Effects of ENU mutations of Zfp804a on behavioural phenotypes

By

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Summary

Genetic variation in the gene *ZNF804A* has been shown to be related to risk for psychopathology, especially schizophrenia and bipolar disorder. The main aim of this thesis was to characterise the behavioural effects of *Zfp804a*, the mouse orthologue of *ZNF804A*, in order to understand more about how this gene influences brain and psychological functioning, and hence provide clues as to its possible role in mediating risk for mental disorders.

Prior to this work, two ENU-mutant mouse lines had been generated from a DNA library of ENU mutagenised mice with two non-synonymous mutations selected as viable candidates for further investigation. The C59X mutation encodes a premature stop codon in exon 2, thought to lead to a functional null of the gene, and the C417Y mutation is missense, substituting cysteine for tyrosine in exon 4. A first series of experiments examined the early development of the ENU-mutant lines and showed no gross developmental abnormalities, although the C59X mutants weighed significantly less than their WT littermate controls at weaning and during adulthood. A comprehensive series of behavioural tests then assayed aspects of emotion, motivation, hedonia, sensorimotor gating and response control. In general, the C59X mutants showed the greatest effects, displaying reduced anxiety, anhedonia, and sensorimotor gating deficits, together with evidence of enhanced response inhibition. The C417Y mutants only showed selective effects in terms of enhanced motivation.

The data dissociate between the effects of the two ENU-induced mutations of *Zfp804a*. Furthermore, the findings with the C59X mutants would suggest, *a priori*, that genetic variance leading to alterations in ZNF804A expression may be an important mechanism contributing to risk for psychopathology.

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List of abbreviations

5-HT	Serotonin
ACC	Anterior cingulated cortex
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
ASR	Acoustic startle response
ASU	Arbitrary startle units
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
Ca.	*Circa*/approximately
CM	Condensed milk
CNV	Copy number variant
CRF	Continuous reinforcement
CRF2	Continuous reinforcement for 2 days after PR
DAT	Dopamine transporter gene
dB	Decibel
ddNTPs	Di-deoxy-dinucleotide-triphosphates
DLPFC	Dorsolateral prefrontal cortex
dNTP	Deoxy-ribonucleotide-triphosphates
DZ	Dizygotic
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
EPM`	Elevated plus-maze
eQTL	Expression quantitative trait loci
EZM	Elevated zero-maze

fMRI	Functional magnetic resonance imaging
GABA	γ-Aminobutyric acid
Go-RT	Go reaction time
GWAS	Genome-wide association study
H_2O	Water
HPC	Hippocampus
IVF	*In vitro* fertilisation
LCA	Lick cluster analysis
LH	Limited hold
LI	Latent inhibition
LMA	Locomotor activity
MAM	Methylazoxymethanol acetate
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
MRC	Medical research council
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MZ	Monozygotic
NaCl	Sodium chloride
NMD	Nonsense mediated decay
NMDA	N-methyl-D-aspartate
qPCR	Quantitative polymerase chain reaction
OCD	Obsessive compulsive disorder
OF	Open field
PCP	Phencyclidine
PCR	Polymerase chain reaction

PET	Positron emission tomography
PFC	Prefrontal cortex
PPI	Prepulse inhibition
PR	Progressive ratio
PRT	Progressive ratio task
PTSD	Post-traumatic stress disorder
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROI	Regions of interest
RT	Reaction time
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Stretch-attend postures
SEM	Standard error of the mean
SHIRPA	SmithKline, Beecham, Harwell, Imperial College, Royal London Hospital Phenotype Assessment
SNP	Single nucleotide polymorphism
SPSS	Statistical package for social sciences
SSRT	Stop-signal reaction time
SSRTT	Stop-signal reaction time task
TE	Tris-EDTA
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultra-violet
VS	Ventral striatum
WT	Wild-type

Contents

Chapter I; General introduction

Schizophrenia is a complex and severely debilitating disorder, with significant medical, societal and economic impacts (Tandon, Keshavan & Nasrallah, 2008). Due to its high incidence in the population, its pervasive nature and its relative resistance to treatment, the World Health Organisation (WHO) has rated schizophrenia as one of the top ten leading causes of disease related disability worldwide (2001), with an estimated societal cost in England of £6.7 billion in 2004/2005 (Mangalore & Knapp, 2007), and an estimated direct economic burden of $62.7 billion in the USA in 2002 (Wu, Birnbaum, Shi et al., 2005). The worldwide median prevalence rate of schizophrenia is estimated to be 15.2 in 100,000 people (Aleman, Kahn & Selten, 2003; McGrath, Saha, Welham, et al., 2004; McGrath, Saha, Chant et al., 2008). Males are more likely to suffer with the disorder than females; the male to female rate ratio (median) is of the order of 1.4:1 (McGrath et al., 2004), with men often having both an earlier age of onset and a more serious and chronic form of the disorder (with poorer outcomes) than women (Jablensky, 2000). Urban living increases the risk of schizophrenia (Pedersen & Mortensen, 2001), as does migration (McGrath et al., 2004; Saha, Chant, Welham & McGrath, 2005).

Schizophrenia not only impacts on the affected individual due to the wide array of disabling symptoms, but also at both a family and broader societal level, as a result of social isolation, poverty and homelessness. These problems are often exacerbated by the residual prejudice, discrimination and stigma attached to the disorder. Despite the high economic and social costs of schizophrenia, and the large amount of research conducted (~5000 publications/year, Tandon et al., 2008) the aetiology and pathophysiology of the disorder still remain largely unknown. At the broadest level, schizophrenia is thought to arise from a complex interaction between environmental factors and biological predispositions, most likely genetic in nature, that impact, in particular, on developmental factors that have multiple effects across diverse brain areas and circuits in the adult.

1.1 Early concepts and more recent thinking

Schizophrenia, as a discrete diagnosis, has been an ever changing concept over the last 100 years. Before the turn of the 20th century, 'psychosis' was thought of as a unitary construct, with all forms viewed as variations of one major disease process. In 1919 the physician Emil Kraepelin (1856-1926) divided psychosis into two distinct forms; 1) affective psychosis (described in modern terms as bipolar disorder) and 2) dementia praecox (1919). Affective psychosis included short term episodic emotional problems which were eventually followed by full remission of symptoms, whereas dementia praecox was seen as a progressive decline in cognitive function, an organic disease of the brain, and regarded as incurable by Kraepelin (Andreasen, 2011). The psychiatrist Eugen Bleuler disagreed with this diagnosis, reformulating dementia praecox as schizophrenia ('split mind'), refuting the idea that it was a type of dementia involving mental decline, but instead suggesting that thought disorder was the key symptom alongside deficits in cognition and emotion (Andreasen, 2011).

The 'Kraepelinian dichotomy' marked the start of the classification of mental disorders based on commonality of symptoms, paving the way to a more clinical approach to mental illness. The dichotomy to this day is a subject of debate, and even though the diagnostic boundary between bipolar disorder and schizophrenia has remained in the clinic, some argue that viewing them as distinct disorders may be hampering research and even clinical care (Craddock & Owen, 2005; Craddock & Owen, 2010). This is in part due to a number of patients presenting with both major mood and psychotic symptoms, but also, crucially, due to the recent emergence of evidence suggesting a shared genetic aetiology between schizophrenia and bipolar disorder (Craddock & Owen, 2005) and increased risks of both disorders that increases with family relatedness (Lichtenstein, Yip, Björk, et al., 2009).

Single nucleotide polymorphisms (SNP) within certain schizophrenia candidate genes revealed in large sample genome-wide association studies (GWAS) also seem to suggest a genetic overlap between schizophrenia and bipolar disorder (Moskvina, Craddock, Holmans, et al., 2009). For example, the SNP in the gene encoding *Zinc finger protein 804A* (*ZNF804A*) which showed robust association with risk for schizophrenia, was found to have an increased association signal when bipolar disorder cases were added to the sample (O'Donovan, Craddock, Norton et

al., 2008). The emerging biological data from the latest generation of genetic studies indicates strongly that the nosology of psychiatric disorders, including schizophrenia and bipolar, should be viewed more on a dimensional basis than as a dichotomy, and that investigations into psychiatric disorders should be aware of the possibility of shared aetiology at the level of biological mechanism(s).

1.2 Diagnosis of schizophrenia and clinical features

In the UK, diagnoses of patients with schizophrenia and bipolar disorder are currently conducted using a combination of the American Psychiatric Association's DSM 5th edition (DSM-V) and the International Classification of Diseases (ICD-10, by the WHO). These manuals lay out a number of criteria for a diagnosis of schizophrenia, which includes the presence of 2 or more of the following symptoms; delusions, hallucinations, disorganised speech, grossly disorganised or catatonic behaviour and negative symptoms such as blunted affect, alogia, reduced pleasure and motivation. In addition to these medical symptoms, the person must also show occupational/social dysfunction, with these disturbances persisting for at least 6 months, including a minimum of 1 month of medical symptoms. A diagnosis of schizophrenia will be ruled out if the person presents with symptoms of bipolar disorder (i.e. persistent bouts of mania/depression), a pervasive developmental disorder (such as autism) or if the symptoms are directly the result of another medical condition or substance.

Schizophrenia is often characterised by a loss of contact with reality, with abnormal perception of external stimuli including the occurrence of delusions and hallucinations. Symptoms of schizophrenia have been grouped into 3 categories; positive, negative and cognitive. Positive symptoms are those which represent an exaggeration of normal function; symptoms which a healthy individual would not experience, including hallucinations, delusions, disorganised or catatonic behaviour and disordered speech. Negative symptoms are characterised by attenuation or lack of function normally seen in healthy individuals, including blunted affect, avolition, poverty of speech and anhedonia (Arango & Carpenter, 2011). Cognitive symptoms include impaired working memory, poor attentional capabilities and overall reduced executive functioning (Arango & Carpenter, 2011).

1.2.1 Positive symptoms

Positive symptoms are those which represent an exaggeration or distortion of normal function. Delusions occur in 90% of schizophrenia patients, with the most common type being delusions of persecution (Cutting, 2003). Hallucinations can occur in any modality, with 50% of patients experiencing auditory hallucinations, 15% visual and 5% tactile (Arango & Carpenter, 2011). Often auditory hallucinations appear in the form of voices talking to the patient or among themselves (Arango & Carpenter, 2011). Other examples of positive symptoms include disorganised thought, manifesting in speech disturbance, tangentiality (failing to get to the point of the conversation), thought blocking (loss in their train of thought) and perseveration (the repetition of an idea) (Arango & Carpenter, 2011). Anti-psychotic drugs are most effective in treating these positive symptoms. When modelling schizophrenia in animals, it is accepted that the positive symptoms are especially challenging, as many cannot be (convincingly) measured, such as hallucinations and delusions. However, sensorimotor effects such as pre-pulse inhibition (PPI) and latent inhibition (LI) have been successfully modelled in animals for many years (Desbonnet, Waddington & O'Tuathaigh, 2009). Sensorimotor functioning as indexed by changes in acoustic startle reactivity and the low level attentional sensorimotor gating phenomenon of prepulse inhibition (PPI) was employed in the current experimental work examining N-ethyl-N-nitrosourea (ENU)-induced mutant lines targeting the mouse orthologue of *ZNF804A, Zfp804a* (detailed in Chapter VI).

1.2.2 Negative symptoms

Negative symptoms, characterised by attenuation or lack of function normally seen in healthy individuals, including blunted affect, avolition, alogia and anhedonia, contribute in a major way to adverse functional outcome and quality of life in schizophrenia patients (Rabinowitz, Levine, Garibaldi et al., 2012). They often present before positive symptoms, and can persist after the positive symptoms have subsided (Arango & Carpenter, 2011). Unlike the positive symptoms of schizophrenia, the treatment of negative symptoms is often unsuccessful (Kirkpatrick, Fenton, Carpenter & Marder, 2006). One possible reason for this may be that the negative symptoms are similar to personality traits, which may be viewed as

more stable and enduring, and less influenced by environmental factors (An der Heiden & Häfner, 2011). Obviously, several of the negative symptoms such as poverty of speech are impossible to model in animals, and indeed negative symptoms in general, though clearly important, have proved difficult to recapitulate in animal models (O'Tuathaigh, Kirby, Moran & Waddington, 2010). One of the main aims of this thesis was to examine emotional functioning, and anhedonia and motivation, using valid approaches in *Zfp804a* mutant mouse lines (Chapters IV and V).

1.2.3 Cognitive deficits

Cognitive impairments have long been recognised as a major part of the presentation of schizophrenia (Arango & Carpenter, 2011). However, despite the clear evidence linking cognitive deficits such as poor working memory, reduced attentional capabilities and diminished executive function to schizophrenia, impairments in cognition still do not form part of the DSM-IV criteria for a diagnosis of the disorder. Lower quality of life and poor functional outcome in patients have been associated with severity of cognitive impairment, perhaps due to the fact that, as with most negative symptoms, there are still no viable drug treatments for cognitive deficits (Green, Kern, Braff and Mintz, 2000).

Cognitive impairments in schizophrenia encompass most functional domains and patients with schizophrenia generally perform worse than healthy controls in almost every cognitive task used (Heinrichs and Zakzanis, 1998). Cognitive deficits may be stable over time (Rund, 1998; Saykin, Shtasel, Gur et al., 1994), and even premorbid, with patients showing lower IQ in childhood and adolescence (Woodberry, Giuliano & Seidman, 2008). However, IQ changes may be transient, with reductions only at the onset of the illness and a gradual reinstatement of premorbid levels through the course of the disease (van Winkel, Myin-Germeys, Delespaul, et al., 2006). Unlike the positive and negative symptoms seen in schizophrenia, cognitive deficits can, arguably, be modelled more easily in animals, investigating domains such as attention, impulsivity, working memory and social cognition (Arguello & Gogos, 2010) with reference to standardised assessment scales such as the Measurement and Treatment Research to Improve Cognition in

Schizophrenia (MATRICS, Nuechterlein, Green, Kern, et al., 2008) or Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia (CNTRICS, Carter & Barch, 2007). These batteries incorporate a number of different tasks, which have mostly been translated for use with primates and rodents, assessing dissociable cognitive functions: e.g. Impulse Control, Speed of Processing, Attention/Vigilance, Working Memory, Verbal Learning and Memory, Visual Learning and Memory, Reasoning and Problem Solving, and Social Cognition (Nuechterlein et al., 2008). Abnormalities in response control are increasingly recognised as an important component of a number of psychiatric disorders (Newman, Widom, & Nathan, 1985; Heerey, Robinson, McMahon & Gold, 2007; Humby & Wilkinson, 2011) and there is some evidence supporting the utility of monitoring impulsivity as a possible biomarker for schizophrenia (Barch, Braver, Carter et al., 2009). Consequently, we pursued this novel angle in the experimental work reported in Chapter VII, examining response control in *Zfp804a* mutant lines in a recently developed murine version of the stop-signal reaction time task (SSRTT, Humby, Eddy, Good et al., 2013).

1.3 Neurobiological substrates of schizophrenia

The neurobiology of schizophrenia remains obscure. Unlike neurodegenerative disorders such as Alzheimer's disease, there are no obvious neuropathological signs of schizophrenia (Harrison, Lewis & Kleinman, 2011). With no known reliable and robust biomarkers for the disorder, treatment options at best alleviate the symptoms with little disease modifying efficacy. Understanding the aetiology and pathogenesis of schizophrenia are key requirements for improved diagnosis and the development of safe and effective therapeutics.

1.3.1 Neuropathology of schizophrenia

Despite the controversial, but initially well supported, statement that there is no neuropathology of schizophrenia, some progress has been made in this area of research. The first major findings based on meta-analyses have been that schizophrenia patients have a reduced brain volume of around 4% (Wright, Rabe-Hesketh, Woodruff & David, 2000) and a 3% reduction in brain weight (Harrison,

Freemantle & Geddes, 2003). Post-mortem studies of schizophrenia patients have shown enlarged lateral ventricles (Brown, Colter, Corsellis et al., 1986; Pakkenberg, 1987), decreased temporal lobe volume (Bogerts, Meertz & Schönfeldt-Bausch, 1985; Brown et al., 1986) and decreased thalamic volume (Pakkenberg, 1990; 1992). Other evidence has shown increased cell packing density in the dorso-lateral prefrontal cortex (DLPFC, Daviss & Lewis, 1995; Selemon, Rajkowska & Goldman-Rakic, 1995; 1998). A potential confound of any neuropathological finding is that antipsychotic drug treatments may contribute, and even cause some, if not all, of the neuropathology observed in the disease. However, studies investigating chronic treatment of non-human primates with antipsychotic drug agents, failed to produce some of the pathologies seen in schizophrenia such as increased cell packing density in the DLPFC, although the study did not examine the full range of schizophrenia neuropathology (Selemon, Lidow & Goldman-Rakic, 1999).

The neuropathological causes of schizophrenia are thought to be either neurodegenerative or neurodevelopmental in nature (Harrison et al., 2011). If neurodegenerative, we would expect to find the hallmarks of these types of disorders, such as neurofibrillary tangles, gliosis or neuronal and synaptic loss gradually increasing over time. However, none of these signs are consistently observed in the schizophrenic brain. On the other hand, the only neuropathological support for a neurodevelopmental hypothesis of schizophrenia remains the lack of findings that actually negate a neurodevelopmental aetiology; some studies describe hippocampal neuron disarray, but without replication of these types of findings, the neurodevelopmental hypothesis of schizophrenia remains unconfirmed (Harrison et al., 2011), although indications of risk in early life also provide support for this hypothesis (See Section 1.4).

1.3.2 Neurochemistry in schizophrenia

The dopamine hypothesis of schizophrenia posits, essentially, that the disorder is caused by an excess of dopamine. This idea stems from the observation that all of the current antipsychotics are D_2 dopamine receptor antagonists. Laruelle and colleagues (1996) provided further support for this hypothesis by demonstrating that acute psychosis could be induced in drug-free schizophrenia patients through

increased striatal dopamine release, bought on by an amphetamine challenge. There is also post-mortem evidence for increased density of striatal D_2, D_3 and D_4 receptors (Abi-Dargham & Grace, 2011), however, many post-mortem and positron emission tomography (PET) studies are unable to rule out potential antipsychotic drug effects (Keshavan, Tandon, Boutros & Nasrallah, 2008).

The excess striatal dopamine associated with the positive symptoms of schizophrenia has been well replicated (Laruelle, Abi-Dargham, Gil et al., 1999; Harrison, 1999a), however, the neurobiological correlates of the cognitive and negative symptoms of schizophrenia are more complex (Keshavan et al., 2008), and have led to a reformulation of the dopamine hypothesis to account for these non-psychotic symptoms. One explanation that has been put forward is that there is a deficit of dopamine in the cortex, which is thought to cause hypo-stimulation of D_1 receptors (Keshavan et al., 2008). This idea that cortical dopamine deficits cause the cognitive symptoms of schizophrenia has been further supported by a clinical study demonstrating that high DLPFC D_1 receptor availability was associated with poorer performance in patients with schizophrenia on a working memory task (Abi-Dargham, Mawlawi, Lombardo et al., 2002) as well as an animal model showing that induced dopamine depletion in the pre-frontal cortex produced cognitive impairments (Abi-Dargham & Grace, 2011). As a result of these findings, it is currently thought that the cognitive and negative symptoms of schizophrenia can be explained by an underactive mesocortical dopaminergic system and that the positive symptoms are due to an over-active mesolimbic dopamine system (Weinberger, 1987).

Serotonin (5-hydroxytryptamine, 5-HT) is another neurotransmitter that has been linked to schizophrenia, originally due to observations of the similarity between serotonin and the hallucinogenic drug lysergic acid diethylamide (LSD), which caused users to experience psychotic-like episodes comparable to schizophrenia (Iqbal & van Praag, 1995). Consequently it was proposed that schizophrenia was due to a 5-HT deficit, although this was later revised, as it was discovered that LSD could be both 5-HT antagonist and agonist, hence both a decrease in serotonin and an increase could produce psychotic like symptoms (Iqbal & van Praag, 1995). The strongest evidence for the role of serotonin in the pathogenesis of schizophrenia is

the fact that atypical antipsychotics such as risperidone and clozapine (Keshavan et al., 2008), show greater affinity for 5-HT-$_{2A}$ receptors in comparison to their affinity for D_2 receptors, potentially working as serotonin antagonists rather than dopamine antagonists; relieving the negative symptoms, as well as the more treatment resistant forms of schizophrenia (Moghaddam & Krystal, 2004). Despite this, direct evidence for serotonergic dysfunction in schizophrenia pathogenesis is lacking, but a potential interaction between serotonin and dopamine remains promising (Keshavan et al., 2008).

The neurotransmitter glutamate has also been implicated in schizophrenia due to the initial observations of reduced glutamate in the cerebrospinal fluid of schizophrenia patients (Kim, Kornhuber, Schmid-Burgk et al., 1980). This theory was supported by the finding that the drug phencyclidine (PCP), an NMDA (*N*-methyl-D-aspartate) antagonist, produces both the positive and negative symptoms of schizophrenia in healthy individuals and exacerbates the symptoms in patients with schizophrenia. Glutamate neurotransmission is the principal method for excitatory communication in the brain, leading to the theory that a deficit in glutamate transmission is responsible for at least part of the pathogenesis of schizophrenia (Krystal & Moghaddam, 2011). Support for this hypothesis comes from pharmacological studies using glutamate transmission agonists to alleviate negative symptoms of schizophrenia (Coyle, Darby, Flood et al., 1996), and from post-mortem analysis of the brains of schizophrenia patients showing reduced expression of glutamate receptors in brain regions such as the prefrontal cortex and the hippocampus (Keshavan et al., 2008).

It is not likely that one neurotransmitter can explain the complete pathogenesis of schizophrenia. What is needed is a better understanding of the interactions between neurotransmitters and how aberrant signals may contribute to some of the mechanisms underlying this disorder.

1.3.3 Structural and functional neuroimaging in schizophrenia

Modern imaging techniques make it possible to visualise the brain, confirming the brain abnormalities seen in patients with schizophrenia from earlier post-mortem studies. Magnetic resonance imaging (MRI) studies measuring regions

of interest (ROI) in the brains of patients with schizophrenia have discovered areas which are more reduced in volume than the brain as a whole (Lawrie, Johnstone & Weinberger, 2004). Findings include a 5-10% reduction in the volume of the PFC and temporal lobes (Lawrie & Abukmeil, 1998; Wright et al., 2000), a 10% reduction in the volume of the amygdala (Wright et al., 2000), and a 6% reduction in both the left and right hippocampi (Wright et al., 2000). Lateral and third ventricle sizes are thought to be increased by as much as 30% in cases with schizophrenia as compared to controls (Lawrie & Abukmeil, 1998; Wright et al., 2000). Variations in structures such as the basal ganglia are thought to be due to the effects of antipsychotic medication, as basal ganglia volume correlates with first episode antipsychotic dose and regresses on second generation treatment (Lawrie & Pantelis, 2011). The Edinburgh high risk study followed young unaffected relatives of schizophrenia patients in Scotland, with a known family history of schizophrenia (Johnstone, Cosway & Lawrie, 2002). Imaging results found that these high risk subjects, in comparison to controls, had a 4% reduction in their amygdala-hippocampal complex, perhaps suggesting that a reduction in medial temporal lobe structures could be a risk marker for schizophrenia (Lawrie et al., 2004). Whole brain gray matter volumes are also reduced by approximately 3-4% in schizophrenia patients, particularly in temporal lobe structures like the hippocampus, amygdala and the superior temporal gyri (Lawrie & Abukmeil, 1998). White matter tract abnormalities have also been found, with whole brain white matter reduced by approximately 2% (Wright et al., 2000), with specific reductions found in the corpus callosum of patients with schizophrenia, as well as their relatives (Keshavan et al., 2008).

Positron emission tomography (PET) scans use radioactive-labelled reagents to measure metabolism (as indexed by glucose levels), ratio of receptors (occupied at a ligand) and blood flow (Meyer-Lindenberg & Bullmore, 2011), with findings indicating elevated D_2 and D_3 receptor density in schizophrenia patients in the PFC, hippocampus and anterior cingulate cortex regions (Vyas, Patel, Nijran et al., 2010). PET imaging studies on patients who were currently experiencing auditory hallucinations revealed increased blood flow in the thalamus, right straitum, left hippocampus, parahippocampus, orbitofrontal, and cingulate areas (Shenton, Dickey, Frumin & McCarley, 2001).

Alongside the findings of structural neuroimaging, functional magnetic resonance imaging (fMRI) can be used to relate potential brain abnormalities to cognitive states and behavioural symptoms. Functional MRI uses blood oxygenation levels as an index of brain activity and has been used to assess the activation patterns of schizophrenia cases and controls at baseline and whilst performing various cognitive challenge paradigms. Using these methodologies, schizophrenia patients have shown hypoactivation, as compared to controls, of the DLPFC during a selective attention task (MacDonald & Carter, 2003 and a working memory task (the n-back test) (Minzenberg, Laird, Thelan et al., 2009). The DLPFC and ventro-lateral PFC were also hypoactivated during the encoding and retrieval of episodic memories, while the left pre-central and right medial frontal gyrus were hyperactivated (Achim & Lepage, 2005). Emotional regulation has also been investigated using imaging techniques. Research has shown hypoactivation of the amygdala when schizophrenia patients (as compared to controls) are presented with emotional stimuli versus neutral stimuli (Aleman & Kahn, 2005). The fMRI investigations into the neurocognitive functions of *ZNF804A* are discussed in detail in Section 1.6.3.

In summary, there is good evidence for structural and functional brain changes in schizophrenia, including in relation to possession of risk alleles of the *ZNF804A* gene, as considered in more detail below, (see section 1.7.4), however, the effects are often subtle and the precise implications of the imaging findings remains an issue.

1.4 Environmental risk factors

Current thinking has it that the aetiology of schizophrenia involves a complex mix of risk factors, of which environmental influences are thought to play a substantial part. As mentioned earlier, migrancy has been shown to increase the risk of developing schizophrenia (Pedersen & Mortensen, 2001; McGrath et al., 2004). Although the reasons for this remain unclear, some have suggested that the incidence rates may be raised due to ethnic disadvantage (the relative deprivation of resources and rewards as a result of being a given race), as opposed to migrancy *per se*; a hypothesis supported by the increased vulnerability to schizophrenia among

the Afro-Caribbean population living in England (Wessely, Castle & Murray, 1991; Castle, Wessely, Der & Murray, 1991). Additionally, it is likely that severe disruptions to the social fabric of life also play a role, especially in first generation migrants (van Os, Kennis & Rutten, 2010). Individuals living in urban areas also have an increased incidence of schizophrenia, perhaps due to the stress of overcrowding and environmental pollutants (McGrath et al., 2008).

Early life trauma, such as obstetric difficulties, including premature birth, foetal growth retardation, foetal hypoxia and prenatal complications are all thought to increase the risk of developing schizophrenia in adulthood (Clarke, Harley & Cannon, 2006). There has also been considerable interest in the impact of malnutrition on the developing foetus, with one ecological study examining the effect of the Dutch Hunger Winter (during World War II) on the development of individuals who were *in utero* during this famine, finding that those individuals had higher rates of schizophrenia and schizophrenia spectrum disorders than the general population (Susser & Lin, 1992). Serum taken from pregnant mothers has also suggested a possible link between low vitamin D levels during the 3rd trimester and increased incidence of schizophrenia (McGrath, Eyles, Mowry et al., 2003b).

Prenatal infections are also thought to confer risk of schizophrenia, originally examined in the context of Winter-borne viruses, but the evidence is inconsistent (McGrath & Murray, 2011). Some studies have found links between the incidence of schizophrenia and non-affective psychosis and maternal exposure to the herpes simplex virus type 2 (Buka, Tsuang, Torrey et al., 2001) and rubella (Brown, Cohen, Greenwald et al., 2000a), although again these findings have not been replicated. Adult exposure to infectious agents such as *Toxoplasmosis gondii* and its links to schizophrenia have been better replicated, with meta-analyses showing that cases with schizophrenia have an increased presence of antibodies to *Toxoplasmosis gondii* (Torrey, Bartko, Lun et al., 2007), although it is as yet unknown whether the presence of these antibodies came before the onset of the illness (and were causative) or contributed to the disease process already in place (McGrath & Murray, 2011).

The precise neurodevelopmental routes to psychopathology in adulthood remain to be established but are likely to involve, at least in part, interactions with genetic background.

1.5 The genetic architecture of schizophrenia

1.5.1 Genetic epidemiology

It has long been recognised that schizophrenia clusters among relatives of patients with schizophrenia to a greater extent than in the general population. The first published systematic study into the heritability of schizophrenia was conducted by Rudin in 1916, who found that schizophrenia was more common in the siblings of affected individuals than in the general population (Riley & Kendler, 2011). This was later extended by Kallman (1938) who found that the offspring of probands also displayed an increased risk of developing schizophrenia (Riley & Kendler, 2011). Much has been learnt about the heritability of schizophrenia from twin studies of monozygotic (MZ) and dizygotic (DZ) twins. The most recent meta-analysis of twin study data found a consistent genetic effect with higher concordance rates among MZ (41-65%) than DZ (0-28%) twins, and a heritability estimate of 80-85% (Cardno & Gottesman, 2000).

Adoption studies have attempted to further the investigation into schizophrenia heritability by examining offspring of probands adopted away from their biological parents, thereby attempting to more definitely separate environment effects from shared genes. The Finnish adoptive family study of schizophrenia looked at adopted-away offspring of mothers with schizophrenia versus adopted away offspring of control mothers. Of the children whose mothers had schizophrenia, 9.1% went on to develop a schizophrenia spectrum disorder and 4.9% went on to develop schizophrenia, compared to only 1.1% of the control offspring who went on to develop schizophrenia (Tienari, 1991), providing further strong evidence for a major genetic contribution to schizophrenia. The methods of uncovering the specific genetic variation at the sub-microscopic level conferring risk for schizophrenia fall into two main approaches; linkage and association, with a third approach involving the study of large chromosomal abnormalities assessed using cytogenetic methods.

1.5.2 Linkage studies

'Mendelian' genetic illnesses are caused by mutations in a single gene (or limited number of genes) (e.g. Huntington's disease). These types of illnesses are comparatively rare, and are in large part passed on faithfully down the generations (unless the mutation is lethal prior to reproduction); these illnesses can also arise spontaneously by *de novo* mutations. Linkage analysis maps functionality of genes to their location on chromosomes. As DNA segments lying near to each other on a chromosome tend to be inherited together, markers are often used as tools for tracking the inheritance pattern of a gene that has not yet been identified but whose approximate location is known. Through this genetic mapping, families where some have the disorder and some don't can be used to identify loci associated with that disorder. Typically, linkage analysis allows rare variants of large effect to be discovered, and has proved a fruitful tool for schizophrenia research, with many putative candidate genes discovered this way, such as *DISC-1* (St Clair, Blackwood, Muir et al., 1990), *Neuregulin 1* (Stefansson, Sigurdsson, Steinthorsdottir et al., 2002)*, and DTNBP1* (Straub, Jiang, MacLean et al., 2002)*.* However, replication of these findings is difficult and as yet no specific risk alleles in these genes have been identified and replicated successfully.

1.5.3 Association studies

Association studies examine genetic variation between populations of individuals with a diagnosed disorder and non-affected controls without the disorder. If one allele is more frequent in people with the disease, the SNP is said to be "associated" with the disease. The associated SNPs are then thought to highlight a region of the genome which influences the risk of disease. The association of the SNP can be explained by one of two possibilities: either that the SNP is directly associated with the disease and has functional relevance, or that the SNP is indirectly associated with the disease through linkage disequilibrium. Recent advances in genotyping technology have meant that high throughput sequencing of whole genomes is possible, leading to the development of genome wide association studies (GWAS). Here, almost 1 million DNA variants can be assessed in a single experiment, providing genome wide coverage of possible genetic variants between cases and

controls. For many common diseases, GWAS have transformed our knowledge of genetic susceptibility by identifying putative causal alleles, often of relatively small effect. It should be emphasised that, despite many of the discovered variants from GWAS having little effect on overall risk burden, functional studies examining their mechanism(s) of action and the pathways they interact with can be highly illuminating in terms of pathogenesis and options for treatments (Willer & Mohlke, 2012).

Early GWAS for schizophrenia failed to implicate specific loci, probably due to small sample sizes (O'Donovan & Owen, 2011). One of the more recent genome wide association studies (discussed in detail in section 1.7.1) utilised a much larger sample and was successful in identifying loci associated with a diagnosis of schizophrenia, with the greatest association stemming from a SNP in the gene *ZNF804A,* an association that, crucially, has been replicated several times (O'Donovan et al., 2008). Schizophrenia genetics was further enhanced by publications in 2009 of three more GWAS, each containing more than 2000 cases and almost 14000 controls. These studies, stemming from three different consortia, combined their datasets enabling meta-analysis, giving almost 13000 cases and 34000 controls (Stefansson, Ophoff, Steinberg, et al., 2009). Four loci emerged as genome wide significant, with 2 mapping to chromosome 6, including the *major histocompatibility complex* (*MHC*) region (P = 1.4 x 10^{-12}), one loci is adjacent to *neurogranin* (*NRGN*) (P = 2.4 x 10^{-9}), and the other is located in an intron of *transcription factor 4* (*TCF 4*) (P = 4.1 x 10^{-9}).

There are limitations with association studies, including the fact that low frequency variants are often not tagged, and even when they are detected they require much larger samples to gain equivalent effect sizes (O'Donovan & Owen, 2011). There is also the fact that there are no biomarkers for schizophrenia, and as such the samples of patients included may be heterogeneous, and findings may therefore be ambiguous (O'Donovan & Owen, 2011). The hope is that some of these limitations, especially in terms of molecular resolution, can be overcome by 'next generation' sequencing methods. However, whilst there is much research activity in this area going on, at the time of writing there were no substantial published reports.

1.5.4 Structural abnormalities

Structural genomic variation occurs in the form of duplications, deletions, sequence inversion and translocation. Copy number variants (CNVs) are structural variants that are at least 1000 bases in size and change the number of copies of a given portion of DNA sequence. Velo-cardio-facial syndrome (VCFS) involves deletions in chromosome 22q11, giving rise to an increased risk of schizophrenia (25-30 times that of the general population). These findings have led to work examining this interval for susceptibility genes and a number of genes have been suggested, notably *Catechol-O-methyltransferase* (*COMT*, see Section 1.5.5.1) and *proline dehydrogenase* (*PRODH*).

Most studies have found that at a genome wide level, cases have a greater load of low frequency CNVs than controls, but there are differences in terms of the effect sizes (Walsh, McClellan, McCarthy et al, 2008; International Schizophrenia Consortium, 2008; Kirov, Grozeva, Norton et al, 2009). In the International Schizophrenia Consortium study, cases had a 1.15-fold excess of low frequency CNVs, increasing to 1.6-fold for deletions >500kb (O'Donovan & Owen, 2011). Three deletions were discovered in two recent genome wide CNV studies, with both studies in agreement over two loci, one at 1q21.1 and one at 15q13.32 (International Schizophrenia Consortium, 2008; Kirov et al, 2009b). A third deletion was discovered by only one of the studies (International Schizophrenia Consortium, 2008), but this locus was later independently confirmed (Kirov et al., 2009).

Although this data suggests that there is greater CNV burden in schizophrenia, some of the associations between certain CNVs are not exclusive to schizophrenia, with the 1q21.1 locus also conferring high risk for autism, mental retardation and epilepsy, and the 15q13.3 locus linked to idiopathic generalised epilepsy (O'Donovan & Owen, 2011). Currently CNVs are thought to occur in only 2% of schizophrenia cases (Kirov et al., 2009b); however this does not mean that they are not meaningful in terms of helping to uncover biological mechanisms involved in the pathogenesis of schizophrenia. Indeed, in conjunction with SNP analysis, they have great potential to unravel the complexity of the disease.

1.5.5 'Functional' schizophrenia candidate genes

The functional candidate gene approach originates from the idea that those genes that have a potential role in the pathogenesis of schizophrenia from *a priori* evidence (such as drug targets for anti-psychotics) may also be statistically associated with this disorder. These studies have struggled to find true positives; with no evidence emerging that is on par with the discovery of *APOE* in Alzheimer's disease or *HLA* in Type I diabetes (O'Donovan & Owen, 2011). The power issues associated with much of the candidate gene literature means that although an association may fail to replicate, it does not necessarily rule out the possibility of a true association. With the technology for genotyping rare high penetrance SNPs ever increasing, there is hope for further discoveries in the future.

1.5.5.1. Dopamine genes

As discussed previously (Section 1.3.2), there is a convincing body of evidence that altered dopaminergic functioning contributes to the aetiology of schizophrenia, and as such, genes which have been shown to influence dopamine function have been posited as potential candidate genes. *COMT* is one such gene, and encodes an enzyme which catabolises dopamine, and is located in a region of chromosome 22q11, which when deleted results in a multifaceted syndrome of which schizophrenia is a common feature (Williams, Owen & O'Donovan, 2007). The gene also contains a polymorphism resulting in a valine-to-methionine substitution (Val/Met locus), thought of as the main source of genetic variation in COMT activity. The association between *COMT* and schizophrenia has been reviewed by Williams and colleagues (2007), with results indicating both positive and negative findings at the Val/Met locus, perhaps due to the low power of some of the studies included in the meta-analysis. There are also some interesting interactions between *COMT* and environmental factors such as cannabis use (O'Donovan & Owen, 2011).

DRD2 is a gene which encodes dopamine 2 receptors, and is a plausible candidate due to the therapeutic effect of antipsychotic drugs with a high affinity for dopamine 2 receptors (O'Donovan & Owen, 2011). One study has found an association between a serine to cysteine polymorphism in *DRD2* in a Japanese population diagnosed with schizophrenia but not suffering from negative symptoms

(Arinami, Gao, Hamuguchi & Toru, 1997), with a meta-analysis confirming the association of this polymorphism with schizophrenia (Glatt & Jönsson, 2006). Another variant, rs6277, has also received statistical support for its association with schizophrenia (Lawford, Young, Swagell et al., 2005; Monakhov, Golimbet, Abramova et al., 2008), suggesting that DRD2 may warrant further investigation.

1.5.5.2 Serotonin genes

Many atypical antipsychotics influence the 5-HT system, and therefore genes implicated in this system have been suggested as likely candidate genes (Section 1.3.2). The first studies to provide evidence for a potential serotonin gene found that the variant rs6313 within the gene *HTR2A* (which encodes the 5-HT_{2A} receptor) was associated with schizophrenia (Inayama, Yoneda, Sakai et al., 1996; Williams, Spurlock, McGuffin et al., 1996); however this association has not been replicated since. The 5-HT transporter gene (SERT) has been vigorously studied (due to its role in the treatment of psychosis) but no variants within this gene were significantly associated with risk for developing schizophrenia (O'Donovan & Owen, 2011). One other gene, *TPH1*, is one of 2 enzymes encoding tryptophan hydroxylase; the rate limiting enzyme in the synthesis of serotonin. One particular SNP in this gene has been associated with schizophrenia in a meta-analysis of 1200 case and 1700 controls (SZGene; Allen, Bagade, McQueen et al., 2008), but as the numbers are fairly small, replication may in fact show this to be a false positive.

1.5.6 Positional candidate genes

Positional candidate genes are those genes that have been linked to schizophrenia based on evidence of their involvement from linkage studies or chromosomal location/abnormalities. *DISC1* has been put forward as a potential candidate gene for schizophrenia due to a translocation (chromosome 1:11, q42.1:q14.3) co-segregating with mental illness in a Scottish family (St Clair et al., 1990), with further research identifying the translocation breakpoint to two genes; *Disrupted in Schizophrenia 1 (DISC1) and Disrupted in Schizophrenia 2 (DISC2).* The translocation disrupts the coding sequence of *DISC1,* hypothesised to result in a truncated protein, with reduced *DISC1* expression in the family members (O'Donovan & Owen, 2011). The translocation appears to be unique to this family,

but other research has attempted to link *DISC1* to schizophrenia and other mood disorders such as bipolar, but with little success. *DISC1* does, however, appear to be linked to brain development, including processes such as neural migration and proliferation, neurogenesis and synaptic plasticity (O'Donovan & Owen, 2011). There is also data suggesting that variants in this gene are associated with performance on various neurocognitive tests, and with functional neuroimaging (Callicott, Straub, Pezawas et al., 2005; Di Giorgio, Blasi, Sambataro et al., 2008)

RGS4 is another positional candidate gene for schizophrenia, mapping to a potential linkage region on 1q21-q22. *RGS* genes are thought to suppress the effects of neurotransmitter-receptor interaction at G protein coupled receptors, including the dopamine receptors (O'Donovan & Owen, 2011). Chowdari and colleagues have also shown RGS4 to be down-regulated in the brains of schizophrenia patients (Chowdari, Mirnics, Semwal et al., 2002), although association studies have failed to provide support for a specific haplotype which survives correction for multiple testing.

1.6 ZNF804A

1.6.1 Discovery of ZNF804A as a potential schizophrenia candidate gene

O'Donovan and colleagues conducted a large-scale GWAS for schizophrenia, incorporating 479 cases and 2937 controls from the UK, genotyped using a mapping array chip containing roughly 500,000 SNPs (O'Donovan et al., 2008). Loci with a moderately strong association to schizophrenia ($p<1 \times 10^{-5}$) were followed up in a more international sample of 6666 cases and 9897 controls (Table 1.1). The strongest association in the replication sample came from the risk allele at rs1344706, found on chromosome 2 in the region of the *zinc finger protein 804A* (*ZNF804A, p*<9.25 x 10^{-5}). This same SNP in *ZNF804A* surpassed the benchmark for a strong association with schizophrenia when bipolar cases were added to the affected sample ($p<9.96 \times 10^{-9}$), suggesting that SNPs in the area of *ZNF804A* may influence risk for a broader psychosis phenotype (O'Donovan et al., 2008).

The genetic association between the *ZNF804A* risk allele and schizophrenia has since been thoroughly replicated; firstly in an Irish Case-Control study with a reported *p*-value of 0.0113 (Riley, Thiselton, Maher et al., 2009), followed by another

replication study showing a *p*-value of 0.0029, which strengthened to 0.00065 when bipolar cases were included (Steinberg, Mors, Børglum et al., 2011). The association has also been replicated in a Han Chinese sample with a *p*-value of 0.00083 (Zhang, Lu, Qiu, et al., 2012), although another study on a different Asian population failed to find an association between rs1344706 and schizophrenia, with a *p*-value of 0.26 (Li, Shi, Shi et al., 2012). Furthermore, recently, the investigators who originally uncovered the association between *ZNF804A* and schizophrenia conducted a large scale meta-analysis (Williams, Norton, Dwyer et al., 2011), which provided yet more compelling evidence that the risk SNP at rs1344706 in *ZNF804A* is associated with schizophrenia in both schizophrenia cases alone (p= 2.5 x 10^{-11}) and schizophrenia and bipolar cases combined (p= 4.1 x 10^{-13}). It appears that *ZNF804A* may be one of the most robustly replicated genes in schizophrenia to date.

Table 1.1: Loci selected for follow up in the O'Donovan et al. GWAS (2008).

			Combined UK samples					Replication 1		Replication 1+2		Meta SZ			
			Cases n = 642 Controls n = 2,937					Cases n = 1,664 Controls n = 3,541		Cases n = 6,666 Controls n = 9,897		Cases n = 7,308 Controls n = 12,834			
Chr./Mb	SNP	Risk allele	SZ	CON	ATT(P)	Adj(P)	OR	CMH(P)	OR	CMH(P)	OR	CMH(P)	Meta-Adj	OR	Locus
2/185.5	rs1344706	T	0.66	0.59	7.08×10^{-7}	1.83×10^{-6}	1.38	0.026	1.09	9.25×10^{-5}	1.09	1.61×10^{-7}	1.95×10^{-7}	1.12	*ZNF804A*
11/29.1	rs1602565	C	0.15	0.11	7.81×10^{-6}	1.70×10^{-5}	1.49	0.005	1.19	3.22×10^{-4}	1.12	2.99×10^{-6}	3.02×10^{-6}	1.16	Intergenic
16/13.0	rs7192086	T	0.30	0.24	3.32×10^{-5}	6.52×10^{-5}	1.33	0.018	1.11	5.10×10^{-4}	1.09	6.08×10^{-6}	1.34×10^{-5}	1.12	Intergenic
11/132.1	rs3016384	C	0.56	0.49	5.82×10^{-5}	1.10×10^{-4}	1.29	0.012	1.10	0.016	1.05	5.63×10^{-4}	1.11×10^{-4}	1.08	*OPCML*
16/52.2	rs9922369	A	0.05	0.03	8.05×10^{-7}	2.05×10^{-6}	2.06	0.015	1.31	0.029	1.14	4.54×10^{-4}	5.01×10^{-6}	1.24	*RPGRIP1L*
12/116.2	rs6490121	G	0.40	0.34	4.33×10^{-6}	9.82×10^{-6}	1.33	0.044	1.08	0.992	1.00	0.109	5.51×10^{-3}	1.04	*NOS1*
2/144.3	rs2890738	A	0.41	0.33	4.96×10^{-9}	1.83×10^{-8}	1.44	0.249	1.03	–	–	–	–	–	Intergenic
3/134.5	rs7624858	A	0.44	0.37	1.15×10^{-4}	2.07×10^{-4}	1.27	0.113	1.06	–	–	–	–	–	*TMEM108*
5/138.5	rs17131938	A	0.07	0.04	2.94×10^{-4}	4.94×10^{-4}	1.64	0.091	0.81	–	–	–	–	–	*SIL1*
10/5.6	rs4750519	T	0.48	0.41	1.07×10^{-4}	1.93×10^{-4}	1.27	0.612	0.98	–	–	–	–	–	Intergenic
15/94.0	rs8031294	T	0.51	0.42	2.29×10^{-5}	4.62×10^{-5}	1.30	0.311	1.02	–	–	–	–	–	Intergenic
18/9.0	rs1893146	A	0.16	0.11	5.40×10^{-7}	1.42×10^{-6}	1.55	0.102	0.89	–	–	–	–	–	Intergenic

SZ and CON; allele frequency in schizophrenia and controls. ATT(P), trend test P value; Adj(P), genomic control adjusted P value; CMH(P), Cochran-Mantel-Haenszel P value; Meta-Adj, genomic control adjusted meta-analysis P value.

Table showing the 12 loci with moderately strong association to schizophrenia selected for follow up in the O'Donovan et al. GWAS (2008).

ZNF804A maps to chromosome 2q32.1 and contains 4 exons which code for a protein consisting of 1210 amino acids. Little is known about the encoded protein, although its sequence highlights a zinc finger domain at the N-terminus end suggesting that it may regulate gene expression through its role in binding DNA (Donahoe, Morris & Corvin, 2010). Steinberg et al. (2011) not only sought to replicate the association between the risk SNP in *ZNF804A* and schizophrenia, but

also set out to examine *ZNF804A* for any gain or loss in copy number. Using 4235 psychosis patients, 1173 patients with other psychiatric disorders and 39481 controls, they identified two psychosis patients with CNVs affecting part of *ZNF804A* and none in controls. These CNVs included a deletion in a schizophrenia patient and duplication in an individual with bipolar disorder. They also uncovered a deletion in *ZNF804A* in an individual with anxiety disorder. These cases contrast with no CNVs at the *ZNF804A* locus in around 40000 controls. Despite this, Steinberg notes that it is hard to draw any conclusions about CNVs in *ZNF804A* and schizophrenia as there is overlap with other psychiatric disorders such as anxiety.

The risk SNP in *ZNF804A*, rs1344706 is an intronic SNP found 47kb from the 3' end of exon 2 20kb from the 5' end, in intron 2 (Okada, Hashimoto, Yamamori et al., 2012), located in roughly 30bp of conserved mammalian sequence (Riley et al., 2009). Given the intronic location of the SNP, any functional effects are thought to be due to splicing or transcriptional factors (Hill & Bray, 2012). It is not known whether the SNP is directly related to schizophrenia in a causative way or if the SNP is indirectly related and merely in linkage disequilibrium with another more informative variant. Williams et al. (2011) conducted extensive mapping of the locus and high density linkage disequilibrium mapping to better localise the association signal, covering 96% of all the known SNPs in *ZNF804A*. Despite this thorough investigation, rs1344706 remained the strongest associated polymorphism in the gene.

1.6.2 ZNF804A variants and risk for disease

Although there has been extensive research into the statistical association between schizophrenia and *ZNF804A*, little is currently known about the biological function of *ZNF804A*, and how the SNP rs1344706 actually confers risk for schizophrenia. *ZNF804A* is expressed widely throughout the adult and developing brain, including the cortex and hippocampus (Johnson, Kawasawa, Mason, et al., 2009; Kang, Kawasawa, Cheng, et al., 2011). Riley et al. (2010) carried out post mortem qPCR expression analyses of brain tissue taken from rs1344706 carriers and non-carriers in schizophrenia cases versus controls, showing that controls carrying the risk allele had significantly higher levels of *ZNF804A* expression in the DLPFC than

controls carrying the non risk allele (p=0.033). Patients with schizophrenia showed increased *ZNF804A* expression in the DLPFC relative to controls; however this failed to reach significance. Williams et al. (2011) also investigated *ZNF804A* expression, using carriers and non carriers of the risk allele (T), and finding that the risk allele was associated with higher expression of *ZNF804A*. As there was no difference in the degree of differential expression in rs1344706 homozygotes compared to heterozygotes, the authors point out that the risk allele was not the expression quantitative trait loci (eQTL) directly responsible for the higher expression (Williams et al., 2011). This may suggest that the SNP affects expression by altering its regulatory sequence, and as such is acting in *cis*; affecting expression of the gene copy that is transcribed from the parental chromosome on which it lies. Both these studies support, ostensibly, the idea of a link between the risk allele, increased expression of *ZNF804A* in adult brain and risk for schizophrenia.

Another allelic expression study looked at the binding of the risk allele to nuclear proteins derived from human neural cell lines, using a synthetic oligonucleotide containing the rs1344706 locus (Hill & Bray, 2011). Here they found that the rs1344706 risk allele (T) resulted in reduced nuclear protein binding; binding 46% less than the non-risk allele (G), suggesting a direct effect of the SNP on *ZNF804A* expression through altered DNA-protein interactions, and support the idea that the SNP might be a *cis*-acting transcriptional regulator of the gene (Okada et al., 2012). A very recent expression study examining foetal brain tissue (Hill & Bray, 2012) has shown that the risk allele is associated with a relative *decrease* in expression of *ZNF804A* during the second trimester of development. These data are important as they emphasise how stage of development needs to be taken into account in formulating ideas about how variants in *ZNF804A* might impact on disease risk and also suggest that models where gene expression is reduced constitutively may be of use in discerning pathogenic mechanisms.

Acute knockdown of *ZNF804A* has marked effects on expression of genes involved in cell adhesion, with the most significant differences observed for *STMN3*, a gene that has been implicated in axonal and dendritic branching adhesion and neurite outgrowth. The fact that *ZNF804A* knockdown affected genes belonging to the cell adhesion category, could mean that *ZNF804A* may have a role in processes

such as synapse formation and neural migration, which are thought to be abnormal in schizophrenia (Hill, Jeffries, Dobson, Price, & Bray, 2012). Moreover, the mouse orthologue of *ZNF804A*, *Zfp804a*, is a target of the gene *Hoxc8.* Chung and colleagues (2008) found that *Hoxc8* bound to an intronic region in the 3rd intron of *Zfp804a*, and that *Hoxc8* upregulated *Zfp804a* mRNA levels. The two genes were also co-expressed in E11.5 mouse embryos. The combined expression of various *Hox* genes are thought to make up the 'Hox code'; the genetic program posited to be responsible for the fate of cells, such as morphogenesis, growth and differentiation during embryonic development. This link with *Hoxc8* could further indicate a role for *ZNF804A* in early neurodevelopment.

In summary, the relatively limited studies carried out so far suggest a direct effect of the risk SNP on *ZNF804A* expression through altered DNA-protein interactions, and support the idea that the SNP might be a *cis*-acting transcriptional regulator of the gene (Riley ey al., 2009; Williams et al., 2011; Hill & Bray, 2011, 2012). *ZNF804A* knockdown studies show that *ZNF804A* may have a role in cell adhesion processes such as synapse formation and neural migration, which are thought to be abnormal in schizophrenia (Hill et al., 2012). Despite the recent developments in understanding the biological function of *ZNF804A*, there are still many unanswered questions on exactly how the risk allele in *ZNF804A* contributes towards the development of schizophrenia, and indeed what the exact biological functions of *ZNF804A* are remain something of a mystery. Finally, it is important to emphasise that there may be as yet unidentified variants at the locus which may also influence susceptibility to schizophrenia.

1.6.3 Neurocognitive functions of ZNF804A

Work looking into the neurocognitive phenotype of the risk allele at rs1344706 began with an fMRI study looking at the connectivity between certain brain regions (Esslinger, Walter, Kirsch et al., 2009). Using 115 healthy German participants, they found that carriers of the risk allele had disturbed functional coupling between certain brain regions whilst performing the n-back task, a well validated working memory cognitive probe. Specifically, risk allele carriers showed reduced connectivity both within the DLPFC and to the contralateral DLPFC whereas

connectivity between the DLPFC and the hippocampus was increased in a dose dependent manner for homozygous risk allele carriers. The risk allele was also associated with increased connectivity from the amygdala to the hippocampus, the medial prefrontal cortex and to the orbitofrontal cortex. Regional brain activation was not related to genotype, nor was overall performance in the task, perhaps indicating that the risk allele may be predominantly influencing the neurobiological phenotype (Esslinger et al., 2009). Due to widespread research of reduced brain function in schizophrenia, and the fact that *ZNF804A* has been associated with schizophrenia, the authors speculated that the reduced connectivity within the DLPFC may contribute to disturbed executive function (an endophenotype of schizophrenia), but that the increased connectivity between the DLPFC and hippocampus could disrupt interactions between the prefrontal and limbic cortices, perhaps pointing towards a more bipolar like phenotype.

Another study offers an alternative theory concerning the role of *ZNF804A* in cognition. Here an independent sample of German and Irish schizophrenia cases and healthy controls were tested on cognitive functions that are impaired in schizophrenia, for example IQ, working memory, episodic memory and attention (Walters, Corvin, Owen et al., 2010). Irish schizophrenia patients homozygous for the rs1344706 risk allele performed relatively better on measures of episodic memory and working memory than patients homozygous for the non-risk allele, with these results replicated in a German sample. The allelic association between *ZNF804A* and schizophrenia further increased when patients with IQs lower than 90 were excluded from the analysis. These findings suggest that the *ZNF804A* risk allele has a protective effect on cognition in schizophrenia cases but not controls, and that it may contribute to risk for a sub-type of schizophrenia not affected by cognitive impairments.

A further study has looked at theory of mind measures and rs1344706 genotype by investigating brain activation in a theory of mind task using healthy volunteers (Walter, Schnell, Erk et al., 2011). Risk allele carriers exhibited a significant risk allele dose effect on brain activity in the theory of mind network, including the dorsomedial prefrontal cortex and the left temporo-parietal cortex. The same effect was found in the left inferior parietal cortex and left inferior frontal

cortex, the human analogue of the mirror neuron system. The study also found altered connectivity between the fronto and temporo-parietal regions in rs1344706 risk allele carriers. This study supports the idea that *ZNF804A* has an impact on the neural activity associated with social cognition, and that the risk variant could be used to test intermediate phenotypes of schizophrenia with a view to developing biological interventions to alleviate social dysfunction in this disorder. There were, however, no risk allele dose effects on performance in the task, with a possible explanation being that behavioural effects of genes are more distal biologically than brain activation effects (Walter et al., 2011).

Esslinger and colleagues sought to investigate interactions between cognitive state and *ZNF804A* genotype on connectivity (Esslinger, Kirsch, Haddad et al., 2011). Continuing on from their earlier study, they set out to examine whether the deficits in connectivity within the DLPFC for risk allele carriers were specific to the working memory task or broadly present and therefore structural. Imaging healthy control subjects during a working memory task, an emotion recognition task and at rest, they found that during all three conditions there was a linear decrease in connectivity within the DLPFC with increasing number of risk alleles. This decrease in interhemispheric prefrontal connectivity in subjects with the risk allele was cognitive state independent, indicating a potential structural abnormality within the working memory network of risk allele carriers, affecting functional connectivity irrespective of task. The second main finding was that risk allele carriers showed increased connectivity between the DLPFC and the hippocampus during the working memory task only, indicating that this finding was cognitive state dependent (Esslinger et al., 2011).

Another study looking at functional brain connectivity and cognition found that *ZNF804A* risk allele homozygotes as well as heterozygotes had increased connectivity between the anterior cingulated cortex (ACC) and the right DLPFC compared to non-risk allele homozygotes during a response inhibition task (Thurin, Rasetti, Sambataro et al., 2012). During the interference and monitoring aspect of the task, risk allele homozygotes and heterozygotes showed decreased right DLPFC and ACC activation compared to non risk allele carriers. These results suggest that *ZNF804A* could be modulating mechanisms which underlie cognitive control and

functional connectivity (Thurin et al., 2012). Another study has looked into how the *ZNF804A* risk allele may modulate neural activity during a working memory task. Alterations in neural activity in *ZNF804A* risk allele carriers were seen during a working memory task which incorporated an emotional facial processing element (Linden, Lancaster, Wolf et al., 2013). Specifically the rs1344706 risk allele carriers demonstrated a reduced activation of the right DLPFC (rDLPFC) during the working memory task for faces. This hypoactivation of the rDLPFC is a common finding in schizophrenia patients, and perhaps suggests that the *ZNF804A* risk allele may influence neural activity during working memory. A subsequent study aimed to replicate the findings of Esslinger and colleagues (2009) and looked into the link between the *ZNF804A* risk allele and functional connectivity during a working memory task. They failed to replicate the decrease in connectivity within the DLPFC, but did find the increased functional coupling between the right DLPFC and hippocampus (Paulus, Krach, Bendenbender et al., 2013).

These results, taken together, do seem to support the role of the *ZNF804A* risk allele in cognition, such as working memory and emotional processing. The majority of the results indicate fronto-hippocampal disconnectivity and hypoactivation of the DLPFC as intermediate phenotypes potentially linking the risk allele to schizophrenia, by way of mirroring the aberrant connectivity seen in schizophrenia patients when performing working memory tasks. However, in terms of the association between cognitive performance and the risk allele, one study implies that rs1344706 has a protective effect on cognition in schizophrenia cases but not controls, as patients with schizophrenia who were homozygous for the risk allele performed relatively better on the cognitive tasks than those patients not carrying the risk allele. The other studies, using healthy participants, did not indicate a relationship between the risk allele and cognitive performance leading to the suggestion that the effects of the risk allele may be influencing the neurobiological phenotype as opposed to the cognitive phenotype per se.

1.6.4 Brain structure and ZNF804A

A structural MRI study examined the effect of the *ZNF804A* risk allele on white and gray matter volumes as well as regional brain volumes in healthy

volunteers (Lencz, Szeszko, DeRosse et al., 2010). It was found that individuals homozygous for the risk allele had larger total white matter volumes than those with at least 1 non-risk allele. Furthermore, Donohoe and colleagues found that schizophrenia patients homozygous for the risk allele had larger gray matter brain volumes, particularly in the superior temporal gyrus, insula and hippocampus (Donohoe, Rose, Frodl et al., 2011), suggesting that *ZNF804A* is contributing to risk for a schizophrenia subtype characterised by relatively intact brain volume (Donohoe et al., 2011). Wassink and colleagues sought to further investigate these structural findings using a larger sample of 335 schizophrenia cases and 198 controls (Wassink, Epping, Rudd et al., 2012). They found that in the schizophrenia group, risk allele carriers had larger volumes and more severe psychotic symptoms, than non-risk allele homozygotes. Whereas in the healthy control group, risk allele homozygotes showed increased total white matter volume compare with non risk allele carriers, concordant with previously reported research (Lencz et al., 2010). It appears that the risk allele is associated with distinct structural phenotypes in both schizophrenia patients and healthy volunteers.

125 Chinese patients with schizophrenia were examined for the effect of *ZNF804A* risk allele genotype on brain white matter integrity (Kuswanto, Woon, Zheng et al., 2012). Patients homozygous for the risk allele had lower white matter integrity in bilateral parietal lobes and left cingulated gyrus compared with non-risk allele carriers. Secondly, compared to healthy controls homozygous for the risk allele, homozygous schizophrenia patients had decreased white matter integrity in these same areas as well as the right medial temporal lobe, perhaps suggesting that the *ZNF804A* risk variant may influence white matter integrity involving cortico-limbic brain regions in schizophrenia (Kuswanto et al., 2012).

1.7 Animal models for schizophrenia

Animal models are a useful means of investigating the biological, and to an extent psychological, mechanisms that may confer risk and can also help in determining the 'biological plausibility' of a potential candidate gene for complex psychiatric disorders (Powell & Miyakawa, 2006). Model studies are driven by the genetic similarity between certain animal and human genomes, and the high level of

conservation between certain species. In particular, animal models have strengths in offering a controlled experimental environment and allowing the use of methods which may be viewed as unethical/impossible with humans, such as those requiring direct access to the brain. Currently, mice are one of the most popular species used in the laboratory, mostly due to their genetic tractability, short breeding cycle and worldwide availability. There are also catalogues of mutant mice already generated and available for testing (e.g. MRC Mammalian Genetics Unit, Harwell, http://www.har.mrc.ac.uk/services).

Mouse models which recapitulate all aspects of schizophrenia are currently non-existent, as many of the symptoms such as disorganised speech, hallucinations and delusions remain unobservable in the murine species. There is also the issue of translatability; how similar are the symptoms and underlying pathological processes seen in schizophrenia similar to the phenotypes tested in mice? Researchers might argue that the deficits in cognition cannot be fully replicated in species which are less cognitively developed. The heterogeneity of schizophrenia also poses a problem for animal models, as patients can experience differing combinations of symptoms, with the course and outcome of the disorder extremely variable (Marcotte, Pearson & Srivastava, 2001).

In view of the formidable complexity of mental disorders, a more systematic approach to mouse models for schizophrenia has been suggested; examining phenotypic indicators which could point to more discrete aspects of the condition (Arguello & Gogos, 2006). The mutant models of *Zfp804a* used extensively in this thesis aim to do just that, looking at how alterations in function in the mutants could be related to components of the complex mix of symptoms seen in schizophrenia and/or bipolar disorder. For example, the *Zfp804a* mutants were tested on prepulse inhibition and assays measuring hedonic responses in an attempt to model core attentional and affective symptoms, respectively.

1.7.1 Non genetic animal models of schizophrenia

These types of animal models have mainly focused on the dopamine system, due to many antipsychotic drug treatments having D_2 receptor antagonist activity, and the observation that dopamine agonists can induce psychotic-like symptoms

(Lipska & Gogos, 2011). The psychosis-inducing effects of amphetamine (a dopamine-releasing agent) are well reported, leading to investigations of chronic administration of amphetamine to rodents, with hyperactivity a common finding (Jones, Watson & Fone, 2011).

