Making a Mouse Model for Schizophrenia:

Using the mouse to model the schizophrenia susceptibility gene *ZNF804A*

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at Cardiff University

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Summary

Schizophrenia is a complex disorder, with several genes putatively associated with the pathogenesis of the disorder. A large genome-wide association study (O'Donovan et al. 2008) identified *ZNF804A* as a candidate gene for schizophrenia (meta-analysis $p = 1.61 \times 10^{-7}$). The association of the gene with schizophrenia (and bipolar disorder) has since been successfully replicated several times, confirming the association (Riley *et al.,* 2010; Steinberg *et al.,* 2010; Zhang *et al.*, 2010, Williams *et al.*, 2011).

The aim of this thesis is to create and provide preliminary assessments of a mouse model of the murine form of *ZNF804A*, *Zfp804a*. A mutagenised DNA archive derived from mice treated with N-ethyl-N-nitrosourea (ENU) held at the MRC Mammalian Genetics Unit, Harwell, was screened for mutations in *Zfp804a*. Two mutations (C59X and C417Y) were selected for re-derivation based upon the estimated impact upon the protein. The mutations were backcrossed onto a C57Bl/6J background for three successive generations using a panel of genetic markers to aid selection for the highest level of C57Bl/6J congenicity (and therefore speed up the backcrossing process). G4 mice were tested in the study.

Preliminary assessments of the fourth generation intercross cohort revealed, most notably, that the mice breed well, have no gross physical deficits and that male *Zfp804a*$^{C59X/C59X}$ mutants appeared less anxious than other groups in the elevated plus maze and performed better than other groups on the RotaRod.

Initial indications show that *Zfp804a* may indeed influence behaviour and cognition however further work is necessary to expand upon these findings with larger samples.

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List of abbreviations

A	Adenosine
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
bb	Beam breaks
C	Cytosine
CNTRICS	Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia
CNV	Copy number variant
COMT	Catechol-*O*-methyltransferase
CSF2RA	Colony stimulating factor 2 receptor, alpha
C_2H_2	2 cysteine, 2 histidine
DAOA	D-amino acid oxidase activator
df	degrees of freedom
DIG	Digoxigenin
DHPLC	Denaturing high performance liquid chromatography
DISC1	Disrupted in schizophrenia 1
DLPFC	Dorso-lateral prefrontal cortex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRD2	Dopamine receptor D_2
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTNBP1	Dystrobrevin-binding protein 1, or dysbindin
DZ	Dizygotic
EDTA	Ethylenediaminetetraacetic acid
EJC	Exon junction complex
ENU	*N*-ethyl-*N*-nitrosourea
EPM	Elevated plus maze

G	Guanine
GABA	γ-Aminobutyric acid
GPATCH8	G patch domain-containing protein 8
GWAS	Genome-wide association study
HF	Hippocampal formation
HRMA	High resolution melting analysis
HSD	Honestly significant difference
ICD	International Statistical Classification of Diseases and Related Health Problems
ID	Identification
IL3RA	Interleukin 3 receptor, alpha
IQ	Intelligence quotient
IVF	*In vitro* fertilisation
LD	Linkage disequilibrium, or in the context of ’Mouse LD Linkage Panel’, low density
LI	Latent inhibition
LMA	Locomotor activity
LOC561947	Zinc finger protein 804A
LOC707018	Zinc finger protein 804A
LSD	Lysergic acid diethylamide
M	Mean average
MANOVA	Multivariate analysis of variance
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MYT1l	Myelin transcription factor 1-like
MZ	Monozygotic
NEP	Neuroepithelium
NMD	Nonsense-mediated decay
NMDA	*N*-Methyl-D-aspartic acid

OCT-6	organic cation/carnitine transporter 6
OF	Open field
PCA	Principal components analysis
PCP	Phencyclidine
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PIL	Personal licence
POU3F1	POU class 3 homeobox 1
PPI	Pre-pulse inhibition
PPL	Project licence
PTC	Premature stop codon
RNA	Ribonucleic acid
RPM	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SED	Standard error of the difference
SEM	Standard error of the mean
SHOX	Short stature homeobox
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
STG	Superior temporal gyrus
SVZ	Sub-ventricular zone
T	Thymine
TE	Tris-EDTA
TGCE	Temperature gradient capillary electrophoresis
TM	Transmembrane
UV	Ultra-violet
VCFS	Velocardial facial syndrome
WM	Working memory

WT	Wild-type
Zfp804a	Zinc finger protein 804a (murine)
ZNF804a	Zinc finger protein 804a (human)
ZNF804b	Zinc finger protein 804b
5-HT	5-hydroxytryptamine, or serotonin

CHAPTER 1: General Introduction

Schizophrenia is a severe and disabling psychiatric disorder that is amongst the top ten leading causes of disability worldwide (Murray & Lopez, 1996; World Health Organisation, 2001). The disorder and the deficits associated with it are frequently life-long in course and current treatments are only, at best, moderately effective.

The problem of schizophrenia in society is not restricted to the disruption in everyday life the symptoms cause the sufferer; problems associated with the disorder are substantial and far-reaching. Schizophrenia, according to Saha *et al.* (2007), reduces lifespan by about 12 to 15 years on average. This is mainly due to suicide but other causes include increased risk of murder (Hiroeh *et al*. 2001) and increased risk of death due to natural causes (Hewer *et al*., 1995; Hewer and Rössler, 1997). There is also a substantial burden on patients, families and communities due to schizophrenia. Patients suffer not only from the vastly debilitating nature of the disorder but also from stigma and discrimination in society associated with mental health problems. The burden on families and close friends is also substantial, encompassing practical difficulties such as disruption of routine, reduced leisure time and restricted social activities, emotional and stress-related reactions, economic considerations and dealing with prejudice associated with mental illness.

Costs associated with schizophrenia are also high with Knapp (1997) estimating that 1.5% of national health expenditure is spent on the disorder, with a high rate of spending being due to the nature of the disorder (e.g. chronic course and frequent readmissions to hospital). Additional costs to the economy

are associated with the economic burden on relatives and caregivers, early retirement and reduced ability to work of the patient. Schizophrenia is a relatively common problem with a high financial cost to society and devastating effects on the lives of the sufferer and those close to them. For such a problematic and prominent disorder, we know very little about the aetiology and pathogenesis of the disorder.

1.1 Schizophrenia epidemiology

Schizophrenia affects approximately 1% of the world population in a lifespan with an incidence of roughly 15 per 100 000 per year (e.g. Tandon *et al.,* 2008). The disorder affects both males and females although males are more frequently affected with McGrath *et al*. (2004) reporting a male to female prevalence rate of 1.40. Schizophrenia is a problem that affects all nationalities and races fairly equally although there are findings indicating that certain groups in the UK are more severely affected than others such as migrants (and their second generation offspring) from Africa and the Caribbean being up to 10 times more likely to develop schizophrenia than in the populations in the countries of origin and in the UK (Eaton & Harrison, 2000). Other factors are also involved in the risk for developing schizophrenia including season of birth, birth complications, immune system and autoimmunity, cannabis use, urbanicity and genetics (discussed further in 1.5 Genetics of schizophrenia).

1.2 What is schizophrenia?

Schizophrenia is a severe mental health disorder that has been recognised since Emil Kraepelin (1896 -1987) described dementia praecox in the fifth

edition of his textbook. Prior to this early work, no clear, consensual demarcation was made between dementia praecox and affective psychosis. Kraepelin's work focused around clustering symptoms commonly co-occurring into a disease classification. He introduced a simple distinction between conditions characterised by mental deterioration and the affective disorders which were more episodic. The mental deterioration (named dementia praecox) was characterised as a psychological rather than physical impairment and included symptoms recognised in schizophrenia today such as auditory hallucinations, delusions, thought disorder and blunted affect (Wing & Agrawal, 2003). The term 'schizophrenia' derives from Eugen Bleuler (1911 -1950). He maintained Kraepelin's ideas of distinguishing schizophrenia from manic-depressive psychosis but pointed out that affective symptoms could co-exist and substantially widened the boundaries of the classification of the disease (Wing & Agrawal, 2003).

In modern psychiatry, schizophrenia is diagnosed solely upon the presentation and reporting of symptoms and a diagnosis is made upon the reported intensity and duration of symptoms (Wing & Agrawal, 2003). In the UK, the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, version DSM-IV-TR is used to provide the criteria of a diagnosis of schizophrenia. Two or more 'characteristic symptoms' must be present, including positive symptoms such as delusions, hallucinations and disorganised speech and behaviour, and negative symptoms such as a flattening of affect, an inability to experience pleasure in daily life and a lack of motivation for a diagnosis of schizophrenia. These symptoms must, in addition, cause a significant impairment in daily functioning socially and/or at work and

there must have been at least a six-month period where behaviour has been disturbed, including a minimum of one month (if untreated) of characteristic symptoms being present.

Schizophrenia cannot be diagnosed if there are apparent symptoms of a mood disorder or pervasive developmental disorder, or the symptoms are the direct result of another medical condition or a substance, such as abuse of a drug or medication. Symptoms of schizophrenia are generally grouped under three subheadings: positive, negative and cognitive symptoms. ‘Positive symptoms’ are features of the disorder that are not present in healthy individuals including hallucinations in any modality, delusional thinking, disorganised speech and behaviour and catatonia. ‘Negative symptoms’ refers to features normally present in healthy people that are missing in people with schizophrenia such as a flattening of affect, avolition and anhedonia. Cognitive symptoms describe the difficulties in processing many people with schizophrenia experience in attention, memory and concentration (Cutting, 2003).

Schizophrenia is clinically understood as a discrete disorder and a distinct diagnosis from schizoaffective disorder and bipolar disorder. The diagnosis given to the presenting patient depends upon nature of the psychosis and mood disturbances present. Essentially schizophrenia is diagnosed where psychosis is present without a prominent depressive, manic or mixed episode. Schizoaffective disorder may be diagnosed with both, one or more prominent mood episode/s and psychosis. Finally, bipolar disorder is diagnosed where mood disturbances are the prominent feature. Psychosis may or may not be

present but in a diagnosis of bipolar disorder, will never meet the DSM IV criterion A requirement for schizophrenia (Cutting, 2003).

The nosology of schizophrenia has recently been called into question in the light of converging genetic evidence that there is some shared susceptibility on the psychosis-mood disorder diagnostic spectrum. Schizophrenia is a syndromic diagnosis. That is, it is diagnosed when a person presents with a collection of symptoms characteristic of schizophrenia. Many, if not all, of these symptoms could be present in other psychiatric disorders, albeit in differing proportions. Owen *et al.*(2007) highlight the need to re-consider traditional diagnostic boundaries and argue persuasively that genetic evidence doesn't comply with current classifications.

Although traditional family studies provide some support to the schizophrenia –bipolar divide (e.g.Gottesman, 1991; Tsuang *et al.* 1990), family studies looking at schizoaffective disorder have found similar rates of the disorder in both families with predominantly bipolar illness (Rice *et al*., 1987) and families with schizophrenia (Kendler *et al*. 1998). Cardno *et al*. (2002) found that in twin studies, results are skewed as schizophrenia-like symptoms tend to "trump" those of mood disorder (forming a diagnostic hierarchy for each individual lifetime diagnosis). When they defined syndromes non-hierarchically, they unveiled a clear overlap between syndromically defined mania and schizophrenia. Moreover, employing linkage analysis Hamshere and colleagues (2005) showed that schizophrenia and bipolar families contributed equally to genome-wide significant linkage at 1q42 and suggestive linkage at 22q11; *DISC1* and *COMT* map to these regions respectively.

Individual gene studies have also been informative in the area of nosology (see Owen *et al*., 2007) and although some genes do provide evidence for specific susceptibility to prototypical schizophrenia (e.g. *DTNBP1*) or prototypical mood disorder (e.g. *DAOA*) there is again clear overlap in genetic susceptibility across the traditional Kraeplinian divide. For example, the recent association of schizophrenia with *ZNF804A*, a strong statistical candidate gene, is strengthened to genome-wide significance when bipolar cases are added to the sample (O'Donovan *et al*., 2008).

Currently, the issue of biological overlap of diagnostic boundaries in brain diseases is a hot topic which may go beyond schizophrenia and bipolar disorder to include other psychopathology, and even extend to certain neurological conditions. Craddock *et al.* (2009) review the evidence from genetic epidemiological, common single-nucleotide polymorphism (SNP) studies and genomic structural variation studies and conclude that there is substantial overlap in possible aetiology between the traditional boundaries for schizophrenia, bipolar disorder, unipolar depression, autism and epilepsy. Whilst far from settled, it is clear that the increasing knowledge about the biological aspects of psychiatric disorder highlights the need for more complex diagnostic models.

Schizophrenia has no known biomarkers meaning it cannot be diagnosed by laboratory tests. This lack of biomarkers for the disorder is indicative of our lack of knowledge about the underlying biological pathways that lead to its clinical presentation. There are numerous theories of the biological pathways impacting on the symptoms of schizophrenia such as the dopamine and glutamate hypotheses. However, none as yet have been shown

to be definitive for schizophrenia and so cannot be exploited for diagnostic purposes. Schizophrenia is currently diagnosed by psychiatrists on the basis of self-report of symptoms, behaviours observed, and reports of those close to the patient.

1.3 Clinical presentation of schizophrenia

The criteria for a diagnosis of schizophrenia encompass the three core symptom-groups of schizophrenia: positive, negative and cognitive symptoms.

1.3.1 Positive symptoms in schizophrenia

Schizophrenia is a heterogeneous disorder characterised by an array of possible symptoms .There is no single prescriptive symptom that every person diagnosed with schizophrenia must exhibit. Between people with schizophrenia therefore, there can be varying degrees of symptom overlap (Cutting 2003). Auditory hallucinations (usually voices), for example, are often thought of as characteristic of schizophrenia yet only 50% of people with schizophrenia studied experienced these (Cutting 1990). Other hallucinations were less common (visual, 15% and tactile, 5%). Delusions are more common; 90% of those with schizophrenia have had delusions at some point (Cutting, 2003). Thought disorder is another major category of symptoms in schizophrenia and refers to aberrant form and content of thoughts. Thought disorder includes some positive (such as inappropriate affect) and some negative (such as derailment and tangentiality) symptoms. The positive symptoms of schizophrenia are generally measured by self-report or tests that require language and/or writing skills (Cutting, 2003). It is therefore the general opinion

in the scientific community that these symptoms, even if they were to exist in non-human animals, cannot be easily measured and are unsuitable for examination in a mouse model for schizophrenia (Desbonnet, *et al*., 2009).

1.3.2 Affect in schizophrenia

Although mood disturbances cannot be the dominant feature of schizophrenia (or the diagnosis would not be one of schizophrenia, see earlier), affective abnormalities can be both predictive of (e.g. Johnstone *et al.*, 2005; Cunningham Owens *et al.*, 2005), and co-morbid with schizophrenia (discussed in Cutting, 2003, Buchanan, 2007). Interesting work emerging from the Edinburgh High Risk Study showed that affective abnormalities are one of the best predictive pre-morbid symptoms for predicting the development of the disorder in those deemed to be at high genetic risk (Cunningham Owens *et al.*, 2005; Johnstone *et al.*, 2005). In a longitudinal study of those people at 'high genetic risk' of developing schizophrenia, those who went on to become ill, had higher pre-morbid levels of situational and social anxiety, nervous tension, depression and withdrawal. Post illness-onset, the affective symptomatology in the group remained high but stable.

Other researchers have also found that affective abnormalities do not disappear or lessen after the onset of schizophrenia. Buchanan (2007) discusses the prominent and persistent nature of negative symptoms throughout the course of the disorder. Furthermore, the negative and affective disturbances in schizophrenia are deemed to be such an integral part of the disorder that Carter *et al*., (2009) as part of the CNTRICS (Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia)

group, recommend using social cognition and affective neuroscience-based measures as part of the assessment of the cognitive deficits of schizophrenia, in particular, looking at the ability to identify and respond to emotions.

1.3.3 Cognitive deficits in schizophrenia

There is a substantial body of recent research that has acknowledged on focused on the role of cognitive deficits in schizophrenia. Wilk *et al.* (2005) suggest that those apparently 'spared' the deficits are those people who had higher or superior pre-morbid abilities. People with earlier-onset schizophrenia (presenting during youth, rather than adulthood) tend to have higher levels of cognitive impairment. Later-onset appears to be associated with preserved cognitive function to some extent (Rajji *et al.*, 2009).

It is debatable whether or not the cognitive profile of an individual with schizophrenia is static throughout the lifespan or whether it is subject to decline during the course of the illness with anti-psychotic treatment and hospitalisation. The literature suggests a mixed picture with some studies (e.g. Heaton & Drexler, 1987) showing evidence for a static picture throughout the lifespan and other studies show a more undulating picture of impairment that varies according to illness status (e.g. Harvey, 2001). Interestingly, in their longitudinal study of IQ in people with schizophrenia, Van Winkel *et al*. (2006) showed that IQ was lower than estimated pre-morbid levels at the onset of illness but 10 years following that, IQ had returned to pre-morbid estimated levels. This finding serves as an indicator that cognitive endophenotypes in schizophrenia need to be defined not only at illness onset but measured at time-points throughout illness course in order to be valid markers. At present,

there are too few longitudinal studies to draw any definitive conclusions as to the stability of cognitive subtypes in schizophrenia (Joyce & Roiser, 2007) and further investigation is needed.

Another matter to be considered is the nature of the cognitive impairments; is there a global cognitive impairment or are specific functions affected more severely than others? Goldberg *et al.*, (2004), suggest that patients with schizophrenia typically exhibit abnormalities in attention, executive function and memory which stand out against a background of diffuse impairment. Fioravanti *et al*., (2005) suggest that cognitive deficits are more widespread and specific than this with significant impairments in people with schizophrenia seen in IQ, memory, language, executive function and attention although there was much heterogeneity between the individual studies examined. A more recent review (Reichenberg, 2010) describes the specific deficits in processing speed, learning, working memory, executive functions and attention that are evident in people with schizophrenia on a background of a general cognitive deficit. These specific impairments are reflected in the design of The Matrics Consensus Cognitive Battery (Nuechterlein *et al*., 2008, Kern *et al*., 2008), a battery of cognitive tasks designed specifically to assess cognitive function in people with schizophrenia.

1.4 Neurobiology of schizophrenia

That schizophrenia may have a neurobiological basis has been suspected for over a century (Kraepelin, 1919; 1971; Spielmeyer, 1930) yet it is still poorly understood. Increased understanding of the neurobiology and underlying pathogenesis of schizophrenia should lead to improved diagnosis and

nosology, prediction of illness course and outcome, identifying causative and secondary mechanisms and, most importantly, identifying valid targets for treatment and developing better drug therapies.

1.4.1 Neuropathological findings

Despite the neuropathology of schizophrenia remaining elusive, the null hypothesis, that is, that there is no neuropathology in schizophrenia can be rejected (Harrison, 1999b). Strong evidence from post-mortem studies exists for overall effects including decreased brain weight (Brown *et al.,* 1986; Pakkenberg, 1987; Bruton *et al*., 1990), shorter brain length (Bruton *et al*., 1990) and reduced volume of the cerebral hemispheres (Pakkenberg, 1987). More specific structures have also been found to be affected in schizophrenia, including enlarged lateral ventricles (Brown *et al.,* 1986; Pakkenberg, 1987; Crow *et al*., 1989), decreased thalamic volume (Pakkenberg, 1990, 1992; Danos *et al*., 1998) and reduced temporal lobe volume (Bogerts *et al*., 1985, 1990; Brown *et al.,* 1986; Falkai & Bogerts, 1986; Falkai *et al*., 1988; Jeste & Lohr, 1989; Altshuler *et al*., 1990; Vogeley *et al.,* 1998). This evidence is largely corroborated by neuroimaging findings taken in life (Harrison, 1999b, see 1.4.3 for a summary).

Some authors such as Stevens (1982) have suggested a role for gliosis in schizophrenia neuropathology. Gliosis is a sign of previous damage or scar tissue (Kreutzberg *et al*., 1997). Such damage would contradict the neurodevelopmental hypothesis of schizophrenia (which would suggest hallmark pathology developed and was not as the result of an insult) and would support an infectious, autoimmune or neurodegenerative hypothesis of

schizophrenia (Harrison, 1999b). Harrison discusses the evidence in his review (1999b) and remarks that many subsequent studies have not found gliosis (e.g. Roberts *et al*., 1986, 1987, Stevens *et al*., 1988; Casanova *et al*., 1990, Arnold *et al*., 1996) and one interesting study, Bruton *et al*. (1990) found that those schizophrenia cases with gliosis had accompanying neurocognitive deficits where gliosis was present. Currently, the evidence against gliosis outweighs the evidence for the process having an involvement in schizophrenia (Harrison, 1999b).

1.4.2 Neurochemistry and neuropharmacology in schizophrenia

The observed psychoactive effects of certain compounds and their similarity to aspects of schizophrenia symptoms have largely driven neurochemical hypotheses in schizophrenia. The dopamine hypothesis of schizophrenia, which is the hypothesis that dopaminergic overactivity contributes to the schizophrenia phenotype, emerged with the observation that all available and effective anti-psychotic drugs are D_2 dopamine receptor antagonists. This was strengthened by the observation that amphetamine, a substance which triggers the release of dopamine, can induce a form of paranoid psychosis (Harrison, 1999a). Roberts *et al*. (1997) review evidence for the dopamine hypothesis and highlight the support for findings of higher density D_2 receptors in schizophrenia. However, as Davis *et al*. (1991) and Joyce and Meador-Woodruff (1997) point out, despite all the correlational data, there is still no consensus on the specifics of the abnormality. D_2 receptors have received the most attention in the dopamine hypothesis and there is some evidence for

involvement of $D_{1,3,4}$ receptors also (reviewed in Harrison, 1999a), although again none of the evidence is conclusive.

Serotonin, or 5-HT, has also been implicated in schizophrenia due to the hallucinogenic effects of the 5-HT agonist, LSD. Interest, as of late, has particularly focussed on the 5-HT_{2A} receptor (Harrison & Burnet, 1997). The gene encoding the 5-HT_{2A} receptors has been found to have an association with schizophrenia (Williams *et al.*1997) and has been also associated with differential responses to the antipsychotic clozapine (Arranz *et al*., 1998). In terms of neurochemical evidence, Harrison (1999b) reviews strong evidence for lowered 5-HT_{2A} receptor expression in the pre-frontal cortices of people with schizophrenia.

The glutamate hypothesis of schizophrenia is another strong neurochemical hypothesis of schizophrenia that is again, driven by the observation that the drug phencyclidine (an NMDA receptor antagonist) given systemically can evoke a schizophrenia-like psychosis (Javitt & Zukin, 1991). The glutamate 'hypothesis' is not straightforward and findings vary between different brain regions (Taminga, 1998 Harrison, 1999b). There is some suggestion (e.g. Carlsson & Carlsson, 1990), that the mechanism by which glutamate acts in schizophrenia is through its interactions with dopamine.

1.4.3 Neuroimaging in schizophrenia

MRI technology has allowed the rapid development of this field over the last 30 years. Structural MRI has confirmed (as previously thought from post-mortem studies) that there is a reduction in overall brain and grey matter volume. Ventricles in the brain have also been found to be larger (e.g. Daniel *et al*.,

1991; Shenton *et al*., 2001; Steen *et al*., 2006; Ward *et al.,* 1996; Wright *et al*., 2000). In addition to a reduction in overall brain volume, certain temporal lobe structures have been found to have notably reduced volume including the hippocampus, amygdala and the superior temporal gyri (STG) (Lawrie & Abukmeil, 1998, Nelson *et al*., 1998). The prefrontal cortex and thalamus (Konick & Friedman, 2001) and the anterior cingulate (Baiano *et al*., 2007) and corpus callosum (Woodruff *et al*., 1995) have also been identified as being reduced in volume in people with schizophrenia.

Most of these findings are however from region-of-interest studies, rather than a whole brain approach. Voxel-based morphometry has helped validate these findings with a reduction in STG volume found (correlating with positive symptoms) and a reduction in medial temporal lobe volume (correlating with memory impairments) (Honea *et al*., 2005; Antonova *et al*., 2004). Brain volume and hippocampal volume findings seem to hold when first episode patients (who have limited exposure to antipsychotics) are compared with controls (Steen *et al*., 2006; Vita *et al.,* 2006). Many other findings described above do not, and some have been demonstrated to be due to drug treatment effects (Liddle & Pantelis, 2003). Brain volume changes are highly heritable (Baare *et al*., 2001; Bartley *et al*., 1997).

One caution with these findings is that many of these imaging changes in schizophrenia such as ventricular enlargement, are also seen in affective disorders (such a as bipolar disorder) although perhaps to a lesser extent (Elkis *et al*., 1995; Strasser *et al*., 2005) rendering brain volume/ventricle size unsuitable as a biomarker as it is a non-specific facet of schizophrenia.

Disorder presentation and course is so diverse in schizophrenia that it is not surprising that the literature reflects this with many different brain regions and systems implicated. There are some areas of convergence however. Harrison and Weinberger (2005) and Owen *et al.* (2005) review the evidence and there is consensus among the literature that whole brain volume is reduced, ventricles are enlarged and thalamic-hippocampal-prefrontal circuitry is altered in schizophrenia. There is additional data from genetic studies that implicate genes involved at the synapse, which has led to the hypothesis that schizophrenia may be a disorder of the synapse; a hypothesis gaining increasing support (reviewed in Owen *et al.,* 2005). More recently, Kirov *et al*., (2012) have again implicated genes involved at the synapse through the observation of copy number variations in schizophrenia cases.

A degree of caution should be taken when approaching neurobiological data alone. Networks and pathways in the brain are inextricably interlinked and interact with each other, with one system bearing an influence upon another. It is therefore very difficult to dissociate primary from secondary effects and support from other fields is needed to validate neurobiological findings.

1.4.4 A neurodevelopmental hypothesis of schizophrenia

The neurodevelopmental hypothesis of schizophrenia is not at odds with the neurodegenerative profile of schizophrenia. In a recent meta-analysis of MRI studies, Olabi *et al*. (2011) state that there is no neurodevelopment-neurodegeneration dichotomy as both processes can co-exist in schizophrenia (Balla and Frecska, 2011). Keshavan and colleagues review pathogenesis theories of schizophrenia in terms of timing, stating that neurodevelopmental

and neuroprogressive deficits in schizophrenia can co-exist and that any integrative approach to understanding schizophrenia needs to account for premorbid abnormalities as well as accounting for observations of the course and progression of the disorder (Keshavan *et al*., 2008).

The neurodevelopmental hypothesis views schizophrenia as the consequences of aberrant neurodevelopment that took place early in life (during pre-, peri-, post-natal, childhood and/or adolescent brain development), processes which take place long before the onset of clinically diagnosed schizophrenia. (Cardno *et al*., 1999, Singh *et al*., 2004). Several diverse lines of evidence support this model (Weinberg *et al*., 2007) including epidemiological studies of environmental insults such as *in utero* influenza infection and stress exposure (reviewed in Cannon *et al*., 2004) and the presence of obstetric complications (McNeil *et al*., 2000; Cannon *et al*., 2002). In addition to environmental pre-natal influences, a major influence on neurodevelopment is genetic variation in genes that influence neurodevelopmental processes such as synapse formation and neuronal migration (e.g. Stahl, 2000, Owen *et al*., 2005, Debnath *et al*., 2012). Many genes that influence such processes have shown association with schizophrenia.

One such gene is *DISC1* or disrupted in schizophrenia 1. A balanced translocation (chromosome 1:11, q42.1:q14.3) was discovered in a large Scottish family, co-segregating with schizophrenia and mood disorders (St Clair *et al*., 1990, Millar *et al*., 2000). *DISC1* is discussed further in 1.5.2.4 and 1.6.

DISC1 has been shown to affect neural development particularly through its role in neurite and dendrite outgrowth (reviewed in Bellon, 2007) and variants in the gene have been associated with deficits in memory and

decreased prefrontal and hippocampal grey matter volume (Cannon *et al*., 2005). *DISC1* is very likely to be a true susceptibility gene for schizophrenia although the extent of its influence on schizophrenia risk is as yet unknown (Walters *et al*., 2011). Although the studies described are interesting in the context of a neurodevelopmental hypothesis of schizophrenia, the lack of knowledge on the extent of its action should be borne in mind.

Neuregulin 1 was identified as a putative susceptibility gene for schizophrenia in a follow-up association study of putative linkage at 8p22-p11 (Stefansson *et al*., 2002) that has since been twice replicated (Stefansson *et al*., 2003, Williams *et al*., 2003). The gene has been shown to have a wide range of roles in development. It has been shown to influence neuronal migration, synaptic development and function, oligodendrocyte function and myelination (Harrison & Law, 2006). Other findings have improved the standing of *NRG1* as a risk gene. Variants have been shown to be associated with altered frontal and temporal cortex activation and white matter structure (Hall *et al*., 2006, McIntosh *et al*., 2008 respectively). These results should however be treated with caution as there have been many negative reports showing no association with schizophrenia or association of different variants in the gene with schizophrenia (reviewed in Munafo *et al*., 2008)

Dysbindin is another putative susceptibility gene that follows a similar pattern of findings to *NR1*. Following discovery through linkage (Straub *et al*., 2002), the gene has been shown to play a role in vesicular transport of neurotransmitters (Li *et al*., 2003) and it may also be involved with dopamine

D2 receptor internalisation (Iizuka *et al*., 2007). There are however, many negative findings (e.g. Williams *et al*., 2005, Mutsuddi *et al*., 2006).

There are many other candidate genes for schizophrenia susceptibility that play a role in neural development including *BDNF* (Gall *et al*., 1992), reelin (Shifman *et al*., 2008), and *NR1* (Eastwood *et al*., 1994), all well-researched schizophrenia candidate genes. This plethora of genes implicated in the disorder that are involved with neurodevelopmental processes provides strong support for the neurodevelopmental hypothesis of schizophrenia.

CNV analysis has also strengthened the case for the neurodevelopment hypothesis of schizophrenia. Many CNVs in schizophrenia also confer risk to other neurodevelopmental disorders such as autism, epilepsy leanring difficulities. (reviewed in Walters *et al*., 2011. Refer to 1.5.2.4 for further discussion on CNVs in schizophrenia).

1.5 Genetics of schizophrenia

1.5.1 Genetic epidemiology

A heritable component to schizophrenia has been suspected for a long time. The first systematic family study implicating genetics was Rudin (1916). Schizophrenia, then known as dementia praecox was found to be more common in siblings of probands than in general population. Kallman, 1938, extended these findings to the offspring of probands. It was not until some years later that quantification of risk for the disorder was estimated. Kendler *et al.*(1985) suggested that the general population have a lifetime risk of developing schizophrenia of 0.2%, (although current estimates of the risk are

close to 1%, see 1.1) whereas in first degree relatives, this risk is elevated to 3.7% and further increased to a risk of 8.6% if non-affective psychosis and schizoaffective disorder were also included. There was however substantial variation in reports of risk to first degree relatives ranging from 3.1% to 16.9% (Gershon *et al*., 1988).

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Figure 1.1: Risk of developing schizophrenia by relation to a proband (taken from Gottesman, 1991)

Sullivan *et al.* (2003) in their meta-analysis more recently demonstrated support for the idea that there are both genetic and environmental influences on developing schizophrenia, concluding that susceptibility to schizophrenia lies 81% in heritability and 11% in shared environmental influences. Thus the majority of variance in susceptibility to schizophrenia lies in genetic effects. Twin and adoption studies are especially useful in studying heritability and

allow the disentanglement and dissociation of genetic and environmental influences by looking at shared phenotypes amongst twins reared apart, offspring adopted from parents with schizophrenia or adopted into a family with schizophrenia and so forth. There is a vast body of literature which has investigated schizophrenia in families. Sullivan (2005) reviews this evidence succinctly, concluding that the smaller but important environmental influences are likely prenatal in origin (due to the higher rates of schizophrenia in fraternal twins compared with non-twin siblings and that schizophrenia is generally assumed to be neurodevelopmental). Sullivan also importantly highlights the need not to over-interpret family data, it merely rationalises genetic studies into schizophrenia and still leaves us in the dark with regards to the mechanism by which schizophrenia develops, or the genes that may influence risk for schizophrenia.

More detailed analysis of the genetic epidemiology of the disorder consistently concluded that monozygotic (MZ) twins were approximately three times more likely than other first degree relatives to develop the disorder. (Tienari, 1971; Kringlen, 1976; Fischer, 1971; Pollin *et al*. 1969; Gottesman & Shields, 1972). The findings were however criticised for their variability which was, in a large part, put down to the failure to employ strict operational diagnostic criteria to diagnose schizophrenia. Researchers called for a more rigorous approach to data collection, allowing other researchers to apply different operational criteria to the same dataset and to allow for meta-analysis (McGuffin & Owen, 1991).

One example of how important definition of the phenotype under study is comes from a Norwegian study looking at concordance in MZ and dizygotic

(DZ) twins. Expanding the phenotype (using DSM-III criteria) to include other psychotic disorders (schizoaffective disorder, atypical psychosis and schizotypy) resulted in a large difference between MZ and DZ concordance rates (concordance rates in MZ twins were higher) whereas when the phenotype included affective and personality disorders and excluded psychotic disorders, this difference decreased as concordance rates in DZ twins increased to narrow the gap (Onstad *et al.*, 1991).

In a frequently cited study, Cardno & Gottesman (2000) pooled their data with four other twin studies (Kläning *et al*., 1996, Cannon *et al*., 1998, Franzek & Beckmann, 1998, Tsujita *et al*., 1992). Concordance rates for MZ twins reached 50%, and 4.1% for DZ twins using the (then current) DSM-III-R criteria for schizophrenia. Using ICD-10 criteria altered concordance rates to 42.4% and 3.9% for MZ and DZ twins respectively. The authors also provided heritability estimates of 88% for DSM-III-R schizophrenia and 83% for ICD-10 schizophrenia. It is important to note that these heritability estimates do not suggest that this proportion of schizophrenia is genetic in origin in any individual; rather that the difference between one person developing schizophrenia and the next, is approximately 80 – 90% due to genetic variation between individuals. In reality however, genetics and environment cannot be separated in this manner and the schizophrenia phenotype is likely to be caused by a complex cascade of interactions between the two.

The evidence for a genetic basis of schizophrenia is nonetheless compelling. Further evidence from Cardno & Gottesman (2000) suggests even unaffected MZ twins of a proband can confer increased risk for schizophrenia to offspring, risk that is as great as would be expected should the proband be the

parent. The same direction of effect was not however seen in DZ twins though. This study is very interesting as it strongly supports genetic susceptibility to schizophrenia, yet it also reminds us of the importance of the environment in the development of the disorder as having genetic variants that could result in schizophrenia, does not mean the onset of the disorder is inevitable. This is an important consideration to bear in mind.

1.5.2 Schizophrenia gene discovery

1.5.2.1 Methodological overview

Linkage analysis has traditionally been the starting point for identifying specific genetic loci involved in a disease. The premise behind linkage analysis is that a genetic marker, or several genetic markers, and the resultant disease phenotype co-segregate within either many independent families, or in an extended pedigree through the principle of genetic linkage. In linkage, genetic material is inherited in a non-random fashion, occurring when homologous chromosomes are paired in gametogenesis. Pairing occurs in such a way that the chromosomes of the gamete consist of alternating segments of chromosome from the mother and father. Segments of chromosomes, rather than individual genes tend to be inherited together. Linkage analysis exploits this phenomenon by examining genotypes at genetic markers (generally microsatellites) at evenly placed locations throughout the genome, looking for the co-segregation of the phenotype with specific genotypes (Sham and McGuffin, 2002, Strachan and Read, 2003). If the phenotype does indeed co-segregate with specific genotypes, then the linkage analysis may be refined to

that region for further investigation (positional cloning). Linkage has been especially promising in identifying rare variants in genes of large effect in single-gene disorders (Owen *et al.*, 2004). Although this situation is unlikely the case in the majority of genetic risk for schizophrenia, the hope in using linkage is that it will detect rare alleles of large effect (Owen *et al.*, 2004).

Association is another method of gene discovery and works on the basic premise of comparing genotypes of markers between one set of people and the next (usually those with a disease or disorder, compared with those without). Association of a particular genotype with a particular state is then tested for statistical significance. Association has largely been used on positional and functional candidate genes to date and has the advantage of being able to pick up common alleles of weak to moderate effect. However, one disadvantage of association is the potential for false negatives. Many associations are often discovered, then fail to replicate. However, Owen *et al.* (2004) warn against considering a negative report a definite exclusion of a marker or candidate.

A third approach researchers have used to locate susceptibility genes for schizophrenia is through looking for cytogenetic abnormalities, such as translocations, insertions, deletions and inversions, which may cause direct disruption of a gene, formation of a new gene, indirect disruption of genes via a position effect or altered gene dosage and are identified through linkage. The field of chromosomal abnormalities has made some positive contributions to schizophrenia genetics but it is prudent to remember that it is possible for a chromosomal abnormality to be merely linked to an anomaly in a family and this doesn't necessary indicate that the chromosomal abnormality is pathogenic for

schizophrenia (Owen *et al.*, 2004). Similar caution must be taken with searching for genomic copy number changes, although when the abnormalities are observed in more than one pedigree, the evidence is persuasive (Owen *et al.*, 2004).

1.5.2.2 Linkage analysis in schizophrenia

Psychiatric genetics began with linkage studies looking for highly penetrant alleles, that is, alleles responsible for a high proportion of disease cases. Many putative candidate genes have been discovered but replication has proved to be very difficult and no strongly replicated findings emerged from the early linkage studies (Owen *et al.,* 2009). One major problem has been the power of the studies, or their intrinsic ability to detect significant linkage. Positional cloning studies, increasingly refining the putative disease region to increasingly small chromosomal segments have however, had more success and identified some promising putative susceptibility genes including *DTNBP1, DAOA,* and *DISC1* (Straub *et al*., 2001, Hattori *et al*., 2003, St Clair *et al*., 1990 respectively). Data to support the involvement of these genes in schizophrenia has come from cognitive (e.g. Burdick *et al*., 2006, Opgen-Rhein *et al*., 2008, Burdick *et al*., 2005 respectively), neuroimaging (e.g Narr *et al*., 2009, Jansen *et al*., 2010, Prata *et al*., 2008 respectively) and animal model studies (e.g. Feng *et al*., 2008, Otte *et al*., 2009, Ma *et al*., 2002 respectively), although no specific risk alleles in these genes have been identified and unambiguously replicated.

1.5.2.3 *A priori* candidate gene approaches

The most investigated a priori candidate genes originate from the neuro-pharmacological literature and are genes involved in dopaminergic and serotonergic neurotransmission. The majority of findings have failed to replicate consistently and confidence in the finding is therefore reduced, with two exceptions. Inayama *et al* (1996) provided the first genetic evidence for the involvement of the serotonergic system in schizophrenia in the gene encoding the 5HT2a receptor. These findings were replicated by Williams *et al*.,(1996) and again by Williams *et al*.,(1997) in their meta-analysis. However, no variants have yet been detected in the genes that alter receptor function or expression.

A second stand-out finding relates to *DRD2*, a D2 receptor gene and a polymorphism in the promoter which affects *in vivo* expression. Three groups have implicated this polymorphism (Arinami *et al*., 1997; Ohara *et al*., 1998; Jonsson *et al*., 1999). However, some studies (e.g. Li *et al*., 1998) have found no evidence for association here with some suggesting that the polymorphism is merely a marker for the true susceptibility variant and that the association is due to linkage disequilibrium.

However, perhaps the best-studied candidate (and arguably best *a priori* candidate, Williams *et al*., 2007) gene is COMT (catechol-*O*-methyl transferase) which codes for an enzyme that catabolises dopamine (Walters *et al*., 2011). The gene is an attractive candidate due to its direct involvement in the regulation of dopamine levels and its position on chromosome 22q11 (see 1.5.2.4 below). The gene has been demonstrated to show linkage with schizophrenia several times (reviewed in Wiliiams *et al.,* 2007). The gene contains a polymorphism resulting in a valine-to-methionine substitution (which

alters the action of the enzyme, Lachman *et al*., 1996) which has shown both positive and negative associations with schizophrenia (Williams *et al*., 2007) although evidence is currently weakening for an association of this polymorphism with schizophrenia (Walters *et al*., 2011). However, the interaction of COMT with other candidate genes and environmental influences, such as smoking and antipsychotics as well as its role in cognitive function (Egan *et al*., 2001) make the gene an interesting candidate nonetheless (see Ŝagud *et al*., 2010 for a review). There have also been many mouse models looking at the effects of altered COMT with some interesting findings (see 1.6 for discussion of these).

1.5.2.4 Chromosomal abnormalities

Copy Number Variation

Copy number variations (CNV) (operationally speaking) refer to deletions, duplications, insertions and inversions that are >1kb in size (Alkan *et al*., 2011, Zhang *et al*., 2011), although structural variations can be much smaller and exist on a continuum (Malhotra & Sebat, 2012). CNVs represent another type (alongside SNPs) of genetic variation between individuals. Sebat *et al*., (2004) demonstrated that small (<500kb) CNVs are widespread in healthy human genomes, with an average of >1000 CNVs accounting for ~4 million base pairs of difference between two 'average' individuals (Conrad *et al*., 2010, Mills *et al*., 2011). Although CNVs are less frequent in humans than SNPs on the whole, the relative contribution to genomic variation (measured in nucleotides) is

similar between SNPs and CNVs due to the larger size of a CNV (Malhotra & Sebat, 2012).

Studies have established a role for rare (<1% frequency), large (>100kb) CNVs in schizophrenia risk in the last decade or so (Sebat *et al*., 2009) with the variations being estimated as 1.1 to 3 times more common in people with schizophrenia compared with controls (ISC, 2008, Walsh *et al*., 2008 respectively).

One well-researched large 3Mb deletion has long been known about (since Karayiorgou *et al*., 1995) at chromosome 22q11.21 as a significant risk factor for schizophrenia. Small interstitial deletions at this locus are associated with velocardiofacial syndrome (VCFS) and approximately 25% of deletion carriers demonstrate psychosis symptoms qualifying for a diagnosis of schizophrenia (Shprintzen *et al.*, 1992; Pulver *et al.*, 1994; Papolos *et al*., 1996; Murphy *et al*., 1999). Some studies have suggested a general schizophrenia susceptibility locus on 22q, although most suggest that this maps outside the 'VCFS region' (Owen, O'Donovan & Gottesman (2004).

There have been several other major regions of the genome CNVs have been found to co-segregate with schizophrenia. Interestingly, CNVs in all of these regions have also been associated with autism (albeit in some cases a duplication in associated with schizophrenia and deletions associated with autism, Walters *et al*., 2011). The table below (Table 1.1) summarises findings relating to these four regions. The rate of CNVs in the region in cases of schizophrenia and controls varies from 0.19 to 0.7% in cases compared with 0.02% to 0.24% in controls. Perhaps the most interesting thing to emerge from these results however, is the prevalence of neurodevelopmental disorders such

as autism, epilepsy and learning difficulties that are also associated with CNVs in this region (and the lack of mood disorders showing association, Grozeva *et al*., 2010).

Earlier studies (Walsh *et al*., 2008) demonstrated that CNVs in schizophrenia tended to impact upon genes involved in neuronal function, particularly synaptic activity and neurodevelopment (Malhotra *et al*., 2011, Walsh *et al*., 2008). Kirov *et al*., 2012 extended this finding by demonstrating that *de novo* CNVs in schizophrenia were particularly enriched for components of the N-methyl-d-aspartate receptor (NMDAR) and post-synaptic signaling complexes. Interestingly for nosology, the prominence of CNVs seems to be one point of difference between schizophrenia and bipolar disorder with CNVs being more frequently discovered in schizophrenia cases compared with bipolar cases (Grozeva *et al*., 2010).

Studies	Chromosomal region	Rate in SZ (average % across studies)	Rate in ctrl (average % across studies)	CNV type	Additional phenotypes associated with this region
Stefansson *et al*., 2008 Walsh *et al*., 2008 ISC, 2008	1q21.1	0.2	0.02	Deletion	Cardiac defects, neuroblastoma, microcephaly, autism, ADHD (Mefford *et al*., 2009).
Kirov *et al*., 2008 Walsh *et al*., 2008 Rujescu *et al*., 2009	2p16.3	0.19	0.04	Deletion	Autism (Weiss *et al*., 2008, Kim *et al*., 2008)
Stefansson *et al*., 2008 Kirov *et al*., 2009	15q11.2	0.6	0.22	Deletion	Speech problems, ADHD, obsessive compulsive disorder, autism, learning difficulties (Mefford *et al*., 2009, Doombos *et al*., 2009)
Stefansson *et al*., 2008 ISC, 2008 Kirov *et al*., 2009	15q13.3	0.2	0.02	Deletion	Epilepsy, learning difficulties, ADHD, autism (Miller *et al*., 2009, Dibbens *et al*., 2009, Sharp *et al*., 2008)
Ingason *et al*., 2009 ISC, 2008 Kirov *et al*., 2009 Ikeda *et al*., 2009	16p13.1	0.3-0.7	0.09-0.24	Duplications associated with schizophrenia, deletions with more severe cognitive phenotypes such as learning difficulties, epilepsy and autism	Autism, learning difficulties (Ullmann *et al*., 2007, Mefford *et al*., 2009)
McCarthy *et al*., 2009	16p11.2	0.3	0.03	Duplications associated with schizophrenia, deletions with more severe cognitive phenotypes such as learning difficulties and autism	Autism, learning difficulties (Weiss *et al*., 2008, Abrahams & Geschwind, 2008, Glessner *et al*., 2009, Mefford *et al*., 2009)

Table 1.1: Summary of findings of major CNV regions for schizophrenia showing the rate of discovery in schizophrenia cases and controls, type of CNV found and additional phenotypes associated with the region.

1.5.2.5 Association studies

Association studies have traditionally followed linkage analysis in the search for putative disease genes. Essentially, association studies look for significantly different marker allele frequencies between people affected by the disease, and healthy controls. The association of the marker, if significant can be explained by one of two mechanisms (provided cases and controls are well-matched for factors such as ethnicity): either that marker is directly associated with the disease and has functional relevance, or the marker is indirectly associated with the disease through linkage disequilibrium. Indirect association means the marker and the disease-causing locus/loci are sufficiently close so that they are inherited together, an association that has not yet been eroded in the population by recombination. Genetic association studies are better placed than linkage analysis to detect small effect sizes due to the smaller critical region detected by association studies. Recently, high-throughput genotyping technology, whereby upwards of a million SNPs and CNVs can now be genotyped on a single array, has drastically improved and genome-wide association studies (GWASs) are now a possibility for disease gene discovery with the advantage that they are not hypothesis driven, an important consideration for a disorder such as schizophrenia where the pathogenesis is highly complex and largely unknown. The HapMap, a public database describing genome-wide known SNPs has greatly aided marker choice and distribution.

The few genome-wide association studies of schizophrenia published to date have not been without problems. Lencz *et al.* (2007), for example reported significant association in an intergenic region between *CSG3RA* and

SHOX. CSG3RA (or colony stimulating factor 2 receptor alpha) is a subunit of a cytokine receptor which regulates granulocytes and macrophages, types of white blood cell. SHOX is thought to be transcription factor which has some influence upon height. The authors sequenced *CSG3RA* and the flanking *IL3RA* in a smaller sample and found several significant associations with schizophrenia. The effect sizes of these associations are however are much larger than would be expected for a locus implicated in a complex disease. Sullivan *et al.* (2008), in their GWAS of 738 cases with DSM-IV schizophrenia and 733 controls, genotyped approximately 500 000 SNPs. The authors failed to discover any putative disease loci showing association surpassing the genome-wide threshold of significance of $p= 5 \times 10^{-8}$. A potential problem here may have been the ethnic heterogeneity of the samples which may have added 'noise' to the study and potentially produced false negative (in terms of not reaching genome-wide significance) results.

GWAS for schizophrenia are notoriously difficult to conduct due to the poor characterisation of the phenotype. As previously mentioned, there are no biomarkers for the disorder that may be used as discrete inclusion/exclusion criteria for a 'case' in a genome-wide association study. There is also growing evidence that schizophrenia is not a unitary disorder and exists on a continuum with affective disorders, autism and mental retardation (Carroll and Owen, 2009). Diagnoses may therefore be overlapping which may cause ambiguity in findings. One way to control for these potential confounds is through replication. Successful replication of association at loci is taken as indicative of true schizophrenia susceptibility loci.

1.5.2.6 Discovery of the schizophrenia risk gene *ZNF804A*

In a large-scale GWAS for schizophrenia, O'Donovan and colleagues genotyped 479 schizophrenia cases and 2 937 controls using a mapping array of 500 000 SNPs (of which 362 532 passed quality control measures). SNPs showing 'moderately strong' association ($p<1 \times 10^{-5}$) were selected for follow-up investigation and put through two replications with larger sample sizes (see results presented in figure 1.2). The table below shows results from the study. The SNP rs1344706 in the gene coding for ZNF804A was the only locus to achieve strong ($p=1.51 \times 10^{-7}$) support in the meta-analysis and surpassed the genome-wide significance

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Figure 1.2: Table of most significant results ('moderately strong association') and follow-up from O'Donovan et al. (2008)

threshold when bipolar cases were added to the disease sample ($p=9.96 \times 10^{-9}$) suggesting the gene variant may confer risk to bipolar disorder as well as schizophrenia.

In addition to replication embedded in the original study, the association of *ZNF804A* variants with psychopathology has since received support from three independent studies. The first replicated the association of rs1344706 with schizophrenia, reporting a p-value of 0.0113 (Riley *et al.* 2010). Steinberg *et al.* (2010) also replicated the association with schizophrenia (p = 0.0029) and with schizophrenia and bipolar disorder (p=0.00065). Genotyping a Han Chinese sample for association of the SNP rs1344706 with schizophrenia, Zhang *et al.* (2010) added to the evidence, reporting a p-value of 0.00083. Thus *ZNF804A* appears to be a true susceptibility locus for schizophrenia (O'Donovan *et al.*, 2009).

1.5.2.7 *ZNF804A*

ZNF804A or *Zinc finger protein 804a* is a 4-exon gene found on chromosome 2q32 of unknown function that is largely uncharacterised and is, at the time of writing, definitely within the 'annotation gap'. The SNP rs1344706 is located in intron 2 of the gene, within a short region that is conserved between mammalian species. High levels of conservation indicate that the sequence has important function and is most commonly found in exonic sequences, rather than intronic sequences.

Although rs1344706 is described as a risk SNP for schizophrenia in *ZNF804A*, it is unknown whether this SNP is directly associated with schizophrenia and is therefore the functional allele conferring risk, or if the association is indirect and rs1344706 is a tagging SNP for the true causative locus. To investigate this issue, Williams *et al.* (2011) re-sequenced this region and conducted fine-scale LD mapping. Almost all the known SNPs in this gene

were covered (96% with a minor allele frequency > 0.01) yet rs1344706 remained the most strongly associated marker in the gene. Meta-analysis (21 274 cases of schizophrenia and bipolar disorder and 38 675 controls) provided further support for the association of this SNP with both schizophrenia ($p=2.5 \times 10^{-11}$) and schizophrenia and bipolar disorder combined ($p=4.1 \times 10^{-13}$), far surpassing the accepted threshold for genome-wide significance.

1.5.2.8 *ZNF804A* gene function

This strong statistical support doesn't, however, provide us with any clues to the function of the protein product ZNF804A or, indeed, how it may confer risk to schizophrenia. As previously indicated, the structure of the ZNF804A protein is largely unknown with the only characterised region being a C2H2 domain occurring early on in the protein. Such domains are characteristic of the zinc finger protein family and suggest that the protein may have some DNA-binding function and therefore, a role in transcription of other genes; indeed, further bioinformatic analysis of the sequence flanking rs1344706 indicates there may be transcription-factor binding sites in this region (Donohoe *et al.* 2010). It is not unusual, however, for C2H2 domains to also have a role in RNA- and protein-binding (Donohoe *et al.* 2010).

