.

6.4.6Anxiety-related behaviours in Map2k7$^{+/-}$ *mice*

Anxiety-related behaviours can be measured in a mouse model by using the innate natural tendency to explore a novel environment, compared to their conflicting preference for dark enclosed space (Lister., 1987). The elevated plus

maze and the open field apparatus exploit this conflict to indicate changes in levels of anxiety between transgenic or knockout mice and their wildtype controls. Mice with high levels of anxiety will avoid the bright open arms of the EPM and the centre of the open field arena, and spend more time in the dark arms or the corners and walls of the open field arena (thigmotaxis). *Map2k7*$^{+/-}$ mice did not show altered anxiety-related behaviours when compared to *Map2k7*$^{+/+}$ mice during the 5 minutes allowed to explore the EPM. However, in the open field arena, after 30 minutes (during the 'test stage'), *Map2k7*$^{+/-}$ mice appeared to spend less time in the thigmotaxis zone compared to *Map2k7*$^{+/+}$ mice. This could possibly indicate disinhibited behaviours, which are thought to be a symptom of 'disorganised schizophrenia' and are also present following drug abuse (Gawin., 1991;Fillmore *et al.*, 2003).

Impairments to the dopaminergic circuitry were explored in *Map2k7*$^{+/-}$ mice using administration of 3 mg/kg amphetamine. Surprisingly, administration of 3 mg/kg amphetamine did not produce an overall effect on duration spent in zones. 3 zones were introduced during open field analysis to fully assess anxiety-related behaviours. Centre zone represents the duration of time the mouse spent in the centre of the arena, indicating exploratory behaviour; the outer zone represents the time spent in the rest of the arena. The outer zone was further split to analyse thigmotaxis. Amphetamine resulted in hyperlocomotor activity in both *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice, a result commonly observed in mice (Ralph et al., 2001), validating the experimental approach. Amphetamine increases hyperlocomotor activity by increasing synaptic dopamine levels in the mesolimbic system (Costall et al., 1987;Pijnenburg et al., 1976), this can occur through a number of different mechanisms (refer to 1.10.1). The excess levels of dopamine result in the increased activation of dopamine D1 and D2 receptors, inducing hyperlocomotion (Sharp et al., 1987;Tenn et al., 2003;Mansbach et al., 1988;Pijnenburg et al., 1975).

In order to investigate fully the effect of amphetamine on velocity, the mean baseline for each individual mouse was taken across time points 5-30 minutes and velocity following amphetamine administration (35-90 minutes) was calculated. There was a significant overall effect of genotype and *Map2k7*$^{+/-}$ mice exhibited significantly increased velocity at time points 35, 40 and 45

minutes, in comparison to *Map2k7*$^{+/+}$ mice. Although this could suggest *Map2k7*$^{+/-}$ mice are more sensitive to amphetamine, implying a potential role of *Map2k7* in the dopaminergic signalling circuitry, further testing such as sensitivity to amphetamine in sensorimotor gating (Cilia *et al.*, 2005) or response to administration of antipsychotic drugs such as clozapine and haloperidol, which are antagonists at the dopamine receptor, would be required to confirm this (Swerdlow and Geyer., 1993).

6.4.7Sensorimotor gating in Map2k7$^{+/-}$ *mice*

Sensorimotor gating is a complex process involving multiple interactions with multiple neurotransmitters including dopamine, serotonin and glutamate (Ojima et al., 2004;Quednow et al., 2004;Egerton et al., 2008). Deficits in sensorimotor gating are a key symptom of schizophrenia and stem from impairments in inhibition in a number of regions in CNS. Imaging, electrophysiological investigations and animal studies have provided evidence for the role of a number of brain regions, such as the hippocampus (Swerdlow et al., 1995;Adler., 1982;Siegel et al., 1984) nucleus accumbens (Swerdlow et al., 1990;Wan et al., 1995) and the striatum (Kodsi and Swerdlow., 1994;Kretschmer and Koch., 1997), in the underlying mechanisms of impaired sensorimotor gating. These impairments result in the oversensitivity to sensory information and the inability to filter important sensory information from 'noise'. *Map2k7*$^{+/-}$ mice did not have deficits in hearing or sensorimotor gating, suggesting *Map2k7* does not play a role in the physiology of hearing or filtering of sensory inputs. The administration of ketamine attenuates sensorimotor gating in both *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice. However ketamine did not induce a significant difference between the genotypes. Ketamine exerts its action via inhibition of the NMDA receptor and is therefore used to explore impairments in glutamatergic signalling despite the up-regulation of the *Grin1* subunit in the PFC. These results suggests Map2k$^{+/-}$ mice do not have alterations in glutamatergic signalling. However it is difficult to conclude whether or not *Map2k7* is implicated in glutamatergic pathways due to the up-regulation of *Grin1* expression, which may be contributing to normal glutamatergic transmission.