As mentioned previously, glutamate has also been implicated in schizophrenia due to the finding that both the drugs phencyclidine (PCP) and ketamine (NMDA antagonists) produce both positive and negative symptoms of schizophrenia in healthy individuals and exacerbate the symptoms in patients with schizophrenia. Acute PCP administration in rodents has been shown to cause hyperactivity (Kalinichev, Robbins, Hartfield et al., 2007), social withdrawal (Sams-Dodd, 1995), and impairment of both PPI (Mansbach and Geyer, 1989) and cognition (Egerton, Reid & McKerchar et al., 2005).

Other models for schizophrenia have focused on the posited neurodevelopmental aspect of schizophrenia including perinatal stress, examined with techniques such as isolation rearing. This environmental manipulation was found to induce hyperactivity of the mesolimbic dopamine systems in rats, with these rats exhibiting reduced PPI (Geyer, Wilkinson, Humby et al., 1993). Rat maternal malnutrition induced enhanced amphetamine locomotion and reduced PPI (Palmer, Printz, Butler et al., 2004). Other models have looked at neonatal damage of certain brain regions in rats. The hippocampus has been one of the targeted regions, a brain area implicated in the neurobiology of schizophrenia (Lipska & Gogos, 2011). Neonatal excitotoxic lesions of the ventral hippocampus in rats has produced promising phenotypes in adolescence and adulthood, including reduced sociability (Sams-Dodd, Lipska & Weinberger, 1997), enhanced sensitivity to PCP agents, deficits in PPI and latent inhibition and working memory problems (Lipska & Gogos, 2011). Crucially, anti-psychotic drugs have helped to normalise some of the phenotypes seen in the lesioned animals (Sams-Dodd et al., 1997).

MAM (methylazoxymethanol acetate), an anti-mitotic agent which methylates DNA, has been given to pregnant dams to investigate its effect on brain development in the affected rodent foetuses. MAM treatment at gestational day 17 was found to produce enhanced sensitivity to PCP and ketamine on locomotor activity, and altered electroencephalogram responses to NMDA receptor agonists

(Phillips, Cotel, McCarthy et al., 2011), as well as disrupted sleep, (Phillips, Bartsch, McCarthy et al., 2012), PPI and LI (Moore, Jentsch, Ghadjarnia et al., 2006).

1.7.2 Genetic animal models for schizophrenia

Many genes identified from linkage and association studies have a largely unknown biological function. Schizophrenia is highly heritable, and polygenic, comprising of a complex genetic architecture with a large number of common alleles of small effect, likely to have escaped the rigours of natural selection, and a number of much rarer but highly penetrant variants (e.g. CNVs), which due to their highly damaging effects are of a much more recent origin, often *de novo* (Xu, Roos, Levy et al., 2008). Genetic mouse models offer a way in to examining the effects of manipulating a 'risk gene candidate' in an intact *in vivo* system. Genetic animal models can be divided into 2 basic categories: those investigated in a candidate-led manner from putative disease-associated mechanisms (e.g. dopamine related genes) and those discovered from 'hypothesis free' investigations of clinical samples e.g. GWAS, and linkage analysis.

1.7.2.1 Genes modelled from putative disease-associated mechanisms

Many of these models have again focused on the dopamine system. As mentioned previously, striatal overexpression of D_2 receptors (D2R) has been linked to the pathogenesis of schizophrenia, and this has been modelled in mice overexpressing D2R in this brain region, with findings indicating increased activation of D1 receptors in the PFC and impaired working memory (Kellendonk, Simpson, Polan et al., 2006), as well as reduced incentive motivation (Drew, Simpson, Kellendonk et al., 2007) in these mice. *COMT*, encoding an enzyme which catabolises dopamine, has been put forward as a potential schizophrenia candidate gene. It contains a polymorphism resulting in a valine-to-methionine substitution (Val/Met locus), and it is this variant which has been overexpressed in mice. These COMT Val-tg mice have shown working memory deficits, and object recognition deficits, which have been rescued with amphetamine treatment (Papaleo, Crawley, Song et al., 2008). *COMT* knockout mice, interestingly, show improved working memory (Papaleo et al., 2008). Models looking into the glutamate hypothesis of

schizophrenia have included transgenic mice with 90% reduction in NMDA receptor 1 expression (NR1), showing hyperactivity, and reduced social and sexual behaviour, with hyperactivity reduced with the administration of antipsychotics (Mohn, Gainetdinov, Caron, et al., 1999).

1.7.2.2 Genetic models from clinical association

Neuregulin 1, discovered originally from linkage analysis in Icelandic families (Stefansson et al., 2002) is a leading candidate gene for schizophrenia. The NRG1 protein gives rise to many different proteins and isoforms, and as a pleiotropic growth factor, homozygous knockout of NRG1 is lethal in mice leading to NRG1 heterozygotes and hypermorphs/conditional knockouts being utilised instead. NRG1 heterozygotes for a mutation at the transmembrane domain showed hyperactivity, PPI deficits and altered sociability (O'Tuathaigh, O'Connor, O'Sullivan et al., 2008). A mouse model of a separate *NRG1* isoform also found PPI deficits, and also working memory impairments and enlarged ventricles characteristic of certain schizophrenia neuropathologies (Chen, Johnson, Lieberman et al., 2008).

DISC-1 was discovered from a translocation which co-segregated with mental illness in a Scottish family (St Clair et al., 1990). One mouse model took a 'knock-in' approach generating mutant mice carrying the translocation seen in humans (Koike, Arguello, Kvajo et al., 2006). These mice were found to have working memory deficits on a number of tasks, as well as altered neuronal distribution in the hippocampus. Hikida and colleagues (2007) used a dominant negative form of DISC1 in mutant mice, finding enlarged lateral ventricles and behavioural phenotypes including disturbed PPI and hyperactivity. Another study used a conditional model to induce expression of the C-terminal section of DISC1, to test the hypothesis that the mutation uncovered in the original Scottish family was developmental in nature (Li, Zhou, Jentsch et al., 2007). They found that early postnatal (but not adult) induction resulted in impaired synaptic transmission, reduced sociability, depressive-like behaviour and altered spatial memory. Other mutations in exon 2 of *DISC-1* (not linked to the translocation), were studied by Clapcote and colleagues (2007), through ENU mutagenesis. The Q31L mutation showed depressive like behaviour in the forced swim test, with the L100P mutant mice showing reduced PPI and LI,

behaviours which were reversed with antipsychotic drugs. Structural abnormalities, such as in the 22q11 locus deletion covering multiple genes, can also be modelled in mice, using chromosomal engineering approaches (Lipska & Gogos, 2011), with these mutants displaying deficits in PPI, fear memory and the acquisition of working memory (Stark, Xu, Bagchi et al., 2008).

Genetic animal models recapitulating (to an extent) aspects of schizophrenia are proving to be a useful tool for understanding the neurobiological pathways involved in schizophrenia. As the molecular techniques become more advanced, so too will the genetic models. We are also at an important juncture in this area of research, insofar as with the increasing detail and veracity of the genetic data from hypothesis free studies in very large clinical samples, we can now model the action of candidate risk genes that have a high probability of actually being involved in pathogenic processes in some way, such as the robust findings with *ZNF804A*.

1.8 Aims of the thesis

The overall aim of the experimental work in this thesis was to further characterise the functions of *Zfp804a*, the mouse orthologue of *ZNF804A*, with the goal of understanding more about how variance in this gene may increase the risk of developing schizophrenia/bipolar disorder. Prior to the current work, two ENU mouse lines had been generated from a DNA library of ENU mutagenised mice (DNA Archive, MRC Mary Lyon Centre, Harwell) by a previous member of the laboratory. In brief, this DNA library was screened for single base pair point mutations in the *Zfp804a* gene (Al-Janabi, 2012, See Chapter II, Section 2.1), with two non-synonymous mutations selected as viable candidates for further investigation and founder mice generated through *in vitro* fertilisation. The first line selected for re-derivation was a nonsense mutation within exon 2 (C59X), encoding a premature stop codon, thought to lead to a functional null of the gene. The second mutation, within exon 4 (C417Y), was missense and predicted significant effects on the 3D structure of Zfp804a due to the substitution of the relatively small, nonpolar cysteine residue with a larger, polar tyrosine residue. Much of the work that follows exploited the availability of these two ENU *Zfp804a* mutant mouse lines.

Specifically the aims of this thesis were to:

1) Utilise the previous work of Al-Janabi (2012) in producing the two ENU mutant lines - but to make use of mice with a more pure (>98%) non-ENU strain genetic background.
2) Make a detailed assessment of the developmental profile and basic sensorimotor capabilities of the *Zfp804a* mutant mice (Chapter III).
3) Examine the effects of *Zfp804a* mutagenesis on anxiety-related behaviours (Chapter IV).
4) Examine the effects of *Zfp804a* mutagenesis on motivational and hedonic responding (Chapter V).
5) Determine sensorimotor gating changes in the *Zfp804a* mutant lines using acoustic startle and pre-pulse inhibition of acoustic startle (Chapter VI).
6) Assess whether *Zfp804a* mutagenesis influences response control and impulsivity (Chapter VII).
7) Compare and contrast between effects observed following *Zfp804a* mutagenesis in exon 2 (nonsense) and exon 4 (missense) with respect to wild-type littermate controls.

It is important to note, at the outset, that this work was not an attempt to explicitly model the human *ZNF804A* risk variant rs1344706 nor to model schizophrenia in a mouse, but to alter the function of *Zfp804a* to gain understanding of how genetic variation in this gene may contribute to risk for schizophrenia.

Chapter II; General materials and methods

This chapter describes the procedures that were performed throughout the course of this thesis, and the behavioural apparatus that were employed. All procedures that involved the usage of live animals were carried out in accordance with the requirements of the U.K. Animals (Scientific Procedures) Act (1986) and in line with the Home Office Project Licence granted to Dr William Davies (PPL 30/2601). Work was carried out under the Home Office Personal License granted to Jessica Eddy (PIL: 30/8796).

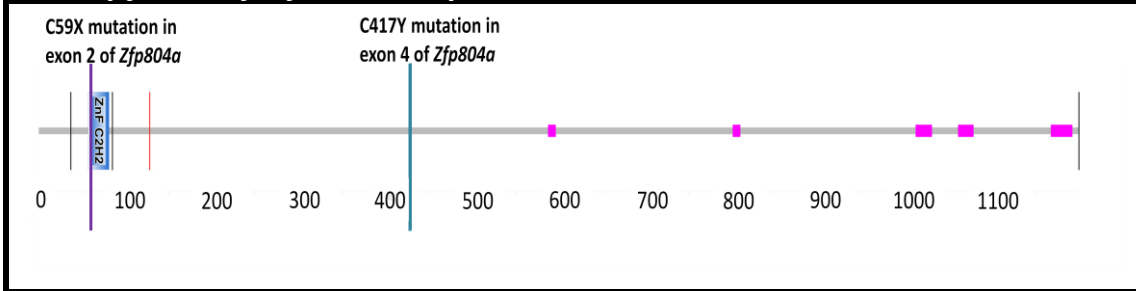
2.1 Production of the *Zfp804a* mutant mouse lines

There are a number of methods for the creation of genetically modified mice, including models with spontaneous or engineered targeted mutations at the loci of interest using knock-in or knock-out strategies, or N-ethyl-N-nitrosourea (ENU) mutagenesis. The approach used here was that of ENU mutagenesis, whereby a DNA library of ENU mutagenised mice (DNA Archive, MRC Mary Lyon Centre, Harwell) was screened for single base pair point mutations in the *Zfp804a* gene. The melting profiles of 5856 samples using Hi-Resolution Melting was determined, resulting in the discovery of ten non-synonymous mutations, which were then sequenced to confirm the presence of a mutation in *Zfp804a* (Al-Janabi, 2012). Two mutations were selected as candidates for further investigation (see below). Frozen sperm from the Harwell archive corresponding to the DNA sequences screened was used to re-derive founder mice by *in vitro* fertilisation (IVF); DNA was collected from the progeny of the mutagenised mice (F1) where mutations had affected the germ line. All of the founder re-derivation was performed by the MRC Centre at Harwell.

The two non-synonymous *Zfp804a* mutations selected for re-derivation, designated C59X and C417Y (Fig. 2.1), were chosen as they were considered (on the very limited functional and bioinformatic data available) likely to lead to functional effects (Al-Janabi, 2012). The C59X nonsense mutation is within exon 2, and encodes a premature stop codon in the gene sequence, predicting a severely truncated protein product, if transcribed/translated, at aa59 of the aa1200 protein. The C417Y mutation is missense, affecting aa417 within exon 4, predicting significant effects on

the 3D structure of Zfp804a due to the substitution of the relatively small, nonpolar cysteine residue with a larger, polar tyrosine residue. Therefore, it was expected that the C59X mutation should lead to a functional null of the gene, whereas the C417Y mutation would lead to altered function of *Zfp804a.*

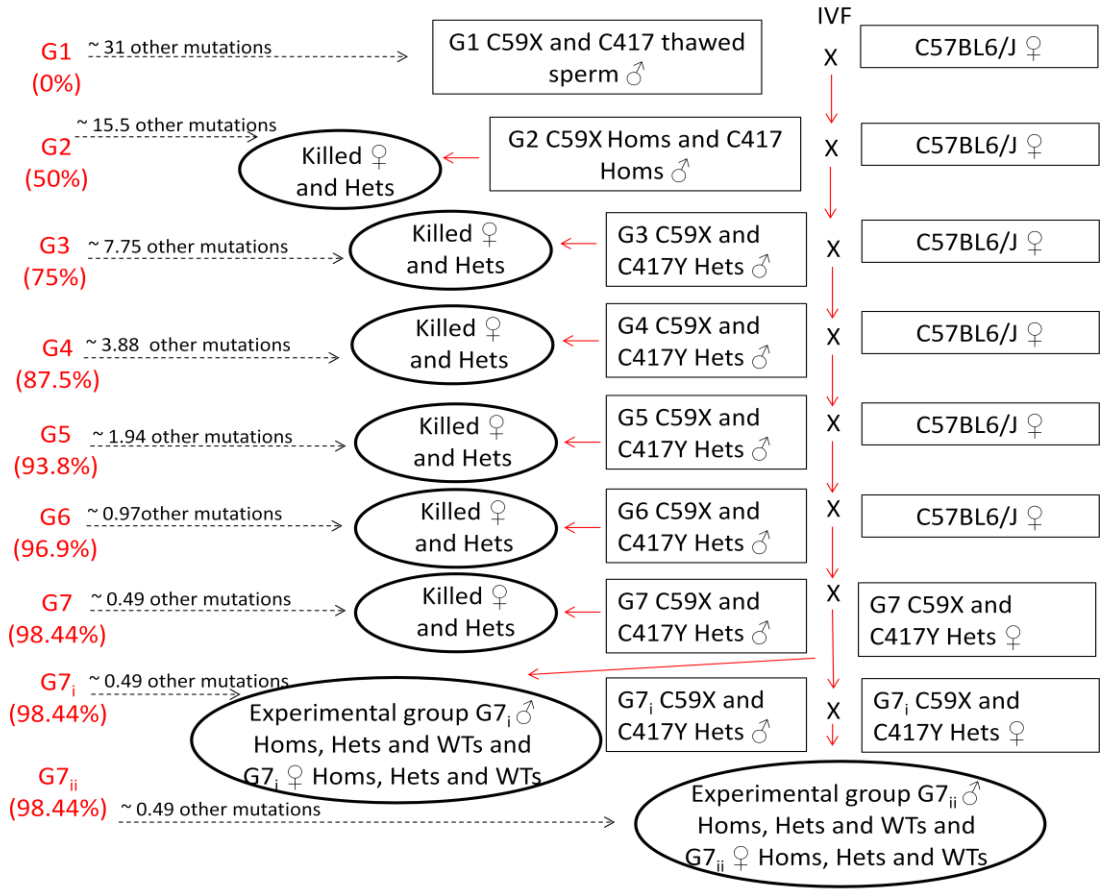
Figure 2.1: Summary of the two Zfp804a mutations in the* Zfp804a *protein selected to carry forward for further analysis.



The C59X mutation is located in exon 2 and encodes a premature stop codon, truncating the 1200aa protein prematurely at 59aa (aa6 of 28 comprising zinc finger domain, ZnF). The C417Y mutation is located in exon 4 (aa417) and substitutes a nonpolar cysteine residue with a larger polar tyrosine residue.

A disadvantage of ENU mutagenesis is that it causes many random mutations, in addition to the target mutation of interest, at unknown locations across the genome; estimated to be approximately 31 in founder mice (see Keays, Clark & Flint, 2006). G2 heterozygous *Zfp804a* founder mice (bred on a BALB/c x C3H/HeJ background, re-derived at Harwell), from both lines (C59X and C417Y), were crossed with wild type C57BL/6JOlaHsd mice, to produce offspring (G3) which were 75% genetically similar to C57BL/6JOlaHsd mice (and would therefore carry ~8 other ENU-induced mutations). Male and female progeny of each line which were positive for the C59X and C417Y mutations were then backcrossed again to C57BL/6JOlaHsd mice (C57BL/6J). Heterozygous progeny from the 6th backcross, with a predominantly C57BL/6JOlaHsd genetic background (average of 98.5% at G7) and where only the *Zfp804a* mutations should be consistently expressed in each mouse, were inter-crossed with other heterozygous mice of the same line to generate *Zfp804a* heterozygous ENU-mutants, homozygous ENU-mutants and C59X and C417Y wild-type (WT) littermate control mice for testing. Two cohorts of mice from both mutant lines were generated for the studies described in this thesis; $G7_i$ and $G7_{ii}$ (Fig. 2.2).

Figure 2.2: Flow diagram to show the breeding programme for the generation of the $G7_i$ and $G7_{ii}$ Zfp804a cohorts.



G1 male mice (0% C57BL/6JOlaHsd mice) were crossed to C57BL/6JOlaHsd females. These progeny (G2) were sent from Harwell and estimated at 50% similarity to C57BL/6J, carrying ~15 other mutations from the ENU mutagenesis. The *Zfp804a* heterozygous ENU-mutant mice (for both C59X and C417Y mutant lines) were then backcrossed to the C57BL/6JOlaHsd mice 5 more times, until the G7 generation were 98.5% similarity to C57BL/6J and carrying less than one other potential ENU mutation. G7 heterozygous ENU-mutant mice (for either the C59X or C417Y mutation) were then inter-crossed with other G7 heterozygous ENU-mutant mice from the same line to generate $G7_i$ heterozygous ENU-mutants, homozygous ENU-mutants WT littermate control mice for the *Zfp804a* behavioural cohorts. The $G7_i$ heterozygous ENU-mutant mice were then inter-crossed with other $G7_i$ heterozygous ENU-mutants from the same line, generating a second experimental group of homozygous ENU-mutants, heterozygous ENU-mutants and WT littermate controls, known as the $G7_{ii}$ cohort. Hom= homozygous, Het= heterozygous, WT= wild-type littermate control.

2.2 Subjects and animal husbandry

Mice from each experimental cohort were group housed (2-5 mice per cage) in environmentally enriched cages (i.e. with cardboard tubes, shred-mats, tissue paper) in a temperature and humidity controlled animal holding room (21 ± 2°C and 50 ± 10%, respectively) with a 12-hour light-dark cycle (lights on at 07:00 hours/lights off at 19:00 hours). Only subjects of the same line were housed together; most of the home cages included at least one mouse of each genotype (i.e. homozygous

ENU-mutant, heterozygous ENU-mutant and WT littermate control mice); birth litters were kept together whenever possible. Standard rodent laboratory chow and water were available *at libitum* unless otherwise stated. Home cages were cleaned and changed once a week, at approximately the same time of the day and on the same day of the week, in order to cause minimal disruption to the behavioural testing. Initially, both males and females of all genotypes and lines were monitored for weight gain and developmental profiling (Chapter III), but after this, testing was limited to just the male homozygous ENU-mutants and their male WT littermate controls. Throughout this thesis mice which were homozygous for the *Zfp804a* mutations are referred to as C59X or C417Y homozygous ENU-mutants, those that were heterozygous were C59X or C417Y heterozygous ENU-mutants and wild-types (WTs) were referred to as WT littermate controls. Experimental animals were regularly monitored and weighed from birth for signs of ill health. Any mice showing signs of illness were immediately assessed by a Veterinarian and, if necessary, withdrawn from the experiment.

2.3 Behavioural methods

2.3.1 Handling

One experimental cohort of mice ($G7_{ii}$) were weighed daily from birth and subjects from both cohorts were handled regularly post-weaning before beginning an experiment. This was for approximately 1 minute each, twice a week.

2.3.2 Measurement of body weight

Body weights of all mice were recorded on a regular basis as an index of growth and development and a measure of general health. Weights were registered daily (pre-weaning; cohort $G7_{ii}$) and fortnightly (post-weaning; all subjects) at the same time of day (at around 10:00). Subjects were also weighed before any behavioural experiments began, and after each behavioural experiment had finished.

2.3.3 Behavioural testing environment

Behavioural testing was carried out in sealed and air-conditioned testing rooms. Testing rooms were lit by fluorescent lights. Temperature and humidity levels

were not strictly controlled, but were generally maintained at around 21 ± 2°C and 50 ± 10% respectively. Rooms were thoroughly cleaned once a week.

2.3.4 Protocol for the water restriction schedule

Prior to testing in behavioural paradigms where condensed milk was used as a reinforcer, subjects were placed on a schedule of reduced home cage water access (in order to enhance motivation for the reinforcer). This schedule lasted 6 days, encompassing 4 days of 4hr access/day and 2 days of 2 hr access/day. Mice were weighed daily during this schedule, as were their drinking bottles to ensure they were drinking during the period of water access. Importantly, subjects losing >10% of their *ad libitum* body weight or showing clinical symptoms of dehydration or ill-health were immediately given *ad libitum* water access until normal *ad libitum* body weight was re-established. Subjects were allowed *ad libitum* access to standard laboratory chow, throughout the duration of the water deprivation schedule. After these 6 days, mice were maintained on a 2hr access/day schedule for the duration of the experiment.

2.3.5 Reinforcer preference test/reactivity to a novel food substance

Subjects undergoing appetitively motivated behavioural tasks (i.e. the progressive ratio task and the stop-signal serial reaction time task, SSRTT), were habituated to the liquid reinforcer before testing (10% condensed milk solution, Nestle Ltd, U.K.). Briefly, the reinforcer preference test (see Humby, Wilkinson & Dawson, 2005) was carried out in 10 small holding cages (285 x 130 x 120mm) with a single subject per cage, during a single 10-minute session per day, across a six-day period. During the first session, subjects were allowed to habituate to the test apparatus, while general water consumption was measured by placing two containers (max vol.= 3ml each) containing an excess of tap water of registered weight, to the rear of each cage. Following each test session, the containers were re-weighed in order to determine the total water consumption.

Over the next four sessions, one of the containers was filled with the condensed milk reinforcer, and the second container was filled with tap water. The

locations of the two containers within the cage were pseudo-randomly switched between days. As before, the containers were weighed prior to, and immediately after, testing in order to determine the consumption of each liquid as well as the preference for condensed milk. Reinforcer preference was defined as the amount of condensed milk (CM) consumed in the final session of testing, as a percentage of the total amount of liquid (i.e. the CM and water collectively) consumed during that session. The final day of testing involved filling both containers with condensed milk, for a 'milk vs. milk' condition. This was to ensure that all of the subjects had sampled the condensed milk reinforcer before testing in the behavioural paradigms. The data on total volume of liquid consumed was normalized for body weight differences using Kleiber's 0.75 mass exponent (Schmidt-Nielsen, 1990). Any subjects failing to reach 70% preference for the condensed milk on the final day of preference, and failing to consume condensed milk on the milk vs. milk day, were excluded from any experiments using condensed milk as a reinforcer.

2.3.6 Behavioural phenotyping: general experimental control measures

Behavioural testing was always conducted in the light phase of the light-dark cycle of the animal holding room (i.e. between 07:00 hours and 19:00 hours), and water access was provided immediately subsequent to any behavioural assessment. Moreover, in order to maintain a constant time period between the time of testing and water access (which might affect motivation for the liquid reinforcer), and also to minimize any behavioural variation due to when the subjects were tested, individual subjects were tested at the same time each day. In addition, possible 'order of experimental run' effects were negated by running the experimental and control subjects in a pseudorandom order. To minimize possible confounds related to cage/litters, the experimental subjects were drawn from as large a number of cages/litters as available.

2.4 Behavioural apparatus

The current section describes the experimental apparatus employed in this thesis. The different procedures used are described in more detail in the subsequent experimental chapters.

2.4.1: SHIRPA (the SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) equipment

A horizontal wire cage lid (28.5cm x 12cm) was used for the visual placing and negative geotaxis tests. The grip strength test and the wire manoeuvre test used a 16cm long (0.5cm diameter) fixed metal bar suspended 35cm above a soft surface.

See **Chapter III** for experimental details.

2.4.2: Locomotor activity chambers

Testing of locomotor activity (LMA) was carried out in an apparatus consisting of twelve clear Perspex chambers (each 21 x 36 x 20cm, width x length x height), with two embedded infra-red beams crossing each cage, 30mm from each end and 1cm from the floor. Beam breaks were recorded as an index of activity, using a computer running custom written BBC Basic V6 programmes with additional interfacing by ARACHNID (Cambridge Cognition Ltd, Cambridge, U.K.). Data stored were the total number of beam-breaks from each 2 hour session, as well as the number of beam-breaks made over each 30 minute quartile.

See **Chapter III** for experimental details.

2.4.3: EthoVision observer system

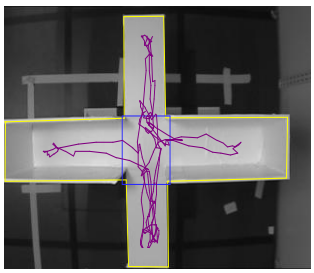
All of the anxiety assays used the EthoVision Observer video tracking software (version 3.0.15, Noldus Information Technology, Netherlands) to track and analyse the behaviour, movement and activity of the mice. EthoVision tracks a subject's movement within previously defined zones within the arena (these zones differ between the different anxiety paradigms), performing calculations over a series of frames (12 frames/sec) and deriving a set of quantitative descriptors about the movement and location of subjects determined by the location of the greater body-proportion of subjects. Tracking of the subject was calibrated using non-experimental mice of the same body size and coat colour as the experimental subjects.

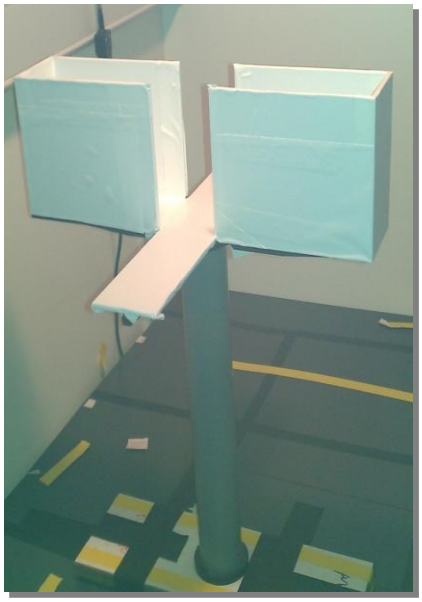
See **Chapter IV** for more details of the virtual zones set up in each anxiety assay.

2.4.4: Elevated plus maze

The elevated plus-maze (EPM) was constructed of dulled black Perspex covered in white tape (to enable visualisation of dark coated mice) and consisted of two exposed open arms (17.5 x 7.8mm, length x width) and two enclosed arms (19 x 8 x 15cm, length x width x height) with an open roof. The maze was positioned 94cm above the floor and illuminated evenly with a 60w bulb. Mice were tracked on the EPM using EthoVision (manufactured), an example of a trace can be seen below.

See **Chapter IV** for experimental details.





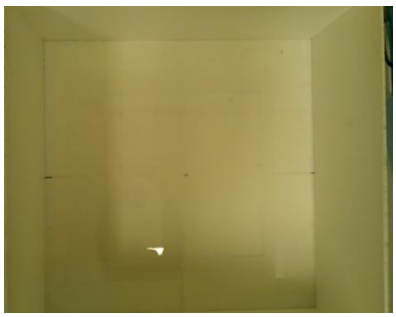
2.4.5: Open field

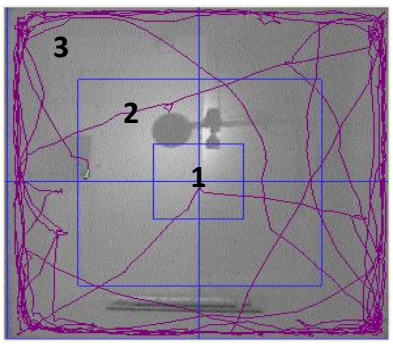
The open field (OF) consisted of a square-shaped arena (75 x 75 cm, length x width), constructed of white plastic and illuminated evenly with a 60w bulb. For tracking purposes, using EthoVision, the OF was divided into 3 virtual zones of concentric squares; an inner zone, a middle and an outer zone. For analysis purposes the two inner zones were combined to become one central zone versus the outer zone.

1. The Inner Zone (20*20cm)
2. The Middle Zone (40*40cm)
3. The Outer Zone (15cm periphery)

The image to the right shows the EthoVision tracking of a subject in the OF.

See **Chapter IV** for experimental details.

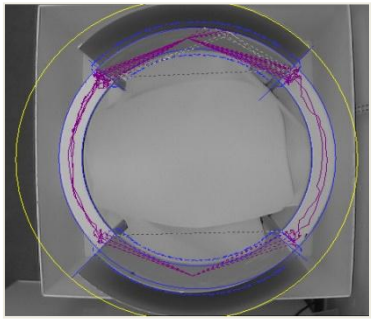


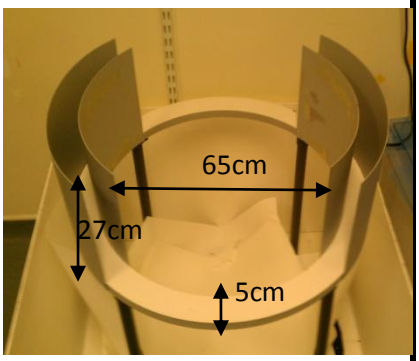


2.4.6: Elevated zero-maze

The elevated zero-maze (EZM) consisted of an elevated circular platform (5cm width) with a diameter of 65cm. Two of the quadrants are open and the other two quadrants are enclosed by 27cm high walls. The maze was constructed from Plexiglas, positioned 940mm from the floor and illuminated by a 60w bulb. The image below shows the EthoVision tracking of a subject on the EZM.

See **Chapter IV** for experimental details.





2.4.7: Lick cluster analysis

Testing took place in 16 home cages (32 x 15 x 12cm) modified to incorporate lickometer equipment (Med Associates Inc., St Albans, VT, USA), including metal grid floors and wire mesh lids. The lickometer accurately detected each lick to the nearest 0.01s. Data was recorded with custom written software using Med-PC-IV, running on a PC (Med Associates Inc., St. Albans, VT, USA). Sucrose solutions were presented through stainless steel drinking spouts attached to 50 ml cylinders. Drinking bottles were weighed before and after testing to assess consumption.

See **Chapter V** for experimental details.

2.4.8: Startle chambers

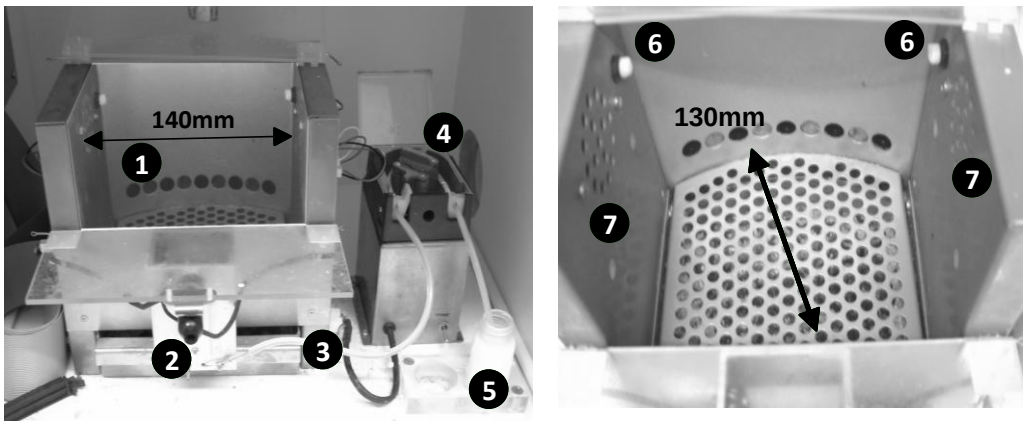
The startle chamber apparatus consisted of ventilated and sound attenuating SR-LAB startle chambers (San Diego Instruments, CA, USA) containing a non-restrictive Plexiglas cylinder (3.5cm in diameter) mounted on a Perspex plinth. Directly beneath the centre of the tube was a piezoelectric sensor that detected flexion in the plinth: the measure of startle reactivity. Above the animal enclosure (12cm) a loud speaker was mounted, via which all white-noise stimuli were presented. The motor responses of the subject were recorded via the piezoelectric accelerometer and values were transduced and digitised by a transducer linked to the computer running SR-LAB software. For each day of testing, the test chambers were calibrated, using mice of equivalent bodyweight to the test subject to equalise the measure of startle response from each chamber. Similarly, the peak amplitude of the stimuli presented in each chamber was made equivalent.

See **Chapter VI** for experimental details.

2.4.9: Apparatus for the progressive ratio and stop-signal reaction time task (SSRTT)

Testing was carried out in '9-hole' operant chambers (Cambridge Cognition Ltd, U.K.) based on a design first used in rats (Carli, Robbins, Evenden et al., 1983) and tailored for use in mice (Humby, Laird, Davies et al., 1999). The chambers were made of aluminium, with a clear Perspex roof, and a curved rear wall (at a distance of 130mm) into which nine 10mm diameter holes were set. Set into each aperture was a 2.5W bulb and for the progressive ratio task, aperture 5 (from the left side) was used, and for the stop-signal reaction time task (SSRTT) apertures 3 and 5 were used. Responses made by subjects into each aperture were measured by a vertically orientated infra-red beam. Opposite the aperture array was a food magazine (accessed through a clear Perspex door), into which condensed milk reward was delivered to a small food well in the floor via 0.8mm silicone tubing (Watson & Marlowe, U.K). This food delivery process was controlled by a peristaltic pump, with standard food delivery of 22µl (equivalent to driving the pump for 1 second). A micro-switch recorded when the door to the food magazine was opened, and two infra-red beams (located 5mm off the floor, emanating from the side wall) detected general movement in the chambers. A loudspeaker was also located in the side wall, as well as two infra-red LEDS (providing background illumination for the CCTV systems) and two 2.5W house lights. The chamber was located in a sound attenuating outer box complete with a fan to keep a constant low background noise and provide ventilation. Infra-red sensitive cameras mounted in the roof of the outer boxes meant that CCTV could be used to observe the mice (Watac WM6, Tracksys Ltd, U.K.), located 100mm above the chambers. Control of the chambers was managed by an Acorn RISC-PC computer running custom-written BBC BASIC V6 programmes with further interfacing by ARACHNID (CeNeS Ltd, U.K.).

See **Chapter V and VIII** for experimental details.



1 response array (9 apertures, 10mm diameter, spanned by vertical infra-red beams and each containing a small light), **2** food magazine (containing tray light and food well) accessed via a hinged panel, **3** silicone tubing, **4** peristaltic pump, **5** reinforcer bottle (10% condensed milk solution), **6** house light and **7** loudspeakers.

2.5 General data presentation and statistical methods

Behavioural data are presented as mean values (*M*) with the standard error of the mean (SEM), unless stated otherwise. Data for each line (C59X and C417Y) were analysed separately, and separate ANOVAs were performed with between subjects factor of GENOTYPE (homozygous ENU-mutant vs. heterozygous ENU-mutant vs. WT littermate control) and occasionally GENDER (male vs. female) as well as within subject factors as appropriate. All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Bonferroni corrections for multiple comparisons were applied where appropriate and Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. Specific statistical designs and analyses are described in the relevant experimental chapter.

2.6 Molecular methods

2.6.1 Standard genotyping and sequencing protocol

To identify the genotype of each mouse, DNA was extracted from tail biopsies (2mm), amplified by Polymerase chain reaction (PCR) and then sequenced to determine the presence/absence of either the C59X or C417Y mutation.

2.6.1.1 Tail lysis

A tail biopsy was collected from each mouse following weaning, at approximately four to six weeks of age. The collected tissue samples were placed in 1.5ml micro-centrifuge tubes and digested in 400µl of lysis buffer (0.2% SDS, 50mM Tris (tris(hydroxymethyl)aminomethane);pH=8.0, 10mM EDTA (ethylenediaminetetraacetic acid), 100mM NaCl (sodium chloride) with 2µl of Proteinase K (Qiagen, Crawley, U.K.) with a final concentration of 0.2mg/ml, at 55-60°C overnight.

2.6.1.2 DNA extraction

Digested tissue samples were spun in a centrifuge (13,000rpm, 14 min) to sediment debris, and the supernatant subsequently transferred into new micro-

centrifuge tubes. Next, an equal volume of cold isopropanol (i.e. 400µl) was added to the obtained supernatant and the samples left on ice for 20 minutes to facilitate DNA precipitation. Samples were then re-centrifuged (13,000rpm, 12 min) and the supernatant discarded. The tubes were then left to air dry for 1 hour to allow the isopropanol to evaporate. A volume of 50µl of TE buffer, (10mM of Tris and 1mM of EDTA, bought to pH=8 with hydrogen chloride to down-regulate the activity of nucleases), was added to stabilize and protect the dissolving DNA from degradation. Samples were then spun at 4°C overnight to allow any DNA adhering to the sides of the tubes to be resuspended into the TE buffer.

2.6.1.3 DNA quantification

DNA was quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific). The absorbance (A) of UV light at wavelengths (λ) of 260nm and 280nm was calculated. Assuming that an A (260nm) of 1 is equivalent to 50µg of DNA, a ratio of A (260nm) to A (280nm) of above 1.8 indicated a suitable amount of clean DNA without contamination by RNA and protein. The spectrophotometer calculated a concentration (in ng/µl) for the sample based on the UV absorbance.

2.6.1.4 Polymerase chain reaction (PCR) for genotyping

DNA was tested at a working dilution of 5ng/µl. PCRs were performed in 96-well skirted plates. Reaction mixtures were produced as per the protocol described in Table 2.1, using primer sets detailed in Table 2.2. The reactions were run in a Peltier Thermal Cycler (MJ Research, U.K.).

2.6.1.5 PCR purification

This purification stage removed unincorporated dNTPs, primers, primer dimers, salts and other contaminants from the samples. The PCR product was mixed with 21.6µl/sample Agencourt AMPure reagent (Beckman Coulter, USA), which contains metallic beads which adhere to the amplimers. Successive washing of the samples in 85% ethanol removed any products that were not adhered to the

magnetic beads and purified the samples. PCR amplimers were then eluted in 100µl of pure water into a new plate.

Table 2.1: PCR reaction protocol.		
***Zfp804a* C59X line**	***Zfp804a* C417Y line**	**Program-cycle**
6.18µl of nuclease-free H_2O	6.18µl of nuclease-free H_2O	**1.** 94°C for 15min
0.96 µl of dNTPs (5mM, Invitrogen, U.K.)	0.96 µl of dNTPs (5mM, Invitrogen, U.K.)	**2.** 95°C for 20sec
1.2µl of 10x Taq buffer (Qiagen, U.K.)	1.2µl of 10x Taq buffer (Qiagen, U.K.)	**3.** 55°C for 30sec
0.56µl of *Zfp804a* C59X Forward +Reverse primer (5uM)	0.56µl of *Zfp804a* C417Y Forward + Reverse primer (5*uM*)	**4.** 72°C for 45sec
0.1 µl of Taq Polymerase buffer (Qiagen, U.K.)	0.1 µl of Taq Polymerase buffer (Qiagen, U.K.)	**5.** Go to steps 2-4; 44 times
3µl of genomic DNA (5ng/µl)	3µl of genomic DNA (5ng/µl)	**6.** 72°C for 3min
12 µl	**12 µl**	**7.** 4°C for 5min

Table 2.2: Primer sets used for genotyping of the Zfp804a gene.			
Primers	**Primer sequence**	**Base pairs amplified**	**Genotype detection**
Zfp804a C59X Forward	**5'**aatatcatagaaaagaatcccact-**3'**	430	*C59X homozygous and heterozygous ENU-mutants and their WT littermate controls*
Zfp804a C59X Reverse	**5'**cttctgttcagagaaaaggtca-**3'**	430	*C59X homozygous and heterozygous ENU-mutants and their WT littermate controls*
Zfp804a C417Y Forward	**5'**tcatgttttcaaacttgttttg-**3'**	397	*C417Y homozygous and heterozygous ENU-mutants and their WT littermate controls*
Zfp804a C417Y Reverse	**5'**ttgattcatcatcacagaattac-**3'**	397	*C417Y homozygous and heterozygous ENU-mutants and their WT littermate controls*

2.6.1.6 Sequencing PCR

The purified and cleaned PCR product was added in a 6µl volume to a 4µl reagent mix consisting of 0.2µl of Big Dye termination mix, 0.8µl of water, 2µl of Big

Dye sequencing buffer and 1µl of Primer (either the Forward or Reverse oligonucleotide primer used in the original PCR reaction (3uM, see Table 2.2). The reactions were then run on the Thermal Cycler as detailed in Table 2.3.

Table 2.3: PCR conditions for the sequencing reactions.
1. 94 °C for 4 minutes
2. 94°C for 45 seconds
3. 61°C for 45 seconds
4. 72°C for 45 seconds
5. Repeat steps 2-4 , 35 times
6. 72°C for 5 minutes

2.6.1.7 Post-sequencing PCR clean-up

Post-sequencing clean-up employed an Agencourt CleanSEQ chemistry protocol (Beckman Coulter, USA) that was semi-automated. The process removed unincorporated dye terminators and other sequencing contaminants. Sequencing reaction product (10µl) was added to 10µl CleanSEQ reagent and 41.59µl of 85% ethanol and aspirated to mix. The sequencing product bound to magnetic beads contained in the CleanSEQ reagent. The non-bound sequencing reaction contaminants were washed off and removed in successive 85% ethanol wash steps. The cleaned sequencing product was then eluted in pure water (75µl) and was ready for analysis using a capillary sequencer.

2.6.1.8 Sequencing and analysis

All sequencing was performed using the fluorescent Sanger sequencing method via Big Dye termination chemistry and analysed using a 3130xl *Genetic Analyzer (Applied Biosystems, USA)*. The fluorescent sequencing reaction involved large amounts of template DNA and then the random incorporation of four fluorescently labelled ddNTPs (di-deoxy-dinucleotide-triphosphates: ddATP, ddCTP, ddGTP and ddTTP) that terminate after extending one base during primer extension. This produced a series of DNA fragments where the chain growth was terminated at each successive position. When electrophoresed in the 4-capillary sequencer and detected by a laser, each base of the sequence was fractioned by size and fluoresced

according to the base at that size. Sequencing required 12μl of PCR product for the genomic region to be sequenced.

The raw data generated by the 3130xl *Genetic Analyzer* was automatically analysed by Sequence Analysis Software (Applied Biosystems). The software reads the amount of fluorescence at each nucleotide as the corresponding base. Sequencher software (Gene Codes, USA) was then used, which aligned multiple sequencing traces and allowed comparison to a reference sequence. The software then highlighted where differences occurred between the traces, allowing the user to visually inspect these and judge whether a polymorphism existed (for each possible trace outcome for both lines, see Appendix 1.1).

2.6.2 Sex/genotype determination protocol for Zfp804a pup survival rates

In order to examine the survival rates, sex ratios and patterns of inheritance of the *Zfp804a* mutant lines it was necessary to determine the gender and genotype of the pups which did not survive to weaning.

2.6.2.1 Genotyping protocol for Zfp804a pup corpses

Pup corpses were genotyped and sequenced as described in the protocol set out in Section 2.6.1. DNA extraction and lysis were performed as described in Sections 2.6.1.1 and 2.6.1.2, except that the tissue used was whole head instead of tails.

2.6.2.2 Sex determination protocol for Zfp804a pup corpses

To determine the gender of the *Zfp804a* pup corpses a multiplex PCR was performed using a Y-linked gene family (*Ssty*) and an autosomal control gene *Om1a* (myogenin). PCR primer sets used can be seen in Table 2.4. The PCR reaction (25μl) contained 17.75μl of sterile water, 2.5μl 10x Taq buffer (Qiagen, UK), 1μl of dNTPs (5mM, Invitrogen, UK), 1μl of *Ssty* Forward primer (10uM), 1μl of *Ssty* Reverse primer (10uM), 0.25μl of *Om1a* Forward primer (500ng/μl), 0.25μl of *Om1a* Reverse primer (500ng/μl), 0.25μl of Taq polymerase (Qiagen, UK), and 1μl of DNA. The PCR conditions can be seen in Table 2.5.

Table 2.4: Primer sets used for sex determination of the Zfp804a mouse pups.			
Primers	**Primer sequence**	**Base pairs**	**Gender detection**
Ssty (Forward)	**5'**-ctggagctctacagtgatga-**3'**	343	Male
Ssty (Reverse)	**5'**-cagttaccaatcaacacatcac-**3'**	343	Male
Om1a (Forward)	**5'**-ttacgtccatcgtggacagcat-**3'**	245	Male and Female
Om1a (Reverse)	**5'**-tgggctgggtgttagtcttat-**3'**	245	Male and Female

Table 2.5: PCR conditions for sex determination of the Zfp804a mouse pups.
1. 94 °C for 4 minutes
2. 94°C for 45 seconds
3. 61°C for 45 seconds
4. 72°C for 45 seconds
5. Repeat steps 2-4 , 35 times
6. 72°C for 5 minutes

Once the reaction had finished 8µl of 6x DNA loading buffer was added to each reaction and a total of 30µl of the reaction/loading buffer mixture was loaded on a 1.2% agarose gel. Finally, 5µl of DNA ladder (Hyperladder IV, Bioline, U.K.) was loaded at both sides next to the reaction samples, and the gel subsequently run at 120 V for 20-25 minutes, allowing the DNA to separate. Once the gel had been run, the amplicons were examined. A single band at 245bp indicated the presence of the X linked gene *Om1a*, and confirmed the correct amplification of the DNA template. A further band at 343bp indicated the amplification of the Y-chromosome gene (*Ssty*), and hence the male gender of the sample.

Chapter III: Developmental and initial behavioural phenotyping of Zfp804a mutant mice

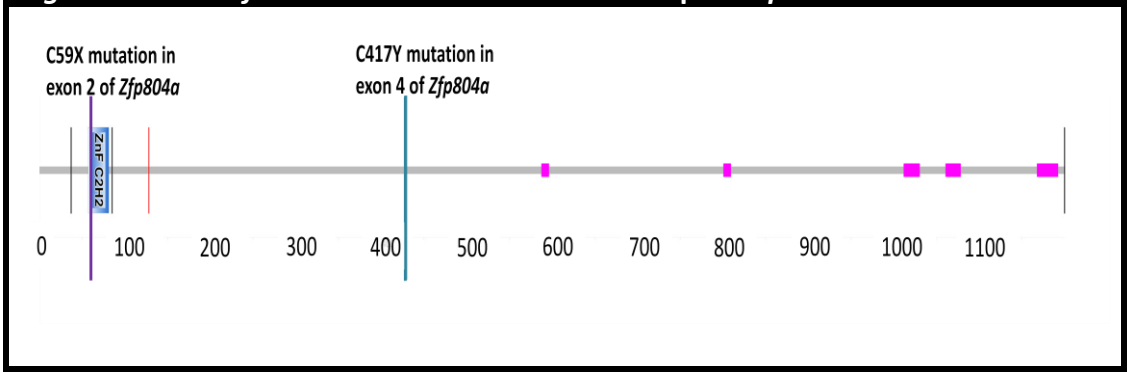
3.1 Introduction

This chapter is concerned with the developmental characterisation and initial behavioural phenotyping of the selected *Zfp804a* mutant lines. These were important investigations for a number of reasons. Firstly, the developmental profile of the ENU-mutants was of general interest, but also of specific interest given the well established hypotheses regarding the neurodevelopmental bases of mental disorders, including schizophrenia and bipolar disorder (Marenco & Weinberger, 2000; Owen, O'Donovan, Thapar & Craddock, 2011). Secondly, it was necessary to determine if there were any pre-existing differences in basic sensory and/or motor performance in the *Zfp804a* ENU-mutants lines which could have confounded the findings from the subsequent in-depth testing reported later in the thesis.

As previously described (Chapter II, Section 2.1) the Harwell DNA library of ENU-mutagenised mice was screened for mutations in *Zfp804a*, on mouse chromosome 2, by comparing the DNA melting profiles of the samples using Hi-Resolution Melting (Al-Janabi, 2012). Ten non-synonymous mutations in *Zfp804a* were discovered, with two selected to carry forward for re-derivation through *in vitro* fertilisation; the C59X mutation and the C417Y mutations were selected based upon the hypothesis that they should lead to functional effects (Fig. 3.1). The C417Y missense mutation located in exon 4 (genomic position 82097223) substitutes cysteine for tyrosine at aa417 (base change TGT>TAT). This mutation is thought to have damaging effects on the 3D structure of *Zfp804a* due to the substitution of the relatively small, nonpolar cysteine residue with a larger, polar tyrosine residue. PolyPhen (http://genetics.bwh.harvard.edu/pph/), a program which predicts the functional effects of human non-synonymous SNPs, but can also be used to predict the effects of non-synonymous SNPs in mice, found the C417Y mutation to be probably damaging. The C59X mutation located in exon 2 (genomic position 82076020) encodes a premature stop codon in the zinc finger domain at aa59 of the aa1200 Zfp804a protein (base change TGT>TAA). The expectation was that the mutant mRNA transcript would not be fully translated due to nonsense mediated

decay (NMD), which selectively degrades mRNAs harbouring premature stop codons (for a review of NMD see Chang, Imam and Wilkinson, 2007). If the aberrant mRNA transcript was to escape NMD and be translated it would produce a truncated protein with possible deleterious gain of function or dominant-negative activity (Chang et al., 2007).

Figure 3.1: Loci of the selected mutations in the* Zfp804a *protein.



Ten non-synonymous *Zfp804a* mutations were discovered in different samples screened from the Harwell mouse ENU DNA library. Two of these were selected for further investigation based on putative function. The C59X mutation, located in exon 2, encodes a premature stop codon, truncating the 1200aa protein prematurely at 59aa (aa6 of 28 comprising zinc finger domain, ZnF). The C417Y mutation, located in exon 4 (aa417), substitutes a nonpolar cysteine residue with a larger polar tyrosine residue. It is hypothesised that the C417Y mutation may affect the 3D structure of the protein. Founder mice which carried each of the mutations were re-derived and used to create lines of mice for behavioural and neurobiological investigation. Summary edited from SMART (Simple Modular Architecture Research Tool).

To generate mice for the experiments, founder mice for each of the lines (G1, bred on a BALB/c x C3H/HeJ background), heterozygous for the target mutations, were backcrossed onto a wild-type background (C57BL/6JOlaHsd) and positive progeny subsequently crossed to the same background, for a number of generations. An important consideration when using mouse lines derived from ENU mutagenesis is the presence of additional residual mutations in the genome, which may be inherited along with the mutation of interest, and could potentially confound any observed findings. Previous work by others in the lab assessed early growth and development of the $G4_i$ generation of C59X and C417Y homozygous ENU-mutant mice (and their WT littermate controls) and found no gross physical or developmental abnormalities (Al-Janabi, 2012), as well as no differences in the birth ratios between genotypes or sex. Initial investigations did, however, reveal that both male and female C59X homozygous ENU-mutant mice stayed longer on the RotaRod

and were lighter in adulthood than their heterozygous and WT littermates. With this earlier work carried out with the $G4_i$ generation there were no differences between genotypes in the C417Y line. Mice of the $G4_i$ generation were hypothesised to be only ca. 96% similar to the C57BL/6J strain, and would be expected to be carrying an average of 3-4 other ENU-induced in addition to the *Zfp804a* ENU mutations of interest (Keays et al., 2006). Thus, in the initial investigations (Al-Janabi, 2012) using *Zfp804a* mutant mice from the $G4_i$ generation, other ENU-induced mutations may have been present which could have, in theory, influenced phenotype. To minimize this potential problem, the current studies utilised mice from the the G7 generation (See Chapter II, Fig. 2.2) which should be >98.4% similarity to C57BL/6J and carrying on average less than 0.49 other residual mutations (as calculated from Keays et al., 2006). In addition to a general dilution of additional mutations, mice from different litters were inter-crossed at each stage of breeding as a way of disrupting the consistent transmission of other mutations in each mouse.

In the present work, heterozygous mice of 7th generation (G7) were then inter-crossed such that litters ($G7_i$ and $G7_{ii}$) would contain male and female, homozygous and heterozygous ENU-mutants and wild-type littermates according to Mendelian inheritance ratios. Using this approach, littermates would have identical rearing conditions and subsequent differences in behaviour could be attributed more confidently to the genomic differences present. Although no major developmental or behavioural effects of the mutations were observed at $G4_i$ (Al-Janabi, 2012) further developmental characterisation and behavioural screening were warranted with $G7_i$ and $G7_{ii}$ mice, as the extra 3 generations of breeding could have unmasked significant effects of the C59X and C417Y mutations. Here, investigations included an assessment of the weight gain, growth rate and emergence of somatic developmental indices in both of the *Zfp804a* ENU mutant lines, as well as the litter sizes and distribution of the different genotypes across gender. The total number of pups born and pre-weaning deaths was measured to assess the survivability of the mutant mice, and Mendelian inheritance patterns. In terms of the initial behavioural phenotyping, different tests were carried out to assess any deficits in sensory-motor functioning. Hence locomotor activity,

habituation to a novel environment, the SHIRPA screen and the reactivity to a novel food substance to test for neophobia were assessed.

The general strategy employed throughout the thesis of comparing the effects of the two ENU mutations relative to wild type littermate controls, was interesting insofar as it allowed investigation of the phenotypic consequences of different variants within the same gene. This strategy was also useful in helping to dissociate the specific effects of the ENU mutations from any effects on phenotype due to co-segregating genes from the original ENU-treated mouse strain which can be transmitted through generations of breeding in association with the gene-mutation of interest as a result of recombination, leading to linkage disequilibrium (Gerlai, 1996; Preuss, Riemenschneider, Wiedmann & Stoll, 2012). Common effects across both the C59X and C417Y ENU mutant mice, relative to wild type littermate controls, could theoretically be due to these 'hitchhiking' flanking genes. However, phenotypic dissociations between the two different lines are highly likely to be due to specific effects of ENU-induced changes in *Zfp804a* function.

3.2 Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods Chapter (Chapter II).

3.2.1 Subjects and animal husbandry

A subset of mice from the total litter cohort, consisting of mice from the *Zfp804a* $G7_{ii}$ C59X and C417Y lines were weighed and examined on several measures of somatic/neurological development during the first 30 days of postnatal life (See Table 3.1). Litters were derived from heterozygous*heterozygous pairings for each ENU-mutant line. At weaning, a tissue biopsy was made and DNA extracted for PCR analysis of genotype. As part of the developmental analysis, tissue from any dead pups found was also analysed for *Zfp804a* genotype and gender (See Chapter II, Section 2.6.2).

Table 3.1: Sample size and genotype of each cohort-subset of the Zfp804a mice assayed on measurements of somatic/neurological development.				
Mutation line	**Gender**	**Genotype**	**Weight and Growth Curves**	**Somatic Indices of development**
C59X line	♀	WT	19	19
		Heterozygous	29	29
		Homozygous	13	13
	♂	WT	10	10
		Heterozygous	35	35
		Homozygous	8	8
C417Y Line	♀	WT	8	8
		Heterozygous	35	39
		Homozygous	9	9
	♂	WT	9	11
		Heterozygous	12	13
		Homozygous	16	16

Initial behavioural testing was carried out on male mice from the C59X and C417Y lines of the $G7_i$ generation. Details of specific mouse numbers per genotype can be seen in Table 3.2. For the SHIRPA screen, mice were on average 6 months old at testing, and for the reactivity to a novel food substance mice were on average 7 months old. For the locomotor activity assay mice were tested in separate batches and had a mean age of 3 months at testing. All mice were housed in littermate groups of two to five animals per cage, under temperature- and humidity controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow and water, unless otherwise stated. Mice were weighed on a regular basis. Any mice dropping 10% of their body weight or more were removed from the experiment. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986.

Table 3.2: Sample size and genotype of each cohort-subset of mice assayed on the initial behavioural tasks				
Behavioural Task	**C59X line**		**C417Y line**	
	WT	**Homozygous**	**WT**	**Homozygous**
SHIRPA	14	16	16	19
Locomotor activity	14	17	15	19
Reactivity to novel food-substance	14	15	14	16

3.2.2 Neonatal health/viability of the lines

3.2.2.1 Assessment of birth weight and somatic/neurological indices of development

Pregnant dams were monitored twice daily so that accurate times of birth and birth litter sizes could be determined; fathers were removed from the cage when the litters were born. Body weights of the newborn pups, from the 2nd cohort of *Zfp804a* mice (termed as $G7_{ii}$), were registered on the day of birth, and subsequently monitored every day for the first 30 days of postnatal life. Somatic indices of development were systematically examined by visual inspection on a daily basis until mice were weaned, and consisted of the following parameters; the first day of ear opening (the unfolding of the ear pinna in both ears), incisor eruption (eruption of the upper incisors), fur growth (the first visible appearance of coat colour) and bilateral eye opening (the first visible break in eye lids and membranes covering the eyes).

3.2.2.2 Assessment of pre-weaning deaths/infanticide

For each cohort of mice, the date of birth and number of pups born was recorded. Litters were subsequently checked daily and the number of pups alive was noted, in addition to the number of pups found dead, mutilated or missing. Whole bodies (if intact) and heads (if found mutilated) were kept for DNA genotyping (see Chapter II, Section 2.6.1). A simple PCR assay was also carried out to determine the gender of the dead pups (see Chapter II, Section 2.6.2).

3.2.3 Initial behavioural phenotypic screen

3.2.3.1 SHIRPA phenotype assessment

SHIRPA (the **S**mithKline Beecham, **H**arwell, **I**mperial College, **R**oyal London Hospital, **p**henotype **a**ssessment), is a set of standardised screens designed to test rodent sensory-motor function (as described in Rogers, Fisher, Brown, Peters, Hunter & Martin, 1997, developed from Irwin, 1968). The range of tests include assessments of locomotor activity, startle responding, pain assessment and anxiety-related behaviours, but for the purpose of these studies 4 behaviours from the primary observational screen were used to assess aspects of sensory and motor function (Irwin, 1968; Rogers et al., 1997); 'Grip Strength', 'Visual Placing', 'Wire Manoeuvre' and 'Negative Geotaxis'. Apparatus for these procedures are described in Chapter II, Section 2.4.1).

The visual placing test assessed muscle and lower motor neuron function, as well as sensory and spinocerebellar function. Subjects were lifted by the base of the tail to a height of 15cm and lowered onto a flat surface. The experimenter then rated at what point the subject extended their forelimbs (scoring criteria for all tests can be found in Appendix 2.1). The grip strength test assessed spinocerebellar function and involved allowing the mouse to grip a horizontal bar. Applying a gentle backwards pull, the subject's resistance was then rated. The wire manoeuvre test assessed muscle strength and lower motor neuron function. For this, subjects were allowed to grasp a horizontal bar with their forelimbs. They were then held in extension and rotated around partially downward and released. Subjects were rated for their ability to grasp the bar with their forelimbs, but also their hindlimbs too. In the test of negative geotaxis, which assays an unlearned orientation response in opposition of cues of a gravitational vector (Fraenkel & Gunn, 1961), and which is thought to measure vestibular and proprioceptive function, the mice were placed on a wire cage lid, which was then raised to vertical orientation with the mouse facing the floor. The mice were rated for their ability to turn and climb the grid, in a 30-sec period.

3.2.3.2 Locomotor Activity

Locomotor activity (LMA) was tested in a battery of twelve clear Perspex chambers, each 210 x 360 x 200mm and equipped with two infra-red sensors projecting across each cage at 30mm from each end and 10mm from the cage floor (see Chapter II**,** Section 2.4.2). All animals were run in the dark between the hours 09:00 and 17:00 on three consecutive days in order to assess their habituation to a novel environment, with each animal run in the same chamber at a similar time on each day. The total number of beam-breaks made within each 2hr session was recorded, as well as the total number of beam breaks in each 30 minute time bin (quartile), using a computer running custom written BBC BASIC V6 programmes with additional interfacing by ARACHNID. Cages were thoroughly cleaned with 2% acetic acid between subjects.

3.2.3.3 Assessment of reactivity to a novel food substance

The reactivity to a novel foodstuff was assessed in the *Zfp804a* mice following a period of 24-hour water restriction as described previously (see Chapter II, Section 2.3.4). The test of food neophobia (a choice between water and the 10% condensed milk solution) was carried out within the reinforcer preference testing, which was implemented prior to behavioural paradigms which required habituation to a reward (the progressive ratio and stop-signal reaction time tasks, see Chapters IV and VII). Briefly, on each of the 6 test days, the mice were individually placed into a small chamber containing 2 small containers, for 10 minutes. The volume consumed from each container was calculated by weighing the containers before and after each test. On the first day of each experiment both containers contained tap water, but between the 2nd and 5th days of testing one of the containers contained the 10% condensed milk solution. On the 6th day of testing, both containers contained the 10% condensed milk solution to ensure that all mice had sampled the reward. The total volume consumed from both containers and the preference for 10% condensed milk solution (days 2 to 5) was calculated for each subject. Consumption data were normalised for potential body weight differences using Kleiber's 0.75 mass exponent (Schmidt-Nielsen, 1990). Further details of this procedure are described in Chapter II, Section 2.3.5.

3.2.4 Statistical analysis

Data were analysed using SPSS (Version 18.0) and presented as mean values (*M*) with the standard error of the mean (SEM), unless stated otherwise. All data were analysed by either ANOVA or t-tests with data for each strain (C59X and C417Y) and gender analysed separately, with separate ANOVAs performed with between-subjects factor of GENOTYPE (homozygous ENU-mutant vs. WT) and within-subject factors as appropriate. All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. T-tests were performed where appropriate. For the SHIRPA phenotype assessment, the non parametric chi-squared statistic was calculated. Outliers which were 2 standard deviations or more from the mean were removed from the analysis.

3.3 Results

3.3.1 Establishing the lines

As described previously (Al-Janabi, 2012 and Chapter II, Section 2.1), heterozygous ENU-mutant founder mice for the C59X and C417Y lines were crossed with C57BL/6J mice and positive progeny subsequently backcrossed to C57BL/6J mice for a further 4 generations. There were no obvious signs of breeding related problems in either line of mice during this time and reasonable numbers of positive mice of both genders were produced in each generation for each line. Heterozygous mice of the 7th generation were inter-crossed so that litters of each line were produced that contained male and female homozygous, heterozygous and WT littermate pups. Consequently, all litters were born to and raised by heterozygous mothers.