There is evidence that rs1344706 may also influence expression of *ZNF804A* itself. Riley *et al.* (2010) showed in *post-mortem* brain tissue analysis using qPCR that the A-allele at rs1344706, the risk allele, shows higher levels of expression in dorsolateral pre-frontal cortex (DLPFC) in controls (and also in patients with schizophrenia, although this increase is non-significant). Bioinformatic analysis predicted that the risk A-allele maintains binding sites for

the brain-expressed transcription factors MYT1l and POU3G2/OCT-6. These transcription factors are known to be involved in oligodendrocyte differentiation and proliferation. Further clues connecting rs1344706 to expression of *ZNF804A* emerge from work by Williams *et al.* (2011) based on the GeneVar database, which again showed that the risk allele was significantly associated with higher levels of expression of *ZNF804A* mRNA; confirming this association with higher expression levels in *post-mortem* samples using a proxy SNP, inferring that this translated as a increase in ZNF804A expression 1.13-fold.

A link between the mouse orthologue of the gene, *Zfp804a* and *Hoxc8*, a gene implicated in neurodevelopment has also been found (Chung *et al.*, 2010). Binding of Hoxc8 to an intronic region in the third intron of *Zfp804a* was reported. Hoxc8 upregulated *Zfp804a* mRNA levels. In the study, Zfp804a and Hoxc8 were coexpressed in E11.5 mouse embryos. Despite these hints at mechanism there is still a huge amount of work to be done to understand the basic brain function of *ZNF804A*; a crucial pre-requisite if we are to ever understand its involvement in schizophrenia pathogenesis.

1.5.2.9 Neurocognitive function of *ZNF804A*

In addition to work done at the molecular and cellular levels, there are a number of studies that have examined the neurocognitive phenotype associated with the risk allele at rs1344706. A study in Science by Esslinger and colleagues (2009) genotyped a cohort of 115 healthy participants for rs1344796 genotype. The authors examined performance on the n-back working memory task and a face-matching task to probe the link with the affective/bipolar disorder. Correlational regional brain activity in the DLPFC and

hippocampal formation (HF) using fMRI was also examined. In the study, regional brain activity was not significantly altered in relation to genotype although indices of connectivity were. The authors found connectivity within the DLPFC and to the contralateral DLPFC was reduced in risk allele carriers. Furthermore, the HF and DLPFC showed normal connectivity in people homozygous for the non-risk allele but there was a risk-allele dose-dependent decrease in DLPFC connectivity in heterozygous and homozygous risk allele carriers. Conversely a dose-dependent increase in HF-DLPFC coupling associated with the risk allele. Interestingly, the genotype at rs1344706 had no impact upon performance in the tasks.

The same research group, following similar methodology, also looked at 'theory of mind' measures in relation to rs1344706 genotype, finding a risk allele dose effect on levels of activity in parts of the mirror neuron system implicated in the theory of mind network (left inferior parietal cortex and left inferior frontal cortex) and also in the medial prefrontal cortex and left tempero-parietal cortex. The authors additionally found evidence for aberrant functional connectivity between the frontal and temporo-parietal regions in risk allele carriers (Walter *et al.*, 2010). *ZNF804A* therefore seems to have an impact on neural networks associated with aspects of cognition, and at least one aspect of social cognition.

Further work on the risk allele was conducted by Walters *et al.* (2010) in a large-scale study looking at the influence of rs1344706 genotype on a number of cognitive functions in both patients and controls. Interestingly, the authors found the risk allele to have a protective effect on cognition in patients but not in controls. An association between rs1344706 and IQ, episodic

memory, working memory, and attention was tested for in an Irish discovery sample. Significant results were then looked at in a German replication sample. In the Irish samples, genotype was associated with better performance on tests for episodic and working memory in patients but not in controls. These findings were replicated in the same direction in the German samples, leading the authors to hypothesise that perhaps the risk variant at the *ZNF804A* locus contributes to risk for a particular sub-type of schizophrenia that doesn't encompass cognitive deficits.

1.6 Animal models for schizophrenia

One means of understanding gene function is by using an animal model. Genetic animal models most commonly exploit the mouse as an organism for several reasons. As with all mammals, there are high levels of genetic similarity between the mouse and the human genomes and extensive synteny (conserved gene order). Mutations that cause certain diseases in humans can cause similar diseases in mice (Beckers, Wurst & de Angelis, 2009). Mice have emerged as the model organism of choice in preference to other mammalian species due to their diminutive size (making them easier and cheaper to manage), the short breeding cycle and gestation period (a new generation can be produced every two to three months) and their amenability to genetic manipulations such as transgenesis and mutagenesis. There is also a large existing catalogue of mutant mice (e.g. The Jackson Laboratory, http://jaxmice.jax.org/query/f?p=205:1:12049464109724088944, MRC Mammalian Genetics Unit, Harwell, http://www.har.mrc.ac.uk/services) and a

compendium of well-validated phenotyping tests for ease of comparison of findings between studies.

Genetic animal models for psychiatric disorders are useful tools, especially since the advent of the GWAS. They allow us to examine functions of uncharacterised putative genes for the disorders including exploring the putative associated neurobiology and observing the effects of knocking out/altering the gene on the behavioural phenotype, gene expression of other genes and the effects of drug therapy (such as antipsychotics) on any deficits or abnormalities observed.

1.6.1 Non-genetic animal models for schizophrenia

Many different types of animal models for schizophrenia have emerged over the years. There are developmental models generally using environmental insults such as isolation rearing (e.g. Wilkinson, *et al*., 1994), influenza challenges (e.g. Fatemi *et al*.,1999; Shi, 2003; Fatemi *et al*., 2005; Fatemi *et al*., 2008; Winter *et al*., 2008) and other various types of prenatal and neonatal immune challenge (e.g. Romero *et al*., 2007; 2008; Pletnikov *et al*., 2000). There are also pharmacological challenge models, most commonly, and successfully using NMDA receptor antagonists such as phencyclidine (PCP) and ketamine, which are known, in humans to cause a psychosis reminiscent of that observed in schizophrenia (e.g. Jentsch & Roth, 1999; Manahan-Vaughn *et al.*, 2008; Zavitsanou *et al.*, 2008; Karasawa *et al.*, 2008). Animal lesion models of schizophrenia have also been popular and yielded some interesting findings, particularly in the regions strongly implicated in schizophrenia such as hippocampal lesions (e.g. Lipska & Weinberger, 2000; Lipska, 2004; Tseng *et*

al., 2006; 2007; 2008; Endo *et al*., 2007; Marquis *et al*., 2008a,b) frontal cortex lesions (e.g. Lazar *et al*., 2008) and prefrontal cortex lesions (e.g. Wilkinson *et al*., 1997; Lipska *et al*., 1998).

1.6.2 Genetic animal models for schizophrenia

Genetic animal models of schizophrenia are currently a big growth area in psychiatric research, in a large part due to the advent of the GWAS and the identification of numerous new putative candidate genes for disorders such as schizophrenia. Many genes identified in such association studies are largely uncharacterised, biologically and patho-physiologically and genetic animal models are invaluable in ascertaining their function, and equally importantly, how this might inform us about the disease mechanism.

1.6.2.1 Creating and characterising genetic models

Methods of creating genetic animal models include deleting (knockout) or inserting (knockin or transgenesis) individual genes. Some inbred strains have naturally occurring mutations or deletions in some genes that may be of interest. For example Clapcote and Roder (2006) discovered that the deletion found by Koike *et al*. (2006) in 129S6/SvEv mice from Taconic Farms is common to all 129 substrains. The mutation is homozygous and 25-bp in exon 6 are deleted, inducing a frame shift in the reading frame of *Disc1*, resulting in the production of 13 novel amino acids, followed by a premature stop codon. Neither full-length Disc1 nor the predicted C-terminally truncated protein were detectable in 129S6 brain tissue (Koike *et al*., 2006), suggesting that the deletion is a null allele. Other mutations found include a deletion of the α-

synuclein locus in a C57Bl/6J substrain in mice supplied by Harlan UK (Specht & Schoepfer, 2001).The creation of conditional mutants adds further to the inventory of methods available for creating genetic animal models by allowing the researcher to study the spatial and temporal effects of the gene affected. More subtle and varied mutations, as exploited in the current work, can also be achieved by using chemical mutagenesis (such as *N*-ethyl-*N*-nitrosourea, ENU) causing random point mutations in the genome which can be identified by phenotype- or genotype-driven screens of mice treated with mutagens.

Desbonnet, Waddington and O'Tuathaigh (2009) review mutant models for genes associated with schizophrenia. When conducting a phenotype screen of a mutant model for a gene of unknown function, it is important to adopt a hierarchical approach and screen at the levels of anatomy, physiology and behaviour. Endophenotypes are also very important (refer to Esslinger *et al.*, 2009, discussed in 5.2.3 Neurocognitive phenotypes associated with *ZNF804A*) as the absence of a phenotype does not indicate the absence of an endophenotype. Desbonnet and colleagues additionally warn that when using an animal model for schizophrenia, the merits of ethologically-based approaches must be considered compared with using more structured tasks that are perhaps better disease-validated in humans. Both approaches have their merits and the compendium of tests selected to characterise a mutant mouse model should reflect a consideration of what is known about the function of the gene on all levels to date.

Genetic animal models for schizophrenia can largely be divided into two categories: those implicated in putative disease-associated mechanisms (e.g. a focus on dopamine-related genes) and those implicated by clinical

association, discovered through methods such as linkage analysis and association methods. In terms of the former approach, it is important to note that the absence of strong genetic evidence does not necessarily exclude genes from an involvement in developing schizophrenia. A strong physiological story may produce features analogous to the disease in mouse models, providing an alternative means of investigating disease pathogenesis. Mohn *et al.* (1999) for example showed that mutant mice expressing only 5% of essential NR1 show hyperactivity that is ameliorated by haloperidol and clozapine in line with evidence that an NMDA receptor gene is altered in schizophrenia.

1.6.2.2 Genetics mouse models for functional candidate genes

Genetic animal models for putative disease-associated mechanisms have largely focused on the dopamine and glutamine hypotheses of schizophrenia and are interesting as they offer an alternative means of investigating genetic susceptibility to schizophrenia by offering a 'bottom-up' perspective and are a departure from traditional gene discovery methods. Kellendonk *et al.* (2006) for example, with their conditional knockout, implicated dopamine receptors (D_2 and D_1) in cognitive symptoms associated with schizophrenia. In their model, striatal overexpression of D_2 receptors resulted in a phenotype of impaired PFC-mediated working memory and marked perseverative behaviour. These effects interestingly persisted after the transgene was switched off which indicates that these observed effects were (at least in part) due to some secondary compensatory mechanism. The striatal overexpression of D_2

receptors also resulted in altered dopamine turnover and D_1 receptor activation in PFC.

COMT (an enzyme that catabolises dopamine), has also been shown through mouse models to have an effect on cognitive behaviours that are associated with schizophrenia with a dissociation between homozygous and heterozygous knockouts/deletions respectively. Babovic *et al*. (2007) found that heterozygous deletion but not homozygous knockout resulted in disrupted exploration of and habituation to a novel environment. Babovic *et al*. (2008) and Papaleo *et al*. (2008) found that the knockout but not the heterozygous deletion resulted in improved spatial learning and working memory. COMT is implicated in schizophrenia through both its pathophysiological influence (association with dopamine) and through association analysis (meta-analyses by Allen *et al.*, 2008).

The glutamate receptor (NMDA, *N*-methyl-D-aspartate) hypo-function hypothesis of schizophrenia is derived largely from observations of the effects of NMDA receptor antagonists (e.g. PCP and ketamine) and their effects on normal people and people with schizophrenia. Relevant animal models have generally supported the hypothesis. Mohn *et al.* (1999) for example created mutants with a 90% reduction in NMDA receptor 1 expression and demonstrated increased LMA and deficits in social and sexual behaviour. Hyperactivity (and to a lesser extent, the social deficits) were ameliorated by the antipsychotics haloperidol and clozapine.

1.6.2.3 Genetics mouse models for candidate genes discovered through clinical samples

The second main approach for genetic animal models, and the approach utilised in the present thesis, is based on manipulations of genes that have been implicated by traditional gene discovery approaches using clinical samples. Animal models for the majority of putative schizophrenia susceptibility genes have been created and tested, some with more success than others.

NRG1

Neuregulin 1 (*NRG1*) has been frequently implicated in the development of schizophrenia, is expressed widely throughout the brain and influences key neurodevelopmental processes such as myelination and neuronal migration (Buonanno, 2010).

Mouse models for *NRG1* are an interesting example and also serve to warn us of some of the difficulties inherent in characterising a mouse model for a complex disorder such as schizophrenia. The majority of NRG1 proteins are synthesised with a transmembrane (TM) domain. Mutant mice with a heterozygous mutation of the TM domain of NRG1 exhibited hyperactivity when examined in anxiety- and exploration-related tasks, facets which were reversed by clozapine. The mice also showed a PPI deficit, selective impairment in social novelty preference and altered patterns of social interaction (O'Tuathaigh *et al.*, 2007). Different types of NRG1 mutants show variance in the pattern of deficits observed. For example, type iii mutant mice (a mutant model of a different isoform) display more pronounced PPI deficits and WM impairment than the

mice described by O'Tuathaigh *et al.* (2007), suggesting the effects of specific mutation of NRG1 isoforms on behavioural phenotype may be masked by the compensatory activity of other genes or environment factors.

DNTBP1

Mutant mice for a schizophrenia candidate gene identified by positional cloning, *DTNBP1*, have also received substantial attention in the field. The Sandy (sdy) mouse (e.g. Murotani *et al* 2007, Bhardwaj *et al* 2008, Takao *et al* 2008, Feng *et al* 2008, Hattori *et al* 2008, Chen *et al* 2008) have a deletion mutation that arose spontaneously in the DBA/2J strain in the gene encoding dysbindin-1 (Dtnbp1). This mutation (Dtnbp1(sdy)) leads to an absence of dysbindin-1 protein in homozygotes, as well as reductions in protein levels of several direct and indirect binding partners of dysbindin-1. Neurobiological effects were also observed in the model with decreases in dopamine levels in the cortex, hippocampus and hypothalamus and some increases in dopamine metabolism (reviewed in Talbot, 2009). Behaviourally, the mice have shown delayed activity in novel environments, and reduced activity in the open field, suggestive of an increase in anxiety. Object recognition was also impaired as well social interaction and long-term and working memory.

DISC1

Various *Disc1* mouse models have also been characterised. These are a good example of a mouse model for schizophrenia due to the heterogeneity of the models in terms of the genetic manipulation. The 'natural' model for the gene exploits the mutation occurring in the 129 strain previously discussed. The

model displays working memory deficits, decreased hippocampal short-term plasticity and altered organisation of neurons in dentate gyrus (Koike *et al*., 2006, Kvajo *et al*., 2008). Clapcote *et al*. (2007) created two *Disc1* ENU mutants (that both showed reduced binding to the known binding partner, Pde4b) and found that one showed depressive-like behaviour (indexed by the forced swim task) and the other showed PPI and latent inhibition (LI) deficits that were reversed by antipsychotic treatment. These mutations have not been biochemically characterised so it is difficult to draw conclusions from this study but the data nevertheless suggest that a reduction in Disc1 can produce some deficits akin (arguably) to psychiatric disorders.

Four groups have now produced *DISC1* transgenic mice (expressing the human mutant form of the gene) Pletnikov *et al.* (2007), Li *et al.* (2007), Hikida *et al.* (2007) and Duan *et al.* (2007). Results between the groups are consistently supportive and all groups found that the mutation affects brain morphology (e.g. mutants have a mild enlargement of the lateral ventricles), as well as some cognitive deficits such as enhanced LMA, disrupted PPI and impaired social interaction. Findings from mouse models for *DISC1* are compatible with *DISC1* playing a role in the pathogenesis of schizophrenia (and other major psychiatric illnesses).

1.7 An ENU mouse model of *Zfp804a* gene function: strengths and cautions

The main aim of this thesis was to create and begin to characterise ENU-based mouse mutants of *Zfp804a*, the mouse orthologue of *ZNF804A*. Details of the specific procedures used and experiments undertaken can be found in the

relevant methods and experimental chapters of the thesis. However, it is important, at the outset, to be aware of the advantages and, in particular, the limitations of modelling human disorders in another species, in this case the mouse. As noted earlier, genetic animal models for schizophrenia are potentially extremely useful in schizophrenia research as they allow us to create mutant gene isoforms and study these effects *in vivo.* There are however, difficulties with genetic animal models for the psychiatric disorders.

First, the major psychiatric disorders are acknowledged to be genetically complex disorders with numerous genes of different effect size interacting in a complex network with each other, and with the environment. In a genetic animal model, in the near future at least, we are only able to model specific genes and their effects, possibly with some informative environmental interactions rather than the actual disorder, in other words, any model is only ever going to be partial. This is before taking into account the fact that psychiatric disorders are essentially 'human' problems, involving distortions in perception, thought, language, emotion and behaviour. It is important to state therefore, that we cannot, with our current understanding, create an animal that has schizophrenia. Indeed, it may be better to say that, as was the case in the present work, most animal models involve determining the functions of a gene involved in some way in the pathogenesis of schizophrenia.

Limiting the ambition of any animal model will hopefully inhibit over-interpretation of the clinical meaning of any experimentally-derived (endo)phenotypes. However, another problem with animal models for psychiatric disease is that processes and observable traits affected by disorders such as schizophrenia that we are able to observe in animals, such

as pre-pulse inhibition (PPI), are not specific to schizophrenia. People with bipolar disorder for example, also have PPI deficits (Thaker, 2008). Additionally, another problem with animal models for genes implicated in schizophrenia is that it is expected that (notwithstanding the above cautions) there will be at least some indications reminiscent of schizophrenia in the model. Given the genetic complexity of schizophrenia and the cascade nature of gene-gene interactions, gene-environment interactions and combinations of the two, a-priori it seems somewhat hopeful to expect that a mutant model of a single gene will have a significant deficit on a particular measure. Failing to find any deficits or abnormalities in a model does not necessarily provide evidence against the involvement of that particular gene in schizophrenia. It also does not mean that the gene being investigated has no impact upon the domains tested as there may be compensatory effects involved, for example, a paralog may take over function.

Despite the fundamental limitations noted above, it is possible to approach an understanding of the function(s) of a gene implicated in a complex disorder such as schizophrenia, and then make cautious connections with the disease condition itself. For example, by starting off with a gene that is definitively involved in schizophrenia – this was the logic in choosing to study *ZNF804A/Zfp804a* – and by using well-validated translatable assays, in particular at the psychological/behavioural levels of analysis. However, in addition to choosing informative genes and functional assays it is also necessary to be aware of a number of technical limitations and pitfalls inherent in using genetic animal models. These are considered in detail in the relevant sections of the thesis but one potential problem in the creation of mutant

models is linkage. Through the principle of linkage, the effects of a closely linked gene may be attributed to the mutation when the mutation is crossed onto a different background strain (Crusio, 2004); although the chances of this happening are small, it remains a consideration.

Epistasis may also be a problem. The effects of other alleles at perhaps unlinked locations may change the effects of a mutation. An example of this is Le Roy *et al.* (2000). *nNOS* knockout mutants showed an aggressive phenotype on a mixed 129S4/SvJae – C57BL/6J background which vanished after five generations of backcrossing onto the C57BL/6J background. This led to the conclusion that the *nNOS* knockout may only cause aggression upon an interaction with some variant in the 129S4/SvJae genome that is not found in the C57BL/6J background. The environment could also have a similar modifying effect to epistasis but it is rarely considered explicitly beyond controlling the environment so it is a homogenous as possible between experimental subjects. For example, Rampon *et al.* (2000) found that a null mutation in the gene for the NMDA receptor 1 subunit in area CA1 of the hippocampus caused learning deficits but no effect when the animals were raised in enriched environments.

1.8 Specific aims

The overall aim of the experimental work in the thesis was to create novel mouse mutants targeting *Zfp804a*, the mouse orthologue of human *ZNF804A* in order to be able to conduct basic functional studies examining the role of *Zfp804a* in brain and behaviour, and thereby gain information of potential relevance to how genetic variance in *ZNF804A* may contribute to risk for

schizophrenia. The main aim was to be achieved via the following practical objectives:

- To identify ENU-induced mutations in *Zfp804a,* in the MRC Mammalian Genetics Unit's archive of DNA from the offspring of ENU-mutagenised mice and select mutations of interest to be re-derived for investigation *in vivo* based upon the estimated impact on the structure and function of the Zfp804a protein.
- To backcross the re-derived mutations onto a C57Bl/6J wild-type background to isolate the mutations of interest and reduce the possibility that any observed phenotypes are due to other residual mutations.
- To assess the general viability of the mutant lines
- To begin the specific functional assessment of the novel *Zfp804a* mutated lines, focusing on behavioural endophenotypes

CHAPTER 2: General Materials and Methods

This chapter describes procedures performed throughout the course of work for this thesis including specific laboratory equipment and descriptions of any behavioural apparatus used. Further details are given in experimental chapters where appropriate. All procedures necessitating the use of live animals were performed in accordance with Animals (Scientific Procedures) Act (1986) and the Home Office Project Licence granted to Dr William Davies (PPL 30/2601). All animal work was performed under the Home Office Personal Licence granted to Tamara Al-Janabi (PIL 30/8107).

2.1 DNA extraction and quantification

A tail biopsy (following the protocol described in 2.7) was taken from experimental subjects for genotyping

2.1.1 DNA extraction

Tail samples (approximately 10mm) were lysed overnight in a solution of tail lysis buffer (0.2% SDS, 100mM Tris HCl pH8.8, 5mM EDTA pH8.0, 200mM NaCl) and 10µg/ml proteinase K in phosphate-buffered saline (10X, Sigma). For every 10ml tail lysis buffer, 50µl proteinase K was added, making enough solution to lyse 20 tail samples. This solution (500 µl/ 10mm tail) was added to tail samples and left in a waterbath overnight (14-18 hours) at 55°C.
The following day, samples were centrifuged for 10 mins at 13 000 RPM. Supernatant was removed and aliquoted into fresh tubes. The pellet of fur left at the bottom of the original tube was discarded. 500 µl per tail of isopropanol

were then added and samples were stored at 4-8°C for 20 minutes. Precipitated DNA was then fished out of the solution using a pipette tip and put into 100 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA). If the DNA was not visible or was unable to be retrieved by this method, samples were centrifuged for 10 minutes at 4°C at 13 000 RPM, the supernatant pipetted off (and discarded), and allowed to air dry until the DNA became 'glassy' in appearance and no obvious liquid could be seen. 100 µl of TE buffer was then added to dissolve the DNA. Genomic DNA was refrigerated (4-8°C) until use. Once used for PCR genotyping, DNA was stored at -20°C.

2.1.2 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 contaminating RNA and protein. The spectrophotometer calculates a concentration (in ng/μl) for the sample based on the UV absorbance.

2.2 Mutation detection

Mutation screening was performed on 5 856 samples of DNA obtained from MRC Harwell Mammalian Genetics Unit. DNA was received at a working dilution of 5ng/μl and corresponded to the first 5 856 samples in the ENU mutagenesis DNA archive for genotype-driven screens.

2.2.1 Mutation detection PCR

Standard PCRs were performed to screen for mutations in *Zfp804a* in 2 regions using 3 sets of primers. Primer sets 2 and 3 overlap to cover a larger region of exon 4 than is possible to reliably amplify in a single PCR. Oligonucleotide PCR primers used in this study were designed *in silico* using the online program Primer3 (http://frodo.wi.mit.edu/primer3/). PCR primers used in this thesis were synthesised by Metabion unless otherwise stated. The following primer pairs were used in this study (for PCR optimisation methods, see Chapter 3: Mutation detection in *Zfp804a* in ENU-mutagenised mouse DNA):

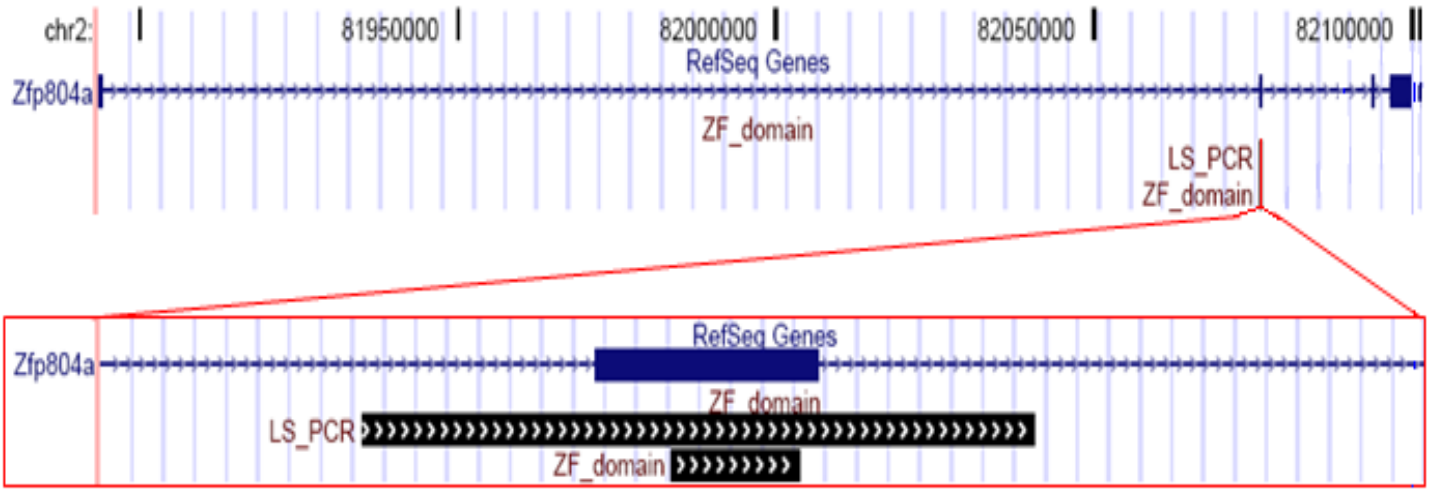
Zfp804a C2H2 G2: tcatgttttcaaacttgttttgctg
Zfp804a C2H2 R2: aaacttgattcatcatcacagaattac

Zfp804a SZ 1A F: CAGCGATGAATCCTCTATGG
Zfp804a SZ 1B R: TTGTGATGTTTTCCTCTGTGG

Zfp804a SZ 2B F: CAAATATCATAGAAAAGAATCCCACTG
Zfp804a SZ 2A R: CCTTCTGTTCAGAGAAAAGGTCA

Figure 2.1 below shows the position of the amplimeres in *Zfp804a* in relation to the the gene and exons 2 and 4. Diagram b) also shows the position of the zinc finger binding domain in relation to exon 2.

(a)



b)

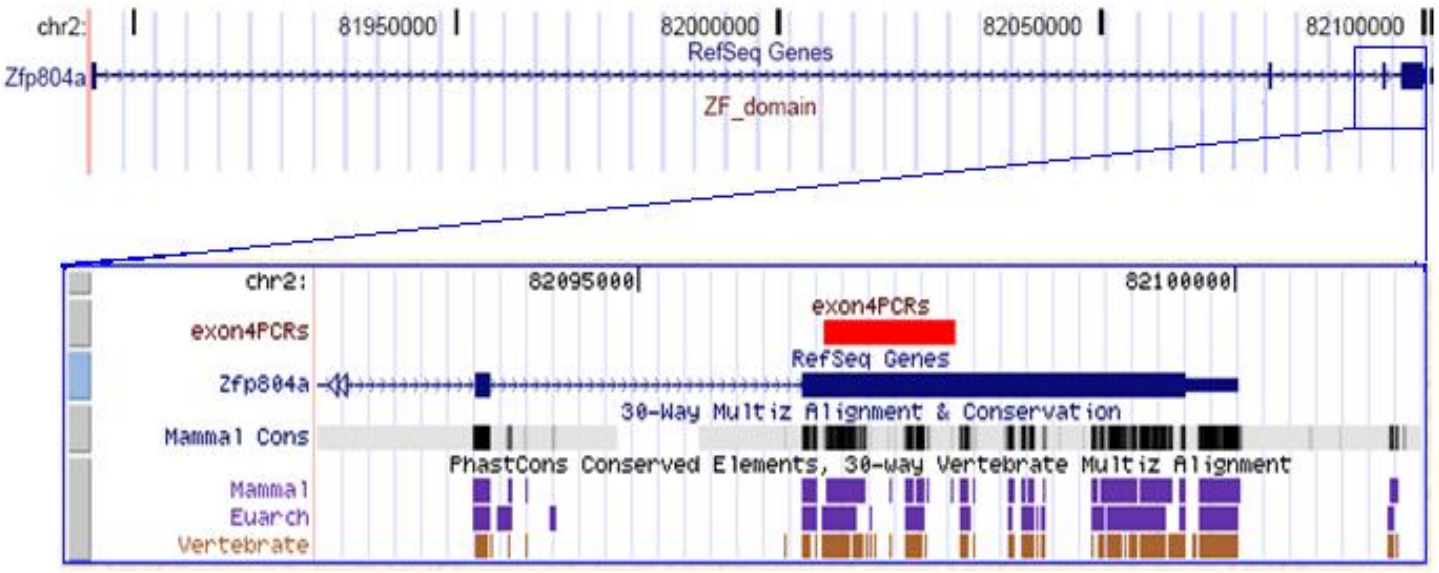


Figure 2.1: A schematic diagram to illustrate the location and coverage of genomic regions screened of Zfp804a for mutations. The gene has 4 exons, denoted by short, dark, blue lines with the exception of exon 4, a larger exon, indicated by a blue square. Exon 2 is magnified in the red box in image a) and exon 4 is magnified in the blue box in image b). (a) shows the region covered by PCR spanning the C2H2 domain of Zfp804a; (b) shows the coverage of the PCRs covering part of exon 4.

PCRs were performed in Framestar 96-well plates (black frame with white wells for imaging on the LightScanner) in a 12μl reaction using 3μl of genomic DNA (at 5ng/μl), 0.28μl of each primer (at 5pmol), 0.96μl dNTPs (5mM) each, 1.2μl LC Green ($MgCl_2$) buffer, 1.2μl LC Green dye, 0.1μl HotStar Taq (Qiagen) and 4.98μl of water. Framestar plates, LC Green and LC Green buffer were all purchased from Cadama Medical Ltd (recommended for use with the LightScanner). HotStar Taq was used as it has a chemically modified site that prevents activity until it has been heated for 15 minutes at 95°C. This prevents non-specific elongation by primer binding as the temperature increases, therefore annealing will only occur at the correct temperature as the reaction cools.

PCR:

1. 94°C for 15 minutes
2. 95°C for 20 seconds
3. 60°C for 30 seconds
4. 72°C for 45 seconds
5. Repeat steps 2-4 for 44 cycles
6. 72°C for 3 minutes
7. 4°C for 10 minutes

Following the PCR, 15μl of Mineral Oil (Sigma) was added to overlay the reaction and prevent evaporation during LightScanner analysis.
Plates were then analysed using High Resolution Melting Analysis (HRMA) on the LightScanner. This involved following the SOP of heating the reactions on a

gradient from 70°C to 98°C (to denature the synthesised DNA) whilst the fluorescence given off is measured. LightScanner analysis (including repeating the PCR) was repeated for samples showing a variant profile on initial analysis. Samples showing a variant profile on two separate LightScanner analyses were sequenced.

2.2.2 Sequencing

All sequencing was performed using the fluorescent Sanger sequencing method via Big Dye termination chemistry (Applied Biosystems) and analysed using the ABI3100 PRISM Genetic Analyser. The fluorescent sequencing reaction involves obtaining large amounts of template DNA (i.e. by PCR) 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 produces a series of DNA fragments where the chain growth has been terminated at each successive position. When electrophoresed in a capillary sequencer and detected by a laser, each base of the sequence will be fractioned by size and fluoresce according to the base at that size. Sequencing required a 12μl PCR of the genomic region to be sequenced. PCRs performed for LightScanner analysis were used for this purpose. The method was automated using a Beckman-Coulter NX liquid handler (programs were written by Sarah Dwyer).

2.2.2.1 PCR clean-up

Clean-up removed unincorporated dNTPs, primers, DNA, polymerase and salts from the PCR. PCR product is mixed with 21.6 AMPure reagent (Agencourt)

per sample. The AMPure contains metallic beads which adhere to the amplimers. Solution and products that are not adhered to the magnetic beads are washed off and removed using successive 85% ethanol wash steps. PCR amplimers are then eluted in 195μl of pure water into a new plate.

2.2.2.2 Sequencing reaction

The cleaned PCR product was added in a 5μl volume to a 5μl reagent mix consisting of 0.166μl of Big Dye termination mix, 1.917μl water, 1.917μl Big Dye sequencing buffer and 1μl of either the forward or reverse orientation oligonucleotide primer used in the original PCR (3pmol/μl). The sequencing reactions were performed on a thermocycler under the following conditions:

1. 96°C for 2 minutes
2. 96°C for 30 seconds
3. 55°C for 15 seconds
4. 60°C for 4 minutes
5. Repeat steps 2-4 for 24 cycles
6. 4°C for 4 minutes
7. 15°C for ever

2.2.2.3 Post-sequencing clean-up

Post-sequencing clean-up employs a CleanSEQ chemistry protocol (Agencourt) that is semi-automated. The process removes unincorporated dye terminators and further contaminants. Sequencing reaction product (10μl) is added to 10μl CleanSEQ reagent and 41.59μl of 85% ethanol and aspirated to

mix. The sequencing product binds to the magnetic beads contained in the CleanSEQ reagent. The non-bound sequencing reaction contaminants are washed off and removed in successive 85% ethanol wash steps. The cleaned sequencing product can then be eluted in pure water (75μl) and is ready for analysis using a capillary sequencer.

2.2.2.4 Sequencing analysis

The raw data generated by the ABI3100 PRISM Genetic Analyser is automatically analysed by Sequence Analysis Software (Applied Biosystems). The software calls the fluorescence at each nucleotide as the corresponding base. Sequencher software (Gene Codes) was then used, which aligns multiple sequencing traces and allows comparison with a reference sequence. The software then highlights where differences occur between the traces, allowing the user to manually inspect these and judge whether a polymorphism exists.

2.3 Generating the mutant lines

Mice were re-derived by *in vitro* fertilisation (IVF) using frozen sperm from an archive corresponding to the DNA archive screened (see Appendix 8.2). All IVF was performed by the MRC Mammalian Genetics Unit at Harwell. Genotyping and sequencing was performed as in 2.2 above except LC Green omitted and standard 10X buffer was used. The LC Green was replaced with double-distilled water.

2.3.1 Mouse LD Linkage Panel

The extent to which the mutations had been successfully backcrossed onto C57BL/6J background was estimated using the Mouse LD Linkage Panel (Illumina). The Mouse LD Linkage Panel consists of 377 SNPs that are polymorphic between different strains. The Mouse LD Linkage Panel is designed to include approximately four SNPs per 27 Mb interval and covers the entire genome. At least one SNP in each interval was selected by Illumina to be informative in crosses involving the C57Bl/6J strain. On average, at least one SNP in each interval is informative for 85% of all possible strain combinations. The linkage panel uses the GoldenGate Genotyping Assay (Illumina), a pre-optimised assay that uses a discriminatory DNA polymerase and ligase to interrogate multiple SNP loci simultaneously. Figure 2.2 gives an overview of the methods employed for genotyping SNPs in the panel. Data analysis was conducted using Microsoft Excel to count alleles corresponding to parental strain genotypes. Further details of the genotyping and data analysis methodology are given in Chapter 6: 'Generating the 59X and 417Y lines'.

Before embarking on the protocol for the Mouse LD Linkage Panel, DNA was normalised to 50ng/μl using PicoGreen (Invitrogen) and a fluorimeter Fluoroskan Ascent (Thermo Labsystems) to quantify the DNA. The PicoGreen reagent interacts with double-stranded DNA specifically (and not single-stranded DNA or RNA) and is therefore more accurate than DNA quantification by spectrophotometry. Samples were first diluted to less than 100ng/μl in sterile water, based on the concentration calculated using the spectrophotometer. The sample was then diluted to 1% in a 1 x TE buffer in a white 96-well cliniplate (Thermo Labsystems). A working PicoGreen solution is produced by adding 5μl

PicoGreen at 200x to 995μl of 1 x TE buffer. The fluorimeter dispenses 100μl PicoGreen working dilution into each sample and measures the DNA concentration using a UV excitation wavelength of 485nm and an emission wavelength of 538nm. The DNA is quantified by comparison to the gradient of a standard curve which can be generated each time by the user using the calibrant (100ng/μl) DNA standard provided, or compared to a standard curve generated recently using the same kit (by Liam Carroll).

Figure 2.2: Overview of Mouse LD Linkage Panel genotyping protocol using GoldenGate Asssay (Reproduced from www.illumina.com)

2.4 Subjects, animal husbandry and general procedures

Once created, subjects were bred in the Behavioural Neurosciences Laboratory, School of Psychology, Cardiff University. All experimental mice were group housed in cages of 2-5 subjects except where fighting or bullying necessitated single caging. Cages were environmentally enriched (with chew sticks and cardboard tubes) and standard laboratory food and water were available *ad libitum* unless otherwise stated. Home cages were cleaned weekly by laboratory staff (not prior to testing on that day however). The light-dark cycle of the holding room was 12 hours with lights on at 07:00 and lights off at 19:00.

Experimental animals were regularly monitored for signs of ill health. Any mice showing signs of illness were immediately assessed by the Named Animal Care and Welfare Officer and, if necessary, the University's vet. If necessary mice were withdrawn from the experiment and treated or sacrificed, depending upon the nature and deemed severity of the illness.

2.4.1 Habituation to handling, test environment and procedures

Subjects were accustomed to being handled daily for two weeks prior to behavioural testing (approximately 1 minute per mouse, per day). Body weight measurements were also taken during this time

2.4.2 Body weight measurement

Body weights were recorded regularly (once per week after initial 2 week period prior to behavioural testing). Weights were taken at the same time each day (approximately 17:00).

2.4.3 Vaginal smearing

Vaginal smearing was performed with a dampened cotton wool swab, and smears were stained in a 0.1% cresyl violet solution for at least 10 minutes. The procedure was performed several times during the first two weeks of handling and immediately following a behavioural procedure thereafter. The swabbing was extremely quick and as uninvasive as possible and did not appear to cause discomfort to the mice. Smears were classified into three types, indicating dioestrus, proestrus and oestrus stages of the oestrus cycle (Fig 2.3).

Figure 2.3: Left-Right: Dioestrus cells, characterised by their small, dense structure. Proestrus cells, characterised by their compacted nuclei. Oestrus cells, characterised by their large size, not compacted nuclei and straight edges. Figure adapted from Mettus and Rane (2003).

2.4.4 Behavioural testing environment

All behavioural testing took place in an air-conditioned testing room adapted solely for this purpose where lighting levels could be adapted to the requirements of the specific experiment. Although humidity and temperature were not strictly controlled as they were in the animal holding rooms, these parameters were generally maintained around 50% and 21°C respectively. Disturbances created by background noise were kept to a minimum and entry to the testing rooms was prohibited whilst experiments were taking place. Testing rooms were cleaned at the end of each day.

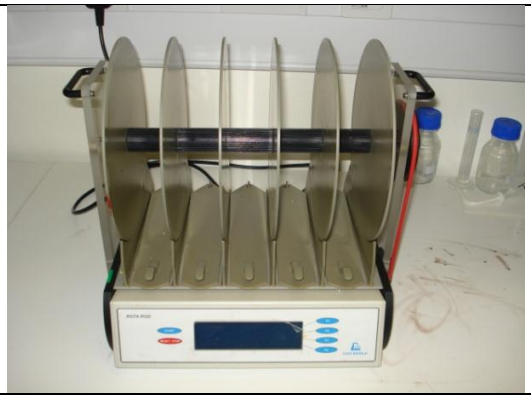
2.4.5 Phenotypic testing: control measures

Methods of production employed in generating the C59X and C417Y mice meant that each line had a different array of residual mutations due to the nature of ENU mutagenesis. We overcame this by breeding the C59X and C417Y mutations onto the C57Bl/6J strain (a strain not used in the ENU mutagenesis) where the possibility of any 2 mice in the same group (genotype, line and gender) harbouring the same residual 'other' mutation was no longer deemed to be likely (discussed further in Chapter 6). In addition to the breeding control measures described above, numerous other control measures were used to ensure consistency of the data. Behavioural testing always took place in the daytime (coinciding with the light phase of the holding room). If the experiment required more than a single test session per subject, subjects were tested at the same time each day, in order to minimise any 'time of testing' effects. 'Order of running' effects were negated through running experimental and control subjects in a pseudorandom order. In order to minimise possible cage/litter effects, experimental subjects were drawn from as large a number of cages/litters as possible. No single-caged animals were used in the production of data from behavioural experiments for this thesis and in every cage there were at least two different genotypes. In addition to these precautions, the data was routinely screened for any such effects; in no experiment in this thesis was there any evidence of systematic differences, which could be attributed to these general factors.

2.5 Behavioural apparatus

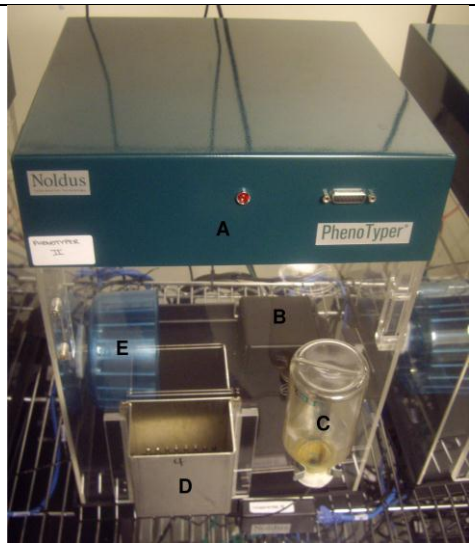
2.5.1 RotaRod

Mice were tested on a 5-lane accelerating RotaRod (Ugo Basile) with a ribbed rubber coating on the rotating rod. Each compartment was separated by a wheel and sensors detected when the mouse fell from the rod and stopped the time. Fixed-speed or accelerating sessions were programmable on the equipment

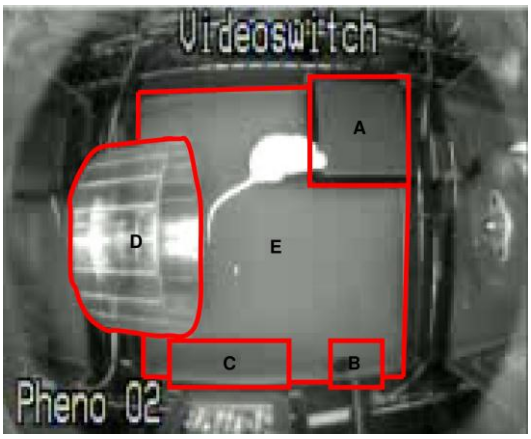


2.5.2 Phenotyper cages

The PhenoTypers (Noldus Instruments) consisted of a Perspex square-base home cage environment with an overhead camera and tracking device (in A). Cages were furnished with wood shaving bedding, a shelter (B), a food hopper (D), a running wheel (E) and a water bottle (C). Mice spent 12 hours in darkness and 12 hours in light in the cages. Cages were separated by a black opaque sheet of card placed between outside walls so mice were unable to see neighbouring mice. Image reproduced with permission from Trent *et al*. (2012)



Plan view of the Phenotyper homecage showing the zones used for data analysis. (A) hidden shelter, (B) drinking zone, (C) feeding zone, (D) exercise wheel, (E) arena floor – general activity. All zones combined comprised the tracking arena. Image reproduced with permission from Trent *et al*. (2012)

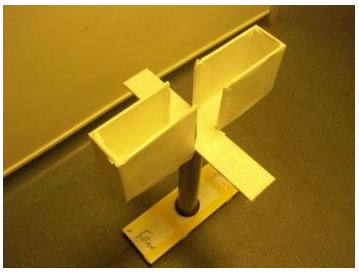


2.5.3 Locomotor activity boxes

Mice were tested in a set of 12 clear perspex boxes (width x length x height = 215mm x 350mm x 200 mm) with two infrared beams crossing each box 30mm from each end and 10mm from the floor of the cage. Beam breaks were recorded by a computer using custom-written programs run by ARACHNID (Cambridge Cognition Ltd., Cambridge, UK)

2.5.4 Elevated plus maze

The elevated plus maze comprised two open arms (length x width = 180mm x 80 mm) and two enclosed arms (length x width x height = 180mm x 80mm x 160mm) with an open roof. The maze was coated in white Benchkote (Camlab) which could be wiped clean and enabled tracking of the contrasting (black or brown) mouse. The plus maze was elevated 500mm from the floor and illuminated by a 60 watt bulb.



2.5.5 Open field apparatus

The open field apparatus was a white perspex walled box (length x width x height = 750mm x 750mm x 470mm). The open field was subdivided into 'centre' (a circle, located centrally, diameter 120mm), 'inner' (a square surrounding the circle measuring 450mm x 450mm) and 'outer', the remainder of the open field.

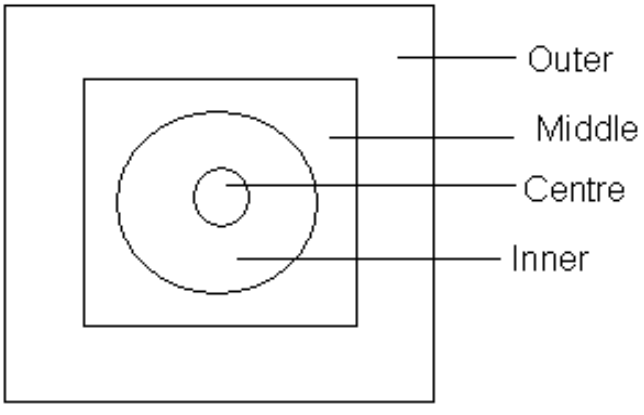


Figure 2.4 Photographs and summaries of the behavioural apparatus used. Theschematic diagram (left) shows the division of the zones in the open field.

2.6 Tail biopsy

A tail biopsy was taken from animals for DNA extraction. Animals were restrained and the tip of the tail was anaesthetised using a freezing anaesthetic spray. Approximately 10mm was removed from the tip of the tail using a sharp pair of scissors and the animals were returned to the home cage and later checked for excessive bleeding and general well-being. The procedure was extremely quick and did not appear to cause excessive stress or discomfort to the animal. Biopsies were taken in accordance with Animals (Scientific Procedures) Act (1986) and relevant Home Office licences.

2.7 Culling protocol

At the end of the breeding/experiment, or if persistent illness/injury occurred, subjects were culled through cervical dislocation or suffocation by CO_2 in accordance with Schedule 1 of Animals (Scientific Procedures) Act (1986). A tail biopsy of experimental animals was taken at death to confirm the genotype of the animal.

2.8 Brain extraction

Brains were obtained from sexually-naïve subjects previously used in behavioural experiments. Immediately after culling, the head was removed and the brain manually extracted. Brains were immediately weighed before being frozen in liquid nitrogen prior to storage at -80°C.

2.9 Data presentation and statistical methods

Behavioural data are presented either as mean values ± standard error of the mean (SEM), calculated from the following formula:

$$\text{Standard error of the mean} = \frac{\text{standard deviation of values}}{\sqrt{\text{number of values}}}$$

or as mean values together with an overall standard error of the differences of the mean (SED). The relevant formula for the calculation of the SED is given in Cochran and Cox (1957). As this parameter may be used as the denominator for *post hoc* statistical comparisons, it represents an appropriate comparator for the visual evaluation for the difference between two mean values.

Behavioural data were analysed using SPSS (SPSS for Windows, Rel. 16.0 2007. Chicago: SPSS Inc.) Data were subject to ANOVA and ANCOVA and MANOVA. All genotype and gender comparisons were planned *a priori*. For all comparisons, p values of <0.05 were regarded as significant.

CHAPTER 3: Mutation detection in *Zfp804a*

3.1 Introduction

The general introduction discusses how O'Donovan *et al*. (2008) implicated *ZNF804A* in the pathogenesis of schizophrenia in their large-scale genome-wide association study ($p=1.61 \times 10^{-7}$ in meta-analysis). The association of this gene has since received support from several independent studies (Riley *et al.,* 2010; Steinberg *et al.,* 2010; Zhang *et al.*, 2010, Williams *et al.*, 2011), with Williams *et al.* reporting more significant results at $p=2.5 \times 10^{-11}$. *ZNF804A* appears therefore, to be a true susceptibility locus for schizophrenia (O'Donovan *et al.*, 2009); however, at present very little is known about the function of the gene, or how it may confer risk for schizophrenia.

As noted previously, the structure of the ZNF804A protein is largely unknown with the only characterised region being a cysteine$_2$-histidine$_2$ (C2H2) zinc finger binding motif occurring early on in the protein (aa57-81 of 1200 in the mouse; discussed in 1.5.2.2). This domain binds DNA (or less frequently RNA) and is the most common DNA-binding domain in transcription factors (Wolfe, Nekludova & Pabo, 2000). Some recent studies have been published investigating the biology and putative function(s) of *ZNF804A* (Donohoe *et al.,* 2010, Riley *et al.,* 2010 and Chung *et al.*, 2010, Williams *et al.* 2010) and gene variants has also been shown to have a role in human cognition (Esslinger *et al.,* 2009, Walter *et al.*, 2010, Walters *et al.* 2010).

As discussed earlier, one way of investigating the function of a gene is using an animal model. Animal models are useful for studying gene function for

several reasons. They allow us to manipulate a gene and observe associated alterations in biology, biochemistry and behaviour in ways that we are not able to in human subjects. There are currently no *in vivo* animal models available for examining *ZNF804A* function. An animal model of the function of a given gene can only be created if that organism has an orthologue of the gene. Orthologues are equivalent genes (i.e. similar function and high similarity between sequences) between species.

The mouse orthologue of *ZNF804A, Zfp804a*, is located on chromosome 2 (2qD) and spans 206kb. The gene has one protein coding isoform (NM_175513.3 (81893815-82100036), ID: 241514, RefSeq) and 4.1kb of coding sequence from 4 exons. The Zfp804a protein is 1200 amino acids. Bioinformatic analysis of Zfp804a *in silico* has shown the protein to have a cysteine$_2$-histidine$_2$ (C2H2) zinc finger binding motif at approximately positions 57-81 (positions vary ±2 depending on resource), encoded in exons 2 and 3 (ProSite, PFam, EMBL-EBI, all programs available online). No other domains or features in the protein are currently characterised.

There are several different methods of creating a genetic mutant mouse model. *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis is one of the most efficient mutagens available; it induces point mutations at a rate far higher than any other chemical mutagen in a random, unbiased fashion (Hitotsumachi, Carpenter & Russell, 1985). As the mutations are random, this gives rise to a multiplicity of potential models including the loss of gene function (by the introduction of a null allele), partial loss of function (hypomorphic) or gain of function (hypermorphic). ENU can therefore produce more varied and subtle

genetic mutants than can be achieved by other methods of gene manipulation (such as knockouts and transgenics; Godinho & Nolan, 2006).

ENU is a chemical mutagen, an alkylating agent that acts by transferring the ethyl group to individual nucleobases, with a preference for thymine (Cordes, 2005). During DNA replication, these alkylated nucleobases are mis-read by the DNA polymerase, resulting in errors being incorporated into the DNA. The resultant mutant DNA may or may not result in a functional mutation, a mutant protein, depending on the position and nature of the point mutation (Bielas & Heddle, 2000, O'Neill, 2000, Acevedo-Arozena *et al*., 2008). ENU mainly targets the spermatogonial stem cells (which later become mature sperm), making the chemical an ideal agent to cause heritable point mutations (Cordes, 2005). Russell *et al.* (1979) found ENU to be more effective than other mutagens such as x-rays and procarbazine producing a higher maximum number of mutations and being simpler to administer. Hitotsumachi *et al.* (1985) showed that repeated but lower (100 mg/kg, weekly for 3 to 4 weeks) doses of ENU yielded a higher rate of mutations than a single dose of 250mg/kg.