6.4.8 Social interaction and social novelty in Map2k7$^{+/-}$ mice

Testing of mouse social behaviours has become a well-established method to study deficits in social interaction and social novelty in models of schizophrenia and autism (Lord et al., 2000;Aghevli et al., 2003;Wing and Gould, 1979). Various behaviours such as social communication, social novelty (social recognition), aggression and juvenile play can be investigated following analysis of behaviours between two mice. These mice could be familiar with one another, related, strangers or differ in sex. Social interaction can be assessed by the duration of time a mouse will spend investigating and interacting with a non-familiar mouse (termed stranger mouse) in an arena containing a cylindrical wire cage which permits visual, olfactory and tactile interaction.

In this study, sociability was defined as the tendency to spend a longer duration of time in the zone containing the stranger mouse, in comparison to the empty zone. Both Map2k7$^{+/+}$ and *Map2k7*$^{+/-}$ mice displayed sociability, however disruption of *Map2k7* did not induce deficits in sociability. Preference for social novelty is interpreted to have occurred when the test mouse spends more time investigating a novel mouse, in comparison to a familiar mouse. In this study, mice were familiarised to the first stranger mouse during sociability testing, a novel mouse was then entered into the 'empty' zone to test preference for social novelty. Interestingly, neither *Map2k7*$^{+/+}$ or *Map2k7*$^{+/-}$ mice displayed a preference for social novelty. The genetic background of the *Map2k7*$^{+/+}$ and *Map2k7*$^{+/-}$ mice is C57BL/6 and in our laboratories has been backcrossed onto the substrain C57BL/6J rcchsd strain. Studies have shown substrains of C57BL/6 do not differ in social interaction levels from one another (Matsuo *et al.*, 2010), furthermore both strains have previously shown a preference for social novelty (refer to Chapter 5 (Matsuo *et al.*, 2010;Moy *et al.*, 2004). In Moy *et al., (2004)* study, 'n' numbers are substantially larger compared to those used in this study (n=20, n=5, respectively), and thus statistical power may not be high enough to have detected social novelty reliably in the present experiments.

6.4.9 Anhedonia

The inability to experience pleasure (anhedonia) is associated with the negative symptoms of schizophrenia (refer to 1.3.2, (Strauss and Gold., 2012;Dowd and

Barch., 2010;Pizzagalli., 2010). Mice have a natural preference for sweet sucrose water over standard water, anhedonia can be detected between wildtype and knockout/transgenic mice by a reduction in the ratio of sucrose water to standard water consumption (Papp et al., 1991). A heterozygous mouse was removed from this study as it did not show any preference for sucrose water over standard water during the 5 days. It could be argued that this is a phenotype of *Map2k7* heterozygous deletion, however the rest of the group (n=4) all showed sucrose preference. Analysis of sucrose consumption as a ratio of total fluid intake revealed *Map2k7*$^{+/-}$ mice do not suffer from anhedonia as tested in this model.

6.4.10 Working memory

Working memory can be assessed using a T maze-based 'delayed non-match to sample' task (refer to 2.10.6). The task requires the retention and manipulation of short term memory in order to perform executive functions (Aultman and Moghaddam., 2001). Working memory requires synchrony between the prefrontal cortex and hippocampus (Sigurdsson *et al.*, 2010;Yoon *et al.*, 2008) and from the expression pattern of *Map2k7* in these regions, it was hypothesised *Map2k7*$^{+/-}$ mice may present impairments in working memory. Collapsing the data revealed that *Map2k7*$^{+/-}$ mice have a working memory impairment in comparison to *Map2k7*$^{+/+}$ mice and on further investigation it was found these impairments derived from increased perseverative errors. In this study, perseverative errors were defined as a mouse not correcting its behaviour following an incorrect trial, leading to at least two consecutive incorrect trials in a row. An increase in perseverative errors has been postulated to be a result of impairments in dopamine in the PFC (Yawata *et al.*, 2012;Pezze *et al.*, 2006;Zahrt *et al.*, 1997) . Moreover, hypofunction of the dopamine D1 receptor has been associated with cognitive deficits and working memory impairments in schizophrenia (Castner et al., 2000;Abi-Dargham et al., 2002;Granon et al., 2000), effects which are not responsive to treatment with typical antipsychotic drugs (Masahiko & Michio., 1997). However, studies have shown that short term treatment using D1 agonists can improve cognitive deficits (Castner et al., 2000;Cai and Arnsten., 1997). It may therefore be of interest to investigate the role of D_1 receptors in the perseverative responding of Map2k$^{+/-}$ mice using D_1 agonists, particularly