3.3.2 Physiological results

3.3.2.1 Neonatal health/viability of the C59X and C417Y mice

Subjects from the $G7_i$ and $G7_{ii}$ cohorts of C59X and C417Y mice were continuously monitored throughout the duration of their lives for general health and wellbeing. The genotype of subjects was ascertained when the mice were 3-4 weeks

old, by the methods described in Chapter II, Section 2.6.1. In the $G7_i$ cohort from 25 C59X and 23 C417Y litters, 188 and 185 mice were born, respectively (Table 3.3) and in the $G7_{ii}$ cohort from 39 C59X and 39 C417Y litters, 207 and 198 mice were born, respectively. Mean birth litter sizes were on average, between 5 and 8 for both lines, consistent with reported birth litter sizes for inbred C57BL/6J mice (Jackson Laboratories, 2009; Harlan Laboratories, UK). All pups appeared normal at birth, with no indications of observable health problems in either the C59X or C417Y lines. However, there were a perceived high number of deaths in the earliest days of postnatal life in both lines of *Zfp804a* mice, with approximately 19% of all mice of the $G7_i$ cohort and 35% of the $G7_{ii}$ cohort not surviving to weaning (combined pup mortality of 27% for both C59X and C417Y mice). It is unclear why there was a difference in survival between the two cohorts of mice, mothers were of the same breeding generation and litters were treated in the same way. Comparison with C57BL/6J mice suggests that these levels of post-natal mortality are, in fact, comparable with the pure inbred strain. One paper looking at murine infanticide found that 28.9% of C57BL/6J whole litters were found dead (Weber, Olsson & Algers, 2007), similar to the 25% and 27% rates found for both the C59X and C417Y lines, respectively, suggesting that *Zfp804a* maternal influences were not an issue in pup survivability. Where possible, the bodies of dead pups were collected. It was unclear as to why pups were dying, but a significant number had been mutilated (ca. 40%), presumably by the mothers. Comparable data for pure inbred C57BL/6J line is not available, so whether this reflects increased maternal aggression by C59X and C417Y heterozygous ENU-mutant mothers is not clear.

There was a noticeable imbalance between the number of male and female C59X mice weaned (male:female ratios for $G7_i$ and $G7_{ii}$ cohorts were 0.64 and 0.77, combined 0.70), suggesting possible pre-natal effects on male foetal development in this line or an increase in post-natal deaths of male mice (Table 3.3). Analysis of tissue from dead C59X pups appeared to confirm the former hypothesis, and showed that less male pups died during the post-natal period, suggesting that the decrease in male offspring is most likely to be a pre-natal effect. There were no gender differences in survivability of C417Y mice (male:female ratios for $G7_i$ and $G7_{ii}$ cohorts at weaning were 1.1 and 0.86, combined 0.98) however, the proportion of

male:female mice found dead pre-weaning was 0.57 for $G7_i$, 0.29 for $G7_{ii}$, and 0.58 combined, hinted that potentially fewer male mice were born for the C417Y line as well, also suggesting a pre-natal effect.

Table 3.3: Birth numbers and survivability of *Zfp804a* mutant mouse lines

Mutation line	C59X			C417Y		
Cohort	$G7_i$	$G7_{ii}$	Total	$G7_i$	$G7_{ii}$	Total
Total number born	188	207	395	185	198	383
Litters	25	39	64	23	39	62
Average litter size at birth	7.5	5.3	6.4	8	5.5	6.8
Total Infant mortalities	34 (18.1%)	71 (34.3%)	105 (26.6%)	35 (18.9%)	72 (36.4%)	107 (27.9%)
Whole litters found dead	3 (12%)	13 (33%)	16 (25%)	3 (13%)	14 (36%)	17 (27%)
Male:female mortalities	0.5	0.26	0.35	0.57	0.29	0.48
Number Mutilated	14	33	47	13	25	38
Number Unmutilated	20	38	58	22	47	69
Mutilated: unmutliated	0.70	0.87	0.81	0.59	0.53	0.55
Total surviving to weaning	154 (81.9%)	136 (65.7%)	290 (73.4%)	150 (81.1%)	126 (63.6%)	276 (72.1%)
Average litter size at weaning	6.2	3.5	4.5	6.5	3.2	4.5
Males weaned	60	59	119	78	58	136
Females weaned	94	77	171	72	68	140
Male:female weaned	0.64	0.77	0.70	1.1	0.85	0.98

Genotype distribution amongst the dead pups was analysed based on expected Mendelian ratios of 25% WT, 50% heterozygous and 25% homozygous with

heterozygous*heterozygous pairings. Analysis of dead C417Y pups (N=80) showed close to the expected Mendelian ratio for the different genotypes, with 24% homozygous ENU-mutants, 17% WTs and 59% of the dead pups revealed to be heterozygous ENU-mutants. Thus, survivability to weaning or maternal aggression to pups did not appear to be significantly moderated by the genotype of the pup, although there was a small tendency for more C417Y WT mice to survive to weaning. Genotype analysis of dead C59X pups was not as clear cut, it appeared that more WT pups were killed (ca. 43%) than homozygous (ca. 28.5%) or heterozygous (ca. 28.5%) pups, although this was based on a significantly smaller sample (N=7). Therefore, it would appear that male C59X and C417Y mice, of any genotype, seemed less likely to be born but, once born, C417Y WTs were more likely to prosper to adulthood, whereas C59X homozygous and heterozygous ENU-mutant mice were more likely to survive to weaning than their C59X WT littermate controls.

Table 3.4: Genotype analysis of weaned C59X mice

Cohort	Gender	♀			♂		
	Genotype	***+/+***	***mut/+***	***mut/mut***	***+/+***	***mut/+***	***mut/mut***
$G7_i$	**Number Weaned**	24	38	29	14	28	16
	Genotype (%)	26.37	41.76	31.87	24.14	48.28	27.59
	Expected n	22.75	45.50	22.75	14.50	29.00	14.50
	Concordance	1.05	0.84	1.27	0.97	0.97	1.10
$G7_{ii}$	**Number Weaned**	23	45	18	12	43	11
	Genotype (%)	26.74	52.33	20.93	18.18	65.15	16.67
	Expected n	21.50	43.00	21.50	16.50	33.00	16.50
	Concordance	1.07	1.05	0.84	0.73	1.3	0.67
Total	**Number Weaned**	47	83	47	26	71	27
	Genotype (%)	26.55	46.89	26.55	20.97	57.26	21.77
	Expected n	44.25	88.50	44.25	31.00	62.00	31.00
	Concordance	1.06	0.94	1.06	0.84	1.15	0.87

Expected n is based on the 1:2:1 Medelian ratio and concordance is the ratio between actual n and expected n, with a concordance value of 1.0 being perfect Mendelian inheritance.

At 3-4 weeks old, the pups were separated from their mothers, split into groups by gender (but littermates were kept together), and a tissue biopsy taken.

Analysis for each line showed that the distribution of the different genotypes (Tables 3.4 and 3.5) were close to Mendelian ratios of 1:2:1 (based on heterozygous*heterozygous pairings), suggesting similar survivability and viability of all C59X and C417Y mice.

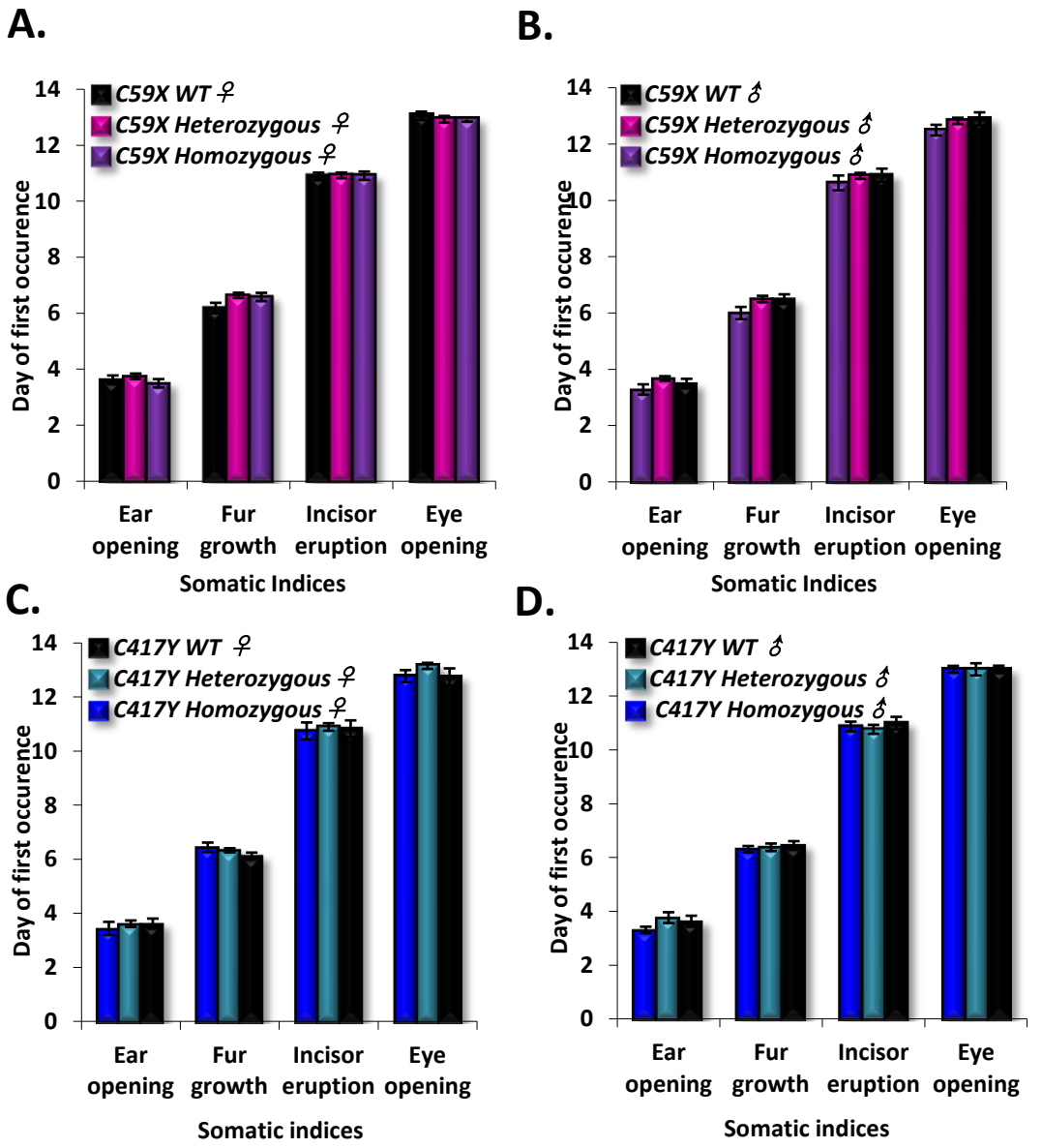
Table 3.5: Genotype analysis of weaned C417Y mice

Cohort	Gender	♀			♂		
	Genotype	***+/+***	***mut/+***	***mut/mut***	***+/+***	***mut/+***	***mut/mut***
$G7_i$	**Number Weaned**	17	33	20	14	44	19
	Genotype (%)	24.29	47.14	28.57	18.18	57.14	24.68
	Expected n	17.50	35.00	17.50	19.25	38.50	19.25
	Concordance	0.97	0.94	1.14	0.73	1.14	0.99
$G7_{ii}$	**Number Weaned**	9	49	12	17	22	21
	Genotype (%)	12.86	70.00	17.14	28.33	36.67	35.00
	Expected n	17.50	35.00	17.50	15.00	30.00	15.00
	Concordance	0.5	1.4	0.68	1.13	0.73	1.4
Total	**Number Weaned**	26	82	32	31	66	40
	Genotype (%)	18.57	58.57	22.86	22.63	48.18	29.20
	Expected n	35.00	70.00	35.00	34.25	68.50	34.25
	Concordance	0.74	1.17	0.91	0.91	0.96	1.17

Expected n is based on the 1:2:1 Medelian ratio and concordance is the ratio between actual n and expected n, with a concordance value of 1.0 being perfect Mendelian inheritance.

Somatic indices of development were monitored from birth and showed the expected order of occurrence with ear pinna opening (ca. postnatal day 3) preceding fur growth (ca. postnatal day 6) and incisor opening (ca. postnatal day 11). The final physical indication recorded was eye opening, which occurred between postnatal days 12 and 13 (Fig. 3.2). There were no significant differences between WT, heterozygous and homozygous ENU-mutants of either gender for either C59X or C417Y mice (See Appendix 2.3 for *p*-values).

Figure 3.2: Development of a number of somatic indices for both Zfp804a mutant lines and genders, cohort $G7_{ii}$.



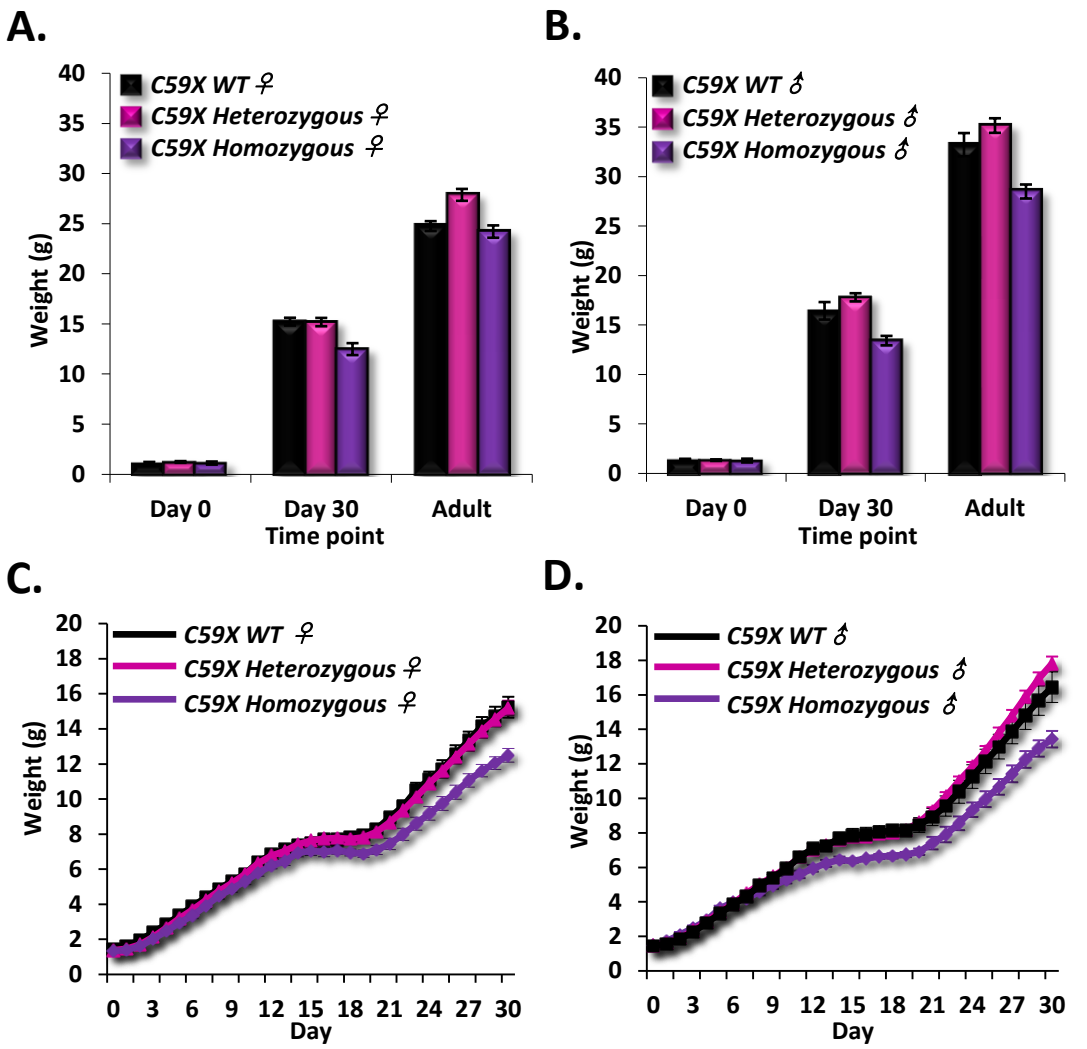
C59X (top) and C417Y (bottom) mice were checked daily from birth and the age at which certain developmental characteristics first appeared recorded. For both lines of *Zfp804a* mutant mice, there was no genotype or gender-related differences for any of these measures. Data shows the mean values ±SEM. For subject N see Table 3.1.

3.3.2.2 Postnatal growth

Postnatal growth was assessed by weighing a subset of C59X and C417Y mice from the $G7_{ii}$ cohort daily during the first 30 days of postnatal life. As expected, male mice in both lines were heavier than female mice, and all mice showed significant weight gain up to weaning. All mice showed growth curves with an initial dramatic increase in the rate of growth between days 1 and 6, and a secondary surge in

growth from approximately day 18 (see Appendix 2.4). For ease of analysis, separate analyses were used for the C59X and C417Y lines of mice and males were analysed separately from females. There were no differences in the birth weight of C59X female homozygous and heterozygous ENU-mutants or WT littermate control females (Fig 3.3A, main effect of GENOTYPE, $F_{2,50}$=1.9, p=0.16) or between male mice of each genotype (Fig. 3.3B, main effect of GENOTYPE, $F_{2,49}$= 0.2, p=0.87). However, when the mice were 30 days old it was noticeable that both female and male C59X homozygous ENU-mutant mice were lighter than their WT and heterozygous ENU-mutant littermates (main effect of GENOTYPE, $F_{2,60}$= 8.9, p=0.0, $F_{2,52}$= 11.1, p=0.00, respectively). This weight difference was maintained when the mice were adults (over 2 months old, main effect of GENOTYPE, $F_{2,42}$= 9.9, p=0.0, and $F_{2,45}$= 8.0, p=0.001), although for the females this effect was driven by the difference between homozygous and heterozygous ENU-mutants. Examination of the profiles of the weight changes in C59X mice showed that the growth characteristics of female and male homozygous ENU-mutant mice diverged from WT and heterozygous ENU-mutant mice at different times. Thus, through the first 10 days of life there were no differences in weight gain between the different genotypes of both female and male C59X mice (Fig. 3.3C and Fig. 3.3D, main effect of GENOTYPE, $F_{2,47}$=1.7, p=0.20, $F_{2,45}$= 0.01, p=0.99, respectively). However, male C59X homozygous ENU-mutant mice then weighed significantly less than their WT and heterozygous ENU-mutant littermates between postnatal days 11-20 and 21-30 (main effect of GENOTYPE, $F_{2,35}$=11.7, p=0.00, $F_{2,50}$=9.4, p=0.00, respectively). In contrast, the C59X females homozygous ENU-mutants weighed significantly less than their heterozygous littermates but had equivalent weights to their WT littermates between postnatal days 11-20 (main effect of GENOTYPE, $F_{2,40}$=3.4, p=0.04), but as with the males, the female C59X homozygous ENU-mutants weighed significantly less than their heterozygous ENU-mutant and WT littermates between postnatal days 21-30 (main effect of GENOTYPE, $F_{2,57}$=6.9, p=0.002).

Figure 3.3: Growth, as indexed by body weight in C59X mice



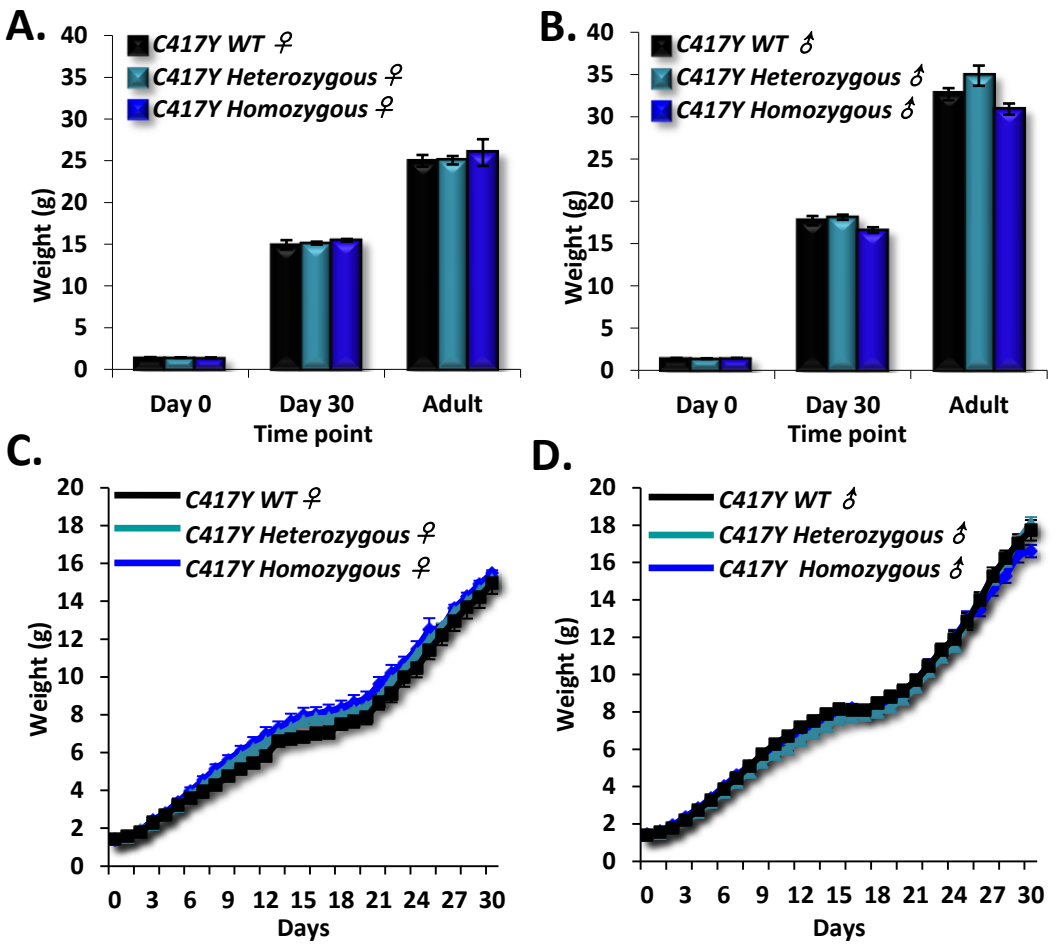
C59X female (A and C) and male (B and D) WT, heterozygous and homozygous ENU-mutant mice were weighed daily from birth until they were separated from their mothers at approximately 30 days old. There was no weight difference between the different groups of subjects at birth, for both female (A) and male (B) mice, but by day 30 C59X homozygous ENU-mutants of both genders were significantly lighter than their WT and heterozygous ENU-mutant littermates. This difference in weight was maintained in adulthood. There was a dissociation between female and male mice as to when the pattern of reduced weight originated, with male homozygous ENU-mutant mice (D) showing a divergence from their WT and heterozygous ENU-mutant littermates earlier than female mice (C); from day 11, thought to be around the time that mice are weaned onto solid food. Data shows the mean values ±SEM. For subject N see Table 3.1.

It is noticeable from Fig. 3.3D that the age at which the weights of the male C59X homozygous ENU-mutants start to diverge corresponds closely with the time when mice wean from their mothers, generally associated with the age at which the eyes are fully open (Silver, 1995), in this case between postnatal days 12 and 13 (Fig. 3.2A and 3.2B). Therefore it could be concluded that the decreased weight of male C59X homozygous ENU-mutant mice resulted from their transfer to solid food from

maternal milk, but the growth deficit maintained for the remainder of their lives. This was probably not the situation for the C59X female homozygous ENU-mutants (Fig. 3.3C), where the reduced growth appeared to occur later in the postnatal period, but still prior to separation from their mother. These differential patterns of growth are also apparent when investigating the percentage weight change/day through the early postnatal period (see Appendix 2.4), where between postnatal days 5-10 the C59X homozygous ENU-mutant males showed significantly reduced percentage growth than their WT littermate controls (main effect of GENOTYPE, $F_{2,50}$=3.5, p=0.04).

There were no differences between C417Y female WT, heterozygous and homozygous ENU-mutant mice at birth (Fig. 3.4A, main effect of GENOTYPE, $F_{2,51}$=0.2, p=0.82), at 30 days old (main effect of GENOTYPE, $F_{2,51}$=0.6, p=0.57) and in adulthood (main effect of GENOTYPE, $F_{2,40}$=0.3, p=0.72). There were also no significant differences between C417Y male homozygous, heterozygous and WT mice at birth (Fig. 3.4B, main effect of GENOTYPE, $F_{2,36}$=0.4, p=0.70), however, at postnatal day 30, C417Y male homozygous ENU-mutant mice weighed significantly less than their heterozygous littermates (main effect of GENOTYPE, $F_{2,36}$=4.9, p=0.014), with this effect still apparent in adulthood (main effect of GENOTYPE, $F_{2,33}$=5.9, p=0.008), although they had equivalent weights to their WT littermate controls. There were no differences in the weight gain between postnatal days 1-10 for both female and male C417Y mice (Fig. 3.4C and Fig. 3.4D, main effect of GENOTYPE, $F_{2,49}$=1.3, p=0.28, $F_{2,34}$= 1.2, p=0.32, respectively). Between postnatal days 11-20 C417Y female homozygous ENU-mutants weighed significantly more than their female WT littermate controls (main effect of GENOTYPE, $F_{2,49}$=4.2, p=0.021, pairwise comparison, p=0.019), however this increase was only transient as growth was equivalent between postnatal days 21-30 (main effect of GENOTYPE, $F_{2,49}$=1.1, p=0.33). C417Y males of all genotype showed equivalent weight changes through postnatal days 11-20 and 21-30 (main effect of GENOTYPE, $F_{2,34}$=1.3, p=0.29, $F_{2,34}$=0.3, p=0.75, respectively). Therefore, growth characteristics and final bodyweights of C417Y females (all genotypes) and C417Y males (all genotypes) were essentially equivalent, further confirmed when the % weight change/day is also considered (see Appendix 2.4).

Figure 3.4: Growth, as indexed by body weight in C417Y mice



C417Y female (A and C) and male (B and D) WT, heterozygous and homozygous mice were weighed daily from birth until they were separated from their mothers at approximately 30 days old. There were no weight differences between the different groups of subjects at birth, or at any other time of life, for both female (A) and male (B) mice, although heterozygous male mice were heavier than their WT and homozygous ENU-mutant littermates in adulthood (over 2 months old). There were equivalent rates of growth for both female (C) and male (D) C417Y mice. Data shows the mean values ±SEM. For subject N see Table 3.1.

Note that from this point on in the thesis only C59X and C417Y male homozygous ENU-mutants and their male WT littermate controls are discussed, although female data from both lines was collected.

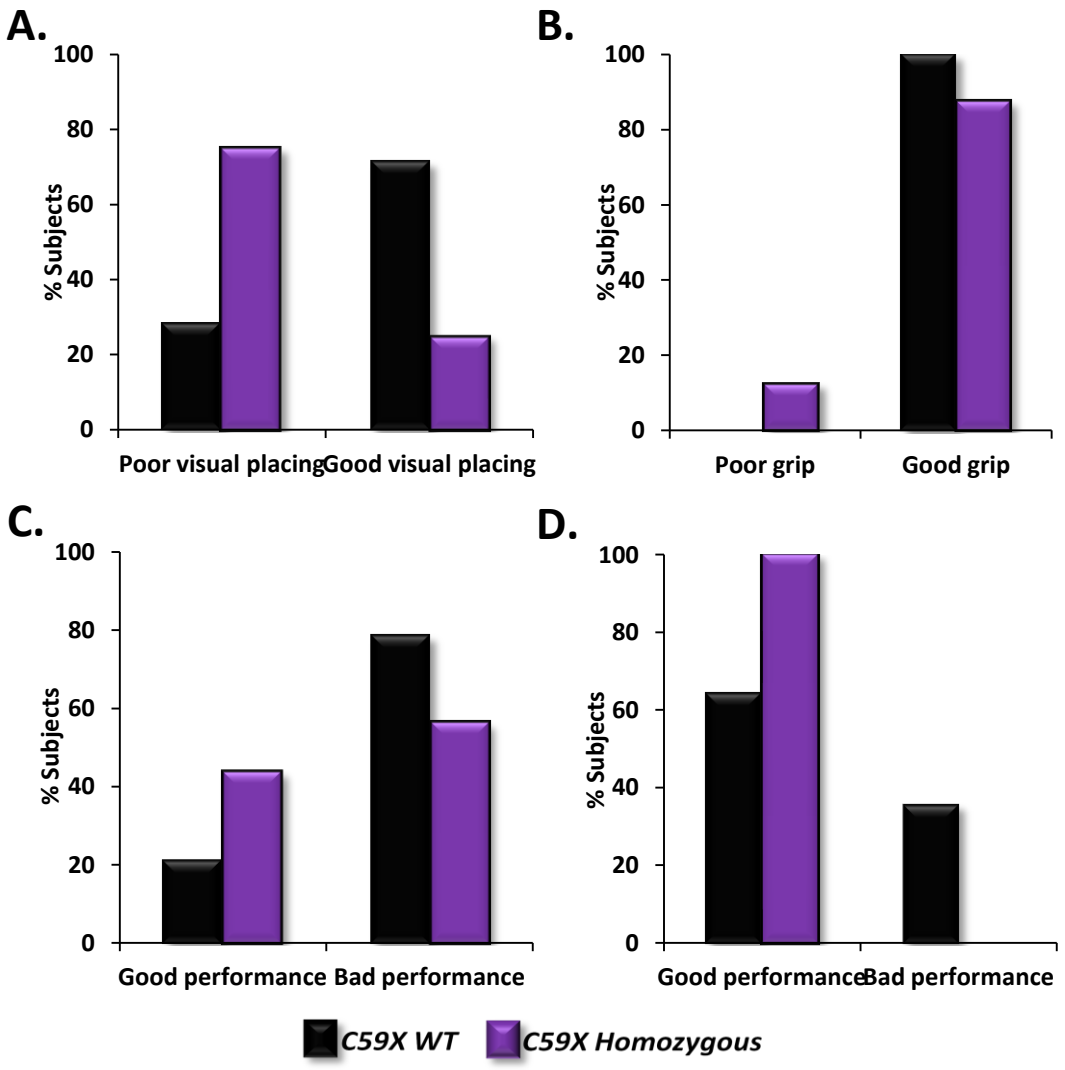
3.3.3 Zfp804a C59X behavioural screen results

3.3.3.1 SHIRPA performance

For the visual placing test (a measure of subject's sensory and spinocerebellar function) there was no relationship between the genotypes of the C59X line and

performance (Fig. 3.5A, x^2=3.45, df =1, p=0.75). There were also no genotype differences in the grip strength test, a different measure of spinocerebellar function (Fig 3.5B, x^2=1.88, df =1, p=0.30) where all mice demonstrated good levels of grasping.

Figure 3.5: Analysis of sensory, spinocerebellar function, muscle strength and negative geotaxis in C59X male mice.



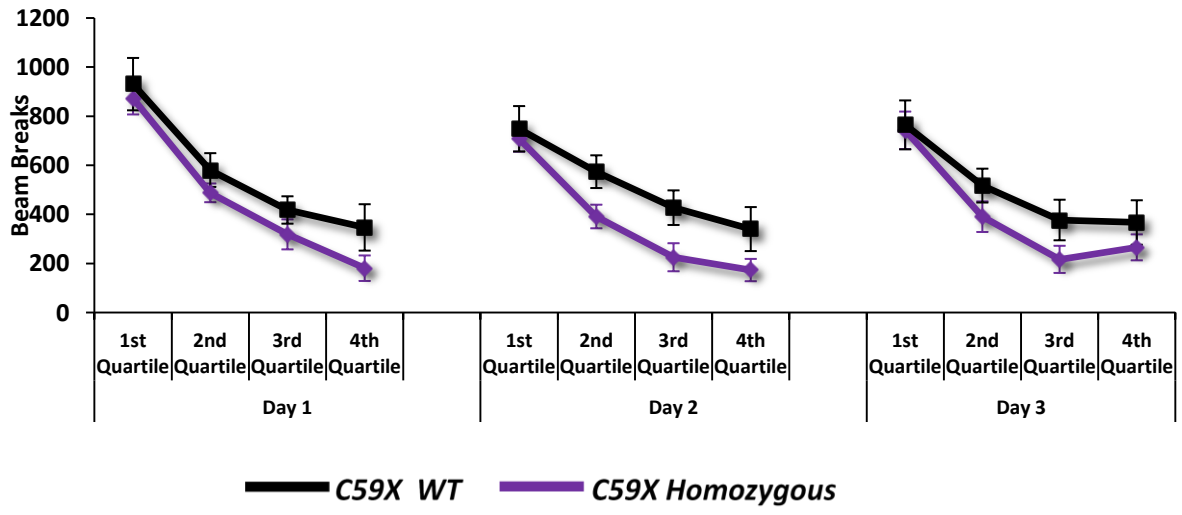
Four tests from the SHIRPA battery were used to assess simple sensory-motor abilities in C59X mice. Although there appeared to be a genotype-related difference in the test of visual placing (A) this was not significantly different, and on average all mice performed the test adequately. All mice showed good grip strength (B) and reasonable performance in the wire manoeuvre test (C). C59X homozygous ENU-mutant mice, however, showed significantly better abilities in the test of negative geotaxis (D) than their WT littermate controls. Data shows the mean values. For subject N see Table 3.2.

In the wire manoeuvre test, which indexes muscle strength and lower motor neuron function, there was no difference in the patterns of behaviour shown by the different groups of mice (Fig 3.5C, x^2=1.67, df =1, p=0.30), however, C59X homozygous ENU-mutant mice showed better performance in the test of negative geotaxis, with more mice better able to turn and climb the wire grid than their WT littermate controls (Fig 3.5D, x^2=6.9, df =1, p=0.01). Results from the SHIRPA screen suggest that mice of the C59X line have equivalent spinocerebellar and lower motor neuron function, as well as equivalent muscle strength. However, it appears that the C59X homozygous ENU-mutant mice have better vestibular and proprioceptive function than their WT littermate controls.

3.3.3.2 Locomotor activity performance

Locomotor activity (LMA) performance, measured as infra-red beam breaks, was assessed over three days, with separate 2-hour sessions/day. Although C59X homozygous ENU-mutant mice appeared to show reduced activity compared to their WT littermate controls, this difference was non-significant (Fig. 3.6, main effect of GENOTYPE, $F_{1,28}$=2.7, p=0.11).

Figure 3.6: Locomotor activity in C59X male mice.



Locomotor activity was assessed over three consecutive days, indexed by infra-red beam breaks. All mice demonstrated habituation within each 2hr session, and there were no significant differences in activity between C59X homozygous ENU-mutant mice and their WT littermate controls. Data shows the mean values ±SEM. For subject N see Table 3.2.

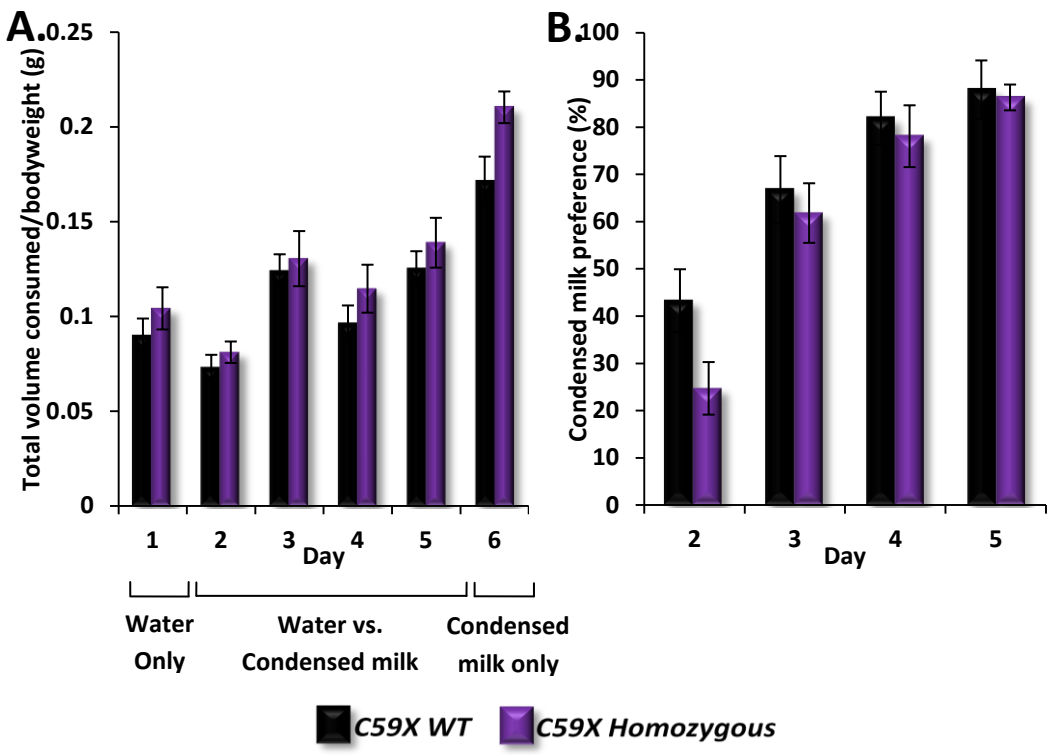
LMA reduced within sessions as all mice habituating to the novel environment (main effect of QUARTILE, $F_{1.5,41.3}$= 77.8, p=0.00), although overall activity was equivalent in each session (main effect of DAY, $F_{1.6,45.4}$= 2.9, p=0.08). There were no significant interactions between genotype and these other measures (DAY*GENOTYPE, $F_{1.6,45.4}$= 0.3, p=0.73, QUARTILE*GENOTYPE, $F_{1.5,41.3}$= 0.5, p=0.55) suggesting that, not only was overall activity equivalent, but that the C59X homozygous ENU-mutant mice and their WT littermate controls habituated to a novel environment in the same way.

3.3.3.3 Assessment of reactivity to novel foodstuff

Neophobia and habituation to a novel foodstuff (10% condensed milk solution, also used as the reward in appetitive conditioning paradigms, see Chapter II, Section 2.3.5) was assessed over 6 sessions. As there was a significant difference in bodyweight between C59X homozygous ENU-mutant mice and their WT littermate controls, Kleiber's 0.75 mass exponent method was used to normalise consumption to bodyweight (Schmidt-Nielsen, 1990). In the first session, where only water was available, the mice drank equally from each container (t_{28}= 1.0, p=0.33) suggesting that they did not show a side bias in which container they sampled solution from. Following the introduction of the novel foodstuff in the 2nd session, all mice increased their consumption (Fig. 3.7A, main effect of DAY, $F_{5,135}$= 36.4, p=0.00, DAY*GENOTYPE, $F_{5,135}$= 0.8, p=0.55), however there was no difference in overall consumption between C59X homozygous ENU-mutant mice and their WT littermates (main effect of GENOTYPE, $F_{1,27}$= 3.6, p=0.07). Initially, all of the C59X mice showed a neophobic response to the novel foodstuff (Fig. 3.7B), demonstrating a preference of less than 50%, although they overcame this and reached >85% preference for the novel foodstuff by the 4th choice session (main effect of DAY, $F_{3,81}$= 64.98, p=0.00, DAY*GENOTYPE, $F_{3,81}$= 1.7, p=0.17). This neophobic response to the reinforcer was most pronounced for the C59X homozygous ENU-mutant mice, having a significantly reduced condensed milk preference on the 1st choice day in comparison to their WT littermate controls (t_{27}= -2.2, p=0.04). When condensed milk preference was looked at over all 4 choice days, there were no genotype differences (main effect of GENOTYPE, $F_{1,27}$= 1.2, p=0.29), nor was there an effect of genotype on condensed milk preference for the final choice day (t_{27}= 0.3, p=0.81) indicating that both the

C59X homozygous ENU-mutants and their WT littermate controls had an equivalent preference for the condensed milk reinforcer over water.

Figure 3.7: Food neophobia and habituation in C59X male mice.



Neophobic responses to a novel foodstuff were assessed in 6 daily sessions. Following habituation to the test environment, where only water was present in the two drinking vessels, all of the mice readily consumed the available solutions (A). The novel foodstuff (10% condensed milk solution) was introduced on the 2nd day of testing, and it was noticeable that all C59X mice showed a neophobic (<50% preference) response on the first day (B), although this effect was significantly greater in the homozygous ENU-mutant mice. All mice then overcame this initial reluctance and showed significant preference for the condensed milk solution over water. Data shows the mean values ±SEM, normalised to bodyweight$^{0.75}$. For subject N see Table 3.2.

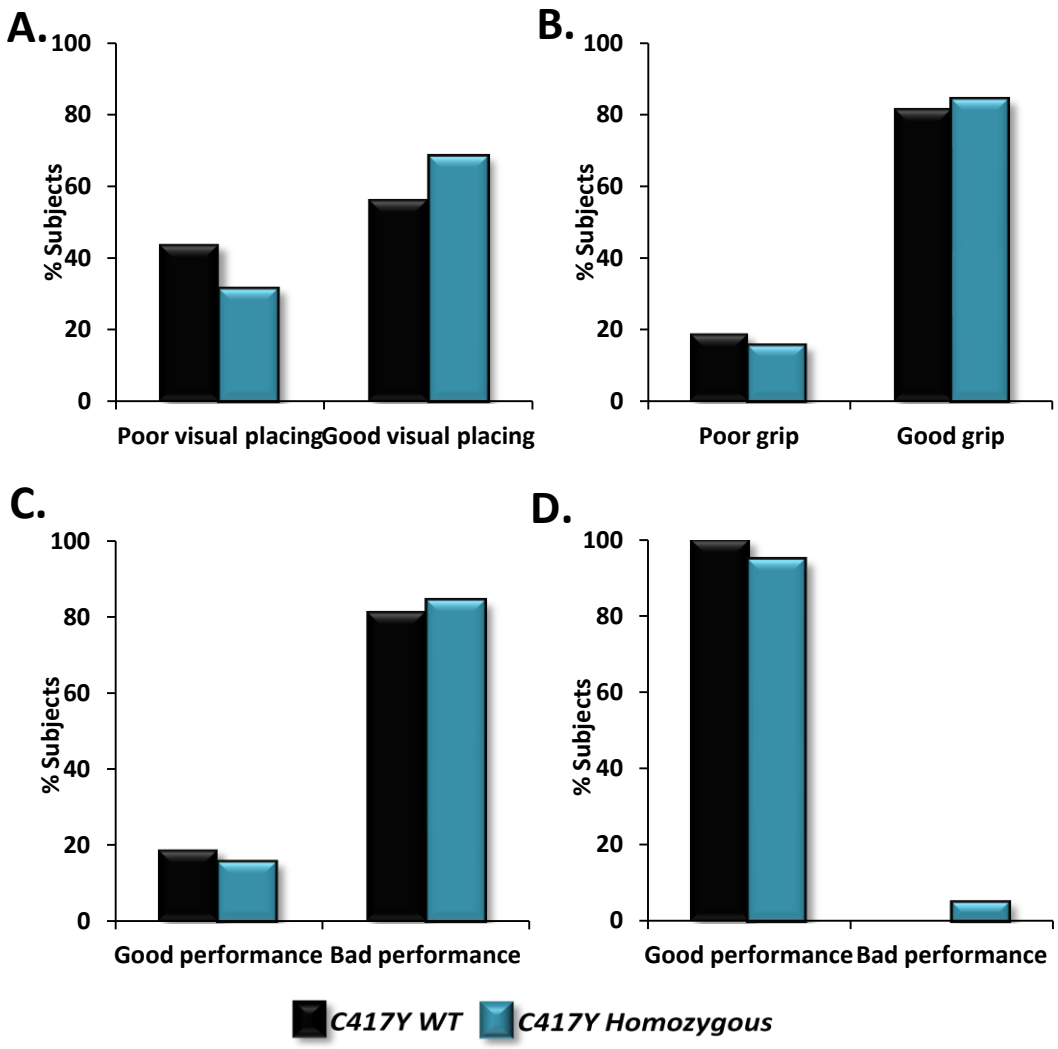
3.3.4 Zfp804a C417Y behavioural screen results

3.3.4.1 SHIRPA performance

Evaluation of C417Y mice on the sensory-motor function tests from the SHIRPA primary observational screen demonstrated no significant differences between C417Y homozygous ENU-mutants and their WT littermate controls in terms of spinocerebellar and lower motor neuron function, muscle strength and vestibular and proprioceptive function. There were no genotype-related differences in the visual placing (Fig. 3.8A, x^2=0.21, df =1, p=0.75), grip strength (Fig. 3.8B, x^2=0.05, df

=1, p=0.75) wire manoeuvre (Fig. 3.8C, x^2=0.05, df =1, p=0.75) or negative geotaxis (Fig. 3.8D, x^2=0.87, df =1, p=0.30) tasks.

Figure 3.8: Analysis of sensory, spinocerebellar function, muscle strength and negative geotaxis in C417Y male mice.



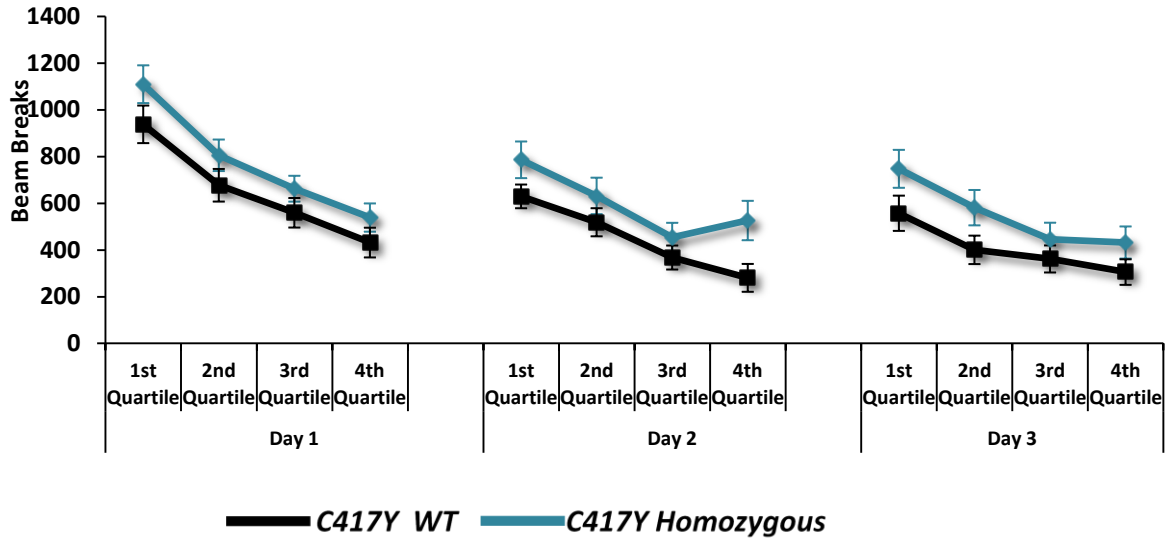
Four tests from the SHIRPA battery were used to assess simple sensory-motor abilities in C417Y mice. All the mice demonstrated good levels of behaviour in the test of visual placing (A), showed good grip strength (B), reasonable performance in the wire manoeuvre test (C) and good abilities in the test of negative geotaxis (D). Data shows the mean values. For subject N see Table 3.2.

3.3.4.2 Locomotor activity performance

There were no differences between C417Y mice in the assessment of LMA over the three 2hr sessions (Fig. 3.9, main effect of GENOTYPE, $F_{1,32}$= 2.8, p=0.1), although there was a tendency for C417Y homozygous ENU-mutant mice to be hyperactive in relation to their WT littermates. Within each session all of the mice showed habituation within sessions (main effect of QUARTILE, $F_{1.6,51.1}$= 90.4, p=0.00,

QUARTILE*GENOTYPE, $F_{1.6,51.1}$= 1.1, p=0.35) and habituation between each of the 3 sessions (main effect of DAY, $F_{2,64}$= 43.8, p=0.00, DAY*GENOTYPE, $F_{2,64}$= 0.1, p=0.91), but these alterations in locomotor activity did not differ between C417Y homozygous ENU-mutants and their WT littermate controls.

Figure 3.9: Locomotor activity in C417Y male mice.



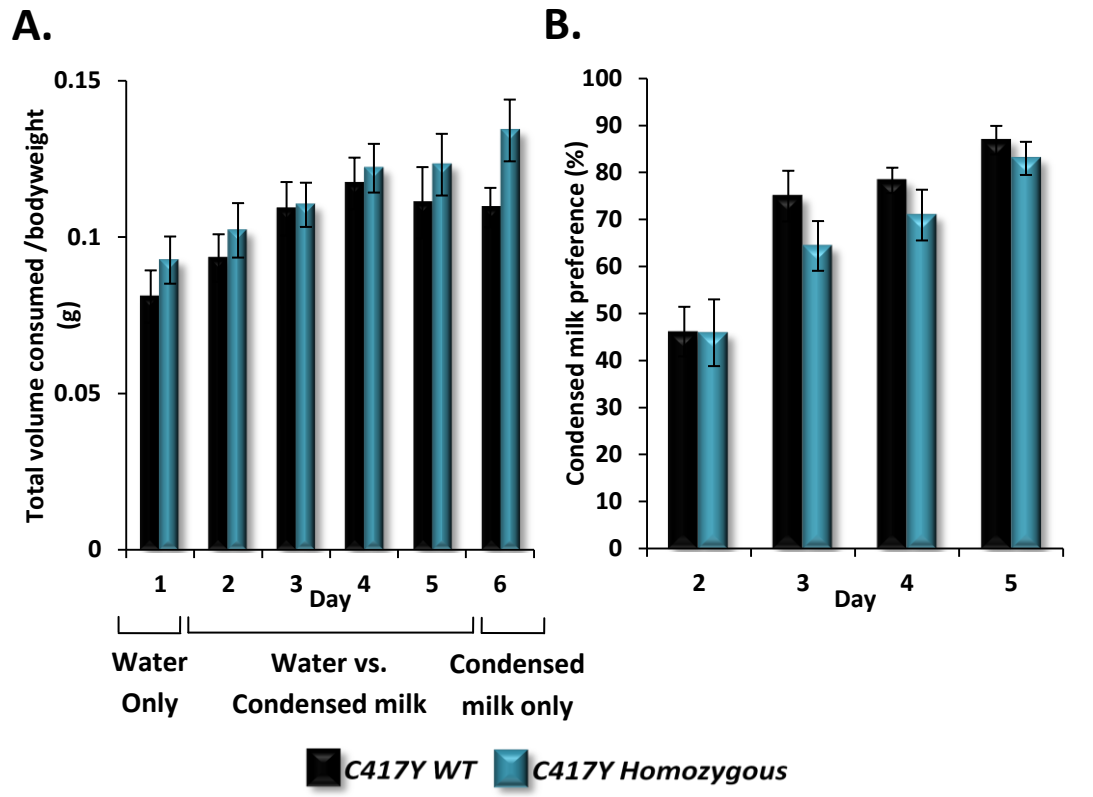
Locomotor activity was assessed over three consecutive days, indexed by infra-red beam breaks. All mice demonstrated habituation within each 2hr session, and there were no significant differences in activity between C417Y homozygous ENU-mutants and their WT littermate controls. Data shows the mean values ±SEM. For subject N see Table 3.2.

3.3.4.3 Assessment of reactivity to novel foodstuff

There were no differences between C417Y homozygous ENU-mutants and WT littermate controls in terms of their total consumption, normalised for bodyweight as previously described, (Fig. 3.10A, main effect of GENOTYPE, $F_{1,28}$= 1.7, p=0.2) and the volume of the solutions consumed increased on each of the 6 days of this test (main effect of DAY, $F_{5,140}$= 7.9, p=0.00, DAY*GENOTYPE, $F_{5,140}$= 0.07, p=0.65). Importantly, as with the C59X subjects, all C417Y subjects displayed an equivalent consumption between the two containers during the first day of testing (t_{29}= 0.2, p=0.87), illustrating that the spatial position of the containers was not a factor in subjects consumption. When the novel foodstuff was introduced from the 2nd session, all C417Y mice (Fig. 3.10B, main effect of GENOTYPE, $F_{1,28}$= 0.97, p=0.33) showed an increased preference for the novel foodstuff (main effect of DAY, $F_{2.3,64.1}$= 42.9, p=0.00, DAY*GENOTYPE, $F_{2.3,64.1}$= 0.8, p=0.5). C417Y homozygous ENU-mutant

mice and their WT littermate controls also showed equivalent neophobic responses (<50% preference) on the introduction of the novel foodstuff (t_{28}= -0.03, *p*=0.98) and had an equivalent preference for the condensed milk on the final choice session (t_{28}= -0.8, *p*=0.42).

Figure 3.10: Food neophobia and habituation in C417Y male mice.



Neophobic responses to a novel foodstuff were assessed in 6 daily sessions. Following habituation to the test environment, where only water was present in the two drinking vessels, all of the mice readily consumed the available solutions (A) and demonstrated increasing preference for the novel foodstuff (10% condensed milk solution), following an initial neophobia (B), with no genotype differences in these measures. Data shows the mean values ±SEM, normalised to bodyweight$^{0.75}$. For subject N see Table 3.2.

3.4 Discussion

3.4.1 General viability of the models

There were no obvious signs of breeding related problems in either mutant line with all pups appearing normal at birth, with no indications of observable health problems in either the C59X or C417Y lines. The mean litter sizes at birth for both the C59X and C417Y lines were consistent with reported data for the inbred C57BL/6J

strain (Jackson Laboratories, USA; Harlan Laboratories, UK), suggesting that the there were no major fertility problems with either of the *Zfp804a* mutant lines. However, there were a significant number of pups found dead in both lines (27%), with a high percentage of these found mutilated (40%); presumably by the mother. This may reflect an increased level of maternal aggression in the heterozygous mutant mothers, although comparison with the pure inbred C57BL/6J strain suggests that this post-natal mortality rate was in fact at normal levels (Weber et al., 2007), and therefore pup survivability was not unduly influenced by *Zfp804a* maternal aggression.

The distribution of gender and genotypes within the surviving *Zfp804a* mice uncovered an imbalance of C59X male mice weaned, with equivalent male to female ratios for the C417Y line. There were no significant differences in the Mendelian distribution of genotypes (1:2:1, for heterozygous* heterozygous pairings) for mice not surviving to weaning. There were, however, fewer males killed pre-weaning for both mutant lines, suggesting that fewer male mice were born in total for both the C59X and C417Y lines. As there is no available data from the pure inbred C57BL/6J line to compare these findings with, it is difficult to conclude whether the C59X and C417Y mutations are influencing pre-natal male survivability or if the fewer males born to both lines were simply a natural occurrence for this ENU-mutant mouse strain. At weaning, Mendelian distribution of genotypes was also seen, further suggesting non-genotype related reasons for post-natal mortality.

There were no birth weight differences between any of the different genotypes of C59X and C417Y mice for either gender, with all mice showing the expected pattern of weight changes with increasing age, as well as a plateau between post-natal days 12 and 19 when they wean from the mothers and begin eating solid food (Silver, 1995). At 4 weeks old, when the mice were separated from their mothers, both C59X male and female homozygous ENU-mutant mice were significantly lighter than their WT littermate controls, a difference which was maintained into adulthood in the male C59X homozygotes. Interestingly, this difference in weight originated at different times, where C59X male homozygous ENU-mutant mice showed reduced growth from the time when they weaned from their mothers (ca. 12 days old) whereas C59X female homozygous ENU-mutant mice

showed significant weight difference from their WT littermates approximately 7 days later. It is thought that complete weaning from their mother's milk occurs when the pups eyes are fully open (ca. 13 days old, Silver, 1995), suggesting that the reduced bodyweight of the C59X male homozygous ENU-mutants may be related to poor weaning in these mice. This could be due to increased neophobia to the solid food, increased anxiety to explore the cage or perseveration of maternal contact. The weight difference seen in the C59X female homozygous ENU-mutants occurs later than this, and is not likely to be related to weaning as eye opening occurred at an equivalent time for all C59X female genotypes, as well as at an equivalent time to the C59X males. There were no differences in the growth characteristics or weight of C417Y male and female homozygous ENU-mutant mice relative to their WT and heterozygous littermates. Furthermore, somatic indices of development, such as ear and eye opening, emerged at equivalent time points between both C59X and C417Y mutants and their WT counterparts. As the early life development of the females in both the C59X and C417Y ENU-mutant lines was not affected by genotype, it was decided that for the behavioural aspects of the thesis, only the males of both lines would be tested.

Early life trauma, such as birth complications, foetal infection and growth retardation were discussed in the General Introduction (see Section 1.4) as factors thought to increase risk for schizophrenia. The C59X and C417Y mutations did not lead to lower birth weights, but the C59X male homozygous ENU-mutants did weigh significantly less than their WT counterparts at weaning and in adulthood, replicating the reduced adult weight found with the C59X homozygous ENU-mutants in the *Zfp804a* $G4_i$ cohort (Al-Janabi, 2012). The C59X homozygous ENU-mutant mice from the $G4_i$ cohort also showed reduced brain weight, albeit not significantly reduced from their WT littermate controls. It is difficult to relate these findings with other mouse models for schizophrenia as these types of analysis have not been presented previously, to our knowledge, but reduced adult body and brain weights have been reported in pups that, for example, were exposed to a low protein diet *in utero* (Barnes, Neely, Kwong et al., 1968; Marichich, Molina, & Orsingher, 1979).

Schizophrenia and bipolar disorder are thought to arise from a complex interaction between genetic predisposition and early life adversity (van Os, Rutten &

Poulton, 2008). These adverse early life events can occur prenatally, for example with maternal malnutrition (Susser & Lin, 1992), and/or postnatally with infection and obstetric difficulties (e.g. McGrath & Murray, 2011). Abnormal early life characteristics have been shown in both of the C59X and C417Y homozygous ENU-mutants, with the reduced bodyweight seen in the C59X homozygous ENU-mutants (relative to their WT littermate controls) potentially due to delayed weaning to solid food and fewer males born to both mutant lines, perhaps due to an unknown gender related pre-natal effect caused by the mutations. It would therefore be interesting to examine a number of litters prenatally to ascertain the number, genotype and gender of pups *in utero* to determine if more male pups are present prenatally and if Mendelian ratios are intact before birth.

3.4.2 Behavioural phenotypic screen

A preliminary SHIRPA screen (Rogers et al., 1997) was conducted assessing the visual placing, grip strength, wire manoeuvre and negative geotaxis capabilities of the *Zfp804a* mutants. WT mice of both lines showed good visual acuity, strength and co-ordination and were able to correctly orient themselves on a vertical grid, respectively. C417Y homozygous ENU-mutant mice did not differ from their WT littermates in any of these tests. C59X homozygous ENU-mutants displayed equivalent performance levels to their WT counterparts on all of the SHIRPA tests except for the negative geotaxis assay, displaying an enhanced ability to turn and orient themselves on a vertical grid, as compared to their WT littermate controls. This, however, may not be due to enhanced vestibular/proprioceptive abilities in the C59X homozygous ENU-mutants but rather point to poorer WT capabilities, where 35% of the C59X WT controls showed poor negative geotaxis performance as compared to 0% poor performance in the C417Y WTs. Previous testing of the $G4_i$ *Zfp804a* mutants uncovered a genotype effect on another sensorimotor task; the RotaRod performance test, which assessed balance, co-ordination, grip and fatigue. Here, C59X male homozygous ENU-mutants were able to stay on the RotaRod longer than their WT and heterozygous counterparts, indicating greater sensorimotor abilities on this task (Al-Janabi, 2012).

Locomotor activity and reactivity to novel foodstuff were also tested in the *Zfp804a* ENU-mutants. Neither the C59X nor the C417Y mutation induced locomotor activity differences in the locomotor activity task (LMA), with all mice habituating to the novel environment at an equivalent rate; reducing their locomotor activity as the sessions progressed. There was a tendency for the C59X homozygous ENU-mutants to display reduced levels of activity as compared to their WT littermate controls, although this was not found to be significant. This pattern of effects is comparable to the previous study (Al-Janabi, 2012), with the $G4_i$ cohort of mice, where C59X homozygous ENU-mutant mice displayed significant reductions in locomotor activity in all sessions with C417Y homozygous ENU-mutant mice not differing from their WT counterparts. The difference in effect size between the two studies may be accounted for by other ENU-derived gene mutations present in the $G4_i$ cohort of mice, in addition to the *Zfp804a* target mutation (on average 3.88 mutations, See Chapter II, Fig. 2.2), whereas in the $G7_{i/ii}$ cohorts other non-target gene mutations had been effectively eliminated. Alternatively, and much more likely, it may be due to slight variations in methodological details.

The reactivity to novel foodstuff did not uncover any neophobia or consumption differences between the C417Y homozygous ENU-mutants and their WT littermates, suggesting no overall differences in thirst, hunger, satiation, anxiety or motivation to freely drink. C59X homozygous ENU-mutants displayed significant neophobia to the novel foodstuff, consuming significantly less than their WT counterparts. This could be related to heightened reactivity in the C59X homozygous ENU-mutants, although this initial reaction disappeared after further presentations of the foodstuff with both C59X homozygous ENU-mutants and their WT controls habituating to the solution and displaying equivalent consumption levels. Overall, these findings indicated there were no major sensorimotor deficits in the *Zfp804a* mutants prior to going on to perform the more complex behavioural assays detailed later in the thesis.

3.4.3. Summary of key results from Chapter III

- Fewer male mice were born to both *Zfp804a* mutant lines.
- A Mendelian genotype inheritance pattern was observed for weaned *Zfp804a* mice from both lines.
- C59X male homozygous ENU-mutants weighed significantly less than their WT littermate controls between PND 11-20, at weaning and in adulthood.
- C59X female homozygous ENU-mutants weighed significantly less than their WT littermate controls between PND 21-30.
- C59X homozygous ENU-mutants demonstrated significantly better performances on the negative geotaxis assay of the SHIRPA screen than their WT littermate controls.
- No locomotor differences between homozygous ENU-mutant mice and their WT counterparts in either *Zfp804a* mutant line.
- C59X homozygous ENU-mutant mice displayed neophobia to a novel foodstuff compared to their WT littermate controls.

Chapter IV: Investigating anxiety-related behaviour in Zfp804a mutant mice; *elevated plus-maze, open field and elevated zero-maze*

4.1 Introduction

Chapter III showed that the *Zfp804a* mutant lines were not compromised by any gross developmental or physiological effects which would undermine further testing. This chapter describes a detailed assessment of emotional functioning in the mutant lines, focusing on anxiety-related behaviours assessed using three separate tests of anxiety.