The MRC Mary Lyon Centre, Harwell has compiled a DNA archive from mice treated with the ENU mutagen over the last decade (Coghill *et al*., 2002). The archive currently consists of over 12 000 DNA samples from ENU-mutagenised male mice and is paralleled by a frozen sperm archive (Dr Pat Nolan, MRC Harwell, personal communication). Quwailid *et al*. (2004) estimated the mutation rate in the samples by performing a PCR-based screen of a gene covering 27.4mb of DNA. Using denaturing high-performance liquid chromatography (DHPLC, discussed later) to detect mutations, Quwailid and

colleagues found an overall mutation rate of 1 in 1.01 Mbp of which 1 in 1.82 Mbp were non-synonymous. The study supports the idea asserted in Coghill *et al*. (2002) that if a sufficient length of DNA is screened in a sufficient number of samples, then the chances of finding a mutation that is likely to affect the structure and/or function of the protein that gene encodes is very high.

There are two different approaches to using ENU mutagenesis for investigating gene function. Godinho and Nolan (2005) describe the phenotype-driven screening approach used at the MRC Harwell Mary Lyon Centre. Mice with unknown mutations are tested using standardised behavioural tasks. Those mice showing a phenotype in the tasks are backcrossed, selecting for the phenotype, onto a wild-type background to isolate mutations and positional cloning and candidate gene analysis are then used to identify the mutation that is believed to be causing the phenotype. The other type of approach is the genotype-driven approach. In this approach, a known candidate gene or genomic region is screened for potentially functional mutations. Once mutations have been selected mice can be re-derived carrying this/these mutation(s) and then phenotyped using behavioural tasks etc. Clapcote *et al*. (2007) created two *Disc1* ENU mutants from mutations discovered in exon 2 of the gene using TGCE heteroduplex analysis. Mice carrying the mutations were bred for four generations onto a wild-type background and then intercrossed. The cohort was then characterised for schizophrenia- and depression-like behaviours.

In the experiments described in this chapter, mutations were detected in *Zfp804a* using high resolution melting analysis (HRMA). HRMA is an effective means of mutation detection and can be used as part of a two-tier strategy to identify samples carrying putative mutations in the first instance.

These samples can then be sequenced (as sequencing all samples would be prohibitively expensive and time-consuming) (Dwyer *et al.* 2010). HRMA is based upon the idea that melting temperature of DNA (i.e. the temperature at which the DNA denatures) depends, at least in part, upon the GC/AT ratio, length and sequence of the DNA (Ririe *et al.* 1997). In the presence of a fluorescent dye that binds specifically to dsDNA (such as LC Green), differences in melting temperature (T_m) between DNA samples can be detected by measuring the fluorescence given off as the samples are heated (and therefore denatured) on a temperature gradient. Graham *et al.* (2005) demonstrate that not only can differences in T_m be produced by samples differing in sequence, but that these T_m differences also produce difference size and shape melting curves when plotting, allowing quick visual examination of data.

The LightScanner™ (Idaho Technology, Utah) operates on the principles described above. A temperature-controlled platform and fluorescent-sensitive camera are combined with Call-IT™ software (Idaho Technology) that automatically plots a graphical representation of the fluorescence given off by a sample as the DNA is melted, a “melting curve”. The software also calculates “difference curves” showing the differences between samples in melting temperature (Figure 3.1). The LightScanner™ platform has been successfully used for mutation detection previously (e.g. Kennerson *et al.*, 2007, Chen *et al.*, 2009, De Leeneer *et al.*, 2009, Li *et al*., 2009, Dwyer *et al.* 2010).

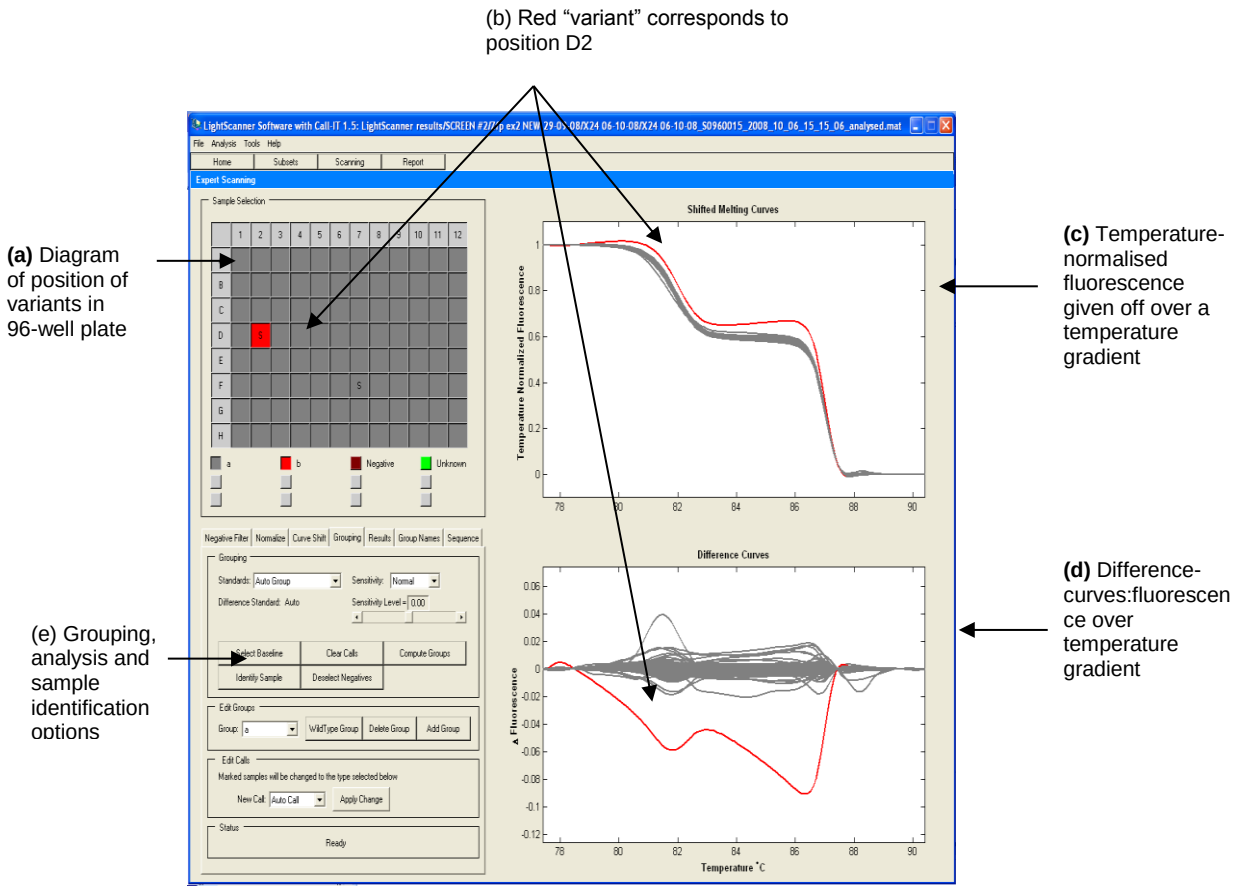


Figure 3.1. Diagram to show melting curves and difference curves output from LightScanner Call-It software. (a) indicates the diagram generated by the software showing the position of all the variants in a 96-well plate layout; (b) points to the sample coloured in red, identified as a ‘variant’ and shows the position of this variant in the 96-well plate, the melting curves and the difference curves; (c) shows a graphical representation of the temperature-normalised fluorescence given off over a temperature gradient by all samples (variant in D2 in red); (d) shows the difference between the melting curves in fluorescence given off over a temperature gradient and (e) indicates options for grouping, analysis and sample identification manual options.

This chapter describes the screening of an ENU-treated DNA library for mutations in the mouse orthologue *Zfp804a* of the human gene *ZNF804A*, and the selection of two mutations to carry forward for functional characterisation.

3.2 Materials and methods

3.2.1 Samples

All (5, 856) DNA samples involved in the screen described in this chapter were obtained from MRC Mammalian Genetics Unit, Harwell. The DNA was extracted (using a DNeasy Blood & Tissue Extraction kit, Qiagen) from liver taken from animals which were male C3H/HeH x BALB/c offspring of mice injected with the ENU mutagen. At the MRC Centre in Harwell, three weekly doses of 100mg/kg of ENU were injected intraperitoneally into both BALB/c male mice from 10 weeks of age. Following a period of sterility these mice were mated to C3H/HeH females. The male progeny were used to provide DNA and sperm for the archive. DNA was at a working concentration of 5ng/µl. (Quwailid, *et al.* 2004). All DNA samples (and corresponding sperm samples) were frozen between 17-10-2001 and 20-01-2004.

3.2.2 Regions selected for screening

Two areas of *Zfp804a* were selected for mutation screening: exon 2 encoding the only characterised domain of the Zfp804a protein and a region of exon 4 that is highly conserved that several non-synonymous SNPs were found in by Dwyer *et al*. (2010). Figure 3.2, below, shows a diagram of the Zfp804a protein with the C2H2 domain highlighted in pink. The Zfp804a (and ZNF804A) protein is largely uncharacterised. The only characterised domain is the C2H2 zinc finger binding motif, one of the most common DNA-binding domains found in transcription factors (Wolfe, Nekludova & Pabo, 2000). These proteins typically consist of several fingers that make parallel contacts along the DNA. Each

finger has a ββα structure where the α-helix is the point of contact with the major groove of the DNA. Each of these fingers has a single zinc ion that is coordinated by two cysteine residues at one end of the two anti-parallel β-sheets and two histidine residues at the C-terminal of the α-helix. A C2H2 zinc finger spans approximately thirty amino acids: (F/Y)-X-C-X_{2-5}-C-X_3-(F/Y)-X_5-ψ-X_2-H-X_{3-5}-H where X represents any amino acid and ψ is one of the hydrophobic residues (Wolfe, Nekludova & Pabo, 2000). Bioinformatic analysis (using PFam, ProSite, UniProt and EMBL-EBI) has shown the zinc finger binding domain to span exons 2 and 3. The cysteine and histidine residues that bind the zinc ion are both coded for in exon 2 of the gene so screening was commenced in this region.

MECYYIVISSTHLSNGHFRNIKGVFRGPLSKNGNKTLDYAEKENTIAKALEDLKAN**FYCELCDKQYYKHQ**
EFDNHINSYDHAHKQRLKELKQREFARNVASKSRKDERKQEKALQRLHKLAELRKETVCAPGSGPMFKST
TVTVRENLNDVSQRESVDPINNQQDLIPSEEKERDGTTALAETETASNCTANNCQIGDQSQAVHRHRIGF
SFAFPKKATVKLESCSAAAFSEYSDESSMEKEFSRKTRFVPSTSHLQLPPPTCELLSSEEKGNSPPPEAM
CTDKATAQTEERKITSNENNTLLTSSFCQLQHYIPTCSEADTCQNLAPFEDQLPMEAVIVNEDGPVSKSN
PNIIEKNPTVPNDRTSAQMTTEENITINDVTKMETRNKIDHEPLTPSSTIEESIRLQKRPDLCKRQCDPF
VPVLNKLGSTVLQWPSEMLAYTTTEPSISYSCNPLCFDFKSTKLNNNQDKNKLTLNDLFSEQKEDCCKGA
HADCKDVPIARVTNDETGHSKNDYPQVTTLSPVHVLSNGCDLGQNENVGQRYKHISCTNRQTNKYKFTRC
QIKRDMLNEKYDKMRLKETREHWFHKSRRKKKRRKLCRYHPGKSSKEPEGSGKTERSRQRTDEARKNPRE
PVLEKHPRSPERASDLHQLPDERPKAASTHLGEKETMNTTVNTESNDAAPGSQNCGGKNATVVNEQAKPL
VIHSVKQNLTYVRTYCCWKARTSRYQEDDGSLASQSNVKGPTQNQPVKRGYSSLTNDSERIHRKRRQHSC
SYSSDESLNQQHHLGEYLKPLSTSLISCQPKKKRRRKRSRLHIGDGTTKMKGNSNYPMKCSSLSQPDELA
KDCIKED
INPQENVSIEKNSEQTEQTEIKGMLHPYNPLLSEPSGEGEHSVTETTPCDSSQTSNDLATPVNVTRDPSN
STTDNTLLEHNQRSQTTNSNEKQTPFKVTNPERNFRHSQAKSYICRYELAETIPQGKTNEASTEWLCYNS
GILNTQPPLQFKEAHVSGHAFVTTEQILAPLPLPEQALLIPLENHDKLKHLPCEVYQHIIQPNMLTNKVK
FTFPPPPLPPPSTPVQPLPLQRPFCSTSVTTIHHTVLQHHAAAAAAAAAAAAAAGTFKVLQPHQQFLPQVP
ALARTSIPQISVGTVGPRLCPGGQPALVASPQMPIIPASVLHPSPLAFPPLPHSFFPSLLSPHPTVIPLQ
PLF

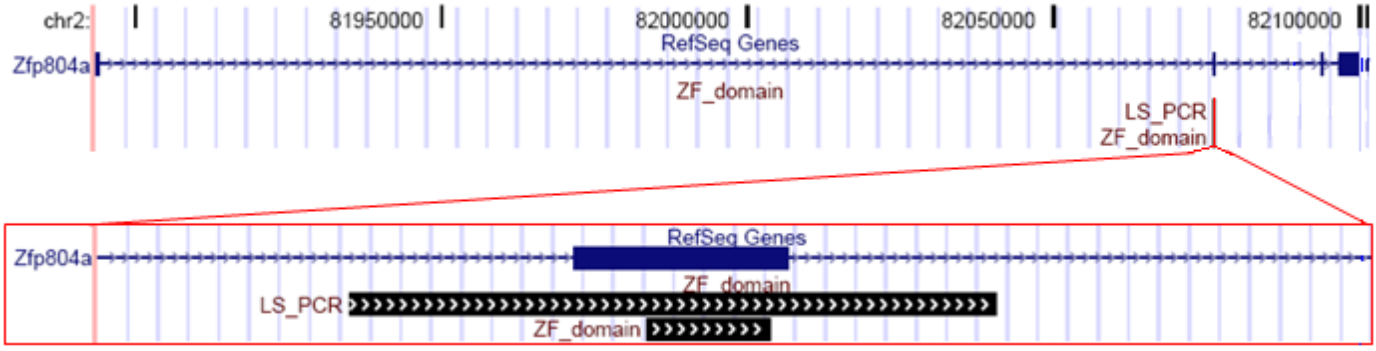
Identity between mouse and human, whole protein: 66.5% identity in 1212 residues overlap

Identity between mouse and human, C2H2 domain: 100%

Figure 3.2. Mouse Zfp804a *protein sequence. C2H2 zinc finger domain in* Zfp804a *(highlighted in pink). Percentage identity between the mouse and human for the whole protein sequence of* Zfp804a/ZNF804A *and for the zinc finger domain is also given*

The second region selected for screening was identified as a particularly well-conserved region of the protein and to potentially provide a contrasting model to an exon 2 mutant line. This region of exon 4 of the gene was identified as rich in non-synonymous SNPs with variants found in both cases and controls (Dwyer *et al.*, 2010). It was not practical to screen the entire gene although a comprehensive screen would have been optimal. Figure 3.3 below shows a schematic diagram of the genomic regions amplified by PCR.

(a)



(b)

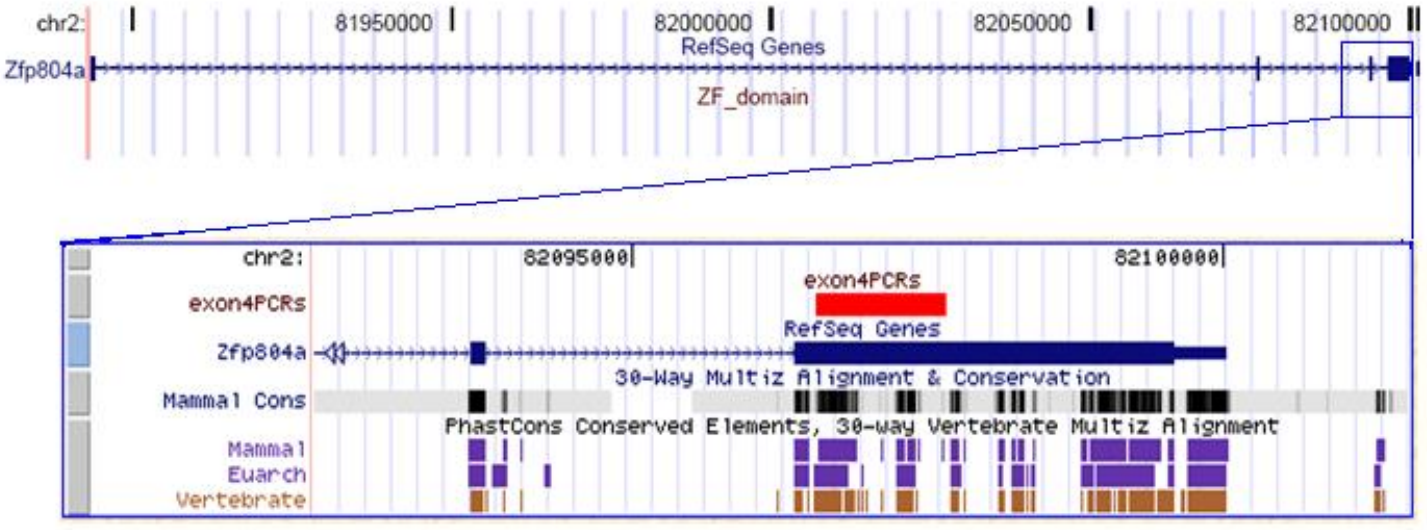


Figure 3.3. A schematic diagram to illustrate the location and coverage of regions screened of Zfp804a for mutations. The gene has 4 exons, denoted by short, dark, blue lines with the exception of exon 4, a larger exon, indicated by a blue square. Exon 2 is magnified in the red box in image a) and exon 4 is magnified in the blue box in image b). (a) shows the region covered by PCR spanning the C2H2 domain of Zfp804a; (b) shows the coverage of the PCRs covering part of exon 4.

3.2.3 Primer Design

Oligonucleotide primers were designed using software available online, Primer3 (http://frodo.wi.mit.edu/primer3/). Primers were all designed to have an annealing temperature of 55 to 65°C and a length of 18 to 22 nucleotides. Primers were checked against catalogued SNPs in the region to ensure the oligonucleotides did not overlap with any known SNPs using the Mouse Genome Informatics database (Blake, Bult *et al.*, 2011). All oligonucleotides were synthesised by Metabion and are listed in 2.2.1.

3.2.4 PCR Optimisation

PCRs were optimised for use with the LightScanner with and without the addition of dimethyl sulfoxide (DMSO, 1.2µl water substituted for DMSO where appropriate), over an annealing temperature gradient of 55-65°C, using different primer combinations for each region.

3.2.5 Mutation Detection

See 2.2.1 for details of PCRs performed. PCRs were analysed on the LightScanner following the protocol described in Dwyer *et al.* (2010). Melting curves generated automatically by the Call-It software were normalised by defining '0%' and '100%' fluorescence manually either side of the prominent change in fluorescence, where the DNA is denatured. For consistency these lower and upper temperature intervals were always normalised to -2°C to -1°C and +1°C to +2°C respectively. Samples were then analysed using the "normal" sensitivity setting of the Call-It software which groups samples by melt curve. All samples were then inspected manually and re-grouped if deemed

necessary. PCRs were repeated for samples showing a different melt curve and re-analysed using the LightScanner. Samples showing an unusual profile on both occasions were sequenced across the region covered by the PCR to identify any mutations present. Details of sequencing methods can be found in 2.2.2.

3.3 Results

3.3.1 Mutations discovered

Mutation detection was carried out in a total of 5,856 putative mutation-carrying DNA samples. Approximately 1200bp were screened in each sample (therefore 5856x1200 = 7 272 000bp were screened for mutations in this study). A summary of results (mutations found) from the screen are shown in table 3.1. Twelve mutations were found with two of these mutations being synonymous and excluded from further analysis. The mutation discovery rate was therefore 1 in 0.61Mb with a non-synonymous mutation rate of 1 in 0.73Mb. Table 3.1 shows the mutations discovered in the 7 272 000bp screened by sample ID, genomic position, *Zfp804a* region, substitution, type of mutation and amino acid change if relevant.

The raw sequencing data is shown for non-synonymous mutations, i.e. those mutations predicted to result in an amino acid substitution (or the introduction of a premature stop codon in the case of the mutation in sample X23, E5) in figures 3.4 to 3.14. Raw data is not shown for the synonymous mutations in X33, E8 and X15, E8 as the mutations were not considered any further.

The quality of the sequencing varies and is in some cases poor. This was particularly the case with output near the 5’ or 3’ end of the amplified sequences. Nonetheless, it is deemed sufficient quality to infer the presence of a mutation (Liam Carroll, personal communication, 2009). The mutations that were carried forward for re-derivation were confirmed in separate PCRs and sequencing reactions.

DNA sample ID	Genomic position	REGION	Base change	Mutation	Amino acid change
X14,E1	82076010	EXON 2	AAT>AGT	Non-synonymous (Missense)	Asparagine > serine (N>S)
X23,E5	82076020	EXON 2	TGT>TAA[†]	Non-synonymous (Nonsense)	Cysteine > terminator (C>*)
X33,E8	82076065	EXON 2	AAT>AAC	Synonymous	N/A
X32,E3	82096730	EXON 4	TTT>ATT	Non-synonymous (Missense)	Phenylalanine > Isoleucine (F>I)
X13,A5	82096776	EXON 4	GAG>GGG	Non-synonymous (Missense)	Glutamic > glycine (E>G)
X13,A5	82097042	EXON 4	ATC>ACC	Non-synonymous (Missense)	Isoleucine > threonine (I>T)
X13,D5	82097056	EXON 4	AAT>AAA	Non-synonymous (Missense)	Asparagine > lysine (N>K)
X13,E2	82097138	EXON 4	ACT>GCT	Non-synonymous (Missense)	Threonine > alanine (T>A)
X42,G9	82097223	EXON 4	TGT>TAT	Non-synonymous (Missense)	Cysteine > tyrosine (C>Y)
X9,G1	82097246	EXON 4	GTA>GCA	Non-synonymous (Missense)	Valine > alanine (V>A)
X29,A12	82097262	EXON 4	AAA>AGA	Non-synonymous (Missense)	Lysine > arginine (K>R)
X15,E8	82097269	EXON 4	GGA>GGG	Synonymous	N/A

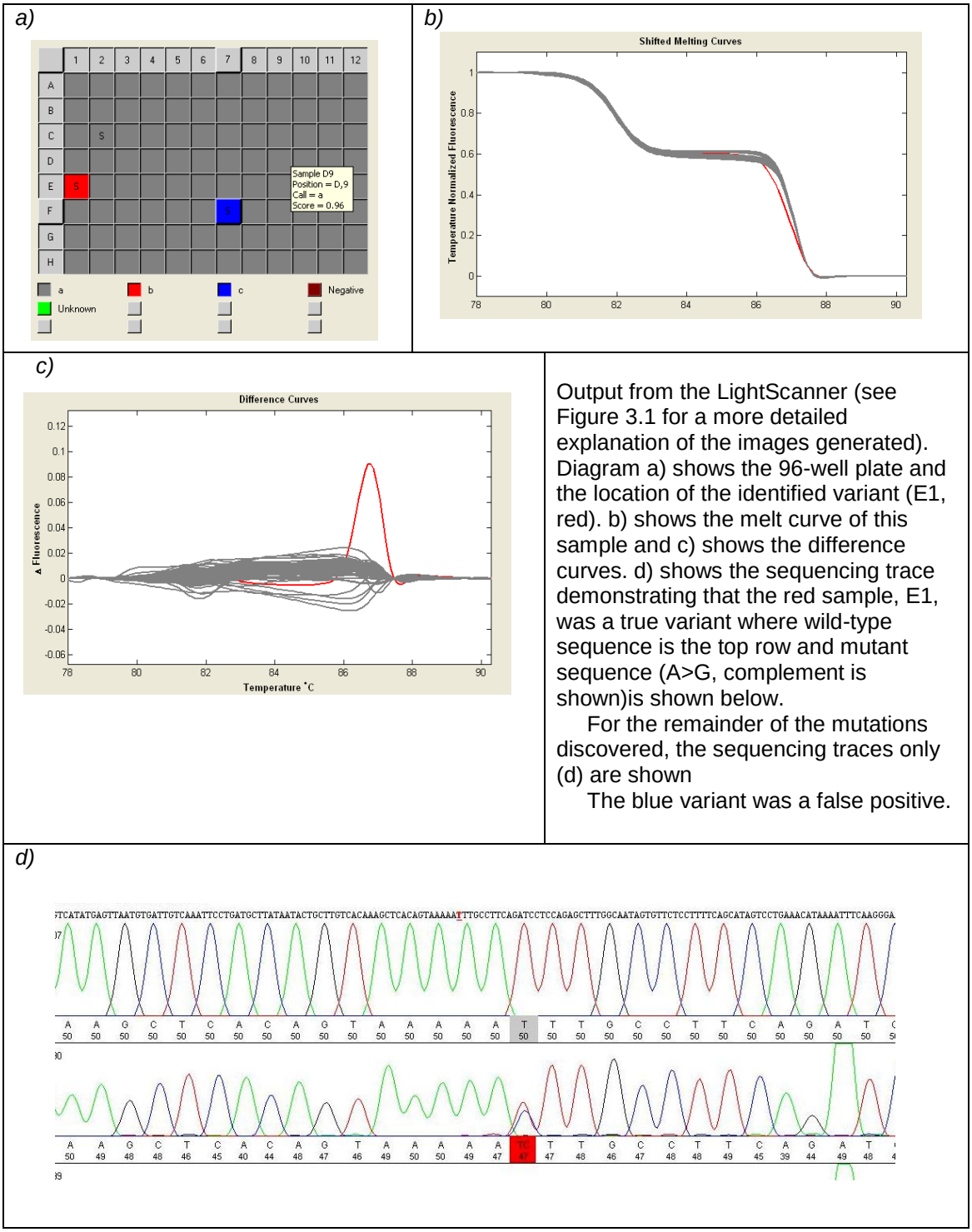
[†] double adjacent mutation

Table 3.1: Summary of results from the mutation screen of Zfp804a

Figure 3.4 overleaf additionally shows the output from the LightScanner CallIt software showing how mutations with a varying melt profile were identified initially. None of the mutations discovered were strain polymorphisms. This was confirmed using the Jackson Laboratory's Mouse Genome Informatics Strains,

SNPs and Polymorphisms database

(http://www.informatics.jax.org/strains_SNPs.shtml) accessed November 2008.

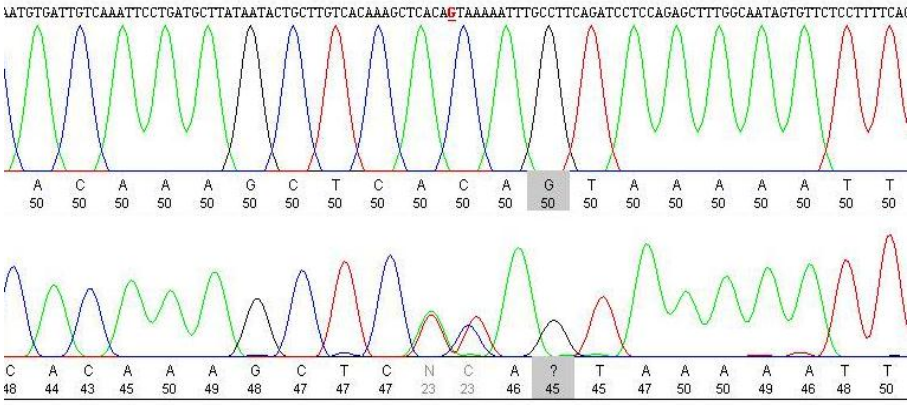


Output from the LightScanner (see Figure 3.1 for a more detailed explanation of the images generated). Diagram a) shows the 96-well plate and the location of the identified variant (E1, red). b) shows the melt curve of this sample and c) shows the difference curves. d) shows the sequencing trace demonstrating that the red sample, E1, was a true variant where wild-type sequence is the top row and mutant sequence (A>G, complement is shown)is shown below.

For the remainder of the mutations discovered, the sequencing traces only (d) are shown

The blue variant was a false positive.

Figure 3.4: Mutation X14,E1



LightScanner traces for this and the remainder of the mutations discovered are shown in Appendix 8.1.

Figure showing sequencing trace of sample X23, E5 - the wild-type (top) and mutant (bottom). Codon TGT>TAA, complement is shown).

Figure 3.5: Mutation X23, E5

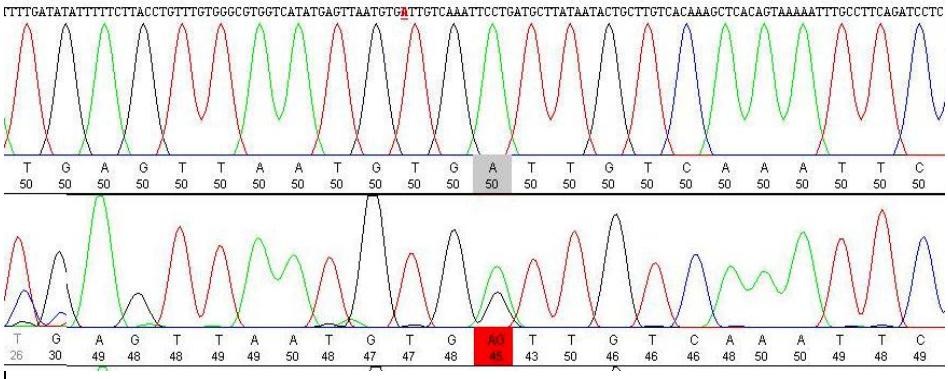


Figure showing sequencing trace of sample X33, E8 - the wild-type (top) and mutant (bottom) (T>C, complement is shown).

Figure 3.6: Mutation X33, E8

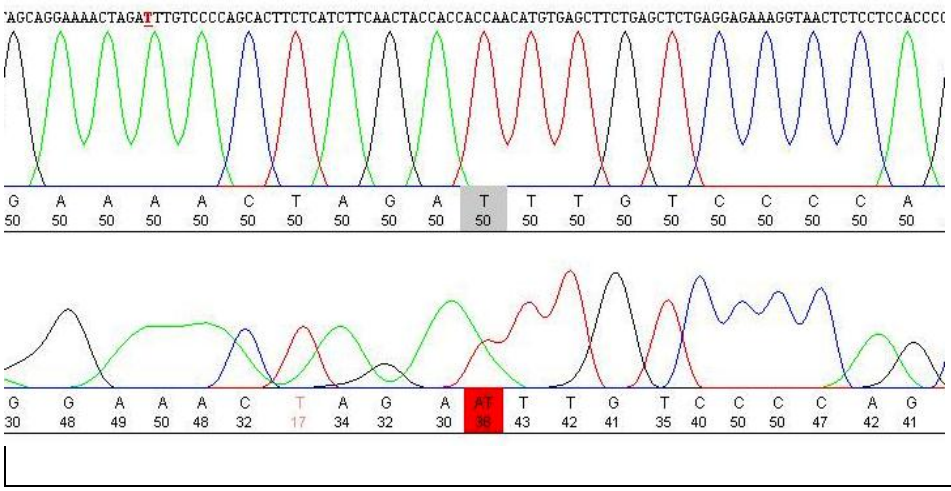


Figure showing sequencing trace of sample X32, E3 and the wild-type (top) and mutant (T>A) (bottom) sequencing trace (b).

Figure 3.7: Mutation X32, E3

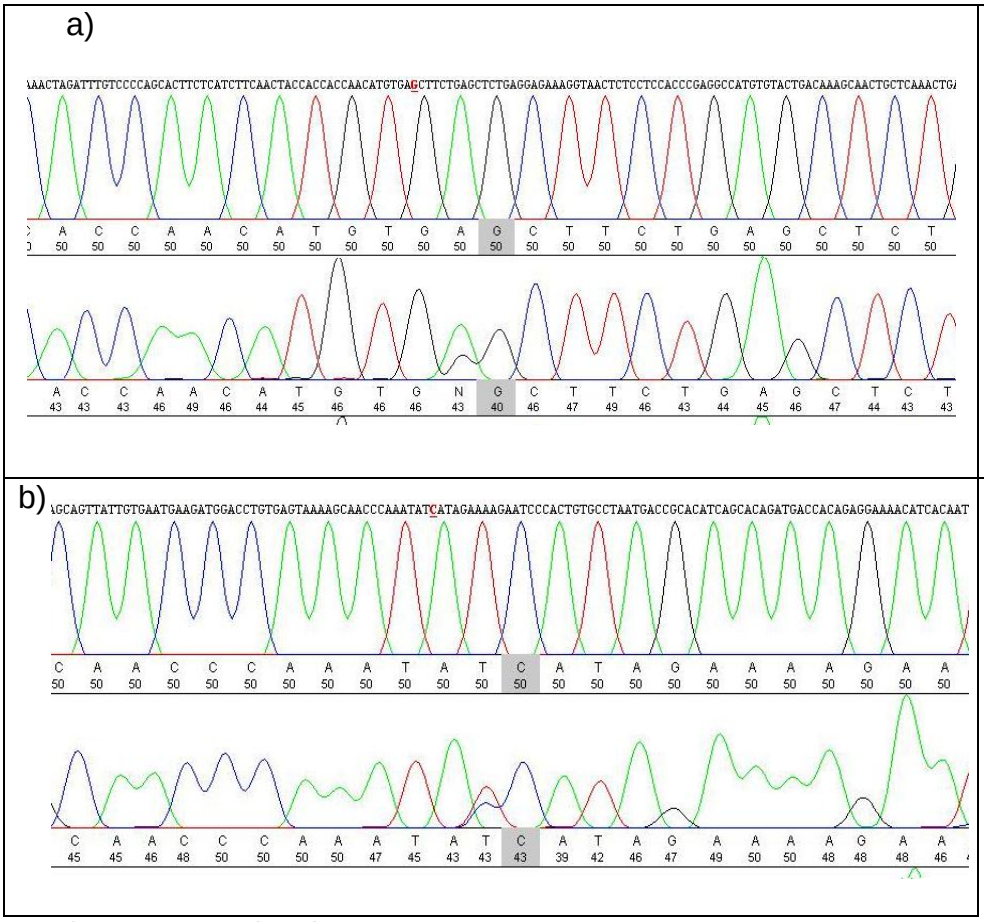


Figure showing sequencing traces of sample X13, A5 - the wild-type (top row a and b) and mutant (bottom row, a and b). a) shows the A>G mutation. The T>C mutation is shown in (b).

Figure 3.8: Mutations in X13, A5

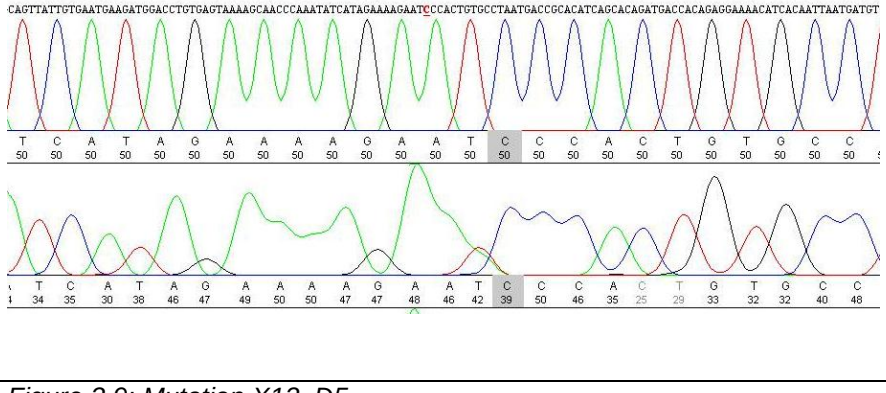


Figure showing sequencing trace of sample X13, D5 - the wild-type (top) and mutant (T>A) (bottom) are shown.

Figure 3.9: Mutation X13, D5

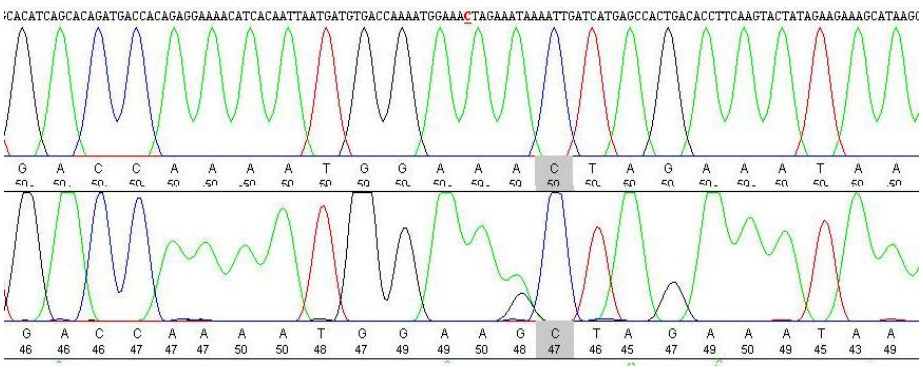


Figure showing sequencing trace of sample X13, E2 - the wild-type (top) and mutant (A>G)(bottom) are shown.

Figure 3.10: Mutation X13, E2

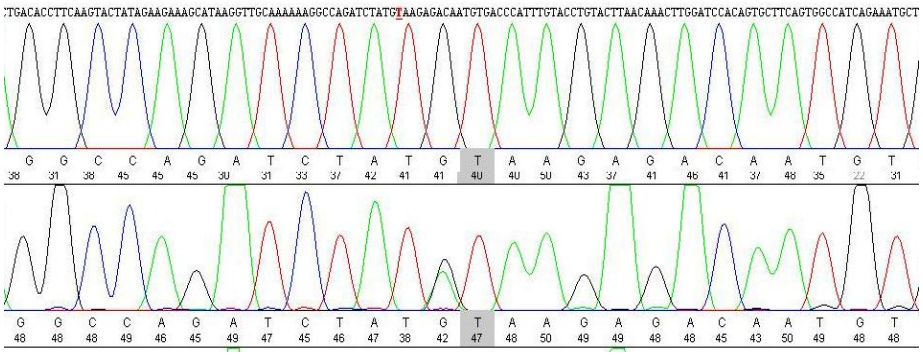


Figure showing sequencing trace of sample X42, G9 - the wild-type (top) and mutant (G>A)(bottom) are shown.

Figure 3.11: Mutation X42, G9

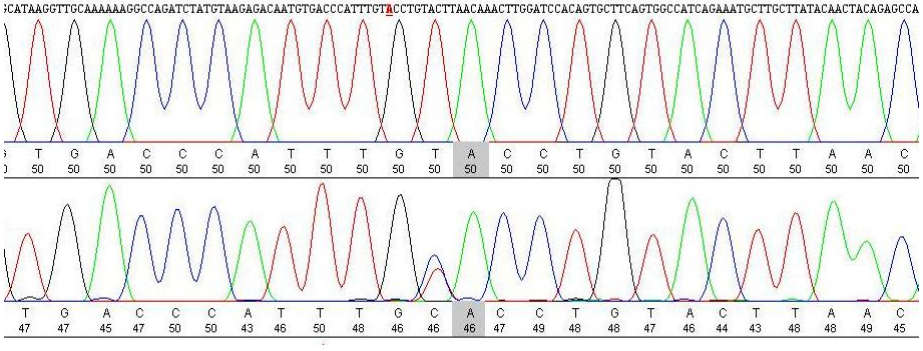


Figure showing sequencing trace of sample X9, G1 - the wild-type (top) and mutant (T>C)(bottom) are shown.

Figure 3.12: Mutation X9,G1

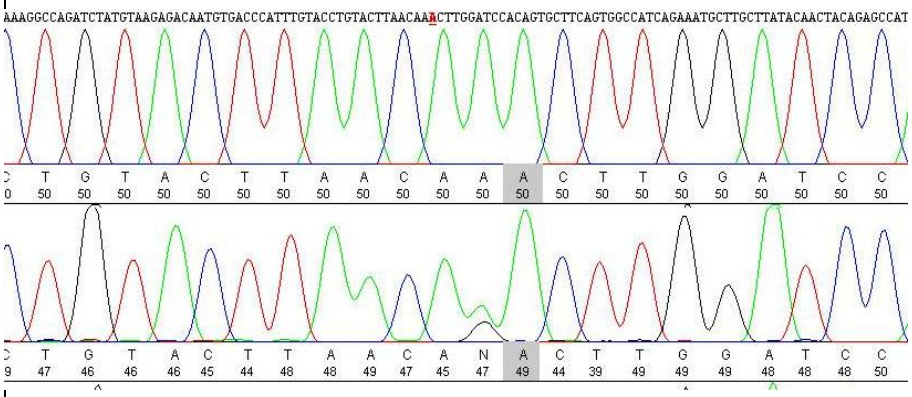


Figure showing sequencing trace of sample X29, A12 - the wild-type (top) and mutant (A>G)(bottom) traces are shown.

Figure 3.13: Mutation X29,A12

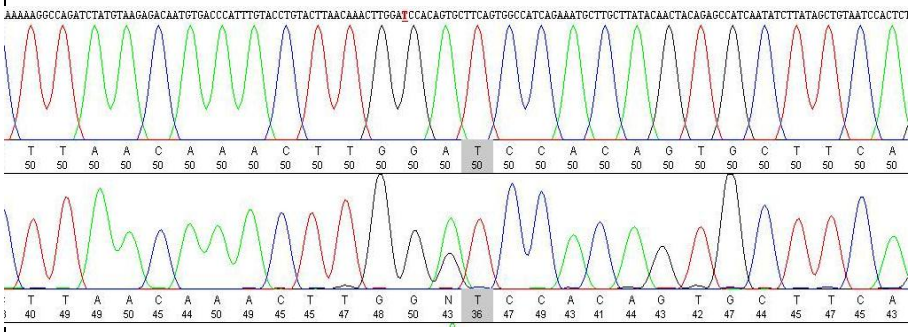


Figure showing sequencing trace of sample X15, E8 - the wild-type (top) and mutant (A>G)(bottom) traces are shown.

Figure 3.14: Mutation X15, E8

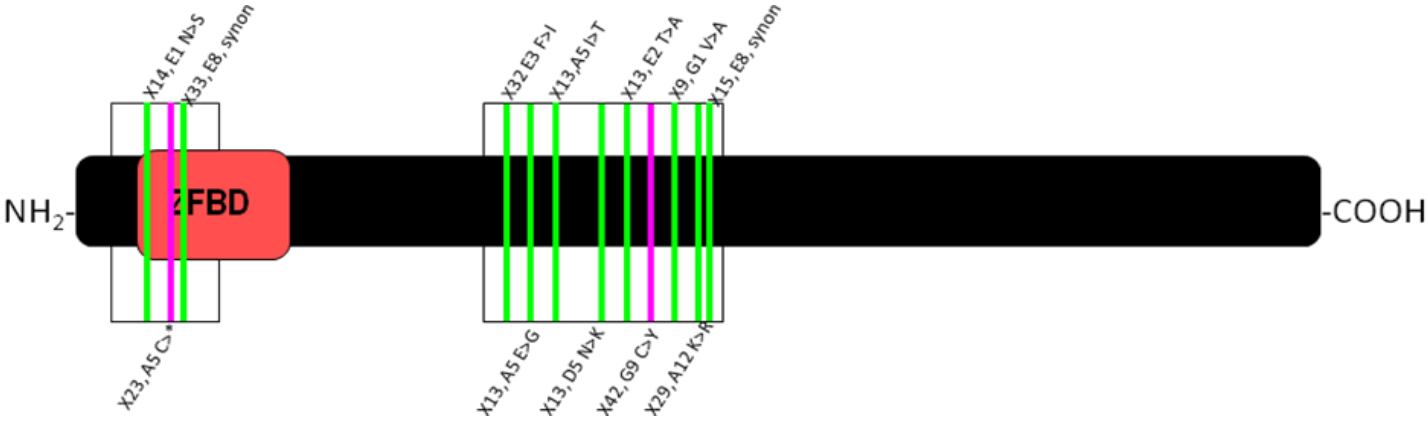


Figure 3.15: Summary of mutations discovered on a schematic diagram of the Zfp804a *protein. Mutations depicted by pink lines were the mutations selected for phenotypic analysis (see below). The green lines depict mutations that were not pursued.*

3.3.2 Mutation analysis

Synonymous mutations were excluded from further analysis as they do not affect protein structure. For each mutation, the predicted magnitude of the substitution was considered by taking into account the chemical properties of the amino acids substituted and the extent of conservation (by phylogenetic protein sequence alignment done *in silico*, see below) both at that particular residue and if the residue was within a block of conservation. Orthologous and paralogous protein sequences were compared using Malign (a multiple alignment program available online) to assess conservation. The yeast GIS1

SPECIES	GENE	HOMOLOGY
Mouse	*Zfp804a*	N/A
Human	*ZNF804A*	Orthologous
Human	*ZNF804B*	Paralogous
Mouse	*Gpatch8*	Paralogous
Human	*GPATCH8*	Paralogous
Rat	*Znf804a*	Orthologous
Chicken	*Znf804a*	Orthologous
Horse	*ZNF804A*	Orthologous
Chimpanzee	*ZNF804A*	Orthologous
Rhesus monkey	*LOC707018*	Orthologous
Zebrafish	*LOC561947*	Orthologous
Yeast	*GIS1*	A zinc finger protein containing a C2H2 domain

Table 3.2: Listof the proteins aligned with Zfp804a. Orthologous sequences in humans, other mammals, and the zebrafish were used. Paralogous sequences in the mouse and human were also selected.

was added as the gene contained a C2H2 zinc finger binding domain. Proteins aligned are shown in the table above (table 3.2).

Table 3.3, below, shows a summary of results from the mutation screen (non-synonymous mutations only), with the amino acid substitution, chemical nature of the substitution and conservation between similar proteins aligned (see table 3.2) around that residue.

Sample ID	Type of mutation & position	Substitution	Nature of change	Conservation
X14E1	Missense aa56	Asparagine> serine (N>S)	Within zinc finger domain and both the original and substituted aa are polar and uncharged N slightly larger than S.	Within a highly conserved block but not conserved in the paralog Gpatch8 or the yeast GIS1
X23A5	Nonsense aa59	Cysteine> terminator (C>*)	Truncates 1200aa protein prematurely at 59aa (aa 6 of 28 comprising zinc finger domain). The mutated cysteine is the first of two vital cysteine residues. Substituting one of these is predicted to have a destructive effect on the zinc finger binding domain. This cysteine residue is involved in binding the zinc ion of the finger and its substitution is predicted to disrupt the binding of the zinc ion (Wolfe *et al*. 2000).	Within a highly conserved block but not conserved in the paralog Gpatch8 or the yeast GIS1
X32E3	Missense aa249	Phenylalanine > isoleucine (F>I)	Both are hydrophobic, not charged. F has a phenyl ring. Both amino acids are similar in size.	F is conserved in 6 of 7 orthologous sequences (not in LOC561947, zebrafish) but not in paralogous sequences. Residue is F in GIS1 (yeast). In a fairly well conserved region of the protein. All mutations found in exon 4 are within this block.

X13A5	MIssense aa264	Glutamate> glycine (E>G)	Glutatmate is negatively charged and highly polar. It is a much bigger amino acid than glycine which is amphiphilic and not charged. G is smaller than E.	The glutamate residue is shared only by the mouse and rat orthologous sequences and the majority residue is valine (rhesus monkey, chimp, horse and human orthologs). Valine has different properties from glutamate. It is nonpolar and not charged and is smaller and differs in shape from glutamate.
X13A5	Missense aa353	Isoleucine> threonine (I>T)	I is non-polar, T is polar, more reactive and slightly smaller	The isoleucine residue is shared only by the mouse and rat orthologous sequences and the majority residue is leucine (rhesus monkey, chimp, horse and human orthologs), although leucine is very similar to isoleucine.
X13D5	Missense aa357	Asparagine> lysine (N>K)	Both are polar although lysine is positively charged. N and K are different shapes and N is slightly bigger	Residue is K in human, chimp, rhesus monkey & horse orthologs and also in yeast GIS1.
X13E2	Missense aa385	Threonine> alanine (T>A)	No charge difference between the amino acids although A is non-polar and T is polar and a bigger amino acid.	The locus is not particularly well-conserved. The majority residue (found in human, horse and chimp) is valine. Valine is non-polar and so differs from threonine and therefore suggests a substantial variability at this locus.
X42G9	Missense aa417	Cysteine> tyrosine (C>Y)	The loss of a cysteine residue may disrupt the 3D structure of Zfp804a. Y is also polar (C is non-polar) and bigger than C.	C is found at this locus in 5 of 6 orthologous sequences (zebrafish doesn't align here and residue is serine (polar) in the horse. Paralogs are leucine (ZNF804B human) which is nonpolar, and threonine in the human and mouse forms of GPATCH8 (polar). The residue is well conserved amongst orthologs but not in paralogs.

X9G1	Missense aa421	Valine> alanine (V>A)	Amino acids are smaller, both non-polar, except valine has a slightly larger hydrocarbon side chain.	Residue is valine in the majority of orthologs and not conserved in paralogous sequences.
X29A12	Missense aa426	Lysine> arginine (K>R)	Both are hydrophilic and positively charged. They are similar in size.	Residue is lysine in all genes compared other than LOC561947 in zebrafish and GIS1 in yeast.

Table 3.3: Summary of non-synonymous mutations, chemical nature of the substitution and conservation at that position between sequences aligned (see table 3.2).

From comparison of the mutations, two were considered to have a greater predicted functional effect than the others and so were taken for further *in silico* analysis:

- The nonsense mutation C59X (genomic position 82076020) prematurely truncates the protein in the zinc finger domain, and it is predicted that the mutant mRNA transcript will not be fully translated and will be destroyed by nonsense mediated decay.
- The cysteine to tyrosine missense mutation C417Y (genomic position 82097223) is predicted to disrupt the structure of the protein. This residue is well conserved in orthologs. From the exon 4 mutations discovered, this mutation was deemed to be the most severe due to the nature of the substitution. Cysteine is known to be important in determining protein structure (Berg *et al*., 2002) and the loss of the residue may damage the 3D protein structure. Tyrosine is also polar (cysteine is non-polar), and bigger than cysteine.

3.3.2.1 In silico analysis of the functional impact of the chosen mutations

The C59X mutation is predicted to result in nonsense-mediated decay of the mRNA transcript. The effect of the C417Y substitution is however more uncertain. There are several bioinformatic resources available online that use algorithms to predict the effect of amino acid substitutions on structure and function. The C417Y substitution in Zfp804a lies in an area of the protein that is uncharacterised which makes prediction of the effects of mutations in this region more difficult, as obviously the function of this part of the protein is unknown. However, programs such as Align GVGD (where GV is the Grantham Variation and GD is the Grantham Deviation) and PolyPhen are nevertheless useful and use other factors to predict the magnitude of mutations.

Align GVGD uses an algorithm based on the Grantham Difference (Grantham, 1974), a formulaic means of analysing amino acid substitutions. Similarly to Grantham (1974), Align GVGD uses the composition, polarity and molecular volume information to estimate difference between amino acids. The program uses two variables to estimate differences, both of which are based on the original Grantham Difference. GV refers to the range of variation observed at a position in a protein multiple sequence alignment and GD is a measure of the magnitude of the difference between the substitution and the range (if any) of variation observed at this position Tavtigian *et al.* (2008) provides further details. Align GVGD generates a prediction of the severity of the substitution based upon the multiple protein sequence alignments entered. Figure 3.16 below shows the output from the program.