considering the trend towards sensitivity to amphetamine, which suggests a potential altered dopaminergic circuitry in these mice.

Due to the modest impairment in working memory, it is difficult to conclude whether *Map2k7*$^{+/-}$ mice have impairments in the manipulation and retention of short term memory and executive functioning. Unusually, during this task there was not an overall affect of duration of retention delay during this task. In working memory tasks, as retention delays are increased, mice would be expected to have decreased correct trials; however this was not observed in both genotypes. This could be concealed from variability in performance stemming from low 'n' numbers. Another possibility for the lack of clear deficits associated with increasing delays could be gender, however male mice only were used in the 'working memory' task due to female mice presenting difficult in reaching criteria (personal correspondence), this could be due to a number of factors affecting learning behaviour, such as circadian rhythm, menstrual cycle, environmental factors such as stimulating housing toys, littermates etc. It is therefore important that during working memory testing, and similar tasks, environmental factors such as housing conditions and handler are kept constant.

6.5 Conclusion

The molecular and phenotypic characterisation of *Map2k7*$^{+/-}$ mice revealed *Map2k7* is expressed in brain regions associated with schizophrenia. The heterozygous deletion of *Map2k7* results in alteration of glutamate receptor *Grin1* expression, a receptor reported to have altered expression in schizophrenia. Similarly, *Map2k7*$^{+/-}$ mice display some phenotypes similar to those reported in schizophrenia, such as working memory deficits, increased perseverative responding, and sensitivity to amphetamine.

As *Map2k7*$^{+/-}$ mice did not exhibit deficits in PPI, social behaviours or neurochemical deficits in GABAergic markers, the functional role of *MAP2K7* as a risk factor in schizophrenia still remains tenuous, however these results do suggest a putative role of *MAP2K7* in impairments in glutamatergic neurotransmission and a potential rodent model of cognitive impairments.

Chapter 7. Discussion.

The overall aims of this PhD study were to investigate the genetic and functional roles of two genes recently associated with schizophrenia: *FXYD6* and *MAP2K7*. A genetic association between *FXYD6* and schizophrenia in a Northern European population was not confirmed in this study, or a role of epistatic interactions between *FXYD6* and *MAP2K7* in the risk of schizophrenia. Despite this, the molecular and phenotypic characterisations of two strains of mice, one homozygous for disrupted *Fxyd6* and one heterozygous for *Map2k7,* reveals an interesting insight into the roles of these genes in the neuronal circuitry associated with schizophrenia and related psychiatric disorders. Furthermore, the phenotypes of these mice could provide potential rodent models for looking at certain aspects of cognition.

7.1 FXYD6 as a risk factor in schizophrenia

This study did not find an allelic or haplotypic association between *FXYD6* and schizophrenia in a north European population. A proportion of the samples analysed derived from a subset of samples from a previous study, in which a genetic association between *FXYD6* and schizophrenia was found (Choudhury et al., 2006; Choudhury et al., 2007). It is well known that difficulty can arise when attempting to confirm a positive association between a gene and a disorder and the lack of replication could be due to a number of reasons such as the generation of false-positive results in the Choudhury studies or factors such as low sample size and statistical power, or the potential presence of rare but highly penetrant alleles. For these reasons, despite not confirming an association between *FXYD6* and schizophrenia, a role of *FXYD6* in susceptibility to schizophrenia was not discarded and instead further explored using a mouse model homozygous for disrupted *Fxyd6*.