As mentioned previously, in a GWAS published in 2008, *ZNF804A* was the top hit for an association with schizophrenia, with this association strengthened when bipolar disorder cases were added to the affected sample (O' Donovan et al., 2008), providing further support for the view that there may be an overlap between schizophrenia and bipolar disorder in terms of their genetic risk architecture (Owen, Craddock and Jablensky, 2007). Moreover, this can be seen in a GWAS undertaken by Steinberg and colleagues (2011), where they found a copy number variant, in the form of a deletion of *ZNF804A,* in both a schizophrenia patient and an anxiety disorder patient. It is also recognised that there can be overlap between schizophrenia and bipolar disorder symptomatology, leading to the idea that the two disorders may be better viewed as lying within a spectrum and not the polarised ''Kraepelinian dichotomy'' previously thought (See Chapter I, Section 1.1). Emotional problems occur in both bipolar disorder and schizophrenia, indeed schizophrenia is in many cases co-morbid with anxiety disorders. A literature review of studies published between 1966 and 2004 found that individuals with schizophrenia spectrum disorders had a lifetime prevalence rate for any anxiety disorder ranging from 30% to 85% with most studies showing rates higher than in the general population (Pokos & Castle, 2006). In more than half of the patients, the anxiety disorder preceded the onset of psychosis, potentially resulting in a misdiagnosis. Another literature review illustrates the variability in prevalence rates of anxiety disorders, but adds further weight to the observation that anxiety co-morbidities are prevalent in schizophrenia and that treatment for anxiety could help alleviate the symptoms in those patients (Braga, Petrides & Figueira, 2004).

Other studies have shown co-morbidity of schizophrenia with panic attacks (Argyle, 1990), obsessive-compulsive disorder (Fenton & McGlashan, 1986; Eisen, Beer, Pato et al., 1997), social anxiety (Pilkonis, Feldman, Himmelhoch & Cornes, 1980; Penn, Hope, Spaulding et al., 1994), posttraumatic stress disorder (Meyer, Taiminen, Vuori, Aijala & Helenius, 1999) and agoraphobia (Braga, Mendlowicz, Marrocos & Figueira, 2005), with a further study illustrating the prevalence of obsessive-compulsive disorder (OCD, 30%) and panic disorder (15%) in bipolar disorder (Cosoff & Hafner, 1998). Despite the co-morbidity between schizophrenia and anxiety disorders, there are very few mouse models which have included a detailed assessment of anxiety phenotypes, with the limited number of studies that have done work in this area giving rise to mixed results. For example, heterozygous neuregulin 1 mice ($NRG1^{+/-}$) showed equivalent levels of anxiety to wild-type controls on the elevated plus-maze (EPM) but demonstrated less anxiety in tests of neophobia or social interaction (Karl, Duffy, Scimone et al., 2007; O'Tuathaigh et al., 2008). *DISC1* knockout mice were no different to wild-types on the elevated plus maze (Clapcote et al., 2007), nor were *DISC1* dominant negative mice different to their WT counterparts in the open field test of anxiety (Hikida et al., 2007). These previous data indicate that anxiety is likely to be an interesting phenotype to examine in the *Zfp804a* mutant lines and that the analysis should utilise a range of methods which tax different facets of anxiety-related behaviour.

The neurobiological underpinnings of anxiety are only partly established, although research has implicated the limbic system, with particular emphasis on the hippocampus and amygdala (Engin & Dallas, 2007; Davis, 1992; Etkin, Prater, Schatzberg et al.,2009) with ventral hippocampal lesions leading to reduced anxiety in the light/dark exploration test (Bannerman, Grubb, Deacon et al., 2003). A number of neurotransmitter systems have also been implicated in anxiety behaviour, including GABA-ergic and serotonergic mechanisms. Indeed, benzodiazepines work by enhancing the effect of GABA, and serotonin reuptake inhibitors are thought to alleviate anxiety-related symptoms, suggesting that reduced serotonin levels may modulate the symptoms of anxiety (Baldwin & Rudge, 1995; Ballenger, 1999). Furthermore, certain GABA-ergic and serotonergic receptor subtypes have been

found to be greatly expressed in the hippocampus (Mehta and Ticku, 1999; Hoyer, Clarke, Fozard et al., 1994).

The assays used in this chapter were the EPM, the open field (OF) and the elevated zero-maze (EZM); all well validated tasks in the study of rodent anxiety (for reviews see Lister 1990; Sousa, Almeida & Wotjak, 2006; Ramos, 2008; Sartori, Landgraf & Singewald, 2011). All 3 anxiety assays tap into unconditioned approach-avoidance conflict situations based on two conflicting innate rodent tendencies: exploring a novel environment and avoiding elevated/open spaces with potential danger, including predator risk. Whilst the relevance of these animal tests of anxiety to human psychology and behaviour is a topic of debate (Sartori et al., 2011), they do appear to give rise to significant overlap in terms of sensitivity to anxiety provoking (anxiogenic) and anxiety relieving (anxiolytic) effects of drugs such as beta-carbolines (File & Baldwin, 1987; Rägo et al., 1988) and benzodiazepines (Treit, Engin & McEown, 2010; Prut & Belzung, 2003; Shepherd, Grewal, Fletcher et al., 1994).

The EPM (Pellow, Chopin, File & Briley, 1985) is the most widely used exploratory paradigm of anxiety in rodents, partly because it is rapid to perform but also because it has been well validated with both anxiolytic and anxiogenic agents (Lister 1987a). The EPM assays aversion to elevated and open spaces, and consists of four arms where two opposing arms are enclosed by high walls and the other two arms are open. Anxiety is determined by measuring the frequency of entries and time spent by subjects in the open arms of the maze relative to the enclosed arms, in addition to a range of other behaviours sensitive to anxiety-provoking stimuli such as head-dips and stretch-attend postures (Rodgers et al., 1997). The OF (Walsh and Cummins, 1976) is an enclosed square arena (80cm in diameter) assaying the aversion of mice to open illuminated spaces. Rodents will spend a large proportion of time around the periphery, with the tendency to remain close to the walls (thigmotaxis) used as an index of anxiety. The EZM (Shepherd et al., 1994) is a modification of the EPM, and consists of two open and two enclosed elevated arms which form a zero/circle, thus lacking the ambiguous central area of the EPM. Like the EPM, the EZM measures the aversion of mice to elevated open spaces.

Due to the multifaceted nature of anxiety, it can no longer be assumed that all rodent anxiety tests are measuring the same component of emotionality (Ramos,

2008; Sousa et al, 2006). One study using factor analysis found that emotionality-related behaviours from the OF and EPM loaded on distinct factors, thus reflecting different psychological/behavioural dimensions (Trullus & Skolnick, 1993). Similarly, it has been shown that variables from the EPM and OF do not produce a common anxiety-related factor in rats (Ramos, Mellerin, Morme`de & Chaouloff, 1998). Despite this, some argue that these anxiety paradigms should not be viewed as measuring separate anxiety related behaviours but as measuring partially overlapping constructs (Ramos, 2008). Whatever view one takes it is important to utilise multiple assays in order to obtain a comprehensive assessment of any changes in emotional functioning in the *Zfp804a* mutant lines

4.2 Materials and Methods

4.2.1 Subjects and animal husbandry

In total, 64 adult male mice were used in these experiments. For the elevated plus-maze (EPM) mice were tested at a mean age of 3 months, for the open field (OF) mice were on average 4 months old and for the elevated zero-maze (EZM) mice had a mean age of 8 months. The EPM was the first anxiety paradigm to be used, followed by the OF (1 month later) and then the EZM (4 months after the OF). A limited number of mice were dropped from the study between the EPM and the EZM, due to illness/death with further mice added in between the OF and EZM when additional genotyping results were obtained. Details of specific mouse numbers per genotype for each anxiety assay can be seen in Table 4.1.

Table 4.1: The sample size and genotype of each cohort-subset of mice assayed in the tests of anxiety

Behavioural Task	C59X line		C417Y line	
	WT	Homozygous	WT	Homozygous
Elevated plus-maze	14	15	15	20
Open field	12	14	15	20
Elevated zero-maze	14	15	14	15

The mice were housed in littermates groups of two to five animals per cage, under temperature- and humidity-controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow and water and were weighed on regular basis and any subjects losing greater than 10% of their body weight were removed from the experiment. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986.

4.2.2 General behavioural methods

The experimental procedures for each of the behavioural assays were well established in the laboratory in previous work (Mikaelsson et al., 2013). Each of the anxiety paradigms involved testing mice individually; therefore, the apparatus was cleaned thoroughly with 1% acetic acid between each mouse, to remove any odours left by the previous occupant. All testing took place between the hours of 09:00 and 18:00, with equal distribution of testing for subjects of different genotypes throughout the day. Prior to testing, mice were habituated to the dimly lit test room for at least 20 minutes. EthoVision Observer software (Version 3.0.15, Noldus Information Technology, Netherlands) was used to collect data for the EPM, OF and EZM. The software tracks the movement of a subject (via an overhead digital camera) within previously defined zones within the arena (these zones differed between the different anxiety paradigms; see Chapter II, Section 2.4.3). A number of measures were automatically calculated for each zone, including duration and entries into each zone, time spent moving, rearing frequency, mean velocity and distance travelled. Data were analysed as total values for each session and in 1 minute intervals to assess performance within a session. Each session was also recorded using DVD HD recorders (Sony Corp, U.K.) for further analysis if required.

4.2.3 The elevated plus-maze (EPM) paradigm

In order to assess the innate aversion of both mutant lines of *Zfp804a* mice to open space and height, subjects were placed at the centre of the EPM apparatus (as previously described in Chapter II, Section 2.4.4) facing one of the exposed open arms and allowed to explore the maze over a single session of 5 minutes. The maze

was illuminated by a single 60 Watt lamp positioned above the arena (facing upwards). For the data analysis, the EPM was divided into 5 virtual zones; 2 open arm zones, 2 closed arm zones and a middle zone from which all the arms originated. The 2 open arm zones were combined to form one open zone value, as were the 2 closed arm zones. In addition to parameters previously mentioned in Section 4.2.2 above, other measures, sensitive to emotional state, were recorded manually. These being the number of stretch attend postures (SAPs defined as stretching forward in an open zone) and number of head dips over the open arm (looking over the edge of an open arm). Data were expressed for the total session and the main parameters used as indices of anxiety-related behaviour were the number of entries onto the open arms, the total duration of time spent exploring the open arms, and the latency of first entry onto the open arms. Other potential indicators of anxiety states included; the number of rears, the number of SAPs and the number of head dips on the open arms. Entries made and time spent in the closed arms was also recorded. The parameters used as measurements of general locomotor activity were; the total distance moved, the total duration of movement and the mean velocity of movement.

4.2.4 The open field (OF) paradigm

Mice of the *Zfp804a* mutant lines were tested in the OF, a white square shaped Perspex arena enclosed by high walls (Chapter II, Section 2.4.5). The maze was illuminated by a single 60 Watt lamp, located directly above the central region (facing upwards). Subjects were placed, facing a wall, at the edge of the arena. The OF arena was divided into virtual zones consisting of 2 concentric squares, a central zone (400*400mm) and an outer zone (the 200mm wide periphery). Data were expressed for the total session and the main parameters used as indices of anxiety-related behaviour were the total duration of time spent exploring the central region, the frequency of entries into the central region, the latency to first entry into the central zone and the number of rears. The parameters used as measurements of general locomotor activity were; the total distance moved, the total duration of movement and the mean velocity of movement.

4.2.5 The elevated zero-maze (EZM) paradigm

Behaviour was assessed in the elevated zero-maze as previously described in Chapter II, Section 2.4.6). The EZM has the advantage over the EPM in containing no central region which can interfere with measurements of exploration of the open and closed arms; the idea being that the EZM will provide data with less noise and therefore be a more sensitive assay (Shepherd et al., 1994). Each subject was placed in one of the closed zones and allowed to explore the maze over a single session of 5 minutes. The inside of the maze was 65 cm in diameter. The circular pathway was divided into four quadrants, two of which had walls (27 cm high) around the edge (closed quadrants); while the other two did not have walls (open quadrants). The 2 closed quadrants were combined to form a single closed zone value, as were the 2 open quadrants to form a single open zone value. As with the EPM, SAPs and head dips were recorded manually. Data were expressed for the total session and the main parameters used as indices of anxiety-related behaviour were the total duration of time spent exploring the open arms, the number of entries onto the open arms and the latency of first entry onto the open arms. Other potential indicators of anxiety states included; the numbers of rears, stretch attend postures (SAPs) and head dips on the open arms. The parameters used as measurements of locomotor activity were; the total duration of movement, the mean velocity of movement, as well as the distance moved.

4.2.6 Statistical analysis

The experimental data was analysed using SPSS (Version 18.0). Behavioural data are presented as mean values (*M*) with the standard error of the mean (SEM), unless stated otherwise. Data for each strain were analysed separately, and separate ANOVAs were performed with between-subjects factor of GENOTYPE (homozygous ENU-mutant vs. WT littermate control) and within subject factors as appropriate. All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. Data were screened for skewed variance with arcsine transformations applied to skewed data

(percentage data in particular) to obtain a normal distribution. Behaviour on all anxiety assays was followed-up by correlation analysis (Pearsons *r*).

4.3 Results

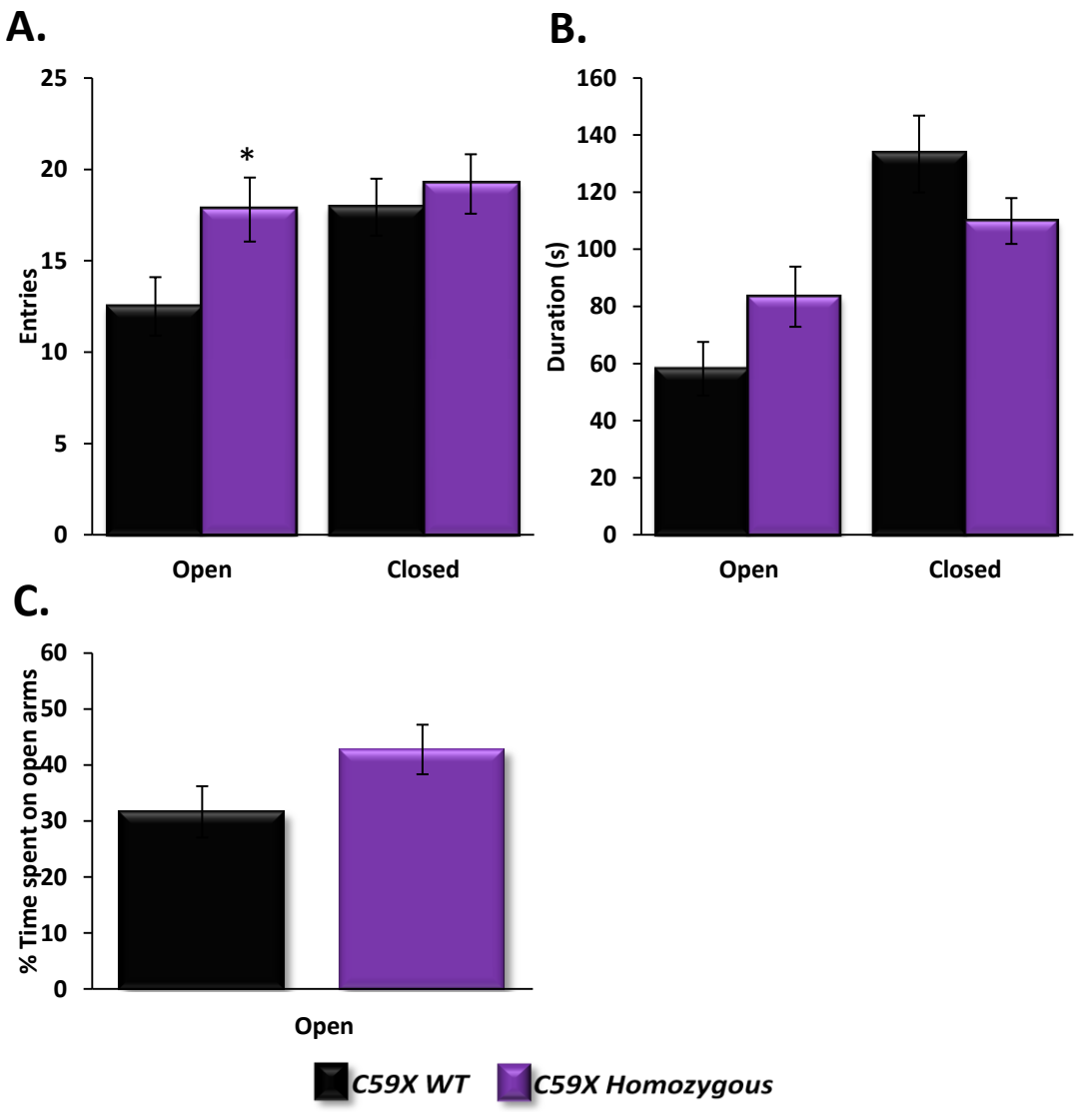
4.3.1 Zfp804a C59X line

4.3.1.1 Elevated plus-maze

Analysis of the data from EthoVision showed that as expected, on average, all C59X subjects made significantly more entries on to the protected closed arms than the exposed open arms (Fig. 4.1A, main effect of ZONE, $F_{1,27}$= 5.5, *p*=0.03, ZONE*GENOTYPE, $F_{1,27}$= 1.9, *p*=0.18), and spent significantly longer on the closed arms than the open arms (Fig. 4.1B, main effect of ZONE, $F_{1,27}$= 14.1, *p*=0.001, ZONE*GENOTYPE, $F_{1,27}$= 3.2, *p*=0.08). As is common with these tests (Lister, 1990), analysis was then focused on open arm behaviour, revealing that the C59X homozygous ENU-mutant mice made significantly more entries on to the open arms than their WT littermate controls (t_{27}= 2.2, *p*=0.04). This pattern, consistent with a reduced level of anxiety, was also seen in the overall length of time spent on the open arms of the maze, with C59X homozygous ENU-mutant mice spending longer on the open arms than their WT littermate controls, both in duration and percentage of time, although these measures were not found to be significant (Fig. 4.1B and 4.1C, t_{27}= 1.9, *p*=0.07, t_{27}= 1.9, *p*=0.07, respectively).

In contrast to behaviour on the open arms, no genotype differences were found for entries on to the closed arms (t_{27}= 0.6, *p*=0.58) or duration of time spent on the closed arms (t_{27}= -1.4, *p*=0.16), suggesting, *a priori*, that the genotype differences seen with respect to behaviour on the open arms were not simply the result of underlying differences in activity (since if this was the case one might anticipate changes in behaviour that were common to both the open and closed arms).

Figure 4.1: Entries made and duration of time spent on the open and closed arms of the EPM for the C59X line



Subjects of the C59X line made more entries on to the closed arms than the open arms, with the C59X homozygous ENU-mutant mice making significantly more entries on to the open arms than their WT littermate controls (A). Similarly, all C59X subjects, independent of genotype, also spent longer on the closed arms than the open arms. Despite the C59X homozygous ENU-mutant mice spending more time on the open arms (raw data (B), percentage of time (C)) than their WT littermate controls (indicative of reduced anxiety) these differences were not significant. Data shows the mean values ±SEM, **p*<0.05 for pairwise differences related to genotype. For subject N see Table 5.1.

The latency to enter the open arms for the first time is sometimes considered an additional index of anxiety, but in this case there were no genotype differences with respect to this measure (t_{27}= -0.8, *p*=0.45). There were however, a number of other ancillary behaviours consistent with a pattern of reduced anxiety in the C59X homozygous ENU-mutant mice, including increased rearing (t_{27}= 2.5, *p*=0.02), stretch

attend postures (SAPs, t_{27}= 3.0, *p*=0.006) and head dips (t_{27}= 2.9, *p*=0.007) on the open arms, Table 4.2.

Table 4.2: Ancillary parameters on the EPM for the C59X line		
Behavioural Parameter	**WT**	**Homozygous**
Latency of first occurrence on the open arms (seconds)	17.4 9 ± 7.1	11.7 ± 2.9
Number of rears on the open arms	8 ± 1.5	13.6 ± 1.7
Number of SAPs on the open arms	24.2 ± 2.7	38.9 ± 4.1
Number of head dips on the open arms	17.1 ± 2.9	32.2 ± 4.2

Data shows mean ± SEM

Confounding influences of activity on the pattern of data were unlikely as differences due to genotype were limited to activity monitored on the open arms. Hence, the C59X homozygous ENU-mutant mice moved greater distances and spent longer moving on the open arms than their WT littermate controls (t_{27}= 2.7, *p*=0.01 and t_{27}= 2.9, *p*=0.007, respectively) but were no different to their WT counterparts when measuring activity in the maze as a whole in terms of the total distance moved and time spent moving (main effect of GENOTYPE, $F_{1,27}$ = 1.6, *p*=0.22, and $F_{1,27}$= 2.7, *p*=0.11, respectively); similarly, the averaged velocity (cm/sec) of locomotion was indifferent to genotype (main effect of GENOTYPE, $F_{1,27}$= 2.9, *p*=0.1, see Table 4.3).

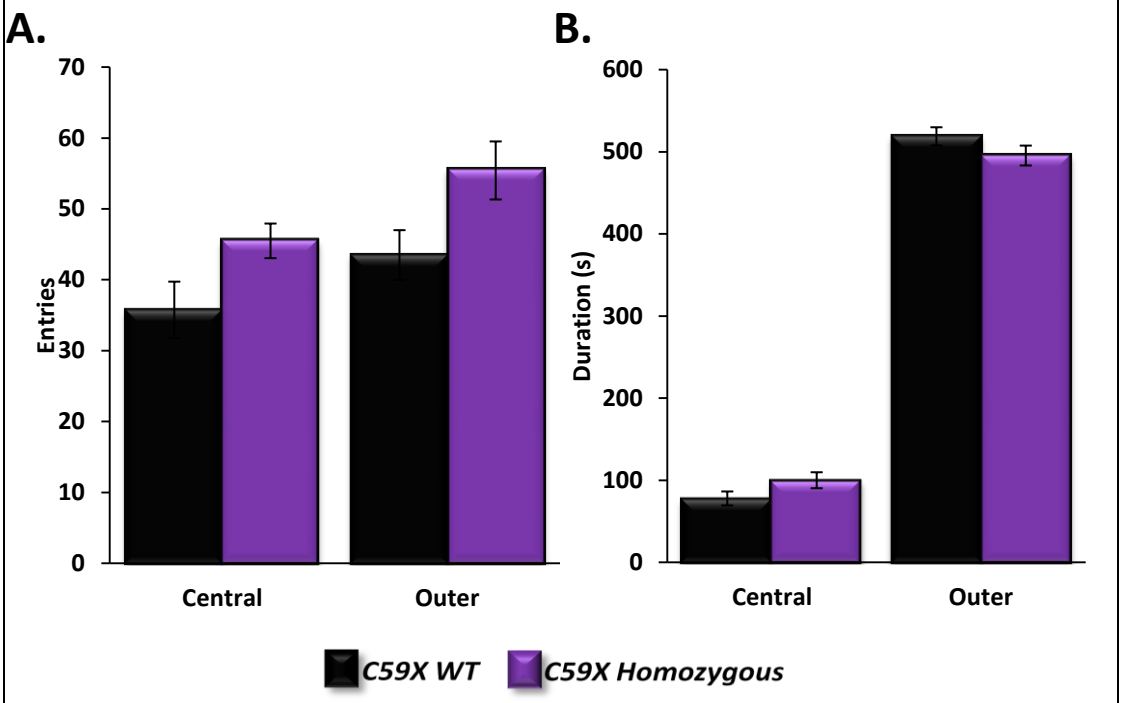
Table 4.3: Locomotor parameters on the EPM for the C59X line		
Behavioural Parameter	**WT**	**Homozygous**
Total distance moved on the open arms (cm)	181.6 ± 27.3	297.4 ± 32.8
Total time spent moving on the open arms (seconds)	32.1 ± 16.5	53.1 ± 21.4
Total distance moved (cm, all arms)	377.8 ± 35.5	440.3 ± 34.3
Total time spent moving (seconds, all arms)	60 ± 4.3	69.9 ± 4.2
Averaged velocity (cm/s)	3.9 ± 0.3	4.5 ± 0.3

Data shows mean ± SEM

4.3.1.2 Open field

The area of the OF arena was subdivided into 2 virtual regions (outer and central) using the EthoVision tracking software. When anxious, rodents are expected to stay in close proximity to the walls of the open field arena (the outer zone). Generally, all subjects demonstrated this thigmotaxic behaviour by making more entries into (Fig. 4.2A, main effect of ZONE, $F_{1,28}$= 15.2, p=0.001, ZONE*GENOTYPE, $F_{1,28}$= 0.2, p=0.63) and spending longer in the outer area as opposed to the more anxiety-provoking central region of the OF (Fig. 4.2B, main effect of ZONE, $F_{1,28}$= 806.5, p=0.00, ZONE*GENOTYPE, $F_{1,28}$= 2.4, p=0.13).

Figure 4.2: Entries made and duration of time spent in the central and outer zones of the OF for the C59X line



All C59X subjects demonstrated thigmotaxic behaviour by making more entries into the outer zone (A) and spending longer in the outer zone as opposed to the more anxiety-provoking central zone (B). Despite the C59X homozygous ENU-mutant mice making more entries into the central zone and spending longer in the central zone than their WT littermate controls, these differences were not found to be significant. C59X homozygous ENU-mutant mice made significantly more entries into the outer zone than their WT littermate controls, but duration of time spent in the outer zone was equivalent between genotypes. Data shows the mean values ±SEM. For subject N see Table 5.1.

Despite C59X homozygous ENU-mutant mice making more entries into the central zone and spending longer in the central zone than their WT littermate controls (Fig. 4.2A and Fig. 4.2B), these differences were not found to be significant ($t_{22.8}$= 2.0,

p=0.053, and t_{28}= 1.4, *p*=0.16, respectively), although they do indicate an anxiolytic tendency in the C59X homozygous ENU-mutants. Inspection of outer zone behaviour revealed that despite the C59X homozygous ENU-mice making significantly more entries into the outer zone than their WT littermate controls (t_{28}= 2.3, *p*=0.03), duration of time spent in the outer zone was equivalent between groups (t_{28}= -1.7, *p*=0.11), suggesting that the C59X homozygous ENU-mutants display a more exploratory phenotype in the OF than their WT littermate controls.

As seen on the EPM, there were no genotype differences in terms of the latency of first occurrence into the central zone of the OF (t_{28}= -1.3, *p*=0.21), however, consistent with a reduced anxiety phenotype, C59X homozygous ENU-mutant mice made more rears in both the central and outer zones than their WT littermate controls (t_{28}= 3.2, *p*=0.003 and t_{28}= 3.1, *p*=0.005, respectively), Table 4.4.

Table 4.4: Ancillary parameters in the OF for the C59X line

Behavioural Parameter	WT	Homozygous
Latency of first occurrence in the central zone (seconds)	33.3 ± 15.2	12.7 ± 5.1
Number of rears in the central zone	24.3 ± 2.7	37.5 ± 3.1
Number of rears in the outer zone	44.8 ± 4.1	64.1 ± 4.8

Data shows mean ± SEM

Confounding influences of activity on the pattern of data were unlikely as there were no genotype differences on locomotion (Table 4.5), this included the total distance moved, time spent moving and the average velocity in the OF as a whole (main effect of GENOTYPE, $F_{1, 28}$= 0.01, *p*=0.93, and $F_{1, 28}$= 0.3, *p*=0.6, $F_{1,28}$= 0.2, *p*=0.64, respectively). Further analysis on locomotion in the more anxiety-provoking central zone also revealed no differences between the two genotypes in terms of total distance moved in the central zone and time spent moving in the central zone ($t_{23.9}$= 2.0, *p*=0.06, and t_{28}= 1.8, *p*=0.08, respectively).

Table 4.5: Locomotor parameters in the OF for the C59X line		
Behavioural Parameter	**WT**	**Homozygous**
Total distance moved (cm, all zones)	3798.8 ± 260.5	3766.2 ± 260.5
Total time spent moving (seconds, all zones)	270.3 ± 5.5	274.5 ± 5.5
Averaged velocity (cm/s)	16.1 ± 1.2	15.3 ± 1.2
Total distance moved in the central zone (cm)	1320.6 ± 12.1	1705.1 ± 9.7
Total time spent moving in the central zone (seconds)	70.8 ± 10.8	97 ± 9.3

Data shows mean ± SEM

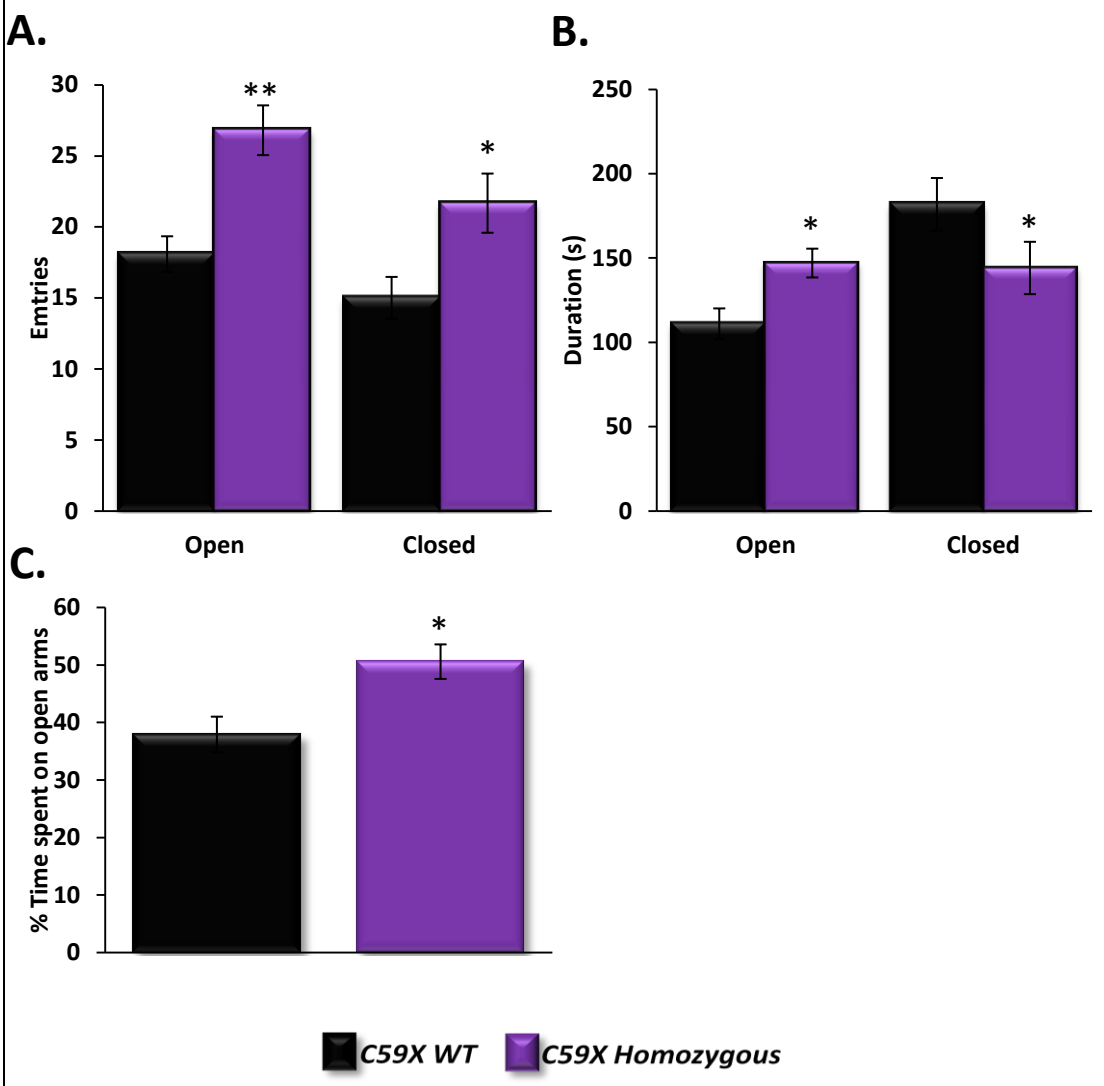
4.3.1.3 Elevated zero-maze

Analysis of the data showed that as expected, on average, all C59X subjects made significantly more entries into the protected closed zones than the exposed open zones (Fig. 4.3A, main effect of ZONE, $F_{1,27}$= 25.4, *p*=0.00, ZONE*GENOTYPE, $F_{1,27}$= 1.6, *p*=0.22), however, subjects spent an equivalent amount of time in both zones (Fig. 4.3B, main effect of ZONE, $F_{1,27}$= 3.8, *p*=0.06). A significant interaction (ZONE*GENOTYPE, $F_{1,27}$= 4.4, *p*=0.045), revealed that C59X homozygous ENU-mutant mice spent more time in the open zone (*p*=0.048) and less time in the closed zone (*p*=0.042) than their WT littermate controls, indicating a reduced level of anxiety in these mice. C59X homozygous ENU-mutant mice made significantly more entries into the open zones (t_{27}= 3.2, *p*=0.003) and spent longer in the open zones than their WT littermate controls, both in duration and percentage of time (Fig. 4.3B and Fig. 4.3C, t_{27}= 2.1, *p*=0.048, t_{27}= 2.1, *p*=0.044, respectively), consistent with a reduced level of anxiety, providing further support that the C59X homozygous ENU-mutants displayed an anxiolytic phenotype in these anxiety paradigms.

Inspection of closed zone behaviour revealed that the C59X homozygous ENU-mutant mice made more entries into the protected closed zone than their WT littermate controls (t_{27}= 3.5, *p*=0.002) but as expected from their open arm behaviour, the duration of time spent in the closed zone was significantly less for the C59X homozygous ENU-mutants than their WT littermate controls (t_{27}= -2.1, *p*=0.04),

again pointing towards an anxiolytic phenotype in the C59X homozygous ENU-mutants.

Figure 4.3: Entries made and duration of time spent in the open and closed zones of the EZM for the C59X line



All subjects from the C59X line made significantly more entries into the protected closed zones than the exposed open zones (A), however, subjects spent an equivalent amount of time in both zones (B). The C59X homozygous ENU-mutant mice made significantly more entries into the open zone and spent longer in the open zone than their WT littermate controls, both in terms of duration (B) and percentage of time spent in the open zones (C). The C59X homozygous ENU-mutant mice also spent significantly less time in the closed zones than their WT littermate controls, providing further support that the C59X homozygous ENU-mutants displayed an anxiolytic phenotype in these anxiety paradigms. Data shows the mean values ±SEM, $^{**}p<0.01$ and $^{*}p<0.05$ for pairwise differences related to genotype. For subject N see Table 5.1.

As observed in the EPM and OF, there was no genotype difference in the latency to first enter the open zones in the EZM (t_{27}= -0.8, p=0.41), there were however, a number of other ancillary behaviours consistent with reduced anxiety in

the C59X homozygous ENU-mutant mice, including increased rearing (t_{27}= 4.0, p=0.001), SAPs (t_{27}= 3.3, p=0.003) and head dips (t_{27}= 3.4, p=0.002) on the open arms, Table 4.6.

Table 4.6: Ancillary parameters on the EZM for the C59X line

Behavioural Parameter	WT	Homozygous
Latency of first occurrence on the open arms (seconds)	2.7 ± 0.8	1.9 ± 0.6
Number of rears on the open arms	23.5 ± 2.4	35.1 ± 1.7
Number of SAPs on the open arms	41.6 ± 5.3	63.1 ± 3.8
Number of head dips on the open arms	23.9 ± 3.2	38.7 ± 2.9

Data shows mean ± SEM

In terms of the locomotion of subjects from the C59X line during the EZM (Table 4.7), the total distance moved and time spent moving on the maze, were higher for C59X homozygous ENU-mutants than their WT littermate controls (main effect of GENOTYPE, $F_{1,27}$= 13.7, p=0.001 and $F_{1,27}$= 6.6, p=0.02, respectively), potentially reflecting hyperactivity in the C59X homozygous ENU-mutants on this assay, however, the averaged velocity (cm/sec) of locomotion was indifferent to genotype ($F_{1,27}$= 3.6, p=0.07). A separate analysis of open zone behaviour between genotypes revealed that the C59X homozygous ENU-mutant mice moved greater distances and spent longer moving in the open zone than their WT littermate controls (t_{27}= 3.3, p=0.003 and t_{27}= 2.2, p=0.04, respectively), behaviour which indicates more exploratory behaviour in the C59X homozygous ENU-mutants in the more exposed open zones.

Table 4.7: Locomotor parameters on the EZM for the C59X line

Behavioural Parameter	WT	Homozygous
Total distance moved (cm, all zones)	851.5 ± 90	1282.7 ± 91.8
Total time spent moving (seconds, all zones)	74.7 ± 10.9	109.5 ± 10
Averaged velocity (cm/s)	4.1 ± 0.3	4.9 ± 0.3
Total distance moved in the open arms (cm)	542.2 ± 0.3	842.1 ± 0.3
Total time spent moving in the open arms (seconds)	60 ± 9.2	88.5 ± 8.9

Data shows mean ± SEM

4.3.2 Zfp804a C417Y line

4.3.2.1 Elevated plus-maze

Analysis of the data collected from EthoVision showed that, as expected, overall, all subjects made significantly more entries on to the protected closed arms than the exposed open arms (Fig. 4.4A, main effect of ZONE, $F_{1,32}$= 76.2, *p*=0.00, ZONE*GENOTYPE, $F_{1,32}$= 0.5, *p*=0.48) and spent significantly longer on the closed arms than the open arms (Fig. 4.4B, main effect of ZONE, $F_{1,32}$= 90.6, *p*=0.00, ZONE*GENOTYPE, $F_{1,32}$= 1.1, *p*=0.3). There were no significant differences between C417Y homozygous ENU-mutant mice and their WT littermate controls in the number of entries made on to the open arms (t_{32}= 1.7, *p*=0.09) or total duration of time and percentage of time (Fig. 4.4C) spent on the open arms (t_{32}= 1.7, *p*=0.09, t_{32}= 1.7, *p*=0.09, respectively) indicating an equivalent level of anxiety between the two genotype groups. Looking at closed arm behaviour also revealed no genotype differences for entries on to the closed arms (t_{32}= 0.9, *p*=0.38) nor for the duration of time spent on the closed arms (t_{32}= -0.4, *p*=0.7).

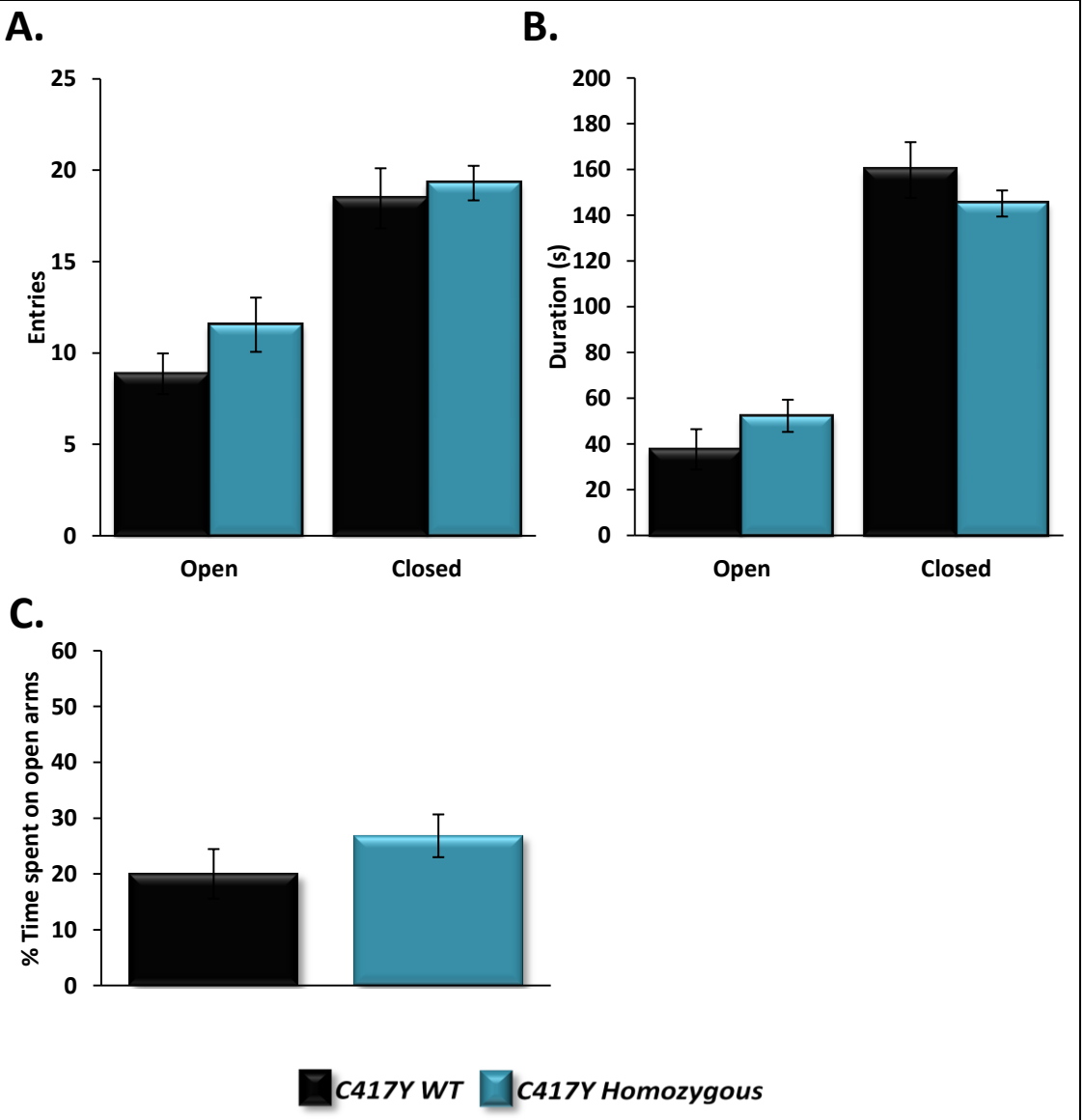
When assessing the latency to first enter the open arms, there were again no genotype differences observed (t_{27}= -0.8, *p*=0.44), nor were there any differences between genotypes in other ancillary measures of anxiety such as the number of rears, stretch attend postures and head dips in the open arms (t_{32}= 1.9, *p*=0.07, $t_{28.8}$= 1.2, *p*=0.26, and $t_{27.2}$= 1.9, *p*=0.06, respectively), Table 4.8.

Table 4.8: Ancillary parameters on the EPM for the C417Y line

Behavioural Parameter	WT	Homozygous
Latency of first occurrence on the open arms (seconds)	30.2± 12.4	20.6 ± 4.9
Number of rears on the open arms	3.9 ± 1	7.4 ± 1.5
Number of SAPs on the open arms	17.5 ± 2	22.3 ± 3.3
Number of head dips on the open arms	8 ± 1.2	19 ± 3.2

Data shows mean ± SEM

Figure 4.4: Entries made and duration of time spent on the open and closed arms of the EPM for the C417Y line



All subjects from the C417Y line made more entries on to the closed arms than the open arms and spent significantly longer on the closed arms than the open arms. C417Y homozygous ENU-mutants and their WT littermate controls made an equivalent number of entries made on to the open arms (A) and spent an equivalent amount of time on the open arms, both in terms of duration (B) and percentage of time (C). Data shows the mean values ±SEM. For subject N see Table 5.1.

In terms of the locomotion of the C417Y subjects on the EPM (Table 4.9), the total distance moved and time spent moving on the maze (as a whole) during the session was equivalent between C417Y homozygous ENU-mutants and WT littermate controls (main effect of GENOTYPE, $F_{1,32}$= 2.0, p=0.17, and $F_{1,32}$= 1.7, p=0.2, respectively), similarly, the averaged velocity (cm/sec) of locomotion was indifferent to genotype (main effect of GENOTYPE, $F_{1,32}$= 1.1, p=0.31). A separate analysis of

open arm behaviour also revealed no differences between the C417Y homozygous ENU-mutants and their WT littermate controls in terms of total distance moved on the open arms and time spent moving on the open arms ($t_{29.7}$= 1.6, p=0.13, and $t_{28.03}$= 1.9, p=0.7, respectively), suggesting that locomotor activity was equivalent between genotypes on all regions of the maze, and unlikely to be a confounding influence on the pattern of data observed.

Table 4.9: Locomotor parameters on the EPM for the C417Y line

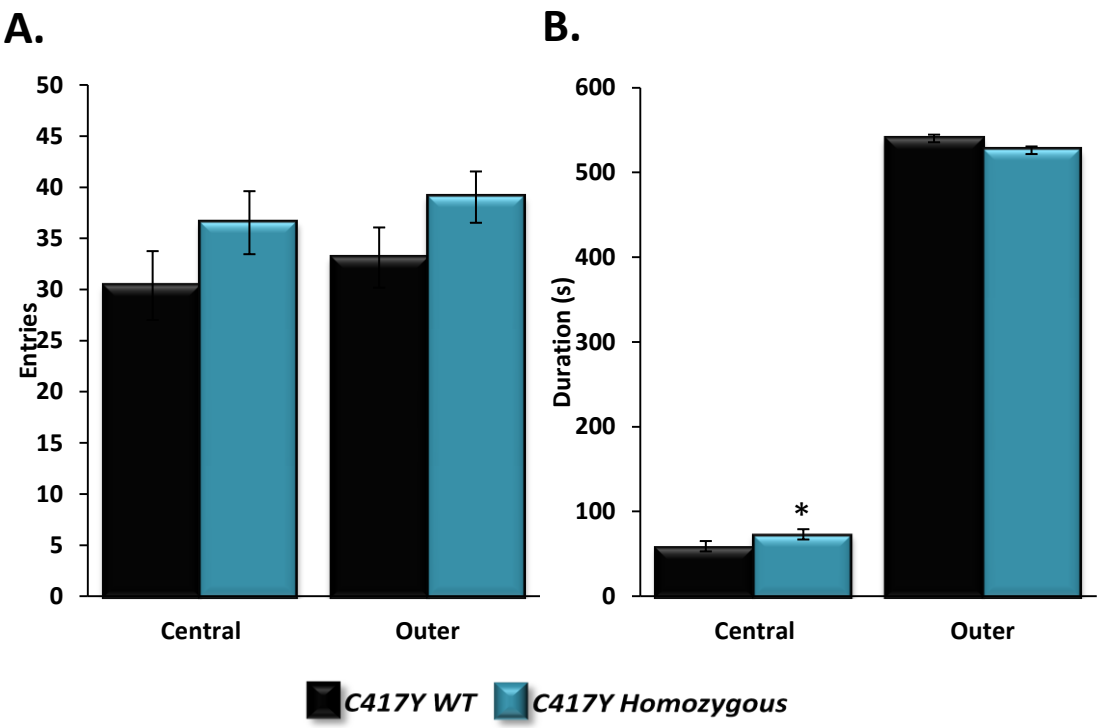
Behavioural Parameter	WT	Homozygous
Total distance moved (cm, all arms)	352.9 ± 30.9	410.7 ± 27.4
Total time spent moving (seconds, all arms)	56.6 ± 4.5	64.6 ± 4
Averaged velocity (cm/s)	3.7 ± 0.3	4.0 ± 0.2
Total distance moved on the open arms (cm)	128.2 ± 18.2	180.5 ± 27.9
Total time spent moving on the open arms (seconds)	22.5 ± 2.7	32.7 ± 4.7

Data shows mean ± SEM

4.3.2.2 Open field

All subjects demonstrated the expected thigmotaxic behaviour by making more entries into the outer zone (Fig. 4.5A, main effect of ZONE, $F_{1,32}$= 38.0, p=0.00, ZONE*GENOTYPE, $F_{1,32}$= 0.01, p=0.91) and spending longer in the outer zone (Fig. 4.5B, main effect of ZONE, $F_{1,32}$= 772.2, p=0.00, ZONE*GENOTYPE, $F_{1,32}$= 0.01, p=0.91) as opposed to the more anxiety-provoking central zone of the OF. All subjects made an equivalent number of entries into the central zone (t_{32}= 1.4, p=0.16) but, perhaps reflecting a reduction in anxiety, C417Y homozygous ENU-mutant mice spent significantly longer in the central zone than their WT littermate controls ($t_{30.7}$= 2.3, p=0.03). There were no genotype differences in the number of entries into the outer zone and total time spent in the outer zone of the OF (t_{32}= 1.3, p=0.21, and t_{32}= 0.4, p=0.69, respectively).

Figure 4.5: Entries made and duration of time spent in the central and outer zones on the OF for the C417Y line



All C417Y subjects demonstrated thigmotaxic behaviour by making more entries into the 'safer' outer zone (A) and spending longer in the outer zone as opposed to the more anxiety provoking central zone (B). All C417Y subjects made an equivalent number of entries into the central zone but the C417Y homozygous ENU-mutant mice spent significantly longer in the central zone than their WT littermate controls, perhaps reflecting a reduction in anxiety in the C59X homozygous ENU-mutants. Data shows the mean values ±SEM, $p<0.05$ for pairwise differences related to genotype For subject N see Table 5.1.

The time taken to first enter the central zone was not significantly different between C417Y homozygous ENU-mutant mice and their WT littermate controls (t_{32}= 0.4, p=0.72), furthermore, there were no genotype differences in the number of rears in either the central or outer zones (t_{32}= 0.9, p=0.37, and t_{32}= 0.3, p=0.74, respectively), Table 4.10, again indicating equivalent levels of anxiety between C417Y homozygous ENU-mutants and their WT counterparts.

Confounding influences of activity on the pattern of data were unlikely as there were no genotype differences in terms of the total distance moved or time spent moving in the OF as a whole, during the session (main effect of GENOTYPE, $F_{1,32}$= 0.7, p=0.42, and $F_{1,32}$= 0.7, p=0.4, respectively) similarly, the averaged velocity (cm/sec) of locomotion was indifferent to genotype (main effect of GENOTYPE, $F_{1,32}$= 0.3, p=0.59, Table 4.11). Further analysis on locomotion in the more anxiety-

provoking central zone revealed no differences between the two genotypes in terms of total distance moved in the central zone ($t_{23.9}$= 1.5, *p*=0.16) but C417Y homozygous ENU-mutants spent significantly longer moving in the central zone than their WT littermate controls (t_{28}= 1.8, ($t_{31.2}$= 2.2, *p*=0.04).

Table 4.10: Ancillary parameters in the OF for the C417Y line

Behavioural Parameter	WT	Homozygous
Latency of first occurrence on in the central zone (seconds)	15.6 ± 4.7	21.8 ± 14.9
Number of rears in the central zone	17.9 ± 2.4	22.3 ± 3.9
Number of rears in the outer zone	30 ± 4.9	32.4 ± 5.1

Data shows mean ± SEM

Table 4.11: Locomotor parameters in the OF for the C417Y line.

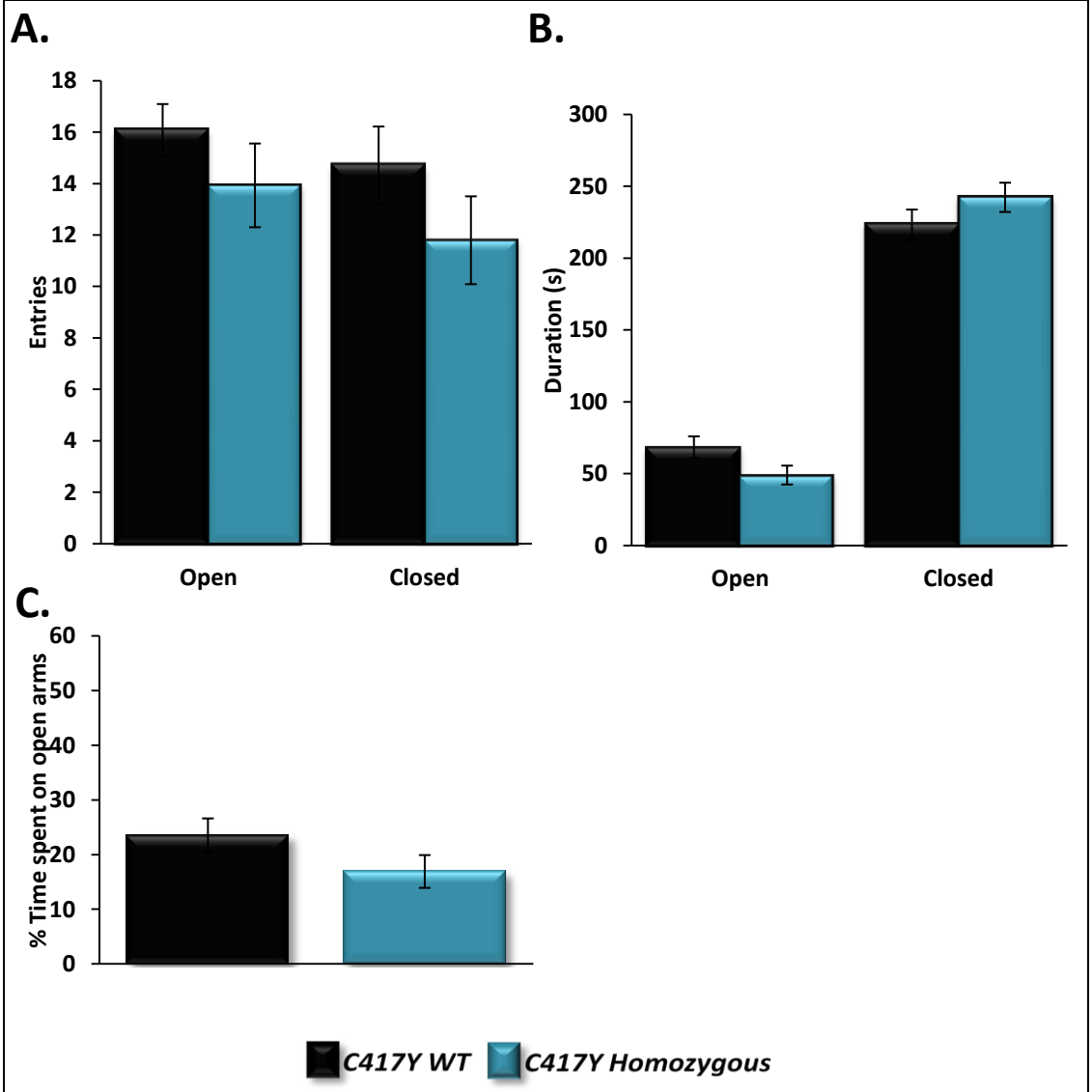
Behavioural Parameter	WT	Homozygous
Total distance moved (cm, all zones)	3029.6 ± 427.4	3499 ± 379.8
Total time spent moving (seconds, all zones)	252.2 ± 15	269.4 ± 13.3
Averaged velocity (cm/s)	13.3 ± 1.9	14.7 ± 1.7
Total distance moved in the central zone (cm)	1017 ± 88.8	1330.3 ± 177.3
Total time spent moving in the central zone (seconds)	57.3 ± 4.3	73.3 ± 5.8

Data shows mean ± SEM

4.3.2.3 Elevated zero-maze

Analysis of the data showed that, unexpectedly, all C417Y subjects, in general, made significantly more entries into the exposed open zones than the protected closed zones (Fig. 4.6A, main effect of ZONE, $F_{1,27}$= 11.4, *p*=0.002, ZONE*GENOTYPE, $F_{1,27}$= 0.6, *p*=0.46), however, following a more typical pattern, all C417Y subjects spent significantly more time in the closed zones than in the more anxiety-provoking open zones (Fig. 4.6B main effect of ZONE, $F_{1,27}$= 197.2, *p*=0.000, ZONE*GENOTYPE, $F_{1,27}$= 2.4, *p*=0.13).

Figure 4.6: Entries made and duration of time spent in the open and closed zones of the EZM for the C417Y line



All C417Y subjects unexpectedly made significantly more entries into the exposed open zones than the protected closed zones (A), however, as expected all C417Y subjects spent significantly more time in the closed zones than the open zones, both in terms of duration (B) and percentage of time (C) spent in the open zones. There were no genotype differences in the number of entries or time spent in the open zone. Data shows the mean values ±SEM. For subject N see Table 5.1.

This could reflect a more exploratory phenotype by C417Y mice in general on the EZM as opposed to the EPM and OF; perhaps due to the loss of the ambiguous middle platform seen in the EPM. There were no genotype differences in the number of entries made into the open zones (t_{27}= -0.9, p=0.437*),* or the duration and percentage of time (Fig. 4.6C) spent in the open zones (t_{27}= -1.6, p=0.12*,* t_{27}= -1.6, p=0.12, respectively), or entries and time spent in the closed zone (t_{27}= -1.6, p=0.12,

t_{27}= 1.5, *p*=0.15) suggesting equivalent levels of anxiety between C417Y mutant and WT mice.

The time taken to first enter the open zone was not significantly different between the C417Y homozygous ENU-mutants and their WT littermate controls (t_{27}= 1.3, *p*=0.22), furthermore, there were no genotype differences in the number of rears, SAPs or head dips in the open zone (Table 4.12, t_{27}= -1.6, *p*=0.12, t_{27}= -1.3, *p*=0.21, and t_{27}= -1.5, *p*=0.15, respectively), again indicating equivalent levels of anxiety between C417Y homozygous ENU-mutants and their WT counterparts.

Table 4.12: Ancillary parameters on the EZM for the C417Y line

Behavioural Parameter	WT	Homozygous
Latency of first occurrence on the open arms (seconds)	3.8 ± 1	8.7 ± 3.6
Number of rears on the open arms	20.8 ± 2.3	16.1 ± 1.8
Number of SAPs on the open arms	28.3 ± 4.1	21.5 ± 3.3
Number of head dips on the open arms	15.6 ± 2	11.7 ± 1.8

Data shows mean ± SEM

In terms of locomotion during the session, the total distance moved and time spent moving on the maze as a whole was equivalent between the C417Y homozygous ENU-mutants and WT littermate controls (Table 4.13, main effect of GENOTYPE, $F_{1,27}$= 2.7, *p*=0.11, and $F_{1,27}$= 2.5, *p*=0.13, respectively), similarly, the averaged velocity (cm/sec) of locomotion was indifferent to genotype (main effect of GENOTYPE, $F_{1,27}$= 0.6, *p*=0.45). A separate analysis of open zone behaviour also revealed no differences between the C417Y homozygous ENU-mutants and their WT littermate controls in terms of total distance moved in the open zones and time spent moving in the open zones (t_{27}=-1.6, *p*=0.13, and t_{27}= -1.5, *p*=0.15, respectively), suggesting that locomotor activity was equivalent between genotypes on all regions of the maze, and unlikely to be a confounding influence on the pattern of data observed.

Table 4.13: Locomotor parameters on the EZM for the C417Y line		
Behavioural Parameter	**WT**	**Homozygous**
Total distance moved (cm, all zones)	735.5 ± 79	578.3 ± 57.6
Total time spent moving (seconds, all zones)	51.4 ± 6.6	38.5 ± 5.3
Averaged velocity (cm/s)	4.8 ± 0.3	4.5 ± 0.3
Total distance moved in the open arms (cm)	439.3 ± 45.3	340.8 ± 43.8
Total time spent moving in the open arms (seconds)	37.1 ± 4.8	27 ± 4.7

Data shows mean ± SEM

4.3.3 Comparison of EPM, OF and EZM performance for both Zfp804a mutant lines

Correlational analyses were carried out using subjects from both lines to determine if performances on the three anxiety measures were related. The parameters examined were entries onto the more anxiety-provoking open arms and time spent on the open arms in the EPM and EZM, as well as entries made into the more anxiety-provoking central zone and time spent in the central zone in the OF.

The results showed moderately strong positive correlations between entries made onto the EPM open arms and entries made into the central zone of the OF (r=0.34, p (two-tailed) =0.007, Appendix 3.1A), and entries made onto the open arms of the EZM (r=0.42, p (two-tailed) =0.001, Appendix 3.1B). Entries made onto the open arms of the EZM and entries made into the central zone of the OF were not significantly correlated (r=0.16, p (two-tailed) =0.22, Appendix 3.1C). The same pattern of results was seen with the duration of time spent in the exposed zones, with moderately strong positive correlations between the duration of time spent on the open arms in the EPM and the duration of time spent in the central zone of the OF (r=0.32, p (two-tailed) =0.01, Appendix 3.1D), and duration of time spent on the open arms of the EZM (r=0.38, p (two-tailed) =0.003, Appendix 3.1E). The duration of time spent on the open arms of the EZM and the duration of time spent in the central zone of the OF were not significantly correlated (r=0.23, p (two-tailed) = 0.08, Appendix 3.1F). These results suggest that performance on the EPM is significantly associated with performance on the OF and EZM, but that behaviour on the OF and

EZM were not related. This adds further weight to the idea that the paradigms may be measuring different aspects of emotionality, further highlighting the need for multiple assays to examine anxiety in rodents.

4.4 Discussion

WT littermate control mice of both the C59X and C417Y lines demonstrated the expected basic patterns of results for each of the assays used, generally showing avoidance behaviour of the most anxiety-inducing regions of each apparatus. C59X homozygous ENU-mutant mice demonstrated consistent findings across all the tests suggestive of reduced levels of anxiety. C417Y homozygous ENU-mutant mice, on the other hand, demonstrated equivalent levels of anxiety to their WT littermate controls. This pattern of results is consistent with a previous study using Z*fp804a* C59X mice on a <98% C57BL/6J background (Al-Janabi, 2012); $G4_i$ C59X homozygous ENU-mutant mice exhibited similar patterns to the current study of reduced anxiety on the EPM. This replication of findings on the EPM would therefore indicate that the reduced anxiety displayed is a result of the mutation of *Zfp804a* at the C59X locus, and not a consequence of mutations in other genes induced by ENU-mutagenesis. Thus, in the current study C59X homozygous ENU-mutant mice spent less time in the periphery of the OF and made more entries into and spent more time on the open arms of the EPM and EZM; further corroborated by increased amounts of ethological behaviours such as SAP's, rearing and head dips. In contrast, although C417Y homozygous ENU-mutant mice were found to have spent significantly longer in the central zone of the OF than their WT littermate controls, indicative of a reduced level of anxiety, this, appears to be an isolated result as none of the other ancillary measures, such as SAPs and rearing, support this suggestion and these mice did not differ from their WT littermates on the EPM or EZM. Therefore, it can be concluded that mutation of the *Zfp804a* gene in exon 2 (C59X mutation) leads to altered anxiety whereas the exon 4 mutation (C417Y) does not affect this function. The lack of effect in C417Y homozygous ENU-mutant mice, however, is consistent with other mouse models for schizophrenia, where equivalent behaviour between WT and mutant mice on the same assays used here has been found (Karl et al., 2007; O'Tuathaigh et al., 2008; Clapcote et al., 2007). Further

discussion about the mechanism of action will be addressed in the General Discussion chapter (Chapter VIII); however, the fact that there is dissociation between the two mutant lines is further evidence for causal effects of the targeted C59X mutation rather than co-segregating genes inherited alongside both mutations (Chapter III, Section 3.1).