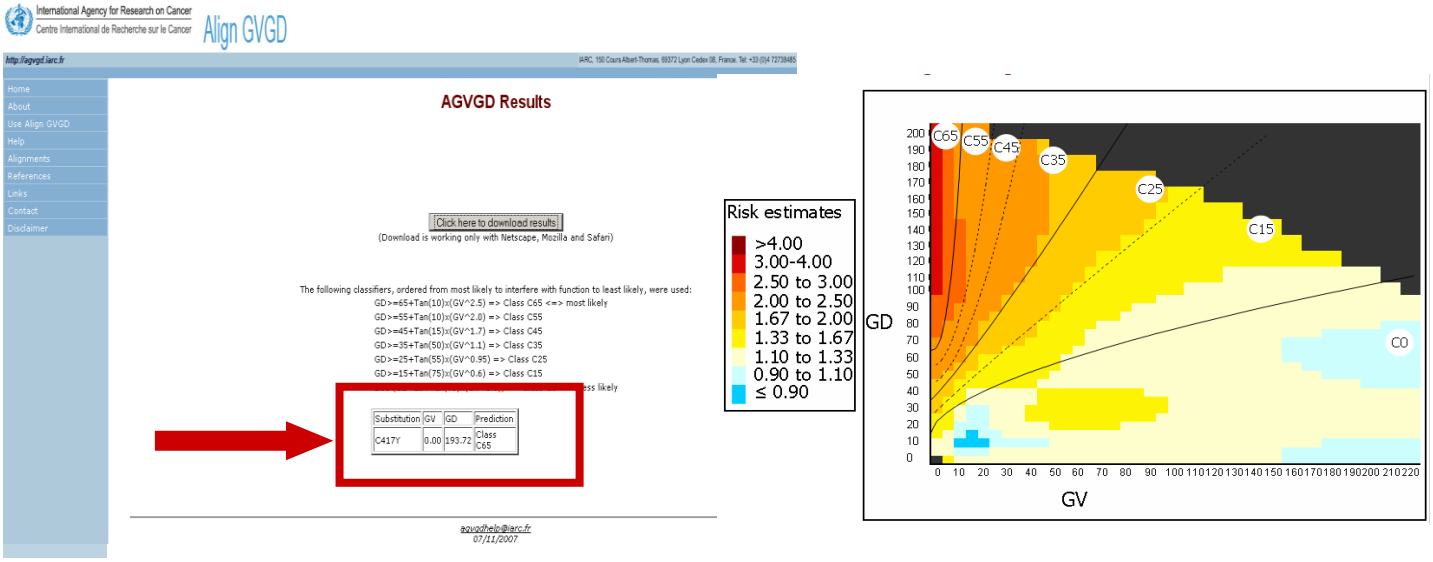


Figure 3.16: Output from Align GVGD on the predicted impact of the C417Y mutation

Align GVGD predicted the mutation to be in class C65. This is the most severe class of mutations.

PolyPhen (http://genetics.bwh.harvard.edu/pph/) predicts the functional effects of human non-synonymous SNPs (nsSNPs) but it is also used for predicting the effect of non-synonymous mutations in the mouse (Reuveni, Ramensky & Gross, 2007). After entering a protein sequence and the substitution, PolyPhen searches the UniProt database for specific features in the sequence such as binding sites. It then calculates a score for the likelihood of the substitution, based upon the amino acid change and information retrieved from database searches. The programme then attempts to map the substitution onto the known 3D structure of the protein to predict the magnitude of the change for example whether or not it interferes with the hydrophobic core or not. Figure 3.17 illustrates the output from PolyPhen for the C417Y mutation.

Query

Acc number	Position	AA_1	AA_2	Description
A2AKY4	417	C	Y	Zinc finger protein 804A OS=Mus musculus GN=Znf804a PE=2 SV=1

Prediction

This variant is predicted to be probably damaging

Prediction	Available data	Prediction basis	Substitution effect	Prediction data
probably damaging	alignment	alignment	N/A	PSIC score difference: 2.991

Details

PSIC PROFILE SCORES FOR TWO AMINO ACID VARIANTS

Score1	Score2	|Score1-Score2|	Observations	Diagnostics	Multiple alignment around substitution position
+2.414	-0.577	**2.991**	6	calculated	Sequences: all Flanks: 25 Show alignment

MAPPING OF THE SUBSTITUTION SITE TO KNOWN PROTEIN 3D STRUCTURES

Database	Initial number of structures	Number of structures
PQS	0	0

Figure 3.17: Output from PolyPhen on the predicted impact of the C417Y mutation

The mutation in exon 4 of *Zfp804a* using this algorithm was predicted to be 'probably damaging'. Although the programme was unable to map the 3D structure of the protein (as the region of the protein in which the C417Y substitution falls is currently uncharacterised), the mutation was predicted to have a damaging effect based on protein sequence alignments entered.

3.4 Discussion

Mutation detection was carried out in a total of 5,856 putative mutation-carrying DNA samples. Approximately 1200bp were screened in each sample (therefore 5856x1200 = 7 272 000bp were screened for mutations in this study). Twelve mutations were found with two of these mutations being synonymous and excluded from further analysis. In this study, the mutation discovery rate was significantly higher than expected (χ^2 = 23.01, 1df, p<0.0001), compared with the rate of mutations found by Quwailid and colleagues (2004). Whilst Quwailid *et al*. discovered 1 mutation in 1.01Mb (in exonic sequence of which 1 in 1.82Mb was functional), mutations were discovered (in the same DNA archive) at a rate of 1 in 0.61Mb (of which 1 in 0.73Mb was functional). Three suggestions of possible explanations for this difference are provided. Nolan, Hugill and Cox (2002) state that AT-rich genes are more likely to be targeted by ENU mutagenesis (as 82% of sequenced mutations discovered by Justice *et al*. (1999) involved transitions/transversions of AT pairs). However, in a large screen of 9.48Mb, Coghill *et al.* (2002) found more GC than AT transitions (although 40% were AT transitions).. In the present study, 10 out of the 12 mutations, or 83% were AT rather than GC transitions, in agreement with the rate discovered by Justice *et al*. (1999). Detailed analysis of the sequences analysed in this chapter compared with the sequences analysed by Quwailid *et al*. (2004) would be useful to investigate the possibility of sequence differences being responsible for the difference in mutation discovery rate further. Another possible reason for the difference in discovery rate is that different mutation detection techniques were used. As discussed in 3.1, the LightScanner has a very low false negative rate (although consequently a high false positive rate).

Quwailid *et al*. (2004), on the other hand, used denaturing high performance liquid chromatography (DHPLC) which has a higher false negative rate and could lead to mutations being missed (Coghill *et al.*, 2002). A third reason for the higher mutation rate discovered in the present study could be that the *Zfp804a* locus is particularly susceptible to ENU-induced mutations, a so-called 'ENU hotspot' (e.g. Kiernan *et al*., 2002). Of the ten non-synonymous mutations considered, two mutations stood out as potentially more severe from *in silico* analysis, the C59X and C417Y mutations. These mutations were selected to be investigated further in a mouse model.

3.4.1 C59X

It is assumed that the introduction of the premature stop codon in *Zfp804a* in this model will cause the mutant mRNA transcript to be destroyed due to nonsense-mediated decay, resulting in no detectable Zfp804a protein in mice homozygous for the C59X substitution. Nonsense mediated decay (NMD) is a highly-conserved function that destroys transcripts encoding premature stop codons (see figure 3.18). A mutant or truncated version of the protein can be more harmful than the complete absence of the protein due to potentially deleterious gain-of-function or dominant-negative effects (Chang *et al.*, 2007). NMD is vital to the survival of a healthy organism. Medghalchi *et al.* (2001) show that mice lacking the up-frameshift 1 (Upf1) protein die at day 7.5 of gestation. Upf1 is a protein heavily involved in NMD and promotes translation and histone mRNA decay.

Nonsense mediated decay occurs when a premature termination codon (PTC) is encountered in the mRNA transcript during translation. The main

method believed to indicate whether a stop codon is premature or not is the presence of an exon junction complex (EJC, a protein complex deposited about 20-24 nucleotides upstream of exon-exon boundaries) downstream from the PTC. As the ribosome passes along the mRNA transcript, EJCs are removed. Translations ends when eukaryotic release factors (eRG2 and eRG3, which also interact with UPG2), attached to the ribosome, recognise a stop codon. When a stop codon is encountered that isn't in the last exon of a gene, EJCs remain on the mRNA. Any remaining EJCs bind to the eRFs and UPG2, initiating nonsense-mediated decay by dissociating the ribosome and causing the cap-binding complex to be removed, which protects the mRNA transcript. This leaves the mRNA transcript susceptible to rapid decay by exonucleases. NMD is believed to happen during the 'pioneer' round of translation (see figure 3.19). Chang *et al.* (2007) review the topic.

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Figure 3.18: Diagram taken from Chang et al. (2007) to illustrate the NMD process. NMD occurs when up-frameshift protein 1 (UPG2, labelled 1) interacts with UPG3 (2), and UPG4b (3b). This interaction does not normally occur as the ribosome (purple) does not ordinarily meet a stop codon until all the EJCs have been removed. PTCs in the last exon of a gene normally escape destruction by NMD.

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Figure 3.19: Diagram taken from Chang et al. (2007) shows the removal of the cap-binding complex and destruction of the mRNA by exonucleases.

It is predicted that NMD will occur with the C59X mutant. The nonsense mutation is not in the last exon of the gene nor is it less than 50 nucleotides upstream of the final splice junction of the gene, two factors which can cause a transcript containing a PTC to escape NMD (Strachan & Read, 2010). There is currently no *Zfp804a* antibody however to verify the decay of the transcript. There are exceptions to NMD, where there may be 'read-through' in the transcription phase, or the stub may not be decayed depending on the position of the premature stop codon in the transcript. Matsuda, Sato & Maquat (2008) review the topic. Whether or not NMD indeed occurs, the loss of the cysteine residue is predicted to have a large effect on the structure of Zfp804a (predicted by both literature and bioninformatic analysis of the protein).The mutated cysteine is the first of two vital cysteine residues. Substituting one of these is predicted to have a destructive effect on the zinc finger binding domain. This cysteine residue is involved in binding the zinc ion of the finger

and its substitution is predicted to disrupt the binding of the zinc ion (Wolfe *et al*. 2000). Thus even if the mutated protein were to escape NMD it is likely that the mutation would disrupt the function of Zfp804a.

3.4.2 C417Y

This mutation was selected to take forward for phenotyping as the cysteine to tyrosine substitution was predicted to have a damaging effect on the protein, the mutation occurred in a highly conserved genomic region and several non-synonymous SNPs were found in this region by work in this laboratory (Dwyer *et al*., 2010). The substitution occurs in a part of the protein that has not yet been characterised and it is unknown what function this part of the protein may fulfil. It is therefore difficult to predict the effect the mutation will have on the protein. Nonetheless cysteine and tyrosine residues are different in size and polarity and the loss of a cysteine residue may disrupt the 3D structure of Zfp804a which may result in a loss or gain of function. Differently folded proteins can dramatically affect protein-protein interactions and consequently their functions. Such effects may be seen in both homozygous and heterozygous mutants (if there is haploinsufficiency of the protein product) which necessitates investigation of phenotypes in wild-types and homozygous and heterozygous mutants to explore the phenotypic effects of the mutation. Cysteine has a sulfhydryl group which makes cysteine highly reactive and gives cysteine an important role in the structure of proteins by forming disulfide links. These bonds enable cross-links between polypeptide chains and are formed by the oxidation of the cysteine residue.

3.4.3 Summary

The selected mutations are predicted to have differing effects on the protein and comparison of the two models will provide more insight into the potential function of *Zfp804a* than a single model. Another benefit of using two parallel models is the reduction of the risk that one (or both) mutant(s) may not be viable, i.e. the mutation may be embryonic lethal in the homozygous form. This is potentially a problem with mutant *Zfp804a* models as Ramensky *et al.* (2002) found that proteins involved in transcription regulation display the highest selective pressure against deleterious non-synonymous SNPs when compared with other classes of proteins such as enzymes.

CHAPTER 4: Creating the mutant *Zfp804a* models

4.1 Introduction

To recapitulate chapter 3, we selected two mutations to re-derive for behavioural characterisation. The first, a nonsense mutation in exon 2 of *Zfp804a,* is predicted to prematurely truncate the protein at the beginning of the zinc finger domain with the transcript predicted to be destroyed by nonsense mediated decay. The other mutation, in exon 4 occurs 417 amino acids downstream from the N-terminal of the protein and is a cysteine to tyrosine substitution, which is predicted to influence the 3-D structure of the protein, given the involvement of cysteine in maintaining protein structure (e.g. Wolfe, Nekludova & Pabo, 1999). Both mutations are expected to disrupt the function of Zfp804a by causing aberrant protein-protein interactions. Comparison of the two models will provide more insight into the potential function of *Zfp804a* than a single model and provides an alternative model should one of the two be embryonic lethal.

Having identified mutations of interest, the next stage is to create mice carrying these mutations. The MRC Mary Lyon Centre, Harwell, hosts a large resource described in Chapter 3 (3.1) containing DNA and corresponding sperm for re-derivation by *in vitro* fertilisation (IVF) of wild-type surrogate mothers. IVF following such methods has been shown to be an effective means of re-deriving ENU mutants (Coghill *et al*., 2002). However, once mice have been created or 're-derived' carrying the mutations of interest, a cohort must be bred suitable for behavioural testing in number, age, genotype, gender etc.

Breeding a cohort of mice carrying mutations selected from ENU mutagenesis can be time-consuming and problematic. ENU is a mutagen that causes multiple mutations in the genome, meaning that these mutations may also be inherited, along with the mutation selected. Just how many mutations are caused by ENU mutagenesis is a matter of some debate although a consensus is now gradually being reached.

The rate of mutations is affected primarily by the dosage of ENU that is administered (subject to a threshold level, Russell and Montgomery, 1982; Hitotsumachi *et al*., 1985; Lewis *et al*., 1991). Keays *et al.* (2006) review mutation rates over several studies. Of the four studies reported, the rates of coding (missense) mutations varied from 1 in 0.42Mb (Wienholds *et al*., 2003) to 1 in 1.82Mb (Quwailid *et al*., 2004). Quwailid and colleagues has been taken as a more reliable estimate as the mutation rate is based upon a larger sequence. The mouse haploid genome is estimated to be 2.6 billion bases (or 2600Mb, Mouse Genome Sequencing Consortium, 2002). 2.3% of this is coding (Keays *et al*., 2006). This gives 59.8Mb of coding bases. Mutation rate of 1 in 1.82Mb of coding DNA gives an estimated mutation rate of 32.86 mutations, or 33 corrected to the nearest integer.

It is important to be able to estimate the number of mutations a mouse may carry in order to calculate how many backcrosses (breeding the mutant to a wild-type background, selecting for the chosen mutation, with the ultimate goal of congenicity) would be required to be confident of studying the mutation of interest in isolation and to estimate the likelihood of other mutations being responsible for any phenotypes observed. Backcrossing is the easiest method of isolating the mutation of interest (Coghill *et al*., 2002) and is relatively simple

(genotyping for the mutation of interest and selectively breeding that mutant with a wild-type). Keays, Clark and Flint (2006) discuss backcrossing and demonstrate that backcrossing is an efficient means of eliminating potentially confounding mutations. Mice in the Harwell DNA archive (G1) are the product of one parent (male) who was exposed to the ENU mutagen (and heterozygous for the selected mutation) and one wild-type mother. This G1 mouse will carry two copies of each chromosome, one copy carrying mutations from the father and one wild-type chromosome from the mother. Assuming 54.89Mb (figure used by Keays *et al.*, 2006, taken from Ensembl) of the genome is coding in a male mouse, and using the rate of mutation of 1 in 1.82Mb (given in Quwailid *et al.*, 2004, used as this study represents the largest [in bases] screen of all published screens), this estimates that the G1 mouse would have 30.16 (given as 31 in Keays *et al.*) potentially functional mutations. With each backcross onto a wild-type background, the number of mutations will decrease on average by 50%. Following this, G2 mice are likely to have 15.5 potentially functional mutations, G3 mice, 7.75 and and G4 3.88. So, after five backcrosses, a mouse (G_6) is likely to harbour just one mutation (the mutation of interest selectively bred for). In this thesis it was decided to examine the G4 generation in order to gain some functional data in the time available. Although individuals in this generation are likely to harbour mutations other than the selected *Zfp804a* mutation, studying the G4 generation is justifiable (as the mice in any one genotype, line or gender group are unlikely to share the same set of additional mutations), especially as a means of preliminary screening for gross abnormalities or indicators of further areas to investigate. If necessary, tests can be repeated on purer animals.

Although estimates of additional mutations harboured are extremely helpful to the researcher, they give a deceptively simplistic picture of confidence in isolating the selected mutation. A major obstacle with residual mutations and backcrossing comes from linkage. Linkage occurs due to the way in which chromosomes recombine during germline cell division (see description of linkage in general introduction, section 1.5.2.2.). Keays *et al.* (2006) estimate the likelihood of another linked mutation being inherited along with the selected mutation for each chromosome. *Zfp804a* is found on chromosome 2 and mice were bred 3 generations from the founder mouse and then intercrossed. For mutations on chromosome 2 and 4th generation mutants, there is estimated to be a 32.8% probability that another mutation is linked (from estimations given by Keays *et al.,* 2006). The chance that this mutation is missense is 64% (Keays *et al.,* 2006) of this, resulting in a 21.0% chance that a missense mutation is linked to either of the mutations selected on *Zfp804a*. The potential for a linked mutations is another reason why it was thought prudent to follow up two mutations of interest, rather than a single model as there is a far lower (21.0% x 21.0% = 4.4%) chance that both selected mutations will be linked to another (which can only reliably be ascertained by sequencing around the mutation of interest) and it is extremely unlikely that both the selected mutations in *Zfp804a* will be linked to the same 'other' mutation.

It is important to note that although G4 mice are predicted to have 3.88 potentially functional mutations (2.88 being residual, unwanted mutations) and there is a 21.0% chance that a missense mutation will be linked to the mutation of interest, these residual mutations do not pose as much of a problem as it

may appear. Individual mice will have different sets of residual mutations and the chances of any one mutation occurring more frequently in any one genotype and gender group are extremeley low. These residual mutations also may not interact with *Zfp804a*, affect brain function and may not have an influence on the performance of the mouse in behavioural tests administered. These issues are discussed in more detail in the Discussion of this chapter.

Backcrossing - although an effective means of isolating the mutation of interest - can be time consuming. For example, backcrossing the mutation onto a wild-type background for five generations (assuming a gestation period of 3 weeks, a further 5 weeks to reach sexual maturity and a further week to conceive, 9 weeks per generation) and then performing an intercross (heterozygous to heterozygous breeding to generate heterozygous and homozygous mutant and wild type littermate controls) would take upwards of a year, assuming no problems are encountered with fertility and breeding. One way to speed this up is through ‘speed congenics’. Speed congenics allows individual mice to be screened (through linkage analysis) for the relative contribution of each parental strain and uses data from mouse genomic analysis for marker-assisted selection of breeders. The mouse/mice with the highest proportion of markers corresponding to the recipient strain genome (and therefore the lowest corresponding to the mutagenised strain genome) can then be selected for breeding at each generation. The method traditionally uses microsatellites as markers (e.g. Markel *et al.*, 1997, reviewed in Wong, 2002), although SNP panels are also readily available and are perhaps more suited for comparison to a reference genome as microsatellites are mutationally less stable than SNPs. Marker-assisted selection can be used on mice either

as pre-selected carriers of a mutation or the mice may have a phenotype of interest such as epilepsy (Legare *et al.*, 2000) that the researchers wish to pinpoint. A search on PubMed yielded only one publication using marker-assisted selection to speed up congenicity in the case of ENU mutants (Ogonuki *et al.,* 2009). Wong (2002) estimates that using the speed congenics approach, full congenicity may be achieved in 5 generations (where the 'best' male could consist of 100% recipient genome but for the mutation of interest). Using the traditional backcrossing method, F5 mice would have 97% recipient genome. Similarly at G4, using speed congenics, mice could be 94% 'pure', as opposed to 87.5% by chance using the traditional method. Although it does speed up breeding, speed congenics can be problematic if sufficient numbers of mice to produce the full range of congenicity (in order to select the purest possible males) are not bred. It is also fairly resource intensive. The C57Bl/6J strain was chosen to be the recipient genome in this study for a number of reasons.

C57Bl/6J is well-characterised genetically and was the first mouse strain to have the genome sequenced (Mouse Genome Sequencing Consortium *et al.*, 2002). The strain is also frequently used for behavioural testing and so using this strain allows greater validity of results when comparing to other findings. The strain also breeds well which is especially important for ENU mutants due to the amount of breeding needing to be done to isolate the mutation of interest on a wild-type background. Mice are also long-lived, genetically tractable and have low comparative susceptibility to tumours. (Mouse Mutant Resource website, The Jackson Laboratory obtained July, 2011).

4.2 Methods

4.2.1 In vitro fertilisation

IVF was performed by MRC Mammalian Genetics Unit (Harwell, Oxfordshire). Full details are provided in the Appendix (8.2). In brief, young sexually mature female mice were superovulated (ovulation was induced by administration of gonadotropin). Oocyte harvest, fertilisation and sperm dispersal dishes were prepared. Sperm was rapidly thawed and oocytes harvested from the superovulated females. Sperm and oocytes were added to the prepared fertilisation dish and incubated at 37°C to allow fertilisation to occur. IVF success was scored and 2-cell embryos were transferred into pseudopregnant females to mature.

4.2.2 Subjects and animal husbandry

Subjects obtained from the MRC Mammalian Genetics Unit, Harwell were housed and bred in the Behavioural Neurosciences Laboratory, School of Psychology, Cardiff University. Mice were group housed in cages of 2-5 subjects except where fighting or bullying necessitated single caging. Cages were environmentally enriched (with chew sticks and cardboard tubes) and standard laboratory food and water were available *ad libitum*. The light-dark cycle of the holding room was 12 hours with lights on at 07:00 and lights off at 19:00.

Experimental animals were regularly monitored for signs of ill health. Any mice showing signs of illness were immediately assessed by the Named Animal Care and Welfare Officer and, if necessary, the University's vet. Only mice

showing no signs of ill health were used for breeding. If necessary, mice were withdrawn from breeding and treated or sacrificed, dependent upon the nature and presumed severity of the illness.

4.2.3 Breeding

Mice were used for breeding from approximately one to three months of age. Females were introduced to the male's home cage. If this was not possible then both male and female animals were introduced to a new cage. Tubing was removed from cages to facilitate breeding. Breeding cages ranged from one to four females with one male and breeding pairs/ harems were left undisturbed for one week. After this period females were checked for signs of pregnancy or a semen plug and left with the male for a further week if required. If any females were not pregnant following two weeks exposure to the male, they were removed from the breeding programme. If the male (by process of elimination) was not thought to be fertile, it was removed from the breeding programme.

4.2.4 Tail biopsy

See 2.7; Tail biopsy in General Materials and Methods

4.2.5 Genotyping

Genotyping methods followed those described in 2.2 used for mutation discovery but for substituting LC Green in the PCR master mix for double distilled H_20. The LightScanner stage was also not performed as it was not

necessary. PCRs were cleaned as per protocol and sequencing was performed.

4.2.6 Culling

Mice no longer required for the breeding programme were culled according to the protocol outlined in 2.8 Culling protocol in General Materials and Methods.

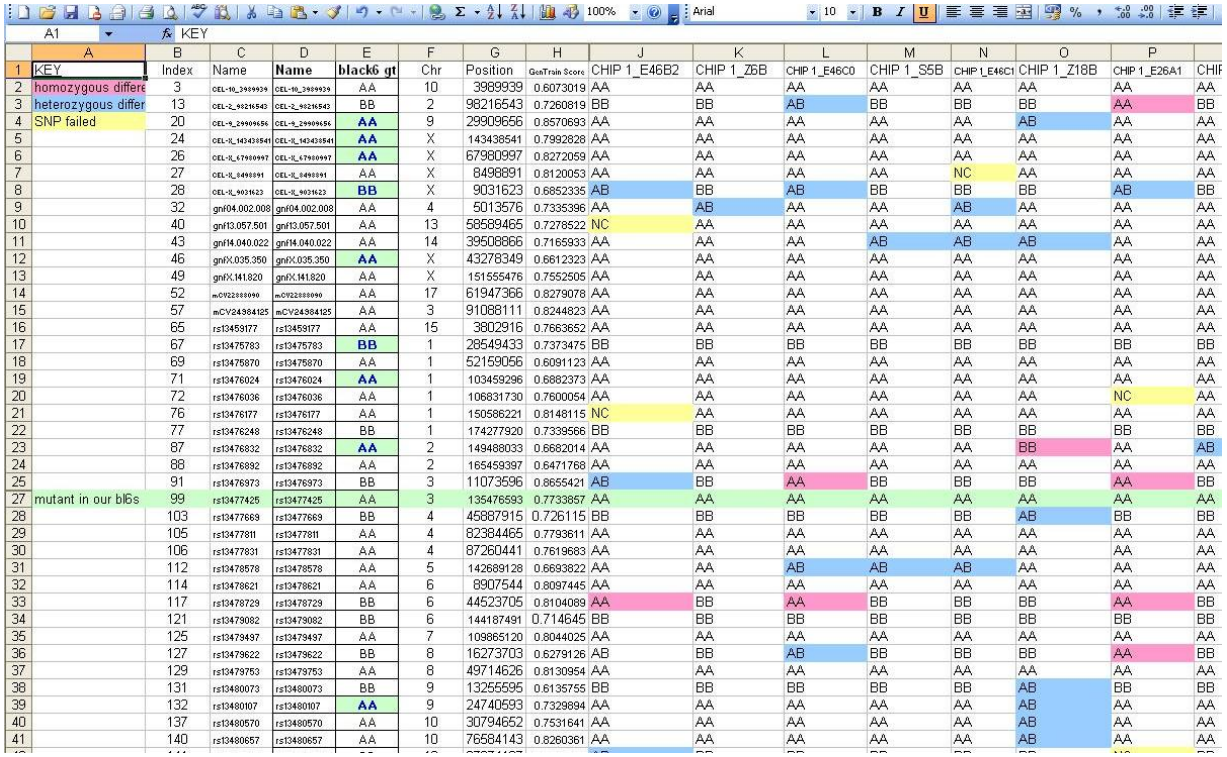
4.2.7 Linkage panel

The Mouse LD (low density) Linkage Panel (Illumina, Inc) was used to genotype 377 SNPs in mice carrying the *Zfp804a* mutations (see 2.3.1 and figure 2.2 for methods). The SNPs were pre-selected by Illumina as they are known to be polymorphic between the 10 most common strains of mouse. Coverage is approximately four SNPs every 27Mb, with at least one of these four SNPs being informative for crosses involving the C57Bl/6J strain. The Panel uses the GoldenGate Gentoyping Assay protocol (Illumina, Inc). Full details of the protocol are provided on Illumina's website (www.illumina.com).

Genomic DNA (gDNA) was normalised to 50 – 60 ng/µl (250ng minimum in total required). DNA was then 'activated' by treating it with streptavidin-biotin to prepare the gDNA for binding to paramagnetic particles. Assay oligonucleotides, hybridisation buffer and the paramagnetic particles were then added to the DNA for the hybridisation stage. Three oligonucleotides are provided for each SNP: two oligos are allele-specific and the third is designed to anneal several bases downstream from the allele that ligates to the extended allele-specific oligos in the next phase following several wash steps to remove

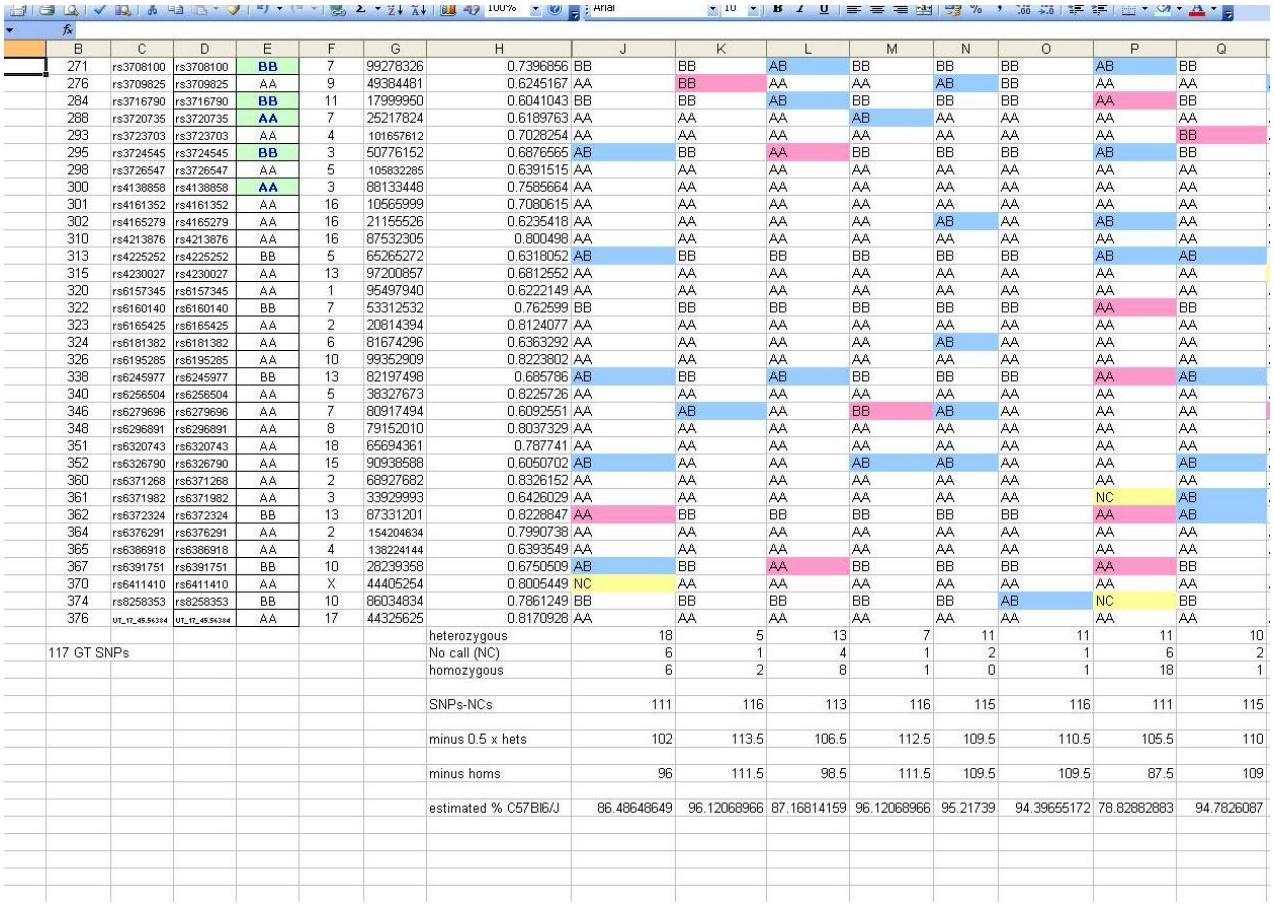
excess and mis-hybridised oligos. Following this, PCR is performed on the samples using three universal PCR primers. Single-stranded DNAs are hybridised to their complement bead. These products were then hybridised onto the BeadChip and dried to isolate the assay products for individual analysis. After the products were hybridised, the SNPs were analysed using the BeadArray Reader (which analyses fluorescence signal). This is then analysed by software for automated genotype clustering and calling. The GenomeStudio Genotyping Module uses a clustering algorithm to call genotypes, depending on the threshold set by the user. Further details of the software are contained in the Technical Note available online (www.illumina.com). Further analysis was performed in Microsoft Excel.

Figures 4.1 and 4.2 below show the layout of the results from the Mouse LD Linkage Panel. Figure 4.1 shows the SNP, the C57Bl/6J genotype, the chromosome, genomic position and quality score (1 is the highest). Columns J to P shown represent individual mice. Figure 4.2 is a different view of the same spreadsheet demonstrating how the estimated percentage C57Bl/6J was calculated (for the formula used, see figure 4.3).



	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q
1	KEY	Index	Name	Name	black6 gt	Chr	Position	GeoTrain Score	CHIP 1_E46B2	CHIP 1_Z6B	CHIP 1_E46C0	CHIP 1_S5B	CHIP 1_E46C1	CHIP 1_Z18B	CHIP 1_E26A1	CHI
2	homozygous differe	3	OEL-10_3989939	OEL-10_3989939	AA	10	3989939	0.6073019	AA	AA	AA	AA	AA	AA	AA	AA
3	heterozygous differ	13	OEL-2_98216543	OEL-2_98216543	BB	2	98216543	0.7260819	BB	BB	AB	BB	BB	BB	AA	BB
4	SNP failed	20	OEL-9_29909656	OEL-9_29909656	AA	9	29909656	0.8570693	AA	AA	AA	AA	AA	AB	AA	AA
5		24	OEL-X_143438541	OEL-X_143438541	AA	X	143438541	0.7992828	AA	AA	AA	AA	AA	AA	AA	AA
6		26	OEL-X_67980997	OEL-X_67980997	AA	X	67980997	0.8272059	AA	AA	AA	AA	AA	AA	AA	AA
7		27	OEL-X_8498891	OEL-X_8498891	AA	X	8498891	0.8120053	AA	AA	AA	AA	NC	AA	AA	AA
8		28	OEL-X_9031623	OEL-X_9031623	BB	X	9031623	0.6852335	AB	BB	AB	BB	BB	BB	AB	BB
9		32	gnl04.002.008	gnl04.002.008	AA	4	5013576	0.7335396	AA	AB	AA	AA	AB	AA	AA	AA
10		40	gnl13.057.501	gnl13.057.501	AA	13	58589465	0.7278522	NC	AA	AA	AA	AA	AA	AA	AA
11		43	gnl14.040.022	gnl14.040.022	AA	14	39508866	0.7165933	AA	AA	AA	AB	AB	AB	AA	AA
12		46	gnlX.035.350	gnlX.035.350	AA	X	43278349	0.6612323	AA	AA	AA	AA	AA	AA	AA	AA
13		49	gnlX.141.820	gnlX.141.820	AA	X	151555476	0.7552505	AA	AA	AA	AA	AA	AA	AA	AA
14		52	mCV22888090	mCV22888090	AA	17	61947366	0.8279078	AA	AA	AA	AA	AA	AA	AA	AA
15		57	mCV24384125	mCV24384125	AA	3	91088111	0.8244823	AA	AA	AA	AA	AA	AA	AA	AA
16		65	rs13459177	rs13459177	AA	15	3802916	0.7663652	AA	AA	AA	AA	AA	AA	AA	AA
17		67	rs13475783	rs13475783	BB	1	28549433	0.7373475	BB	BB	BB	BB	BB	BB	BB	BB
18		69	rs13475870	rs13475870	AA	1	52159056	0.6091123	AA	AA	AA	AA	AA	AA	AA	AA
19		71	rs13476024	rs13476024	AA	1	103459296	0.6882373	AA	AA	AA	AA	AA	AA	AA	AA
20		72	rs13476036	rs13476036	AA	1	106831730	0.7600054	AA	AA	AA	AA	AA	AA	NC	AA
21		76	rs13476177	rs13476177	AA	1	150586221	0.8148115	NC	AA	AA	AA	AA	AA	AA	AA
22		77	rs13476248	rs13476248	BB	1	174277920	0.7339566	BB	BB	BB	BB	BB	BB	BB	BB
23		87	rs13476832	rs13476832	AA	2	149488033	0.6682014	AA	AA	AA	AA	AA	BB	AA	AB
24		88	rs13476892	rs13476892	AA	2	165459397	0.6471768	AA	AA	AA	AA	AA	AA	AA	AA
25		91	rs13476973	rs13476973	BB	3	11073596	0.8655421	AB	BB	AA	BB	BB	BB	AA	BB
27	mutant in our bl6s	99	rs13477425	rs13477425	AA	3	135476593	0.7733857	AA	AA	AA	AA	AA	AA	AA	AA
28		103	rs13477669	rs13477669	BB	4	45887915	0.726115	BB	BB	BB	BB	BB	AB	BB	BB
29		105	rs13477811	rs13477811	AA	4	82384465	0.7793611	AA	AA	AA	AA	AA	AA	AA	AA
30		106	rs13477831	rs13477831	AA	4	87260441	0.7619683	AA	AA	AA	AA	AA	AA	AA	AA
31		112	rs13478578	rs13478578	AA	5	142689128	0.6693822	AA	AA	AB	AB	AB	AA	AA	AA
32		114	rs13478621	rs13478621	AA	6	8907544	0.8097445	AA	AA	AA	AA	AA	AA	AA	AA
33		117	rs13478729	rs13478729	BB	6	44523705	0.8104089	AA	BB	AA	BB	BB	BB	AA	BB
34		121	rs13479082	rs13479082	BB	6	144187491	0.714645	BB	BB	BB	BB	BB	BB	BB	BB
35		125	rs13479497	rs13479497	AA	7	109865120	0.8044025	AA	AA	AA	AA	AA	AA	AA	AA
36		127	rs13479622	rs13479622	BB	8	16273703	0.6279126	AB	BB	AB	BB	BB	BB	AA	BB
37		129	rs13479753	rs13479753	AA	8	49714626	0.8130954	AA	AA	AA	AA	AA	AA	AA	AA
38		131	rs13480073	rs13480073	BB	9	13255595	0.6135755	BB	BB	BB	BB	BB	AB	BB	BB
39		132	rs13480107	rs13480107	AA	9	24740593	0.7329894	AA	AA	AA	AA	AA	AB	AA	AA
40		137	rs13480570	rs13480570	AA	10	30794652	0.7531641	AA	AA	AA	AA	AA	AB	AA	AA
41		140	rs13480657	rs13480657	AA	10	76584143	0.8260361	AA	AA	AA	AA	AA	AB	AA	AA

Figure 4.1 (above): Layout of results from G4i linkage panel showing the SNP, chromosome, position, C57Bl/6J genotype and genotypes of individual mice (per column). Each row represents a SNP and each column represents a mouse (J-P).



	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q
	271	rs3708100	rs3708100	BB	7	99278326	0.7396856	BB	BB	AB	BB	BB	BB	AB	BB
	276	rs3709825	rs3709825	AA	9	49384481	0.6245167	AA	BB	AA	AA	AB	BB	AA	AA
	284	rs3716790	rs3716790	BB	11	17999950	0.6041043	BB	BB	AB	BB	BB	BB	AA	BB
	288	rs3720735	rs3720735	AA	7	25217824	0.6189763	AA	AA	AA	AB	AA	AA	AA	AA
	293	rs3723703	rs3723703	AA	4	101657612	0.7028254	AA	AA	AA	AA	AA	AA	AA	BB
	295	rs3724545	rs3724545	BB	3	50776152	0.6876565	AB	BB	AA	BB	BB	BB	AB	BB
	298	rs3726547	rs3726547	AA	5	105832285	0.6391515	AA	AA	AA	AA	AA	AA	AA	AA
	300	rs4138858	rs4138858	AA	3	88133448	0.7585664	AA	AA	AA	AA	AA	AA	AA	AA
	301	rs4161352	rs4161352	AA	16	10565999	0.7080615	AA	AA	AA	AA	AA	AA	AA	AA
	302	rs4165279	rs4165279	AA	16	21155526	0.6235418	AA	AA	AA	AA	AB	AA	AB	AA
	310	rs4213876	rs4213876	AA	16	87532305	0.800498	AA	AA	AA	AA	AA	AA	AA	AA
	313	rs4225252	rs4225252	BB	5	65265272	0.6318052	AB	BB	BB	BB	BB	BB	AB	AB
	315	rs4230027	rs4230027	AA	13	97200857	0.6812552	AA	AA	AA	AA	AA	AA	AA	AA
	320	rs6157345	rs6157345	AA	1	95497940	0.6222149	AA	AA	AA	AA	AA	AA	AA	AA
	322	rs6160140	rs6160140	BB	7	53312532	0.762599	BB	BB	BB	BB	BB	BB	AA	BB
	323	rs6165425	rs6165425	AA	2	20814394	0.8124077	AA	AA	AA	AA	AA	AA	AA	AA
	324	rs6181382	rs6181382	AA	6	81674296	0.6363292	AA	AA	AA	AA	AB	AA	AA	AA
	326	rs6195285	rs6195285	AA	10	99352909	0.8223802	AA	AA	AA	AA	AA	AA	AA	AA
	338	rs6245977	rs6245977	BB	13	82197498	0.685786	AB	BB	AB	BB	BB	BB	AA	AB
	340	rs6256504	rs6256504	AA	5	38327673	0.8225726	AA	AA	AA	AA	AA	AA	AA	AA
	346	rs6279696	rs6279696	AA	7	80917494	0.6092551	AA	AB	AA	BB	AB	AA	AA	AA
	348	rs6296891	rs6296891	AA	8	79152010	0.8037329	AA	AA	AA	AA	AA	AA	AA	AA
	351	rs6320743	rs6320743	AA	18	65694361	0.787741	AA	AA	AA	AA	AA	AA	AA	AA
	352	rs6326790	rs6326790	AA	15	90938588	0.6050702	AB	AA	AA	AB	AB	AA	AA	AB
	360	rs6371268	rs6371268	AA	2	68927682	0.8326152	AA	AA	AA	AA	AA	AA	AA	AA
	361	rs6371982	rs6371982	AA	3	33929993	0.6426029	AA	AA	AA	AA	AA	AA	NC	AB
	362	rs6372324	rs6372324	BB	13	87331201	0.8228847	AA	BB	BB	BB	BB	BB	AA	AB
	364	rs6376291	rs6376291	AA	2	154204634	0.7990738	AA	AA	AA	AA	AA	AA	AA	AA
	365	rs6386918	rs6386918	AA	4	138224144	0.6393549	AA	AA	AA	AA	AA	AA	AA	AA
	367	rs6391751	rs6391751	BB	10	28239358	0.6750509	AB	BB	AA	BB	BB	BB	AA	BB
	370	rs6411410	rs6411410	AA	X	44405254	0.8005449	NC	AA	AA	AA	AA	AA	AA	AA
	374	rs8258353	rs8258353	BB	10	86034834	0.7861249	BB	BB	BB	BB	BB	AB	NC	BB
	376	UT_17_45.56384	UT_17_45.56384	AA	17	44325625	0.8170928	AA	AA	AA	AA	AA	AA	AA	AA
							heterozygous	18	5	13	7	11	11	11	10
	117 GT SNPs						No call (NC)	6	1	4	1	2	1	6	2
							homozygous	6	2	8	1	0	1	18	1
							SNPs-NCs	111	116	113	116	115	116	111	115
							minus 0.5 x hets	102	113.5	106.5	112.5	109.5	110.5	105.5	110
							minus homs	96	111.5	98.5	111.5	109.5	109.5	87.5	109
							estimated % C57Bl6/J	86.48648649	96.12068966	87.16814159	96.12068966	95.21739	94.39655172	78.82882883	94.7826087

Figure 4.2: Screenshot of the spreadsheet used to calculate % C57Bl/6J of the mice, demonstrating how the % congenicity was arrived at.

The percent congenicity of the mice (or % C57Bl/6J) was estimated by the proportion of successfully genotyped alleles that were of C57Bl/6J origin (see Figure 4.3). The equation below illustrates how the percent C57Bl/6J congenicity for each mouse was then calculated, where N=number of SNPs successfully genotyped, het= number of SNPs heterozygous (1 allele C57Bl/6J, 1 allele BALB/c or C3H/HeJ), hom=number of SNPs homozygous (both alleles non-C57Bl/6J),

$$\%C57Bl6/J = \frac{N-(0.5het)-(\text{hom})}{N} \times 100$$

Figure 4.3: Equation to demonstrate the calculation of percentage congenicity for C57Bl/6J

Due to comparatively low numbers of markers used, the data were not suitable to be analysed by programs such as PLINK (a whole-genome data analysis toolset).

4.3 Results

4.3.1 IVF

In vitro fertilisation attempts for both the C59X and the C417Y lines were successful. One hundred embryos for each line were transferred into pseudopregnant female mice. IVF for the C417Y line was more successful than for the C59X line although there was no significant difference. The proportion of male to female pups was fairly equal for both lines. Table 4.1, below, summarises the results of the IVF.

Mutation	**Number of embryos transferred**	**Number of pups received**	**Males**	**Females**
C59X	100	44	22	22
C417Y	100	61	26	35

Table 4.1: Summary of IVF results for C59X and C417Y lines

4.3.2 Breeding the C59X and C417Y lines

Generation	Line	Males	Females
G2	C59X	5 *Zfp804a*$^{C59X/+}$	10 *Zfp804a*$^{+/+}$
G2	C417Y	9 *Zfp804a*$^{C417Y/+}$	18 *Zfp804a*$^{+/+}$
G3	C59X	6 *Zfp804a*$^{C59X/+}$	24 *Zfp804a*$^{+/+}$
G3	C59X	2 *Zfp804a*$^{+/+}$	2 *Zfp804a*$^{C59X/+}$
G3	C417Y	4 *Zfp804a*$^{C417Y/+}$	16 *Zfp804a*$^{+/+}$
G3	C417Y	2 *Zfp804a*$^{+/+}$	2 *Zfp804a*$^{C417Y/+}$
G4	C59X	11 *Zfp804a*$^{C417Y/+}$	13 *Zfp804a*$^{C59X/+}$
G4	C417Y	9 *Zfp804a*$^{C417Y/+}$	10 *Zfp804a*$^{C417Y/+}$

Table 4.2 Summary of mutant breeding for generations G2 to G4 by line and gender

The table above (Table 4.2) shows the numbers of mutants at each generation used for breeding. Of the 44 mice (G2) of the C59X line received from MRC Mary Lyon Centre, only 5 of these mice were heterozygous mutant males and 9 of the 61 mice received for the C417Y line were heterozygous mutant males carrying the C417Y mutation. These figures are surprising as it would be expected by Mendelian inheritance that 11 of the 22 males received from the C59X line would be homozygous for the mutation and 13 of the 26 males received for the C417Y line would be homozygous for that mutation. These differences were not however significant in a chi-squared test. These mice received from the MRC Mammalian Genetics Unit were used to breed the G3 with C57Bl/6J wild-type females and so forth as detailed in the table. The diagram below (Figure 4.4), outlines the breeding programme used to generate the experimental cohort, the $G4_i$ (intercross) generation.

All G2 progeny received from the MRC Mammalian Genetics Unit, Harwell were genotyped and sexed. All females and any male wild-types were culled and homozygous mutant males were bred with wild-type C57Bl/6J females. The resultant G3 offspring were again genotyped and all wild-types killed. Heterozygous mutants were crossed with C57Bl/6J wild-types. G4 offspring were again genotyped and heterozygous intercrosses (within the same line) were set up to produce the experimental cohort containing heterozygous and homozygous mutants and wild-type littermate controls.

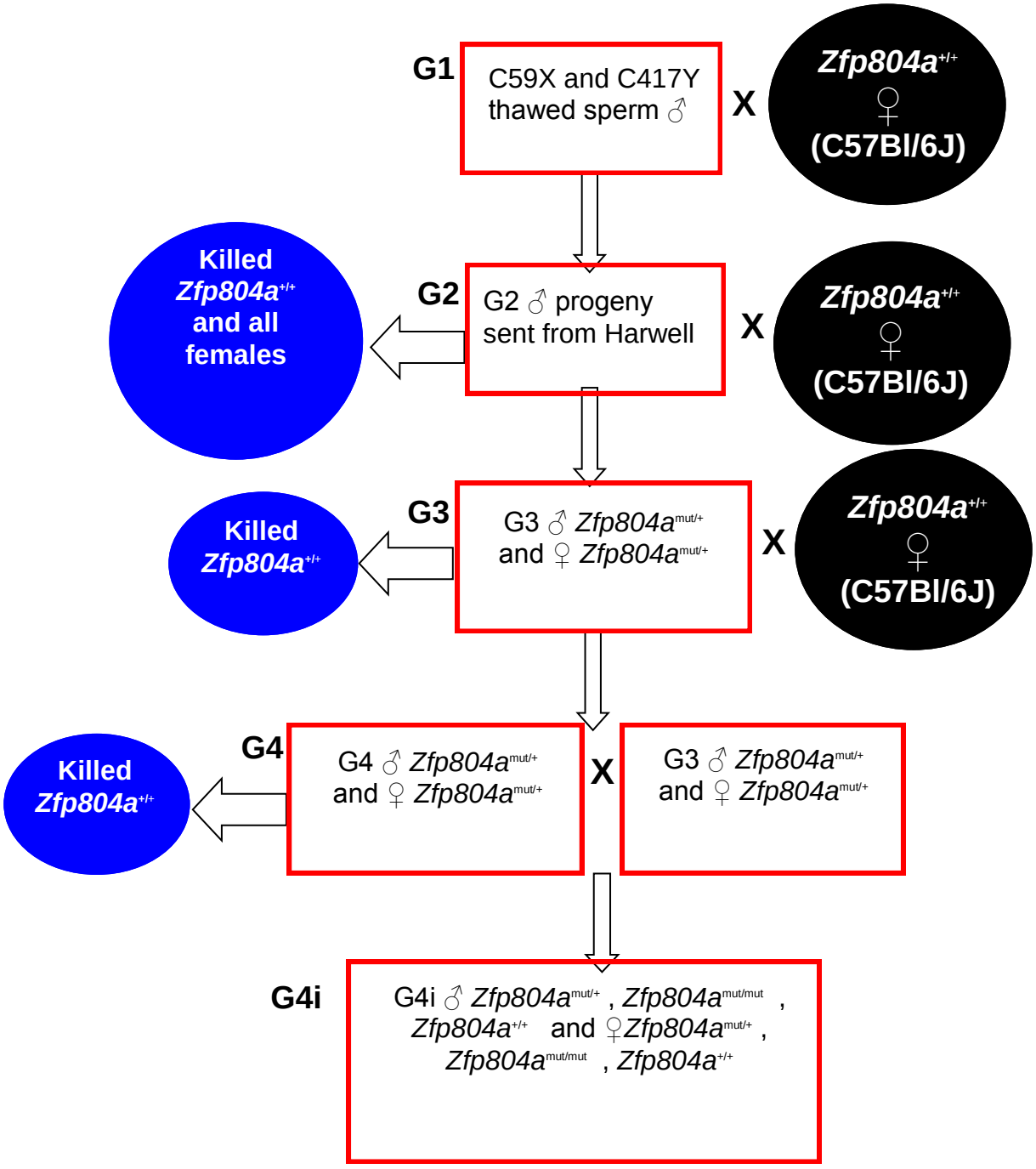


Figure 4.4: Flow diagram to show breeding programme for the generation of the G4$_i$

4.3.3 Speed congenics

Two linkage panels were used in this chapter, one on the G_3 generation to assist selection of breeders and one on the G_{4i} generation to confirm estimates of percentage congenicity. The rate of successful SNPs genotyped by generation (Table 4.3) and the results from each linkage panel are provided (Tables 4.4 and 4.5).

Number of SNPs genotyped	Generation	Number SNPs successful	% SNPs successful
377	G_3	337	89.3
377	G_{4i}	117	31

Table 4.3: Summary of SNPs successfully genotyped in the linkage panel by generation

In the G4i linkage panel, far fewer SNPs were successfully genotyped than in the G3 linkage panel, thought to be due to reagent degradation as reagents were used after nine months of the receipt of the reagents. However, the successful SNPs (in both linkage panels) all were rated with a Gentrain score (a score of confidence of the genotyping score) of >0.64 (where 1 is the maximum). This confidence threshold is used by others in the laboratory (Kiran Mantripragada, personal communication, 2009), indicating that the successful SNPs in this linkage panel were reliable.

Tables 4.4 – 4.6 show the results of the linkage panels for G3, G4i and C57Bl/6J controls respectively. The average percentage of SNPs conforming to the Illumina C57Bl/6J genotype at that locus is given and is used to infer an estimate of C57Bl/6J congenicity.