Until now, the molecular role of FXYD6 was unknown. This study not only utilised the *in situ* hybridisation technique to provide further evidence of the expression of *Fxyd6* in the schizophrenia-related brain regions, the PFC and the hippocampus, but also found expression of both *Fxyd6* and Na^+ K^+ *ATPase* in the hippocampus; a region involved in synaptic plasticity and learning and memory. In addition, this study confirms for the first time that, like other members of the

FXYD family, FXYD6 is a tissue-specific modulator of the Na^+ K^+ ATPase pump. This is particularly interesting as alterations to the Na^+ K^+ ATPase pump activity have been associated with psychiatric disorders whilst mice lacking the Na^+ K^+ ATPase α2 and α3 subunits both exhibit increased anxiety-related behaviour and impaired learning and memory where as alpha1 isoform mice showed increased locomotor in response to methamphetamine (Moseley et al., 2007).

The role of FXYD6 in neurochemical pathways associated with schizophrenia was also explored, however no evidence of a role of FXYD6 in glutamatergic signalling was found at the molecular level, as observed by no change in glutamatergic receptor expression. However increased expression of GABAergic interneuron marker, and parvalbumin expression in *Fxyd6*$^{-/-}$ mice suggests that FXYD6 might interact with GABAergic neuronal activity. To investigate neurotransmission pathways further in *Fxyd6*$^{-/-}$ mice, amphetamine and ketamine were both used to explore impairments in the dopaminergic and glutamatergic pathways (respectively) and their effects on locomotor activity. *Fxyd6*$^{-/-}$ mice do not exhibit altered sensitivity to ketamine or amphetamine, despite this, subtle deficits observed in prepulse inhibition suggests potential deficits in neurotransmission may be present.

Due to the expression of *Fxyd6* in the PFC and the expression of both *Fxyd6* and *Na^+ K^+ ATPase* in the hippocampus, as observed by *in situ* hybridisation, it was hypothesised that memory and learning may be disrupted in *Fxyd6*$^{-/-}$ mice. This was explored by looking at impairments in working memory. Working memory is a key symptom associated with the cognitive deficits of schizophrenia (Goldman-Rakic., 1994;Manoach., 2003), and an important treatment target in schizophrenia, as an effective treatment is yet to be discovered. The *Fxyd6*$^{-/-}$ mice displayed deficits in working memory at delays of 5 seconds, this is encouraging to further investigate *Fxyd6*$^{-/-}$ a potential mouse model of cognitive disorders and provides further evidence for a role of FXYD6 dysfunction in the aetiology of schizophrenia.

7.2 *MAP2K7* as a risk factor in schizophrenia.

A recent genetic association study and an expression study have both implicated *MAP2K7* as a risk factors in the susceptibility to schizophrenia (Winchester et al., 2012); however this study reveals *MAP2K7* does not interact with *FXYD6* to increase the risk to susceptibility in a north European population.

The brain expression of *Map2k7* was previously unknown, however this study utilised the *in situ* hybridisation technique to show expression in regions associated with schizophrenia, including the PFC and the hippocampus. The role of *MAP2K7* in the susceptibility to schizophrenia was explored using mice heterozygous for the disruption of *Map2k7*. The homozygous disruption of *Map2k7* is embryonically lethal. RTqPCR and *in situ* hybridisation techniques were used to assess the effect of heterozygous disruption of *Map2k7* RNA and protein levels. A modest but significant reduction was observed in the RTqPCR data; however this was not observed using *in situ* hybridisation. This could be due RTqPCR being a more sensitive technique, compared to *in situ* hybridisation. Wada et al., 2004 have confirmed heterozygous expression of *Map2k7* in *Map2k7*$^{+/-}$ mice using northern and southern blotting which revealed a clear reduction in *Ma2pk7* genomic mRNA and DNA (respectively). These results combined with analysis from this study suggest it is highly likely *Map2k7* has reduced expression in *Map2k7*$^{+/-}$ mice.

These mice were also used to confirm the lack of interaction between *Fxyd6* and *Map2k7* which was evidenced by no change in RNA or protein expression of FXYD6 and the Na^+ K^+ ATPase pump in *Map2k7*$^{+/-}$ mice compared to Mapk7$^{+/+}$ mice.

The heterozygous deletion of *Map2k7* results in alteration of glutamate receptor *Grin1* RNA expression, a receptor reported to have altered expression in schizophrenia. Furthermore, *Map2k7*$^{+/-}$ mice display increased perseverative responding in the working memory task and sensitivity to amphetamine, suggesting alterations in dopaminergic circuitry. Despite this, *Map2k7*$^{+/-}$ mice did not exhibit deficits in PPI, social behaviours or neurochemical deficits in GABAergic markers, and so the functional role of *MAP2K7* as a risk factor in schizophrenia still remains unclear.