As mentioned, the WT littermate controls from both lines showed the expected patterns of behaviour, and were relatively consistent in each of the different tasks, suggesting that order effects or repeated testing did not affect performance and would not account for the differences between C59X homozygous ENU-mutant mice and their WT littermate controls. Prior exposure to any anxiety-provoking task can make subsequent anxiety tasks less fear inducing and produce a more anxiolytic phenotype (Holmes, 2001); however this did not seem to be the case here. Similarly, it is well known that levels of motor activity can influence anxiety-related behaviours (Lister, 1990). As demonstrated in Chapter III (Section 3.3.3.2) there were no significant differences between C59X homozygous ENU-mutant mice and their WT littermates in a specific test of locomotion, with the trend actually pointing towards hypoactivity. In the anxiety tests used here, of much shorter duration (5-10 minutes), C59X homozygous ENU-mutant mice also showed equivalent levels of motor activity to their WT counterparts, including, measures of the total distance moved, time spent moving and mean velocity of movement on the EPM and OF tests, although on the EZM the C59X homozygous ENU-mutants moved greater distances and spent longer moving than their WT littermates. However, the locomotor results from the EZM were mainly driven by behaviour in the open zones (Table 4.7) illustrating that the C59X homozygous ENU-mutants were not hyperactive across all the maze *per se*, but spent longer moving and moved greater distances in the open zones, indicating an exploratory (not hyperactive) phenotype in the EZM for these mice. Therefore, it would appear that differences in locomotion were not influencing anxiety in these tests and that the differences in behaviour shown by C59X homozygous ENU-mutant mice were due to alterations in innate anxiety responsiveness.

Anxiety is a multi-faceted construct; therefore the different tests used here may tax different, or partially overlapping, components of emotionality (Ramos,

2008; Sousa et al, 2006). For example, the OF and EPM did not produce a common anxiety-related factor in a principal component analysis (Trullus & Skolnick, 1993; Ramos et al., 1998), however, this premise was not supported by the current data where entries and durations in the EPM were significantly correlated with entries and durations in the OF. Furthermore, entries and durations in EZM correlated with EPM behaviour but not the OF, suggesting (*a priori*) that the EPM was measuring the same component of anxiety as the OF and EZM, but that the OF and EZM were measuring separate forms of anxiety and that the consistent differences shown by C59X homozygous ENU-mutant mice reflect altered anxiolytic effects by these mice across a range of anxiety sub-types.

Decreased anxiety, or anxiolysis, can occur in animal models using a variety of different paradigms, e.g. treatment with benzodiazepines (Treit et al., 2010), amygdaloidal lesions, local infusions into the amygdala (Davis, 1992) or other parts of the brain, such as the hippocampus (Bannerman et al., 2003). In terms of genetic approaches there has been a focus on serotonin receptor overexpression and knockout models (Jennings, Loder, Sheward et al., 2006; Holmes, Yang, Lesch et al., 2003) perhaps due to the link between serotonin and anxiety, and it's role in the treatment of certain anxiety disorders (Baldwin & Rudge, 1995; Ballenger, 1999). Anxiolytic behaviour has also been observed in knockout mouse models of dopamine receptor 3 (Steiner, Fuchs & Accili, 1997), Corticotropin releasing factor receptor 1 (Smith, Aubry, Dellu et al., 1998; Contarino, Dellu, Koob et al., 1999), Protein kinase Cε (Hodge, Raber, McMahon et al., 2002) and *Neurabin* (Kim, Wang, Li et al., 2011). Thus, there are a variety of neurobiological mechanisms through which the *Zfp804a* C59X mutation could be mediating effects on anxiety-related behaviours.

Although not widely reported, many patients with schizophrenia also report altered levels of anxiety (Braga et al., 2004; Pokos & Castle, 2006) including symptoms of OCD, PTSD, phobias and panic attacks (Pilkonis et al., 1980; Fenton & McGlashan, 1986; Argyle, 1990; Penn et al., 1994; Eisen et al., 1997; Meyer et al., 1999; Braga et al., 2005). Furthermore, one study (Steinberg et al., 2011) has found a copy number variant, in the form of a deletion of *ZNF804A,* in both a schizophrenia patient and an anxiety disorder patient and the link between *ZNF804A* variants and bipolar disorder (Owen et al., 2007; O' Donovan et al., 2008; Steinberg et al. 2011)

suggests that emotional functionality could be affected in these individuals, if investigated. Bipolar disorder is characterised by deficits in emotional regulation (Townsend & Altshuler, 2012); consequently, we might expect to see phenotypes relating more to the symptomology of bipolar disorder in the *Zfp804a* mutant mouse lines.

The effects observed are consistent and relevant to both schizophrenia and bipolar disorder, but it is difficult to directly predict the effects found in the *Zfp804a* mutants to clinical studies. However, this should not obscure the key finding that disruption of normal *Zfp804a* functionality leads to consistently altered anxiety in C59X mutant mice.

4.4.1. Summary of key results from Chapter IV

- WT littermate controls of both mutant lines showed the expected basic pattern of behaviour in response to anxiety-provoking stimuli.
- C59X homozygous ENU-mutant mice demonstrated a consistent anxiolytic phenotype, in comparison to their WT littermates, in three different anxiety assays.
- Overall, C417Y homozygous ENU-mutant mice showed equivalent anxiety-related behaviour in comparison to their littermate WT controls.

Chapter V: Assessment of motivational and hedonic function in the Zfp804a mutant lines; *progressive ratio and lick cluster analysis*

5.1 Introduction

Schizophrenia and other major mental illnesses, such as bipolar disorder, have a very complex variety of symptoms, including a range of emotional and motivational deficits, sometimes termed 'negative symptoms' (Bleuler, 1911), manifesting as avolition, withdrawal and a reduced ability to experience pleasure (anhedonia). These emotional and motivational deficits have been linked to the cognitive impairments seen in schizophrenia, with the suggestion that perhaps a general lack of motivation in these patients contributes to the cognitive dysfunction observed in psychometric tests (Barch, 2005). Avolition has long being described as a core symptom of schizophrenia (Kraepelin, 1919), however, there have been relatively few studies examining motivation in mental disorders, with one possible reason being that motivation is seen as difficult to quantify and is a complex notion to study empirically (Barch, 2008). Notwithstanding these difficulties, recent research has found that avolition, and other negative symptoms in schizophrenia patients, are strong predictors of functional outcome and quality of life (Rabinowitz et al., 2012), and that self-generated motivation can promote cognitive improvement, and therefore contribute to the positive relationship between cognition and psychosocial functioning in schizophrenia (Nakagami, Xie, Hoe & Brekke, 2008).

Deficits in motivation in schizophrenia have been linked to the dopamine system (Berridge, 2004), with the idea that dopamine is involved with 'wanting' (working towards achieving a desirable goal; thought to be part of motivational processes) but not necessarily 'liking' (the pleasurable consummatory response). Hence, abnormalities in dopamine function may lead to changes in incentive motivation towards achieving a goal, but when the goal is achieved, the hedonic response remains unaltered (Barch, 2005). A progressive ratio task (PRT) can be used to measure the motivation of rodents to continue to work for a reward, when the effort required to earn the reward is progressively increased (Hodos, 1961). This task was originally developed for use with rats, utilising lever presses as the 'work'

element of the task. Since then, the PRT has been adapted for use with mice incorporating an increasing schedule of nose-pokes. Eventually the mouse will reach a ratio at which it will no longer nose-poke, and it is this 'breakpoint' that is held to index the subject's incentive motivational drive to obtain the reinforcer. In the current work we used a mouse version of the PRT to assess motivation in the *Zfp804a* ENU-mutant lines. To our knowledge, there have been no previous mouse models based on manipulations of confirmed psychiatric risk genes that have utilised the PRT. However, one study looking at dopamine receptor subtypes found that mutants that over-expressed subcortical D_2 receptors exhibited lower incentive motivation on a progressive ratio schedule (Drew, Simpson, Kellendonk et al., 2007).

The second task described in this chapter was a lick cluster analysis designed to assess the hedonic reactions of mice to various solutions. Anhedonia, defined as a diminished ability to experience pleasure, is also a common symptom of schizophrenia (Kraepelin, 1919), with research showing that patients with schizophrenia report experiencing lower levels of pleasure in general than controls in both interviews and self-report questionnaires (reviewed in Horan, Kring & Blanchard, 2006b). However, other research has also shown that patients report similar experiences of positive and negative emotions, in comparison to controls, in response to evocative emotional stimuli (Berenbaum & Oltmanns, 1992; Kring & Neale, 1996). This discrepancy in the research findings has led to the idea that, arguably, schizophrenia patients experience normal levels of pleasure when actually engaged in an enjoyable activity (consummatory pleasure) but experience anhedonia in anticipation of future pleasurable activities (anticipatory pleasure), an idea which has received some support (Gard, Kring, Gard-Germans, Horan & Green, 2007). Gard et al. (2007) examined daily reports of consummatory and anticipatory pleasure, finding that patients anticipated less joy from goal-directed activities than controls, but reported similar consummatory pleasure as controls. However, yet another study has found the opposite pattern, with the main differences between patients and controls being seen in reported consummatory pleasure (Strauss, Wilbur, Warren, et al., 2011). Clearly, the exact nature of the hedonic deficits in schizophrenia remains to be established, especially as much of the data is obtained from self-report questionnaires which are notoriously noisy and subject to bias.

Lick cluster analyses have been used previously in a number of settings to index 'pleasure' in rodent models. It is important to appreciate that other assays, which merely measure consumption, are limited in their ability to reflect hedonic function. Typically in consumption-based tests, total consumption of a solution is taken as a measure of palatability; however, the inverted-U shaped function for consumption of palatable solutions confounds this approach (since the same amounts of solution can be consumed at low and high concentrations of, say, sucrose). Consequently, this approach has been criticised as providing an insufficient method for analysing the hedonic reactions of animals (Dwyer, 2012).

An alternative approach to monitor hedonic components of behaviour is to exploit automated technical approaches that allow fine-grained analysis of licking behaviour. This approach, first established in rat models, has demonstrated that rodents produce rhythmic sets of licks that can be grouped into lick clusters based on the interval between licks; critically, the microstructure of the licking behaviour, in particular the size of the lick cluster (licks per cluster) provides an indication of the perceived palatability and concentration of the solution (Davis, 1973; Davis & Levine, 1977; Davis & Smith, 1992). Thus, lower lick cluster sizes have been found for unpalatable solutions such as quinine, and higher lick cluster sizes have been found for palatable solutions such as sucrose (Hsiao & Fan, 1993), suggesting that lick cluster size provides a valid index related to hedonic components of behaviour. Importantly, measurements of lick cluster size and the total amount of solution consumed are dissociable, with maximal levels of consumption at moderate concentrations of palatable solutions, but maximum lick cluster sizes at high concentrations, illustrating that despite consuming less of the higher concentrations of sucrose, they actually like it more; this cannot be discerned from the consumption data alone (Dwyer, 2012).

There are several examples in the literature where lick cluster analysis has been used to examine behavioural changes in relation to factors that may be of relevance to vulnerability for brain disorders. One attempt was to use 16 days of psychosocial cage-change stressor treatment in rats to model the predisposing effects of chronic stress. Every day, rats from the stress treatment group were either placed in a new cage alone, a cage with one other rat or a cage of two other rats,

with bedding soiled by another groups of rats. The stressor treatment group gained weight but also demonstrated anhedonia as evidenced by reduced lick cluster size in response to a 5% sucrose solution (Dwyer, 2012). In contrast, systemic dosing with the NMDA antagonist phencyclidine (PCP), either acutely or sub-chronically in rats, had no reliable effects on hedonic reactivity to sucrose (Lydall, Gilmour & Dwyer, 2010). These unexpected findings could be due to PCP treatment modelling more of the positive symptoms of schizophrenia than the negative (Dwyer, 2012).

5.2 Materials and Methods

5.2.1 Subjects and animal husbandry

In total, 55 adult male mice were used in this series of experiments (age range 7 to 12 months at beginning of testing, see Table 5.1). The progressive ratio task (PRT) was run first followed by lick cluster analysis (LCA) two months later.

Table 5.1: Sample size and genotype of each cohort-subset of mice assayed on paradigms of hedonic and motivational function

Behavioural Task	C59X line		C417Y line	
	WT	Homozygous	WT	Homozygous
Progressive Ratio Task (PRT)	13/13	14/14	13/13	14/15
Lick cluster Analysis (LCA)	13/13	14/14	13/13	15/15

Mice were housed in littermate groups of two to five animals per cage, under standard temperature- and humidity controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow and water until behavioural procedures commenced. For the PRT mice were on a partial water restriction schedule, allowing *ad libitum* access to laboratory chow and 2 hours access to water per day following testing. For the LCA experiment, subjects were on a standardised feeding schedule of 8 hours access/day, with water freely available, which ensured that the subjects had not eaten for 16 hours prior to testing. For both schedules, food or water was presented following testing. Mice were weighed on a regular basis and any subjects losing greater than

10% of their body weight were removed from the experiment. For details of the restrictions schedules used see below. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986.

5.2.2 General Behavioural methods

All testing took place between the hours of 09:00 and 18:00, with equal distribution of testing for subject of different genotypes throughout the day. Prior to testing, mice were habituated to the test rooms for at least 10 minutes.

5.2.3 Progressive ratio task

Mouse nine-hole operant chambers were used for this experiment (Humby et al., 1999), with eight of the nine nose-poke holes in the response array covered, leaving just the central aperture open. A more detailed description of the apparatus can be found in Chapter II, Section 2.4.9. The main measures of interest were the number of rewards collected, the breakpoint of nose poking (the maximum number of nose-pokes a mouse would perform for a single food delivery), the overall session duration, the average latency to make the first nose-poke for each reward, the latency to collect a reward and the intra nose-poke duration.

5.2.3.1 Restriction procedure

Before any behavioural procedures, subjects had to complete a water restriction schedule to motivate behaviour in the PRT (See Chapter II, Section 2.3.4). This schedule lasted 6 days, encompassing 4 days of 4hr access/day and 2 days of 2 hr access/day. Mice were weighed daily during this schedule, as were their drinking bottles to ensure they were drinking during the water access. After these 6 days, mice were maintained on a 2hr access/day schedule.

5.2.3.2 Habituation to the reinforcer

Habituation to the condensed milk reinforcer used in the PRT took place as previously described in Chapter II, Section 2.3.5. Subjects not reaching a 70%

preference for the condensed milk (CM) reward over water (on the last day of testing) were not used in the PRT.

5.2.3.3 Initial behavioural shaping

Mice were given one training session per day at approximately the same time of day, and mice were always tested individually in the same operant boxes each day. Following habituation to the test chambers and shaping to learn that reward was available in the apparatus, the mice were trained over 5 sessions whereby they had to make a single nose-poke to initiate delivery of the CM reward (22µl, 10% concentration). These continuous reinforcement schedule (CRF) sessions terminated after collection of 100 rewards or if 30 minutes had elapsed. In the analysis of these CRF sessions, the last 3 days at CRF were averaged together.

5.2.3.4 Progressive ratio schedule

After the 5 sessions at CRF, subjects were moved to a progressive ratio (PR) schedule of reinforcement. Under this schedule, the nose-poke requirement for food delivery was increased with the following progression: 1, 2, 4, 6 (then further incrementing by 2 nose-pokes/ratio) with two trials at each ratio. The progression continued until the subject failed to complete the ratio requirements, determined by the failure to make a further nose-poke within 4 minutes of the previous response (timed out). Sessions could also be terminated if 100 rewards had been delivered or if 30 minutes had elapsed, as per the CRF sessions. This PR schedule was administered for 3 consecutive days, with performance averaged across the 3 sessions (See Appendix 4.1-4.3). After these 3 PR sessions, subjects were returned to the CRF schedule for 2 days (CRF2); performance here was averaged over the 2 days.

5.2.3.5 Task manipulations

To assess the impact of different levels of reward on the motivation to perform the PRT, a series of probe tests were performed, counter-balanced with standard PR sessions. Thus, the concentration of the 22µl condensed milk reward was decreased from 10% to 0%. Table 5.2 illustrates the different reward

manipulations used. Mice were tested on alternate days with a single session per day.

Table 5.2: The amount and concentration of the reward manipulation.			
Session	**Reward type**	**Amount**	**Concentration of condensed milk**
Standard	Condensed Milk	22µl	10%
Decreased concentration	Water	22µl	0%

5.2.4 Lick cluster analysis

For the LCA experiment, subjects were on a standardised feeding schedule of 8 hours access/day, with water freely available, which ensured that the subjects had not eaten for 16 hours prior to testing. For all testing subjects were placed in a cage similar to their home cage, modified to incorporate the lick cluster analysis equipment (as previously described in Chapter II, Section 2.4.7) with the experimental rooms illuminated. Subjects were placed individually into the test cages and given 10 minutes to drink the available solutions from bottles (maximum volume: 50 ml). Each chamber was equipped with a single test bottle, which was attached to a lickometer. The lickometer accurately detected any contact made with the spout of the bottle, and allowed the measurement of parameters such as the total number of licks, lick clusters (>single lick/cluster), single lick clusters, average inter-lick interval and volume consumed/1000 licks. In the current experiment, a lick cluster was defined as a series of licks where the intra-lick interval did not exceed 500ms, therefore any lick occurring >=500ms after the previous would be recorded in a new lick cluster. Other parameters such as total consumption of sucrose and lick cluster size were also derived from these initial measures. Sucrose consumption was normalised to body weight using the following equation: $Vol^{Nor}=Vol/weight^{0.75}$ (Schmidt-Nielsen, 1990).

5.2.4.1 Training to baseline performance

Mice were given 6 sessions, with a single session each day, to habituate to the test environment and learn to drink from the lickometer apparatus. In each of these sessions, 8% sucrose solution was present in the drinking bottles, and the mice could consume as much of the solution as they would like (ca.1-2ml). As was expected, the size of the lick clusters was low (typically 0-15) and the inter-lick interval quite high (ca.150ms), but on consuming a similar amount of the solution for 3 days running and achieving less than 40 single lick clusters, subjects were moved on to the next stage of testing. Performances across the final 3 sessions of training were averaged together for analysis. Data from sessions where delivery of the sucrose had been impaired by blockage of the spout or leaking from the bottle was removed from the analysis.

5.2.4.2 Test phases

To test the sensitivity of the pattern of drinking to the palatability of the reward solution, animals were tested with 2 further sucrose solutions at 4% and 16% (baseline training was at 8% sucrose concentration). As during baseline sessions, subjects were placed in the testing cages and given 10 minutes to lick the sucrose solution from the testing bottles. Consumption of each solution was assessed over 4 consecutive days, with a single 10 minute session each day, and performance was averaged across these sessions. Days where bottles had leaked or where bottles were blocked were taken out of the analysis. The order of presentation of the 2 sucrose solutions was counter-balanced, hence, some subjects received the lower concentration first, and the higher concentration second, and others in the reverse order. As before, days where bottles had leaked or where the drinking spouts had blocked were removed from the analysis.

5.2.5 Statistical analysis

The experimental data was analysed using SPSS 18.0. Behavioural data are presented as mean values (*M*) along with the standard error of the mean (SEM), unless stated otherwise. All data were analysed by either ANOVA or t tests. All significance tests were performed at alpha level of 0.05 and where significant

interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. Data were screened for skewed variance with arcsine transformations applied to skewed data (percentage data in particular) to obtain a normal distribution. Each parameter (see above for the PRT and LCA) was analysed separately for each strain, with between subjects factor of GENOTYPE (homozygous ENU-mutant vs. WT littermate control) and within subject factors as appropriate (i.e. SESSION, REWARD TYPE and REWARD CONCENTRATION).

5.3 Results

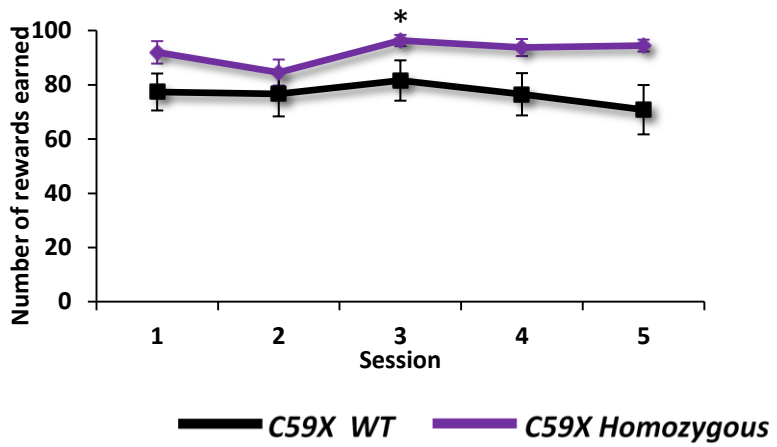
5.3.1 Zfp804a C59X Line

5.3.1.1 Progressive Ratio Task

Mice were initially trained to respond on a continuous reinforcement (CRF) schedule that allowed the animals to collect a maximum of 100, 20µl rewards within each 30min test session. Mice of both genotypes readily achieved high levels of performance, collecting >75 rewards (out of 100 available) per session from the onset of training (Fig. 5.1, main effect of SESSION, $F_{3.1,78.4}$= 1.8, p=0.13). As the C59X homozygous ENU-mutant mice showed higher levels of nose-poke responding, they consequently earned significantly more rewards than their WT littermate controls in this initial phase of training, which did not explicitly tax motivation (main effect of GENOTYPE, $F_{1,25}$= 4.5, p=0.04). There were similar patterns of behaviour for other parameters during the CRF training phase, with no significant session, genotype differences or interactions in the overall session duration (main effect of SESSION, $F_{4,100}$= 0.6, p=0.65, main effect of GENOTYPE, $F_{1,25}$ = 0.9, p=0.36, SESSION*GENOTYPE, $F_{4,100}$ = 0.5, p=0.74) or the latency to collect the reward (main effect of SESSION, $F_{1.5,36.5}$ =2.0, p=0.11, main effect of GENOTYPE, $F_{1,25}$= 0.9, p=0.36, SESSION*GENOTYPE, $F_{4,100}$ = 0.3, p=0.87). The latency to make the first nose-poke for each reward did not significantly differ between CRF sessions (main effect of SESSION, $F_{2.94,73.5}$ = 0.8, p=0.51), however, C59X homozygous ENU-mutants were significantly quicker to initiate the first nose-poke than their WT littermate controls

(main effect of GENOTYPE, $F_{1,25}$= 6.0, p=0.02, SESSION*GENOTYPE, $F_{4,100}$ = 1.4, p=0.26).

Figure 5.1: Acquisition of CRF behaviour by C59X mice.

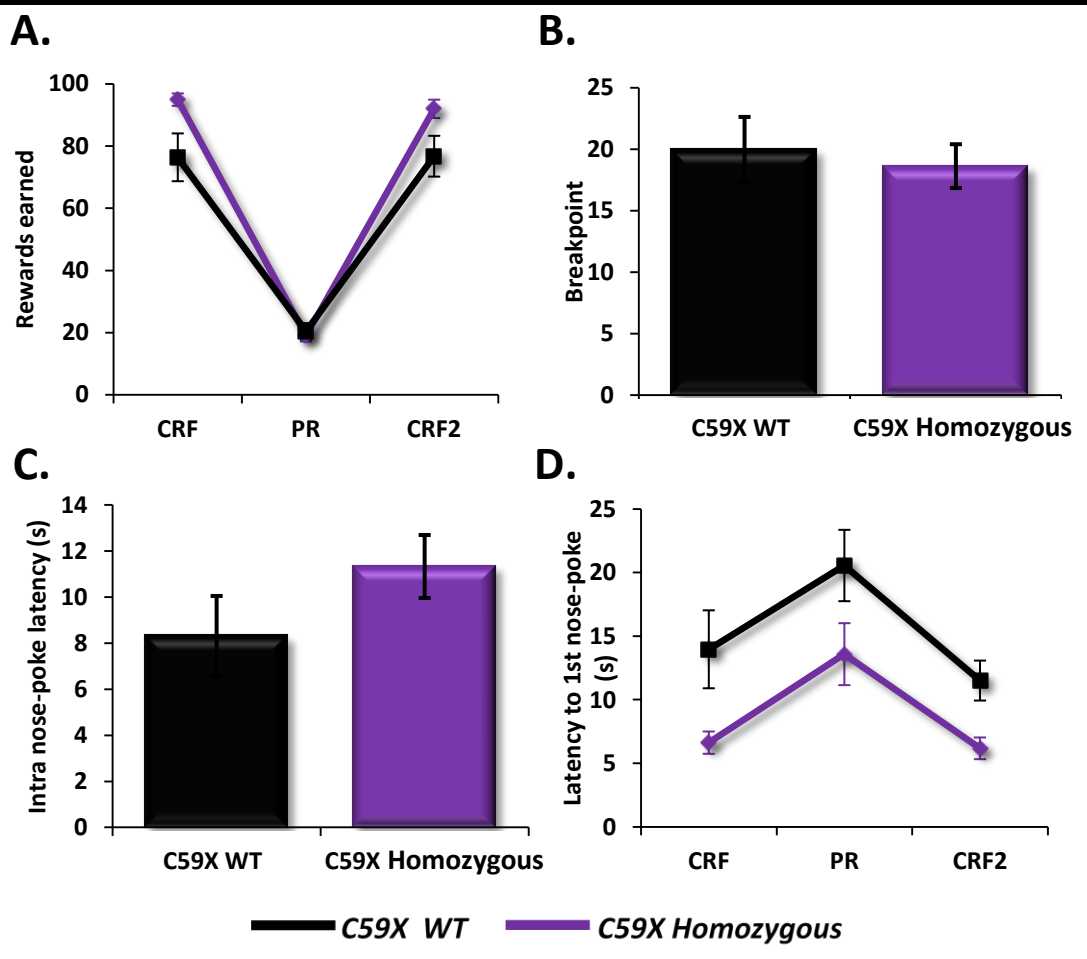


All mice from the C59X line readily learnt to make a single nose-poke to earn a 22µl delivery of 10% CM solution, reaching near the maximum of 100 available rewards within the 30min session. C59X homozygous ENU-mutant mice completed more trials and therefore earned significantly more rewards than their WT littermate controls during this initial phase of training. Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 5.1.

Following CRF training the mice were switched to the progressive ratio schedule (PR), where the number of nose-pokes required to initiate a 22µl reward delivery was gradually increased throughout the session (see Section 5.2.3.4 for details). Performance across the 3 PR sessions was averaged together, and to demonstrate the effects of the imposition of this schedule, PR performance was compared with the average of the last 3 CRF sessions prior to, and the average of 3 CRF sessions following PR testing (See Appendix 4.4 and 4.6). Imposition of the PR schedule led to a significant reduction in the number of rewards earned within a session (Fig. 5.2A, main effect of SESSION, $F_{2,50}$= 382.5, p=0.000); this reduction was equivalent between the C59X genotypes (main effect of GENOTYPE, $F_{1,25}$= 3.9, p=0.06) although a significant interaction (SESSION*GENOTYPE, $F_{2,50}$= 7.8, p=0.001) revealed that the C59X homozygous ENU-mutant mice (as before) earned significantly more rewards than their WT littermate during the CRF sessions before the PR schedule (p=0.04) and the CRF sessions after the PR schedule (CRF2, p=0.02).

The decrease in rewards earned during the PR sessions was not due to the mice running out of time to collect all the available rewards, as the average PR session did not run for the full 30 minutes available, and there were no significant differences in session duration between the PR and CRF sessions (main effect of SESSION, $F_{1.3,\ 33.0}$= 0.9, p=0.42). In fact, most mice were timed out midway through a PR session by the failure to make a further nose-poke within 5 minutes of the previous response as a result of the increased number of nose-pokes required to earn a reward, as illustrated by the maximum breakpoint achieved by the mice (Fig. 5.2B).

Figure 5.2: C59X performance in CRF and PR sessions



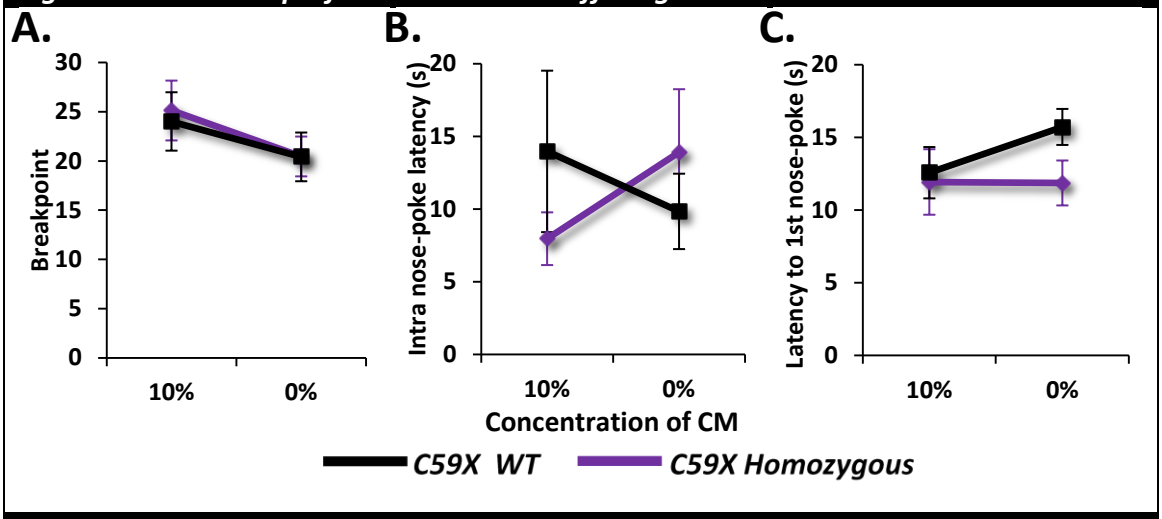
Imposition of the PR schedule led to a large reduction in the rewards earned (A), as a result of the mice having to make increased numbers of nose-pokes for each reward (B). There were no differences in the number of rewards delivered or maximum number of nose-pokes/reward (breakpoint) or in the intra nose-poke latency (C) between the C59X homozygous ENU-mutants and their WT littermate controls. However, the C59X homozygous ENU-mutant mice were quicker to make the first nose-poke in each ratio (D). Data shows the mean values ±SEM. For subject N see Table 5.1.

There was no significant genotype differences in the PR breakpoint (t_{25}= -0.4, *p*=0.66), suggesting an equivalent level of motivation between C59X homozygous ENU-mutant mice and their WT littermate controls as indexed by this key measure. In addition to the main breakpoint measure, further evidence for a lack of genotype effects on motivational processes in the PRT was shown by equivalent intra nose-poke latencies between the C59X homozygous ENU-mutant mice and their WT littermate controls (Fig. 5.2C, t_{25}= 1.4, *p*=0.18). Imposition of the PR schedule did, however, lead to a general slowing of performance compared to the speed of responding in CRF sessions, with an increase in the latency to make the first nose-poke of each ratio sequence (Fig. 5.2D, main effect of SESSION, $F_{1.4,\ 34.7)}$ = 14.9, *p*=0.000). C59X homozygous ENU-mutant mice were quicker overall than their WT counterparts to make this first response (main effect of GENOTYPE, $F_{1,25}$= 8.1, *p*=0.009, SESSION*GENOTYPE, $F_{1.4,\ 34.7}$= 0.2, *p*=0.8); the precise interpretation of these effects in the homozygous ENU-mutants is unclear, though it is unlikely they were the result of being more motivated to earn the reward given the previous pattern of data. As expected, there was also an increase in the total number of nose-pokes made in the PR session, compared to the CRF sessions (main effect of SESSION, $F_{1.02,25.4}$ = 13.3, *p*=0.000, See Appendix 4.6).

Overall, the data were consistent with there being no reliable differences in the motivation to earn reward between C59X homozygous ENU-mutants and their WT littermate controls. However, to test this further we additionally compared PR performance under differing reward conditions (Hodos, 1961). This was done over 2 sessions (the order counter-balanced between genotypes), where the concentration of reward was altered (see Table 5.2). As anticipated, when the concentration of the reward was decreased to 0% CM (water), the breakpoints of all the C59X mice, both homozygous ENU-mutants and their WT littermate controls, were significantly reduced (Fig. 5.3A, main effect of SESSION, $F_{1,17}$= 10.3, *p*=0.003), illustrating that devaluing the reward reduces motivation to work. Importantly, in terms of the breakpoint reduction, both the C59X homozygous ENU-mutants and their WT counterparts responded equally to this manipulation (main effect of GENOTYPE, $F_{1,17}$= 0.03, *p*=0.87, SESSION*GENOTYPE, $F_{1,17}$= 0.2, *p*=0.68). Furthermore, there was no effect of reward type on the pattern of nose poking, as illustrated by the intra

nose-poke latency (Fig. 5.3B, main effect of SESSION, $F_{1,17}$= 0.1, p=0.74, SESSION*GENOTYPE, $F_{1,17}$= 3.6, p=0.08), and the latency to initiate the 1st nose-poke (Fig. 5.3C, main effect of SESSION, $F_{1,17}$= 1.1, p=0.31, SESSION*GENOTYPE, $F_{1,17}$= 1.2, p=0.29), with no genotype differences in these measures (main effect of GENOTYPE, $F_{1,17}$= 0.04, p=0.85, $F_{1,17}$= 1.3, p=0.27, respectively). There was also no effect of reward type on the latency to collect the reward (main effect of SESSION, $F_{1,17}$= 1.1, p=0.14), nor were there any genotype differences in this measure (main effect of GENOTYPE, $F_{1,17}$= 0.04, p=0.45, SESSION*GENOTYPE, $F_{1,17}$= 0.2, p=0.65). The data from the reward manipulation sessions confirmed the lack of genotype effects on motivation in the C59X mice as assayed in the PRT.

Figure 5.3: C59X PR performances under differing reward conditions.



Decreasing the reward concentration to 0% CM decreased the breakpoint value (A). This effect was indifferent to genotype, suggesting that when less motivated to receive the reward, all of the C59X mice, both ENU-mutants and their WT littermate controls, worked less. There were also no differences between the C59X homozygous ENU-mutants and their WT littermate controls in the maximum amount of nose-pokes made to earn a reward delivery, or in the pattern of nose poking, as shown by the intra nose-poke latency (B) and the latency to make the 1st nose-poke of each ratio (C). Data shows the mean values ±SEM. For subject N see Table 5.1.

5.3.1.2 Lick Cluster Analysis

For the LCA, the mice were first trained to drink from the lickometers over 6 initial sessions with 8% sucrose solution available. As performance was stabilised by the final 3 sessions, parameters from these sessions were averaged together so that the behaviour of the C59X homozygous ENU-mutants and their WT littermate controls could be compared (Table 5.3).

Table 5.3: C59X performance in the 8% sucrose solution training stage of the LCA		
Behavioural Parameter	**WT**	**Homozygous**
Total Consumption (normalised)	0.13 ± 0.005	0.13 ± 0.009
Total single lick clusters	14 ± 2.2	25.7 ± 6.1
Average Inter-lick interval	135.4 ± 2	142.2 ± 2.6
Lick cluster size	16.7 ± 1.8	11 ± 0.67

Data shows mean ± SEM

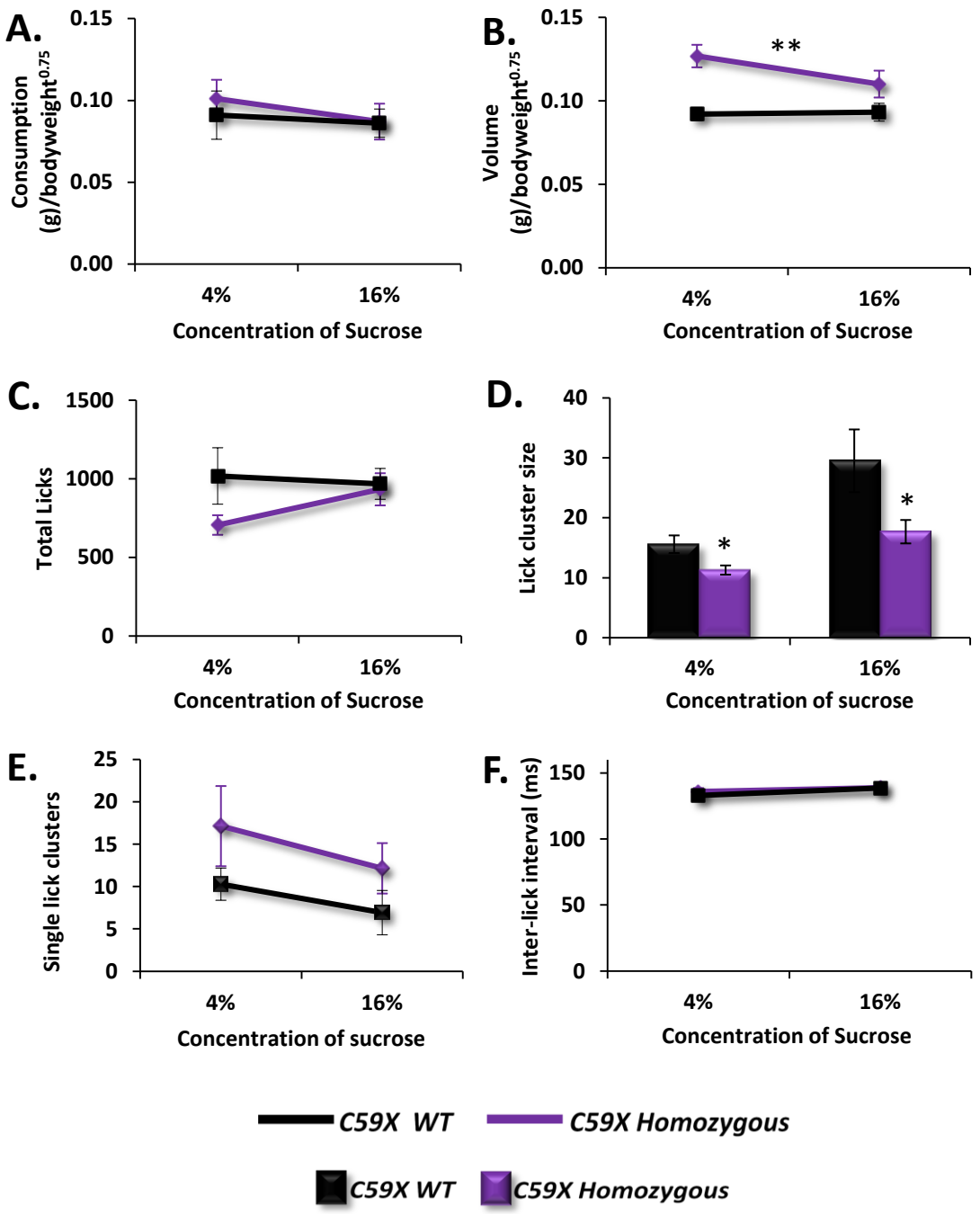
There was no genotype effects in the amount of sucrose consumed in this initial phase of training ($t_{18.58}$= 0.6, *p*=0.53), with mean volumes consumed in the 10 minute session between 1.5 and 1.7ml. There were also no differences due to genotype in the total number of single licks made (t_{24}= 1.8, *p*=0.09), but the C59X homozygous ENU-mutants did show a longer inter-lick latency than their WT littermate controls (t_{24}= 2.1, *p*=0.048). During the initial training phase there were indications of reduced lick cluster size in the C59X homozygous ENU-mutant mice ($t_{15.2}$= -3.0, *p*=0.007). Overall, the average inter-lick intervals for all mice ranged from 135 to 145ms, which meant that they had met the predefined criteria (see Section 5.2.4.1) and were moved on to the main test phase of the LCA with 4% and 16% sucrose solutions. The order of presentation of the 4% and 16% sucrose tests was counter-balanced between the different groups of mice, and data from the 4 sessions of each concentration were averaged together. To ensure that differences in bodyweight could not bias data analysis, the volumes of the different solutions consumed were normalised to body weight on each day of testing (see Section 5.2.4).

In the main test phase there was no difference between C59X homozygous ENU-mutants and their WT littermate controls in the total amount of sucrose consumed (Fig. 5.4A, main effect of GENOTYPE, $F_{1,24}$= 0.4, *p*=0.56) or in consumption of the different sucrose solutions presented (main effect of CONCENTRATION, $F_{1,24}$= 0.1, *p*=0.82, CONCENTRATION*GENOTYPE, $F_{1,24}$= 0.002, *p*=0.96). The C59X homozygous ENU-mutants did show an increased level of consumption per lick (Fig. 5.4B, main effect of GENOTYPE, $F_{1,24}$= 13.1, *p*=0.001), drinking more sucrose/1000

licks than their WT littermate controls. This effect was consistent for both sucrose solutions (main effect of CONCENTRATION, $F_{1,24}$= 2.2, p=0.15, CONCENTRATION*GENOTYPE, $F_{1,24}$= 2.6, p=0.12), although the effect appeared to be driven mainly by the 4% sucrose solution. Both C59X genotype groups made equivalent overall numbers of licks in each session (Fig. 5.4C, main effect of GENOTYPE, $F_{1,24}$= 0.3, p=0.12), and again sucrose concentration did not affect this behaviour (main effect of CONCENTRATION, $F_{1,24)}$= 0.5, p=0.49, CONCENTRATION*GENOTYPE, $F_{1,24}$= 0.9, p=0.32). As expected, lick cluster sizes were significantly greater for the 16% sucrose as opposed to the 4% (main effect of CONCENTRATION, $F_{1,24}$= 20.8, p=0.000, CONCENTRATION*GENOTYPE, $F_{1,24}$= 2.9, p=0.1). Importantly, the C59X homozygous ENU-mutants exhibited reduced lick cluster sizes in comparison to their WT littermate controls (Fig. 5.4D, main effect of GENOTYPE, $F_{1,24}$= 5.4, p=0.03). *Post-hoc* tests showed the genotype effect to be a stronger when the 4% sucrose solution was present (p=0.016), although they still showed smaller lick cluster sizes with the 16% solution (p=0.045). Lick cluster size is the main index of perceived palatability (Davis & Smith, 1992); therefore, these data are consistent with a mutation-related reduction in hedonic reactivity to the palatable solution.

There was no significant difference in the number of single lick clusters made by the mice, although there was a strong tendency for C59X homozygous ENU-mutants to make more single lick clusters than their WT littermate controls (Fig. 5.4E, main effect of GENOTYPE, $F_{1,24}$= 3.5, p=0.075), as might be expected in relation to the overall reduced size of their lick clusters. This pattern of licking was consistent for both sucrose solutions (main effect of CONCENTRATION, $F_{1,24}$= 1.7, p=0.21, CONCENTRATION*GENOTYPE, $F_{1,24}$= 0.06, p=0.8). The average inter-lick interval was found to be significantly increased when the 16% sucrose solution was available (Fig. 5.4F, main effect of CONCENTRATION, $F_{1,24}$= 8.4, p=0.008, CONCENTRATION*GENOTYPE, $F_{1,24}$= 0.9, p=0.37), which may be a result of increased viscosity of the 16% sucrose solution, and so may be more difficult to drink at speed than the 4% sucrose. Importantly, there were no genotype differences for the average inter-lick interval at either concentration (main effect of GENOTYPE, $F_{1,24}$= 0.2, p=0.63).

Figure 5.4: C59X mice LCA at 4% and 16% sucrose concentrations



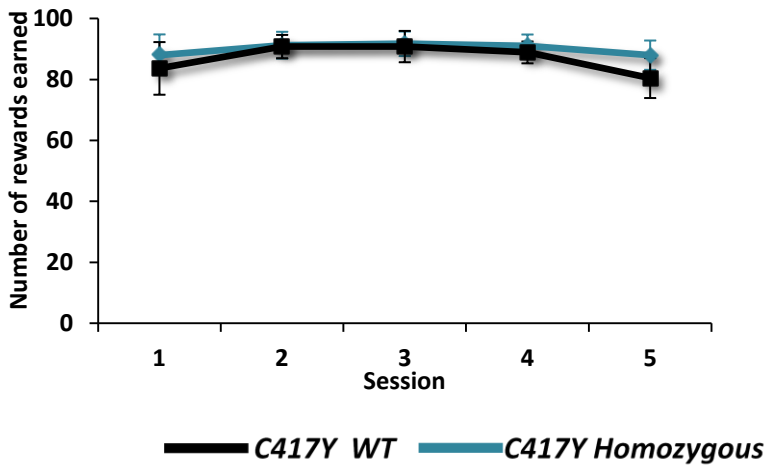
There was no significant difference in the total amount of different sucrose solutions consumed by the C59X homozygous ENU-mutants and their WT littermate controls (A, normalised to bodyweight$^{0.75}$), but the C59X homozygous ENU-mutant mice showed an increased volume per 1000 licks for each solution (B). Similarly, although there was no overall difference in the total number of licks made by the mice (C), the C59X homozygous ENU-mutants had significantly reduced lick cluster sizes (D), and a related increase in single lick clusters (E) relative to their WT littermate controls. There were no genotype differences in the intra-lick latency with either 4% or 16% sucrose solutions (F). Data shows the mean values ±SEM, $**p<0.01$ and $*p<0.05$ for pairwise differences related to genotype. For subject N see Table 5.1.

5.3.2 Zfp804a C417Y Line

5.3.2.1 Progressive ratio task

As with the C59X line, the C417Y line achieved high levels of performance during CRF training sessions, acquiring approximately 85% of the maximum available number of 22µl rewards in each session (Fig. 5.5). All the mice operated at these high levels (main effect of SESSION, $F_{2.1,53.5}$= 1.3, p=0.27, SESSION*GENOTYPE, $F_{2.1,53.5}$= 0.3, p=0.89) and performance between C417Y homozygous ENU-mutants and their WT littermate controls was equivalent during this initial phase of training which did not explicitly tax motivation (main effect of GENOTYPE, $F_{1,25}$= 0.3, p=0.58). Other parameters such as the overall session duration, latency to make the first nose-poke for each reward and the latency to collect the reward also showed no genotype differences (main effect of GENOTYPE, $F_{1,25}$ = 0.3, p=0.6, $F_{1,25}$ = 0.002, p=0.97, $F_{1,25}$ = 0.8, p=0.78, respectively).

Figure 5.5: Acquisition of CRF behaviour by C417Y mice.

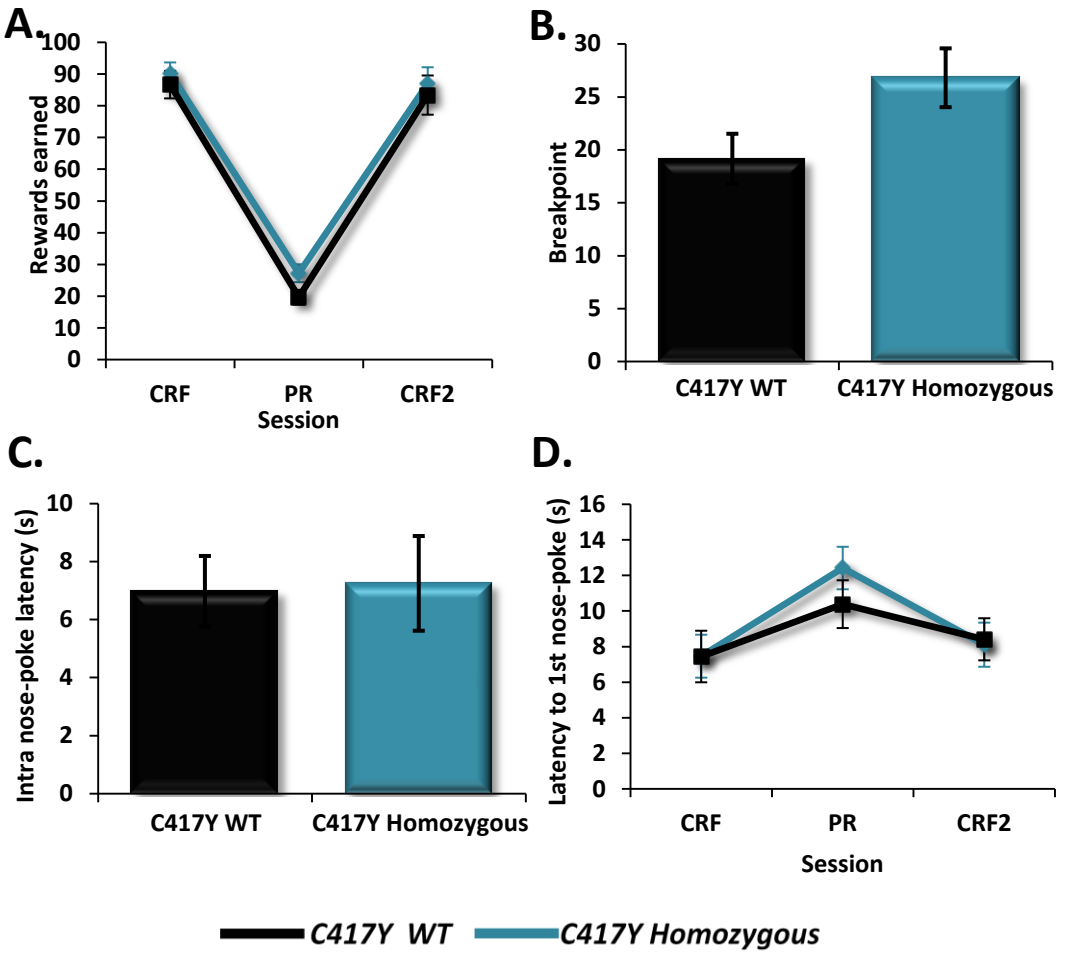


Both the C417Y homozygous ENU-mutants and their WT littermate controls collected near maximum numbers of the 22ml 10% CM solution reward in each 30min session of the CRF training period. Performance of C417Y mice during these sessions was equivalent. Data shows the mean values ±SEM. For subject N see Table 5.1.

Following CRF training, subjects were moved on to the PR schedule, where the number of nose-pokes required for each reward steadily increased through the sessions. For comparison, averages of the final 3 CRF sessions before PR testing, the 3 PR sessions and 3 CRF sessions were calculated (See Appendix 4.5-4.6). As expected, imposition of the PR led to a significant decrease in the amount of rewards

earned by the mice, in comparison to CRF responding (Fig. 5.6A, main effect of SESSION, $F_{2,50}$= 387.8, p=0.000, SESSION*GENOTYPE, $F_{2,50}$= 0.4, p=0.69). Consistent with the C59X line, this is most probably due to the increased load of increasing nose-pokes to earn reward during the PR schedule.

Figure 5.6: : C417Y performance in CRF and PR sessions.



Imposition of the PR schedule led to a significant decrease in the amount of rewards earned by the mice (A) in comparison with CRF responding. The C417Y homozygous ENU-mutants showed a strong tendency for increased motivation to work in PR sessions, as shown by a propensity to make more nose-pokes to earn a reward, reaching higher breakpoints than their WT littermate controls (B). There was no difference in the intra nose-poke latency (C), although there was a general slowing to make the first nose-poke of each ratio in PR sessions (D). Data shows the mean values ±SEM. For subject N see Table 5.1.

There was no genotype difference for the number of rewards earned between the CRF, PR and CRF2 sessions (main effect of GENOTYPE, $F_{1,25}$= 0.9, p=0.35). Again, similar to the C59X mice, PR sessions were more likely to be terminated by the mice discontinuing their nose poking behaviour rather than reaching the end of the 30

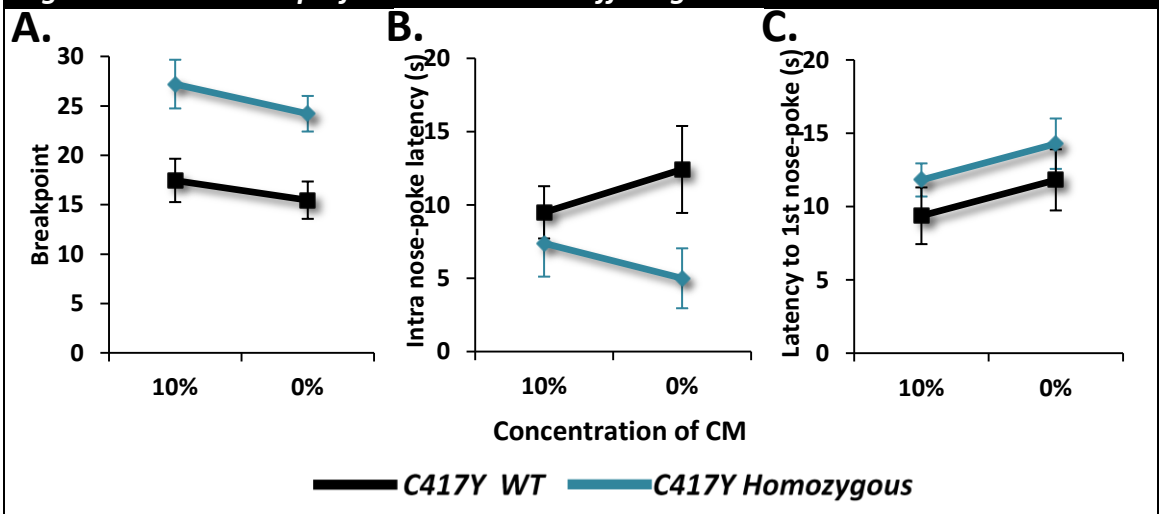
minute sessions, with a mean PR session duration of 24 minutes (data not shown). CRF sessions lasted on average 21 minutes, with no significant difference in session duration between CRF and PR (main effect of SESSION, $F_{1.39,34.7}$= 2.9, *p*=0.06). Importantly, and in contrast to the C59X mice, there was a strong tendency (t_{25}= 2.04, *p*=0.053) for the C417Y homozygous ENU-mutant mice to achieve higher breakpoints in the PR sessions than their WT littermate controls (Fig. 5.6B), suggesting that these mice may be more motivated to work for a reward, at least as indexed by this baseline measure. Intra nose-poke latencies (Fig. 5.6C, t_{25}= 0.1, *p*=0.9) and the latency to make the first nose-poke (Fig. 5.6D, main effect of GENOTYPE, $F_{1,25}$= 0.2, *p*=0.67) did not differ between the C417Y homozygous ENU-mutant mice and their WT littermate controls, although there was a significant decrease in the speed with which they started each ratio in the PR sessions compared to CRF sessions (main effect of SESSION, $F_{1.6,40.5}$= 8.2, *p*=0.001, SESSION*GENOTYPE, $F_{1.6,40.5}$= 0.8, *p*=0.48). As expected**,** overall the C417Y mice made significantly more nose-pokes in the PR session than in CRF sessions (main effect of SESSION, $F_{1.01, 25.2}$= 20.0, *p*=0.000) for the reasons outlined previously.

These initial data suggested that the C417Y homozygous ENU-mutants may have had a higher level of motivation to work for a reward, but to assess this further the mice were then tested under different reward conditions (see Table 5.2). This was done over 2 sessions (the order counter-balanced between genotypes), where the concentration of reward was altered. As expected, when the concentration of the reward was decreased to 0% CM (water), the breakpoints of the C417Y mice were, in general, reduced, albeit not quite significantly (Fig. 5.7A, main effect of SESSION, $F_{1,19}$= 4.3, *p*=0.052, SESSION*GENOTYPE, $F_{1,19}$= 0.2, *p*=0.68). Importantly, the C417Y homozygous ENU-mutants maintained their significantly increased breakpoints, in comparison to WT littermate controls, across both reward conditions (main effect of GENOTYPE, $F_{1,19}$= 11.6, *p*=0.003), providing further evidence that that these animals were more willing to work for a reward, even when the reward was devalued, as indexed by this key measure of motivation. It should be noted that, *a priori*, several factors may have been contributing to the rewarding properties of the two reinforcers used in this part of the study (in addition to palatability), including degree of thirst and rate of satiation. However, it is unlikely that gross effects on

thirst or satiation could explain the effects on breakpoints seen in the C417 homozygous ENU-mutant mice since the CRF performance data (where animals consumed much larger volumes of liquid reinforcer compared to the PR sessions) did not show any effects due to genotype. This issue will be discussed further in the Discussion section below.

The intra nose-poke latency was not affected by reward type (Fig. 5.7B, main effect of SESSION, $F_{1,19}$= 0.02, p=0.9, SESSION*GENOTYPE, $F_{1,19}$= 1.6, p=0.22) nor by C417Y genotype (main effect of GENOTYPE, $F_{1,19}$= 3.6, p=0.07). However, the latency to initiate the 1st nose-poke in each ratio was significantly increased for the 0% CM reward condition (Fig. 5.7C, main effect of SESSION, $F_{1,19}$= 5.6, p=0.03, SESSION*GENOTYPE, $F_{1,19}$= 0.0, p=0.99), perhaps indicating a reduced motivation to initiate trials when the reward is devalued. C417Y homozygous ENU-mutants and their WT littermate controls had equivalent initiation latencies (main effect of GENOTYPE, $F_{1,19}$= 1.1, p=0.3).

Figure 5.7: C417Y PR performances under differing reward conditions.



Altering the reward conditions by reducing the reward concentration to 0% CM reduced the amount of nose-pokes subjects were will to make to earn a reward (A). During these manipulation sessions, the C417Y homozygous ENU-mutants were more motivated, having significantly higher breakpoints than their WT littermate controls. There were no differences in the intra nose-poke latency (B), but latency to make the first nose-poke of each ratio was significantly slowed when the concentration of reward was reduced (C). Data shows the mean values ±SEM. For subject N see Table 5.1.

There was no effect of reward type on the latency to collect the reward (main effect of SESSION, $F_{1,19}$= 2.7, p=0.12, SESSION*GENOTYPE, $F_{1,19}$= 3.4, p=0.08, data not

shown), nor were there any genotype differences on this measure (main effect of GENOTYPE, $F_{1,19}$= 0.13, *p*=0.72). There were also no differences in the way in which these different reward sessions were terminated with all C417Y subjects of both genotypes being timed out for not making consecutive nose-pokes within the criteria time, and equivalent session durations for both reward types (main effect of SESSION, $F_{1,19}$= 2.5, *p*=0.13, SESSION*GENOTYPE, $F_{1,19}$= 0.2, *p*=0.67, data not shown) and genotype (main effect of GENOTYPE, $F_{1,19}$= 0.6, *p*=0.46). In general, the data from the reward manipulation sessions tended to confirm that the C417Y homozygous ENU-mutants showed evidence of increased motivation to work for reward relative to their WT littermate controls, as assayed in the PRT.

5.3.2.2 Lick cluster analysis

Mice were trained over 6 initial sessions with 8% sucrose solution available, data from the final 3 sessions were averaged together and used for comparison (Table 5.4). There was no differences in this initial phase of training between C417Y homozygous ENU-mutants and their WT littermate controls in the consumption of sucrose in the 10 minute sessions (t_{25}= -0.7, *p*=0.5), with average volumes consumed ranging between 1.5 to 1.6ml in 10 minutes. All mice of the C417Y line also made a similar amount of single lick clusters ($t_{18.8}$= -0.6, *p*=0.53), and took an equivalent amount of time between licks (t_{25}= 0.8, *p*=0.42), with inter-lick intervals ranging from 136 to 141ms.

Table 5.4: C417Y performance in the 8% sucrose solution training stage of the LCA

Behavioural Parameter	WT	Homozygous
Total Consumption (normalised)	0.13 ± 0.005	0.13 ± 0.008
Total single lick clusters	16.3 ± 3.5	13.8 ± 1.9
Average Inter-lick interval	137.7 ± 2.7	140.6 ± 2.3
Lick cluster size	20.8 ± 2.1	18.6 ± 1.7

Data shows mean ± SEM

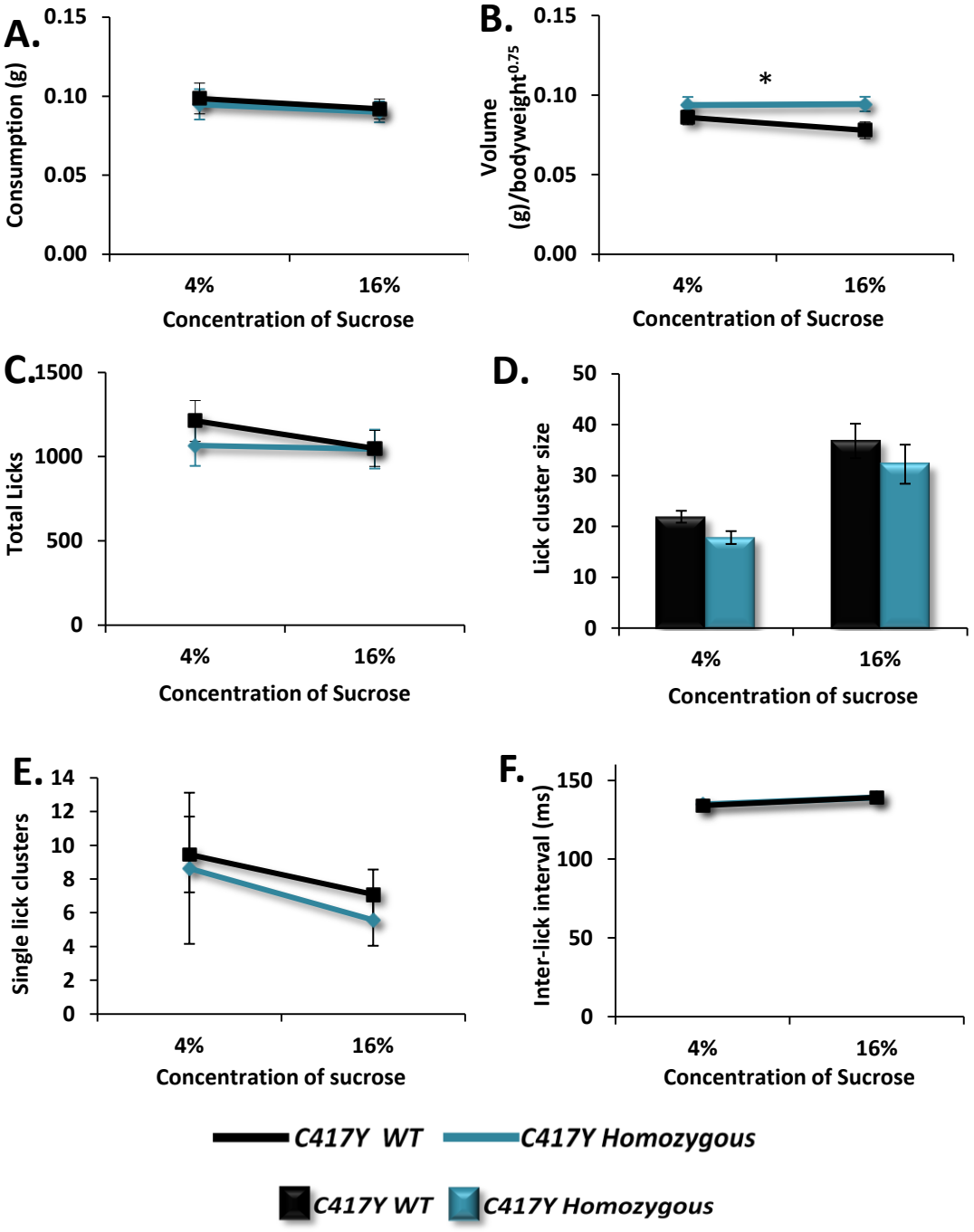
As the values for these parameters were within criteria (see Section 5.2.4.1), all of the mice were moved on to the main test phase of the LCA with 4% and 16% sucrose

solutions, the order of which was counter-balanced between different genotype groups. Drinking of each solution was averaged across 4 consecutive days, and any session where the drinking spout was blocked or the reservoir had leaked was removed from the analysis. As previously, to ensure that differences in bodyweight could not bias data analysis, the volumes of solution consumed were normalised to the individual bodyweight of each mouse (see Section 5.2.4).