Generation	Mouse ID	Estimated % C57Bl/6J	Generation	Mouse ID	Estimated % C57Bl/6J
G3	F3.2	88.18	G3	F15.1	80.03
G3	F5.3	88.98	G3	F19.7	84.35
G3	F11.4	86.42	G3	F4.3	86.74
G3	F15.6	81.15	G3	F7.4	85.30
G3	F1.6	88.34	G3	F21.5	84.35
G3	F5.6	88.98	G3	F19.9	85.94
G3	F12.1	85.30	G3	F4.5	87.86
G3	F18.2	84.50	G3	F3.5	84.03
G3	F1.7	77.96	G3	F20.3	87.86
G3	F5.7	84.66	G3	F7.2	87.54
G3	F12.4	83.87	G3	F14.5	86.42
G3	F1.8	82.75	G3	F19.6	90.58
G3	F6.2	83.55	G3	F3.7	83.87
G3	F12.5	82.75	G3	F7.3	89.14
G3	F18.6	86.26	**G3**	**AVERAGE**	**85.45**
G3	F3.6	84.98			

Table 4.4: Summary of results by mouse for G3 linkage panel

Generation	Mouse ID	Estimated % C57Bl/6J	Generation	Mouse ID	Estimated % C57Bl/6J
$G4_i$	Z6B	96.12	$G4_i$	Z9C	96.85
$G4_i$	S5B	96.12	$G4_i$	S16A	98.25
$G4_i$	Z18B	94.4	$G4_i$	S17A	94.78
$G4_i$	S10D	94.78	$G4_i$	Z2E	97.81
$G4_i$	S8D	96.88	$G4_i$	S14B	94.30
$G4_i$	S4C	96.05	$G4_i$	S5C	89.38
$G4_i$	S5A	97.83	$G4_i$	Z17D	94.2
$G4_i$	S9A	97.83	$G4_i$	Z15B	98.67
$G4_i$	S2B	97.41	$G4_i$	S4D	89.47
$G4_i$	S14A	97.39	**$G4_i$**	**AVERAGE**	**96.13**
$G4_i$	Z16A	97.39			

Table 4.5: Summary of results by mouse for $G4_i$ linkage panel

Generation	Mouse ID	Estimated % C57Bl/6J
C57Bl/6J control	BL627	99.57
C57Bl/6J control	BL628	100
C57Bl/6J control	BL629	100
C57Bl/6J control	BL630	100
C57Bl/6J control	BL631	100
C57Bl/6J control	BL632	100

Table 4.6 Summary of results by mouse for C57Bl/6J controls

4.4 Discussion

This chapter provides further evidence that mutant mice can be successfully created from ENU mutagenesis, and that the procedures adopted in this protocol for mutation detection and re-derivation were successful. Mice for both mutant lines were successfully created and bred to third generation, and an estimate made of congenicity. G4, although some argue is not ideal to study, is valid, especially as a means of preliminary screening for gross abnormalities or indicators of further areas to investigate. If necessary, tests can be repeated on purer animals.

4.4.1 Residual mutations

As previously mentioned (4.1), the chances of any two mice in the same group having the same residual mutation are extremely low. Using the mutation rate adopted by Keays *et al.* (2006) assuming the founder mouse had 31 mutations, there is therefore a 'pool' of 31 mutations which any offspring from that mouse may have. On average, a G4 mouse will have approximately 4 mutations (3 of which are unwanted; see the introduction of this chapter for an explanation). This gives a probability of a specific mouse having any specific unwanted mutation of 0.1 (3/30=0.1). This gives a probability of any two specific mice having the same specific unwanted mutation of 0.01 (0.1x0.1=0.01). This figure becomes smaller with the addition of the likelihood that the mice are in the same experimental group (by gender, genotype and line). It can be seen therefore that the issue of potential confounding residual mutations is minimal. This however, is not taking into account the potential problem of other linked mutations. The issue of how likely mice are to have the same residual

mutations is further complicated by other issues such as gender, parentage, and the chances of any one mutation. However, the chance of recurring residual mutations in groups is extremely unlikely. Standard errors obtained from behavioural tasks and whether or not the wild-type mice can be grouped statistically will give an idea of whether or not the other residual mutations are causing problems. It is important to state that this issue of other mutations is not unique to ENU-derived models and other approaches to a genetic mouse model are equally likely to have confounding factors. Background variation is apparent in the creation of all genetic mouse models as all strains of mice have variation at SNPs and many strains carry a mutation such as the 129 strain's *Disc1* deletion or Harlan's deletion in the α-synuclein locus in a C57Bl/6J substrain (discussed in 1.6.2.1). Moreover, any crossing of a mutation onto a different background strain will result in some variance in genetic background between subjects in the same experimental group. Backcrossing, as well as a means of isolating the mutation of interest, is also a strategy to minimise differences in genetic background between subjects, making the background as homogeneous as possible. With ENU mutants, because of the residual mutations, researchers are often more cautious with background genetics and so tend to backcross for more generations than perhaps researchers looking at other models such as knockout models who may only backcross for two generations (Pat Nolan, personal communication, 2012).

One point to note is that mice, and all wild species, have naturally occurring mutations and it could be argued that it is more ecologically valid (i.e. more akin to the human population) to have some genetic differences between the subjects (although these populations are not suited to studying in small

groups, such as this study). Additionally, not every gene is expressed in the brain (although the majority are). It was recently found that a minimum of 82% of genes are expressed in the brain and so therefore may influence brain function (Human Brain Map, Allen Brain Atlas, 2009).

As previously stated, there is a 20.1% chance that there will be a linked mutation that is inherited along with the C59X or C417Y mutations, or a 4.4% chance that there will be a mutation linked to both the C59X and C417Y mutations. Additionally, the chances that the mutation linked to the C59X and C417Y mutations is the same are extremely small. With the little currently known about *ZNF804A* and *Zfp804a*, it is extremely difficult and not useful to speculate on what we might observe (behaviourally) if there is indeed a linked mutation. Until we are able to characterise the lines molecularly with the use of a reliable antibody, it is a consideration not easily excluded short of sequencing large regions either side of the mutations.

Searching for genes surrounding *Zfp804a* (UCSC Genome Browser, http://genome.ucsc.edu/) revealed many genes and did not prove particularly helpful in terms of guessing the influence of potential linked mutations as it is not known what region of chromosome 2 is likely to be inherited through linkage in the $G4_i$ generation.

4.4.2 Speed congenics

This chapter presents the development of the Zfp804a mutant lines with a trial of the 'speed congenics' approach described in Ogonuki *et al.* (2009) although

the protocol was not followed. The approach used in this thesis had both successes and failures as a means of effectively speeding up congenicity.

The speed congenics approach used in this chapter provided scientific assessment of and confidence in the level of congenicity of the mice as opposed to the conventional probabilistic assessment of congenicity used in simple backcrossing. It has demonstrated that a novel method could work, provided sufficient numbers of mice are bred to make the approach work well, i.e. sufficient numbers of mice to generate a spread of levels of inheritance of the C57Bl/6J parental genome so the 'best' mice can be selected.

Ogonuki *et al*. (2009) bred to 100% congenicity in 5 generations using microsatellite marker-assisted selection, whereas 96.13% was achieved in this chapter with 4 generations. However, due to the numbers of mice the linkage panel was carried out on, the most C57Bl/6J-congenic mice were not able to be selected in the breeding program, instead the lowest congenicity were excluded. As well as increased numbers of mice, confidence in estimates of congenicity would have been improved with a higher density linkage panel, i.e. a linkage panel containing upwards of 1000 SNPs rather than a 377 SNP panel, especially if there is not a high success rate of successfully genotyped SNPs.

To conclude, this chapter has successfully created 2 parallel *Zfp804a* mutant lines harbouring the C59X and C417Y mutations described in Chapter 3. There are some potential problems with the models but characterisation and investigation of these models will provide some insight into whether these are in fact issues.

CHAPTER 5: Initial characterisation of *Zfp804a* mutants; assessment of general viability and preliminary behavioural screen

5.1 Introduction

In Chapter 4, the creation of two novel mouse lines carrying mutations in *Zfp804a* was described. This chapter is concerned with the initial physiological and behavioural characterisation of the mutants. These mutant lines are the first available animal models for human *ZNF804A,* a largely unknown gene in terms of function. Preliminary investigations with any novel mouse model are important in several ways. They give an indication of the viability of the model including any physical deficits the model may have which could confound future analyses, in particular with respect to behavioural tasks. The screen can also provide indications of phenotypes that warrant further investigation *per se*. These were the primary goals of Chapters 5 and 6. Once the presence of confounding deficits are excluded or noted however, it will be of further interest to examine the differences between the genotypes and between the two lines, C59X and C417Y. The latter will be of especial interest as there is, at the time of writing, no biochemical analysis of the mutations so it will give us some initial pointers as to whether or not the two mutations are impacting on function differently.

Characterisation of the mutants at an early stage is also important to assess the validity of one of the potential criticisms of this work, inherent in using ENU-derived animal models in the first few generations of backcrossing (as noted in Chapter 4, in order to gain some functional data in the thesis it was

decided to analyse mice from the $G4_i$ generation). Measures of standard deviation in behavioural tasks for animals within groups and the similarity between wild-types from the two separate lines (as wild-types should carry the same amount of residual mutations as the 'mutants' but not the *Zfp804a* mutations) might be informative.

There has been mixed evidence regarding the interaction between biological sex and *ZNF804A*. Sex does not appear to affect association with rs1344706 (O'Donovan, personal communication) whereas another SNP, rs7597593, shows association with schizophrenia in females, but not in males (Zhang *et al.*, 2011). As both males and females were investigated in the present work, the analysis of the data obtained in these preliminary studies may also give us an idea of whether or not sex interacts with the mutations selected for study. With female mice, it is important to be aware of the potential effects of the stage of oestrus cycle on behaviour (e.g. Marcondes *et al.,* 2001; Sousa, Almeida & Wotjak, 2006) and to conduct analyses to investigate whether or not this has an impact on results, and could explain any differences observed amongst the female mice. In order to do this, it is necessary to obtain vaginal cells at the time of each test, which can be done with a simple and relatively non-invasive smearing procedure.

This chapter assessed the initial viability and some preliminary behaviour of the $G4_i$ generation. As discussed in Chapter 4, this generation is not ideal due to the possibility of residual, potentially confounding 'non-specific' ENU mutations. However, it was considered valid to exploit this early generation in order to get advance notice of the general viability of the mutant lines and provide some indications of possible behavioural phenotypes for

further investigation. Necessarily, most of the G4 generation (used to breed the $G4_i$ line) were needed for further backcross breeding. Additionally, even a 'preliminary' analysis of the type reported here required substantial numbers of experimental animals; 12 groups (2 lines, 3 genotypes [wild type, heterozygous mutants and homozygous mutants], male and female). Together with time constraints, these practical issues dictated that some of the group sizes in this section were relatively small.

Throughout this chapter, all 12 groups of mice were treated identically with the exception, that females underwent vaginal smearing on the day of testing, post-experiment. For clarity, the data are described in terms of the main experimental groups (i.e. C59X and C417Y, genotype, males and females). Wild-types for both lines were treated as separate groups. The groupings were planned *a priori*.

5.2 Materials and methods

5.2.1 Subjects and animal husbandry

An experimental cohort of $G4_i$ (G4, heterozygous x heterozygous intercross) C59X and C417Y mice (aged 3-9 months) and littermate wildtype controls were subjected to general observation and a number of discrete physiological and behavioural tests. General housing, handling and behavioural testing conditions were as described in chapter 2, General Materials and Methods (2.5). The number of mice (*n*) used in each assay is summarised in the tables below (table 5.1 and 5.2 for the C59X and C417Y lines respectively):

C59X	Genotype and *n*					
Test	♂ *Zfp804a*$^{+/+}$	♂ *Zfp804a* +/C59X	♂ *Zfp804a* C59X/C59X	♀ *Zfp804a* $^{+/+}$	♀ *Zfp804a* +/C59X	♀ *Zfp804a* C59X/C59X
Weights from birth	6	5	1	1	5	2
Brain weights (adult)	5	4	4	5	5	5
Body weights (adult)	8	9	4	10	10	10
PhenoTyper cages	8	-	4	-	-	-
Rotarod	8	9	4	10	10	10

Table 5.1: Number of mice, byexperimental group (genotype [wild-type, heterozygous mutant, homozygous mutant] and sex [male, female]) used in each measure in the C59X line

C417Y	Genotype and *n*					
Test	♂ *Zfp804a* $^{+/+}$	♂ *Zfp804a* +/C417Y	♂ *Zfp804a* C417Y/C417Y	♀ *Zfp804a* +/+	♀ *Zfp804a* +/C417Y	♀ *Zfp804a* C417Y/C417Y
Weights from birth	6	6	2	1	4	3
Brain weights (adult)	5	5	5	5	5	5
Body weights (adult)	7	14	8	8	12	9
PhenoTyper cages	7	-	8	-	-	-
Rotarod	7	14	8	8	12	9

Table 5.2: Number of mice, byexperimental group (genotype [wild-type, heterozygous mutant, homozygous mutant] and sex [male, female]) used in each measure in the C417Y line

Physiological measures:

5.2.2. Body weight assessments

A subset of mice was weighed daily for the first 6 weeks of life and identified by a coloured spot on the abdomen, back or the base of the tail (dependent on stage of development). Final body weight was measured at death for all mice. Mice had access to *ad libitum* water and standard laboratory chow throughout their life course.

5.2.3 Brain weight assessment

Brains were extracted as described in chapter 2.9 and weighed immediately following death, with the olfactory bulbs removed.

5.2.4 Oestrus cycle status

Oestrus status was determined by vaginal smearing immediately following a behavioural procedure (as described in the General Methods, 2.5.3). The data from the behavioural tests was then analysed to assess whether or not there

was a correlation between the behavioural data and the stage of oestrus of a particular mouse.

Behavioural measures:

5.2.5 'PhenoTyper' homecage environment

A subset of subjects was observed in PhenoTyper cages (Noldus, described in chapter 2.6.2) for 24 hours from between 07:00 and 09:00 with 12 hours of light and 12 hours of darkness. These cages are designed to replicate a temporary home cage environment and can provide a useful initial behavioural screen across a wide variety of measures, including general activity, consumatory behaviour, bouts of activity on an exercise wheel and general indices of circadian function and sleep. Data was recorded by automated software and the mouse was tracked for the duration using a heat-sensitive camera.

5.2.6 Rotarod

Subjects were tested on the Rotarod apparatus (as described in chapter 2.6.1) to assess motor learning, motor function, balance and coordination. Subjects underwent 3 trials separated by at least 30 minutes (to allow for recovery). In the trial, the rotating rod constantly accelerated over 5 minutes, up to a maximum of 48rpm. The latency to fall off the apparatus was noted up to a maximum of 500 seconds.

5.2.7 Statistical analyses

Experimental data were analysed using SPSS PASW Statistics, release 18.0 (IBM SPSS Inc., 2009, Chicago). Data were subjected to One Way, Two Way or multivariate AN(C)OVA (if normal or One Way Kruskal-Wallis ANOVA if data did not conform to normality estimates, Levene's test, $p>0.05$). All comparisons were planned *a priori* and corrections for multiple testing were not made. Significance was defined as $p<0.05$.

5.3 Results

5.3.1 Generation of experimental cohort

Table 5.3 shows the number of animals paired to generate the $G4_i$ cohort. There were no significant differences observed in the birth ratios for males and females or by genotype. From the animals paired, 3 *Zfp804a*$^{+/C59X}$ and 2 *Zfp804a*$^{+/C417Y}$ mice failed to conceive. Additionally, 2 of the C417Y litters were killed by their mothers. Thus the average litter size for the C59X line was 10.9 and 11.3 for the C417Y line. Not all of the animals born were used in the experimental cohort.

Line	Pairings (*n*)		Offspring: genotype (*n*)					
	G4 ♂ +/mut	G4 ♀ +/mut	♂ +/+	♂ +/mut	♂ mut/mut	♀ +/+	♀ +/mut	♀ mut/mut
C59X	11	13	15	25	4	21	27	17
C417Y	9	10	8	14	9	8	16	13

Table 5.3: Number of (G4) heterozygous mutant animals paired to breed the G4i (experimental) cohort for each line and consequent births observed. Mut is used to denote the mutation, C59X or C417Y.

5.3.2 Early development of mutant lines

Weight gain from birth until 6 weeks post-natal was used as a general proxy of early development (see Figures 5.1 and 5.2). Statistical analyses was not performed on these data as group sizes in some instances were too small to analyse (i.e male *Zfp804a*$^{C59X/C59X}$ and female *Zfp804a* $^{+/+}$ for both lines had *n*=1), however, from simple by eye observations, males seem to be heavier than females, which would be expected. This sex difference in weight seems to emerge at about 3 weeks.

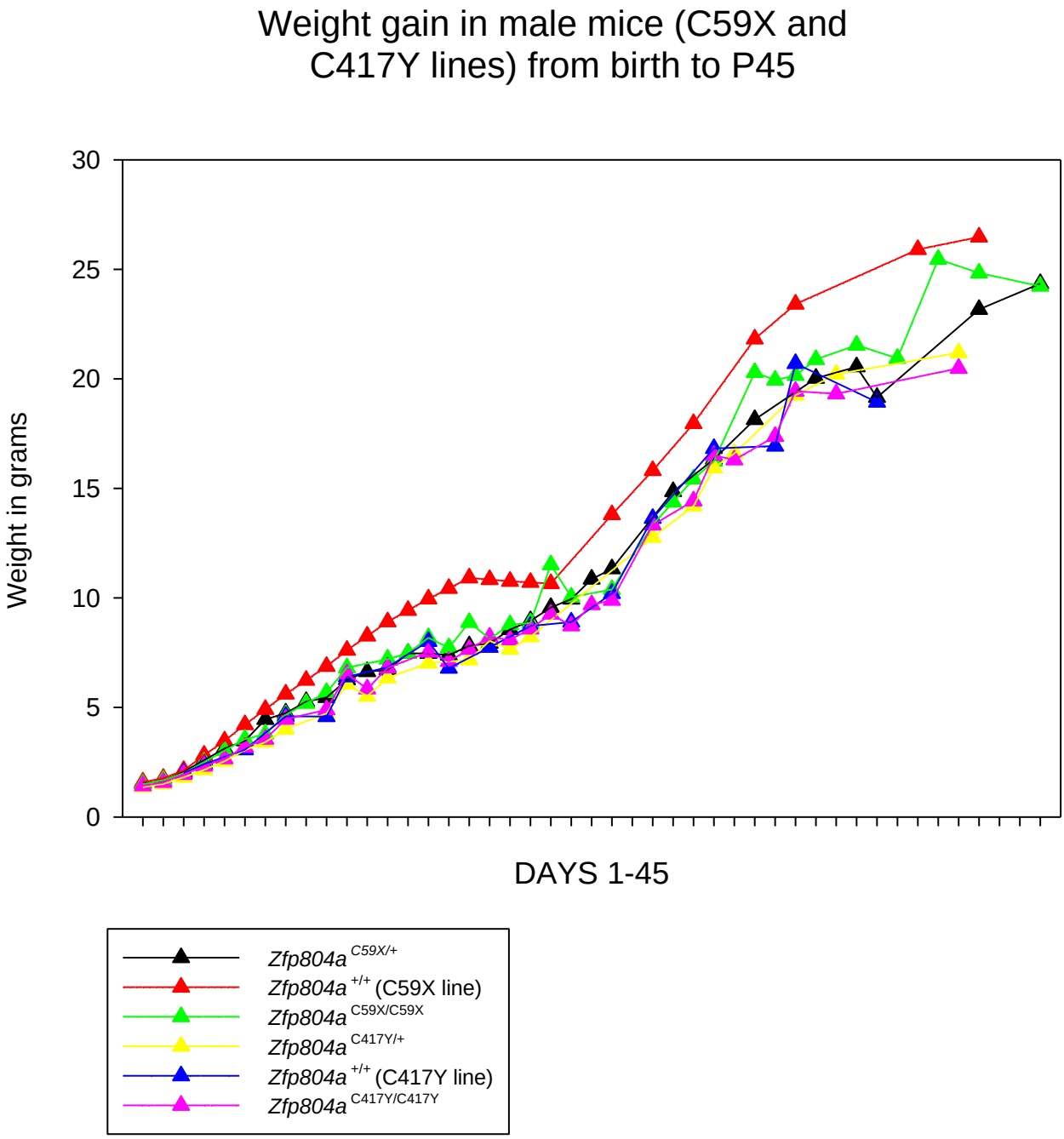


Figure 5.1: Mean average body weight gain from P1 to P45 in males (C59X and C417Y lines)

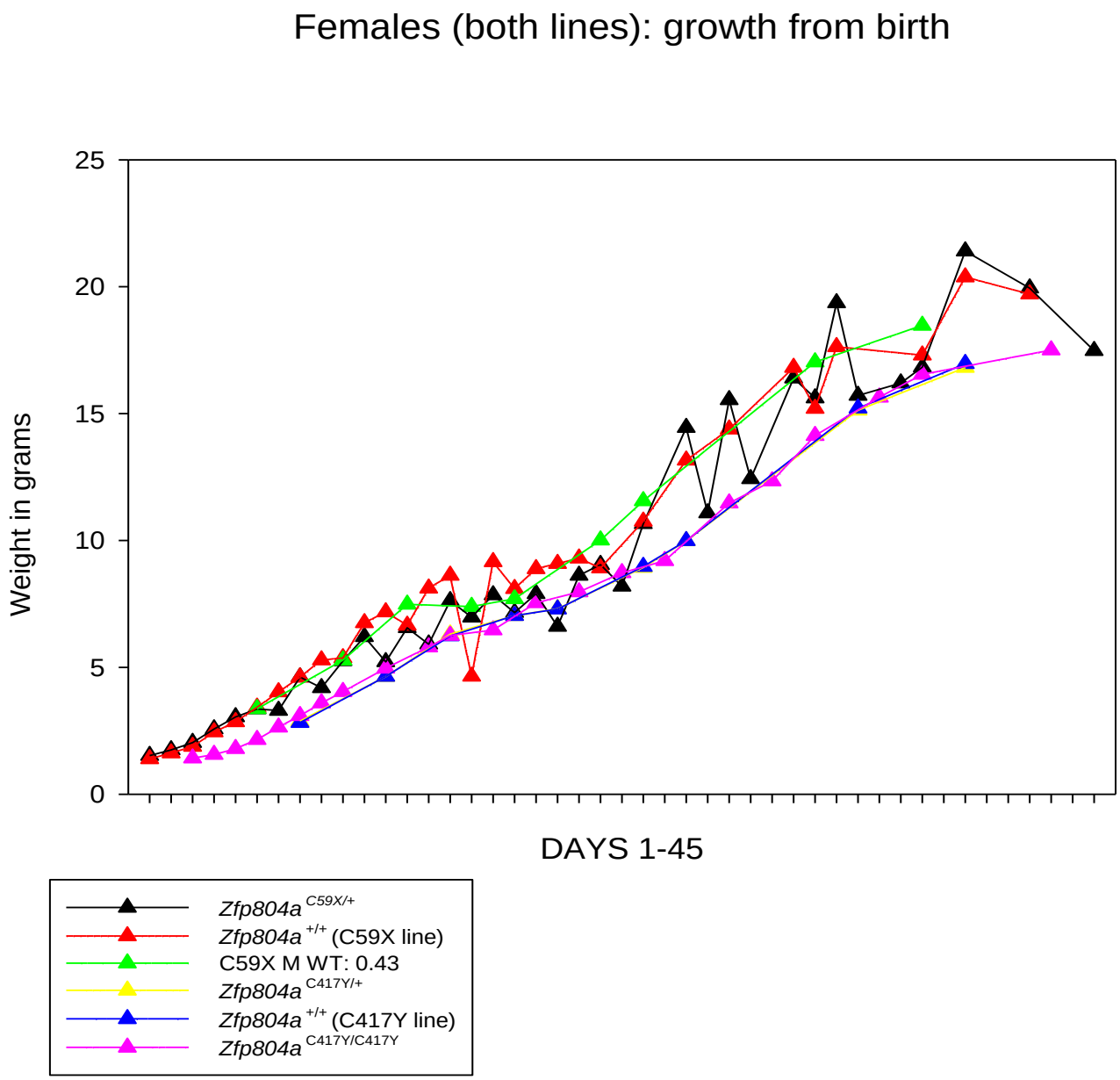
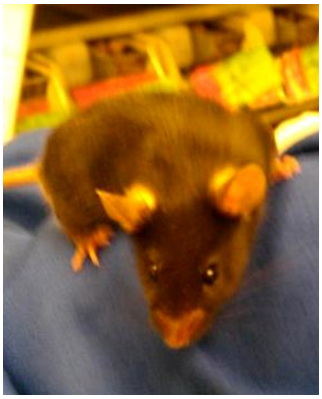


Figure 5.2: Mean average body weight gain from P1 to P45 in females (C59X and C417Y lines)

5.3.3. Gross physical appearance of adult mutants and general observation

The photos in figure 5.3 show the gross physical appearance of the mutant lines, (males only shown), when adult. There were no gross observable physical deficits found in the mutant mice and on general observation the mutants appeared identical to their wild-type littermate controls. As the mutation was bred from a Balb/c x C3H/HeJ cross onto a C57Bl/6J background, there is some variation between the mice in terms of coat colour. The majority of the mice were black with the remaining mice having agouti coats.

a) *Zfp804a*$^{C59X/C59X}$ ♂



b) *Zfp804a*$^{+/+}$ ♂ (C59X line)

c) *Zfp804a*$^{C417Y/C417Y}$ ♂

d) *Zfp804a*$^{+/+}$ ♂ (C417Y line)

Figure 5.3: The physical appearance of the mutants, from left to right, top to bottom: a) Zfp804a$^{C59X/C59X}$ *male b) Zfp804a*$^{+/+}$*male(C59X line) c) Zfp804a*$^{C417Y/C417Y}$ *male d) Zfp804a*$^{+/+}$ *male(C417Y line)*

Continuous monitoring of health and welfare of the animals by the experimenter and animal technicians throughout the periods of both development and experimentation revealed no abnormal health problems segregating with any particular group. During the testing period the mortality rate of the mutant lines did not differ from that of WT littermate controls (1 female mouse was found dead, *Zfp804a*$^{C59X/C59X}$). As the mice were not allowed to age to the point of natural death (subjects were culled at c. 8 months of age average) the effects of the mutations on frank ageing remain unknown to date. Animals were excluded from further testing if they became sick and did not show signs of improving after two days of illness (2 males, *Zfp804a*$^{C417Y/+}$ and *Zfp804a*$^{C59X/+}$and 1 female, *Zfp804a*$^{+/+}$ from the C417Y line). Mice which were bullied to the point of detrimental health effects were also removed from further testing (1 male mouse, *Zfp804a*$^{+/+}$) as were any pregnant mice (1 female mouse, *Zfp804a*$^{C417Y/+}$). Few mice were excluded in practice and, overall, exclusions did not segregate with any line, genotype or sex.

5.3.4. Adult body and brain weights

Differences in adult body and brain weights taken at death, around 8 months on average, were observed (see figure 5.4 for mean average body weights at death, by experimental group). A 2-way ANOVA with factors sex (male/female) and genotype (wild-type, heterozygous mutant, homozygous mutant) for each line, revealed significant differences between the experimental groups in terms of body weight. As would be expected, there was a main effect of sex in both lines. Males (C59X mean [*M*] = 36.10g, standard deviation [*SD*]= 3.89, C417Y *M*=34.63, *SD*=3.52) were significantly heavier than females (C59X *M* = 29.07g,

SD = 4.26, C417Y *M*=27.66, *SD*=3.82), *F*(1,51) = 41.43, *p*<0.0001 for the C59X line and *F*(1,57) = 47.59, *p*<0.0001 for the C417Y line. There was also a main effect of genotype on body weight in the C59X line, *F*(2,51) = 16.32, *p*<0.0001. The homozygous mutants independent of sex (*M* = 26.74g, *SD* = 3.66) were significantly lighter than both the heterozygous mutants (*M* = 34.3g, *SD* = 4.13) and the wild-type mutants (*M* = 33.67g, *SD* = 5.04). There were no differences observed between the heterozygous and wild-type mutants in this line. In the C417Y line, there was no impact of genotype on body weight (*F*(2,57) = 3.013, *p*>0.05.

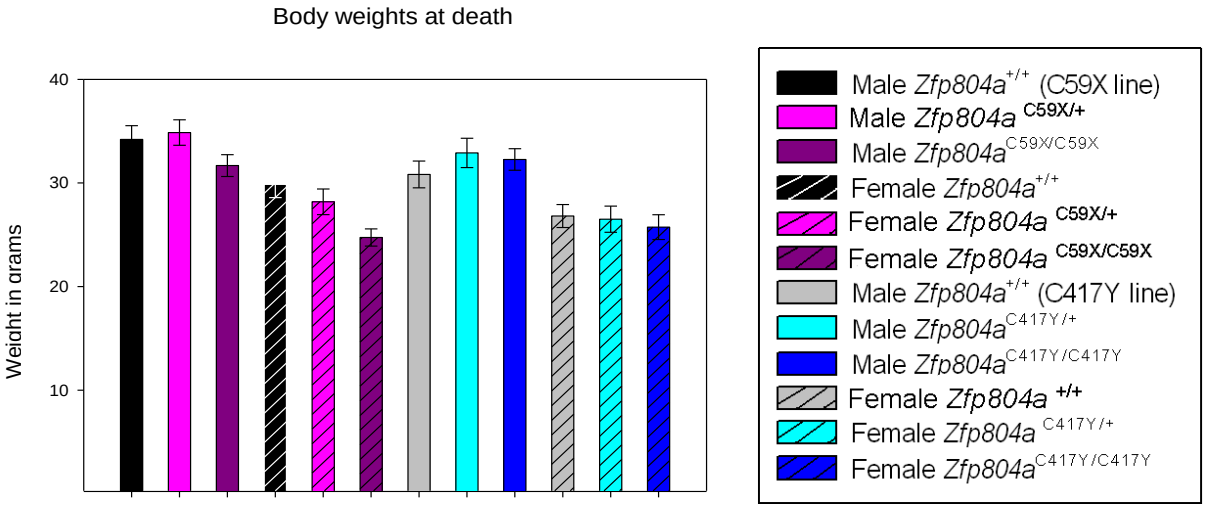


Figure 5.4: Graph (above) showing mean average body weights at death, by experimental group (sex, line and genotype), with key (right of figure). Standard error of the mean bars are shown

From looking at the graph overleaf (figure 5.5), it would appear that the homozygous male mutants in the C59X line had lighter brain weights than their heterozygous mutant and wild-type littermate controls. However, an ANCOVA, with factors sex (male/female) and genotype (wild-type, heterozygous mutant, homozygous mutant) for each line, co-varying for body weight revealed that this was not the case (*F*(2,28)=0.96, p>0.05). Although body weight had no

significant impact on brain weight (not reported) in the C59X line, in the C417Y line it did ($F(1,30)=4.86$, $p=0.037$). Additionally, in the C417Y line, there was a main effect of gender which revealed that females had heavier brains than the males, independent of body weight ($F(1,30)=10.20$, $p=0.004$, females $M=0.43$, $SD=0.02$, males $M=0.41$, $SD=0.02$).

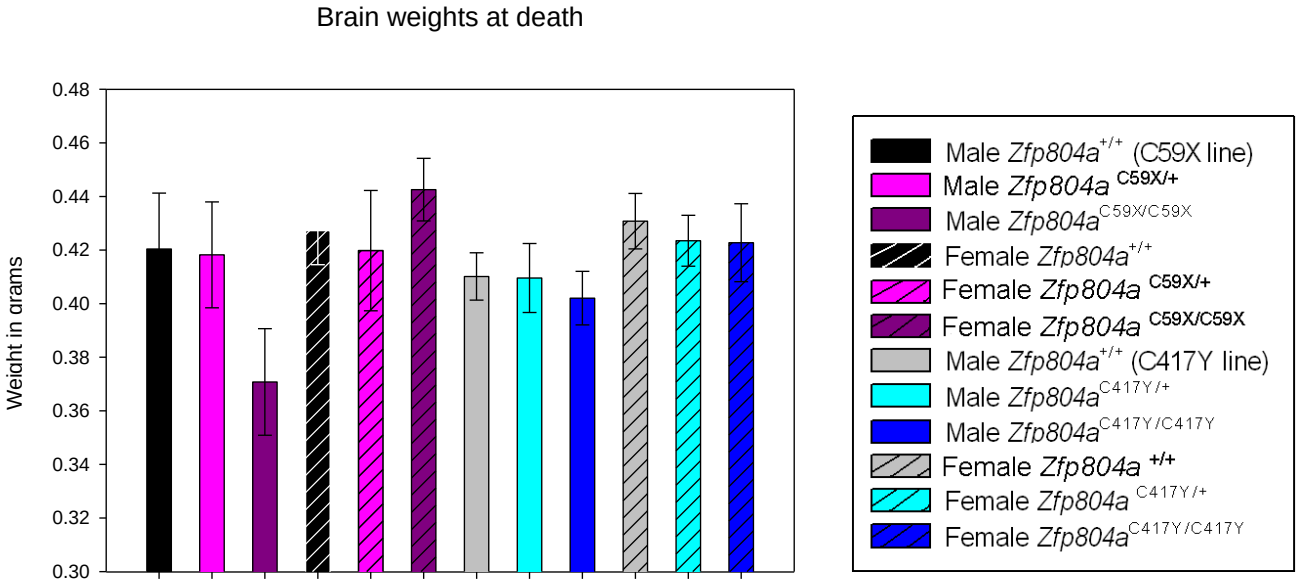


Figure 5.5: Graph (above) showing mean average brain weights at death, by experimental group (sex, line and genotype), with key (right of figure). Standard error of the mean bars are shown

5.3.5. Reactivity of the mutant lines to general handling

All subjects were handled from weaning age, at least weekly. Most animals showed some signs of distress during initial handling (including vocalisation, fecal deposition, urination and struggling). There were no apparent sex, line or genotype effects on these behaviours with all subjects reacting to careful handling in a similar way. Only one mouse from the experimental cohort showed particular signs of aggression or anomalous behaviour, taking the opportunity to bite the experimenter whenever possible. This mouse was a male heterozygous for the C59X mutation and was the only mouse from the

group of 10 mice of the same sex, line or genotype, meaning any systematic relation of the aggression to genotype was likely to be spurious. Prior to behavioural testing mice were handled daily for at least two weeks. Testing began at approximately 8 weeks and mice were well accustomed to handling in general by this point. All female mice responded with signs of distress (unsurprisingly) to the vaginal smearing procedure with some habituation to the procedure over time. Habituation to the procedure did not appear to segregate with any particular line or genotype.

5.3.6 Behaviour monitored in the PhenoTyper cages

Due to time constraints, only a subset of mice was examined in the PhenoTyper homecage environment. Details of animals examined are provided in tables 5.1 and 5.2. In brief, up to 8 homozygous male mutants and wild-types of each line were tested and behaviour was recorded.

5.3.6.1 General activity

Following an initial spurt of activity in the first 3-hour bin measured in the Phenotyper Home Cage Environment (due to exploration of a novel environment), mice habituated somewhat and the level of activity reduced (see figure 5.6). There was another increase in activity after the lights went out after 12 hours in the cages. A significant difference in activity during this 3-hour period in the C417Y line was discovered using MANOVA (factor: genotype) to examine distance travelled in each of the 8 3-hour bins in the 24 hours between the genotypes.Homozygous mutant mice were more active than wild-types (indexed by cm travelled on the cage floor, *M*=25 657.73cm, *SD*=8364.37,

M=16 293.08cm, *SD*=7870.46 respectively, *F*(1,26)=8.93, p=0.006. There were no other differences between the two genotypes examined in the C417Y line and none in the C59X line in the 24 hours spent in the phenotypers in general activity in the cages, data not reported.

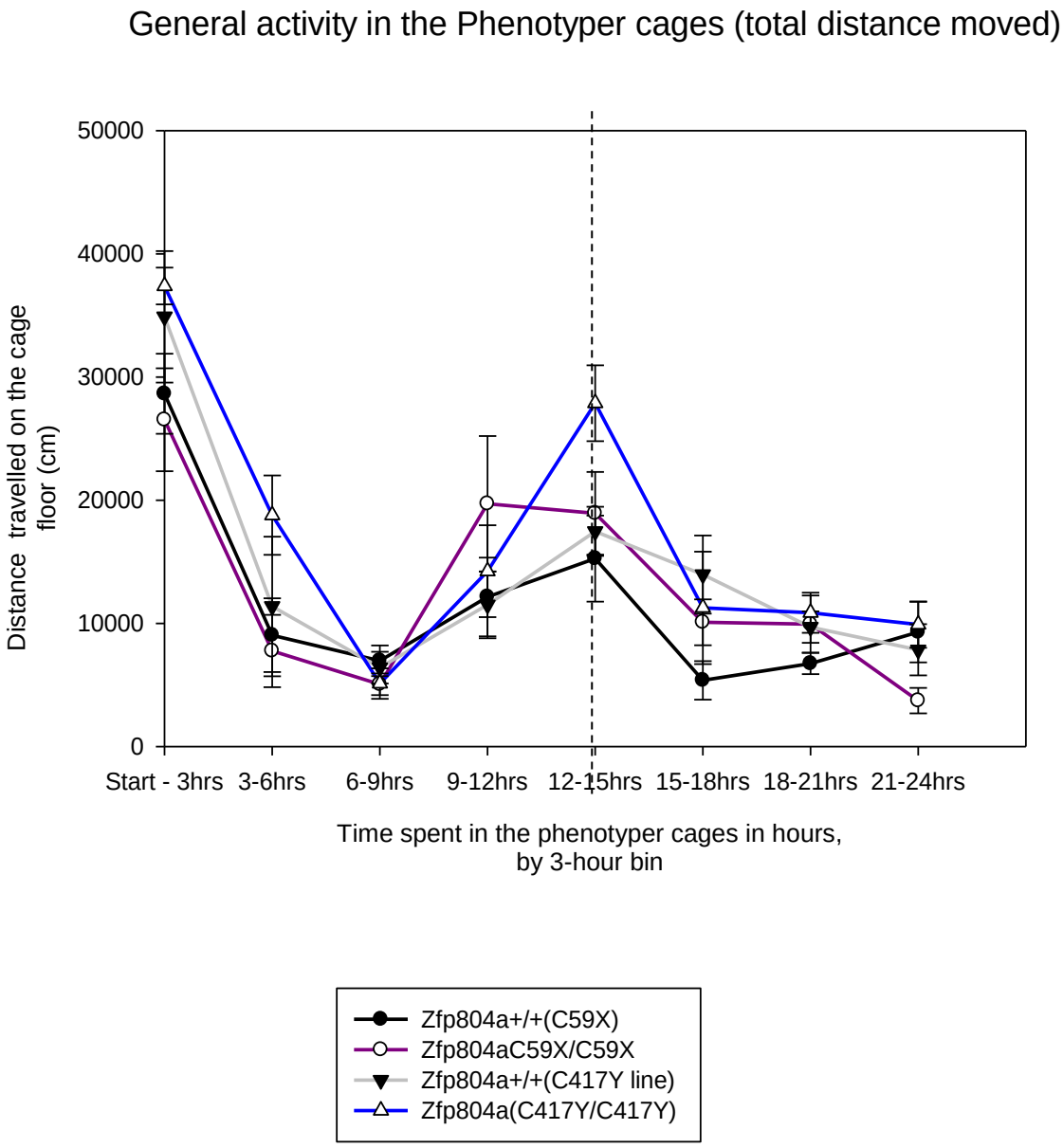


Figure 5.6: Graph (above) showing general activity (indexed by distance travelled on the cage floor in cm) in the PhenoTyper cages over 24 hours by line and genotype. Dashed line indicates when lights were turned off, after 12 hours in the phenotyper cages. Mean averages of the experimental group and SEM (error bar) are shown.

5.3.6.2 Exercise wheel

In each of the lines, bouts of activity, measured by time spent on the exercise wheel, were measured in homozygous mutants and wildtypes using MANOVA (factor: genotype) to look at total time spent on the exercise wheel in each of the 8 3-hour bins. There were no significant differences in bouts of activity between the genotypes for either of the lines in any of the 3-hour bins (data not reported). From examination of the graph (figure 5.7) showing the mean average time (seconds) spent on the activity wheel with standard error of the mean marked, this lack of difference between the genotypes is likely due to fairly large size of the standard errors.

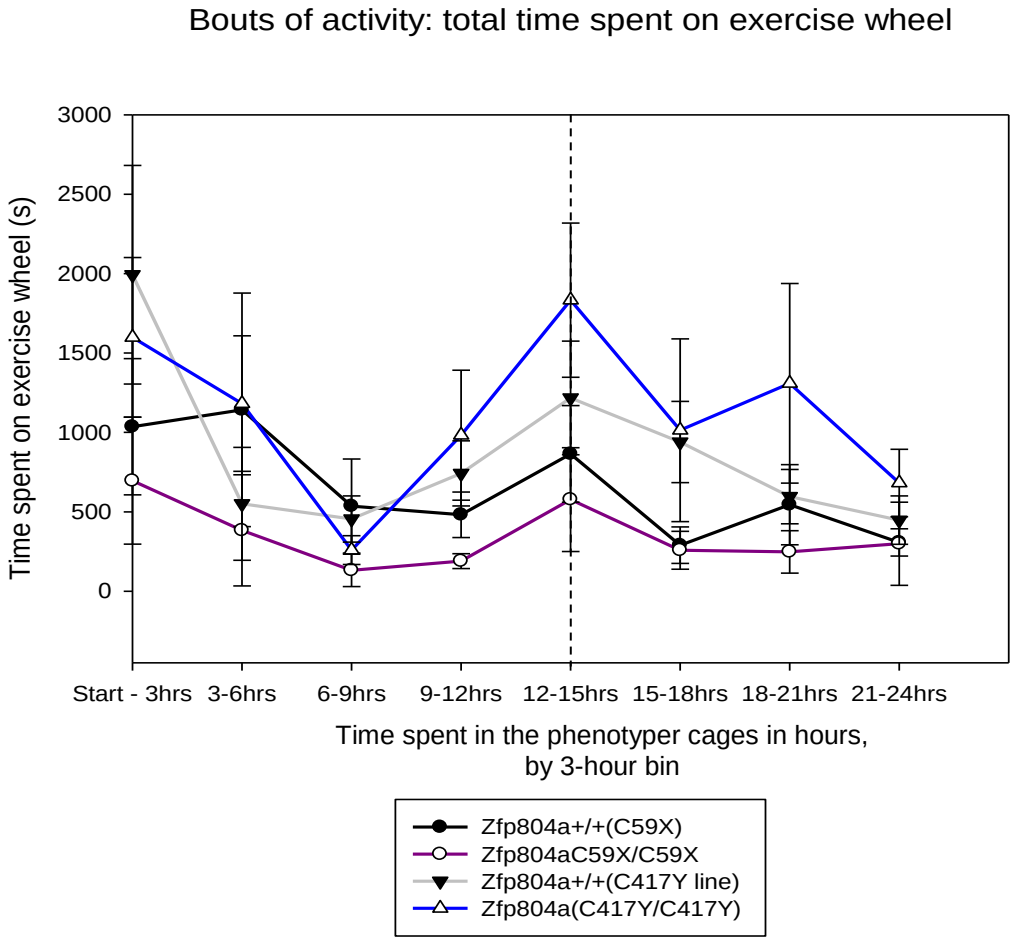


Figure 5.7: Graph (above) showing time (s) spent on the exercise wheel in the PhenoTyper cages over 24 hours by line and genotype. Dashed line indicates when lights were turned off, after 12 hours in the cages. Mean averages of the experimental group and SEM (error bar) are shown.

5.3.6.3 Feeding and drinking behaviour

Figure 5.8 shows the total amount of food and water consumed in the phenotyper cages during the 24 hours for both genotypes of both lines. As is apparent in the graph, there were no differences in food or water consumed between the genotypes for either the C59X line (food: $F(1,11)=0.164$, $p>0.05$, water: $F(1,11) = 0.118$, $p>0.05$) or the C417Y line (food: $F(1,26)=0.142$, $p>0.05$, water: $F(1,26) = 0.361$, $p>0.05$), mean grams consumed and standard deviations are given in the table below (table 5.4).Data analysed by MANOVA (factor: genotype x total amount of food consumed and total amount fo water consumed). It is interesting to note that the homozygous mutants in the C59X line did not consume less food than their wild-type counterparts as their body weights were significantly lower (see 5.3.4).Data are shown in figure 5.8.

	C59X line				C417Y line			
	Zfp804a$^{+/+}$		*Zfp804a*$^{C59X/C59X}$		*Zfp804a*$^{+/+}$		*Zfp804a*$^{C417Y/C417Y}$	
	M (grams)	SD	M (grams)	SD	M (grams)	SD	M (grams)	SD
Food	5.88	2.47	5.25	2.63	5.6	3.33	5.08	3.78
Water	5.63	1.92	6	1.41	5.27	1.94	4.83	1.75

Table 5.4: Table showing mean grams and standard deviations of food and water consumed by homozygous mutants and wild-types in 24 hours in the phenotyper cages, by line and genotype.

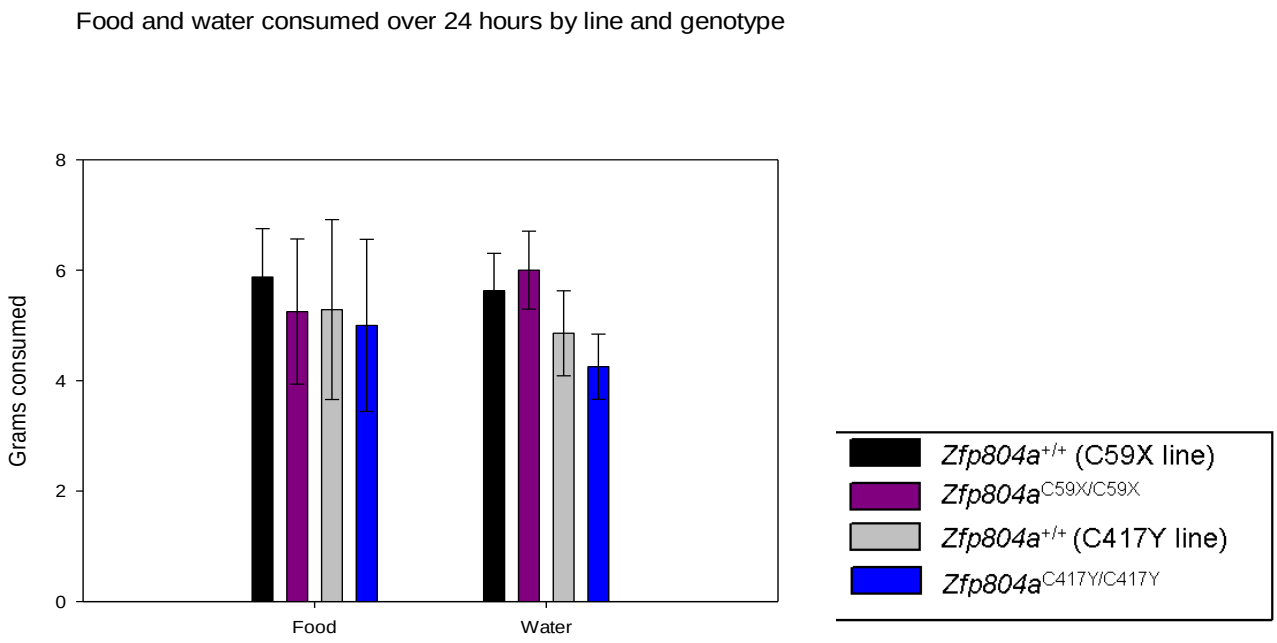


Figure 5.8: Graph (above) showing mean average food and water consumed in the PhenoTyper cages over 24 hours by line and genotype. SEMs (error bars) are shown.

5.3.6.4 Time spent in the hidden shelter

Figure 5.9 shows a striking picture of results of total time spent in the hidden shelter (presumed to be sleeping) by genotype and line in the phenotyper cages in 24 hours. MANOVA (factor: genotype x total time spent in the hidden shelter in each of the 8 3-hour bins) revealed that there were however, no differences between the homozygous mutants and the wild-type mice in time spent in the hidden shelter for either line (statistics not reported). The most striking observation is the time spent in the shelter by C417Y line wild-types, apparently almost three times more time than their homozygous mutant counterparts. However, this result was largely produced by 2 outliers (out of 8 subjects in this experimental group). For example, in bin 2, representing 3-

6hours in the phenotyper cages, the mean average for the wild-types of the C417Y line was 1248.42 seconds spent in the hidden shelter over 3 hours (*SD*=2985.91), whereas if the 2 outliers were removed, the mean average time spent in the shelter was reduced dramatically to 123.22 seconds (*SD*=71.31), mirroring the trend shown by the C417Y homozygous mutants and both genotypes in the C59X line. This finding would be interesting to investigate further in the two outlier mice.

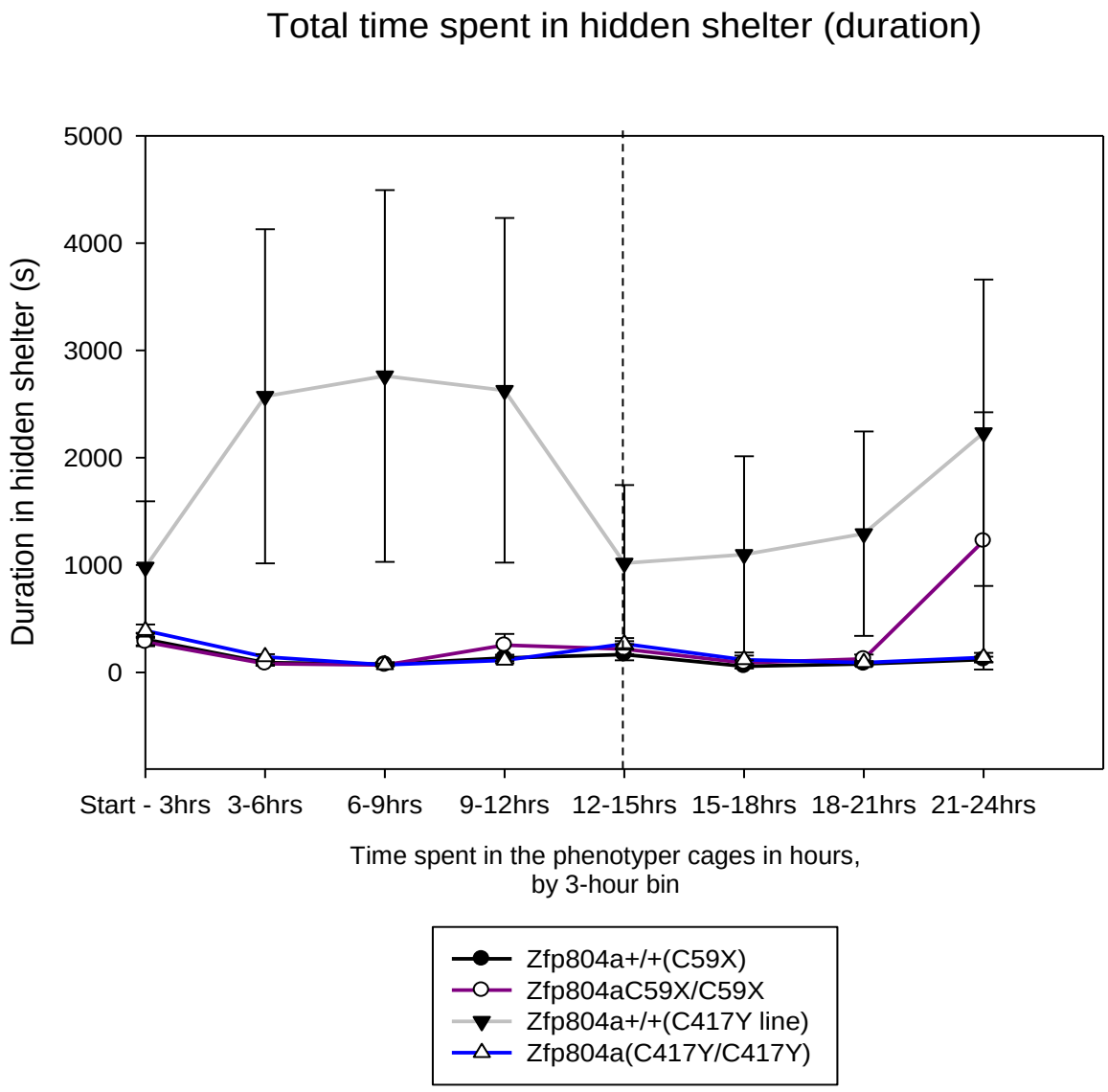


Figure 5.9: Graph (above) showing mean average time spent in the hidden shelter zone in the PhenoTyper cages over 24 hours by line and genotype. Dashed line indicates when lights were turned off, at 12 hours. SEM error bars are also shown.