7.3 Future 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.

Despite this study providing molecular and behaviour characterisations of *Fxyd6* and *Map2k7,* their roles in susceptibility to schizophrenia still remain ambiguous. It would be interesting to investigate hypofrontality in both mouse strains. Hypofrontality has been associated with deficits in working memory and is observed in schizophrenia brain (Berman et al., 1988;Carter et al., 1998;Glahn et al., 2005). As both strains of mice exhibit impairments in components of the working memory task it would be interesting to carry out 2 deoxyglucose experiments to assess whether there is altered metabolism in the prefrontal cortex (Weinberger et al., 1986, Callicott et al., 2000, Manoach et al., 2000). Further behaviour tasks aimed to investigate learning and memory in these strains would also be interesting in order to investigate fully their roles as models of cognition. Executive memory could be investigated using attentional set-shifting task, or spatial learning could be assed using the Morris water maze.

Following the increased expression of *Pvalb* in *Fxyd6*$^{-/-}$ mice, it would be interesting to investigate the role of FXYD6 in GABAergic neurotransmission activity further. Initially, it would also be interesting to investigate further the regions where FXYD6 and parvalbumin are both expressed to see if co-localisation occurs at cellular level. RTqPCR, *in situ* hybridisation and western blot techniques could also be used to investigate the expression of GABA receptors in *Fxyd6*$^{-/-}$ mice. It would also be appealing to investigate serotonin and related neurotransmisson pathways in these mice in a similar manner, in order to elucidate reason for the subtle alterations observed in PPI. The levels of brain-expressed FXYD7 would also be fascinating to investigate to assess if expression is up-regulated in order to compensate for reduced modulation of the Na^+ K^+ ATPase pump by FXYD6.

It would be interesting to investigate further the glutamatergic signalling system in the *Map2k7*$^{+/-}$ mice. This could be done by investigating the expression of further NMDA receptor subunits, and by exploring the effects of PCP and/or

ketamine on processes such as locomotor activity. Furthermore, using western blotting technique, it would be beneficial to investigate the effect of heterozygous *Map2k7* disruption of the expression of the downstream target, JNK2 and related members of the MAPK cascade.

These studies would further investigate the functional roles of both *Fxyd6* and *Map2k7* genes in the neuropathology of schizophrenia and provide further evidence to determine if they would be a suitable model of schizophrenia-related symptoms

7.4 Conclusions

These studies provide an invaluable insight into the molecular functions of FXYD6 and MAP2K7, and their potential roles in schizophrenia.

A genetic association between *FXYD6* and schizophrenia in a Northern European population was not confirmed in this study, or a role of epistatic interactions between *FXYD6* and *MAP2K7* in the risk of schizophrenia. Despite this, this study reveals *Fxyd6* is expressed in regions of the brain associated with schizophrenia and the targeted disruption of *Fxyd6* results in prefrontal cortex and hippocampal- related behaviour deficits, such as deficits in working memory. FXYD6 may also have a potential role in GABAergic signalling, as observed by increased *Pvalb* expression in *Fxyd6*$^{-/-}$ mice. FXYD6 does not appear to have a role in glutamatergic signalling as evidenced by the unaltered expression of glutamate receptors and normal response to ketamine in the open field task. However indication of deficits in neurotransmission in *Fxyd6*$^{-/-}$ mice comes from subtle deficits in prepulse inhibition.

I have also shown for the first time that FXYD6 modulates brain Na^+ K^+ ATPase activity in the frontal cortex.

The molecular and phenotypic characterisation of *Map2k7*$^{+/-}$ mice revealed *Map2k7* is also expressed in brain regions associated with schizophrenia and display some phenotypes similar to those reported in schizophrenia, such as working memory deficits, increased perseverative responding, and sensitivity to amphetamine.

The heterozygous deletion of *Map2k7* also results in alteration of glutamate receptor *Grin1* expression, suggesting a potential role in glutamatergic signalling however a role in GABAergic neurotransmission was not found.

The functional roles of FXYD6 and MAP2K7 as risk factors in schizophrenia still remains equivocal, however these results provide evidence of a putative role for both genes in some areas of the underlying neuronal activity associated with schizophrenia and associated psychiatric disorders. Furthermore, results from this study suggest both strains of mice are potential rodent models of cognitive impairments.

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