In the main test phase of the LCA, there was no difference in the total amount of 4% and 16% sucrose consumed by the mice (Fig. 5.8A, main effect of CONCENTRATION, $F_{1,25}$= 0.6, p=0.44, CONCENTRATION*GENOTYPE, $F_{1,25}$= 0.02, p=0.9) or in consumption between C417Y homozygous ENU-mutants and their WT littermate controls (main effect of GENOTYPE, $F_{1,25}$= 0.1, p=0.77). There were also no differences in the amount of sucrose consumption per lick between the different solutions (Fig. 5.8B, main effect of CONCENTRATION, $F_{1,25}$= 1.1, p=0.3, CONCENTRATION*GENOTYPE, $F_{1,25}$= 1.1, p=0.3), although C417Y homozygous ENU-mutant mice consumed more sucrose/1000 licks than their WT counterparts (main effect of GENOTYPE, $F_{1,25}$= 4.7, p=0.04); this effect appeared to be driven mainly by the 16% sucrose solution. The total number of licks performed by the mice at each concentration were also not found to be significantly different (Fig. 5.8C, main effect of CONCENTRATION, $F_{1,25}$= 0.4, p=0.54, CONCENTRATION*GENOTYPE, $F_{1,25}$= 0.1, p=0.75) and again, there were no significant genotype differences (main effect of GENOTYPE, $F_{1,25}$= 0.9, p=0.635). As expected, the lick cluster size was found to be significantly higher when the mice were drinking the 16% sucrose solution as opposed to the 4% sucrose solution (Fig. 5.8D, main effect of CONCENTRATION, $F_{1,25}$= 52.2, p=0.000, CONCENTRATION*GENOTYPE, $F_{1,25}$= 0.7, p=0.42), as the 16% solution is thought of as more palatable. Importantly, there were no differences in lick cluster size between C417Y homozygous ENU-mutant mice and their WT littermate controls (main effect of GENOTYPE, $F_{1,25}$= 0.8, p=0.37), suggesting that, in contrast to the C59X mutants, hedonic reactivity to a palatable solution was equivalent in the C417Y mice. Furthermore, the number of single lick clusters did not differ between C417Y homozygous ENU-mutants and their WT littermate controls (Fig. 5.8E, main effect of GENOTYPE, $F_{1,25}$= 0.25, p=0.63) or between the different

sucrose solutions (main effect of CONCENTRATION, $F_{1,25}$= 2.5, p=0.13, CONCENTRATION*GENOTYPE, $F_{1,25}$= 0.04, p=0.84).

Figure 5.8: C417Y mice LCA at 4% and 16% sucrose concentrations.



There was no significant differences in the total amount of the different sucrose solutions consumed by the C417Y homozygous ENU-mutants and their WT littermate controls (A, normalised to bodyweight$^{0.75}$), or in the rate of licking for each solution (B). There was also no overall difference in the total number of licks made by the mice (C), with all C417Y mice showing increased lick cluster size for the more palatable 16% sucrose solution (D). The number of single licks did not differ between genotypes (E), nor did the intra-lick latency (F). Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 5.1.

When the 16% sucrose solution was available the mean intra-lick interval was increased, relative to when the mice were drinking the 4% solution, (Fig. 5.8F, main effect of CONCENTRATION, $F_{1,25}$= 7.98, p=0.009, CONCENTRATION*GENOTYPE, $F_{1,25}$= 0.04, p=0.85) although this was behaviour was not affected by C417Y genotype (main effect of GENOTYPE, $F_{1,25}$=0.01, p=0.92).

5.4 Discussion

5.4.1 Motivation, assessed by the progressive ratio task

In general all mice in the C59X and C417Y lines demonstrated the expected basic patterns of behaviour in the progressive ratio task (PRT), achieving high levels of performance in the initial continuous reinforcement phase (where a reward was earned after every nose-poke), with approximately 90 rewards earned, and a reduction in the number of rewards earned (ca.25) following the subsequent imposition of the progressive ratio (PR) schedule. In other words when the number of nose-pokes required to earn a reward increased the subjects became less inclined to work, reaching breakpoints in the 15-25 nose-pokes/reward range. As anticipated, devaluing the reward by reducing the concentration led to a reduction in the breakpoints achieved, although the attenuation in breakpoints was not as large as might be expected. This could be explained by the water restriction schedule the mice were placed on, potentially causing these mice to work equally for any liquid reinforcement, however concentrated. Taken together, this pattern of behaviour would demonstrate that PRT performance in WT mice was governed by the motivation to earn a reward, either in terms of the effort required or by the value of the reward given.

There were no significant effects of C59X genotype in PRT performance, with the only consistent difference being that C59X homozygous ENU-mutant mice were significantly quicker to make the first nose-poke in each ratio than their WT counterparts. The precise interpretation of these effects is unclear, though it is unlikely they were the result of being more motivated to earn the reward as the breakpoint data demonstrated that C59X homozygous ENU-mutants and their WT littermate controls worked equally hard to achieve rewards, even in the face of reward devaluation. Overall, this would suggest that the C59X mutation does not

lead to demonstrable changes in motivation. In contrast, C417Y homozygous ENU-mutant mice demonstrated near significant tendencies to achieve higher breakpoints than their WT littermate controls during the PRT schedule at standard reward conditions (p=0.053). This increased level of motivation became significantly different to their WT littermate controls when the reward was devalued (p<0.01). There were no latency differences between C417Y homozygous ENU-mutants and their WT counterparts under any test condition. Together, these data are consistent with the conclusion that the C417Y mutation was associated with increased motivation to work for liquid reinforcers.

The breakpoints obtained in the progressive ratio task are generally taken as a measure of motivation to earn rewards. Of interest was the fact that the C417Y homozygous ENU-mutants maintained their increased breakpoints across both reward conditions (10% condensed milk and water). This raises the possibility that the pattern of effects could have been influenced by thirst *per se*. However, the idea that the consistently increased breakpoints could have been due to the C417Y homozygous ENU-mutants simply being thirstier than their WT littermate controls is unlikely, as under free drinking conditions (i.e. no requirement to work for the reinforcer, See Chapter III, Section 3.3.4.3) consumption was equivalent between the ENU-mutants and WT littermate controls. Another possibility that may also have led to general effects across the two reinforcers was that the C417Y homozygous ENU-mutants could have been less sated, perhaps due to having larger stomachs than their WT littermate controls. However, confounds due to satiation would also seem unlikely as the much greater amount of reinforcer consumed in general during the initial continuous reinforcement sessions, compared to the PR sessions, showed that satiation was not a factor controlling behaviour in the PR sessions.

It would seem, therefore, that an increased motivation to work for liquid reinforcers remains the most plausible explanation for the increase in breakpoints demonstrated by the C417Y homozygous ENU-mutant mice. This is contrary to what might be expected in a mouse model for schizophrenia, where decreased motivation (avolition) is the more common observation (Barch, 2008) and in the few studies utilising animal models (e.g. Drew et al., 2007). The neural mechanism(s) mediating the changes in motivation remain unknown but, as mentioned previously, one study

has found that mice over-expressing subcortical striatal D_2 receptors had lower incentive motivation on a progressive ratio schedule (Drew et al., 2007). Furthermore, dopamine has been put forward as an important neurotransmitter in motivation (Berridge, 2004). It is possible that altered dopaminergic functioning in the C417Y homozygous ENU-mutant mice is somehow contributing to the increased level of motivation seen in the progressive ratio task, an idea discussed further in the General Discussion (Chapter VIII).

5.4.2 Hedonia, assessed by lick cluster analysis

Mice of both the C59X and C417Y lines demonstrated the expected basic patterns of results in the licking assay, with equivalent consumption of sucrose at both the 4% and 16% concentrations, and greater lick cluster sizes at 16% than 4% (due to the greater palatability of the 16% sucrose solution), although the total number of licks made did not differ. Changes in the size of lick clusters, based on prescribed intra-lick intervals, have been shown to be sensitive to the consumption of more or less palatable solutions (e.g. Hsiao & Fan, 1993), which suggests that lick cluster size is the main determinant of palatability and can therefore provide an index related to hedonic components of behaviour (Davis & Smith, 1992). This can be further demonstrated in terms of the dissociation between total consumption and lick cluster size, where subjects may consume greater amounts of low concentration solutions with smaller lick cluster sizes than more concentrated rewards (lower total consumption, larger lick cluster sizes, Dwyer, 2012). In the current study, the fact that consumption of 4% and 16% sucrose was equivalent would suggest that these concentrations were at approximately equivalent positions on the ascending and descending limbs of the inverted-U shaped function for consumption of palatable solutions, indicating that simple consumption tests would not be sensitive enough to dissociate differences in palatability, and hence hedonia, further supporting the efficacy of lick cluster analysis in assessing rodent palatability (Dwyer, 2012).

Lick cluster analysis did not reveal any significant genotype differences within the C417Y mice, with both C417Y homozygous ENU-mutant mice and their WT littermate controls demonstrating equivalent lick cluster sizes at both sucrose concentrations. (Note, these data add further evidence that the effects seen in the

PRT were not the results of general effects on thirst, satiety or other physiological changes in the C417Y homozygous ENU-mutant mice). In contrast, the C59X homozygous ENU-mutants displayed reduced lick cluster sizes at both concentrations of sucrose in comparison to their WT littermate controls. Lick cluster size has been discussed as the main determinant of palatability (Davis & Smith, 1992). Hence, these effects might be interpreted as the C59X homozygous ENU-mutant mice showing an anhedonic phenotype. To our knowledge, this is the first time such effects have been demonstrated in animal models probing genetic risk factors for mental disorder and would be consistent with findings of anhedonia in both schizophrenia (Horan et al., 2006b; Gard et al., 2007) and bipolar disorder (Serretti & Olgiati, 2004). The specificity of these effects to negative symptoms is underlined by previous data showing that PCP treatment in rats, thought to model the positive symptoms of schizophrenia more than the negative (Dwyer, 2012), did not affect hedonic responding as indexed by lick cluster sizes (Lydall et al., 2010a).

There was some evidence of motoric changes in the C59X homozygous ENU-mutants, insofar as they displayed increased volume per lick for both of the 4% and 16% sucrose solutions in comparison to their WT counterparts. Although total consumption and the total number of licks made for each sucrose solution was equivalent (suggesting that overall motivation to collect and consume the rewards was the same, it was just the manner in which the C59X homozygous ENU-mutant mice drank the sucrose that differed) the possibility that the reduced lick cluster size may have been confounded by altered buccal/tongue physiology as a result of the C59X mutation has to be considered. However, there is strong evidence to refute this possibility due to the dissociation between the increased volume per lick, which was only present at 4% sucrose, and reduced lick cluster size, which was observed at both 4% and 16% sucrose. This pattern of effects is consistent with the C59X mutation affecting licking behaviour in a manner that reflects anhedonia, rather than any motoric consequence of the mutation (D. Dwyer, personal communication).

A number of possible mechanisms might underlie the anhedonia observed with the C59X homozygous ENU-mutants. Chronic stress, for example, leads to anhedonia in rats using lick cluster analysis (Dwyer, 2012), but the extent to which these effects may overlap with changes in brain function in the mutants is unknown.

Much of the research into hedonic reactivity has focused on opiate systems in the brain (Peciña, Smith & Berridge, 2006). Systemic treatment with opiate agonists in humans and animals increases affective (oro-facial liking) hedonic reactions to palatable sucrose (Parker, Maier, Rennie et al., 1992) and infusions into the nucleus accumbens shell or the ventral pallidum increase the hedonic reactions to sucrose in rats (Peciña & Berridge, 2005; Peciña et al., 2006). GABA blockade in the ventral pallidum was also found to stimulate eating but was not accompanied by enhanced 'liking' behaviours (Smith & Berridge, 2005a). Dopamine antagonists also lead to reduced lick cluster sizes (D'Aquila, 2010) suggesting a role for dopamine in the 'liking' of rewards as well as the 'wanting', despite Berridge's argument to the contrary (Berridge, 2004). It would appear that mutation-related changes in several neurotransmitter systems may be of potential relevance to the behavioural findings in this chapter, as discussed further in the General Discussion (Chapter VIII).

5.4.3. Summary of key results from Chapter V

- WT littermate control mice from both lines showed the expected basic patterns of behaviour on the progressive ratio task and lick cluster analysis.
- The results suggest that the C59X mutation caused a dissociation between pleasure and motivation, whereby the C59X homozygous ENU-mutant mice were equally driven to earn rewards on the progressive ratio task but did not find them as pleasurable as their WT littermate controls.
- The C417Y mutation also hinted at a dissociation between pleasure and motivation, but in the opposite direction to the C59X mice, whereby the C417Y homozygous ENU-mutant mice were more motivated to earn rewards but found them equally as pleasurable as their WT littermate controls.

Chapter VI: Assessment of sensorimotor gating in Zfp804a mutant mice; *acoustic startle and prepulse inhibition of startle*

6.1 Introduction

The startle response is the result of brainstem reflexes elicited by sudden intense stimuli, which can facilitate an escape from a potentially dangerous situation (Geyer & Swerdlow, 2001). It has been used as a behavioural measure of central nervous system activity, and can be measured in many species via acoustic, tactile or visual stimuli; the most common methodology employed in the clinic being the eye-blink response to an air puff (Geyer & Swerdlow, 2001). Startle magnitude can be attenuated when the startle stimulus is preceded by a weaker non-startling pre-stimulus (a prepulse), with startle stimuli not needing to be in the same modality (Hoffman & Searle, 1968; Ison, McAdam & Hammond, 1973). This inhibition of a motor response, termed prepulse inhibition (PPI), has been held to reflect the operation of low level attentional processes required to filter out extraneous information (Braff, Geyer & Swerdlow, 2001).

PPI deficits are seen in a number of disorders, including conditions in which frontal brain regions are affected, such as schizophrenia (for reviews see Geyer, Krebs-Thomson, Braff & Swerdlow 2001; Braff et al., 2001) and bipolar disorder (Giakoumaki, Roussos, Rogdaki et al., 2007; Perry, Minassian, Feifel, & Braff, 2001). However it should be emphasised that PPI deficits are also seen in other conditions such as Huntington's disease (Swerdlow, Paulsen, Braff et al., 1995b), obsessive compulsive disorder (Swerdlow, Benbow, Zisook et al., 1993) and ADHD (Ornitz, Hanna, & de Traversay, 1992), so effects on PPI can be sensitive to pathology but are not selective. Most of the studies conducted on schizophrenia patients have found a reduction in PPI (in percentage terms) regardless of what modality the startle response and prepulse are generated in (Braff et al., 2001). Furthermore, a possible link between PPI deficits and schizophrenia is suggested by the finding that antipsychotics drugs (e.g. dopamine D_2 receptor antagonists), which are used clinically to attenuate the primary symptoms of schizophrenia, also reduce the PPI deficiency in patients (for a review see Geyer et al., 2001). Interestingly, one study not only found reduced PPI in schizophrenia patients, but also in the unaffected first

degree relatives of the schizophrenia (Cadenhead, Swerdlow, Shafer, Diaz & Braff, 2000) and bipolar (Giakoumaki et al., 2007) probands, suggesting a genetic element to the PPI deficits seen in both disorders. Patients with schizophrenia have also demonstrated slower rates of habituation to repeated administrations of the startle stimulus than normal controls (Geyer & Braff, 1982; Braff, Grillon & Geyer, 1992,), perhaps suggesting a generalised habituation deficit to startling stimuli.

The startle response and PPI are relatively easily modelled in rodents with common methodologies employing acoustic startle and prepulse stimuli, and an assessment of the whole body startle response. Furthermore, it is possible to use stimuli parameters in animals that are directly comparable to human studies, such as acoustic startle amplitude and prepulse interval manipulations (Swerdlow, Weber, Qu, et al., 2008). In attempting to develop plausible animal models for schizophrenia, a deficit in PPI has become a recognised endophenotype (Turetsky, Calkins, Light, et al., 2007). Thus, animal models have sought to recapitulate the deficits seen in schizophrenic patients. Pharmacological challenges such as dopamine agonists, 5-HT_2 agonists, and NMDA antagonists all impair PPI in rodents, revealing some degree of overlap between neurotransmitter systems important in mediating prepulse inhibition and the robust effects on PPI seen in schizophrenia (for a review see Geyer et al., 2001). Furthermore, the rat social isolation model of early life deprivation, a putative neurodevelopmental model for schizophrenia, also causes a reduction in PPI (Geyer et al., 1993; Wilkinson, Kilcross, Humby, et al., 1994) in addition to altered forebrain dopaminergic systems (Wilkinson et al., 1994), further suggesting that early life stressors may have important repercussions on brain function and behaviour in later life. Studies involving specific brain lesions have implicated the hippocampus, the medial prefrontal cortex and the striatum as brain regions thought to be important in mediating PPI in rodents (see Swerdlow, Geyer & Braff, 2001 for a review); these regions have also been implicated in the pathophysiology of schizophrenia (Swerdlow et al., 2001).

Sensorimotor gating effects have been found in a number of genetic mouse models for psychiatric disorders. For example, mice carrying an ENU-induced missense mutation of DISC1 showed a reduced startle response as well as displaying PPI deficits which were reversed by antipsychotic treatment (Clapcote et al., 2007).

PPI deficits, although less pronounced than the ENU-induced mutant strain, were observed in another *DISC1* mutant over expressing a truncated form of *DISC1* under the control of the CaMKII promoter (Hikida et al., 2007). Similarly, other mouse models of candidate genes for schizophrenia and other mental conditions have shown PPI impairments (e.g. NRG1, Stefansson, et al., 2002), although the findings are not always clear cut (e.g. NRG1, Karl, Burne, Van den Buuse & Chesworth, 2011 and others). These differences in results are not due to any debate about the reliability of the PPI deficit in schizophrenia and other conditions *per se*, which is fully accepted, but are more likely due to the differences between the creation of the different mouse lines and variable testing methods. In the case of the present work, the experiments in this chapter examined acoustic startle and prepulse inhibition of acoustic startle in the two *Zfp804a* ENU mutant lines to further investigate endophenotypes of possible relevance to the pathogenicity of *ZNF804A* variants in people.

6.2 Materials and Methods

Full methodological descriptions can be found in the relevant sections of the General Methods (Chapter II, Section 2.4.8).

6.2.1 Subjects and animal husbandry

In total, 50 adult male mice were used in these experiments (mean age 13 ± 0.21 months). Details of specific mouse numbers per genotype can be seen in Table 6.1. Mice were housed in littermate groups of two to five animals per cage, under temperature and humidity controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow and water. Mice were weighed on a regular basis and any mice dropping 10% of their body weight or more were removed from the experiment. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986.

Table 6.1: Sample size and genotype of each cohort of mice assayed on acoustic startle response and PPI.

Behavioural Task	C59X line		C417Y line	
	WT	Homozygous	WT	Homozygous
Startle/PPI	14	12	11	13

6.2.2 General behavioural methods

All testing took place between the hours of 09:00 and 18:00, with equal distribution of testing for subjects of different genotypes throughout the day. Subjects were habituated to an adjacent holding room at least 30 minutes before testing. Subjects were then collected from the holding room just prior to testing, and immediately placed into the startle apparatus. Following testing the mice were weighed and returned to their home cage.

6.2.3 Acoustic startle and prepulse inhibition

The acoustic startle response (ASR) and prepulse inhibition (PPI) were assayed using SR-Lab apparatus (San Diego Instruments, U.S.A), according to previous methods (Geyer & Dulawa, 2003). ASR and PPI were measured in a single session lasting 30 minutes where subjects were placed in the sound attenuating chambers described in Chapter II, Section 2.4.8. In brief, mice were placed into a 35mm diameter, Perspex tube mounted on a Perspex plinth. Directly beneath the centre of the tube was a piezoelectric sensor that detected flexion in the plinth: the measure of startle reactivity. Above the animal enclosure (12cm) a loud speaker was mounted, through which all white-noise stimuli were presented. The apparatus was thoroughly cleaned with 1% glacial acetic acid solution between subjects to remove any aversive odour cues.

A startle session consisted of exposure to 49 acoustic stimuli, presented in a pseudorandom manner every 10s, against a background noise of 70dB (A scale, (A)). Sessions started with a 5 minute acclimatisation period at background followed by 2 blocks of acoustic stimuli. The first block of 20 trials (See Appendix 5.1) consisted of a pseudorandom distribution of pulse-alone (startle) trials of different amplitude (3 each at 80, 90, 100, 110 and 120 dB and 5 no-stimulus trials). The second block (29

trials), consisted of a pseudorandom distribution of pulse-alone (at 120dB), prepulse+pulse-alone (prepulses at 8 and 16dB above background) and no-stimulus trials. There were 13 pulse-alone trials, and 6 trials for each prepulse+pulse-alone combination and 4 no-stimulus trials. All pulse-alone trials consisted of a 30ms startle stimulus. Prepulse+pulse-alone trials consisted of a 20ms prepulse followed by a 30ms startle stimulus, 70ms after the prepulse stimulus offset. Responses were monitored for 65ms following the onset of the startle stimulus. The whole body startle response to the pulse-alone trials and the inhibition of responding due to the presentation of prepulse stimuli was recorded (as arbitrary startle units, ASU, related to the voltage change detected by the piezoelectric sensor).

6.2.4 Statistical analysis

The experimental data was analysed using SPSS (Version 18.0). Behavioural data are presented as mean values (*M*) along with the standard error of the mean (SEM), unless stated otherwise. Data for each strain were analysed separately, and separate ANOVAs were performed with between subject's factor of GENOTYPE (homozygous ENU-mutant vs. WT littermate control) and within subject factors as appropriate. The mean startle amplitude for each trial type was calculated for trials from the first block and analysed with factor STIMULUS INTENSITY (no-stimulus trials and pulse-alone trials at 80, 90, 100, 110 and 120 dB). Data from the second block were analysed with within subject factors of TRIAL NUMBER (13x 120dB pulse-alone stimuli), TRIAL TYPE (mean pulse-alone, 8dB and 16dB prepulse trials) and amount of PPI (8dB and 16dB prepulse trials). PPI was calculated from data collected in the second block; as the percentage reduction in mean startle amplitude between prepulse trials and pulse-alone trials for each prepulse intensity (at 8 and 16dB prepulse amplitude above background), using the following calculation:

$$\%\ \text{prepulse inhibition} = 100 \times \frac{(\text{mean pulse-alone trial} - \text{mean prepulse trial})}{(\text{mean pulse-alone trial})}$$

All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. Outliers

which were 2 standard deviations or more from the mean were removed from the analysis.

6.3 Results

6.3.1 Zfp804a C59X line

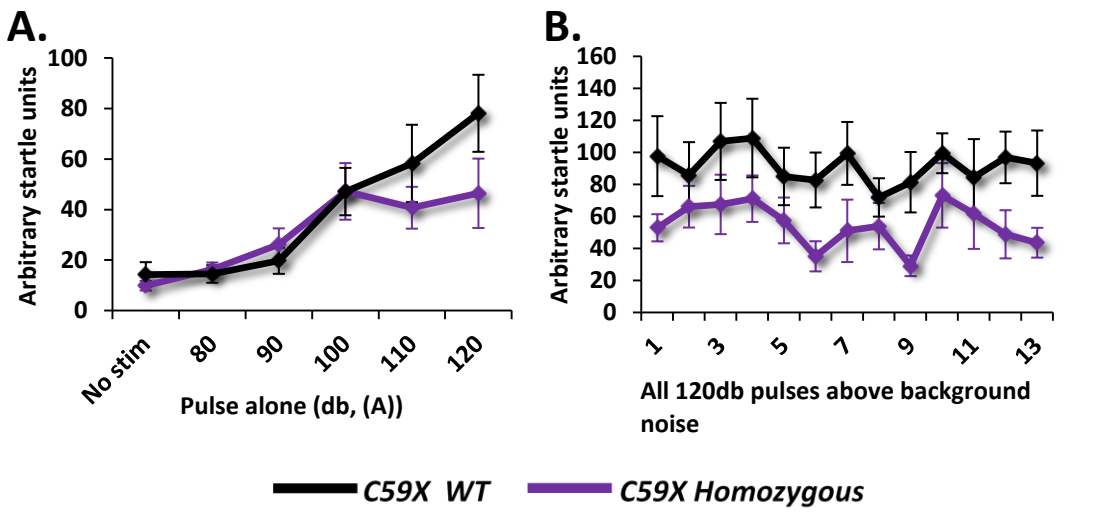
6.3.1.1 Startle reactivity

To assess startle reactivity, the mice were tested with a range of increasing levels of stimulus amplitude, ranging from 70dB to 120dB, against a background noise of 70db, thus the lowest stimulus volume was equivalent to background (Fig. 6.1A). Background activity was also assessed by recording movement in the absence of a specific stimulus presentation (No stim trials). As expected, both C59X homozygous ENU-mutants and WT littermate controls *s*howed an appropriate increase in startle reactivity as the stimulus intensity increased (main effect of STIMULUS INTENSITY, $F_{2.2,43.9}$= 15.5, p=0.000, STIMULUS INTENSITY*GENOTYPE, $F_{2.2,43.9}$= 1.9, p=0.11). There were no overall differences in the startle response between C59X homozygous ENU-mutants and their WT littermate controls (main effect of GENOTYPE, $F_{1,20}$= 0.7, p=0.41), suggesting that auditory sensitivity was equivalent between C59X subjects.

In the second block of trials, 13 pulse-alone trials at 120dB were interspersed with 12 prepulse/pulse-alone trials (6 trials each with 8dB and 16dB prepulse stimuli above background) in the sequence of stimuli presentations. Within this timeframe there was no overall change in the startle response to these stimuli, with all mice maintaining good levels of reactivity throughout the session (Fig. 6.1B, main effect of TRIAL NUMBER, $F_{5.9,117.6}$= 1.5, p=0.12, TRIAL NUMBER*GENOTYPE, $F_{5.9,117.6}$= 0.5, p=0.89). Examination of the pattern of startle reactivity at a constant 120dB pulse suggested that C59X homozygous ENU-mutant mice startled less to the 120dB stimuli than their WT littermate controls, although this did not quite reach statistical significance (main effect of GENOTYPE, $F_{1,20}$= 4.2, p=0.054). *A priori* analysis excluding the first 5 120dB pulse trials, where the startle response was greatest and most anxiogenic (t_{20}= -1.6, p=0.12), demonstrated that the average startle response for C59X homozygous ENU-mutants was indeed significantly reduced in comparison

to their WT littermate controls (Fig. 6.2A, t_{20}= -2.2, p=0.04), perhaps indicating reduced startle reactivity in the C59X homozygous ENU-mutants.

Figure 6.1. Startle reactivity in C59X mice



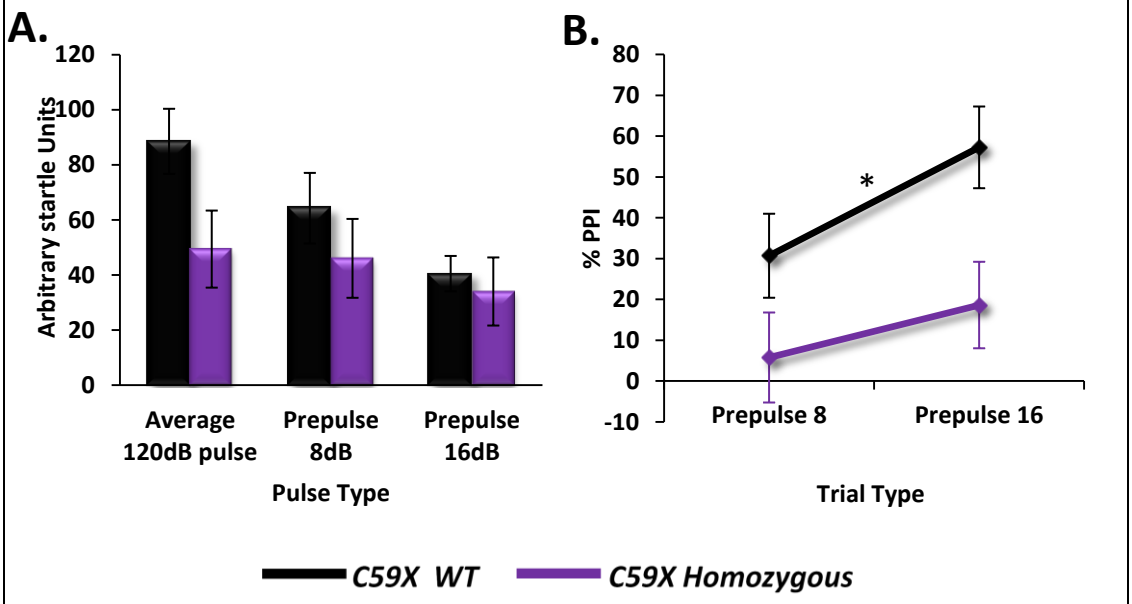
C59X homozygous ENU-mutant mice and their WT littermate controls showed an increased startle response with increasing pulse alone intensity (A). On the 13 120dB pulse alone trials (B) both the C59X homozygous ENU-mutants and their WT littermates showed consistent responding across multiple presentations of the 120dB pulses; the C59X homozygous ENU-mutant mice appeared to startle less than their WT littermate controls, although this did not reach significance. Data shows the mean values ±SEM. For subject N see Table 6.1.

6.3.1.2 PPI

In the presence of prepulse stimuli at either 8 or 16dB, the startle response to 120dB stimuli was significantly reduced from pulse-alone levels (Fig. 6.2A, main effect of TRIAL TYPE, $F_{2,40}$= 17.0, p=0.000). C59X homozygous ENU-mutant mice showed reduced levels of startle for each type of trial in comparison to their WT littermate controls, but this was not found to be significant (main effect of GENOTYPE, $F_{1,20}$= 1.8, p=0.2). PPI was calculated as the difference in mean startle reactivity between pulse-alone and prepulse trials and, as expected, all subjects showed significantly greater PPI when the prepulse was at 16dB than at 8dB above background (Fig. 6.2B, main effect of PREPULSE INTENSITY, $F_{1,20}$= 12.1, p=0.002, PREPULSE INTENSITY*GENOTYPE, $F_{1,20}$= 1.5, p=0.24). Importantly, C59X homozygous ENU-mutant mice showed reduced levels of PPI compared to their WT counterparts (main effect of GENOTYPE, $F_{1,20}$= 5.2, p=0.03), which may reflect an impairment in sensorimotor gating in these mice. As the PPI calculation takes into account baseline

startle levels, it was important to show that the PPI deficits found with the C59X homozygous ENU-mutants was not confounded by the reduced acoustic startle response found with these mice. The results of an ANCOVA with baseline startle levels as the covariate showed no interaction between baseline startle and PPI (p=0.166), although the overall significant difference between C59X homozygous ENU-mutant mice and their WT littermate controls was reduced from the previous analysis (main effect of GENOTYPE, $F_{1,19}$= 3.7, p=0.07).

Figure 6.2. Startle reactivity and PPI in the C59X mice.



The C59X homozygous ENU-mutant mice showed significantly reduced startle when all the 120dB pulse alone trials were averaged together, as compared to their WT littermate controls (A). All C59X mice showed an equivalent amount of startle in trials with 8dB and 16dB prepulses (A). All subjects showed a significantly greater percentage PPI when the prepulse was at 16dB than at 8dB above background (B), however, C59X homozygous ENU-mutant mice show reduced PPI compared to their WT littermate controls at both prepulse amplitudes. Data shows the mean values along with the ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 6.1.

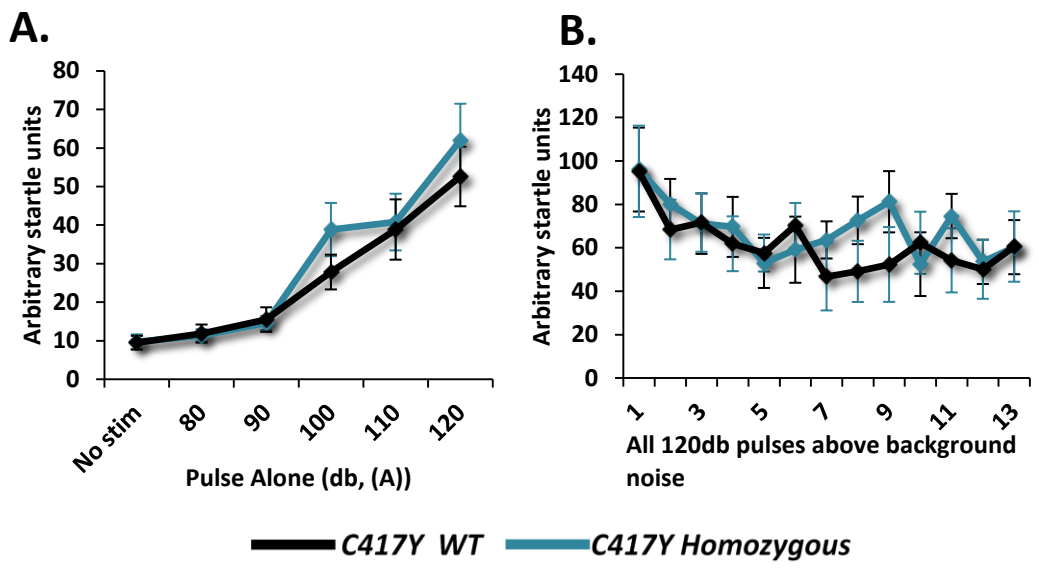
6.3.2 Zfp804a C417Y line

6.3.2.1 Startle reactivity

In response to increasing startle stimuli intensities, all subjects showed the expected increase in startle response (Fig. 6.3A, main effect of STIMULUS INTENSITY, $F_{2.7,51.3}$= 24.8, p=0.000, STIMULUS INTENSITY*GENOTYPE, $F_{2.7,51.3}$= 0.6, p=0.7). C417Y homozygous ENU-mutants and WT littermate controls showed comparable levels of startle to these stimuli (main effect of GENOTYPE, $F_{1,19}$= 0.5, p=0.48), suggesting that

auditory sensitivity is equivalent for all the C417Y subjects. In the second block of trials, 13 120dB pulse alone trials were distributed randomly between the prepulse trials. There was no overall change in the startle response to these stimuli, with all mice maintaining good levels of reactivity throughout the session (Fig. 6.3B, main effect of TRIAL NUMBER, $F_{5.4,102.7}$= 1.8, p=0.12, TRIAL NUMBER*GENOTYPE, $F_{5.4,102.7}$= 0.5, p=0.78) with no difference in startle responses between C417Y homozygous ENU-mutants and their WT littermates (main effect of GENOTYPE, $F_{1,19}$= 0.1, p=0.75). Comparison of startle reactivity between mice of the C417Y line for the first 5 120dB pulse alone trials (t_{19}= 0.3, p=0.75) and the average of all 120dB pulse alone trials (t_{19}= 0.96, p=0.35) showed no significant differences, suggesting that all C417Y subjects startled at equivalent levels throughout the session and habituated to the pulse alone trials at an equivalent rate.

Figure 6.3. Startle reactivity in C417Y mice.



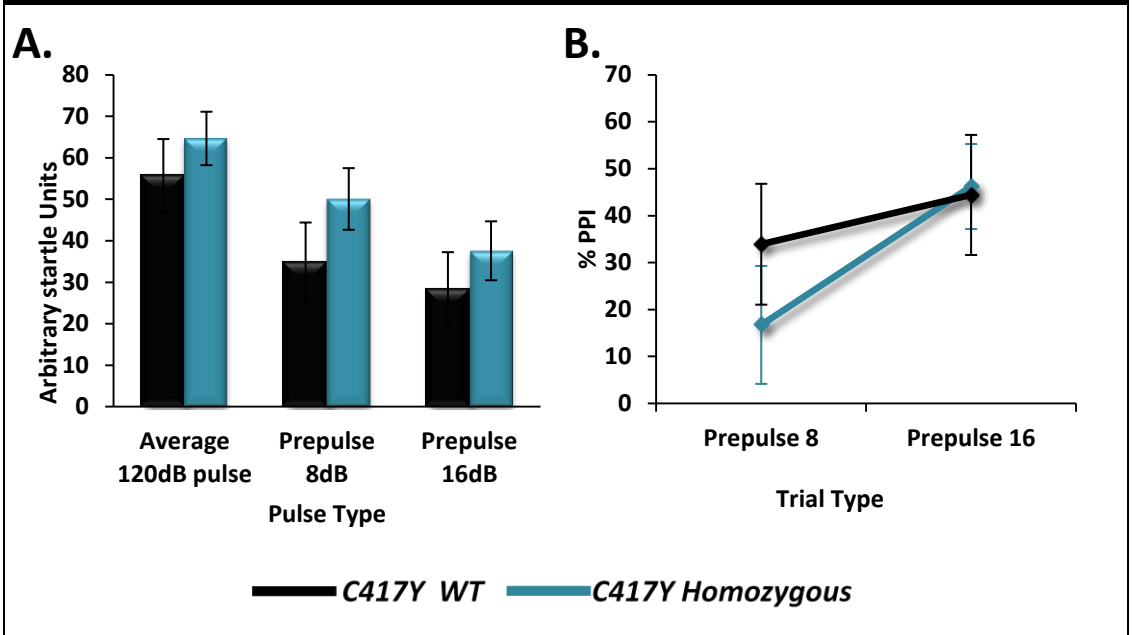
C417Y homozygous ENU-mutant mice and WT littermate controls showed an increased startle response with increasing pulse alone intensity (A). On the 13 120dB pulse alone trials (B) both the C417Y homozygous ENU-mutants and their WT littermate controls showed consistent responding across multiple presentations of the 120dB pulses, with no genotype differences. Data shows the mean values ±SEM. For subject N see Table 6.1

6.3.2.2 PPI

Sensorimotor gating was assessed using 2 different levels of prepulse amplitude, 8 and 16dB above background and compared to the mean startle pulse

alone response to 120dB stimuli. As expected there was a significant attenuation in startle reactivity when a prepulse stimulus was present (Fig. 6.4A, main effect of TRIAL TYPE, $F_{2,38}$= 12.7, p=0.000) with no difference between C417Y homozygous ENU-mutant mice and their WT littermate controls in these measures (main effect of GENOTYPE, $F_{1,19}$= 1.1, p=0.31). This reduction in startle was greatest following the presentation of a 16dB prepulse than an 8dB prepulse (p=0.018), with higher PPI following the 16dB prepulse than the 8dB prepulse (Fig. 6.4B, main effect of PRE PULSE INTENSITY, $F_{1,19}$= 7.9, p=0.01, PREPULSE INTENSITY*GENOTYPE, $F_{1,19}$= 3.4, p=0.08). There were no significant differences in PPI between the C417Y homozygous ENU-mutants and their WT littermate controls under both prepulse conditions (main effect of GENOTYPE, $F_{1,19}$= 0.09, p=0.77).

Figure 6.4. Startle reactivity and PPI in the C417Y mice.



C417Y homozygous ENU-mutant mice startle at an equivalent rate to their WT littermate controls when all the 120dB pulse alone trials were averaged together (A). They also startle at an equivalent rate to both 8dB and 16dB prepulses (A). All subjects showed a significantly greater % PPI when the prepulse was at 16dB than at 8dB above background (B), with no genotype differences observed in percentage PPI. Data shows the mean values ±SEM. For subject N see Table 6.1.

6.4 Discussion

WT littermate control mice of both the C59X and C417Y lines demonstrated the expected basic pattern of results, showing increasing startle responses to

increasing acoustic startle stimulus amplitudes (in pulse-alone trials). They also showed reduced startle responses (prepulse inhibition, PPI), when presented with prepulse trials in which an acoustic prepulse stimulus 8dB or 16dB above background noise preceded the 120dB startle stimulus. Responses to pulse-alone or prepulse trials were equivalent between C417Y homozygous ENU-mutant mice and their WT littermate controls. Responses to increasing startle amplitudes, habituation to 120dB pulse-alone stimuli and the degree of PPI shown with 8dB and 16dB prepulse stimuli were also equivalent between C417Y homozygous ENU-mutant mice and their WT littermates. In contrast, C59X homozygous ENU-mutant mice showed reduced levels of startle at 120dB pulse-alone stimuli and, importantly, reduced PPI, although startle reactivity to increasing startle intensities was equivalent to their WT littermate controls. This pattern of results would suggest, *a priori,* an altered acoustic startle response and impaired sensorimotor gating in the C59X homozygous ENU-mutant mice.

The pattern of data could potentially be influenced by a number of factors. Firstly, as all the stimuli used in the current study were acoustic, hearing impairments could, in theory, be accountable for the reduced levels of startle shown by the C59X homozygous ENU-mutant mice. However, deafness can be discounted as the C59X homozygous ENU-mutant mice demonstrated equivalent startle responses (in relation to their WT littermate controls) to increasing amplitudes of the startle stimuli, especially at the lower end of the range used (80 to 100dB, Fig. 6.1A) suggesting a degree of sensitivity to these stimuli that was equivalent to their WT counterparts. Likewise, data from the next series of experiments in the stop-signal reaction time task would further support this, where C59X homozygous ENU-mutant mice demonstrated improved responding to the 105dB white noise stimuli used to indicate a stop trial, see Section 7.3.1.3. Startle responses and PPI can also be induced using visual and tactile stimuli alone, or in combination with acoustic stimuli, and it would therefore be of benefit to assess startle reactivity and PPI in C59X mice in other sensory modalities, in particular a tactile modality, which is thought to be optimum for rodent models (Geyer & Swerdlow, 2001).

The PPI calculation is not independent of baseline startle responding and therefore the reduced pulse alone startle response of the C59X homozygous ENU-

mutants may have influenced the PPI deficits shown in these mice. However, there is no agreement on how the startle response and PPI levels are correlated, and as such, reduced startle effects could be accompanied by PPI deficits or PPI enhancements (Csomor, Yee, Vollenweider, et al, 2008). Furthermore, the reduced startle response in the C59X homozygous ENU-mutant mice was not found to co-vary with the PPI deficits shown.

There might have also been a possible 'floor effect' in the startle response of the C59X homozygous ENU-mutant mice. As mentioned previously, these mice showed a significantly reduced startle response in 120dB pulse-alone trials, as compared to their WT littermate controls. It could therefore be a possibility that any further reductions in startle responding could not be reliably measured either due to the sensitivity of the apparatus and/or some biological minimum response in these mice, hence their reduced percentage PPI (in comparison to their WT littermate controls) could have been a result of this potential floor effect. To counteract this idea, it can be shown that the C59X homozygous ENU-mutant mice were capable of lower startle responses than those generated when prepulses were present; with Fig. 6.2A showing that even further reduced startle could be reliably assessed in these mice when 70, 80 and 90dB stimuli were presented.

Attenuated PPI is considered an endophenotype of schizophrenia (Geyer et al., 2001; Braff et al., 2001) but is also seen in bipolar patients (Giakoumaki et al., 2007; Perry et al., 2001). This impairment in sensorimotor gating can also be demonstrated as a result of specific brain lesions related to the pathophysiology of schizophrenia and bipolar disorder, such as the prefrontal cortex and hippocampus (Swerdlow et al., 2001), but also manipulation of the major forebrain neurotransmitter systems and by NMDA receptor blockade (Geyer et al., 2001) and altered early life experience (Geyer et al., 1993; Wilkinson et al., 1994). Thus, there are a number of different mechanisms by which the effects of the C59X *Zfp804a* mutation could give rise to the PPI deficit observed, occurring from prenatal differences through to altered adult brain function.

Reduced startle reactivity is not commonly observed in schizophrenia, instead patients are more likely to show reduced rates of habituation to repeated administrations of the startle stimulus than normal controls (Geyer & Braff, 1982;

Braff et al., 1992), an effect that was not observed in the current study, most likely due to the reduced number of presentations of the startle stimuli and the interpolation of the prepulse trials. This blunted startle response is also seen in post-traumatic stress disorder, depression and bipolar disorder (Beck & Catuzzi, 2013), suggesting a potential similarity between the sensory reactivity of these mice to patients with certain psychiatric disorders. In animal models startle reactivity and anxiety have previously been shown to be correlated (Mikaelsson, Constância, Dent et al., 2013), thus, the reduced startle reactivity demonstrated by C59X homozygous ENU-mutant mice could also be related to the reduced anxiety phenotype shown by these mice, (Chapter IV).

The data in this chapter indicate PPI deficits that are specific to the C59X line, providing evidence of an endophenotype that may prove to be of relevance to the risk for mental disorder associated with *ZNF804A* gene variants (Turetsky et al., 2007). As with work done in other models, such as *DISC1* mutants (Clapcote et al., 2007; Hikida et al., 2007) and *NRG1* mutants (Stefansson et al., 2002) it would be interesting to see if the PPI deficits could be reversed with antipsychotic drugs. Additionally, these data may point to underlying neurobiology that are common to startle and PPI, and pathways sensitive to *ZNF804A* function/dysfunction, in particular modulatory effects of the monoaminergic pathways on limbic and cortical circuitries (Powell, Zhou & Geyer, 2009); ideas which are discussed further in the General Discussion (Chapter VIII).

6.4.1. Summary of key results from Chapter VI

- WT littermate controls both mutant lines demonstrated the expected startle and PPI responses.
- C59X homozygous ENU-mutant mice displayed attenuated startle reactivity and reduced PPI in comparison to their WT littermate controls, indicating an impairment of sensorimotor gating in these mice.
- The C417Y mutation did not have an effect on the mutant's ability to startle, habituate or attenuate to a startling stimulus.

Chapter VII: Assessment of response control in Zfp804a mutant mice:
stop-signal reaction time task (SSRTT)

7.1 Introduction

The ability to inhibit thoughts and actions when they are no longer in keeping with current goals is an essential requirement of human behaviour (Logan & Cowan, 1984). Impulsivity on the other hand, is a lack of behavioural inhibition, where actions are 'poorly conceived, prematurely expressed, unduly risky, or inappropriate to the situation and that often result in undesirable outcomes' (Evenden, 1999a). Maladaptive deficits in response control are found in several psychiatric illnesses including schizophrenia, bipolar disorder, attention-deficit hyperactivity disorder (ADHD) and OCD.

Response control forms part of the umbrella of cognitive processes known as 'executive functioning', which essentially act in unison to monitor the world, generate and assess appropriate responses and co-ordinate actions accordingly (Badcock, Michie, Johnson & Combrinck, 2002). Patients with schizophrenia show deficits in elements of executive functioning in areas such as attention (Braff, 1993; Heinrichs & Zakzanis, 1998), impulsivity (Newman et al., 1985; Heerey et al., 2007) and working memory (Goldman-Rakic, 1994; Barch, 2006) and perform poorly on tasks that place a heavy load on executive functioning such as the Wisconsin Card Sorting Test and the Tower of Hanoi problem (Fey, 1951; Bustini, Stratta, Daneluzzo, et al., 1999; Badcock et al., 2002). Patients with bipolar disorder also show similar deficits in executive function, with reduced attention, impairments in working memory and increased impulsivity (Swann, 2010).

Impulsivity is not a unitary concept. It has been proposed that impulsivity actually encompasses a variety of phenomena that may yet have separate biological mechanisms (Evenden, 1999b). Measuring behavioural inhibition in the laboratory has benefitted from this definition, with tasks measuring impulsivity divided into two major categories; those measuring impulsive choice and those measuring impulsive action (Winstanley, Eagle, Robbins, 2006). Impulsive choice paradigms involve measuring impulsive decisions and have, for example, utilised delay-discounting tasks which measure intolerance (or otherwise) to delay-of-gratification, typically

offering subjects a choice between smaller immediate rewards or larger delayed rewards (Ainslie, 1975). Picking the smaller immediate reward is seen as an impulsive choice. Impulsive action paradigms usually measure the ability to withhold making a pre-potent response, such as, for example, in go/no-go paradigms and the stop-signal reaction time task (SSRTT, Logan & Cowan, 1984). In go/no-go tasks, subjects learn to make a response when cued to do so by a 'go' signal, however on some trials a 'no-go' signal is presented simultaneously or just prior to the 'go' signal indicating to subjects to withhold their response. The SSRTT is an elaboration of the go/no-go task with the difference being that the stop- (no-go) signal is presented after the presentation of the go signal, thus measuring the ability to inhibit a response once it has already been initiated, in other words the ability to stop (Winstanley et al., 2006). The stop-signal can be presented close to the start of the go response, making stopping easy, whereas presentation later in the go response would make stopping more difficult (Logan & Cowan, 1984). It is hypothesised that the go and stop responses are independent processes, which compete to control the final behavioural output, described as the 'horse-race' model (Logan & Cowan, 1984). Thus, a key consideration is the point where competition is greatest, i.e**.** at 50% stopping where it is presumed the go and stop responses are equal.

A main measure of interest in the SSRTT is the stop-signal reaction time (SSRT); the time required to successfully stop. This is a calculated measure as it is impossible to accurately measure the latency of a response that never occurs, however estimations of the SSRT can be generated from the race model, using the subjects' mean go reaction time and the stop-signal delay when correct stopping is at 50%. In human studies adult SSRTs are close to 200ms (Logan & Cowan, 1984) but these have been found to be around 200ms longer (slower) for children (Schachar & Logan, 1990) and the elderly (Kramer, Humphrey, Larish et al., 1994). Research has also found that children with ADHD tend to take longer to respond to the stop-signal and as such have SSRTs which are around 100ms slower than normal children (Oosterlaan, Logan, & Sergeant, 1998; Overtoom, Kenemans, Verbaten, et al., 2002). Frontostriatal brain systems, thought to be involved in the pathophysiology of schizophrenia and bipolar disorder have been implicated in the neural substrates of response inhibition (Rubia, 2002; Rubia, Russell, Bullmore et al., 2001; Rubia, Smith,

Brammer & Taylor, 2003; Townsend, Bookheimer, Foland-Ross et al., 2012), with lesions to the right inferior frontal gyrus (Aron, Fletcher, Bullmore et al., 2003), the frontal lobes and the basal ganglia (Rieger, Gauggel & Burmeister, 2003) all producing slower SSRTs in schizophrenia patients on the SSRTT.

While there have been few investigations into the response control of schizophrenia and bipolar patients using the SSRTT, this task has been proposed as a potential biomarker for schizophrenia (Barch et al., 2009). There is debate as to the consistency of findings when schizophrenia patients are tested on the SSRTT, with some evidence showing that subjects have slower inhibitory processes, achieving SSRTs of around 245ms compared to 191ms for controls (Enticott, 2008; Huddy, Aron, Harrison et al., 2009), But other research showing no differences between patients and controls in inhibitory performance (Rubia et al., 2001) or only showing a lateralised deficit in response inhibition (Bellgrove, Chambers, Vance et al., 2005). One study suggests that there are indeed impairments in the triggering of inhibitory processes in schizophrenia patients, but that speed of inhibitory processes (when triggered) are relatively unaffected (Badcock et al., 2002). With bipolar disorder patients, response inhibition, as measured by the SSRTT, was poorer in patients versus normal controls (Strakowski, Fleck, DelBello et al., 2010), but more research using the SSRTT is needed to confirm this finding.

The SSRTT paradigm has also been developed for use in rats (Eagle & Robbins, 2003a,b), using a double lever press as the go response. The rat SSRT has been found to be around 300ms (Eagle & Robbins, 2003a); similar to results in humans. We have recently developed the SSRTT for mice (Humby, Eddy, Reichelt et al., 2013), using a double nose-poke methodology, which, as expected, demonstrated systematic changes in stopping ability as the stop-signal position was altered; and also SSRTs in keeping with data from humans (350ms), illustrating a degree of translational relevance of the mouse SSRTT to clinical settings. Stopping in the mice was improved using drugs known to improve inhibition in humans, i.e. methylphenidate (a dopamine reuptake inhibitor) and atomoxetine (a noradrenaline reuptake inhibitor), whilst lesions to the medial prefrontal cortex (mPFC) impaired stopping (Humby et al., 2013). Consistent with our data in mice, studies in rats have also found that stopping in the SSRTT is affected by lesions to the orbitofrontal

cortex and the subthalamic nucleus (Eagle, Baunez, Hutcheson et al., 2008), and that amphetamine and methylphenidate improved stopping in animals with poor baseline inhibitory performance, including rats with medial striatal lesions (Eagle & Robbins, 2003a,b; Eagle, Tufft, Goodchild & Robbins, 2007). The SSRTT has, therefore, been shown to be a useful translational tool for studying impulsive action in animal models including very recently (and uniquely in our own laboratory) mice, and hence was used in the present chapter to assess whether the *Zfp804a* mutant lines showed any evidence of changes in response control.

7.2 Materials and Methods

Full methodological descriptions can be found in the relevant sub-sections of the General Methods (Chapter II).

7.2.1 Subjects and animal husbandry

In total, 58 adult male *Zfp804a* mice were used in these experiments (mean age of 7 months). Details of specific mouse numbers per genotype can be seen in Table 7.1.

Table 7.1: Sample size and genotype of each cohort-subset of mice assayed on the Stop-signal reaction time task (SSRTT).

Behavioural Task	C59X line		C417Y line	
	WT	Homozygous	WT	Homozygous
SSRTT	14	15	14	15

All mice were housed in littermate groups of two to five animals per cage, under temperature and humidity controlled conditions, with a 12-hour light: 12-hour dark cycle (lights on at 07:30). All subjects had *ad libitum* access to standard laboratory chow but only 2hrs access to water per day during testing. Mice were weighed on a regular basis. Any mice dropping 10% of their body weight or more were removed from the experiment. All experimental procedures were conducted under licenses issued by the Home Office (U.K.) in compliance with the Animals (Scientific Procedures) Act 1986. All testing took place between the hours of 09:00

and 14:00, with equal distribution of testing for subjects of different genotypes throughout the day. Prior to testing, mice were habituated to the test rooms for at least 40 minutes.

7.2.2 General behavioural methods

All testing took place between the hours of 07:00 and 13:00, with equal distribution of testing for subjects of different genotypes throughout the day. Prior to testing, mice were habituated to the test rooms for at least 20 minutes.

7.2.3 Reinforcer habituation

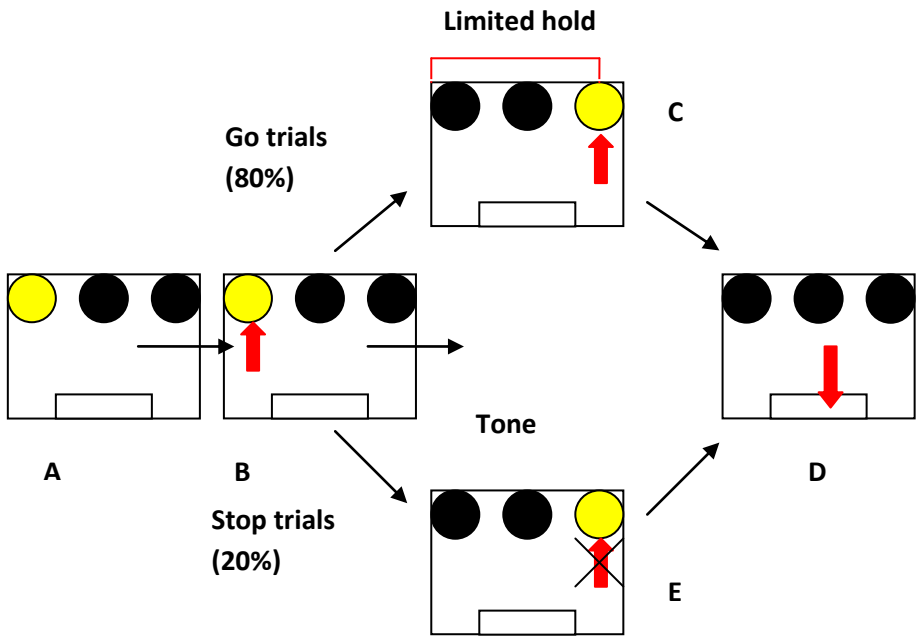
Habituation to the condensed milk (CM) reinforcer used to motivate performance in the SSRTT took place as previously described in Chapter II, Section 2.3.5. Once body weight had stabilised on the water restriction schedule (ca.10 days) the mice were habituated to the reinforcer (10% condensed milk solution, Nestle Ltd, U.K.). This was done so that any neophobic (fear of the novel) responses to the solution could be reduced before SSRTT testing got underway, and also to assess any pre-existing individual subject differences in reinforcer preference. Any subjects failing to reach 70% preference for the CM on the final day of preference or failing to consume CM on the milk vs. milk day were excluded from the SSRTT.

7.2.4 The stop-signal reaction time task (SSRTT)

Testing was carried out in “9-hole” operant chambers (Cambridge Cognition Ltd, U.K.) based on a design first used in rats (Carli et al., 1983) and tailored for use in mice (Humby et al., 1999). A full description of the SSRTT apparatus can be found in Chapter II, Section 2.4.9. In the SSRTT, the presentation of a stop-signal indicated that the response had to be cancelled. As described in detail below, the task (Fig. 7.1 allowed an explicit behavioural measure of stopping performance (successful stopping) and a calculated determination of the latency to stop (SSRT). In addition, the task provided a wide range of ancillary behaviours that, importantly, allowed dissociations between stopping and going behaviours, and also gave some within-task indications of sensorimotor and motivational functions. Subjects performed one 20-minute session per day, with a maximum of 100 trials per session. In sessions

where stopping was assessed a minority of stop trials (20%) were randomly distributed amongst the majority go trials (80%).

Figure 7.1: The stop-signal reaction time task (SSRTT) procedure.



The left stimulus light is illuminated signaling the beginning of the trial (A). The mouse makes a left nose-poke (B) which in Go trials triggers the right stimulus light to be illuminated for a limited time (the limited hold) and the mouse to make a right nose-poke (C), which is then rewarded with a food delivery (D). In stop trials, a burst of white noise was played after the left nose-poke, indicating to subjects that they should withhold their response at the right aperture (E) in order to be rewarded. Incorrect responses (failing to nose poke on Go trials or going on Stop trials) incurred both a timeout period and a lack of reward.

7.2.4.1 Initial behavioural training

Initial training involved animals being trained to push the food magazine panel to retrieve the CM reinforcer. These training sessions occurred consecutively over 5 days, with mice consistently allocated to the same chamber over the course of the SSRTT testing duration. During the first three sessions of this training, the panel to the food magazine was wedged open, and the apertures in the response array were blocked with clear plastic film. Subjects were presented with 22µl of condensed milk in the food magazine every 30 seconds for twenty minutes, indicated by the illumination of the tray light coincident to the food delivery. After the session, food magazines were checked to see whether subjects had been

drinking. During the last 2 days of shaping, the food magazine panels were not wedged open, so subjects had to learn to panel push to gain access to the food. Mice that failed to panel-push or had not collected the reward were given additional training. Once all subjects had learnt to panel-push, they were moved onto the single nose poke phase of the SSRTT training.

7.2.4.2 Initial shaping of behaviour for the SSRTT

In the first phase of SSRTT training, subjects were trained to make a single nose-poke response, the 'initiation nose-poke', at the initiation stimulus location (3rd aperture from the left of the nose-poke array). Each session began with a 22µl condensed milk delivery into the food magazine. On collection of this reward, a trial was started with the illumination of the initiation stimulus. When a nose-poke was made into this illuminated aperture, the light stimulus was extinguished and the reward was delivered; on collection the next trial was started. The initiation stimulus duration was initially set to 30s, and failure to make a nose-poke within this period was punished with a time-out period of 5 seconds indicated by illumination of the house-light, and those trials were recorded as non-started. As subjects became quicker at responding to the initiation stimulus, the presentation time was reduced until stimuli durations of 20s were achieved. Within a session following 4 consecutive responses the stimulus duration was reduced by 5s, and new sessions started with the final value achieved in the previous session for each individual mouse (minimum duration was 20s). Subjects moved to the next stage of training when they completed >70% of trials with a 20s initiation stimulus duration, for 3 consecutive sessions.

In the second phase of training, the subjects had to make a second nose-poke (the 'go' response) after the initiation nose-poke to earn reward. Thus, following a correct initiation nose-poke, the go stimulus aperture (6th aperture from the left of the nose-poke array) was illuminated. A correct nose-poke at this location now initiated delivery of the reward. Failure to make an initiation nose-poke or a go nose-poke were punished by a 5s time-out. The initiation stimulus duration was fixed at 20 seconds, and the initial go stimulus duration was set to 30s. Within a session following 4 consecutive correct go responses the go stimulus duration was reduced

by 5s, until it was equivalent to the go response latency (the go reaction time, Go-RT) of each individual mouse, plus 0.3s. New training sessions started with the final value achieved in the previous session for each individual mouse. Subjects moved to the next stage of training when they completed >70% of trials and made >70% correct go responses with a Go stimulus duration equivalent to their Go-RT, for 3 consecutive sessions.

7.2.4.3 Shaping stopping behaviour

During shaping of stopping behaviour a stop-signal (65 decibels of white noise for 0.3s) was presented after the initiation nose-poke on 20% of the trials (the other 80% being go trials) and the mice had to learn to cancel their ongoing response and wait for a set amount of time in order to earn a reward. In this phase of training stopping was relatively easy since the stop-signal was presented very close to the initiation nose-poke, i.e. at the start of the go response and was classed as a 0% stop-signal position. The initiation stimulus duration was fixed at 20s, the Go stimulus duration was equivalent to the individual mouse's Go-RT and the initial stop limited hold (LH) period was set to 0.35s, a period slightly longer than the duration of the white noise burst, enabling the mice to hear the reward delivery. The stop LH was increased in 0.1s increments following 4 consecutive correctly performed stop trials, and new sessions started with the final value achieved in the previous session for each individual mouse. Subjects moved to the next stage of testing when they completed >70% of trials, made >70% correct go responses with a Go stimulus duration equivalent to their Go-RT and made >70% correct stop trials, for 3 consecutive sessions.

7.2.4.4 Manipulation of the stop-signal delays

Baseline was determined as stable performance for 3 consecutive sessions at the following criteria: >70% of trials initiated, >70% correct go responses with a Go stimulus duration equivalent to individual Go-RTs and >70% correct stop trials at a stop LH equivalent to the Go stimulus duration with 0% stop-signal presentations. To further assess stopping behaviour, the mice were tested in probe sessions where the stop-signal was presented at different positions within the individual Go-RT of each

subject. Thus, stopping was made increasingly more difficult by presenting the stop-signal 10, 20, 30, 40, 50, 60, 70, 80 and 90% of the way through the Go-RT; the nearer the stop-signal was to the execution of the go response, the harder it would be to stop the response. The SSRTT study utilised software that provided an on-line monitoring of the Go-RT for each mouse and calculated the stop-signal position as a percentage of the mean on-going Go-RT for each individual subject. Therefore, if the subject's Go-RT changed during the session then the stop-signal position was recalculated for the next stop trial. Stop-signal positions were changed between sessions but not within them. Each probe session was implemented following a baseline session where subjects had performed to criterion.