5.3.7 Behaviour monitored on the RotaRod

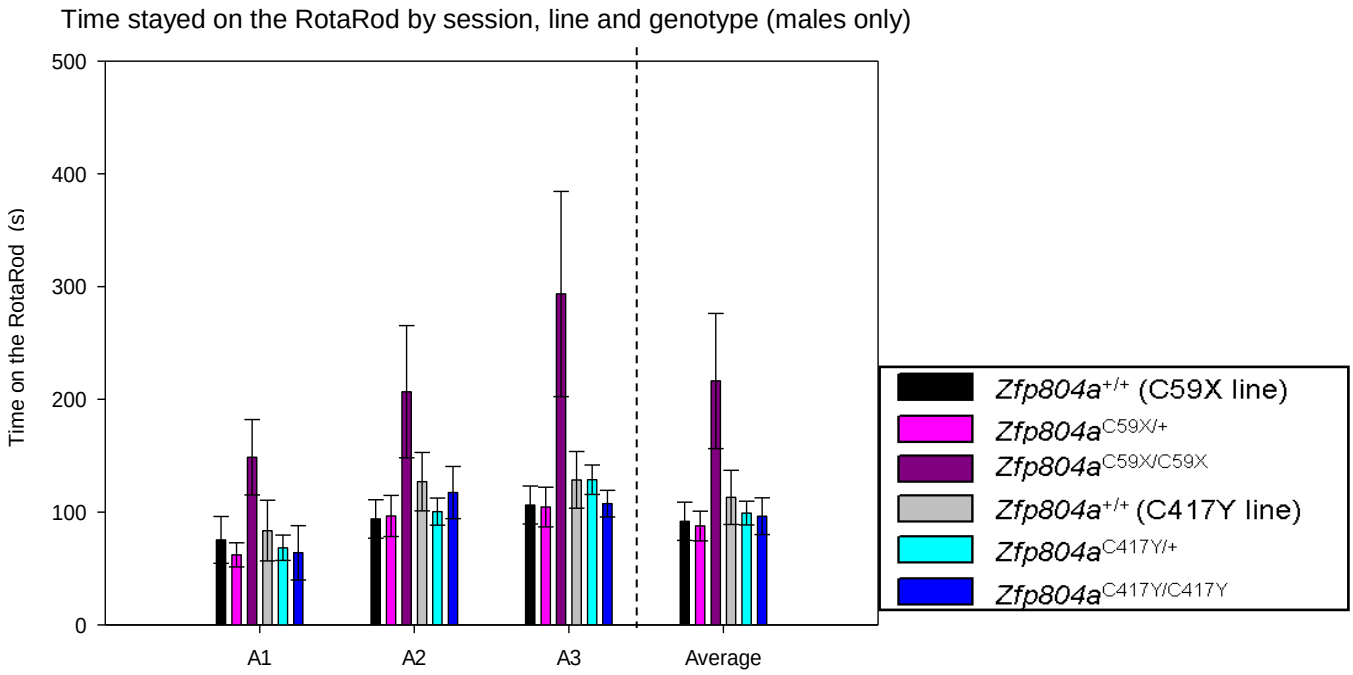


Figure 5.10: Mean average time stayed on the RotaRod (males) over sessions A1, A2 and A3 and combined sessions. SEM error bars are shown

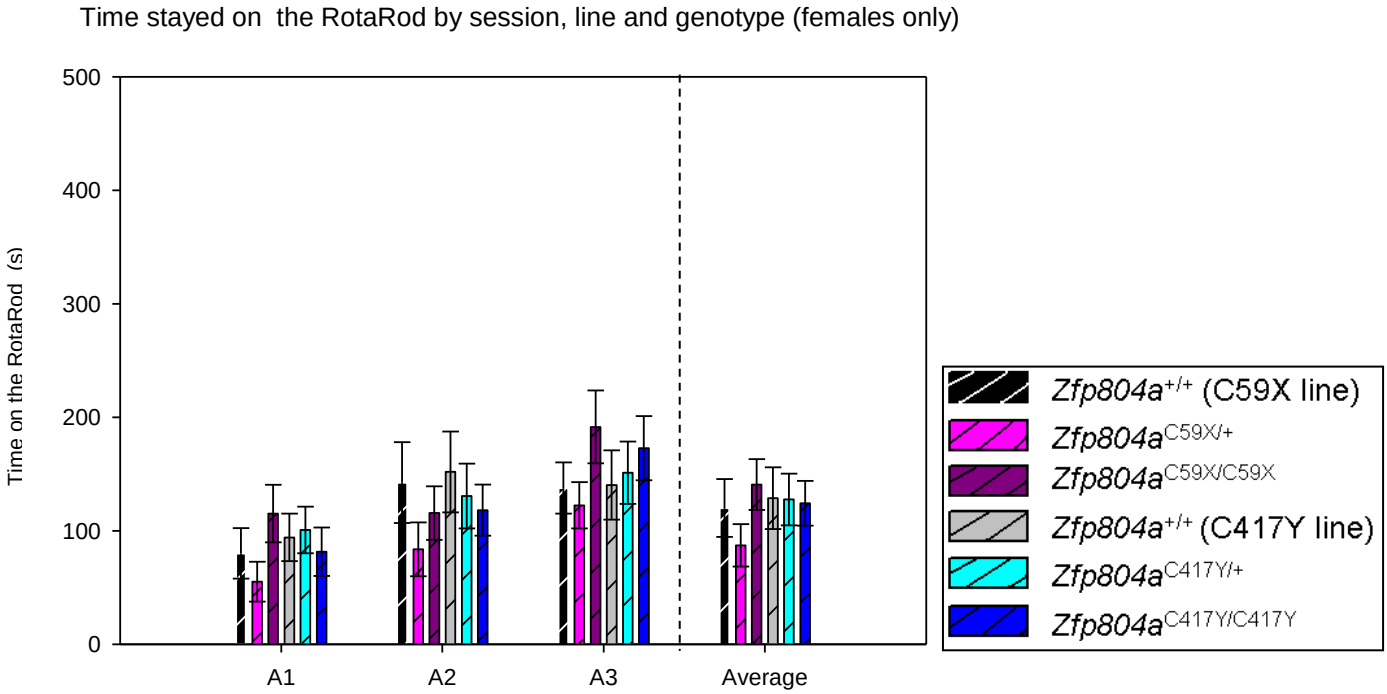


Figure 5.11: Mean average time stayed on the RotaRod (females) over sessions A1, A2 and A3 and combined sessions. SEM error bars are shown

The RotaRod task revealed some interesting differences between the subjects. There were no differences between the male and female (homozygous and heterozygous) mutants and wild-types in the C417Y line (see figure 5.10 for performance of the male mice and figure 5.11 for performance of the female mice), although several differences were revealed by MANOVA (factors: genotype and sex x seconds stayed on the RotaRod in conditions A1, A2, and A3) in the C59X line (stage of oestrus was taken into account in both analyses). Means and standard deviations for this line across the sessions are given in table 5.5. Not surprisingly, there was a main effect of session ($F(3,105)=19.66$, $p<0.0001$) with mice performing better (staying on the RotaRod for a longer time) with practice, which is to be expected (although there was no effect of session in the C417Y line [$F\{3,75\}=1.76$, $p=0.161$]). Interestingly, homozygous C59X mutant mice stayed on the RotaRod longer than their heterozygous mutant and wild-type cage mates with a main effect of genotype ($F(2,45)=8.36$, $p=0.001$). Homozygous mutant mice in this line also improved more in ability to stay on the RotaRod with practice than did their cage mates with a session x genotype interaction ($F(6,135)=4.22$, $p=0.001$).

Although from looking at figures 5.10 and 5.11, it seems that the male C59X homozygous mice performed better than the female C59X homozygous mice and improved more with practice, this was not apparent in the analysis. There was no effect of gender ($F(1,45)=2.62$, $p=0.112$) in the C59X line on performance in the RotaRod task. No influence of stage of oestrus was seen on seconds on the RotaRod in either line.

		A1		A2		A3	
		Mean (s)	SD	Mean (s)	SD	Mean (s)	SD
Male	*Zfp804a*$^{+/+}$	75.38	58.63	93.88	48.03	106.25	47.60
	Zfp804a$^{+/mut}$	65.00	31.68	96.70	51.31	108.10	50.85
	Zfp804a$^{mut/mut}$	162.75	78.06	234.00	134.02	331.50	213.39
	Total	86.55	61.82	120.64	86.35	148.05	127.30
Female	*Zfp804a*$^{+/+}$	80.00	70.65	142.30	112.47	137.60	71.43
	Zfp804a$^{+/mut}$	55.10	55.36	83.60	74.99	122.40	64.82
	Zfp804a$^{mut/mut}$	115.10	80.02	115.50	74.51	191.40	101.51
	Total	83.40	71.51	113.80	89.39	150.4	83.62
Total	*Zfp804a*$^{+/+}$	77.94	63.75	120.78	90.89	123.67	62.38
	Zfp804a$^{+/mut}$	60.05	44.19	90.15	62.89	115.2	57.18
	Zfp804a$^{mut/mut}$	128.71	79.61	149.36	105.24	231.43	148.17
	Total	84.73	66.96	116.69	87.32	149.44	103.20

Table 5.5: Means and standard deviations (SD) of seconds stayed on the RotaRod in the 3 accelarating conditions in the C59X line

5.4 Discussion

This chapter was intended as an initial preliminary characterisation of the novel Zfp804a mutant mice using an experimental group $G4_i$ (third generation, intercross), focusing on possible effects of genetic background and sex. The preliminary assessment was also designed to pick up, at an early stage, any gross deficits that may interfere with further, more complex behavioural and neurobiological testing in future work exploiting the mutant lines.

5.4.1 The effects of background genetics and sex

All groups of mice were bred onto the C57Bl/6J background (~97% C57Bl/6J average in the experimental cohort). The cohort is therefore not inbred but a mixed inbred strain, carrying a proportion (albeit a minimal amount) of the ancestry from the founder parents (BALB/c and C3H/HeH) as well as the selected ENU mutation (in mutants, and likely another one or two residual random [i.e. non-selected] mutations in all the mice, as discussed in chapter 4). The mice are therefore unlikely to be genetically homogeneous at all individual loci (as they are not 100% C57Bl/6J), which may have given rise to a degree of variability within the data. This level of 'impurity' is however small, given the low estimated number of residual ENU mutations in the first place, and additionally the chances of these mutations being functional, and furthermore affecting brain function. This degree of genetic variation between subjects in an experimental cohort of animals is common to all forms of genetic mutants and is not unique to ENU models. Moreover, these models are intended to help characterise a human disease gene; humans are not inbred and it could therefore be argued that inbred mouse models looking at human disease genes

have limited ecological validity. Additionally, it is worth noting the data generated by the wild-type control mice in these experiments do not show excessive degrees of variation and are not excessive when compared with mice on a 'purer' C57Bl/6J background (Eddy, personal communication). It is unlikely therefore, that any group differences were either observed or obscured due to lack of genetic homogeneity.

Female mice are generally avoided in studies due to potential effects of the stage of oestrus cycle on behaviour (particularly emotional-related behaviours) (Gray and Cooney, 1982). Some evidence was found in these experiments that such a correlation existed, with stage of oestrus having an impact upon performance in the RotaRod task. Such differences are easily taken into account in the analysis of data however. Female mutant mice showed normal oestrus cycling patterns (described by Parkes, 1928), when compared with wild-types. Determining (and accounting for, where appropriate) the stage of oestrus in female mice, controls for the possibility that any effects observed in females may be influenced by this factor.

5.4.2 Comparability of the lines

By comparing the two lines, C59X and C417Y, it could be argued that any differences observed between the two lines were not directly due to genotype *per se,* but due to differences in maternal behaviour between the mothers of the different genotypes. The optimal counter to this criticism would be to cross-foster the litters between the different lines. The data at this early stage of testing shows that, there is little, if any difference between the wild-type groups of the two lines, allowing them to be combined in analysis, suggesting that

there are no persistent differences between the lines in terms of mothering behaviour. Additionally, to minimise the effect of the genotype of the mother, the G3 crosses to C57Bl/6J to produce the G4 cohort were alternated so that some litters in the cohort had a wild-type mother and a heterozygous father and other litters had a heterozygous mutant father and a wild-type mother.

5.4.3 Behavioural comparison of the lines

All the groups tested showed intact motor and neurologic competence and no obvious deficits on physical examination nor in more specific assessments (PhenoTyper cages and the RotaRod apparatus). Sensory abilities were not formally assessed although mice handled normally and appeared to behave normally in their home cage environments. Pre-pulse inhibition (Eddy, personal communication) suggested the mice are able to hear and in the RotaRod showed no deficits in strength, co-ordination, grip or fatigue. Anecdotal evidence from casual observation of the animals in their home cage environment indicated that social interaction appeared to be fairly normal. There was no excessive barbering (where a dominant mouse plucks out hairs from the face of a more submissive mouse) of any one genotype group for example, and no excessive fighting going on in any of the cages.

Two formal methods of assessment of behaviour were used in the preliminary characterisation of the *Zfp804a* mutant lines: the PhenoTyper 'homecage' environment and the RotaRod. No differences were observed between the lines or genotypes (wild-types and homozygous mutants only were tested) in the PhenoTyper homecage environment. However, for this task, due to practicalities and time constraints, a sub-sample only of the mice was used.

This reduced the power of the analysis and consequently could mean differences were missed (false negatives). However, even the limited analysis possible gave some indication that there are unlikely to be any large differences between the mice in terms of activity, sleeping behaviour, or feeding and drinking behaviour. There have been no other published data in behaviour in the Phenotyper Home Cage Environment in a mouse model for schizophrenia. However, the circadian rhythm disturbances in schizophrenia have long been known about (reviewed in Pritchett, 2012) with an estimated 30-50% of people with schizophrenia experiencing some disturbances in sleep and/or circadian rhythms (Cohrs, 2008). In genetic mouse models for schizophrenia, few researchers have investigated sleep and circadian rhythms in mutant models. However, there are four schizophrenia-relevant genetic models that have: *Snap-25* (Oliver *et al*. 2012), *Vipr2* (Hughes and Piggins 2008), *Nrg1* (Johnson *et al*. 2002) and *Cckar* (Shimazoe *et al*. 2008). In all of these models, significant disturbance in sleep and circadian rhythms was observed, providing a case for further investigation with the phenotyper cages and other methods in the *Zfp804a* mutants.

Measuring motor agility and balance, the RotaRod data gave rise to reliable differences between the experimental groups. Homozygous mutant mice from the C59X line (males and females) performed ‘better’, i.e. stayed on for longer, than did the C59X heterozygous mutants and wild-types and the C417Y line. A search on PubMed revealed that this finding of improved performance may be the first in genetic mouse models for schizophrenia as no other studies reported better performance in mutants than wild-types on the RotaRod task, although Benoit *et al*. (2010), reported enhanced RotaRod

performance in a quinone reductase 2 (QR2) knockout model. QR2 overexpression in this study was also associated with learning deficits. Several studies looking at well-characterised schizophrenia genes have assessed performance on the RotaRod. Takao *et al* (2008) for example looked at the Sdy mouse with a deletion in *Dtnbp1* and found that the mutants had impaired motor learning capabilities on the RotaRod, compare with wild-types. Deakin *et al*. (2009) found that mice over-expressing *Nrg1* were impaired on the accelerating RotaRod. Pharmacological studies have also reported deficits in the RotaRod with the NMDA receptor antagonist, phencyclidine (PCP) being demonstrated to impair performance (Beraki *et al*., 2008) and the partial D2 receptor agonist aripiprazole impairing performance on the RotaRod in more challenging accelerating conditions (Nordquist *et al*, 2008). Very few studies have shown substances to enhance RotaRod performance. Shiotsuki *et al*. (2010) however demonstrate the dopamine-uptake inhibitor nomifensine to improve learning on the RotaRod. Further investigation of the mechanism(s) by which the *Zfp804a*$^{C59X/C59X}$ mutants show enhanced RotaRod performance could prove to yield very interesting findings about the gene and it's neurochemical and behavioural influences.

5.4.4 Body and brain weight phenotypes

There were no obvious differences between the groups in early development as indexed by initial body weight gain from birth, although sample sizes were small and could lack power to detect differences. Later on in the adult though, there were differences between groups in body weight. Homozygous mutant mice of both lines tended to be lighter than the others, more notably so in the C59X

line, despite assumed equal access to *ad libitum* food and water. This is an observation commonly seen in mutant models (Wilkinson, personal communication).

Despite there being an apparent trend of a reduced brain weight in male *Zfp804a*$^{C59X/C59X}$ mutants, the finding was non-significant. Further investigation of brain weight differences with larger cohorts would help to elucidate whether this trend exists or not. There is substantial *post mortem* evidence from human studies showing reduced brain weight in people diagnosed with schizophrenia (as discussed in the General Introduction, 1.5.3). Moreover, it is not unknown for variations in a putative schizophrenia susceptibility gene to lead to alterations in brain weight in both humans (e.g. Hashimoto *et al*., 2010, alterations in *DTNBP1*) and in mice (e.g. in study looking at both an SREB2 over-expressing model and an SREB2 knockout, Matsumoto *et al*., 2008 found that brain volumes were reduced and increased respectively).

More specifically to *ZNF804A*, Donohoe *et al.*, (2011) compared rs1344706 risk allele carriers with non-carriers (both schizophrenia patients and controls). They found that for patients, but not for controls, the homozygous risk carriers had relatively larger grey matter volumes than heterozygous and homozygous non-carriers, particularly for hippocampal volumes. Cousijns *et al*.'s (2012) findings were not incompatible with this result, demonstrating no differences in brain volume in healthy participants between *ZNF804A* risk and non-risk allele carriers. Clearly, more work, confirming differences in brain weight in the *Zfp804a* mutant lines, and extending the analysis into morphology might be of use.

5.4.5 Genetic explanation for the findings

Genetic explanations for these initial functional data are speculative, as there is, at present, no biochemical characterisation available for the models. It is unknown at the time of writing whether the C59X mutation does indeed cause nonsense mediated decay of the transcript, if it truncates the protein at 59aa, or if something else occurs. The effect of the C417Y mutation on the protein is also unknown and merely predicted to be 'probably damaging' to the protein and disrupt the normal functioning of the Zfp804a protein in some way. It would be premature therefore, on the basis of the data in this chapter to conclude that the behavioural differences observed were definitely related to distinct, dissociable effects of the two mutations. More analyses, both of the behavioural and the biochemical consequences of the mutations are required.

5.4.6 Summary

In summary the work in this chapter has provided important information on the utility of the models created in this thesis and has provided some important indications of areas for further work and investigation.

- There was no excessive variation observed in the animals (due to other, residual mutations).
- There were no differences observed with regard to mortality and general health, in the young and in adults
- There were no differences amongst the mice in activity in the PhenoTyper home cage environment
- C59X homozygous male mutants performed better on the RotaRod

- C59X homozygous mutants were also lighter in body weight
- C59X homozygous mutants (females only) had heavier brains than the heterozygous mice but not the wild-types, although a trend in the other direction in the C59X males makes the picture somewhat unclear.

CHAPTER 6: Further behavioural phenotyping of the *Zfp804a* mutant lines: emotion-related behaviours

6.1 Introduction

Chapter 5 involved the first ever characterisation of two novel mutant mouse lines carrying non-synonymous mutations predicted to affect the structure and therefore function of the Zfp804a protein, the mouse orthologue of the human ZNF804A protein. From an initial screening we have seen that the mutant lines breed, the mutations are not embryonic-lethal and nor do they lead to any grossly observable physical abnormalities in post-natal life. We have therefore confirmed the basic utility of the mutant lines for further functional analysis. Additionally, from an initial analysis of behaviour we have gained preliminary evidence of changes in the behaviour of the mutants.

This chapter extends the basic functional screen with a focus on behaviour that indexes emotionality. As previously noted, *ZNF804A* is a robustly replicated candidate gene for schizophrenia, and also for bipolar disorder (Williams *et al*., 2011). Research to date, in terms of influence of the gene at a phenotypic level, has focused on altered cognition. Schizophrenia and bipolar disorder (as discussed in the General Introduction, 1.2) are syndromic disorders, which can have many symptoms that overlap and so the diagnosis can be complex. There is a growing school of thought, especially with the generation of increasing genetic evidence for an overlap between the disorders, or the existence of the disorders on a spectrum (e.g. Owen *et al*., 2007).

Given this evidence, it is conceivable that *ZNF804A* may influence shared domains known to be aberrant in schizophrenia and bipolar disorder other than cognition, such as emotion. Consequently, this chapter assays differences in emotional-type behaviour between the experimental groups. Three behavioural assays were used, (i) activity in and habituation to a novel environment, locomotor activity (LMA); (ii) the 'Open Field' (OF) test and (iii) the 'Elevated Plus Maze' (EPM). All three assays are well established (reviewed in Sousa, Almeida & Wotjak, 2006; Ramos, 2008; Sartori, Landgraf & Singewald, 2011) means of assessing components of emotional functioning, in particular anxiety, as they all make use of the conflict between the natural instinct to explore new environments and the fear of exposure. As before, female mice were smeared after testing in order to be able to take account of any effects of oestrus cycle on behaviour.

6.2 Methods

6.2.1 Subjects and animal husbandry

The same experimental cohort of $G4_i$ (third generation, intercross) C59X and C417Y mice as described in Chapter 5 (see 5.2.1) were used in the experiments. General housing, handling and behavioural testing conditions were as described in chapter 2, General Materials and Methods (2.5). The number of mice (n) used in each behavioural task is summarised in the tables below (tables 6.1 and 6.2).

C59X	Genotype and n					
Test	♂ *Zfp804a*$^{+/+}$	♂ *Zfp804a* +/C59X	♂ *Zfp804a* C59X/C59X	♀ *Zfp804a* +/+	♀ *Zfp804a* +/C59X	♀ *Zfp804a* C59X/C59X
Locomotor activity	8	8	4	11	10	11
Open field	9	9	4	10	12	11
Elevated plus maze	8	9	4	9	8	12

Table 6.1: Number of mice, by experimental group, used in each task: C59X line

C417Y	Genotype and n					
Test	♂ *Zfp804a* +/+	♂ *Zfp804a* +/C417Y	♂ *Zfp804a* C417Y/C417Y	♀ *Zfp804a* +/+	♀ *Zfp804a* +/C417Y	♀ *Zfp804a* C417Y/C417Y
Locomotor activity	7	14	8	7	11	12
Open field	7	14	9	8	16	11
Elevated plus maze	8	13	8	7	12	13

Table 6.2: Number of mice, by experimental group, used in each task: C417Y line

6.2.2 Oestrus cycle status

Oestrus status was determined by vaginal smearing immediately following a behavioural procedure (see General Materials and Methods, 2.5.3 for a

description). The data from the behavioural tests was then analysed to assess whether or not there was a correlation between the behavioural data and the stage of oestrus of a particular mouse.

6.2.3 Locomotor activity

The apparatus used to assess locomotor activity is described in Chapter 2 (2.6.3). Locomotor activity throughout a specified period was used as an index of general movement ability/volition, willingness to explore a novel environment and the degree to which mice habituated to this novel environment (both during the session and over subsequent sessions the following days, as indexed by the difference in performance upon repeated exposure to the test apparatus). There are two infra-red beams in the cages one crossing either end of the cage. The total number of infra-red beam breaks made by each mouse, every 5 minutes was recorded by a computer running custom-written BBC BASIC V6 programmes with additional interfacing by ARACHNID (Cambridge Cognition, Cambridge, UK) over a 2-hour sessions (i.e. 24 bins in total). Breaking one of the beams by moving through it resulted in a beam break and breaking each of the beams in succession resulted in a 'run'. Cages were cleaned thoroughly with 1% acetic acid to mask the scent of the previous animal. All animals were run in darkness between the hours of 07:00 and 18:00 on three consecutive days, with each animal run at a similar time each day.

6.2.4 Elevated plus maze (EPM)

In a further test for fear responses, subjects were tested in an elevated plus maze apparatus (described in Chapter 2, 2.6.4). At the time of testing, subjects

were habituated to the testing room environment (dimly lit with some low-level noise from background ventilation) for a minimum of 20 minutes prior to the experiment. To conduct the test, subjects were transferred to the centre of the plus maze where they were placed facing an open arm. The experimenter stood back approximately 2 metres and the mice were allowed to freely explore the maze for 5 minutes. Between subjects, the maze was cleaned thoroughly with 1% acetic acid and all faecal boli removed to mask the smell of the subject for the next mouse. All subjects were run between the hours of 09:00 and 18:00. From automated Ethovision analysis, the following parameters were recorded: time spent in the open and closed arms of the maze, entries into the open and closed arms of the maze, distance moved in the zones and overall movement. Time and entries into the arms were recorded according to where the greatest proportion of the subject's body was positioned. Traces depicting the total journey of the mouse in the maze were also recorded. In the EPM, anxiolytic behaviour indicating lower levels of anxiety in the mice is indexed by exploring the open, more fear-inducing arms of the maze and anxiogenic behaviour indicating higher levels of anxiety in the mice is judged as staying in the closed, more secure arms of the EPM.

6.2.5 Open field

Subjects were investigated for fear reactivity in an open field paradigm (described in General Materials and Methods, 2.6.5). After habituation to the general testing environment (i.e. the room, dimly lit with some low-level noise from background ventiliation) for a minimum of 20 minutes, subjects were placed in the corner of the open field arena and allowed to explore freely for 10

minutes. Between subjects, the open field was cleaned thoroughly with 1% acetic acid and all fecal boli removed. All mice were tested between 08:00 and 18:00. For analysis, the arena was subdivided into 4 quadrants, and 2 concentric circles corresponding to radii of 200 and 275mm respectively. A number of parameters were recorded: the time spent in each circle, the number of quadrants entered. Increased entries into and time spent in the centre part of the apparatus is usually interpreted as behavioural evidence of anxiolysis, reduced anxiety in the mice, whilst increased levels of thigmotaxis (running round the perimeter of the apparatus close to the walls) is usually interpreted as the opposite.

6.2.6 Statistical analyses

Experimental data were analysed using SPSS PASW Statistics, release 18.0 (IBM SPSS Inc., 2009, Chicago). Data were subjected to One Way, Two Way or multivariate AN(C)OVA (if normal or One Way Kruskal-Wallis ANOVA if data did not conform to normality estimates, Levene's test, $p>0.05$). All comparisons were planned *a priorii* and corrections for multiple comparisons were therefore not made. Significance was defined as $p<0.05$.

6.3 Results

6.3.1 Oestrus cycle and behaviour

Data were analysed using ANCOVA for any effects of stage of oestrus on behaviour and any significant findings in relation to stage of oestrus are reported in the results for individual tasks.

6.3.2 Locomotor Activity

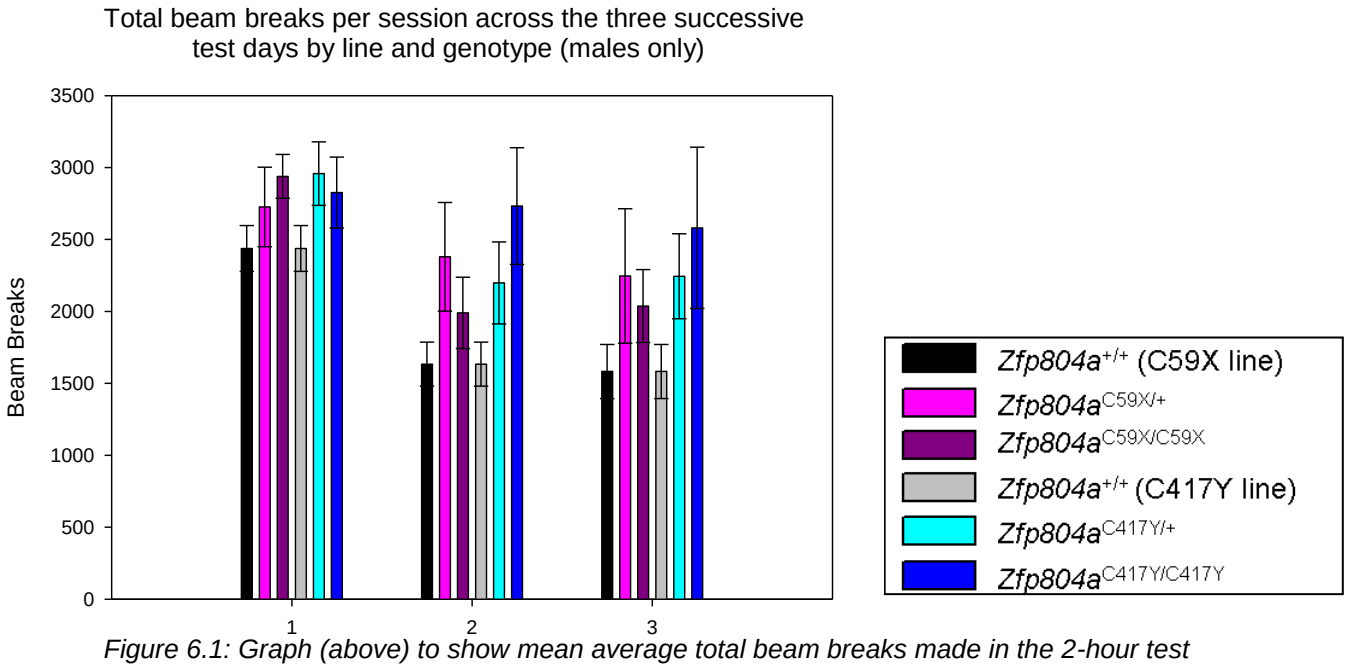


Figure 6.1: Graph (above) to show mean average total beam breaks made in the 2-hour test sessions across the three successive test days (x axis) in males, both lines, by genotype and line. SEM error bars are shown

To look at locomotor activity and habituation to a novel environment, 2 measures were examined in the locomotor activity task: beam breaks (breaking one of two beams at either end of the activity box) and runs (breaking the two beam breaks consecutively). Figures 6.1 (males) and 6.2 (females) show the mean average total beam breaks per session by line and genotype.

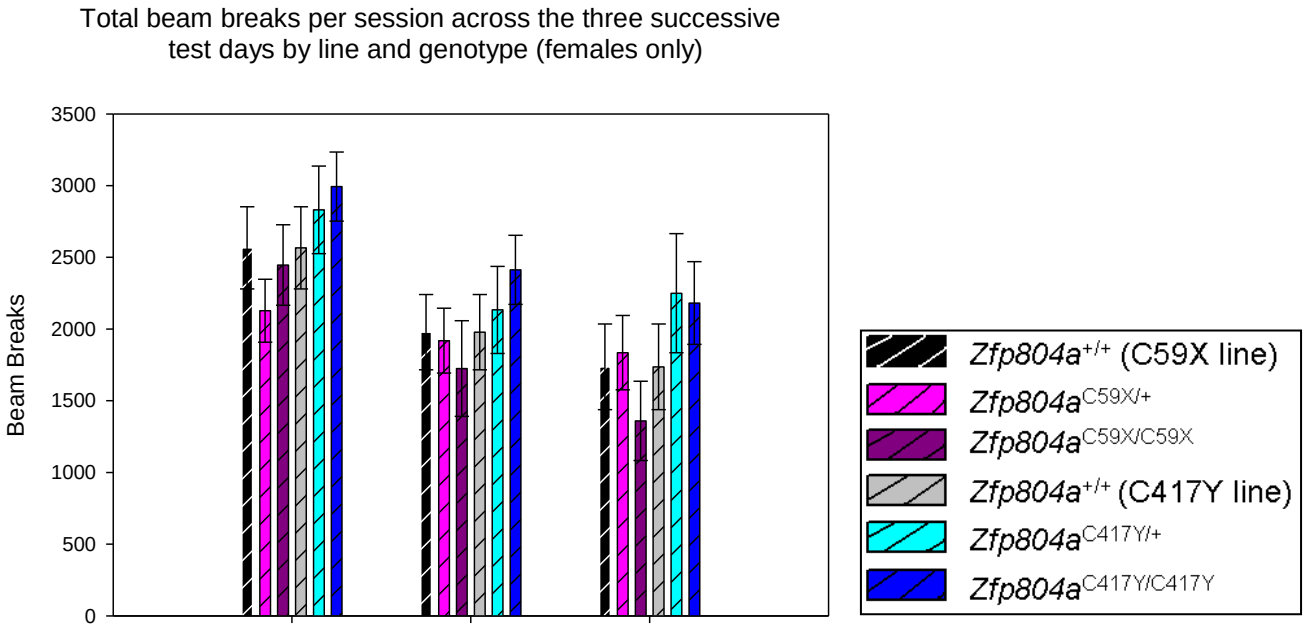


Figure 6.2: Graph (above) to show mean average total beam breaks made in the 2-hour test sessions across the three successive test days (x axis) in females, both lines, by genotype and line. SEM error bars are shown

MANOVA (factors genotype and sex x total beam breaks per session for days 1, 2 and 3 and total runs per session for days 1, 2 and 3), co-varying for stage of oestrus for the female mice was performed on the total beam breaks and runs per 2-hour session (i.e. day) for each line. The beam breaks of the females in neither line was influenced by stage of oestrus (C59X line: $F(1,25)=0.59$, $p=0.45$; C417Y line: $F(1,19)=0.003$, $p=0.960$). Findings for stage of oestrus and runs were similar with no effect of oestrus (data not reported). There were no significant differences between the sexes for either line, or between the genotypes (data not reported) in total beam breaks or total runs, indicating that the mutations have little effect on locomotor activity in such a situation. In the C59X line, there was an effect of day on runs ($F(1,50)=4.26$, $p=0.035$) whereby the mice became increasingly inactive over the three days, indicating habituation to the locomotor activity boxes. There was no effect of

day on beam breaks in this line or on runs or beam breaks in the C417Y line (data not reported).

6.3.3 Elevated plus maze

As would be expected, both lines spent more time in the more secure closed arms of the EPM than they did in the more fear-inducing open arms (duration in closed arm:C59X, *M*=204.73s, *SD*=38.72, C417Y *M*=205.87s, *SD*=33.79, duration in open arm: C59X *M*=120.35s, *SD*=43.77, C417Y *M*=98.35s, *SD*=36.71).

There were no differences in behaviour in the C417Y line on the elevated plus maze with both sexes and all genotypes behaving similarly (data not reported). There were however, more varied results with the C59X line, revealed by MANOVA (factors sex, genotype x time spent on the open arms, frequency of entry to the open arms, total distance moved on the open arms, duration on the closed arms, latency to enter the open arms, latency to enter the closed arms, percentage of time moving in open zone, maximum distance moved in open zone, maximum distance moved in closed zone). There was a main effect of gender on several parameters in the elevated plus maze shown in table 6.3. On all the parameters, the females demonstrated more anxiogenic behaviour than the males by tending to stay in the closed arms of the maze and explore the open arms less.

Measure	Degrees of freedom	*F* statistic	*p* value	Male		Female	
				M	*SD*	*M*	*SD*
Frequency in open zone	1,48	8.84	0.005	21.64	9.04	16.81	5.86
Total distance moved in open zone (cm)	1,48	7.22	0.010	526.94	167.20	449.49	116.09
Duration in closed zone (s)	1,48	13.69	0.001	189.03	39.92	217.53	33.20
Latency in closed zone (s)	1,48	12.01	0.001	3.36	7.37	1.16	3.73
Percentage of time moving in closed zone	1,48	4.55	0.039	43.64	7.04	47.26	7.60

Table 6.3: Statistics derived from MANOVA relating to parameters in the elevated plus maze where males and females showed a difference in behaviour in the C59X line

Measure	Degrees of freedom	*F* statistic	*p* value	*Zfp804a*$^{+/+}$		*Zfp804a*$^{+/C59X}$		*Zfp804a*$^{C59X/C59X}$	
				M	*SD*	*M*	*SD*	*M*	*SD*
Frequency in open zone	2,48	3.49	0.039	17.71	4.21	17.94	9.52	21.86	8.05
Total distance moved in open zone (cm)	2,48	3.78	0.031	475.32	86.97	450.85	180.82	538.10	144.48
Percentage of time moving in open zone	2,48	3.64	0.035	27.58	4.64	26.64	9.44	31.75	6.52
Frequency of entry to closed zone	2,48	4.90	0.012	23.53	5.04	18.89	6.18	18.29	3.56
Duration in closed zone (s)	2,48	6.00	0.005	216.17	31.82	209.22	42.52	185.07	36.19
Latency in closed zone (s)	2,48	16.86	<0.0001	0	0	1.06	2.49	6.16	9.32
Total distance moved in closed zone (cm)	2,48	5.78	0.006	916.33	113.09	801.52	131.51	758.80	144.85
Maximum distance moved in closed zone (cm)	2,48	3.44	0.041	5.43	0.96	4.96	0.48	4.71	0.66
Percentage of time moving in closed zone	2,48	4.93	0.012	49.48	4.84	44.49	7.59	42.42	8.44

Table 6.4: Statistics derived from MANOVA relating to parameters in the elevated plus maze where males and females showed a difference in behaviour in the C417Y line

Table 6.4, above, shows the parameters where there was a main effect of genotype in the elevated plus maze in the C59X line. In fact, there was a main effect of genotype in this line in 9 out of the 12 parameters measured in the maze. All of the effects were in the same direction, with the Zfp804a$^{C59X/C59X}$ mice showing more anxiolytic behaviour than the more timid wild-types.

Total duration in the open arms of the EPM, by line, gender and genotype

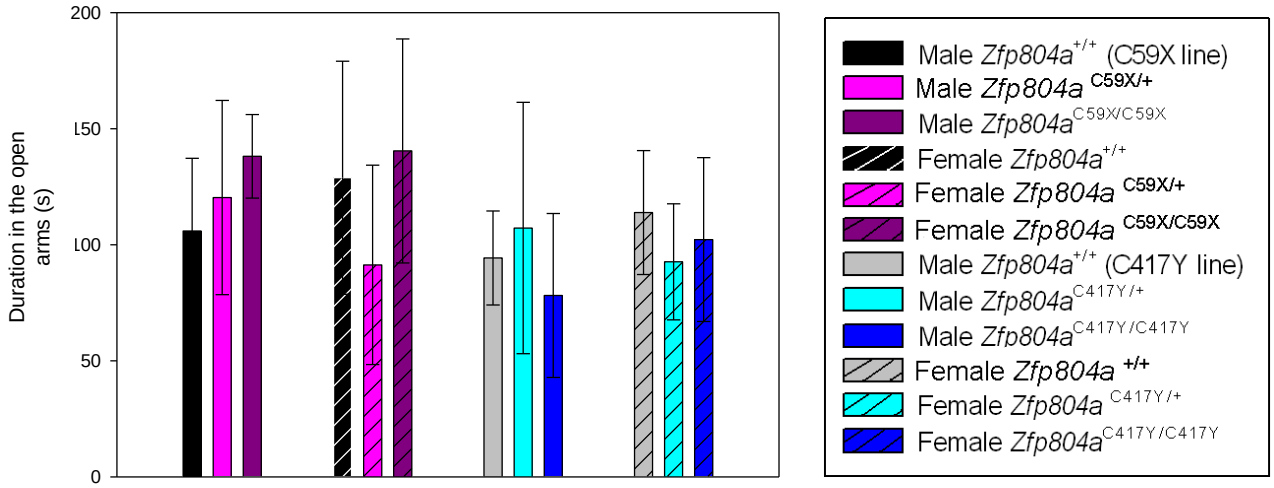


Figure 6.3: Graph to show mean average time spent in the open arms of the EPM, by line, sex and genotype. SEM error bars are shown

Figures 6.3 – 6.6 illustrate the data (mean averages and standard error) for the experimental groups on time spent in the open arms (figure 6.3), frequency of entry to the open arm (figure 6.4), total duration spent in the closed arms (figure 6.5) and total distance moved in the open arms (figure 6.6).

Frequency of entry into the open arms of the EPM, by line, sex and genotype

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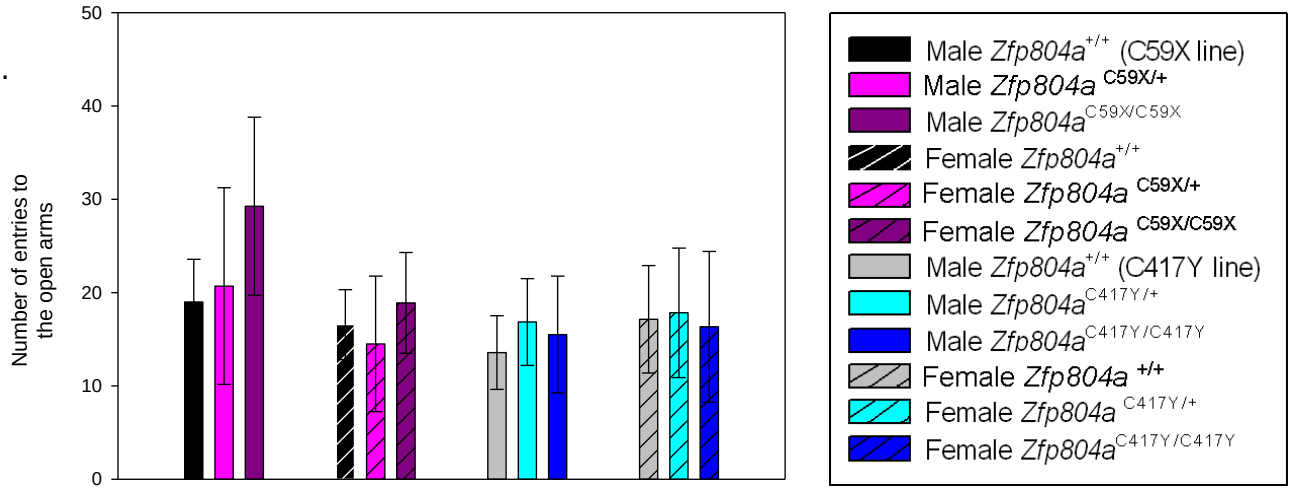


Figure 6.4: Graph to show mean average number of entries to the open arms of the EPM by line, sex and genotype. SEM error bars are shown

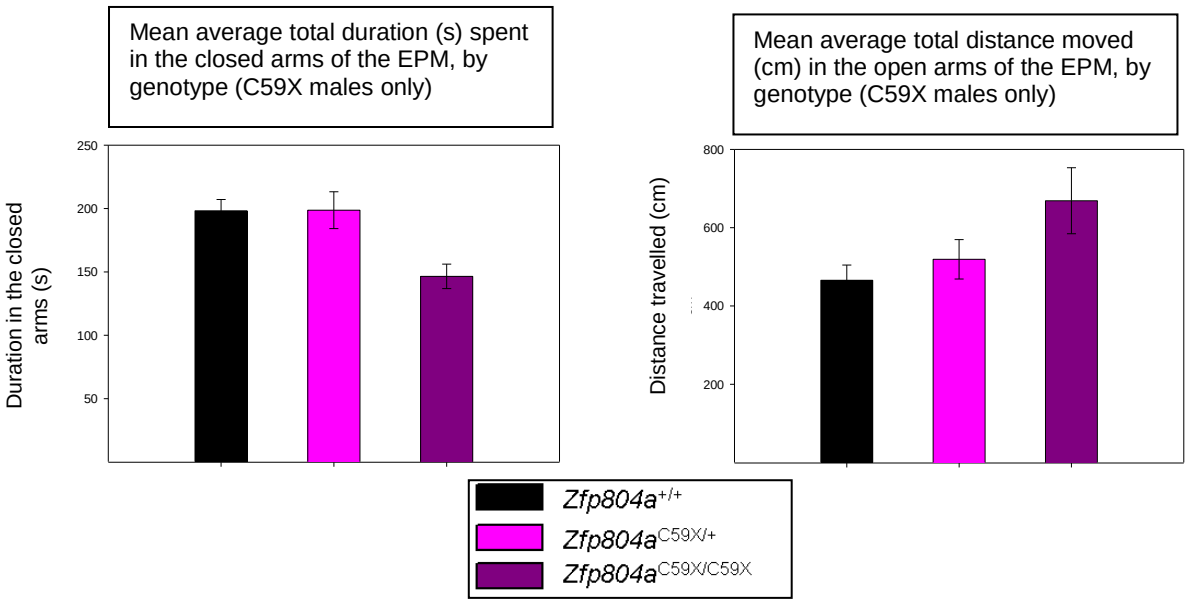


Figure 6.5: Graph (above, left) showing mean average total time spent in the closed arms of the EPM by genotype (C59X males only).SEM error bars are shown. Figure 6.6: Graph (above, right) to show mean average total distance moved in the open arms in the EPM by genotype (x axis) in the C59X line, males only. SEM error bars are shown

6.3.4 Open field

In the Open Field (OF), it is expected that mice will spend longer in the 'safer' edges of the field, close to the walls than they spend in the middle of the field which is more fear-inducing. This general effect was observed here with mice spending almost 10 times as long on the edges (outer zone) as in the centre (outer zone: C59X *M*=527.88s, *SD*=57.05, C417Y *M*=485.66s, *SD*=48.36; centre zone: C59X *M*=59.74s, *SD*=33.00, C417Y *M*=60.52s, *SD*=38.81).

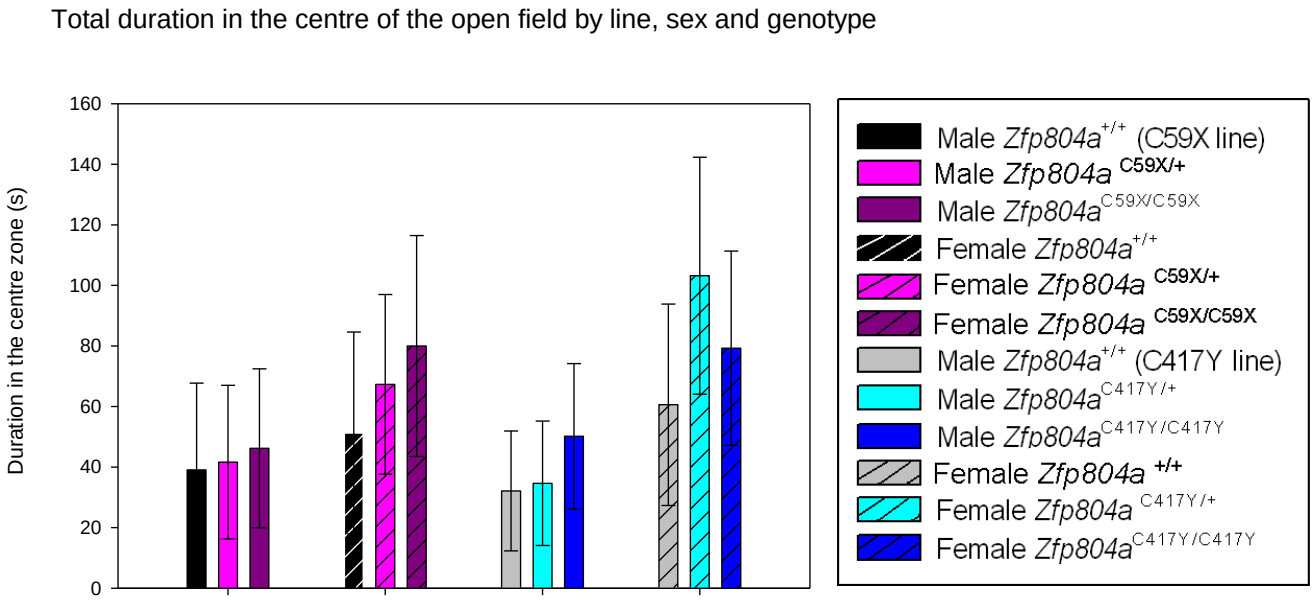


Figure 6.7: Graph (above) to show the mean average total duration in the centre, most aversive, area of the field by line, sex and genotype. SEM error bars are shown

Both lines showed differences in behaviour in some of the parameters between the sexes and the genotypes, revealed by MANOVA (factors: sex and genotype x frequency of entry to zone, total duration in zone, latency to enter zone and maximum distance moved in zone for each of the four zones [outer, inner, middle and centre], for each line). For example, figure 6.7, above, shows an overview of behaviour in the central, most anxiogenic zone of the field, indexed by total duration in the zone, across the genotypes, genders and lines examined. Due to the number of effects seen, it is not practical to discuss all the findings here. Instead, the main trends will be discussed. Tables 6.5 – 6.10, below, list the statistics for all the main effects and interactions seen by line.

In the C59X line, there were several parameters showing a main effect of sex (listed in table 6.5). The data however, present a complex picture as the effects are not all in the same direction with females showing a more anxiogenic phenotype on some parameters (e.g.moving shorter distances in

the zones) than males and a more anxiolytic phenotype (e.g. remaining in the centre and inner zones for longer) than others.

Zone	Measure	Degrees of freedom	*F* statistic	*p* value	Male		Female	
					M	*SD*	*M*	*SD*
Outer	Frequency of entry	1,52	72.17	<0.0001	90.55	26.06	35.10	16.77
Outer	Latency to first entry (s)	1,52	5.46	0.024	0.17	0.73	0	0
Outer	Maximum distance moved (cm)	1,52	18.41	<0.0001	13.68	14.07	7.92	1.36
Middle	Total duration (s)	1,52	6.50	0.014	37.94	22.77	59.29	30.29
Middle	Maximum distance moved (cm)	1,52	11.61	0.001	12.47	11.47	9.26	1.99
Inner	Total duration (s)	1,52	8.27	0.006	3.58	3.66	6.89	4.42
Inner	Maximum distance moved (cm)	1,52	53.23	<0.0001	10.00	3.63	5.77	1.55
Centre	Total duration (s)	1,52	7.00	0.011	41.52	25.59	66.18	34.28
Centre	Maximum distance moved (cm)	1,52	19.00	<0.0001	13.79	11.04	9.27	1.97

Table 6.5: Statistics summary of parameters showing a main effect of sex in the C59X line, data analysed by MANOVA co-varying for stage of oestrus

As these differences in anxiety were not observed in the EPM and the data are inconsistent in the OF, it is possible that these measures could be assaying sex differences in anxiety-related behaviour rather than anxiety *per se*. Further investigation on anxiety with the sexes would be warranted.

Table 6.6 lists measures that showed a main effect of sex in the C417Y line in the open field. The table presents a similar picture of results to the C59X line. Females spent longer in the outer ('safer') zone than males but also longer in the central, most anxiety-provoking zone. Again, further investigation of these findings would be interesting.

Zone	Measure	Degrees of freedom	*F* statistic	*p* value	Male		Female	
					M	*SD*	*M*	*SD*
Outer	Frequency of entry	1,56	74.70	<0.0001	93.93	25.24	41.04	19.29
Outer	Total duration (s)	1,56	31.47	<0.0001	459.99	37.62	513.24	38.68
Outer	Maximum distance moved (cm)	1,56	7.20	0.010	13.98	14.02	7.48	1.12
Middle	Frequency of entry	1,56	11.68	0.001	26.10	15.01	45.89	24.03
Middle	Total duration (s)	1,56	25.28	<0.0001	35.97	20.37	75.76	34.24
Middle	Latency in zone (s)	1,56	6.60	0.013	90.67	109.82	27.28	28.15
Middle	Maximum distance moved (cm)	1,56	6.66	0.013	8.50	2.28	9.87	1.69
Inner	Frequency of entry	1,56	8.85	0.004	5.90	4.10	10.04	5.28
Inner	Total duration (s)	1,56	17.03	<0.0001	3.04	2.01	8.61	6.37
Inner	Maximum distance moved (cm)	1,56	28.00	<0.0001	27.31	20.56	6.34	1.01
Centre	Frequency of entry	1,56	8.77	0.005	21.97	12.24	35.96	19.31
Centre	Total duration (s)	1,56	27.01	<0.0001	38.32	21.92	84.37	39.10
Centre	Latency in zone (s)	1,56	6.61	0.013	85.36	96.47	27.28	28.15
Centre	Maximum distance moved (cm)	1,56	21.79	<0.0001	27.82	20.03	9.87	1.69

Table 6.6: Statistics summary of parameters showing a main effect of sex in the C417Y line, data analysed by MANOVA co-varying for stage of oestrus

Zone	Measure	Degrees of freedom	*F* statistic	*p* value	*Zfp804a*$^{+/+}$		*Zfp804a*$^{+/C59X}$		*Zfp804a*$^{C59X/C59X}$	
					M	*SD*	*M*	*SD*	*M*	*SD*
Outer	Latency in zone (s)	2,52	3.93	0.027	0.02	0.94	0	0	0.24	0.91
Outer	Maximum distance moved (cm)	2,52	10.39	<0.0001	8.99	2.84	8.46	2.27	14.80	17.58
Middle	Maximum distance moved (cm)	2,52	11.44	<0.0001	8.95	2.14	9.11	1.88	14.93	13.90
Inner	Maximum distance moved (cm)	2,52	8.17	0.001	6.61	2.95	7.68	2.94	8.60	4.21
Centre	Maximum distance moved (cm)	2,52	12.42	<0.0001	9.64	2.17	9.73	1.99	15.25	13.76

Table 6.7: Statistics summary of parameters showing a main effect of genotype in the C59X line, data analysed by MANOVA co-varying for stage of oestrus

Several differences were also seen between the genotypes in the C59X line in behaviour (listed in table 6.7, above). The most striking difference here between the genotypes is the difference in maximum distance moved at ony one time in all of the zones. *Zfp804a*$^{C59X/C59X}$ mice moved significantly further than counterparts, suggestive of anxiolytic behaviour (consistent with findings from the EPM, see 6.3.3). Figure 6.8, below illustrates this finding in males, in the centre zone.

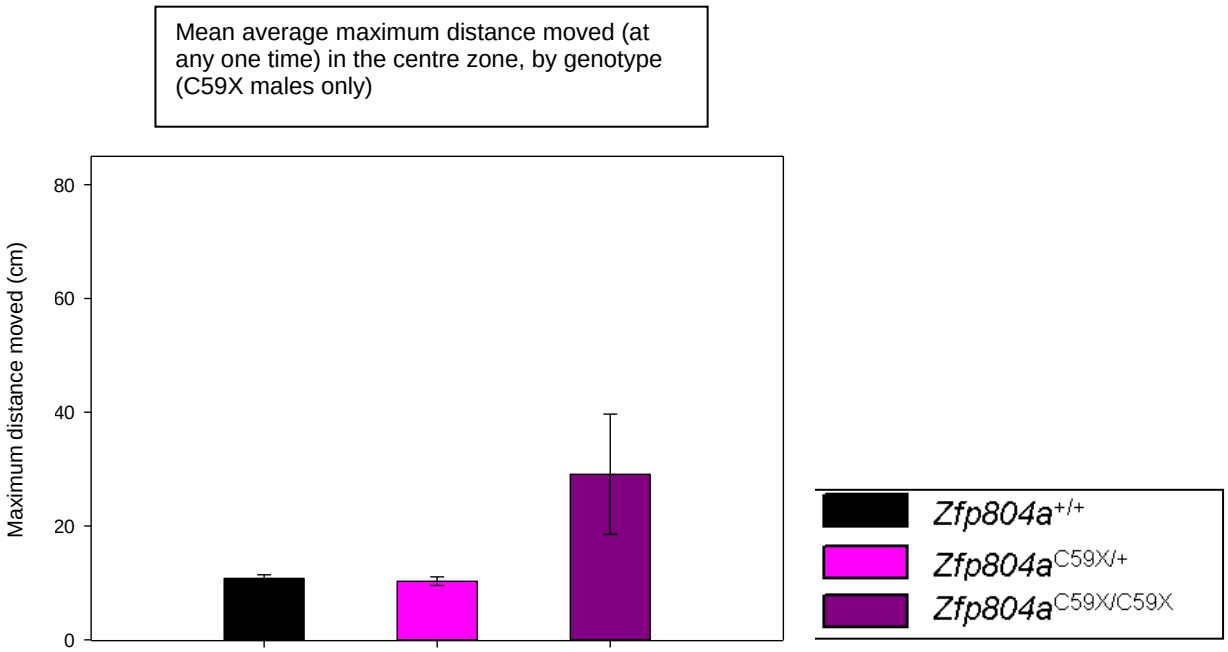


Figure 6.8: Graph to show the mean average maximum distance moved (cm) in the centre zone (x axis) in the C59X line, males only. SEM error bars are shown

In the C417Y line, there were fewer main effects of genotype (see table 6.8), with mutants spending less time in the outer zone, and homozygous mutants moving more (at any one time) in the outer zone. Both main effects are suggestive of lower anxiety in the mutant groups than the wild-type groups but there were no significant findings suggestive of the same trend in the more anxiogenic, middle, inner and central zones.

Zone	Measure	Degrees of freedom	*F* statistic	*p* value	*Zfp804a*$^{+/+}$		*Zfp804a*$^{+/C59X}$		*Zfp804a*$^{C59X/C59X}$	
					M	*SD*	*M*	*SD*	*M*	*SD*
Outer	Total duration in zone (s)	2,56	3.77	0.030	510.08	44.94	475.93	41.12	478.11	50.27
Outer	Maximum distance moved (cm)	2,56	3.27	0.046	7.40	1.14	9.72	7.95	16.23	16.65

Table 6.8: Statistics summary of parameters showing a main effect of genotype in the C417Y line, data analysed by MANOVA co-varying for stage of oestrus

The main effects of sex and genotype described above in the C59X line were found by MANOVA to interact on the maximum distance moved measure in all the zones. All interactions were in the same direction, with male *Zfp804a*$^{C59X/C59X}$ mice showing a more anxiolytic phenotype (by moving further in the open field at any one time, e.g. see figure 6.9). These interactions support findings from the EPM (see 6.3.3).

Zone	Measure	Degrees of freedom	*F* statistic	*p* value
Outer	Latency in zone (s)	2,52	3.93	0.027
Outer	Maximum distance moved (cm)	2,52	10.77	<0.0001
Middle	Maximum distance moved (cm)	2,52	9.96	<0.0001
Inner	Maximum distance moved (cm)	2,52	3.93	0.027
Centre	Maximum distance moved (cm)	2,52	10.82	<0.0001

Table 6.9: Statistics summary of parameters showing an interaction of sex x genotype in the C59X line, data analysed by MANOVA co-varying for stage of oestrus.For means and standard deviations, see tables 6.5-6.8.

There was a different set of results in the C417Y line. Genotype interacted with sex in the maxium distance moved in the outer zone parameter, with male *Zfp804a*$^{C471Y/C417Y}$ mice moving more in this zone and no difference between the genotypes in females. For the total duration in the middle and centre zone parameters, genotype interacted with sex differently, particularly in the centre zone with male *Zfp804a*$^{C471Y/C417Y}$ mice spending longer in the centre zone than their counterparts, whereas female *Zfp804a*$^{C471Y/C417Y}$ mice spent less time in the centre zone than their coutnerparts. Statistics are shown in table 6.10.

Zone	Measure	Degrees of freedom	*F* statistic	*p* value
Outer	Maximum distance moved (cm)	2,56	3.59	0.035
Middle	Total duration in zone (s)	2,56	3.29	0.045
Centre	Total duration in zone (s)	2,56	3.22	0.048

Table 6.10: Statistics summary of parameters showing an interaction of sex x genotype in the C417Y line, data analysed by MANOVA co-varying for stage of oestrus.For means and standard deviations, see tables 6.5-6.8.

6.4 Discussion

6.4.1 Main findings

The experiments gave rise to complex data. The analysis of locomotor activity revealed a difference between the lines with the C59X line emerging as less active than the C417Y line. Additionally, the homozygous mutants were more active than the wild-type mice (independent of line). In the elevated plus maze (EPM), there were behavioural differences influenced by sex and line with the C59X males exploring the open (and more anxiety-provoking) arms more than the C417Y males. Homozygous mutants were also different between the lines, again with those in the C59X line more willing to explore than those in the C417Y line. Interestingly this effect did not persist in differences between the lines in terms of male homozygous mutants but perhaps this is due to the samples being underpowered (male homozygous mutants in the C59X line were only n=4). There were no effects of line in the open field, although there were genotype differences independent of line with mutants (both heterozygous and homozygous) being more active in the inner (and more exposed, therefore more anxiety-provoking) zones of the open field than wild-type mice. Females also explored the open field (inner zones) in general more than males.

6.4.2 Activity

It was interesting that the C59X homozygous mutants performed better on the RotaRod (Chapter 5) yet they were not more active in the locomotor activity cages. This finding sheds doubt to some extent on the proposal put forward in Chapter 5 that perhaps C59X homozygous mutants performed better on the

RotaRod as they had a lower body weight, were more nimble, and therefore found activity easier. The similarity of the genotypes in locomotor activity (which is in agreement with the findings in terms of general activity in the phenotyper cages) makes the anxiety tasks simpler to interpret. It is less likely that any differences seen between animals in those tasks are due to differences in activity rather than anxiety *per se*. As with the other behavioural tasks reported, it is likely the investigation was under-powered, by having small sample sizes. Nonetheless, false negatives are far more likely than false positives.

There were no group differences between sessions on days 1, 2, and 3 on activity measures and no interactions involving day and line or genotype. That is, the anticipated general reductions in activity over the three days of testing as the mice habituated to the novel surroundings of the activity cages (Leussis & Bolivar, 2006) occurred but was common to all groups.

LMA in mice has been used by many investigations as a proxy for positive symptoms in schizophrenia and is has a well-documented positive correlation with dopaminergic activity (described in van den Buuse, 2010). Interestingly, given the well known association of hyperactivity with bipolar disorder (psychomotor agitation can contribute to a diagnosis of a manic episode in bipolar using DSM-IV guidelines) LMA has been used as a simple way of assessing behaviour in mouse models of manic conditions (Young, *et al.*, 2011).

Due to its simplicity and informativeness, many genetic mouse models for schizophrenia have assayed LMA. With *Dtnbp1* mutants, there have been mixed findings with several reports of no change in LMA (reviewed in van den Buuse, 2010) but one recent report of hyperactivity in the Sdy mouse (Cox

et al., 2009). Findings with *Nrg1* mutant mice are more consistent with several authors finding Nrg1 transmembrane mutants mildly hyperactive in the open field (Stefansson *et al*., 2002; O'Tuathaigh *et al*., 2006; Karl *et al*., 2007; van den Buuse *et al*., 2009), which could be partially reversed by clozapine (Stefansson *et al*., 2002).

The literature presents positive, negative and mixed findings in terms of LMA. Koike *et al*.(2006) for example, found no differences in LMA between mice with an endogenous Disc1 deletion in the 129 strain, nor when the deletion was crossed onto the C57Bl/6J strain. Another group (Yee *et al*., 2005) only saw differences in activity when the mutants (with a deletion in *Comt* which increased endogenous dopamine levels 2-3 fold) were given amphetamines (when compared with the wild-type response to amphetamine).

One way of investigating LMA in *Zfp804a* mutants further would be to see if a differential response to pharmacological interventions LMA is known to be affected by such as amphetamine, PCP and some antipsychotics (van den Buuse, 2010).

6.4.3 Anxiety

The data from the behavioural assays of anxiety were similarly complex and again have to be considered in the light of the preliminary nature of the experiments. Nonetheless, in the EPM there was a clear difference in behaviour in the open (and more anxiety-provoking) arms of the apparatus. The findings are consistent with the view that, the C59X mutation in *Zfp804a* affects some anxiety-mediated behaviours, and acts differently between the sexes, the mutations and the mutation dosages. The interactions with sex are worthy of

comment, insofar as females are under-researched in animal models of psychiatric disorders, despite mood disorders being more common in females than males (Palanza, 2001). This is mainly due to perceived problems due to stage of oestrus effects on behaviour and physiology. We controlled for this factor by routine smearing of the mice. As it was, we found no effect of stage of oestrus in the data.

It is important to note at this point that neither the EPM nor the open field can be described as monolithic 'tests of anxiety'; rather, they assay aspects of anxiety-mediated behaviour (e.g. see Turri *et al*., 2001). This is relevant to the increased activity of the mutants (both heterozygous and homozygous, both lines) in the inner zones of the open field. These data are congruous to some extent with the findings from the EPM; however this means that C417Y mutants were also more active (in addition to the C59X mutants) in the open field, indicating more willingness by all the mutants to go into an anxiety-provoking environment. In similar circumstances in the EPM however, the C417Y line were not as willing as their C59X counterparts.

There are several possible explanations for the apparently divergent effects seen across the tasks. Beyond the group samples being too small to elucidate reliable effects, there is the possibility that the tasks are in fact measuring different things. Indeed, all we have truly measured is the exploration of an elevated plus maze apparatus and the middle of a large open –topped box. Neither task is directly or solely measuring anxiety so it could be suggested that it is not unexpected to achieve different results on the different tasks. In fact, order effects could have an influence on the results. Holmes (2001) in his review of anxiety-relevant phenotypes in genetic mouse models,

suggests that prior exposure to any anxiety-provoking task can make subsequent anxiety tasks less fearsome and produce an apparently more anxolytic phenotype. Much literature demonstrates that it is not uncommon to yield different, even contradictory findings from the elevated plus maze and the open field (e.g. Carola *et al*., 2002). In previous work by others, the two tasks have been shown, using principle component analysis to measure different dimensions and components of anxiety in mice, with only some of these in common (Carola *et al.*, 2002). Archer (1973), File (1985); and more recently, Turri *et al.,* (2001) warn that open field alone is not a robust test for anxiety-related behaviours, this task reflecting changes in activity and exploration as well as in anxiety. An additional possible explanation for the apparent inconsistency of the findings could be technical reasons. The open field or the EPM may not have been set up as a sufficiently fear-inducing environment for the mice (unlikely from the data) or they may have differed in the amount of anxiety they induced in the animals. Experiment effects (such as the distance of the experimenter from the test, background noise in the lab etc) could also have influenced the results (discussed in Holmes (2001).

Other genetic mouse models of schizophrenia have also had some inconsistent anxiety-related phenotypes. In the *Dtnbp1* Sdy mouse for example, although researchers generally report no alteration in anxiety with the Sdy model (reviewed in Talbot, 2009), other research (e.g. Hattori *et al*., 2008) has found heightened anxiety-like responses in the model with mutants spending less time in the centre of the open field, which was thought to be due to reductions in forebrain dopamine transmission. Earlier research tended to focus on neurotransmitter receptors and produced mixed findings with some positive,

negative and neutral reports. For example, researchers looking at the 5-$HT1_B$ receptor knockout found both anxiolysis on the elevated plus maze (Brunner *et al*., 1999) and no anxiety-related phenotype on the elevated plus maze (Malleret *et al*., 1999) or the open field (Malleret *et al*., 1999, Zhuang *et al*., 1999), demonstrating the delicate and perhaps volatile nature of laboratory anxiety assays.

Aside from replicating the results in an experiment, one way in which reliability of results from anxiety-related assays in mouse models for schizophrenia can be improved is through congruency with human findings. Due to the early stage of research into ZNF804A/Zfp804a function, it is difficult to draw parallels. However, it is worth noting that ZNF804A may be involved in anxiety behaviour as Steinberg's association study of risk variants in *ZNF804A* revealed a CNV in the gene in an anxiety patient (Steinberg *et al*., 2011). Recent work in the Institute of Psychological Medicine and Clinical Neurosciences in Cardiff with the *Zfp804a* has shown promise as the phenotypes described in this study, of reduced anxiety in male *Zfp804a*$^{C59X/C59X}$ mutants have been replicated in subsequent studies (Eddy, unpublished data).

6.4.5 Genetic explanation for the findings

The behavioural findings with the Zfp804a mutant lines to date are intriguing. There are clear differences between the lines and differences that are mediated by the dosage of the mutation. There also may be differences in the way these mutations interact with sex. These new data are interesting in view of a relevant paper that looked at the effects of 60 donor B23 cM DBA/2J genome segments in a C57Bl/6J wild-type mouse. Gale *et al.* (2009) then screened these mice for

behavioural quantitative trait loci using measures of activity, anxiety and novel object tests. The mice where the donor segment spanned the *Zfp804a* region (2d - distal) were hyperactive in the LMA, open field and novel object task. There were no differences between the mice in the EPM other than the maintenance of hyperactivity, although the mice didn't have the same 'freeze' responses as other mice when confronted with anxiety-inducing stimuli. This data is interesting in the context of findings in this thesis as it suggests that the B23cM segment of genome containing the *Zfp804a* locus is influential in activity and reactivity to fear-inducing stimuli. Of course it is not known what genes or precisely what loci were responsible for these effects and, indeed the effect the donor segment had on *Zfp804a* expression.

We have seen that *Zfp804a* is likely to have some impact on emotion-led behaviour although firm conclusions cannot really be drawn from the present data and investigations are very much in preliminary stages. Both activity and anxiety are potentially influenced by the gene and this requires further work. These findings should also be taken into account when interpreting future behaviour of these mice.

6.4.6 Summary

In summary the work in this chapter has provided a preliminary insight into some emotion-related behaviour in the *Zfp804a* mutant mice. A picture is beginning to emerge that this gene has some important influences on behaviour, and possibly affect, although the data so far is inconclusive. Continued research with these mice with larger cohorts, further characterising their behavioural profile is needed.

- The C59X line were less active than the C417Y line in the locomotor activity task
- Homozygous mutant mice were more active than wild-type mice, independent of line in the LMA task
- C59X males appeared less anxious than C417Y males by exploring the open arm of the EPM more. C59X homozygous mutants were more willing to explore open arms than C417Y homozygous mutants
- Mutant mice were more active in the inner zones of the open field (independent of line)

CHAPTER 7: General Discussion

The experiments described in this thesis identified, developed and screened novel mouse mutants to investigate the function of *Zfp804a,* a mouse orthologue of *ZNF804A*, the schizophrenia susceptibility gene. Two ENU-derived mutations in coding regions of *Zfp804a* were identified and developed to compare the behaviour in mice carrying those mutations with wild-type littermate controls. Largely, the studies described herein address the experimental aims stated in the General Introduction (1.8): to identify, select and re-derive two lines of mutant mice carrying mutations in *Zfp804a* and to carry out a preliminary assessment of these lines.

7.1 Utility of the models: advantages and limitations

The discovery and development of the mouse mutants described in this thesis presented an excellent opportunity to investigate the relatively unknown function of *ZNF804A*. As discussed in the General Introduction, using the mouse as a model species for studying the effects of genetic variation brings a number of opportunities for manipulating and examining gene function and the effect on behaviour. Biochemical characterisation of the models created in this thesis would greatly aid translating findings from these mutant lines into information and knowledge about *Zfp804a* and indirectly, *ZNF804A*. The possibility that the phenotypes observed in *Zfp804a* mutants reflects the presence of unwanted (linked or not linked) mutations was argued fully in the Discussion of Chapter 4. Althought residual mutations may produce some unwanted noise in the behavioural data, they are extremely unlikely to

confound the results of the experiments discussed in this thesis. In terms of the behavioural characterisation of the models, there are two criticisms that could be levied at the present studies. Firstly, the group sizes are small, particularly the male homozygous mutants of the C59X line. Second, the existence of the phenotypes seen would be supported by reversing these effects by pharmacological means.

The male homozygous mutants of the C59X line are low in number compared with the other testing groups (n=4). This was the first cohort of homozygous mutant mice of either line bred. Further backcrossing of the mutants and generation of F7 male homozygous mutants of the C59X line has seemed to be hindered by similar difficulties in generating a group size comparable to other genotype, line and gender groups (Eddy 2012, personal communication), with indications of increased infanticide by *Zfp804a*$^{+/C59X}$ mothers. As already mentioned, further work with F7 mice on the same tasks will increase the overall group size and so increase the power. The fact that some effects were observed with the mice is very telling however.

Rescuing phenotypes by pharmacological (often antipsychotic) means is considered the ‘gold standard’ for a mouse model for schizophrenia (e.g. reviewed by Lu *et al*., 2011). However, it is questionable whether this pharmacological reversal or rescue is in fact informative to a model’s validity. Mouse models for schizophrenia (as discussed in 1.6), are only partial models as some symptoms of schizophrenia, particularly the positive symptoms, are unique to humans and we are not able to assay them reliably in mice. Antipsychotics (in humans) are most effective on the positive symptoms and are well-documented as being ineffective for the cognitive and negative

symptoms of schizophrenia in humans (reviewed in Miyamoto *et al*., 2012). Given this evidence and the evidence from this study of disrupted emotional behaviour (anxiolytic behaviour), it is questionable whether the use of antipsychotics with the *Zfp804a* models would be valid or informative.

Finally, there is of course the limitation frequently levied at such research: we are studying mice to inform us of a uniquely human disorder, how many useful inferences can be drawn between the species? To answer this understandable question, firstly it should be again emphasised that the models studied in this thesis are not intended to be models of schizophrenia by any means. These models are investigating the function of the relatively unknown schizophrenia candidate gene, *ZNF804A*. As the gene is a candidate gene for schizophrenia and bipolar disorder and is known to be highly expressed in the brain, the main focus of investigations is logically cognition and behaviour. There are many successes of using mouse models for such means as investigating gene function (some of the successes relating to schizophrenia are reviewed in Young, Zhou & Geyer, 2010), demonstrating the utility of the mouse as a model species. Whilst it may not be ideal, it serves as a good approximation and is never intended to be the sole means of investigating gene function in a thorough investigation of gene function. Used in tandem with *in vitro* investigations and parallel studies in human participants, mouse models are an invaluable and versatile tool, that are able to contribute to molecular, cellular and behavioural level investigations.

7.2 Main findings

7.2.1 Development of the mutants

Mutations were discovered at a rate of 1 in 0.61Mb (non-synonymous mutation discovery rate was 1 in 0.73Mb). This was a higher than expected rate of discovery (with the expected rate of 1 mutation in 1.82Mb taken from Quwailid *et al.*, 2004). From the 12 mutations discovered, 2 were selected to take forward for phenotyping as these mutations had the most severe predicted effects on the protein. The C59X mutation introduces a premature stop codon early on in Zfp804a, in the zinc finger binding domain of the protein. The C417Y mutation substitutes a cysteine for a tyrosine residue in a highly conserved region of the protein. These mutations were then backcrossed onto the C57Bl/6J background to isolate the mutations for phenotyping as models of *Zfp804a* function.

7.2.2 Preliminary phenotype screen

Following the creation of the mutant lines, the mice were then screened for any gross physical deficits that could interfere with further behavioural and cognitive testing. The tests revealed no major physical deficits and no differences in terms of mortality and general health between the lines and wild-types. From the assessments done, the C59X homozygous mutants performed better on the RotaRod than wild-type and heterozygous counterparts (and were also lighter in body weight) but there were no other behavioural differences observed in this chapter. Importantly for the validity of the model, there was no excessive variation observed in behaviour between animals in the same (gender, line and

mutation) group, which would have been expected if other, residual mutations were interfering with the behaviour assayed. This chapter (5) provided important evidence that the mutations breed well and the (G4) mutants are suitable for behavioural testing.

7.2.2.1 Performance on the RotaRod

The finding of enhanced performance on the RotaRod in *Zfp804a*$^{C59X/C59X}$ male mice appears to be unique to genetic animal models for schizophrenia. As mentioned in 5.4.3, another genetic mutant study (Benoit *et al*., 2010) found enhanced RotaRod performance in QR2 knockout mice. These mice also had enhanced learning abilities in the Morris water maze and object recognition. It would be interesting to investigate the cognitive phenotype (particularly learning abilities) of the *Zfp804a* mutants in further studies. In this thesis, the LMA task and observation in the phenotyper cages offer opportunities to assay aspects of learning. No differences were seen between the mice indicative of differences in learning on these tasks although there are other factors involved that may complicate interpretation (such as baseline activity, anxiety etc).

Dopamine, 5-HT and GABA have all been demonstrated to influence performance in mice on the RotaRod task (e.g. Shiotsuki *et al*., 2010; Peng *et al*., 2008; Rustay *et al*., 2003 respectively). Given this, no one neurochemical hypothesis to explain the findings from the *Zfp804a*$^{C59X/C59X}$ males is immediately obvious. The effect of dopamine on motor activity and co-ordination is perhaps the best documented in the literature (reviewed by Sasa *et al*., 2003). A recent study (Shiotsuki *et al*., 2010) found that increased availability of dopamine in a mouse model (by administering nomifensine, a

dopamine-uptake inhibitor) improved learning on the RotaRod whereas apomorphine, a dopaminergic toxin impaired learning in the task.Other researchers have found substances not directly acting on the dopaminergic system to also influence performance on the RotaRod. For example, ethanol in small doses can improve performance (Rustay *et al*., 2003). Ethanol has been shown to act on NMDA, 5-HT(3) and GABA(A) receptors.Sertraline, an SSRI that increases availability of serotonin has also been shown to enhance performance in the RotaRod, but only in R6/2 mice (a model for Huntington's disease (HD), carrying a transgene with a portion of *HTT (Huntingtin)*, the human HD gene and not in controls (Peng *et al*., 2008). This finding was interesting as it reveals a potential interaction of neurotransmitter systems in determining RotaRod performance. Given the different neurotransmitter systems that influence RotaRod performance and the lack of knowedge of the mechanism of action of *Zfp804a/ZNF804A*, further evidence is necessary to hypothesise about the neurochemical basis of the findings with the *Zfp804a* mutant models (see 7.5).

7.2.3 Emotion-related behavior

The work in Chapter 6 provided a preliminary insight into some emotion-related behaviours in the *Zfp804a* mutant mice. In terms of activity, homozygous mutant mice (independent of line) overall were more active in the locomotor activity boxes, although this same finding was not seen in the phenotyper home cage environment (Chapter 5) or other measures where activity could be measured. Similarly, the C59X line was less active than the C417Y line (in the locomotor activity task) although this finding wasn't supported by other

behavioural tasks. The findings from tasks looking at anxiety were interesting. C59X males appeared to be less anxious than C417Y males by exploring the open arm of the EPM more. The same findings were not observed in the open field although mutants (independent of line), were more active in the inner zones of the open field. There is some uncertainty however about whether or not exploration of the open field and the elevated plus maze are measures of the same underlying behavioural process (discussed in Chapter 6). The data at present is not clear-cut but it does suggest that the *Zfp804a* gene has some important influences on behaviour, and possibly affect. Continued research with these mice with larger cohorts and further characterising their behavioural profile will improve our understanding of some of the influences the gene might have at a behavioural level in mice.

It is interesting to note that several other researchers have found an anxiolytic effect of mutations in schizophrenia-relevant genes in mouse models, particularly in genes affecting the glutamatergic system. Labrie *et al*. (2009), found an anxiolytic phenotype on the EPM, OF and novel object test in GRIN1(D481N) mutant mice that displayed a 5-fold reduction in NMDA receptor glycine affinity. These effects were reversed by pharmacological means. Deletion of the glutamate receptor *Grik4*, which has been associated with susceptibility to depression, bipolar and schizophrenia (Catches *et al*., 2012) has also been robustly shown to have have an anxiolytic effect on the 0-maze, marble-burying and novelty-induced suppression of feeding tasks. Halene *et al*. (2009), found similar effects in NMDA receptor NR1 mutants ($Nr1[neo]^{-/-}$) mice (Mohn *et al*., 1999), with mutants showing a preference for the open arms of the 0-maze and the centre of the open field although no differences in LMA,

similar findings to those presented in this thesis.There have been several reports of an anxiolytic phenotype in *Nrg1* mutants including Karl *et al*. (2007), Long *et al*. (2010) and Desbonnet *et al*. (2012). However, overexpressing *Nrg1* does not necessarily lead to increased anxiety in the mice (Deakin *et al*. (2009). Findings of anxiolysis co-segregating with involvement of the glutamatergic system are interesting. Further investigation of the mechanism by which the mutation has an anxiolytic effect, perhaps by using pharmacological interventions with the *Zfp804a*$^{C59X/C59X}$ males would prove interesting and may be informative to the function and mechanisms of action of this gene.

7.3 Interpretation of findings

The findings of this thesis contribute to the growing body of data on the function of *ZNF804A/Zfp804a*. Research so far on the neurocognitive role of *ZNF804A* has shown that the gene is likely to play a role in cognition but the role it plays is somewhat unclear. This research is discussed in the Introduction (1.5.2.9). Some research has suggested that the risk allele at rs1344706 influences patients but not controls (Walters *et al.,* 2010; Hashimoto *et al.,* 2010, Donohoe *et al,* 2010), some research has suggested it influences patients and close (unaffected) relatives (Rasetti *et al.,* 2011) and some research has suggested the gene influences controls but not patients (Hargreaves *et al.,* 2012). None of the studies, however, explicitly measured the same thing so the findings are not necessarily conflicting. The mouse research presented in this thesis is not incompatible with the human research to date and does indeed suggest the gene plays a role in behaviour. Parallels between the mouse and human work beyond this, at this stage are not possible to draw. Further behavioural genetic

investigations with the C59X and C417Y lines and with human participants is warranted before any hypotheses on the gene's role in cognition and behaviour can be formed.

7.3.1 Comparison of the *Zpf804a* models to other genetic models for schizophrenia

To accurately compare the *Zfp804a* mutant models with other genetic mouse models for schizophrenia, further characterisation of the mutants with a larger group of the *Zfp804a*$^{C59X/C59X}$ males is needed. Nonetheless, important findings from the models have emerged, the mutants are viable and to date, are the only available animal models of altered *Zfp804a* function. The pattern of results seen warrant further investigation of the RotaRod and anxiolytic phenotypes. As discussed previously (7.2.2.1), dopamine, 5-HT and GABA all have been demonstrated to have an influence on performance in the RotaRod task. Pharmacological intervention with antagonists/agonists for these neurotransmitters would be interesting and may help to elucidate the mechanism by which the the *Zfp804a*$^{C59X/C59X}$ mutation aids RotaRod performance. In terms of the findings from the EPM and OF, in the literature, anxiolysis in genetic mouse models seems to be associated with alterations to the glutamatergic system (see 7.2.3). Glutamate agonists and antagonists would prove interesting to investigate the anxiolytic phenotype of the *Zfp804a*$^{C59X/C59X}$ mice further and the mechanism by which it works.

To date, most genetic mouse models for schizophrenia have been knockout models or transgenic animals. Despite the merits of ENU mutagenesis to generate models, there are (to date) only two other ENU

mutant models for schizophrenia: Q31L and L100P *Disc1* mutants (Clapcote *et al*., 2007). In the Neuron paper, the two models showed different phenotypes with the Q31L model showing depressive-like behaviour (deficits in the forced swim task, reversed by bupropion) and the L100P model showing PPI and latent inhibition deficits that were reversed by antipsychotics. However, a more recent study, using the same model but bred to C57Bl/6J for a further two generations, failed to replicate the findings finding no differences between the Q31L mice and wild-types and the L100P mice and wild-types, but for an increase in locomotor activity (consistent with Clapcote *et al*., 2007), (Shoji *et al*., 2012).These studies emphasise the need for findings to be replicated and add strength to the anxiolytic findings in this study, which have been successfully replicated in the same tasks and another task (0-maze) after the mutants have been bred onto C57Bl/6J for a further two generations (Eddy, personal communication, 2011).

7.4 Recent work

The association of the schizophrenia susceptibility gene, *ZNF804A*, was first described by the (then) Department of Psychological Medicine, Cardiff University (O'Donovan *et al.*, 2008). Since the discovery of the gene's potential involvement in schizophrenia pathogenesis, a multi-discilinary program of work has grown in the Department, to investigate the gene's function at a molecular, cellular and behavioural level (in both humans and mice) and how it may confer risk to schizophrenia and bipolar disorder.

7.4.1 Molecular biology

In terms of the gene's molecular function, the central hypothesis is that the gene may have a role in regulating gene expression based on sequence homology with other genes that regulate expression (Knight, personal communication, 2012). Currently, the function of this gene is being investigated by Debbie Knight (PhD student) by using the C59X line generation to examine the consequences of altered *Zfp804a* expression in the brains of mice carrying a nonsense mutation. To identify genes which are differentially expressed between C59X homozygous mutant mice and wild-type controls, Knight is also using microarray analysis and RNA-sequencing. Genes that show differential expression may be involved in mediating the effect of Zfp804a on behaviour and warrant further investigation both molecularly and statistically (in terms of their association with schizophrenia in humans). As well as this molecular genetic work, other ongoing work with *ZNF804A,* led by Professor Derek Blake, includes the development of a ZNF804A antibody, yeast two-hybrid screening to look for binding partners and developing stable cell lines expressing ZNF804A.

7.4.2 Animal models

Parallel behavioural work with the C59X and C417Y lines (F7) is also ongoing, led by Professor Lawrence Wilkinson and Jessica Eddy. A developmental assessment of the mice looking at body weight from birth and somatic indices such as ear development, eruption of teeth, appearance of fur and whiskers etc is being analysed, adding to the data generated in this thesis. The anxiety tasks in this thesis have also been repeated and the findings replicated, suggesting

the *Zfp804a*^{C59X} mutation indeed has an anxiolytic effect (see figure 7.1, Eddy, unpublished data, reproduced with permission). Other ongoing experiments with the mice include looking at impulsivity, working memory, social interaction and anhedonia.

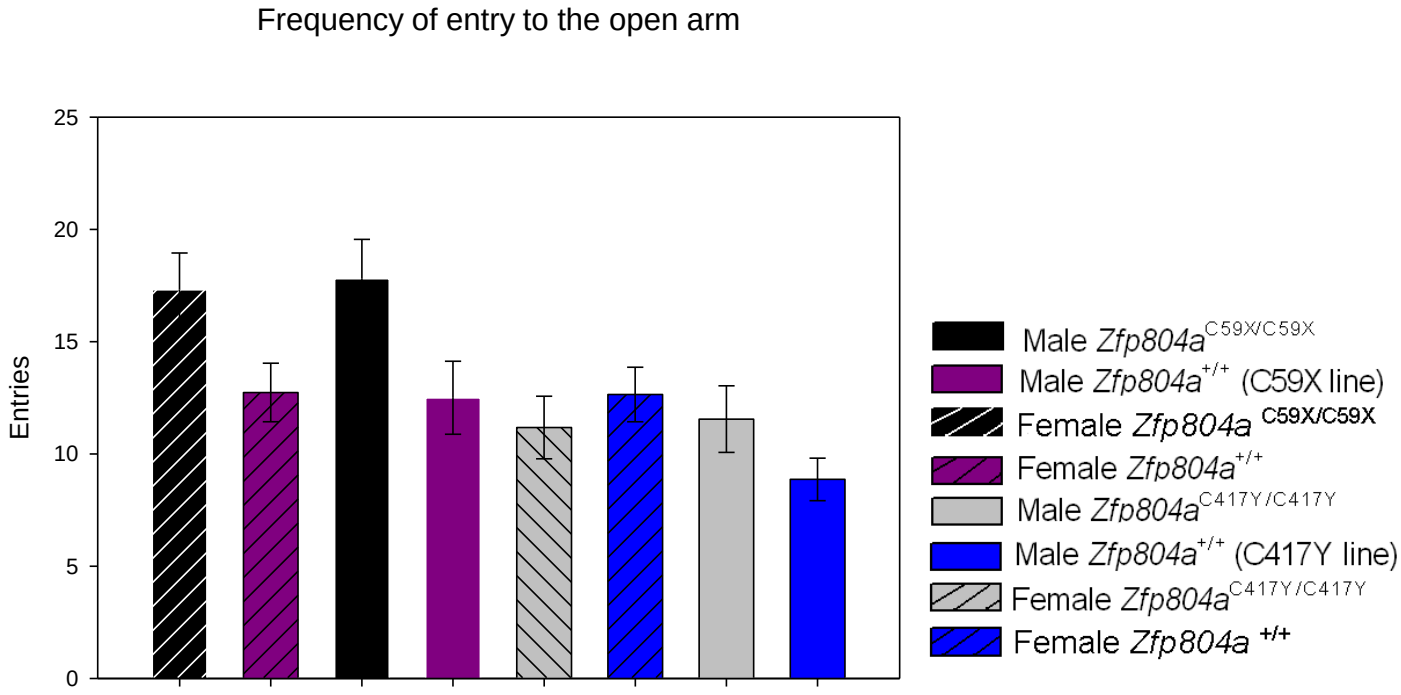


Figure 7.1: Frequency of entry to the open arms of the EPM, Eddy (unpublished data), reproduced with permission

7.4.3 Clinical work

Since the discovery of the gene's involvement with schizophrenia, clinical work in the Department has looked at clinical features and endophenotypes segregating with the risk allele, rs13447606 in patient and control populations, led by Dr James Walters.Findings so far have been interesting. The risk allele in *ZNF804A* seems to segregate with a subgroup of the (schizophrenia) patient population with relatively intact cognition (compared with other schizophrenia patients, Walters *et al*., 2010). The authors tested for an association in two samples (Irish and German) between the risk allele, A, at rs1344706 and

cognitive domains known to be affected in schizophrenia: IQ, episodic memory, working memory and attention. In the Irish sample, in patients but not in controls, the risk allele had a protective effect on episodic and working memory. The results replicated in the German sample supporting the protective action of the risk allele at rs1344706. Slightly elevated manic symptoms associated with the risk allele were also found in this population (Donohoe *et al.,* 2010).

7.4.4 In situ hybridisation

Other ongoing work (by Tamara Al-Janabi and Dr William Davies) is the *in situ* hybridisation of *ZNF804A* in human foetal/embryonic brain tissue. The MRC-Wellcome Trust Human Developmental Biology Resource (HDBR), based at UCL has a tissue bank of human foetal tissue and an in-house gene expression service (IHGES) that can be used to gather *in situ* hybridisation data on gene expression. In adult mice, the *Zfp804a* transcript (also known as *C630007C17Rik*) is most highly expressed in the septum, the thalamus and the hippocampus, and is weakly expressed elsewhere (Allen Brain Atlas, 2011). The brain expression throughout development in mice is largely unknown as is also the case in humans.

According to Unigene, *ZNF804A* is expressed in human brain (10 transcripts per million), and in foetal tissue (15 transcripts per million), but as yet no published studies have explicitly assessed its distribution throughout the brain. As the function and developmental expression of *ZNF804A* is currently unknown, *in situ* hybridisation was performed on the tissue bank by the IHGES. Three anti-sense DIG-labelled probes were prepared from *ZNF804A* to cross exon-exon boundaries to ensure the probe did not hybridise to DNA. Full

details of the methods used for the *in situ* hybridisation can be seen in Appendix 8.3. Figures 7.2 and 7.3, from a 12-week embryo, show specific staining in the posterior ganglionic neuroepithelium and subventricular zone and in the cortical plate. The pattern of expression in these images is very interesting due to some similarities between the specific staining and some research by Yokota *et al.*, (2007) that demonstrated that a particular type of GABAergic interneurons show a pattern of migration via these zones. Furthermore, GABAergic neuronal activity in the frontal cortex (which develops from the cortical plate) is thought to be involved in some of the cognitive deficits observed in schizophrenia (Lewis *et al*., 2005; Lewis & Moghaddam 2006). However, from the images, we cannot tell what type(s) of cells are stained so this hypothesis is merely speculative.

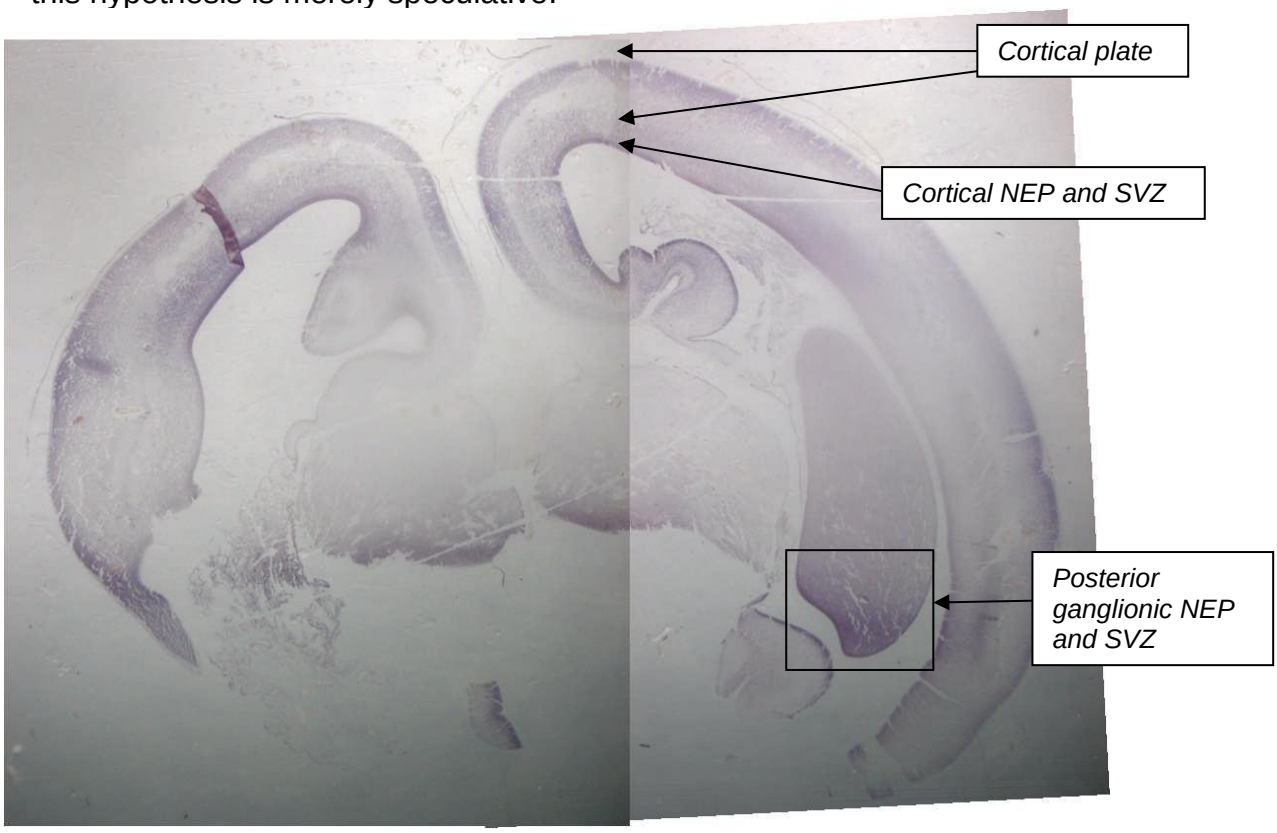


Figure 7.2: Probe -ZNG2, F4 (12 developmental weeks), dorsal coronal section, showing staining (indicated by arrows) in the cortical plate, the cortical neuroepithelium (NEP) and subventricular zone (SVZ) and the posterior ganglionic NEP & SVZ, Al-Janabi, Davies, Fok, Gerelli and Owen (unpublished data)

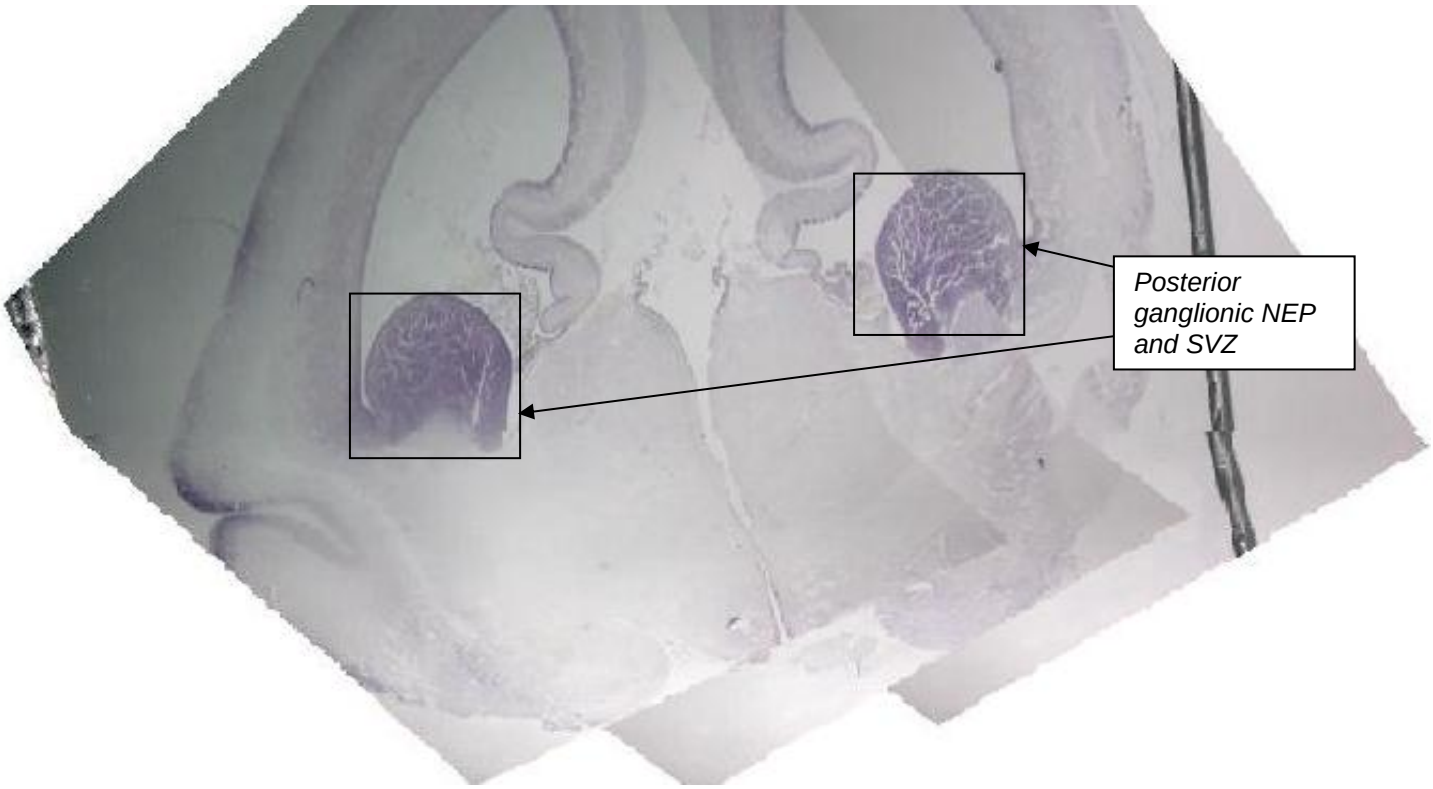


Figure 7.3: Probe - ZNF 1, F4 (12 developmental weeks), coronal ventral section, showing staining (indicated by arrows) in the posterior ganglionic NEP & SVZ Al-Janabi, Davies, Fok, Gerelli and Owen (unpublished data)

In situ hybridisation in an 8 week embryo didn't follow the same pattern but showed very specific staining in Rathke's pouch (which becomes the anterior pituitary, see figures 7.4 and 7.5). One of the anterior pituitary's functions is the production of prolactin. The anterior pituitary houses dopamine receptors and it is well-known that antipsychotics (which tend to block dopamine receptors in the brain, reviewed in Baumeister & Francis, 2002) are associated with hyperprolactinaemia (reviewed in Inder & Castle, 2011, Holt & Peveler, 2011). However this may, of course, be coincidental as the anterior pituitary has several functions other than housing dopamine receptors and producing prolactin (such as regulating stress, growth and reproduction). The results from the *in situ* hybridisation are nevertheless interesting and certainly warrant further investigation.

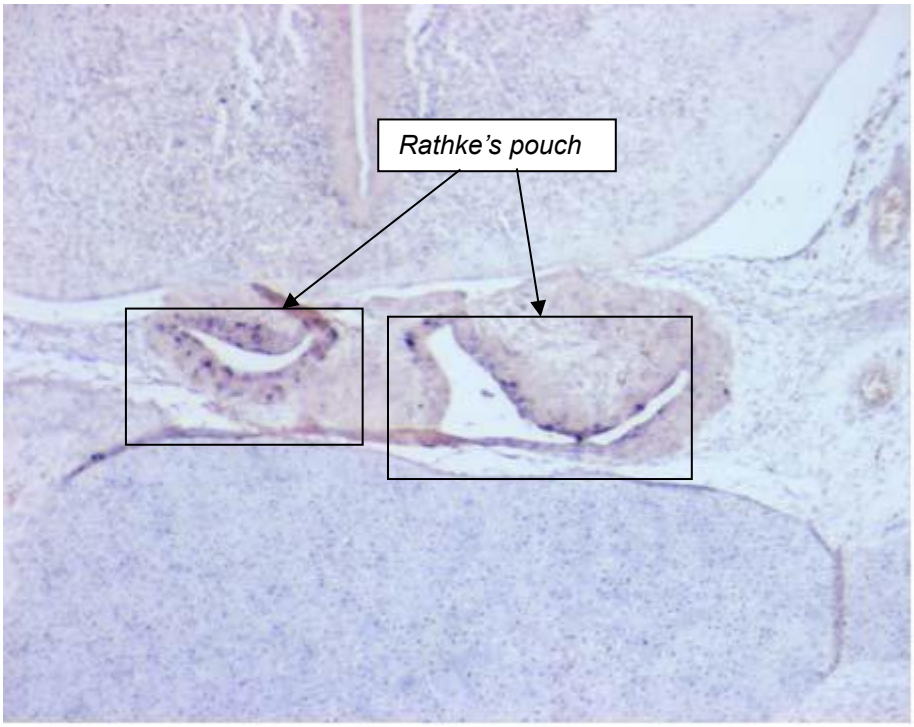


Figure 7.4: A magnified image (x8.0)(CS23, 8 weeks, coronal section,probe - ZNF 3), above of specific staining in Rathke's pouch, Al-Janabi, Davies, Fok, Gerelli and Owen (unpublished data)

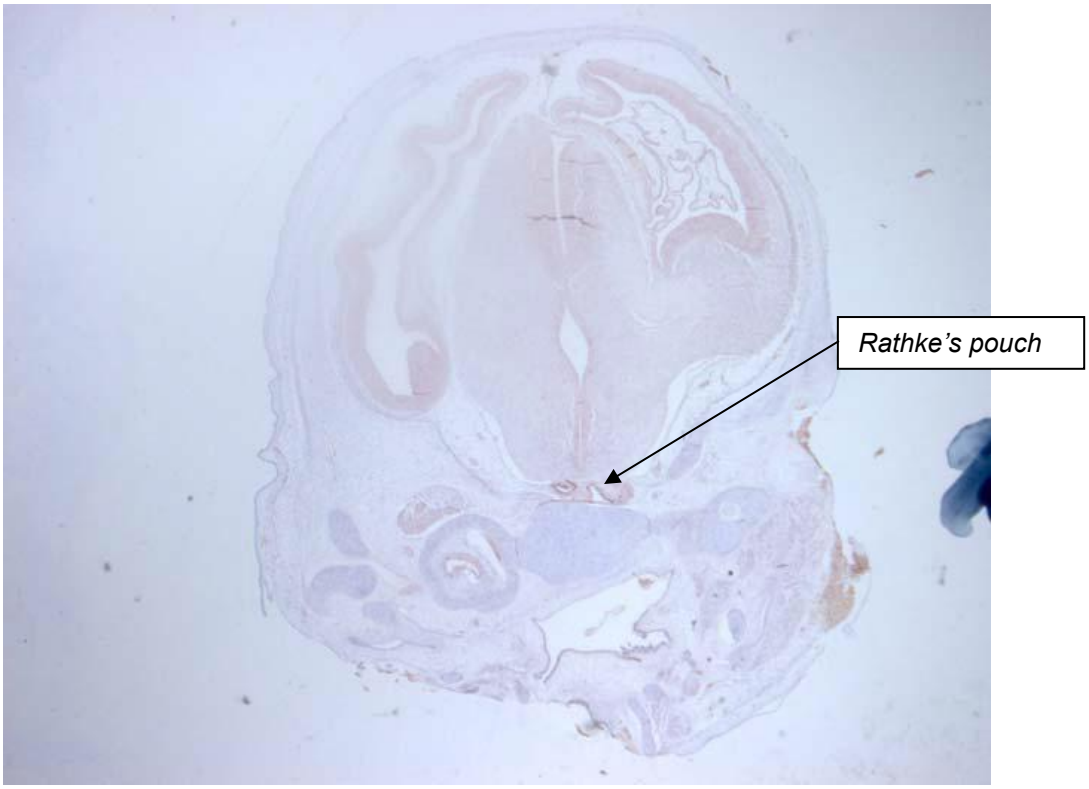


Figure 7.5: An unmagnified image (x1.0)(CS23, 8 weeks, coronal section,probe - ZNF 3) of specific staining in Rathke's pouch, Al-Janabi, Davies, Fok, Gerelli and Owen (unpublished data)

7.5 Possible Clinical Relevance

Data suggesting a role for *ZNF804A* in cognition and behaviour is beginning to emerge although further work is needed to elucidate the function of *ZNF804A* and particularly, the role the gene plays in schizophrenia risk. In terms of the mouse models, the creation of two parallel lines that survive and breed successfully and show indications of behavioural phenotypes is encouraging.