7.2.4.5 Calculating the stop-signal reaction time (SSRT)

SSRTs were estimated using the procedure described in Logan & Cowan (1984) from data where the correct stopping of each subject was 50±10% (a worked example is given in Appendix 6.1). Individual data for each subject from the sessions where the stop-signal position was altered were ranked and individual means calculated from sessions where the mice performed 50% correct stopping ±10%; any subject with mean % correct stopping which were greater than ±10% from 50% were excluded from further analysis of the SSRT. The stop-signal delay (SSD) was calculated for each subject as the stop-signal position*Go-RT, where the mean stop-signal position was calculated for the different sessions specified above. To calculate the SSRT, Go-RTs were rank ordered and the nth Go-RT was obtained (where n was the number obtained by multiplying the number of Go-RTs in the distribution by the probability of responding on stop trials at that stop-signal delay). This value was the estimated time where the stopping process finished, relative to the onset of the go stimulus. The SSRT (the time where the stopping process finished relative to the stop-signal) was then obtained by subtracting the stop-signal delay (SSD) from this value (see Humby et al., 2013).

7.2.4.6 Behavioural measures

Task acquisition of the SSRTT was determined by the number of sessions required for each subject to reach SSRTT baseline performance, and the number of

sessions needed to complete each of the SSRTT training phases. A number of different behavioural measures were recorded for each session: correct go responses, Go-RT, correct stop responses, percentage of trials initiated (indexing levels of motivation), the latency to initiate a trial and latency to collect reward. The final initiation and go stimuli durations, and the stop LH, were also assessed as overall SSRTT performance could be affected by differences in these parameters. The SSD and the SSRT were also calculated when the mice showed 50% correct stopping, according the rationale of Logan & Cowan (1984).

7.2.5 Statistical analysis

The experimental data was analysed using SPSS (Version 18.0). Behavioural data are presented as mean values (*M*) with the standard error of the mean (SEM), unless stated otherwise. As the key point of interest was whether the *Zfp804*a mutant mice demonstrated altered behavioural information, particular focus was placed upon the point at which the go and stop responses were at maximal competition (Logan & Cowan, 1984); i.e. where stopping was ~50%. Data for each strain were analysed separately, and separate ANOVAs were performed with between subjects factor of GENOTYPE (homozygous ENU-mutant vs. WT littermate control) and within subject factors as appropriate, i.e. SESSION (first, last) for acquisition data, STOP-SIGNAL POSITION (Baseline, 50%) for stop-signal position manipulations and SESSION (baseline, 50%) for the comparison of 50% stop responding. All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. Data were screened for skewed variance with arcsine transformations applied to skewed data (percentage data in particular) to obtain a normal distribution. Greenhouse-Geisser degrees of freedom (df) corrections were applied as required to repeated-measures factors. Outliers which were 2 standard deviations or more from the mean were removed from the analysis.

7.3 Results

7.3.1 Zfp804a C59X line

7.3.1.1 Task acquisition

There was no difference in the final CM preference between C59X homozygous ENU-mutants (86%) and their WT littermate (88%) controls (t_{27}= 0.3, *p*=0.81). Of the 29 C59X subjects that commenced the SSRTT, 28 subjects managed to complete the task, with only one C59X homozygous ENU-mutant mouse failing to reach training criteria performance (Section 7.2.4.2). There were no differences between the genotype groups of C59X mice in terms of the number of sessions (ca.7) required to reach stable baseline performance at criteria responding (t_{26}= -0.4, *p*=0.71). During the initial shaping stages of training when the mice were learning to make the initiation nose-poke, the number of completed trials increased by almost 20% (main effect of SESSION, $F_{1,27}$= 12.3, *p*=0.002, SESSION*GENOTYPE, $F_{1,27}$= 0.04, *p*=0.84), and the latency to initiate a trial was over 100% quicker between the first and last sessions (main effect of SESSION, $F_{1,27}$= 189.5, *p*=0.000, SESSION*GENOTYPE, $F_{1,27}$= 0.002, *p*=0.96). There were no differences between C59X homozygous ENU-mutant and WT littermate control mice for either of these measures at this stage of training (main effect of GENOTYPE, $F_{1,27}$= 0.5, *p*=0.61, and $F_{1,27}$= 0.3, *p*=0.51, respectively).

In the next stage of training, the go response was introduced, whereby the mice had to make a 2nd nose-poke after the initiation nose-poke in order to earn a reward. Both C59X homozygous ENU-mutants and their WT littermate controls completed this stage of training in ca.9 sessions (t_{26}= 1.3, *p*=0.21), acquiring the go response readily (Table 7.2), and reaching high levels of correct responding (main effect of SESSION, $F_{1,26}$= 0.00, *p*=0.99, SESSION*GENOTYPE, $F_{1,26}$= 0.003, *p*=0.96). As might be expected, and reflecting the increasing stimulus control as training progressed, the speed of responding improved through training for the first (*M*= 5267ms) and last (*M*= 859ms) sessions respectively, main effect of SESSION, $F_{1,25}$= 73.7, *p*=0.000, SESSION*GENOTYPE, $F_{1,25}$= 1.1, *p*=0.3), although no difference was observed between the two C59X genotype groups of mice (main effect of GENOTYPE, $F_{1,25}$= 0.69, *p*=0.41).

In the final stage of training, the stop-signal was introduced at the start of the go response (i.e. 0% position) in 20% of the trials. It took all mice from the C59X line ca.10 sessions to reach stable performance criteria and again there were no significant differences between C59X homozygous ENU-mutants and their WT littermate controls (t_{26}= 0.3, p=0.77). There was a reduction in the percentage of correct stopping during training, with C59X mice achieving 100% correctly stopped trials in the first sessions compared to 85% correctly stopped trials on average in the last session through training (Table 7.2, main effect of SESSION, $F_{1,26}$= 49.14, p=0.000), most likely as a result of the increase in stop LH. A significant interaction between SESSION and GENOTYPE ($F_{1,26}$= 7.93, p=0.009) demonstrated that C59X homozygous ENU-mutant mice achieved higher levels of performance than their WT counterparts by the end of training (p=0.009). As predicted by the theory that going and stopping are dissociable but competing processes (Logan & Cowan, 1984), the introduction of the stop-signal stimulus in training had no effect on go responding, which remained unchanged from the previous training stage and at high levels of correct performance (main effect of SESSION, $F_{1,26}$= 0.02, p=0.89, SESSION*GENOTYPE, $F_{1,26}$= 0.4, p=0.55), although there was a further increase in the speed of go responding by all of the mice (main effect of SESSION, $F_{1,25}$= 10.35, p=0.004, SESSION*GENOTYPE, $F_{1,26}$= 0.4, p=0.53). These patterns of behaviour were equivalent between C59X homozygous ENU-mutants and WT littermate controls (main effect of GENOTPYE, $F_{1,26}$= 3.33, p=0.08, and $F_{1,25}$= 0.12, p=0.73, for correct go responses and Go-RTs, respectively). Taken together, the data from the training to baseline of the SSRTT indicated no differences between C59X homozygous ENU-mutants and their WT littermate controls in the way that they acquired criteria performance for the different components of the SSRTT.

Table 7.2: Task acquisition in Zfp804a C59X mice.							
Behavioural Parameter	**C59X Genotype**	**Single nose-poking**		**Double nose-poking**		**Learning to stop**	
		First	**Last**	**First**	**Last**	**First**	**Last**
End Initiation stimulus duration (s)	**WT**	24.6 ± 1.5	11 ± 0.7	20 ± 0	20 ± 0	20 ± 0	20 ± 0
	Homozygous	23.9 ± 1.4	10 ± 0.7	20 ± 0	20 ± 0	20 ± 0	20 ± 0
Number of trials initiated (%)	**WT**	44.3 ± 5.7	62.0 ± 4.5	60.8 ± 4.6	67.9 ± 4.0	67.8 ± 2.9	75.4 ± 3.8
	Homozygous	32.6 ± 5.3	54.0 ± 6.2	61.6 ± 5.4	67.2 ± 3.4	67.3 ± 3.3	73.5 ± 3.5
Latency to initiate a trial (s)	**WT**	13.3 ± 0.9	4.4 ± 0.3	3.5 ± 0.4	7.7 ± 0.8	6.5 ± 0.5	5.2 ± 0.4
	Homozygous	13.6 ± 0.9	4.8 ± 0.3	4.7 ± 0.4	7.0 ± 0.7	7.6 ± 0.4	6.6 ± 0.4
End Go stimulus duration (s)	**WT**	.	.	8.4 ± 2.1	1.2 ± 0.05	9.5 ± 0.4	9.5 ± 0.4
	Homozygous	.	.	11.7 ± 1.9	1.1 ± 0.04	9.6 ± 0.4	9.6 ± 0.4
Correct go trials (%)	**WT**	.	.	80.8 ± 3.1	79.1 ± 2.4	77.7 ± 3.2	79.3 ± 3.8
	Homozygous	.	.	78.7 ± 3.6	82.3 ± 2.0	78.7 ± 3.2	77.7 ± 2.3
Correct go reaction time (Go-RT, ms)	**WT**	.	.	4765 ± 791	904 ± 43.1	852 ± 31.7	760 ± 32.4
	Homozygous	.	.	5768 ± 708	813 ± 38.5	823 ± 30.5	762 ± 31.2
Latency to collect the reward (s)	**WT**	.	.	1.9 ± 1.1	3.1 ± 0.8	2.5 ± 1.5	2.6 ± 0.6
	Homozygous	.	.	3.3 ± 1.0	2.7 ± 0.7	5.5 ± 1.4	2.7 ± 0.6
End stop limited hold (ms)	**WT**	.	.	.	.	504 ± 24.9	712 ± 28.8
	Homozygous	.	.	.	.	497 ± 23.2	680 ± 26.8
Correct stop trials (%)	**WT**	.	.	.	.	100 ± 0.0	86.7 ± 3.8
	Homozygous	.	.	.	.	100 ± 0.0	82.3 ± 3.5

Data shows mean ± SEM

7.3.1.2 SSRTT performance at baseline

Baseline performance was calculated by averaging parameter values across all sessions which preceded each of the stop-signal manipulation sessions. During baseline sessions, the stop-signal was played concurrently with the onset of the go response (i.e. 0% of the individual Go-RT for each subject). There were no significant differences between C59X homozygous ENU-mutant mice and their WT littermate controls in terms of the key task parameters which dictated how the mice performed the SSRTT, such as the duration of the initiation and go stimuli and the stop LH (Table 7.3).

Table 7.3: Baseline SSRTT performance in Zfp804a C59X mice.

	WT	**Homozygous**		
Behavioural Parameter	**Mean**	**Mean**	**T statistic**	***p*-value**
Initiation stimulus duration (s)	20.00±0.00	20.00±0.00	n.a.	n.a.
Go stimulus duration (ms)	997.01±32.65	919.33±41.59	t_{26}=1.4	*p*=0.16
Stop limited hold (ms)	676.92±30.28	673.33±21.75	t_{26}=0.1	*p*=0.92
Number of trials initiated (%)	82.75±2.25	87.35±1.66	t_{26}=-1.7	*p*=0.11
Latency to initiate a trial (s)	5.47±0.40	4.36±0.30	t_{26}=-2.3	*p*=0.03
Correct go trials (%)	85.88±1.12	84.90±1.35	t_{26}=0.4	*p*=0.67
Correct stop trials (%)	86.47±2.19	88.42±2.04	t_{26}=-0.6	*p*=0.54
Correct go reaction time (Go-RT, ms)	726.19±20.22	650.48±17.46	t_{26}=2.9	*p*=0.008
Latency to collect the reward (s)	1.67±0.25	1.48±0.17	t_{26}=0.6	*p*=0.53
Initiation nose-pokes/trial (n)	0.97±0.03	0.97±0.02	t_{26}=0.01	*p*=0.10
Go nose-pokes/trial (n)	0.78±0.04	0.76±0.02	t_{26}=0.6	*p*=0.58
Panel-pushes/trial (n)	1.57±0.29	1.00±0.06	t_{26}=2.03	*p*=0.053
Beam-breaks/trial (n)	7.63±0.70	8.26±0.71	t_{26}=-0.6	*p*=0.54

Data shows mean ± SEM

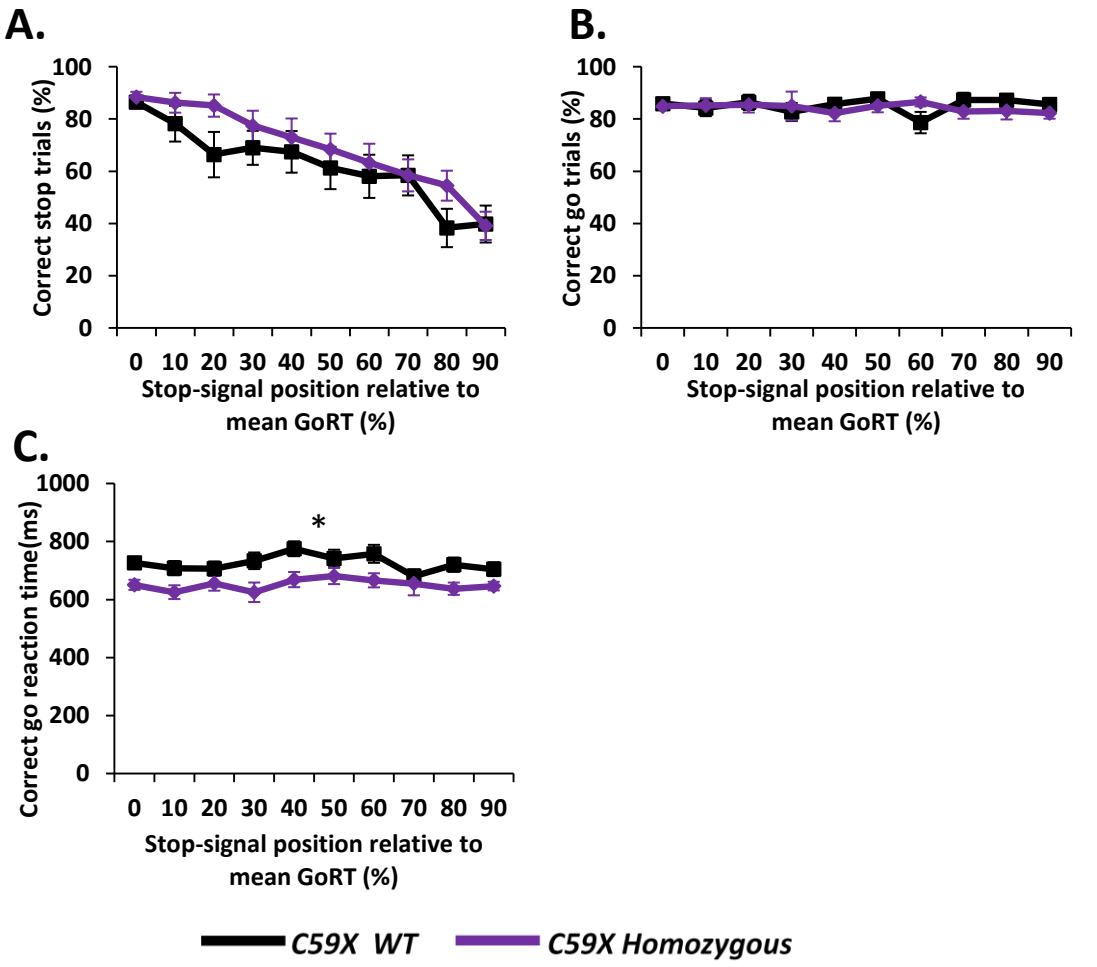
In general both genotypes of the C59X line initiated the same amount of trials within each session, but homozygous ENU-mutant mice were noticeably quicker to do this than their WT littermates (t_{26}=-2.3, *p*=0.03). Likewise there were no differences in the amount of correct go (t_{26}=0.4, *p*=0.67) and stop trials (t_{26}= -0.6, *p*=0.54), but again, C59X homozygous ENU-mutant mice had shorter response latencies when making the go response (t_{26}=2.9, *p*=0.008). There were no genotype-related differences in other task measures, such as the latency to collect the reward, numbers of nose pokes or number of beam breaks suggesting that there were no differences in motivation or locomotion between C59X homozygous ENU-mutant mice and WT littermate controls at baseline in the SSRTT.

7.3.1.3 The effects of varying the stop-signal delay on SSRTT performance

Once the mice had attained a stable baseline performance, where the stop-signal was presented at the start of the go response, they were then moved onto sessions where the stop-signal was presented at different positions to assess the ability to stop the response. As expected, there was a systematic decrease in the amount of correctly stopped trials made by all genotype of the C59X line as the stop-signal position was moved progressively into the individualised Go-RTs of the mice (Fig. 7.2A, main effect of STOP-SIGNAL POSITION, $F_{5.2,133.1}$= 18.7, *p*=0.000, STOP-SIGNAL POSITION*GENOTYPE, $F_{5.2,133.1}$= 0.8, *p*=0.57). The specificity of this manipulation to stopping was confirmed by an absence of any significant effects on the proportion of correct go trials (Fig. 7.2B, main effect of STOP-SIGNAL POSITION, $F_{5,130.2}$=0.7, *p*=0.66, SIGNAL POSITION*GENOTYPE, $F_{5,130.2}$= 1.0, *p*=0.45), and the latency of correct go responding (Fig. 7.2C, main effect of STOP-SIGNAL POSITION, $F_{5.3,136.7}$=2.1, *p*=0.07).

Consistent with the pattern of behaviour shown at baseline, C59X homozygous ENU-mutant mice were quicker at making go responses than their WT littermate controls (main effect of GENOTYPE, $F_{1,26}$=7.6, *p*=0.01), but overall responding in go trials was not different (main effect of GENOTYPE, $F_{1,26}$=0.1, *p*=0.71). By observation, C59X homozygous ENU-mutant mice appeared to better at stopping (Fig. 7.2A, however, this apparent effect did not reach significance (main effect of GENOTYPE, $F_{1,26}$=1.1, *p*=0.31).

Figure 7.2. Effect of varying the stop-signal position on SSRTT performance in Zfp804a C59X mice.



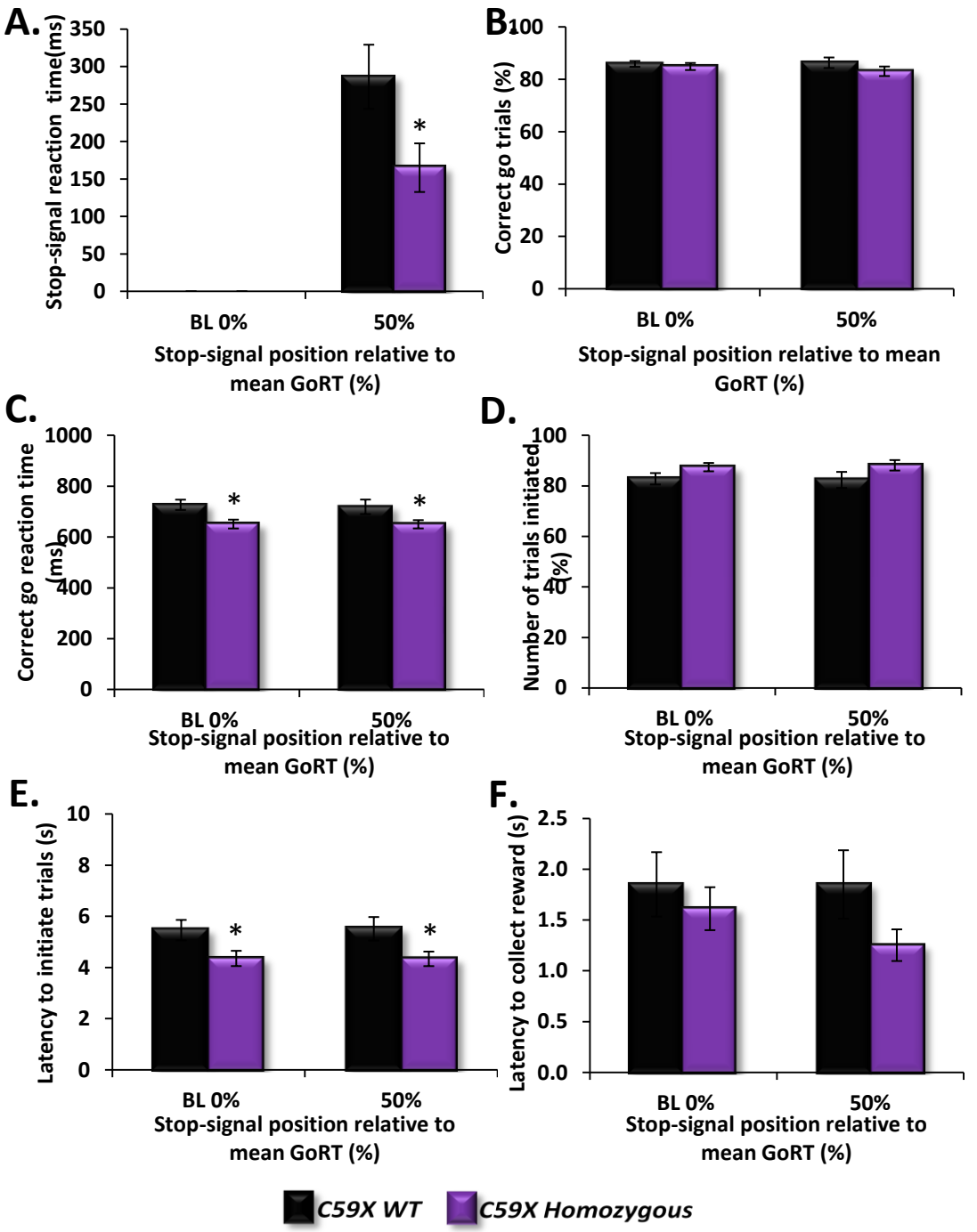
Moving the position of the stop-signal within the individualised go responses of each subject led to a systematic decrease in the ability of the mice to withhold responding, as shown by the proportion of correctly stopped trials (A), however this manipulation did not significantly affect correct responding in go trials (B). Although there was a tendency for C59X homozygous ENU-mutant mice to show better stopping behaviour, this was not significant, nor was the percentage of correct go trials made by the two groups of mice. However, C59X homozygous ENU-mutant mice were quicker than their WT littermate controls to make a response in go trials (C). Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 7.1 and text.

As mentioned previously, a key consideration in interpreting the SSRTT was to assess performance of this task at the point where competition between the go and stop responding was at its greatest, estimated to be where the proportion of correct responses were equal to 50%, which also permits calculation of the latency to stop responding (SSRT). To determine this, the data for each subject from the sessions in which the stop-signal position was moved was ranked by the proportion of correct responses and those between 40% and 60% (i.e. 50%±10%) were

averaged. Any subject outside of this range was removed from the analysis. One C59X homozygous ENU-mutant mouse (mean stopping of 71%) and one WT mouse (mean stopping of 74%) were therefore removed leaving final subject numbers of 14 and 12 for the C59X homozygous ENU-mutant (mean stopping = 51.63±1.38%) and WT (mean stopping = 47.63±2.51%) groups, respectively. C59X homozygous ENU-mutant mice had significantly shorter SSRTs than their WT littermate controls (Fig. 7.3A, t_{24}=2.3, p=0.03) which would be consistent with improved inhibitory control by these mice (increased SSRTs are associated with worse stopping, e.g. Humby et al., 2013). When the mice were showing 50% stop responding, there were still no differences in the proportion of correct responding between C59X homozygous ENU-mutant and WT mice (Fig. 7.3B, main effect of GENOTYPE, $F_{1,24}$= 0.7, p=0.4) and performance did not differ from baseline (main effect of SESSION, $F_{1,24}$= 0.5, p=0.49, SESSION*GENOTYPE, $F_{1,24}$= 1.5, p=0.23). C59X homozygous ENU-mutant mice, however, were consistently quicker in making go responses than their WT littermate controls (Fig. 7.3C, main effect of GENOTYPE, $F_{1,24}$= 9.3, p=0.005), although response speed did not differ from baseline (main effect of SESSION, $F_{1,24}$=0.3, p=0.62, SESSION*GENOTYPE, $F_{1,24}$= 0.6, p=0.44).

There were no differences between the two groups of C59X mice in a number of ancillary behaviours including the amount of trials initiated (Fig. 7.3D, main effect of GENOTYPE, $F_{1,24}$=2.9, p=0.1) or the latency to collect the reward (Fig. 7.3F, main effect of GENOTYPE, $F_{1,24}$=1.6, p=0.23), but C59X homozygous ENU-mutant mice were significantly quicker to initiate a trial than their WT counterparts (Fig. 7.3E, main effect of GENOTYPE, $F_{1,24}$=5.5, p=0.03). These measures did not differ between baseline performance and when the mice were showing 50% stop responding (main effect of SESSION, $F_{1,24}$=0.07, p=0.8, $F_{1,24}$=0.04, p=0.85, and $F_{1,24}$=0.03, p=0.86, for proportion of trials initiated, latency to initiate a trial and latency to collect reward, respectively). There were no significant differences between C59X homozygous ENU-mutant mice and WT littermate controls for other task measures such as the number of initiation and go nose-pokes, food magazine panel-pushes and locomotion in the test chambers, measured as infra-red beam breaks (data not shown).

Figure 7.3. SSRTT performance in C59X mice at 50% stopping.



Normalising performance to 50% correct stopping, where the go and stop responses were equivalent, allowed calculation of the SSRT; the latency to stop a response. C59X homozygous ENU-mutant mice had significantly shorter SSRTs than their WT littermate controls (A), suggesting improved stopping, i.e. enhanced response inhibition. There was no difference in the go response (B), and going was equivalent to baseline responding. There was also no difference in the Go-RT (C), amount of trials started (D), latency to initiate trials (E) or latency to collect the reward (F) between baseline and performance at 50% stopping. The pattern of C59X homozygous ENU-mutant mice making quicker go responses was maintained, however, and these mice were also faster to initiate a trial than their WT littermate controls. Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 7.1 and text.

7.3.2 Zfp804a C417Y line

7.3.2.1 Task acquisition

There was no difference in the final CM preference between C59X homozygous ENU-mutants (83%) and their WT littermate (87%) controls (t_{28}= -0.8, *p*=0.42). Of the 29 C417Y subjects that commenced the SSRTT, 28 mice managed to complete the task, with a single C417Y homozygous ENU-mutant subject failing to reach baseline. There was no differences between the genotype groups of C417Y mice in terms of the number of sessions (ca.7) required to reach stable baseline performance at criteria responding (t_{26}= 0.3, *p*=0.74). The overall percentage of trials that were completed increased between the first and last session of this phase of training (Table 7.4), although this increase was not found to be significant (main effect of SESSION, $F_{1,27}$= 2.4, *p*=0.13, SESSION*GENOTYPE, , $F_{1,27}$= 0.1, *p*=0.73), but the latency to initiate a trial had significantly decreased (main effect of SESSION, $F_{1,27}$= 61.5, *p*=0.000, SESSION*GENOTYPE, , $F_{1,27}$= 0.001, *p*=0.97). C417Y homozygous ENU-mutant mice and their WT littermate controls did not significantly differ for either of these measures (main effect of GENOTYPE, $F_{1,27}$= 0.2, *p*=0.63, and $F_{1,27}$= 1.3, *p*=0.26, respectively).

All mice from the C417Y line achieved criteria and completed the double nose-poke phase, 2nd stage, of training in ca.9 sessions, with no significant difference observed between genotypes (t_{26}= -0.3, *p*=0.77). Although C417Y homozygous ENU-mutants made significantly less correct go responses than their WT counterparts (main effect of GENOTYPE, $F_{1,26}$= 4.7, *p*=0.04), overall performance by both groups was consistently greater than criteria (ca.78%) from the first session of this stage of training (main effect of SESSION, $F_{1,26}$= 0.03, *p*=0.87, SESSION*GENOTYPE, , $F_{1,26}$= 0.4, *p*=0.55). There was a significant reduction in the latency to make a correct go response between the first (*M*= 5628ms) and the last session (*M*= 854ms) of this phase of training (main effect of SESSION, $F_{1,22}$= 65.7, *p*=0.000, SESSION*GENOTYPE, , $F_{1,22}$= 0.8, *p*=0.39, Table 7.4), although no difference was observed between the different C417Y genotype groups of mice (main effect of GENOTYPE, $F_{1,22}$= 0.5, *p*=0.49). There were also no group differences in the amount of trials initiated during this stage of training (main effect of GENOTYPE, $F_{1,26}$= 0.13, *p*=0.72), although all

mice from the C417Y line showed an increase in the number of trials completed (main effect of SESSION, $F_{1,26}$= 5.05, *p*=0.03, SESSION*GENOTYPE, $F_{1,26}$= 0.2, *p*=0.69).

Once at criteria responding, the mice were moved to the final stage of training where a stop-signal was presented at the start of the go response (i.e. 0% position) for 20% of the trials. C417Y homozygous ENU-mutant and WT littermate controls demonstrated good stopping behaviour from the first to the last session, with consistently high correct stop responses (~86%) throughout training (main effect of SESSION, $F_{1,26}$= 3.5, *p*=0.07, SESSION*GENOTYPE, $F_{1,26}$= 0.6, *p*=0.46), with no significant differences between genotypes ($F_{1,26}$= 1.5, *p*=0.24). The amount of correct go responses made by the C417Y mice continued to increase (main effect of SESSION, $F_{1,26}$= 4.9, *p*=0.04, SESSION*GENOTYPE, $F_{1,26}$= 1.71, *p*=0.2) and the speed of go responding increased (main effect of SESSION, $F_{1,24}$= 15.4, *p*=0.001), although the previous difference in correct go responses between C417Y homozygous ENU-mutants and WT littermate controls had now disappeared (main effect of GENOTYPE, $F_{1,26}$= 0.2, *p*=0.65); go reaction times were not different between the groups of mice (main effect of GENOTYPE, $F_{1,24}$= 0.5, *p*=0.48). All mice from the C417Y line (t_{26}= 1.3, *p*=0.21) completed the learning to stop stage of SSRTT in ca.11 sessions. Therefore, there were no fundamental differences between C417Y homozygous ENU-mutant and WT littermate controls in the way that they acquired criteria performance for the different components of the SSRTT.

7.3.2.2 SSRTT performance at baseline

As before, baseline performance was calculated by averaging parameter values across all sessions which preceded each of the stop-signal manipulation sessions. During baseline sessions, the stop-signal was played concurrently with the onset of the go response (i.e. 0% of the individual Go-RT for each subject). There were no significant differences between C417Y homozygous ENU-mutants and their WT littermate controls in terms of the critical task parameters which dictated how the mice performed the SSRTT, the initiation and go stimuli as well as the stop LH (Table 7.5). Mice from the C417Y line initiated the same amount of trials, with equivalent latencies, but C417Y homozygous ENU-mutant mice made more correct go response than their WT littermate controls (t_{26}=-2.2, *p*=0.04).

Table 7.4: Task acquisition in Zfp804a C417Y mice.							
Behavioural Parameter	**C417Y Genotype**	**Single nose-poking**		**Double nose-poking**		**Learning to stop**	
		First	**Last**	**First**	**Last**	**First**	**Last**
End Initiation stimulus duration (s)	**WT**	22.9 ± 1.5	11.1 ± 0.8	20 ± 0	20 ± 0	20 ± 0	20 ± 0
	Homozygous	22.4 ± 1.5	10.3 ± 0.7	20 ± 0	20 ± 0	20 ± 0	20 ± 0
Number of trials initiated (%)	**WT**	47.4 ± 5.7	60.1 ± 3.7	62.1 ± 3.9	71.9 ± 2.7	69.5 ± 3.1	75.3 ± 3.4
	Homozygous	48.9 ± 6.4	52.3 ± 5.2	56.7 ± 4.9	64.0 ± 3.8	64.4 ± 4.0	71.9 ± 3.7
Latency to initiate a trial (s)	**WT**	13.6 ± 0.9	6.0 ± 0.8	5.7 ± 0.8	6.7 ± 0.5	7.1 ± 0.4	6.1 ± 0.6
	Homozygous	12.7 ± 0.9	5.2 ± 0.8	4.9 ± 0.7	6.9 ± 0.4	7.1 ± 0.7	6.2 ± 0.5
End Go stimulus duration (s)	**WT**	.	.	9.8 ± 1.8	1.1 ± 0.08	9.8 ± 0.8	9.8 ± 0.8
	Homozygous	.	.	10.0 ± 2.0	1.2 ± 0.09	9.9 ± 0.5	9.9 ± 0.5
Correct go trials (%)	**WT**	.	.	81.0 ± 2.5	80.7 ± 3.0	64.6 ± 6.6	79.7 ± 2.6
	Homozygous	.	.	74.3 ± 5.5	80.4 ± 1.9	79.2 ± 3.8	81.2 ± 2.8
Correct go reaction time (Go-RT, ms)	**WT**	.	.	6110 ± 925	814 ± 84.3	781 ± 50.3	723 ± 26.0
	Homozygous	.	.	5146 ± 782	893 ± 71.3	870 ± 46.5	700 ± 24.1
Latency to collect the reward (s)	**WT**	.	.	1.6 ± 1.1	2.7 ± 0.7	3.5 ± 0.7	2.5 ± 0.6
	Homozygous	.	.	3.7 ± 1.0	3.1 ± 0.6	2.5 ± 0.6	2.8 ± 0.6
End stop limited hold (ms)	**WT**	.	.	.	.	473 ± 35.1	650 ± 23.1
	Homozygous	.	.	.	.	483 ± 32.7	710 ± 21.5
Correct stop trials (%)	**WT**	.	.	.	.	92.9 ± 7.1	85.7 ± 3.5
	Homozygous	.	.	.	.	91.4 ± 7.2	75.0 ± 6.0

Data shows mean ± SEM

There were no differences in the speed of responding to the go stimuli or in the amount of correctly stopped trials. Other task measures, such as the latency to collect the reward, numbers of nose pokes and beam breaks also did not differ between the different genotype groups of the C417Y line, suggesting that there were no differences in motivation or locomotion between C417Y homozygous ENU-mutants and their WT littermate controls at baseline in the SSRTT.

Table 7.5: Baseline SSRTT performance in Zfp804a C417Y mice.

	WT	**Homozygous**		
Behavioural Parameter	**Mean**	**Mean**	**T statistic**	***p*-value**
Initiation stimulus duration (s)	20.00±0.00	20.00±0.00	n.a.	n.a.
Go stimulus duration (ms)	917.69±49.20	1037.33±78.29	t_{26}=-1.3	*p*=0.22
Stop limited hold (ms)	657.69±15.86	690.00±22.47	t_{26}=-1.2	*p*=0.25
Number of trials initiated (%)	81.64±2.74	84.18±2.11	t_{26}=-0.7	*p*=0.49
Latency to initiate a trial (s)	4.89±0.41	4.96±0.51	t_{26}=-0.1	*p*=0.92
Correct go trials (%)	81.91±1.57	86.16±1.24	t_{26}=-2.2	*p*=0.04
Correct stop trials (%)	87.28±2.26	85.92±2.59	t_{26}=0.4	*p*=0.71
Correct go reaction time (Go-RT, ms)	671.73±33.89	668.79±17.13	t_{26}=0.08	*p*=0.94
Latency to collect the reward (s)	1.54±0.15	1.46±0.14	t_{26}=0.4	*p*=0.71
Initiation nose-pokes/trial (n)	0.95±0.03	0.97±0.03	t_{26}=-0.6	*p*=0.58
Go nose-pokes/trial (n)	0.72±0.03	0.79±0.03	t_{26}=-1.8	*p*=0.09
Panel-pushes/trial (n)	0.88±0.06	1.30±0.19	t_{26}=-2.0	*p*=0.06
Beam-breaks/trial (n)	7.26±0.67	7.41±0.66	t_{26}=-0.2	*p*=0.87

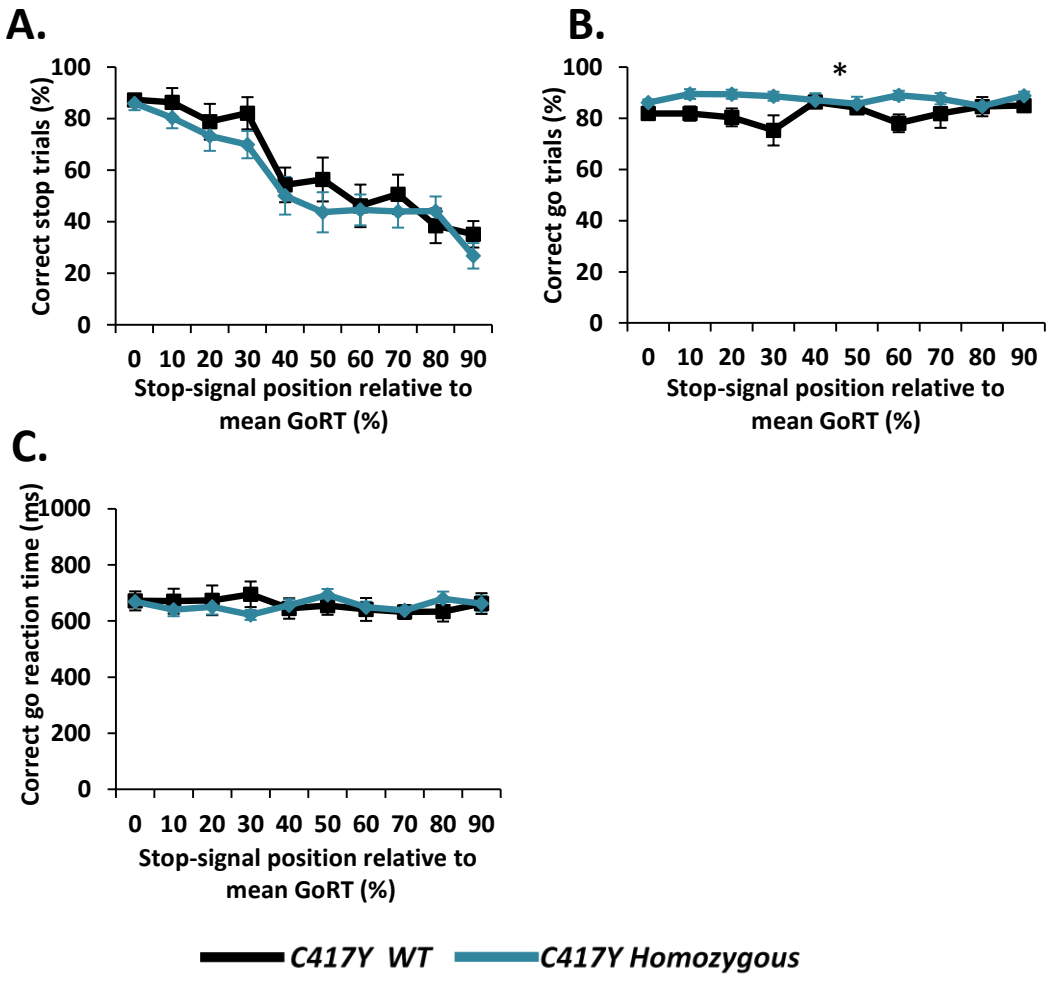
Data shows mean ± SEM

7.3.2.3 The effects of varying the stop-signal delay on SSRTT performance

Once mice from the C417Y line had attained stable baseline performance in the SSRTT, their ability to stop the go response was assessed in sessions where the stop-signal was presented at different positions within the individualised Go-RTs of the mice. As expected, moving the stop-signal position led to a systematic decrease in the amount of

correctly stopped trials made (Fig. 7.4A, main effect of STOP-SIGNAL POSITION, $F_{5.5,141.9}$=22.9, *p*=0.000, STOP-SIGNAL POSITION*GENOTYPE, $F_{5.5,141.9}$= 0.8, *p*=0.55), without affecting the go response in terms of the amount of correct go trials (Fig. 7.4B, main effect of STOP-SIGNAL POSITION, $F_{5.2,134.6}$=0.9, *p*=0.5, STOP-SIGNAL POSITION*GENOTYPE, $F_{5.2,134.6}$= 1.5, *p*=0.18), nor speed of responding (Fig. 7.4C, main effect of STOP-SIGNAL POSITION, $F_{5.3,137.1}$=0.9, *p*=0.51, STOP-SIGNAL POSITION*GENOTYPE, $F_{5.3,137.1}$= 1.96, *p*=0.09).

Figure 7.4: Effect of varying the stop-signal position on SSRTT performance in Zfp804a C417Y mice.



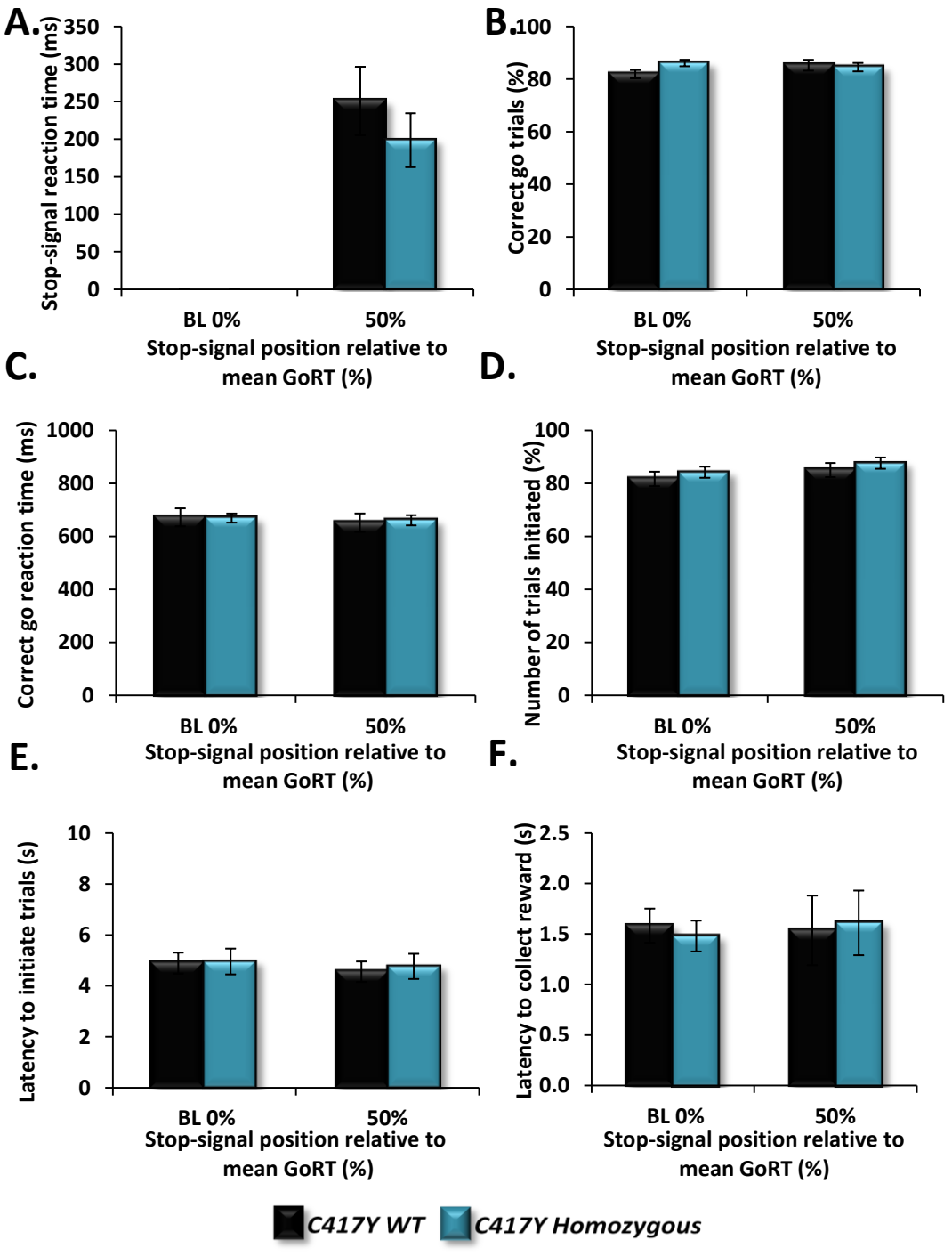
Moving the position of the stop-signal within the individualised go responses of each subject led to a systematic decrease in the ability of the mice to withhold responding, as shown by the proportion of correctly stopped trials (A), however this manipulation did not significantly affect correct responding in go trials (B) or in the time required to make the go response (C). There was no significant genotype difference in the number of correctly stopped trials nor in the time required to make a go response, however, C417Y homozygous ENU-mutants made significantly more correct go responses than their WT littermate controls across the range of manipulation sessions. Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 7.1 and text.

Moving the stop-signal position did not generate any specific differences between C417Y homozygous ENU-mutants and WT littermate controls, a finding consistent with baseline performance. Thus, there were no genotype-related differences in stopping (main effect of GENOTYPE, $F_{1,26}$=1.8, p=0.19), or the latency to make a go response (main effect of GENOTYPE, $F_{1,26}$=0.004, p=0.95). However, C417Y homozygous ENU-mutant mice made significantly more correct go responses than their WT littermate controls across the range of manipulation sessions (main effect of GENOTYPE, $F_{1,26}$=5.3, p=0.03).

To investigate stopping performance at the point where competition between the go and stop responding was at its greatest (i.e. where the proportion of correct responses were equal to 50%), the data for each subject from the sessions in which the stop-signal position was moved was ranked by the proportion of correct responses and those between 40% and 60% (i.e. 50%±10%) averaged, any subject outside of this range was removed from the analysis. One C417Y homozygous ENU-mutant mouse (mean stopping of 33%) and two WT mice (mean stopping of 78% and 38%) were therefore removed from the analysis leaving final Ns of 14 and 11 for the C417Y homozygous ENU-mutant (mean stopping = 51.18±1.21%) and WT (mean stopping = 49.07±1.12%) groups, respectively. There was no difference between the two groups of mice in the SSRT, the latency to stop a response, when the mice were showing 50% correct stopping, (Fig. 7.5A, t_{23}=0.9, p=0.37) suggesting that there was no difference in inhibition between C417Y homozygous ENU-mutants and their WT littermate controls.

When C417Y mice were showing 50% stop responding, there were no differences in the go response in terms of the proportion of correct responding (Fig. 7.5B, main effect of GENOTYPE, $F_{1,23}$=0.33, p=0.57) nor the speed of responding (Fig. 7.5C, main effect of GENOTYPE, $F_{1,23}$=0.27, p=0.61) and performance did not differ from baseline for either of these measures (main effect of SESSION, $F_{1,23}$=0.6, p=0.47, and $F_{1,23}$=0.1, p=0.77, for percentage go correct and Go-RT, respectively). There were no differences between the two groups of C417Y mice in the amount of trials initiated (Fig. 7.5D, main effect of GENOTYPE, $F_{1,23}$=0.5, p=0.48), the latency to initiate a trial (Fig. 7.5E, main effect of GENOTYPE, $F_{1,24}$=0.2, p=0.69) or the latency to collect the reward (Fig. 7.5F, main effect of GENOTYPE, $F_{1,23}$=0.1, p=0.72).

Figure 7.5: SSRTT performance in C417Y mice at 50% stopping.



Normalising performance to 50% correct stopping, where the go and stop responses were equivalent, allowed calculation of the SSRT; the latency to stop a response. C417Y homozygous ENU-mutants and their WT littermate controls had equivalent SSRTs, and therefore comparable inhibitory control (A). There was no difference in the go response (B), and going was equivalent to baseline responding. There was no difference in the Go-RT (C), latency to initiate trials or latency to collect the reward (F) between baseline and performance at 50% stopping, although a significantly greater number of trials were initiated when performance was at 50% stopping (D). There were also no significant genotype differences on any of these measures. Data shows the mean values ±SEM. For subject N see Table 7.1 and text.

There was also no difference in the latency to initiate a trial and the latency to collect a reward between baseline performance and when the mice were showing 50% stop responding (main effect of SESSION, $F_{1,23}$=1.6, p=0.22, and $F_{1,23}$=1.4, p=0.25, respectively) but there was a small but significant increase in the proportion of trials initiated when the mice were showing 50% stop responding (main effect of SESSION, $F_{1,24}$=8.4, p=0.008). There were no significant differences between C417Y homozygous ENU-mutants and their WT littermate controls for other task measures such as the number of initiation and go nose-pokes, food magazine panel-pushes and locomotion in the test chambers, measured as infra-red beam breaks (data not shown).

7.4 Discussion

WT mice from both the C59X and C417Y lines acquired and performed the SSRTT essentially as demonstrated in previous work using inbred C57BL/6J mice (Humby et al., 2013). As expected, moving the stop-signal position progressively into the individualised go responses of each subject led to a systematic decrease in the ability of the mice to cancel the go response. This led to a reduction in the number of correctly stopped trials, supporting the 'horse-race' model's assumption that the later the stop-signal delay is presented the less chance that the stopping process will win the race (Logan & Cowan, 1984).

Analysis of stopping behaviour at the 50% stop-position, where the race is predicted to be at its greatest, yielded stop-signal reaction times (SSRT) of ca. 230ms; these were close to previous data in the C57BL/6J mouse strain (350ms, Humby et al., 2013) and rats (300ms, Eagle & Robbins, 2003a), and were also consistent with human studies using this task (200ms, Logan & Cowan, 1984), adding further weight to the translational potential of the murine SSRTT. Behaviour in the SSRTT was dissociable, in that responding in go trials (amount of correct go trials and the go reaction time), motivation (latency to collect reward, proportion of trials initiated, latency to initiate a trial) or locomotion (beam breaks) were not affected by stopping behaviour at the 50% (or any other) stop-signal position, further supporting the race-model which describes stopping and going as parallel, competing, independent processes (Logan & Cowan, 1984).

C59X and C417Y homozygous ENU-mutant mice acquired the task at the same rate as their respective WT littermate controls and demonstrated equivalent performance in

measures of motivation and locomotion throughout the different phases of testing. There were also no genotype-related differences between mice of the C417Y line for any of the main measures of the SSRTT, such as proportion of correct go responses, initiation and go latencies and SSRT at baseline, as well as in sessions when the stop-signal position was altered, and also when the mice were showing 50% correct stopping. Thus, the C417Y mutation, at least within the task parameters used in the present work, does not appear to have an effect on response control assayed in the SSRTT.

C59X homozygous ENU-mutant mice displayed improved stopping compared to their WT littermate controls, an indication the C59X mutation may actually give rise to enhanced response control. This effect was consistent, being apparent during acquisition when the stop-signal was first introduced, and when the stop-signal was moved to different positions within the individualised go responses of each subject. Furthermore, at 50% correct stopping, C59X homozygous ENU-mutant mice had significantly reduced SSRTs compared to their WT littermate controls, again indicative of enhanced response inhibition/reduced impulsivity. Despite this apparent reduction in impulsivity, C59X homozygous ENU-mutants also demonstrated significantly quicker initiation latencies and go reaction times (there were no differences in the number of correct go trials made) than WT littermate controls, both during baseline sessions and during manipulation of the stop-signal position, indicating both improved going and stopping processes in these mutants.

The enhanced response control, or decreased impulsivity, shown by the C59X homozygous ENU-mutant mice is contrary to what might be expected from a mouse model for schizophrenia, where slower SSRTs, indicative of increased impulsivity, have been observed in patients (Enticott, 2008; Huddy et al., 2009). However, these findings are equivocal (Rubia et al., 2001; Bellgrove et al., 2005), nevertheless there are no studies (to our knowledge) that have reported decreases in impulsivity as measured with the SSRTT, as was found here. Although there are minimal studies investigating SSRTT performance in bipolar disorder, in general these patients similarly show reduced response inhibition (Strakowski et al., 2010). To our knowledge, there are no other mouse models for schizophrenia (or bipolar disorder) that have utilised response inhibition tasks, and certainly none with the SSRTT. Other rodent models using the SSRTT have commonly found poorer stopping behaviour following pre-frontal cortex, orbitofrontal cortex and subthalamic nucleus lesions (Humby et al., 2013; Eagle et al., 2008), with improvements in this function

after administration of methylphenidate, atomoxetine and the $5HT_{2C}$ receptor antagonist SB242084 (Humby et al., 2013; Eagle & Robbins, 2003a,b; Eagle et al., 2007). Thus, SSRTT performance appears dependent on frontostriatal brain systems, which are also involved in the pathophysiology of schizophrenia (Rubia, 2002; Rubia et al., 2001; Rubia et al., 2003), with the involvement of forebrain dopaminergic, noradrenergic and serotonergic systems. It would also be interesting to manipulate the stop-signal itself, for example via degradation of the stop-signal and/or increased/decreased stop-signal length. This would tax the attentional function of the mice and potentially draw out further differences between the C59X homozygous ENU-mutants and their WT littermate controls.

Reconciling the present results in terms of schizophrenia or bipolar disorder is somewhat difficult, however, the data do indicate that manipulation of *Zfp804a* does alter a disease-related phenotype and therefore suggests that forebrain neurotransmitter systems or frontostriatal brain systems have been affected in C59X homozygous ENU-mutant mice. It also difficult to reach a general conclusion in how overall mechanisms of impulse control may be affected in C59X and C417Y ENU-mutant mice, as the SSRTT primarily assesses only one facet of impulsivity: impulsive action (Winstanley et al., 2006). Therefore, investigating mechanisms of impulsive choice, which have dissociable neurobiological systems (Evenden, 1999b; Winstanley et al., 2006) may yield different patterns of effects in the two mutant lines of mice.

7.4.1. Summary of key results from Chapter VII

- All mice from the C59X and C417Y lines were capable of learning a complex operant task, and were quick to acquire the various stages of the SSRTT.
- Moving the stop-signal position within the individualised go responses of each subject led to a systematic decrease in the ability of the mice to cancel the go response.
- The C59X homozygous ENU-mutants demonstrated enhanced response inhibition (quicker SSRTs) as compared to their WT littermate controls when stopping behaviour was normalised to 50% correct stopping.
- There were no significant differences in inhibitory, or other task-related behaviour between C417Y homozygous ENU-mutants and their WT littermate controls.

Chapter VIII; General discussion

In a recent GWAS, a single nucleotide polymorphism within *ZNF804A* was found to be associated with risk for developing schizophrenia (O'Donovan et al., 20008), with this association strengthened when bipolar cases were added to the affected sample. Furthermore, the genetic association between the *ZNF804A* risk allele and schizophrenia has since been thoroughly replicated (Riley et al., 2009; Steinberg et al., 2011; Williams et al., 2011). The aims of the present thesis were to further characterise the function(s) of *Zfp804a*, the mouse orthologue of *ZNF804A*, with the goal of understanding more about how variance in this gene may increase the risk of developing schizophrenia/bipolar disorder. This was achieved by testing mouse models bearing ENU-mutations in *Zfp804a* on a wide range of behavioural tasks.

8.1 Utility of the models: limitations and advantages

A potential limitation of testing mutants derived from ENU mutagenesis is that this technique causes multiple mutations in the genome, meaning that many other residual ENU-induced mutations may be inherited along with the mutation of interest (this has been estimated to be approximately 31 in founder mice, see Keays et al., 2006), leading to possible confounds in interpreting genotype-phenotype relationships. However, after backcrossing the *Zfp804a* lines onto the C57BL/6JOlaHsd strain (C57BL/6J) they were estimated to be 98.5% genetically identical. It was therefore calculated that our $G7_{i/ii}$ *Zfp804a* mice should be carrying on average less than 0.49 other residual mutations (as calculated from Keays et al., 2006). Furthermore, the chances of this very low level of 'off target' mutations being distributed systematically, as opposed to randomly, were extremely slim in any case.

There is also the potential for other 'hitchhiking genes' derived from the original ENU treated mouse strain that flank *Zfp804a* to co-occur with the selected mutation despite the backcrossing protocol if they are in linkage disequilibrium, leading to the possibility that phenotypic differences between the mutants and wild type controls may be influenced by polymorphisms in these flanking genes occurring between the ENU strain (BALB/c x C3H/HeJ) and C57Bl/6J. This can be a general problem in the interpretation of data using many genetically modified animal models (both ENU and knockouts made using

homologous recombination methods) and is only solved, definitely, by specific genetic rescue of the phenotype. However, the same ENU strain derived flanking genes and any polymorphisms within them should be inherited with both the C59X and C417Y mutation. Thus, the general strategy employed throughout the thesis of comparing the effects of the two ENU mutations relative to wild type littermate controls, not only revealed the effects of variance in two parts of the *Zfp804a* gene, but also acted as a control; insofar as common effects across both the C59X and C417Y ENU-mutant mice, relative to wild type littermate controls, could theoretically be due to these 'hitchhiking' flanking genes, the many instances of clear phenotypic differences between the C59X and C417Y homozygous ENU-mutants pointed towards specific effects of ENU-induced changes in *Zfp804a* function. Additionally, the background strain used for this work was the C57BL/6JOlaHsd strain (Harlan, Bicester, UK) which carries a deletion spanning the *alpha-synuclein* (*Snca*) locus (Specht & Schoepfer, 2001). This was not thought to pose a problem with the *Zfp804a* mice tested in this work, as they were estimated to have c.98.5% C57BL/6JOlaHsd background; and should therefore all have the *Snca* deletion (Knight, 2013), with a sample of PCRs for the *Snca* gene showing this to be the case.

It has been argued that ENU-mutagenesis best mimics the naturally occurring single-base pair mutations found in the human genome (Jiao, Cai, Kermany et al., 2009), and as such, mutants derived from ENU-mutagenesis versus mutants derived from knockout technologies may be better placed to investigate the genetics behind certain clinical disorders (Acevedo-Arozena, Wells, Potter et al., 2008). The work in this thesis is based, in general, upon the GWAS which identified SNPs in *ZNF804A* as having an association with increased risk for a schizophrenia/bipolar disorder diagnosis. Recently, the complexity of the genetic architecture of mental disorders has been added to by the discovery of a number of rare, highly penetrant copy number variants (CNVs). It might be argued that rather than studying evolutionary conserved, common, low penetrant risk variants such as those found in *ZNF804A*, models based on more highly penetrant (often de-novo) CNVs, would be the optimal rationale. However, it should be emphasised that, despite many of the discovered variants from GWAS having little effect on overall risk burden, functional studies examining their mechanism(s) of action and the pathways they interact with can be highly illuminating in terms of pathogenesis and options for treatments (Willer & Mohlke, 2012).

The two mutations in *Zfp804a* selected for re-derivation, the C59X (nonsense) and the C417Y (missense), were chosen due to their perceived functional effects. The C59X mutation in exon 2 encodes a premature stop codon, which was predicted to serve as a functional null of the gene. Reverse transcription PCR analysis (RT-PCR) conducted in parallel to this work, and subsequent RT-PCR presented in Section 8.4.3 below, confirms that the C59X mutation has escaped nonsense-mediated decay (NMD), and as such, cannot technically be described as a null. An antibody to characterise the C59X mutation at a *Zfp804a* proteomic level is needed to confirm the assumed influence of the mutation on the *Zfp804a* protein, as without this the nature of the C59X mutation remains unclear. The C417Y mutation is missense and constitutes a cysteine to tyrosine substitution in exon 4. This mutation was predicted to change the structure of the protein, and was deemed 'probably damaging' by PolyPhen, a bioinformatics program which can be used to predict the effects of non-synonymous SNPs in mice. It has recently been posited that the C417Y mutation may be polymorphic (D. Blake, personal communication). Internet programs such as BLAST (Basic Local Alignment Search Tool) have been used to examine the protein sequence around the mutation, finding the region to be highly conserved in humans and rodents, but not conserved across other species, with one species, the ferret, showing the cysteine to tyrosine substitution naturally. It could be argued this makes the C417Y polymorphism a good control for the C59X mutation, and as predicted, it was the C59X mutation that had a greater effect on phenotype (though not exclusively).

The most obvious limitation to the current work is the translatability of mouse models to study uniquely human disorders such as schizophrenia and bipolar disorder. However, the aim of this thesis was not to study a mouse model of schizophrenia, nor to model the human *ZNF804A* risk variant rs1344706 in mice. The purpose was to understand the function of *Zfp804a* at a behavioural level, using tasks that tap into the symptoms of schizophrenia and bipolar disorder. Mouse models are a crucial first step in understanding gene function, and contribute greatly to our understanding of genes when used in conjunction with molecular, cellular and clinical work.

8.2 Main findings

Monitoring of the early development of the *Zfp804a* mutants did not uncover any gross developmental phenotypes. There were no obvious signs of breeding related

problems with pups appearing normal at birth and somatic indices of development emerging within normal time frames. A Mendelian inheritance pattern for the three genotypes of both mutant lines was observed, but fewer males (of any genotype) were born to both mutant lines, perhaps indicating that both mutations were influencing the pre-natal survivability of males. It would therefore be interesting to examine a number of litters prenatally to ascertain the number, genotype and gender of pups *in utero* to determine if more male pups are present prenatally and if Mendelian ratios are intact before birth.

The other main developmental finding was that C59X male homozygous ENU-mutants weighed less than their WT littermate controls between postnatal days 11-20, as well as between postnatal days 21-30 (weaning), and in adulthood. The C59X female homozygous ENU-mutants also weighed less than their WT littermate controls but only between postnatal days 21-30. The reduced weight seen in the C59X male homozygous ENU-mutants appears at around the same time that mice are weaned from their mother's milk and switch to solids, coinciding with the point at which the pup's eyes are open and they become more independent around the cage (Silver, 1995), potentially implying a delayed weaning in these mutants, with the effects of this continued into adulthood. This delayed weaning could be due to increased neophobia to the solid food or perseveration of maternal contact, although both are speculation at this point. These reduced adult weights were also found in the C59X homozygous ENU-mutants of the *Zfp804a* $G4_i$ cohort supporting the idea that the C59X mutation might be having an effect on the growth of these mutants.

Table 8.1 shows the summary behavioural results of homozygous ENU-mutants from both lines, in comparison to their WT littermate controls. Initial sensory-motor testing utilising four tests from the SHIRPA battery (Rogers et al., 1997) found that C59X homozygous ENU-mutants showed enhanced vestibular and proprioceptive function as measured on the negative geotaxis test (Chapter III). This could be a genuine result, but might also be due to poorer WT capabilities, where 35% of the C59X WT controls showed poor negative geotaxis performance as compared to 0% poor performance in the C417Y WTs, so the enhanced abilities of the C59X homozygous ENU-mutants were more likely to be significantly different from their WT littermate controls. If it is a genuine result, then it corresponds with the *Zfp804a* $G4_i$ C59X male homozygous ENU-mutants displaying enhanced sensorimotor abilities on the RotaRod, with mutants staying on the rotating

apparatus for significantly longer than their WT littermate controls (Al-Janabi, 2012). These improved sensorimotor abilities in the C59X homozygous ENU-mutants have not been shown (to our knowledge) in any other mouse model for schizophrenia/bipolar disorder, with other mouse models for schizophrenia displaying impaired sensorimotor capabilities, for example, in mice overexpressing *Nrg1* (Deakin, Law, Oliver et al., 2001) or Sdy mice carrying a deletion in *Dtnbp1* (Takao, Toyama, Nakanishi et al., 2008).

Table 8.1: Summary of behavioural results for homozygous ENU-mutant mice from both lines, in respect to their WT littermate controls.