The findings with the C59X mutants, in terms of the EPM were interesting. We saw more exploratory behaviour in a fear-inducing environment, indicative of lower anxiety levels than the wild-type littermate controls. Presuming (although this is not known at this stage) that the C59X mutation reduces or destroys the Zfp804a protein, and that risk allele rs1344706 is associated with an increase in ZNF804A production (Williams *et al*., 2011) then we may expect to see increased anxiety in risk-allele carriers. Further work is necessary to explore this interesting finding, especially in the light of findings from the Edinburgh High Risk Study (Johnstone *et al.,* 2005) that anxiety can predict the development of schizophrenia. In humans, the risk allele at rs1344706 is common. Any intervention/screening strategies based on this risk allele therefore not practicable. However, the replicated findings regarding differences in anxiety are nevertheless interesting and informative to future work.

There are also other potential clinical implications of the work contained in this thesis although these are speculative at this stage and would need substantial further investigation to support them. We observed that the C59X male homozygous mutants performed better on the RotaRod than other mutants. RotaRod performance has been shown by numerous studies to be

affected by dopamine (e.g. Mizoguchi *et al*., 2002; Sasa *et al*., 2003; Ogura *et al*., 2005; Shiotsuki *et al*., 2010) and dopamine is strongly associated with motor co-ordination (reviewed in Côté & Crutcher, 1991). The findings from the *in situ* hybridisation (see figures 7.3 and 7.4) suggest that *ZNF804A* is highly expressed in Rathke's Pouch, the developing anterior pituitary, a region rich in dopaminergic neurons. A third line of evidence for the involvement of *ZNF804A* in the dopaminergic system comes from clinical work. Three independent studies have shown that antipsychotic response is linked to *ZNF804A* genotype (Xiao *et al*., 2011; Zhang *et al*., 2012; Mössner *et al*., 2012). Zhang *et al* and Mössner *et al*. both demonstrated that antipsychotics in patients either carrying the risk allele (Zhang *et al*.) or homozygous for the risk allele (Mössner *et al*.) showed poorer response to antipsychotic therapy than those patients either carrying (Mössner *et al*.) or homozygous for (Zhang *et al*.) the non-risk allele. Xiao *et al*. (2011) found that although rs1344706 didn't affect antipsychotic response in a Han Chinese population, minor alleles at rs35676856 and rs61739288 in *ZNF804A* both predicted a slower response to antipsychotics. It is plausible therefore that *Zfp804a/ZNF804A* may therefore have some influence on the dopaminergic system although this area would need substantial further investigation before any treatment implications could be drawn. Similarly there are the findings from the *in situ* hybridisation that could relate to GABAergic interneurons (and it is known that GABAergic interneurons modulate dopamine release, reviewed in Fink & Göthert, 2007, see 7.4.3 and figure 7.2 and 7.3) although this again would need substantial further investigation before any treatment implications could be considered.

7.6 Future directions

As highlighted in section 7.5, there is plenty of scope for further work developing and extending upon the findings in this thesis. Further work with the *Zfp804a* C59X and C417Y lines is focused on in this section, although there is a plethora of important work to be done beyond mouse models of *Zfp804a* to elucidate the mechanisms by which this gene confers risk to schizophrenia.

In terms of probing the findings relating to anxiety further, there are numerous behavioural assays that would yield further information. The EPM and OF findings would be supported by repeating the results with larger cohorts (particularly larger numbers of *Zfp804a*$^{C59X/C59X}$ males). Results from other anxiety-assaying tasks including the 0-maze, novelty-place preference and social anxiety tasks would also be interesting, particularly, perhaps, social anxiety tasks given Johnstone *et al.*'s (2005) findings that it is social anxiety rather than other forms of anxiety that best predicts the development of schizophrenia in those deemed to be a high genetic risk.

To explore the idea of a possibly altered dopaminergic system in *Zfp804a* mutant mice, pharmacological manipulations on behavioural tasks, particularly the LMA and RotaRod tasks involving movement and motor co-ordination would likely yield the most interesting results. Antagonist drugs such as antipsychotics and agonists such as L-DOPA, mephadrone and methamphetamine would likely be the best pharmacological candidates.

The possibility that *ZNF804A* may be highly expressed in GABAergic interneurons first and foremost needs confirmation that this is the case before it may be explored. Cell-staining techniques in tissue samples would be the easiest method of doing this (Gerrelli, personal communication, 2012). If this is

confirmed then exploration of behaviour (such as LMA) using GABAergic antagonists such as $GABA_A$, bicuculline and agonists such as benzo/nonbenzodiazipines, barbiturates and ethanol would prove useful.

Aside from behaviourally, these mice are also of great use biologically. Histology using the brains of the mice would be useful to further investigate indications of differences in brain weights between the lines and genotypes, as would the technology now available of using MRI to image the brains of the mice.

However, before any further work on the models is truly informative, the biochemical characterization of the mutations is imperative. Without this, we can only estimate the effects the mutations are having and thus can only draw conclusions that any phenotypes are associated with a change on the Zfp804a protein, rather than anything more concrete. Discovering the effect the mutations have on the protein would add to the utility of these models enormously.

Beyond the areas raised for further investigation in this thesis, there is ample opportunity to extend the research. One such area that would be useful to explore would be creating alternative mouse models such as a conditional knockout and a mouse model with up-regulated *Zfp804a*. In conditional knockout models, *Zfp804a* could be spatially and temporally manipulated to refine where and when the gene has the most influence on phenotype. This would begin to give better indications of how this gene may confer risk of schizophrenia.

7.7 Final comments

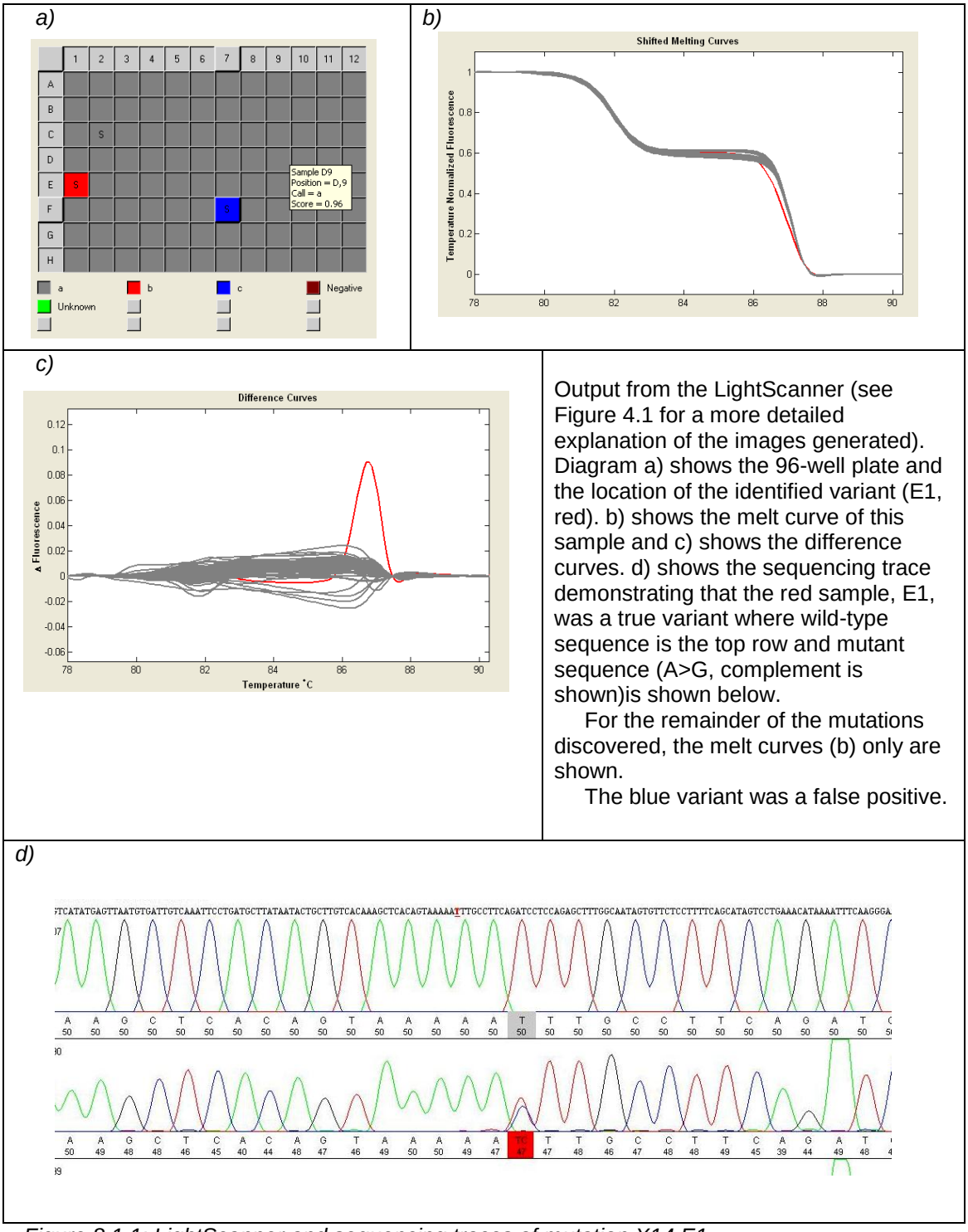
Although work to understand the mechanisms by which ZNF804A confers risk for schizophrenia (and therefore can translate laboratory findings to clinical utility) has only just begun, initial investigations are promising, both with the mouse lines created in this thesis and beyond. The ultimate goal is however not scientific, or intellectual, it is to improve the lives of people with schizophrenia and those close to them. This research, whilst it cannot have any direct clinical impact, makes a small but useful contribution to the understanding of *ZNF804A*, which will in turn, improve our understanding of schizophrenia. These steps, albeit small, are necessary if we are to understand and therefore satisfactorily classify, treat and prevent schizophrenia.

7.8 Summary

The results reported in this thesis contribute to a rapidly growing and rapidly developing field of schizophrenia genetics. As the first mouse models available for *Zfp804a*, the lines created within this thesis will be of great use for understanding the function of *ZNF804A.* The mutant lines will not only be useful in understanding how *Zfp804a* may influence behaviour but can also be used at molecular and cellular levels. Preliminary indications suggest that *Zfp804a* may have a role in anxiety. However, substantial further work is necessary with these models to investigate this and other findings from this thesis further.

CHAPTER 8: Appendices

8.1: Sequencing traces of mutations discovered



Output from the LightScanner (see Figure 4.1 for a more detailed explanation of the images generated). Diagram a) shows the 96-well plate and the location of the identified variant (E1, red). b) shows the melt curve of this sample and c) shows the difference curves. d) shows the sequencing trace demonstrating that the red sample, E1, was a true variant where wild-type sequence is the top row and mutant sequence (A>G, complement is shown)is shown below.

For the remainder of the mutations discovered, the melt curves (b) only are shown.

The blue variant was a false positive.

Figure 8.1.1: LightScanner and sequencing traces of mutation X14,E1

a)

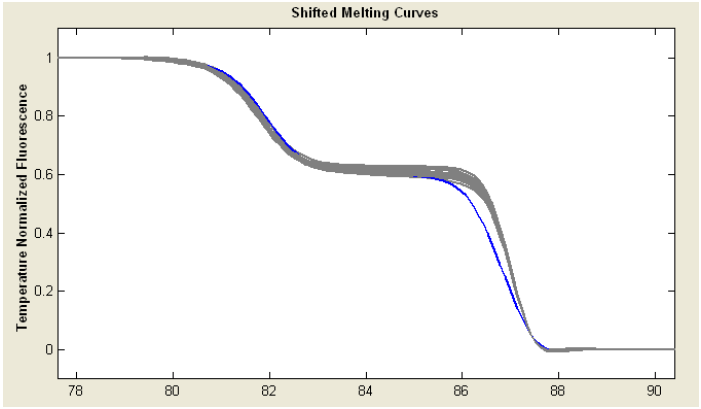


Figure showing melt profile (a) of sample X23, E5 (blue) and the wild-type (top) and mutant (bottom) sequencing trace (b, TGT>TAA, complement is shown).

b)

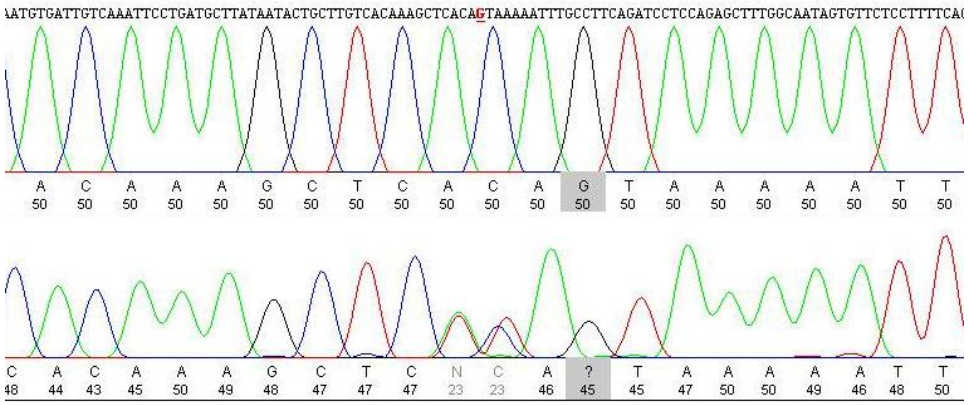


Figure 8.1.2: LightScanner and sequencing traces of mutation X23, E5

a)

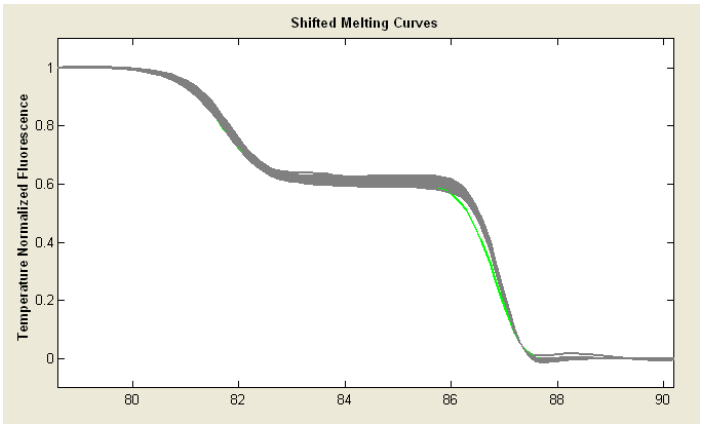


Figure showing melt profile (a) of sample X33, E8 (green) and the wild-type (top) and mutant (T>C, complement is shown) (bottom) sequencing trace (b).

b)

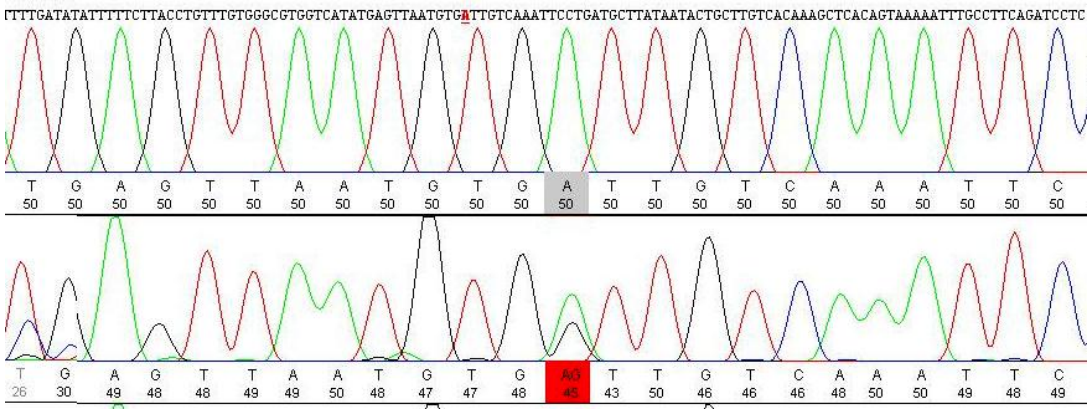


Figure 8.1.3: LightScanner and sequencing traces of mutation X33, E8

a)

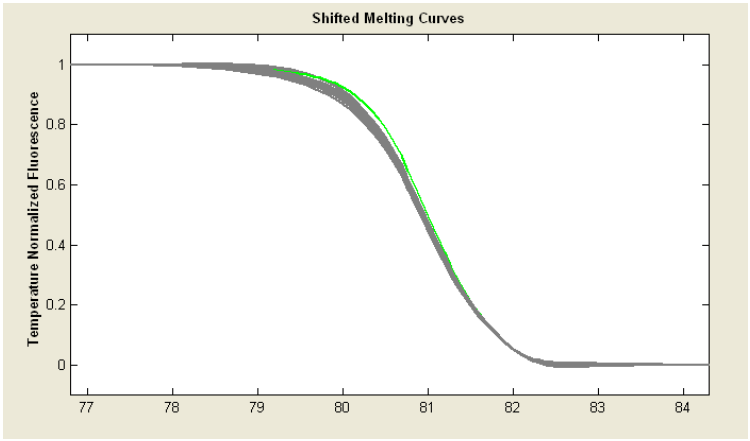


Figure showing melt profile (a) of sample X32, E3 (green) and the wild-type (top) and mutant (T>A) (bottom) sequencing trace (b).

b)

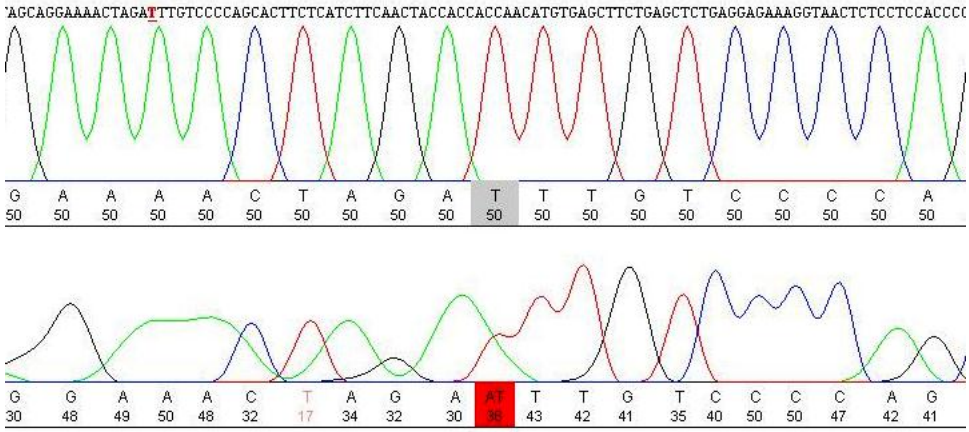


Figure 8.1.4: LightScanner and sequencing traces of mutation X32, E3

a)

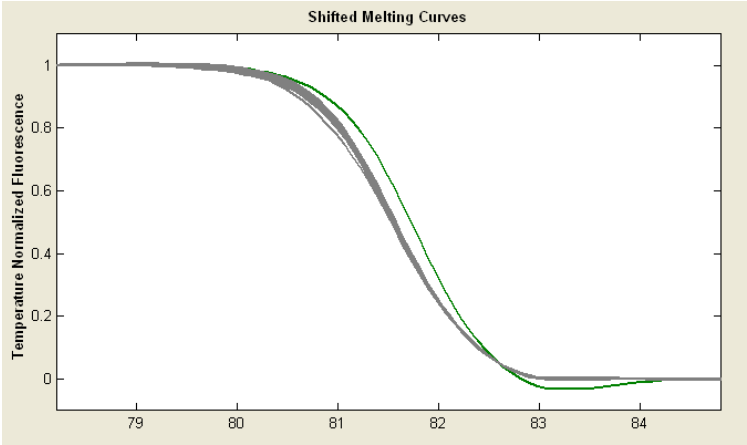
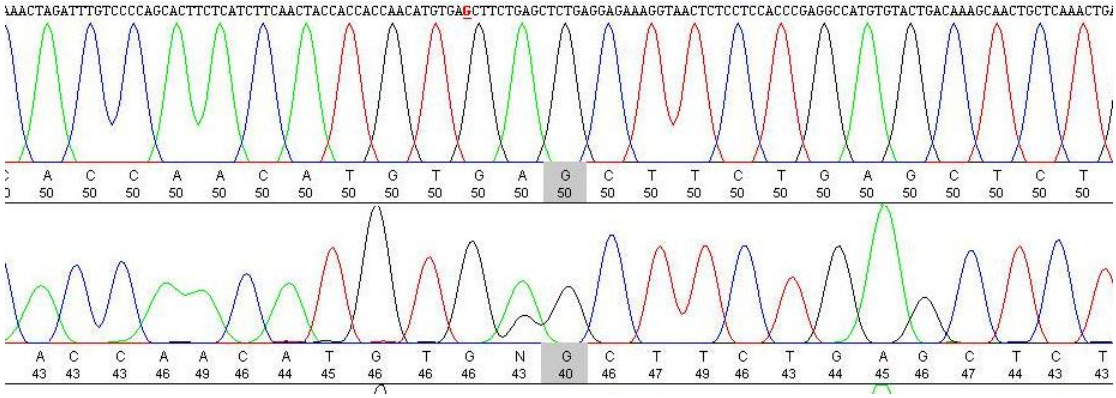


Figure showing melt profile (a) of sample X13, A5 (green) and the wild-type (top) and mutant (bottom) sequencing trace (b) of the A>G mutation. The sequencing trace of the wild-type and mutant sample showing the T>C mutation is shown in (c).

b)



c)

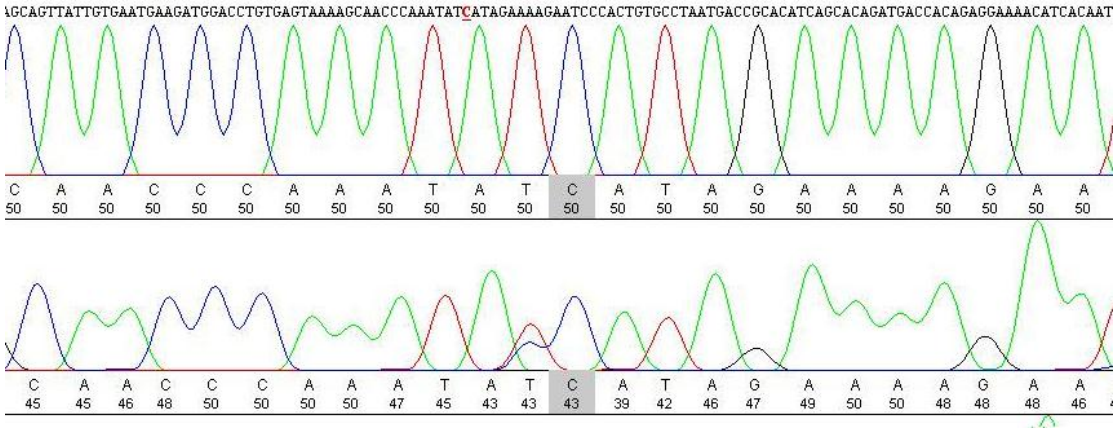


Figure 8.1.5: LightScanner and sequencing traces of mutations in X13, A5

a)

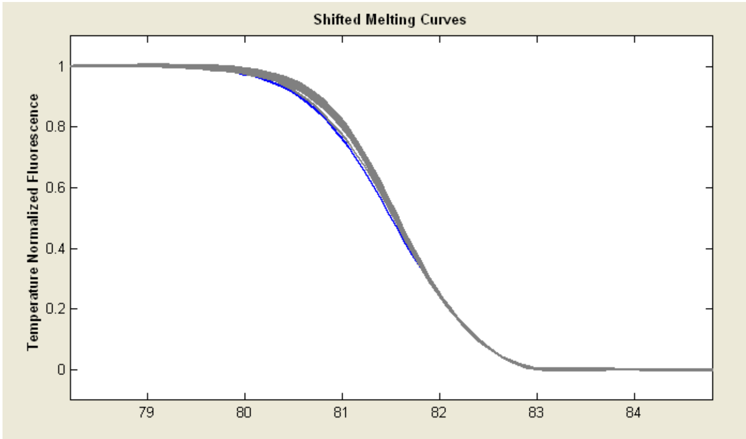


Figure showing melt profile (a) of sample X13, D5 (blue) and the wild-type (top) and mutant (T>A)(bottom) sequencing trace (b).

b)

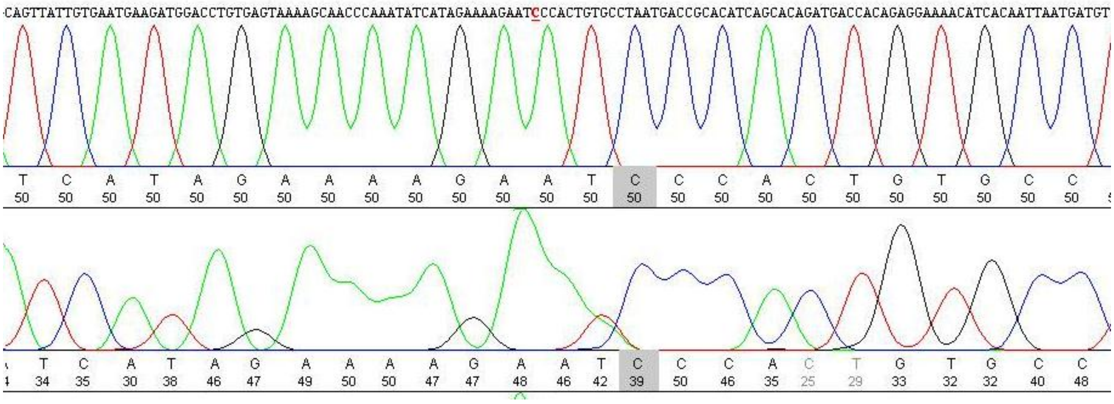


Figure 8.1.6: LightScanner and sequencing traces of mutation X13, D5

a)

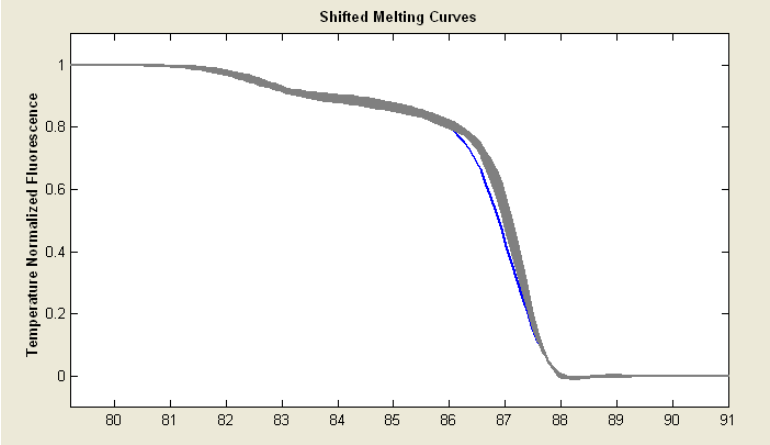


Figure showing melt profile (a) of sample X13, E2 (blue) and the wild-type (top) and mutant (A>G)(bottom) sequencing trace (b).

b)

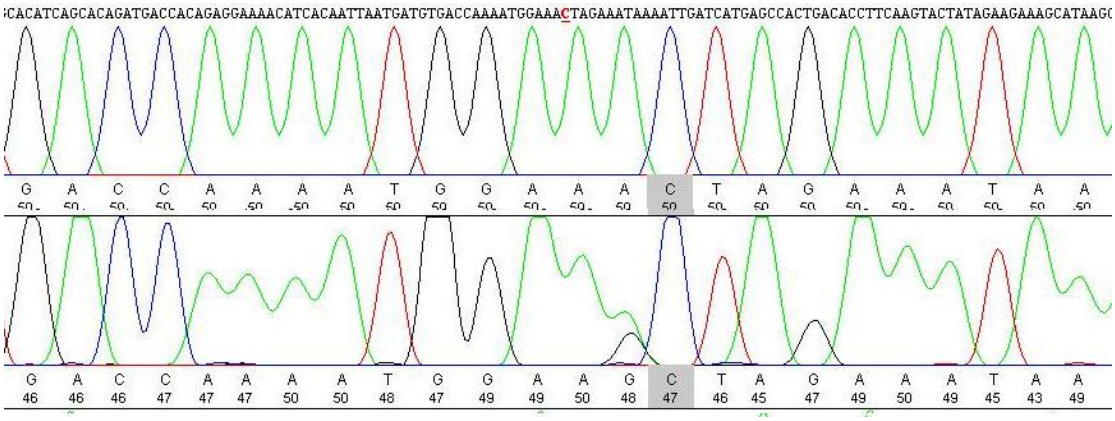


Figure 8.1.7: LightScanner and sequencing traces of mutation X13, E2

a)

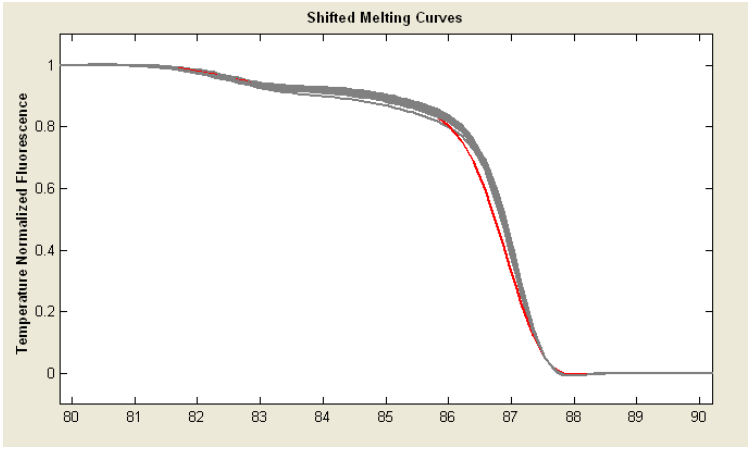


Figure showing melt profile (a) of sample X42, G9 (red) and the wild-type (top) and mutant (G>A)(bottom) sequencing trace (b).

b)

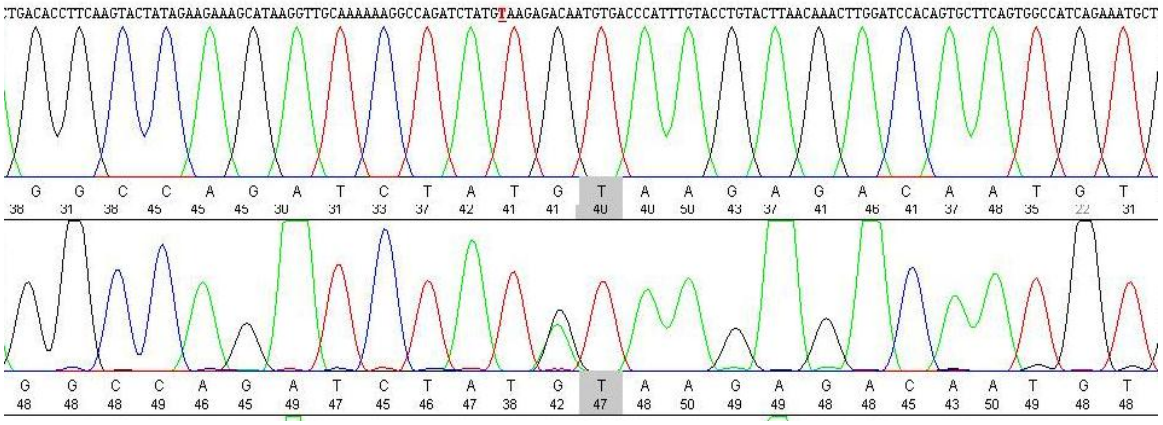


Figure 8.1.8: LightScanner and sequencing traces of mutation X42, G9

a)

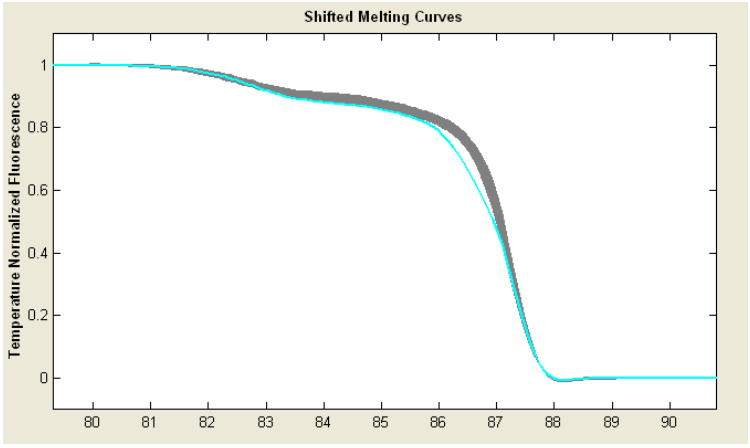


Figure showing melt profile (a) of sample X9, G1 (aquamarine) and the wild-type (top) and mutant (T>C)(bottom) sequencing trace (b).

b)

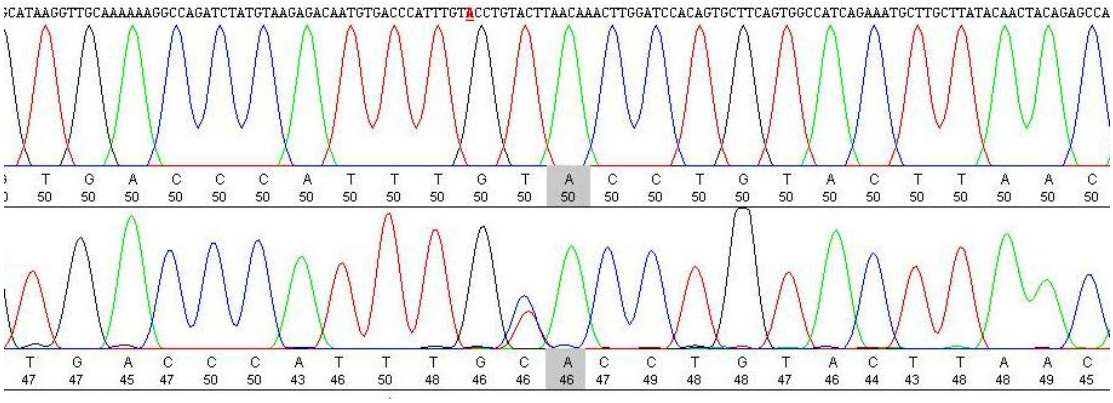


Figure 8.1.9: LightScanner and sequencing traces of mutation X9,G1

a)

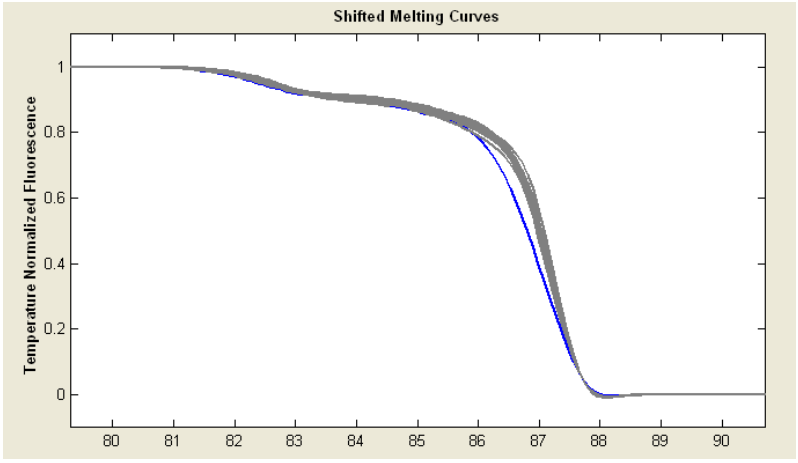


Figure showing melt profile (a) of sample X29, A12 (blue) and the wild-type (top) and mutant (A>G)(bottom) sequencing trace (b).

b)

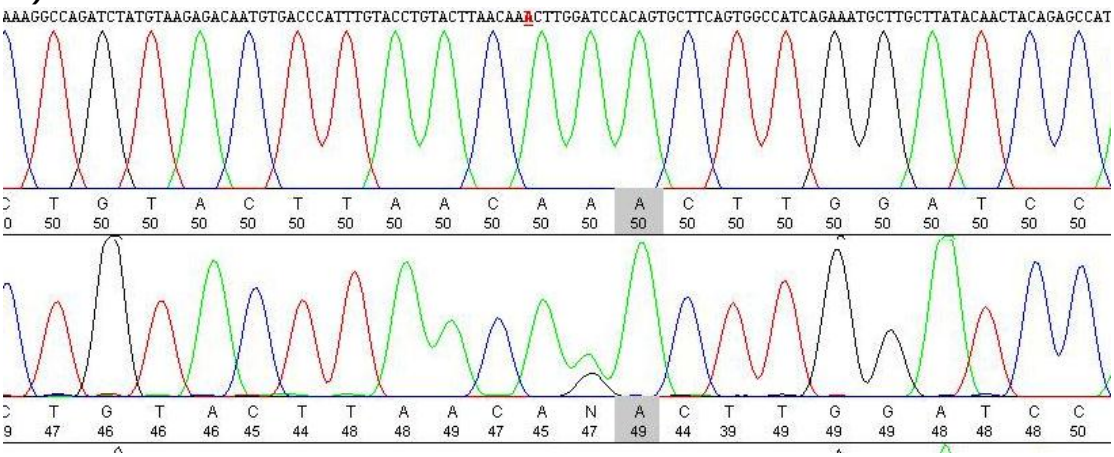


Figure 8.1.10: LightScanner and sequencing traces of mutation X29,A12

a)

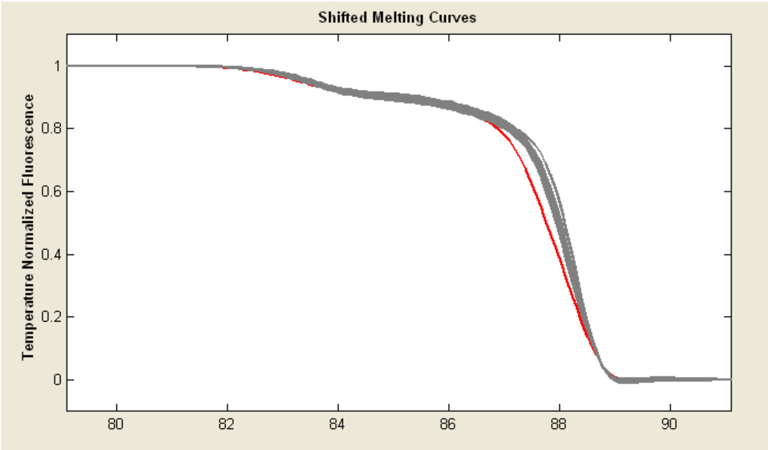


Figure showing melt profile (a) of sample X15, E8 (red) and the wild-type (top) and mutant (A>G)(bottom) sequencing trace (b).

b)

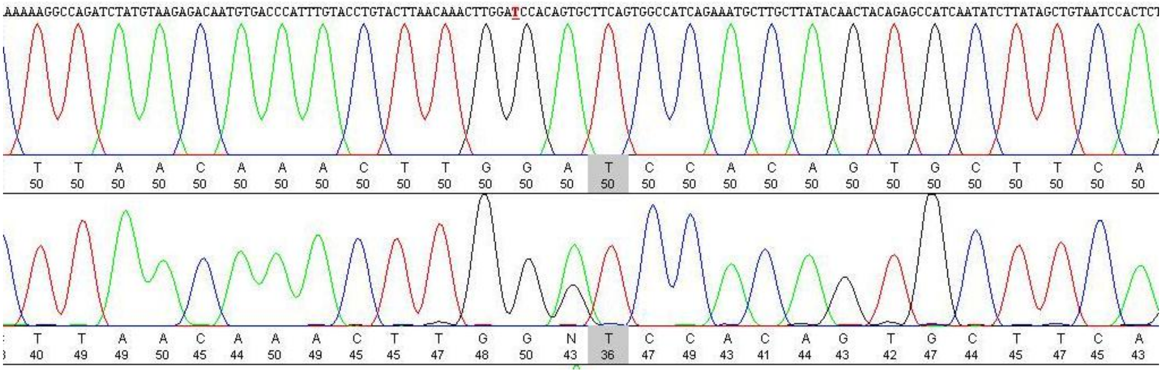


Figure 8.1.11: LightScanner and sequencing traces of mutation X15, E8

***8.2 In vitro* fertilisation of mouse oocytes using HTF medium (from MRC Mammalian Genetics Unit, Harwell)**

EMMA and FESA, MRC Harwell, 11th – 14th February 2008

A. Preparation of oocyte harvest dishes

1. Add approximately 2-3ml of the HTF (with Pen/Strep/BSA) to a numbered 35mm Petri Dish (Falcon 351008). One dish is required for every three superovulated females.

2. Place the dishes in the incubator overnight at 37°C, in 5% CO_2 in air to equilibrate.

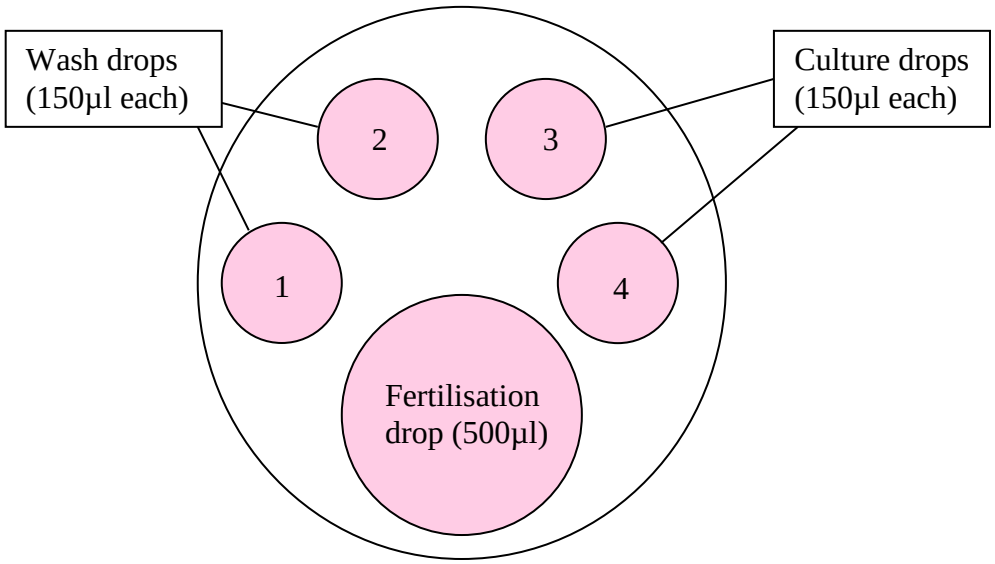
B. Preparation of Fertilisation/Wash/Culture dishes

1. Prepare one 60mm Petri Dish (Falcon 351016) for every three superovulated females.

2. Into each dish, carefully pipette 5 drops of HTF (with Pen/Strep/BSA) as follows:

   1 x 500µl for fertilisation

   4 x 150µl for washing and overnight culture



3. Carefully overlay the drops with Mineral Oil (Sigma Chemical Co.; Cat. No. M8410, embryo culture tested) ensuring that they do not run together.

4. Equilibrate the dishes overnight at 37°C, in 5% CO_2 in air.

C. Preparation of sperm dispersal dish (for freshly harvested sperm)

1. Pipette 500µl HTF into the centre of a 60mm Petri Dish (Falcon 351016).

2. Overlay with Mineral oil and equilibrate overnight at 37°C, in 5% CO_2 in air.

D. Preparation of sperm samples:

Freshly harvested sperm

1. The selected male should be at least 8 weeks old, and not have been used for mating for at least 3 days before sperm collection.

2. Sacrifice the male and dissect the cauda epididymides.

3. Place on a clean paper tissue, and remove as much adipose and vascular tissue as possible, using size 5 watchmakers' forceps. Work under a dissecting microscope lit from above, dissecting quickly to prevent the material from desiccating.

4. Place the cauda epididymides into the oil next to the dispersal drop. Nick the tip of the cauda epididymides with a pair of fine scissors. Gently palpate the tissue to expel the sperm.

5. Drag the blebs of sperm into the dispersal drop.

6. Allow the sperm to disperse into the medium for between 10 and 90 minutes, at 37°C in the CO_2 incubator.

7. Place IVF dishes on a heated stage or hot pad, then pipette 10µl of the sperm suspension into each fertilisation drop using a wide-bore tip and return the dishes to the incubator until the oocytes are harvested.

Cryopreserved sperm

1. Using forceps, hold the cryotube in air for 30 seconds. If liquid nitrogen is present in the cryotube, wait for it to evaporate and escape by rolling the cryotube around on the bench.

2. Take **special care** that the cryotube is not filled with liquid nitrogen before plunging into the water bath (such tubes may explode).

3. Thaw the sperm sample rapidly by placing in a 37°C water bath.

4. Once thawed, pipette 10µl of the sperm suspension into each fertilisation drop using a wide bore pipette tip and return the dishes to the incubator.

E. Harvesting oocytes

1. Dissect the oviducts from three superovulated female mice and place into a preincubated dish of HTF (for superovulation methods, see the protocol for harvesting and cryopreservation of *in vivo* derived embryos and the table below).

2. Under a dissecting microscope, hold each oviduct down with forceps and gently tear the swollen ampulla with a second pair of forceps to release the cumulus masses into the HTF, then remove the oviduct from the dish.

3. When all the cumulus masses have been extracted, gently draw them up into a wide bore pipette tip in a maximum of 30µl HTF. To stop them from sticking to the plastic, first wet the inside of the pipette tip by drawing up and expelling some HTF. Hold the pipette close to vertical to ensure the cumulus masses are aspirated with a minimal amount of medium.

4. Transfer the cumulus masses to a fertilisation drop (containing sperm) being careful to transfer as little HTF as possible.

5. Incubate the dishes at 37°C, in 5% CO_2 in air for approximately 5-7 hours to allow fertilisation to occur.

6. Repeat steps 1-5 for each fertilisation dish in succession (i.e. complete all of the steps from collecting the oviducts to returning the fertilisation dishes to the incubator for one batch of females before starting the next batch). Aim to take no more than 5 minutes from collecting the oviducts to returning the fertilisation drop (including oocytes) to the incubator.

F. Washing and culturing the fertilised oocytes

1. Between 5-7 hours after the cumulus masses were placed in the fertilisation drop, remove all of the oocytes and place them in wash drop 1 (see diagram).

2. Move the good quality oocytes from wash drop 1 to wash drop 2, cleaning the oocytes as much as possible in the process. Leave poor quality oocytes in wash drop 1.

3. Divide the washed oocytes approximately equally between the two culture drops (3 and 4).

4. Incubate overnight at 37°C, in 5% CO_2 in air.

G. Preparing fertilised oocytes for embryo transfer or freezing

1. Next morning, separate the 2-cell embryos from those which have not fertilised or cleaved, or have degenerated. Place all the 2-cell embryos in drop 4 and the 1-cell or degenerated oocytes/embryos in drop 3.

2. Prepare a drop of hyaluronidase solution (300µg/ml made up in M2) in a Falcon 351008 petri dish. **NB: This hyaluronidase washing step is only required when it is necessary to remove the adherent cumulus cells from the zona pellucida.**

3. Collect the 2-cell embryos into a drop of M2 in a Falcon 351008 petri dish.

4. Transfer these embryos into the drop of hyaluronidase.

5. Incubate at 37°C for a few minutes with gentle agitation at intervals using a glass pipette until any adherent cells or sperm have fallen off.

6. Wash the embryos through two drops of M2.

7. Either transfer the 2-cell embryos to the oviducts of 0.5d pseudopregnant foster mothers, or:

8. Prepare the 2-cell embryos for cryopreservation according to the standard protocol for *in vivo* derived embryos, or:

9. Culture the embryos in KSOM.

Timetable of events for IVF

Day -3 (e.g. Saturday)	**Day -1** (e.g. Monday)	**Day 0** (e.g. Tuesday)	**Day 1** (e.g. Wednesday)
Superovulate between ten and thirty 3-4 week old females by injecting 0.1ml (5iu) PMS at 17.30-18.00.	Prepare dishes for oocyte harvest, fertilisation/wash/ culture and sperm dispersal (if using a freshly harvested sample).	07:45 Thaw cryopreserved sperm sample, or collect and disperse freshly harvested sperm.	Morning: score the IVF success (2-cell vs others).
	Induce ovulation in the females by injecting 0.1ml (2.5iu) hCG at 18:00	08:00-09:00 Harvest oocytes and place into diluted sperm preparation.	Prepare the 2-cell embryos for cryopreservation, embryo transfer or culture.
		15:00 Wash the presumptive zygotes and place into culture drops.	

This timetable assumes that the mice are exposed to 12 hours of darkness between 19:00 and 07:00.

8.3 *In situ* hybridisation methods

In situ hybridisation was carried out on human brain tissue for *ZNF804A* expression and on mouse brain tissue for *Zfp804a*. The *in situ* hybridisation on human tissue for *ZNF804A* was carried out by the MRC-Wellcome Human Developmental Biology Resource In-House Gene Expression Service. The probes were prepared by Dr Wiliiam Davies and myself.

8.3.1 In situ hybridisation of ZNF804A in human embryonic and foetal tissue

In situ hybridisation was carried out as described by Wilkinson (1992). Human fetal tissue, at 9 weeks of gestation, was dissected and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. Following fixation, tissues were dehydrated and embedded in paraffin wax. Sagital sections of the head where cut at 8 µm using a standard microtome and attached to Superfrost Plus microscopic slides (VWR). Before hybridization, tissue sections were de-waxed, hydrated, fixed in 4% PFA/PBS and rinsed twice with PBS. Proteins were removed by incubation with proteinase K (20 mg/ml) in PBS. After washing with PBS, the sections were re-fixed in the same PFA solution, and treated with 0.1 M triethanolamine containing 0.25% acetic anhydride. Slides were dehydrated through an alcohol series and air-dried.

Three probes were prepared from *ZNF804A* (Genbank accession number and position of inserts are as follows: ZNG2: NM_194250.1, bp1049-1755; ZNG3: NM_194250.1, bp2072-2787; ZNG4: NM_194250.1, bp 3322-4062) ligated into pGEM –T Easy (Promega). Anti-sense and sense probes were prepared by

linearising plasmids with SaII and NcoI respectively. Digoxigenin-UTP was incorporated into riboprobes during in vitro transcription using the DIG RNA labelling mix (Roche) according to the manufacturer's instructions. Antisense and sense probes were generated using T7 and SP6 polymerase respectively.

Hybridisation solution contained riboprobe (300ng DIG-labelled RNA probe), RNAguard (1 ml/ml) and tRNA (0.5 mg/ml) in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 10% dextran sulphate and 1x Denhardt's solution). A 100 µl aliquot of hybridization probe was added to each slide, which was incubated in a sealed chamber moistened with 50% formamide/1x standard saline citrate (SSC) overnight at 65°C. Stringency washes were performed in the following order: 2x SSC (twice at 65°C); 50% formamide/2x SSC (twice at 65°C); 2x SSC (twice at 65°C); 0.2x SSC (65°C) and 0.2x SSC (65°C cooled to room temperature). Slides were then incubated for 1 h in 150 mM NaCl and 100 mM Tris-HCl pH 7.5 containing 10% fetal calf serum (FCS). For antibody detection, slides were incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase (anti-Dig antibody diluted 1:1000, containing 2% FCS) overnight at 4°C. Expression patterns were visualized using the NBT/BCIP system (Roche). Sections were mounted in VectaMount (Vector Labs) and analysed using the Axioplan 2 imaging system (Zeiss).

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