Behavioural Task	C59X Homozygous	C417Y Homozygous
SHIRPA (sensorimotor)	↑	↔
Locomotor activity	↔	↔
Reactivity to novel food	↑	↔
Anxiety	↓	↔
Motivation	↔	↑
Hedonia	↓	↔
Acoustic startle	↓	↔
Pre-pulse inhibition	↓	↔
Response control	↑	↔

Locomotor activity did not significantly differ between homozygous ENU-mutants and WT littermate controls in either mutant line in the LMA (Chapter III), although the C59X homozygous ENU-mutants appeared to be less active than their WT littermate controls, in line with previous data from the *Zfp804a* G4i cohort (Al-Janabi, 2012). Reactivity to a novel food substance uncovered neophobia to the novel condensed milk substance on the 1st day of testing in the C59X homozygous ENU-mutants, compared to their WT littermate controls. This neophobia had disappeared by the 2nd day of testing, and both C59X homozygous ENU-mutants and their WT counterparts had equivalent preference for the condensed milk (over water) by the last day of testing. Neophobia on this task has been shown to be related to heightened anxiety on standardised tests such as the elevated plus-maze and open field (Mikaelsson et al., 2013). Based on this, one might hypothesise that the C59X homozygous ENU-mutants would show anxiogenic responses on various rodent tests of anxiety, but this

was not found to be the case (Chapter IV). The apparent neophobia therefore could be due to the C59X homozygous ENU-mutants displaying anhedonic tendencies (Chapter V); i.e. the C59X homozygous ENU-mutants did not 'like' the condensed milk solution as much as their WT littermate controls on their first encounter with it.

The reduced anxiety phenotype seen with the C59X homozygous ENU-mutants (as indexed by increased time in the more anxiety-provoking zone) was significantly different to their WT littermate controls on the elevated zero-maze (EZM) and backed up by tendencies in the elevated plus-maze (EPM) and the open field (OF), and further corroborated by increased amounts of ancillary ethological behaviours such as stretch-attend postures, rearing and head dips (Chapter IV). Importantly, the reduced anxiety was not simply a result of hyperactivity in the C59X homozygous ENU-mutants, as they were not hyperactive in the LMA or in the anxiety tasks as compared to their WT littermate controls.

Chapter V revealed the only behavioural phenotype found with the C417Y homozygous ENU-mutants; increased motivation. This increased motivation was hinted at when the mutant lines were required to work for a 10% CM reward but was further substantiated when the reward was devalued (0% CM), with the C417Y homozygous ENU-mutants achieving significantly higher breakpoints than their WT littermate controls. Motivation in the C59X homozygous ENU-mutants was not significantly different to their WT littermate controls, however, in the lick cluster analysis, these mutants showed reduced lick cluster sizes to the 4% and 10% sucrose solutions in comparison to their WT counterparts, indicating a reduced pleasure in these palatable solutions (anhedonia). There was no effect of genotype in lick cluster size for the C417Y mutant line, suggesting a dissociation between the two mutations in terms of motivation and hedonia, with the C59X homozygous ENU-mutants equally driven to earn rewards but finding them less pleasurable than their WT littermate controls, and the C417Y homozygous ENU-mutant mice more motivated to earn rewards but finding them equally as pleasurable as their WT counterparts.

In terms of sensorimotor gating (Chapter VI), the C59X homozygous ENU-mutants showed reduced startle to a loud acoustic stimulus and displayed reduced prepulse inhibition (PPI). This failure to attenuate to a startling stimulus when preceded by a weaker stimulus is considered an endophenotype of schizophrenia and has been shown in other animal models for this disorder (Hikida et al., 2007; Clapcote et al., 2007; Stefansson et al.,

2002). There were no differences between C417Y homozygous ENU-mutants and their WT littermate controls on acoustic startle response or PPI.

Response control exhibited by the C59X and C417Y lines was tested using the stop-signal reaction time task (Chapter VII). It was found that all subjects acquired the task to an equivalent level but that the C59X homozygous ENU-mutants displayed a pattern of enhanced response inhibition, achieving quicker latencies to stop a response which had already been initiated than their WT counterparts. Behaviour between the C417Y homozygous ENU-mutants and their WT littermate controls was equivalent.

All but one of the behavioural phenotypes were found with the C59X and not the C417Y homozygous ENU-mutants, adding weight to the idea that the C417Y mutation is more likely a polymorphism, with the C59X mutation a more damaging genetic insult. The observable differences between the C59X and C417Y homozygous ENU-mutants means that the phenotypes found with the C59X mutants are most likely due to the C59X mutation itself, and not co-segregating genes around the mutation.

8.3 Further interpretation of key findings

8.3.1 Reduced anxiety in C59X homozygous ENU-mutants

Anxiety is not commonly modelled in mouse models for schizophrenia, although schizophrenia and bipolar patients often report altered levels of anxiety (Braga et al., 2004; Pokos & Castle, 2006; Keller, 2006). The effects observed in the C59X homozygous ENU-mutants are consistent and relevant to both schizophrenia and bipolar disorder, but it is difficult to directly translate the effects found in the *Zfp804a* mutants to clinical studies. It would have been interesting to have tested the corticosteroid release of the C59X homozygous ENU-mutants and their WT littermate controls, with the hypothesis being that the C59X mutants would have shown a reduced level of the 'stress hormone' cortisol in their blood immediately after the anxiety assays.

The reduced anxiety in the C59X mutants mimics the anxiolysis seen in treatment with benzodiazepines which enhance the effect of the $GABA_A$ receptor (Treit et al., 2010). Mouse models targeting the glutamatergic system have also shown reduced anxiety, with GRIN1 (D481N) mutants, which display a 5-fold reduction in NMDA receptor glycine affinity, showing anxiolysis on the EPM and OF (Labrie, Clapcote & Roder, 2009), with these effects reversed by administration of a direct activator of the NMDA receptor glycine site. Other

mouse models manipulating the serotonin (5-HT) system have shown anxiolysis on the EPM through over-expression of the 5-HTT transporter gene (Jennings et al., 2006), and anxiogenesis observed in mutants with the 5-HTT transporter gene knocked out (Holmes et al., 2003). It is unclear what the neurobiological causes are for the reduced anxiety in the C59X mutants although the GABAergic, serotonergic and glutamatergic systems may have a role. Further investigation into the mechanisms behind the reduced anxiety is required in the C59X mutants, perhaps using pharmacological manipulations to further elucidate the role of certain neurotransmitters in the anxiolysis phenotype.

8.3.2 Anhedonia in C59X homozygous ENU-mutants

The observation of anhedonia in the C59X homozygous ENU-mutants is, to our knowledge, the only incidence of anhedonia in a genetic mouse model for schizophrenia. Anhedonia is seen in both schizophrenia and bipolar disorder (Horan et al., 2006b; Gard et al., 2007; Serretti & Olgiati, 2004) and remains, as a negative symptom, difficult to treat (Kirkpatrick et al., 2006). Research has linked hedonic reactions to opiate systems in the brain and to hedonic hot spots within the brain such as the nucleus accumbens shell and ventral pallidum (Peciña et al., 2006). Hedonic reactions to taste in rats have been measured with affective taste reactivity tests that measure orofacial liking reactions, which are similar between species to certain flavours i.e. sweet and bitter. Much of the research into hedonic reactivity has focused on opiate systems in the brain (Peciña et al., 2006), with micro-injection of DAMGO (an opiod agonist) into the rostral half of the medial nucleus accumbens shell elevating the hedonic reactions to sucrose in rats (Peciña & Berridge, 2005). DAMGO administration in other parts of the nucleus accumbens did not increase hedonic reactions to sucrose in rats, although it did increase 'wanting' of foods as demonstrated by increases in consumption (Peciña & Berridge, 2005). μ-Opioid agonists elevate the consumption of sucrose and μ-Opioid antagonists suppress the consumption of sucrose in the wider accumbens region of a rat (Peciña & Berridge, 2000), hence individuals with increased μ-opioid activity in the hedonic hot spot of the nucleus accumbens shell may eat because food tastes nicer, but individuals with higher μ-opioid activity in the surrounding areas of the nucleus accumbens shell may eat more because of motivational reasons, i.e. they want it more but don't necessarily like it more (Pecina et al., 2006).

This hypothesis has implications for the anhedonia shown in the C59X homozygous ENU-mutants, perhaps suggesting a reduction of opiod activity in the hedonic hot spot of the nucleus accumbens shell in the C59X mutants. To test if this is the case, the anhedonia in the C59X homozygous ENU-mutant mice might be reversed using micro-injection of μ-opioid agonists in the hot spot of the nucleus accumbens shell. Although the mouse brain is exceptionally small, and the likelihood of hitting the hedonic hot spot is fairly minimal. Dopamine antagonists have been shown to produce reduced lick cluster sizes in rats (D'Aquila, 2010) suggesting a role for dopamine in the 'liking' of rewards as well as the 'wanting', despite Berridge's argument to the contrary (Berridge, 2004). It would therefore be interesting to administer dopamine agonists to the C59X homozygous ENU-mutants to see if the anhedonia in these mutants can be reversed.

8.3.3 Increased motivation in C417Y homozygous ENU-mutants

The increased breakpoints demonstrated by the C417Y homozygous ENU-mutants can be interpreted as an increased motivation to work for a reward. This enhanced motivation, however, could also be seen as perseverative responding, manifest as an inability to inhibit the nose-poke response and so a reflection of a facet of impulsivity (O'Tuathaigh et al., 2010). However, against this idea, there were no differences in the intra-nose-poke latency during the PRT; but also, in Chapter VII, this same line did not show any effects of genotype in the stop-signal reaction time task, a specific test of impulsivity. The fact that the C417Y homozygous ENU-mutants were even willing to work harder for a reward that was devalued (less sweet), could indicate that these mice were actually more thirsty, as opposed to being more motivated to work specifically for the sweet condensed milk solution. However, the idea that the consistently increased breakpoints could have been due to the C417Y homozygous ENU-mutants simply being thirstier than their WT littermate controls is unlikely, as under free drinking conditions (i.e. no requirement to work for the reinforcer, See Chapter III, Section 3.3.4.3) consumption was equivalent between the ENU-mutants and WT littermate controls. Thus, increased motivation to work for a reward remains the most plausible explanation for the increase in breakpoints demonstrated by C417Y homozygous ENU-mutant mice, even when the reward is devalued. However, this could be further validated by investigations using different reward concentrations or different sizes of reward. The concentration used in this study (10%) was the optimum

concentration of condensed milk for mice (Humby et al., 2005), potentially causing the *Zfp804a* mice to operate at ceiling performance. Thus, future studies could start with lower baseline concentrations and smaller reward sizes to provide a more complete investigation of the effects of motivational levels on PRT performance.

Putting forward a neurobiological mechanism for the enhanced motivation in the C417Y homozygous ENU-mutants is difficult, particularly as schizophrenia and bipolar patients show reduced levels of motivation (Barch, 2008). The incentive salience hypothesis seems to suggest that dopamine may play a role in the 'wanting' of rewards, but not necessarily in the 'liking' (Berridge, 2004). The mesolimbic and striatal regions are seen as crucial areas for dopamine related reward reactivity, with many studies using dopamine receptor blockade to demonstrate reduced motivation in rodents (Berridge & Robinson, 1998). One recent study using mice over-expressing subcortical D_2 receptors showed that these mutants had lower incentive motivation on a progressive ratio schedule (Drew et al., 2007). It appears that increased striatal D_2 activity was most likely not sufficient by itself to reduce motivation as D_2 antagonists in schizophrenia patients are not enough to improve the motivational deficits seen (Ward, Simpson, Kandel et al., 2011), clearly additional neurobiological mechanisms are at work.

The fact that the C417Y homozygous ENU-mutants showed increased motivation but equivalent hedonic reaction to reward as their WT littermate controls also fits with a dopamine related hypothesis, as dopaminergic functioning is not thought to influence the hedonic reactions to reward (Berridge, 2004). Research using microdialysis and in vivo electrochemistry has shown that dopaminergic systems are activated before animals receive a pleasurable incentive, not during as might have been predicted by the anhedonia hypothesis (reduction of dopamine induces anhedonia) (Martel & Fantino, 1996). Dopamine transporter gene (DAT) knockdown mice show 70% elevated levels of dopamine and demonstrated higher levels of incentive motivation on a runway task, but did not show increased orofacial 'liking' reactions to sucrose in an affective taste reactivity test (Peciña, Cagniard, Berridge et al., 2003). It could be argued that altered dopaminergic activity in the C417Y homozygous ENU-mutants contributed to their enhanced motivation. If this is the case it would be interesting give the C417Y mutant mice amphetamine and then run them on the locomotor activity test, if the C417Y homozygous ENU-mutants are

hyperdopaminergic they should have an attenuated response to the amphetamine than their WT littermate controls.

8.3.4 Reduced acoustic startle response and prepulse inhibition in C59X homozygous ENU-mutants

The reduced prepulse inhibition demonstrated by the C59X homozygous ENU-mutants is in line with the reduced PPI found in schizophrenia and bipolar patients (Geyer et al., 2001; Braff et al., 2001; Giakoumaki et al., 2007; Perry et al., 2001). Work with other mouse models for schizophrenia have shown similar effects, particularly with DISC1 (Clapcote et al., 2007; Hikida et al., 2007) and *Nrg1* mutants (Stefansson et al., 2002). PPI is thought to be regulated by frontal, striatal and mesolimbic circuitries, and potentially the disrupted PPI shown by the C59X homozygous ENU-mutants indicates that *Zfp804a* may be involved in brain regions thought to play a role in sensorimotor gating (Powell et al., 2009). To test this hypothesis, and see if the reduced PPI found with the C59X mutants is a true result it is necessary to reverse the PPI deficit by administering antipsychotics to the C59X mutants.

Dopamine transporter gene (DAT) knockout mice demonstrate increased levels of dopamine and marked reductions in sensorimotor gating, with these effects reversed by administration of D_1/D_2 receptor antagonists (Ralph, Paulus, Fumagalli et al., 2001). Other neurotransmitter systems also produce deficits in PPI in rats, including 5-HT_{1A} receptor agonists (Rigdon & Weatherspoon, 1992), NMDA receptor antagonists (PCP) (Mansbach & Geyer, 1989) and GABA antagonists (Swerdlow, Braff & Geyer, 1990). Further investigation into the mechanisms behind the reduced PPI seen in the C59X mutants is required, perhaps using pharmacological manipulations to further elucidate the role of key neurotransmitters in the sensorimotor phenotype.

8.3.5 Enhanced response inhibition in C59X homozygous ENU-mutants

The enhanced response inhibition shown by the C59X homozygous ENU-mutants is difficult to reconcile in terms of schizophrenia and bipolar disorder as these illnesses are characterised by deficits in response control (Newman et al, 1985; Heerey et al., 2007; Swann, 2010). It is also difficult to interpret how neurobiological mechanisms in the C59X mutants may be contributing to the enhanced response inhibition seen, although research

has indicated that frontostriatal brain systems are involved in impulsivity and response inhibition (Rubia, 2001; 2002; 2003; Townsend et al., 2012) and that pharmacological manipulations via the dopaminergic, noradrenergic and serotonergic systems have impacts on SSRTT performance (Humby et al., 2013; Eagle & Robbins, 2003a,b; Eagle et al., 2007). Further work investigating other aspects of impulsivity would be interesting, as other facets (i.e. impulsive choice) are thought to have dissociable neurobiological systems (Evenden, 1999b; Winstanley et al., 2006); therefore additional tests could help to clarify the mechanism by which the C59X mutation is affecting response inhibition, and may yield possible effects in the C417Y mutant line.

8.4 Recent work

The work on *Zfp804a* extends beyond the behavioural analyses conducted in this thesis. Colleagues at the MRC centre of Neuropsychiatric Genetics and Genomics have been investigating the expression and splicing patterns of *Zfp804a* in the C59X mutant mouse line (Dr Knight) as well looking into the function of the human orthologue *ZNF804A* (Dr Chapman). I have also conducted further initial molecular work to ascertain the nature of *Zfp804a* expression in the C59X mutant line. The following section focuses on some of the interesting findings on *Zfp804a/ZNF804A*.

8.4.1 Zfp804a expression in WT C57BL/6J mice

Previous RT-PCRs performed by a colleague Dr Tinsley found that *Zfp804a* mRNA transcript was expressed in the brains of WT male mice, but not in other organs such as the heart, liver, lungs, spleen, kidney, muscles or testes (See Appendix 7.1A). This led to a further RT-PCR examining *Zfp804a* expression in differing brain regions of a male WT mouse with findings indicating that *Zfp804a* mRNA was present in each of the regions investigated (See Appendix 7.1C), with highest expression in the hippocampus (HPC), ventral striatum (VS) and prefrontal cortex (PFC), regions thought to be important neural correlates of schizophrenia and bipolar disorder (for reviews see Goghari, Sponheim & MacDonald, 2010; Strakowski, DelBollo & Adler, 2005).

8.4.2 Alternative transcripts in Zfp804a

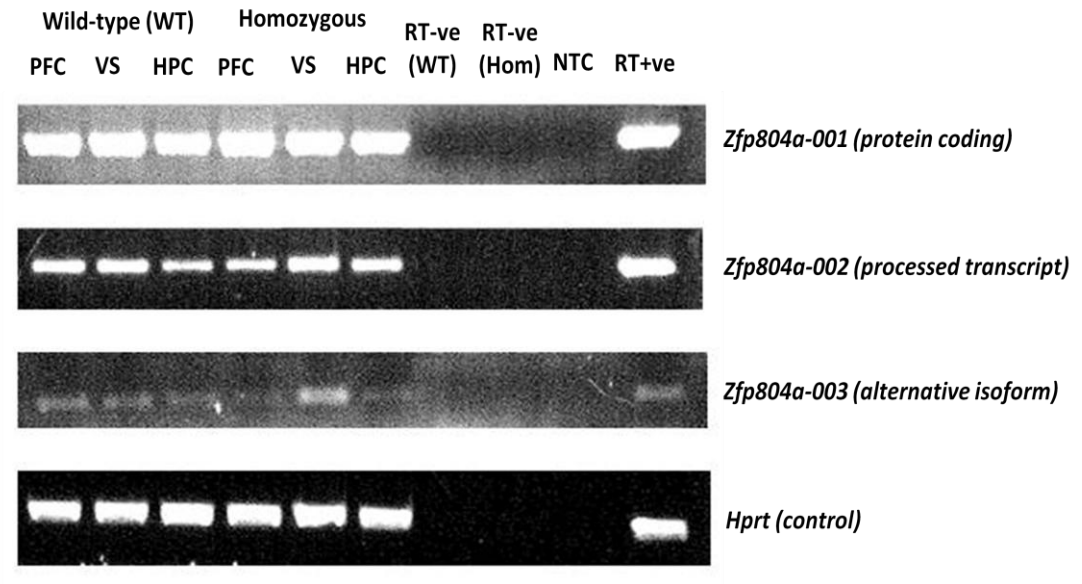
At the outset of this work the predicted effects of the two ENU mutations was based on the presence of a single protein coding *Zfp804a* transcript. However, recently an additional alternate full length non-protein coding transcript has been reported in Ensemble (http://genome-euro.ucsc.edu/cgi-bin/hgTracks). Very recently, a third alternate non-coding transcript has been discovered by colleagues in the lab. This third alternative isoform is thought to contain a novel exon upstream to the Reference sequence exon 1 appearing to skip the constitutive exon 1 and splice to exon 2 of *Zfp804a*. RT-PCR and sequencing validated this transcript in both C59X homozygous ENU-mutants and their WT counterparts and confirmed that the C59X mutation was also present in exon 2 of both of the C59X mutants used in the analysis (Knight, 2013). This discovery informed later RT-PCR work comparing *Zfp804a* expression in C59X homozygous ENU-mutants versus WT littermate controls.

8.4.3 Analysis of Zfp804a isoform gene expression in WT and C59X homozygous ENU-mutant mice

The C59X mutation encodes a premature stop codon and it was thought initially that the mutant mRNA transcript will not be fully translated due to nonsense mediated decay (NMD), which selectively degrades mRNAs harbouring premature stop codons (Chang et al., 2007). Previous work from RT-PCR studies of C59X homozygous and heterozygous ENU-mutants and WT control brain tissue revealed *Zfp804a* mRNA transcript in C59X homozygous ENU-mutants, suggesting that the C59X stop codon mutation has escaped NMD (C. Tinsley, personal communication, see Appendix 7.2).

The discovery of an alternative *Zfp804a* transcript (Knight, 2013) informed later expression analyses conducted in parallel to the work undertaken in this thesis. RT-PCR was performed on the C59X ENU-mutant line to assess the expression of *Zfp804a* in all three transcripts in brain regions shown to have high expression in WT C57BL/6J mice (HPC, VS and PFC). It was thought that this would confirm the *Zfp804a* expression in WT mice and assess the extent to which *Zfp804a* expression in all 3 (known) *Zfp804a* transcripts was affected by the C59X mutation. Within the limitations of RT-PCR (semi-quantitative) it appeared that all three transcripts were expressed in both the C59X homozygous ENU-mutant mouse and the WT control (Fig. 8.1).

Figure 8.1: RT-PCR showing expression of the three Zfp804a transcripts in different brain regions of a C59X homozygous ENU-mutant and a WT littermate control.



RT-PCR, using specific primers specific to each of the 3 *Zfp804a* transcripts and a *Hprt* control, was performed on samples dissected from different brain regions of a C59X male homozygous ENU-mutant and a WT littermate control. Results show that *Zfp804a* mRNA was present in each of the brain regions investigated, although the three transcripts were expressed at differing levels. This semi-quantitative assay did not suggest that expression levels of any of the *Zfp804a* transcripts differed between C59X genotype. Arrows show bands of interest. RT+ve: reverse transcriptase positive control and RT-ve: reverse transcriptase negative control, NTC: no template control, PFC: prefrontal cortex, HPC: hippocampus and VS: ventral striatum.

The three *Zfp804a* transcripts were expressed in all of the brain regions looked at (HPC, VS and PFC), although they were expressed at differing levels, with highest expression in the protein-coding transcript, lower expression in the processed transcript (identified on Ensemble) and even lower expression in the alternative transcript most recently identified (Knight, 2013). There did not appear to be any C59X genotype differences in expression of the three transcripts but a more in-depth analysis using quantitative PCR (qPCR) would be useful. These results further support the hypothesis that the C59X stop-codon mutation has escaped NMD, which is not an uncommon finding in these types of mutations (C. Tinsley, personal communication) and suggests that the C59X mutation should have a more damaging effect at a proteomic rather than transcriptional level.

There are currently no viable Zfp804a antibodies available to verify the presence/absence of the Zfp804a protein in tissue from C59X homozygous ENU-mutant mice; however, even if the mutation has escaped NMD, substituting the lost cysteine

residue with anything but another cysteine or histidine residue should result in loss of function (Wolfe, Nekludova & Pabo, 1999). Further confirmation of the effect of C59X mutation on the Zfp804a protein is required before a definitive conclusion about the precise molecular nature of the C59X mutation can be reached but irrespective of the molecular consequences of the C59X mutation the multiple behavioural phenotypes observed in the C59X homozygous ENU-mutants support the assumption that the C59X mutation is causing behavioural phenotypes of relevance to schizophrenia and bipolar disorder.

8.4.3 ZNF804A

Yeast two-hybrid screens have shown that *ZNF804A* interacts with known transcription and splicing factors, supporting the supposed role of *ZNF804A* in regulating transcription (Chapman, 2013). Exon arrays on the effect of *ZNF804A* knockdown on expression showed effects on genes thought to be involved in nervous system development, such as synaptic contact and axon guidance (Chapman, 2013). Other work on cell lines over-expressing *ZNF804A* has shown that the commercial antibodies currently available for ZNF804A either do not work (in cell lines overexpressing ZNF804A), or that the ZNF804A protein is only expressed transiently and may be rapidly degraded by the proteosome (Professor Derek Blake, personal communication). Their work supports the latter assumption as when the proteosome was inhibited; the ZNF804A protein was visible in cell lines overexpressing *ZNF804A* (Chapman, 2013).

8.4.4 Recent behavioural work

Recent work with the *Zfp804a* mutant lines has included marble burying for anxiety/compulsivity, biconditional learning and the tube test for social dominance. The C417Y homozygous ENU-mutants were no different to WTs in terms of the amount of marbles buried or movement around the arena. However, the C59X homozygous ENU-mutants buried and touched more marbles and moved more around the cage, as compared to their WT littermate controls. There are two interpretations for this behaviour, one being a sign of anxiety, and the other a compulsive phenotype (Thomas, Burant, Bui et al., 2009). It could be argued that this behaviour is more likely due to compulsivity in the C59X mutants rather than increased anxiety, as these mice were significantly less anxious than their WT littermate controls on assays of anxiety and also show reduced startle to a loud stimulus.

This compulsivity phenotype is interesting as obsessive compulsive disorder has been shown to be co-morbid with bipolar disorder (Cosoff & Hafner, 1998), and is seen as on a spectrum with impulsivity (Hollander & Wong, 1995), known to be a problem in schizophrenia (Newman et al., 1985; Heerey et al., 2007).

The behavioural work in this thesis mainly focuses on tasks thought to tax frontal regions of the brain, e.g. the SSRTT, and is relatively lacking on tasks which focus on hippocampal functioning (another brain region associated with schizophrenia and bipolar disorder). The biconditional learning paradigm is one such task, and is based on certain combinations of stimuli being rewarded and other combinations not. The discrimination cannot be solved by selecting individual elements, as these can be equally reinforced and not reinforced, and as such, only the combinations of stimuli are relevant to solving the discrimination, with this type of learning thought to be heavily reliant on hippocampal functioning (Sanderson, Pearce, Kyd et al., 2006). This task is being run in the *Zfp804a* mice with results expected soon, although initial observations suggest that the C417Y homozygous ENU-mutant mice are unable to learn the biconditional discriminations (J. Haddon, personal communication).

Reduced social functioning is often seen in schizophrenia patients (Couture, Penn & Roberts, 2006). Social cognition is a term used to describe how people think about themselves and the social world (Penn, Sanna & Roberts, 2008), and includes concepts such as theory of mind, emotional processing and social perception, with deficits in these areas associated with reduced social functioning in schizophrenia (Couture et al., 2006). The *Zfp804a* mutants were run on the tube test, a task designed to assess the social dominance of mice (Lindzey, Winston & Manosevitz, 1961). Here two mice are placed at opposite ends of a clear plastic tube and are forced into an interaction where one mouse (the dominant) forces the other mouse to back out of the tube (subordinate). After several 'battles', one can ascertain the social hierarchy within a cage. Initial results indicate that both the C59X and C417Y homozygous ENU-mutant mice are no different to their WT littermate controls in terms of social dominance.

8.5 Future directions

8.5.1 Molecular work

The future direction of the molecular work remains heavily reliant on the availability of viable *Zfp804a* and *ZNF804A* antibodies. Until then it would be sensible to repeat the RT-PCR done on the C59X line with more C59X homozygous ENU-mutants and WTs, and extending the work using quantitative PCR (qPCR) methods to be absolutely sure that there are no subtle effects of the C59X mutation on the expression of *Zfp804a* transcripts. It would also be interesting to look at expression in the C417Y line, as although the C417Y line was not predicted to have any gross effects on transcript expression it would make an ideal comparison to the C59X mutation. It would also be logical to follow up a developmental angle and analyse the foetal profile of *Zfp804a* transcript expression in the C59X mutant line (using RT-PCR and qPCR methods), not only because of the neurodevelopmental hypothesis of schizophrenia, but also as the *ZNF804A* risk allele (rs1355706) in human foetal brain tissue was associated with a relative decrease in expression of *ZNF804A* during the second trimester of development (Hill & Bray, 2012).

Once a *Zfp804a* antibody is available there are plans to culture primary cell lines of embryonic mouse tissue. Recent research suggests that *ZNF804A* may be degraded rapidly by the protesome (Chapman, 2013), and as such, *Zfp804a* may also be degraded rapidly, therefore the proteosome in *Zfp804a* would have to be inhibited to enable visualisation of *Zfp804a* in primary cell lines. Hippocampal and cortical neurons could be cultured from both C59X homozygous ENU-mutant mice and their WT littermates with the aim of uncovering whether the C59X stop codon mutation does or does not produce a protein. If a protein is produced, various *Zfp804a* antibodies could be used to characterise the size of the translated protein in the C59X homozygous ENU-mutants, as a truncated protein could also have a damaging effect on *Zfp804a* functioning.

8.5.2 Further behavioural work

Several suggestions on future behavioural experiments were put forward in Section 8.3 as potential ways of elucidating the neurobiological mechanisms behind the phenotypes uncovered with the *Zfp804a* mutants. As an extension of this and a concept not looked at in the mutants before, it would be interesting to manipulate the early life environment of the *Zfp804a* mutants, for example using maternal separation or isolation rearing as an

environmental stressor, to look at the interaction between genetic and environmental manipulations. Perhaps the lack of behavioural phenotypes found in the C417Y homozygous ENU-mutant mice are due to the need for an early life environmental factor to bring the genetic manipulation out. For example, whole C417Y litters could undergo isolation rearing and then be tested on acoustic startle and PPI, as well as an anxiety assay. If PPI deficits or anxiety phenotypes were then observed it would be the result of an interaction between the genetic and environmental manipulations. Similarly, the effects seen in the C59X mutants may be moderated, either exacerbated or attenuated, by environmental stimuli.

Circadian effects in the *Zfp804a* mutants have not been considered previously. Sleep disturbances (Chouinard, Poulin, Stip & Godbout, 2004) and circadian rhythm disruption (Wulff, Dijk, Middleton et al, 2012) are often observed in patients with schizophrenia. It is hypothesised that these sleep disturbances may contribute to the cognitive deficits shown (Phillips et al., 2012). Reduced anxiety and depressive-like behaviour have been shown in *Afh* (after hours) ENU-mutants who show a mutation in a circadian clock gene *Fbxl3*, causing them to have extended circadian rhythms of 27 hours (Keers, Pedroso, Breen et al., 2012). Perhaps the reduced anxiety shown in the C59X homozygous ENU-mutants is due to an altered circadian rhythm in these mice. It would therefore be interesting to observe the sleeping patterns of the *Zfp804a* mutants to ascertain whether the sleeping cycles of the C59X and C417Y homozygous ENU-mutants are any different to their WT littermate controls.

The most sophisticated way of observing the effect a gene may have on behavioural and molecular phenotypes is to knock it out, but also to be able to knock it back in. Termed a conditional knockout, this method would be an ideal way to study the effect of *Zfp804a* in an *in vivo* system, as the gene could be manipulated both spatially and temporally, i.e. in certain brain regions at certain developmental time points. This would most certainly further our understanding of the behavioural phenotypes observed with the *Zfp804a* mutants, and potentially help in understanding more about how variance in *Zfp804a* may increase the risk of developing schizophrenia/bipolar disorder.

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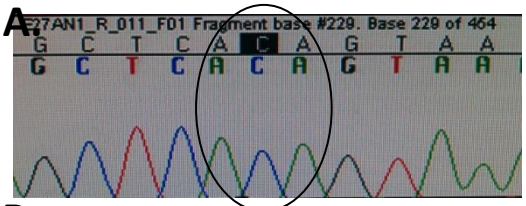
Appendices

Appendix 1: Chapter II

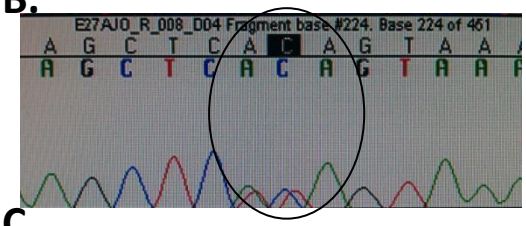
Appendix 1.1: Sequencing traces for all genotypes from the C59X and C417Y lines.

A.



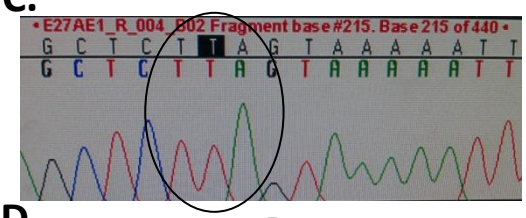
A reverse primer sequencing trace depicting a C59X WT mouse. ACA = WT.

B.



A reverse primer sequencing trace depicting a C59X heterozygous ENU-mutant mouse. The double peaks indicate a mouse heterozygous for the double C59X mutation.

C.

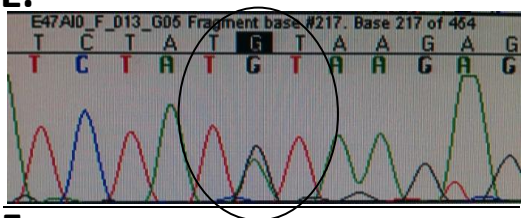


A reverse primer sequencing trace depicting a C59X homozygous ENU-mutant mouse. TTA = Homozygote.

D.

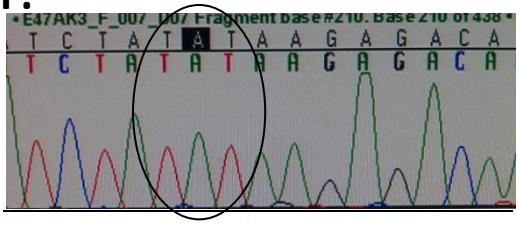
A forward primer sequencing trace depicting a C417Y WT mouse. TGT = WT.

E.



A forward primer sequencing trace depicting a C417Y heterozygous ENU-mutant mouse. The double peak indicates a mouse heterozygous for the C417Y mutation.

F.



A forward primer sequencing trace depicting a C417Y homozygous ENU-mutant mouse. TAT = Homozygote.

Appendix 2: Chapter III

Appendix 2.1: Scoring sheet for the SHIRPA phenotype assessment

A. **Visual Placing (Extension of the forelimbs)**
0 = None
1 = Upon nose contact
2 = Upon vibrasse contact
3 = Before vibrasse contact (18mm)
4 = Early vigorous extension (25mm)

B. **Grip Strength**
0 = None
1 = Slight grip, semi-effective
2 = Moderate grip, effective
3 = Active grip, effective
4 = Unusually effective

C. **Wire Manoeuvre**
0 = Active grip with hind legs
1 = Difficulty to grasp with hind legs
2 = Unable to grasp with hind legs
3 = Unable to lift hind legs, falls within seconds
4 = Falls immediately

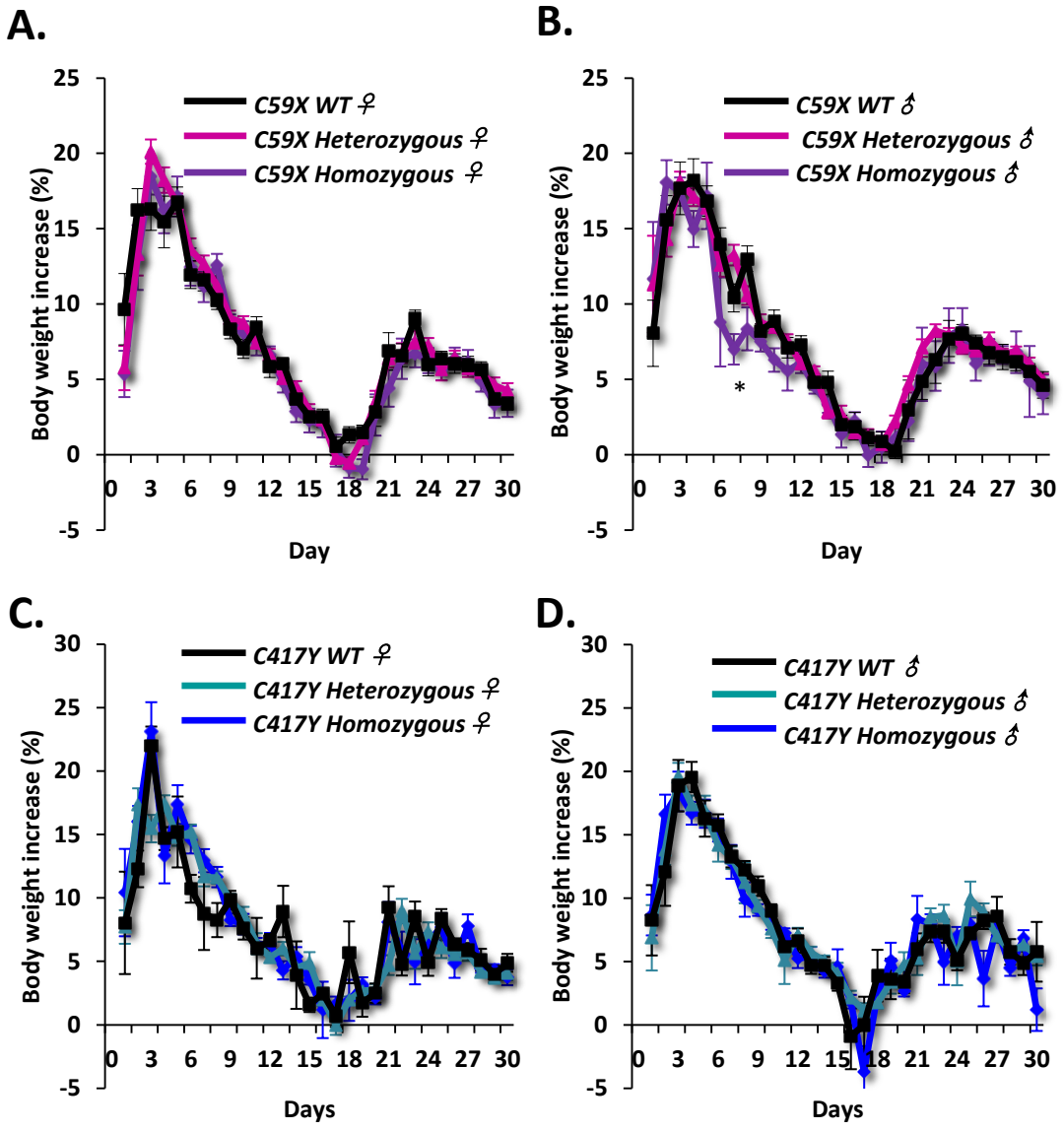
D. **Negative Geotaxis**
0 = Turns and climbs the grid
1 = Turns but then freezes
2 = Moves, but fails to turn
3 = Does not move within 30 seconds
4 = Falls off

Appendix 2.2: The post hoc results from a repeated measures analysis of early postnatal body weight (Days 21-30) for the males of the C59X line.

Postnatal day 21	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.05) and heterozygous ENU-mutant (*p*=0.001) littermates.
Postnatal day 24	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*= 0.05) and heterozygous ENU-mutant (*p*= 0.001) littermates.
Postnatal day 25	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.04) and heterozygous ENU-mutant (*p*=0.001) littermates.
Postnatal day 26	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.03) and heterozygous ENU-mutant (*p*=0.00) littermates.
Postnatal day 27	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.03) and heterozygous ENU-mutant (*p*=0.00) littermates.
Postnatal day 28	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.05) and heterozygous ENU-mutant (*p*=0.00) littermates.
Postnatal day 29	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.04) and heterozygous ENU-mutant (*p*=0.00) littermates.
Postnatal day 30	C59X male homozygous ENU-mutant mice weighed significantly less than their WT (*p*=0.03) and heterozygous ENU-mutant (*p*=0.00) littermates.

Appendix 2.3: The ANOVA result for overall genotype effects in the emergence of somatic indices.	
C59X females	$F_{8,112}$=1.25, p=0.28
C59X males	$F_{8,96}$=1.01, p=0.44
C417Y females	$F_{8,102}$=1.05, p=0.40
C417Y males	$F_{8,68}$=0.77, p=0.63

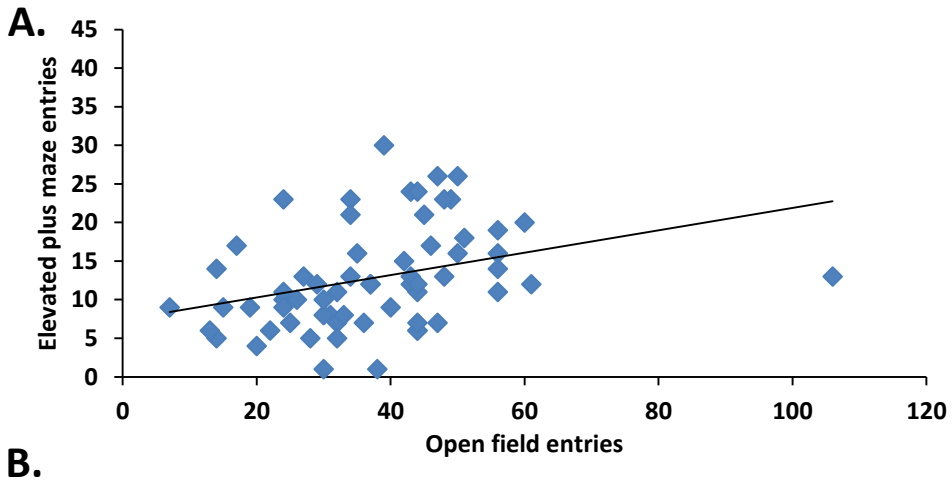
Appendix 2.4: Percentage of body weight increase over the first 30 days of postnatal life for both the C59X and C417Y line, cohort $G7_{ii}$.

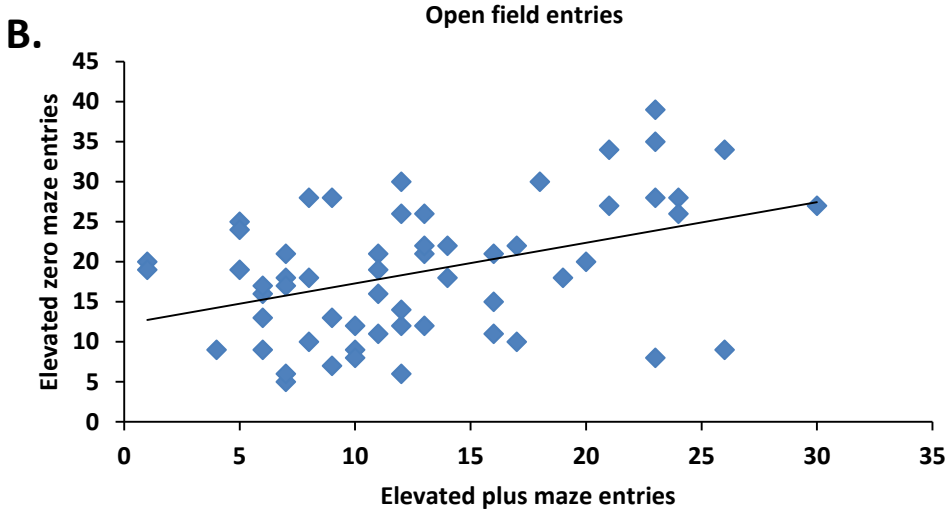


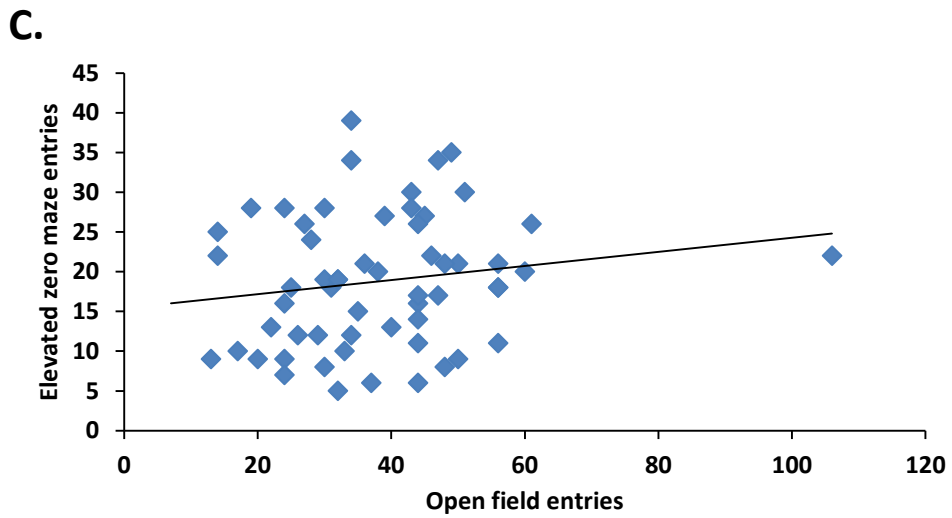
There were no significant differences in percentage of body weight change over the first 30 days of postnatal life for the C59X females (A), the C59X males (B), the C417Y females (C) or the C417Y males (D). However, when only days 5-10 were looked at, the C59X homozygous ENU-mutant males showed significantly reduced % growth than their male WT littermate controls ($F_{2,50}$=3.5, p<0.05). Data shows the mean values ±SEM, *p<0.05 for pairwise differences related to genotype. For subject N see Table 3.1.

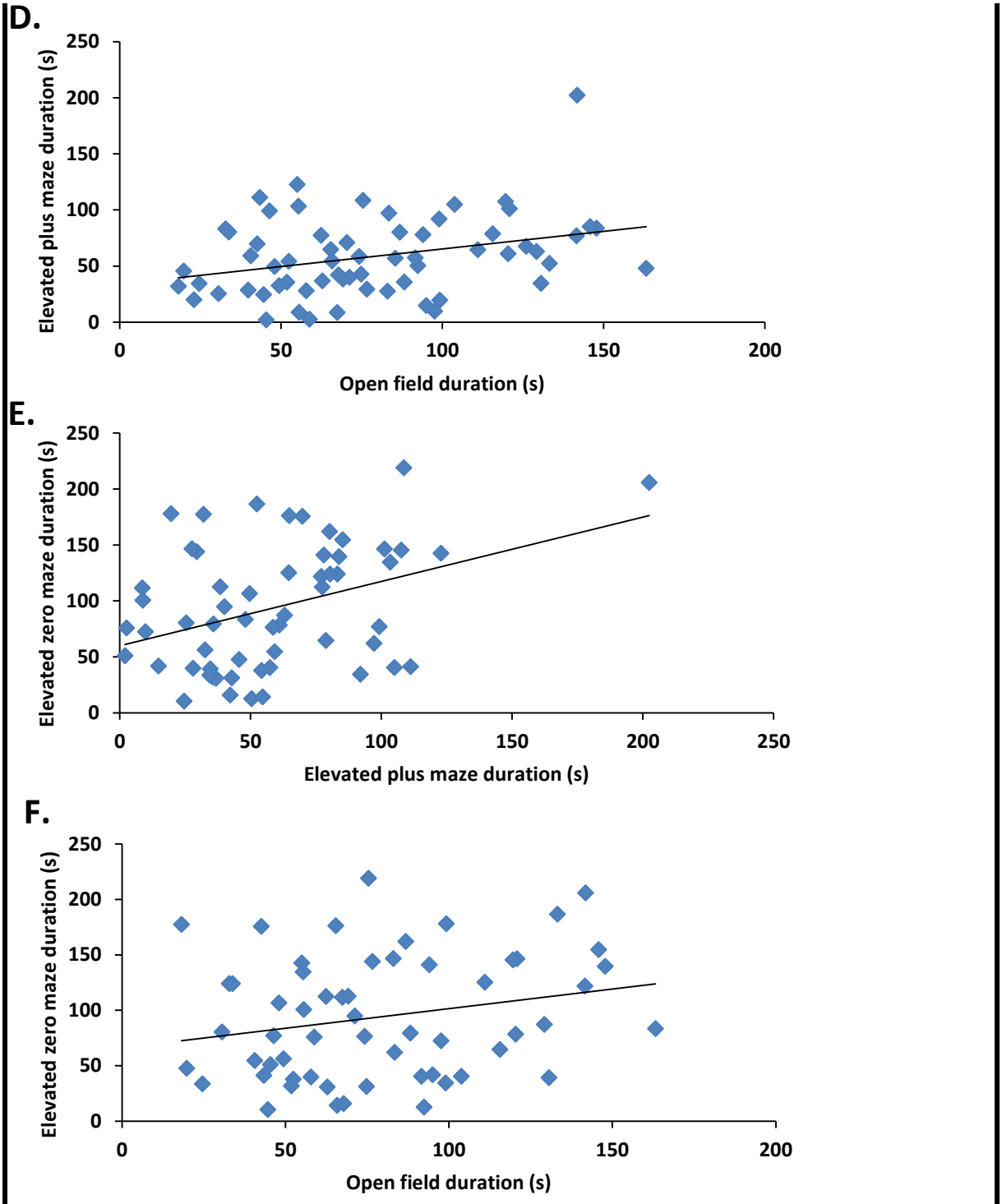
Appendix 3: Chapter IV

Appendix 3.1: Correlational analyses between behaviour on the EPM, OF and EZM.









Scatter plots showing the strong positive correlation between entries into the open zone of the EPM and entries into the central zone of the OF (A), and between entries into the open zone of the EZM and EPM (B). There was no correlation between entries into the open zone of the EZM and entries into the central zone of the OF (C). Scatter plots also show the moderately strong positive correlation between the duration of time spent in the open zone of the EPM and time spent in the central zone of the OF (D), and between time spent in the open zone of the EPM and EZM (E). There was no correlation between time spent in the open zone of the EZM and time spent in the central zone of the OF (F).

Appendix 4: Chapter V

Appendix 4.1: The results from a repeated measures ANOVA to test for differences between the 3 PR sessions in the progressive ratio task for the C59X line.			
Behavioural Parameter	**WT**	**Homozygous**	**Repeated measures ANOVA**
Rewards	20.4 ± 2.3	18.9 ± 2.2	$F_{(1, 25)} = 0.2$, $p=0.64$
Breakpoint	20 ± 2.3	18.6 ± 2.2	$F_{(1, 25)} = 0.2$, $p=0.69$

Data shows mean ± SEM

Appendix 4.2: The results from a repeated measures ANOVA to test for differences between the 3 PR sessions in the progressive ratio task for the C417Y line.			
Behavioural Parameter	**WT**	**Homozygous**	**Repeated measures ANOVA**
Rewards	19.4 ± 3.1	26.5 ± 2.7	$F_{(1, 25)} = 3.0$, $p=0.1$
Breakpoint	18.9 ± 3.0	26.1 ± 2.7	$F_{(1, 25)} = 3.1$, $p=0.1$

Data shows mean ± SEM

Appendix 4.3: The results from a repeated measures ANOVA to test for differences between the 3 PR sessions in the progressive ratio task.		
Parameters over the 3 sessions	**Repeated measures ANOVA**	
	C59X line	**C417Y line**
Rewards earned	$F_{(2, 50)} = 7.18$, $p=0.002$, *post hocs* showed significantly greater rewards earned during PR1 than PR2 ($p=0.01$) and PR3 ($p=0.05$)	$F_{(1.629, 40.715)} = 0.25$, $p=0.7$
Breakpoint	$F_{(2, 50)} = 6.33$, $p=0.004$, *post hocs* showed PR1 significantly higher breakpoint than PR2 ($p=0.02$)	$F_{(2, 50)} = 0.15$, $p=0.9$

Appendix 4.4: The results from a repeated measures ANOVA for the CRF, PR and CRF2 sessions in the progressive ratio task for the C59X line.			
Behavioural Parameter	**WT**	**Homozygous**	**Repeated measures ANOVA**
Total Nose Pokes	146.5 ± 20.1	138.8 ± 19.4	$F_{(1, 25)} = 0.08$, $p=0.78$
Total Panel Pushes	106.4 ± 19.8	117.8 ± 19.1	$F_{(1, 25)} = 0.17$, $p=0.68$
Total Beam Breaks	536.8 ± 108.4	758.7 ± 104.5	$F_{(1, 25)} = 2.17$, $p=0.15$
Session Duration	23.6 ± 1.1	22.4 ± 1.1	$F_{(1, 25)} = 0.57$, $p=0.50$
Volume Consumed	1.3 ± 0.09	1.5 ± 0.08	$F_{(1, 25)} = 3.94$, $p=0.06$

Data shows mean ± SEM

Appendix 4.5: The results from a repeated measures ANOVA for the CRF, PR and CRF2 sessions in the progressive ratio task for the C417Y line.			
Behavioural Parameter	**WT**	**Homozygous**	**Repeated measures ANOVA**
Total Nose Pokes	140.7 ± 29.3	213.1 ± 26.2	$F_{(1, 25)} = 3.40$, $p=0.08$
Total Panel Pushes	82.7± 9.5	106.3 ± 8.4	$F_{(1, 25)} = 3.45$, $p=0.07$
Total Beam Breaks	665 ± 101	613.4 ± 90.3	$F_{(1, 25)} = 0.15$, $p=0.71$
Session Duration	22.2 ± 1.1	22.3 ± 0.98	$F_{(1, 25)} = 0.01$, $p=0.94$
Volume Consumed	1.4 ± 0.09	1.5 ± 0.08	$F_{(1, 25)} = 0.91$, $p=0.35$

Data shows mean ± SEM

Appendix 4.6: The results from a repeated measures ANOVA to test for differences between the CRF, PR and CRF2 sessions in the progressive ratio task.

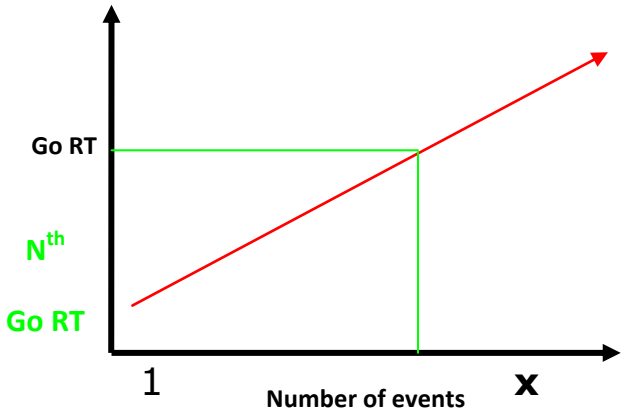
Parameters over the 3 sessions	**Repeated measures ANOVA**	
	C59X line	**C417Y line**
Total Nose Pokes	$F_{(1.017, 25.416)}$ =13.25, *p*=0.001, *post hocs* showed significantly greater nose pokes during PR than CRF (*p*=0.003) and CRF2 (*p*=0.004)	$F_{(1.008, 25.188)}$ =20.02, *p*=0.00, *post hocs* showed significantly greater nose pokes during PR than CRF (*p*=0.00) and CRF2 (*p*=0.00)
Total Panel Pushes	$F_{(1.263, 31.57)}$ =2.5, *p*=0.12	$F_{(1.349, 33.736)}$ =41.8, *p*=0.00, *post hocs* showed significantly less panel pushes at PR compared to CRF (*p*=0.00) and CRF2 (*p*=0.00)
Total Beam Breaks	$F_{(2, 50)}$ =0.42, *p*=0.66	$F_{(1.505, 37.636)}$ =1.08, *p*=0.33
Session Duration	$F_{(1.32, 33.005)}$ =0.89, *p*=0.38	$F_{(1.388, 34.697)}$ =2.93, *p*=0.08
Volume Consumed	$F_{(2, 50)}$ =382.51, *p*=0.00, *post hocs* showed significantly less volume consumed at PR than at CRF (*p*=0.00) and CRF2 (*p*=0.00)	$F_{(2, 50)}$ =387.75, *p*=0.00, *post hocs* showed significantly less volume consumed at PR than at CRF (*p*=0.00) and CRF2 (*p*=0.00)

Appendix 5: <u>Chapter VI</u>

Appendix 5.1: Table to show the trial presentation in the acoustic startle/PPI			
Trial Number	**Trial Type**	**Trial Number**	**Trial Type**
1	120 dB pulse	29	8 dB prepulse
2	120 dB pulse	30	120 dB pulse
3	120 dB pulse	31	8 dB prepulse
4	120 dB pulse	32	4 dB prepulse
5	120 dB pulse	33	16 dB prepulse
6	120 dB pulse	34	120 dB pulse
7	4 dB prepulse	35	No stimulus
8	8 dB prepulse	36	No stimulus
9	16 dB prepulse	37	No stimulus
10	120 dB pulse	38	No stimulus
11	8 dB prepulse	39	80 dB pulse
12	No stimulus	40	90 dB pulse
13	4 dB prepulse	41	100 dB pulse
14	120 dB pulse	42	110 dB pulse
15	16 dB prepulse	43	120 dB pulse
16	8 dB prepulse	44	120 dB pulse
17	4 dB prepulse	45	110 dB pulse
18	120 dB pulse	46	100 dB pulse
19	No stimulus	47	90 dB pulse
20	8 dB prepulse	48	80 dB pulse
21	16 dB prepulse	49	100 dB pulse
22	120 dB pulse	50	120 dB pulse
23	4 dB prepulse	51	90 dB pulse
24	16 dB prepulse	52	80 dB pulse
25	No stimulus	53	110 dB pulse
26	120 dB pulse	54	No stimulus
27	16 dB prepulse	55	No stimulus
28	4 dB prepulse		

Appendix 6: Chapter VII

Appendix 6.1: Calculation of the SSRT.



Ranked Go Reaction Times

1-750
2-760
3-770
4-780
5-790
6-800
7-810
8-820
9-830

To find SSRT, need to find Nth Go Reaction Time when ranked in order from smallest (see graph). The following formula illustrates the calculation:

Nth=(X*Pf)

X=number of correct go trials
Pf=probability of failing to stop on stop trials

SSRT=(Nth GoRT – SSD)

Example

In a session with 20 trials

- 16 go trials (from which GoRT can be measured)

4 stop trials (1 correct stop and 3 incorrect stop) => Pf=0.75

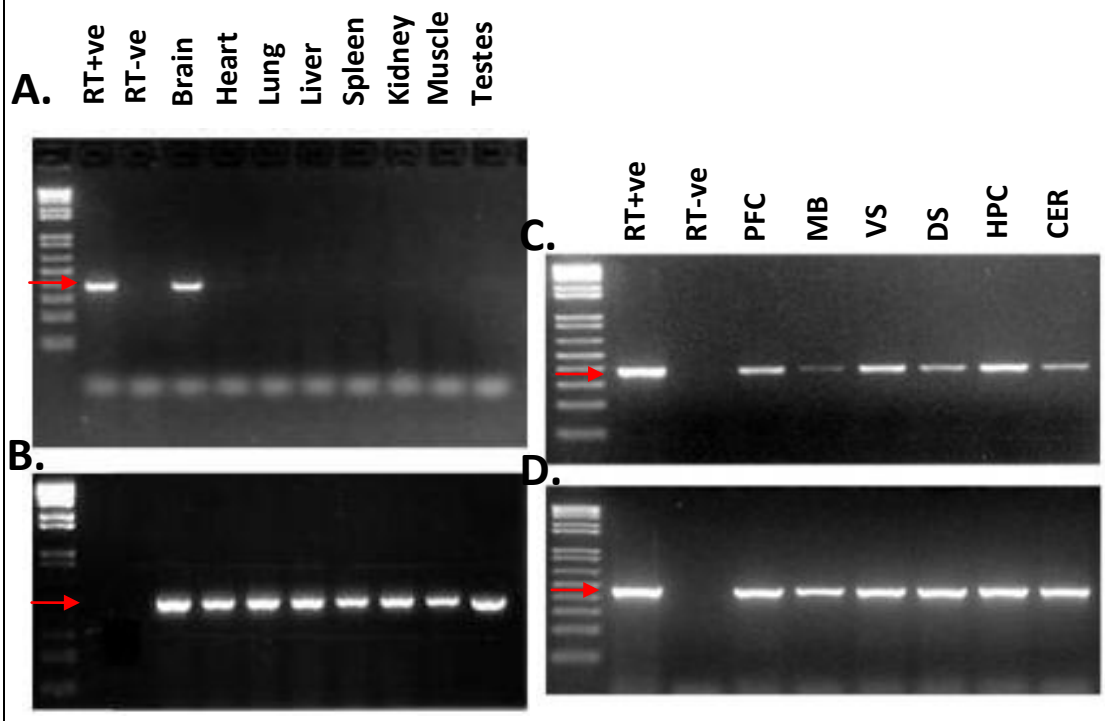
- probability of correctly stopping = 0.25
- SSD = 550 ms

To find the nth reaction time:

- n = number of Go Reaction Time's * probability of responding on stop trials
- n = (16*0.75) = 12
- 12th reaction time in Go Reaction Time distribution = 860 ms
- Therefore, it is estimated that the stop process finished 860 ms after the onset of the go stimulus
- If we subtract the delay to the stop-signal from this value (860 - 550 = 310), we get an estimate of SSRT of 310 ms

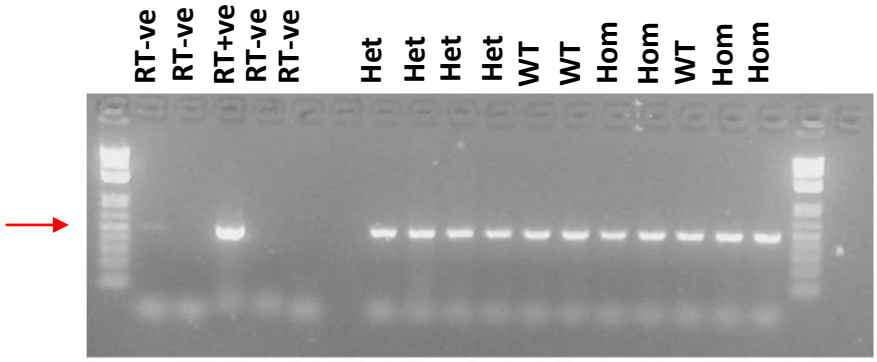
Appendix 7: Chapter VIII

Appendix 7.1: RT-PCRs showing Zfp804a transcript in different organs and discrete brain regions of C57BL/6J WT mice.



RT-PCR using non-specific *Zfp804a* primers was performed on samples dissected from different organs of a male WT C57BL/6J mouse (A). Results show that *Zfp804a* expression was limited to the brain only. A separate RT-PCR for calmodulin mRNA was used for reference (B). A further RT-PCR was then performed with non-specific *Zfp804a* primers on samples dissected from different regions of the brain of a male WT C57BL/6J mouse (C). Results show that *Zfp804a* mRNA was present in each of the regions investigated, and that there appeared to be varying levels of relative expression. From this semi-quantitative study, highest *Zfp804a* expression occurred in the hippocampus (HPC), ventral striatum (VS) and prefrontal cortex (PFC), with lower levels in the dorsal striatum (DS) and cerebellum (CER). *Zfp804a* mRNA was also detected in the hind/mid-brain (MB) but at much lower levels of expression than the other brain regions. A separate RT-PCR for calmodulin mRNA was again used for reference (D). Arrows show bands of interest. RT+ve: reverse transcriptase positive control and RT-ve: reverse transcriptase negative control. Figure reproduced with kind permission of Tinsley, Humby and Wilkinson.

Appendix 7.2: RT-PCR showing the presence of Zfp804a transcript in whole brain tissue of C59X mice.



RT-PCR with non-specific primers for *Zfp804a* was performed on whole brain samples from male C59X homozygous, heterozygous and WT mice (A). Results from this semi-quantitative assay show that, overall, *Zfp804a* expression was relatively equivalent in each genotype. Arrow shows band of interest, RT-ve: reverse transcriptase negative control and RT+ve: reverse transcriptase positive control. Image reproduced with kind permission from Professor Blake & Dr Tinsley.